ABSTRACT

GLUCOSE METABOLISM BY BACILLUS POPILLIAE

by Rollin E. Pepper

Investigations were conducted to determine the metabolic products resulting from the catabolism of glucose, the metabolic pathways used for glucose catabolism, and the types of electron transport systems used by Bacillus popilliae. Efforts were made to elucidate the reasons for the relatively low cell yields, limited viability, and the lack of sporulation in vitro by this organism.

The major glucose products were shown to be lactic acid, acetic acid, and CO_2; the minor products were found to be glycerol, ethanol, acetoin, and acetaldehyde. The ratios of lactate to acetate may be controlled by adjusting the oxygen levels available to the cell.

The enzymes in the upper half of the Embden-Meyerhof pathway were demonstrated in cell free extracts and the hexose monophosphate pathway was implicated by the dissimilation of ribose-5-phosphate with the reduction of TPN. Essential enzymes for the Entner Doudoroff and

phosphoketolase routes were found absent. Inhibitor and radioactive isotope data were consistent with enzyme assays. The percent participation of the hexose monophosphate pathway is dependent upon oxygen availability.

The strain of B. popilliae used in most of these studies is apparently a mutant which oxidizes acetate through the TCA cycle. The selection for such a mutant was possible because of the continuous cultivation in liquid media in which acetate was present as the major product of glucose metabolism. Another variety of the same strain maintained on solid medium did not oxidize acetate. Both strains proved capable of producing the typical "milky disease" of the Japanese beetle larvae. The lack of the acetate oxidizing characteristic may be one of the reasons for the organism's not sporulating in vitro, since this characteristic has been shown to be necessary for sporulation in other bacilli.

An electron transport system through cytochrome oxidase to oxygen was demonstrated in cell-free extracts. Sensitivity to cyanide, azide, and carbon monoxide was demonstrated as well as characteristic cytochrome peaks in a spectrophotometric analysis. Hydrogen peroxide is produced but no catalase or peroxidase was found. Production

of hydrogen peroxide combined with the accumulation of organic acids may be basic causes of low cell yields and limited viability.

GLUCOSE METABOLISM BY *BACILLUS POPILLIAE*

By

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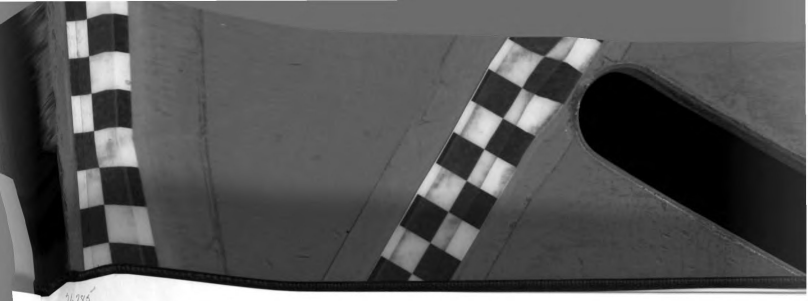
I am grateful to my wife, Lucille, for her encouragement throughout my graduate studies and for the patience of our children, Roger, Barbara, and Susan whose special interest in this thesis was the final punctuation mark.



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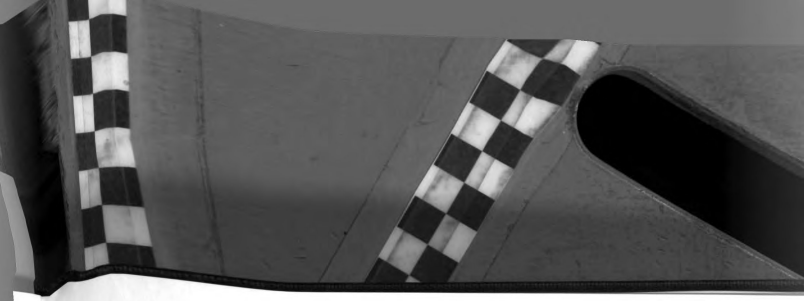

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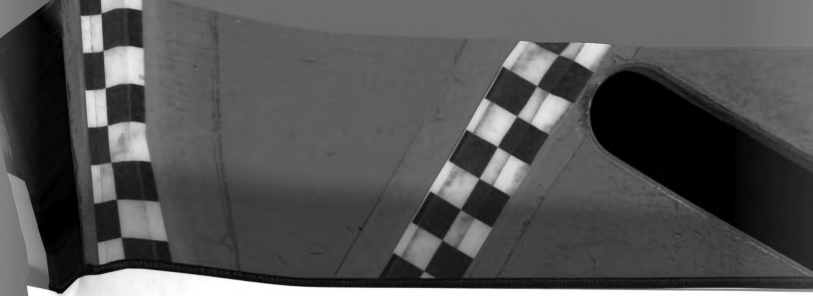

Figure

INTRODUCTION

Bacillus popilliae and *Bacillus lentimorbus* are unique in that they are selectively pathogenic for the Japanese beetle and some related insect pests. The infection, causing what is known as the "milky disease," usually involves the larval stage of the insects (Angus and Heimpel, 1960). Since these bacteria are not pathogenic for mammals or many other insects, particularly beneficial types, great interest has been generated in the propagation of these two bacilli for insect control. Unfortunately, great difficulty has been encountered in growing and maintaining viability of these organisms *in vitro* even though growth is easily effected in artificially inoculated beetle larvae; and efforts to sporulate these bacilli *in vitro* have failed.

Reports concerning these organisms have been mostly concerned with the milky disease itself (Beard, 1945; and Angus and Heimpel, 1960) or growth characteristics and conditions *in vitro* (Dutky, 1947; and Steinkraus, 1957). Both Dutky and Steinkraus list carbohydrates which these organisms will utilize and both indicate that acids accumulate by their references to the drop in pH and the need for highly buffered media to obtain optimal growth. Other than this, little has

been reported on substrate metabolism.

This study involved the characterization of metabolic products appearing from glucose, the tracing of metabolic pathways, and determining the type of electron transport system used by B. popilliae. Certain environmental factors were studied as controlling influences on product ratios and metabolic pathways. An effort was made to elucidate factors which might be limiting growth and cell viability. It is hoped that the findings detailed herein will supply basic information which will aid in future investigations with B. popilliae.

REVIEW OF LITERATURE

The literature was reviewed in the light of surveying the various pathways of glucose catabolism to pyruvate; the types of metabolic products produced, and the nature of various electron transport systems in microorganisms. Attention was focused on methods of demonstrating pathways as well as on factors which influence their usage. A brief review of the environmental and nutritional factors which cause a qualitative or quantitative alteration of glucose metabolic products was made as well as the cultural variations which influence the make-up of the electron transport system. The review is not intended to be complete since it is so voluminous, but an attempt was made to give representative examples for the areas mentioned.

Metabolic Pathways

The longest known and most frequently used route for glucose dissimilation is the Embden-Meyerhof pathway (EMP). Some tissues and organisms use the EMP exclusively. In mammalian tissues, for example, Bloom, Stetten and Stetten (1953) reported that there is no evidence for a non-glycolytic pathway in rat diaphram slices. In support of this, Bloom

and Stetten (1953) measured ratios of $C^{14}O_2$ from glucose labeled in the 1 and 6 positions and found these to be in equal amounts. However, they reported that $C^{14}O_2$ from glucose 1-C^{14} is preferentially higher when kidney and liver slices are used indicating a non-glycolytic pathway is used to at least some extent with these tissues.

Departures from the EMP pathway came to light when some data could not possibly fit known patterns. Evidence for the phosphoketolase cleavage was reported by DeMoss et al. (1951) who found that Leuconostoc mesenteroides produced 1 mole each of lactate, ethanol, and CO_2 from 1 mole of glucose; and that aldolase, triosephosphate isomerase, and carboxylase were absent. These workers (1953) later found a glucose-6-phosphate (G-6-P) dehydrogenase in L. mesenteroides and they postulated that G-6-P was oxidized to 6-phosphogluconate (6-PG) which, after decarboxylation to a 5 carbon compound, was converted to lactic acid and an ethanol precursor upon a 3:2 split. DeMoss (1954) found the 6-PG dehydrogenase to be DPN dependent in this organism and identified ribulose-5-phosphate as one of the compounds resulting from the 6-PG decarboxylation.

Krichevsky et al. (1955) demonstrated EMP enzymes in Microbacterium lacticum but also reported an alternative pathway leading to a direct cleavage of a pentose which

involved riboas-5-phosphate (R-5-P) or ribulose-5-phosphate (Ru-5-P). Evidence for the phosphoketolase cleavage appeared when R-5-P 1-C^{14} yielded unlabeled pyruvate, and Ru-t-P-2,3-C^{14} yielded carboxy labeled pyruvate. Data indicating a phosphoketolase in Acetobacter xylinum were reported by Schramm et al. (1958). Washed suspensions of this organism synthesized cellulose from glucose in the presence of concentrations of iodoacetate which completely inhibit glyceraldehyde-3-phosphate (G-3-P) dehydrogenase. With the phosphoketolase mechanism, energy for synthesis was obtained through the formation of acetyl phosphate. Schramm also reported that an induced phosphoketolase appears in Lactobacillus plantarum grown on pentose which will cleave xyulose-5-phosphate (Xu-5-P) to G-3-P and acetyle phosphate but will not cleave fructose-6-phosphate (F-6-P). Purified F-6-P phosphoketolase will cleave Xu-5-P. Inhibitors such as fluroide, iodoacetate, and arsenite will not affect organisms dependent upon the phosphoketolase mechanism.

Entner and Doudoroff (1952) described another pathway which departs from the traditional EMP route through a G-6-P dehydrogenase, as does the phosphoketolase route. However, in the Entner Doudoroff (ED) pathway, there is no decarboxylation prior to the formation of pyruvate. Instead, the 6-PG is transformed by a 6-PG dehydrase to a 2-keto-3

deoxy-6-phosphogluconate (KDPG) which is split by a KDPG aldolase to pyruvate and G-3-P. Pyruvate is formed from the first three carbons and G-3-P from the last three. The pyruvate resulting from the KDPG action has carboxyl carbons originating from both carbons 1 and 4 of glucose. This route was reported by Claridge and Werkman (1954) in Pseudomonas aeruginosa. These workers found this to be a strictly aerobic mechanism. According to Holzer (1959) pseudomonads metabolize 70 to 100% of glucose by this route.

Evidence for the hexose monophosphate pathway (HMP) was provided by de la Haba and Racker (1952) when they demonstrated triose phosphate formation from a phosphate ester of ribose and ribulose with yeast extracts. They also reported hexose phosphate generation. This deviation from the EMP pathway which also involves G-6-P dehydrogenase and 6-PG dehydrogenase with glucose as substrate was shown operative in Erwinia (Sutton and Star, 1960) based on the reduction of TPN through the oxidation of R-5-P. This suggested the formation of hexose phosphate through transketolase and transaldolase. Enzymes for the EMP pathway were also found to be present. Cochrane et al. (1953) found that Streptomyces coelicolor and Streptomyces scabies lack early steps in the EMP system, but the HMP sequence is present. They also mention that an alternate route exists in which pentose phosphate is

split into triose phosphate and an unidentified two carbon fragment. Their data include the information that 0.01 M iodoacetate and 0.02 M fluoride did not inhibit G-6-P oxidation. This, particularly the inhibitor data, indicates that an active phosphoketolase mechanism is operating by-passing inhibited enzymes. Santer et al. (1955) found EMP and HMP enzymes active in Pasteurella pestis. Their evidence shows that the HMP is particularly active during growth.

In a study of several phytopathogenic bacteria, Katznelson (1955) found that only Erwinia used the EMP pathway; the others used strict oxidative routes. Agrobacterium, Xanthomonas, and Pseudomonas produced pyruvate from 6-PG by the ED route or the HMP route or both. Corynebacterium apparently uses the HMP. Later, Katznelson (1958) found that only Erwinia possess an intact glycolytic system. The others lack one single enzyme, phosphohexokinase, to complete this sytem. It is possible, however, that this enzyme was present but not found since it is notably unstable.

Allen and Powelson (1958) reported that Escherichia coli oxidized glucose by both the HMP and EMP during the growth phase and shifts to the EMP exclusively during the stationary phase. Daws and Holms (1958) found that Sarcina leutea oxidized glucose by both the EMP and HMP pathways and employed the tricarboxylic acid (TCA) cycle in terminal

oxidation. Glucose was not utilized anaerobically and the ED pathway was not used. Doi and Halvorson (1959) demonstrated the HMP in vegetative cells and spores of Bacillus cereus var terminalis. They reported hexokinase and glucokinase were absent in spores. A direct oxidation of glucose to 2-ketogluconate yields 2-keto-6-phosphogluconate upon phosphorylation. The latter is either converted to pyruvate via an unknown pathway or reduced to 6-PG and oxidized via the HMP. The vegetative cells possess a complete glycolytic system as well as a TCA cycle. Goldman (1961), however, reported the key EMP enzymes hexokinase, phosphohexoisomerase, phosphofructokinase, and aldolase to be present in spores of Bacillus subtilis at various stages.

Wang et al. (1958) examined several organisms for pathway participation. He found that E. coli, Saccharomyces cerevisiae, B. subtilis, Aspergillus niger, Penicillium digitatum, Penicillium chrysogenum, and Streptomyces griseus utilize the EMP and HMP routes to various degrees; Pseudomonas reptilivora uses the HMP and the ED routes; and Pseudomonas saccharophilia the ED route only.

Metabolic Products

Products from glucose range all the way from CO_2 and H_2O to a variety of metabolic intermediates. Incomplete

degradation of glucose is due to oxygen availability, enzyme blockage, pH, nutrition of cells, and substrate concentration.

Puziss et al. (1957) found that Bacillus anthracis and B. cereus anaerobically dissimilated glucose to lactate, succinate, acetate, formate, acetoin, 2,3 butanediol, and glycerol. While examining aerobic and anaerobic glucose products of B. subtilis, Neish et al. (1945) observed that air increased the production of acetoin, acetate, and CO_2 but decreased 2,3 butanediol, glycerol, ethanol, lactate, and formate. Ehrlich and Segel (1959) examined glucose products of Bacillus megaterium under conditions of continuous and intermittent shaking. Increased aeration depressed lactate and increased CO_2. They found no striking changes in acetoin, 2,3 butanediol, glycerol, pyruvate, and acetate. Gray and Bard (1952) grew B. subtilis cells on complete and simple media and allowed resting cells to dissimilate glucose aerobically and anaerobically. They found a homolactic fermentation with both groups under anaerobic conditions. Aerobically, the cells from the simple medium oxidized glucose completely to CO_2 and H_2O; cells from the complete medium also accumulated acetate and acetoin but no lactate.

Pierce (1957) reported that pH affected glucose fermentation products of Streptococcus pyogenes. At pH 5.0, most of the glucose was converted to lactate; at pH 9.0,

lactate diminished and more acetate, formate, and ethanol were produced. Similarly, Paege (1961) found that E. coli fermented glucose principally to lactate at pH 5.0 and 8.0. However, with the increased pH, lactate diminished from the pH 5.0 value and increased amounts of ethanol, formate, succinate, and CO_2 appeared.

Christensen (1958) noted that sugar concentration had an effect on acid ratios produced by lactic acid bacteria. He found that an increased sugar concentration decreased the acetate/lactate ratio.

Electron Transport

Dehydrogenation of a substrate results in the reduction of an organic acceptor, a non-organic acceptor such as nitrate, or one or more pigments (cytochromes and/or flavin) leading to oxygen. Normally, anaerobes will reduce other organic compounds with the hydrogen transported by the pyridine nucleotides. However, they have the potential to reduce oxygen to H_2O_2 through flavins (M'Leod and Gordon, 1923). Lactic acid bacteria will transport hydrogen to oxygen in this manner and will dispose of the H_2O_2 via a peroxidase. Most aerobes possess a cytochrome electron transport system to oxygen. Although the H_2O_2 potential is present, most of these organisms produce catalase which converts this harmful

product into H_2O and O_2.

Several workers have studied cytochromes by reducing them with various substances and observing their representative peaks with split beam spectrophotometry. Chance (1952) recognized cytochrome a_3 after cells had become reduced by their own respiration. Smith (1954) using the same method stated that bacterial cytochromes may differ from those found in yeasts and mammalian cells in that (a) a broad band of cytochrome b_1 may replace those for b and c and (b) cytochrome a is often replaced by a_1 or a_2. Wood (1955) reduced cytochromes in Pseudomonas fluorescens with glucose, gluconate, or sodium hydrosulfite and found α peaks for cytochrome c and b (558 and 565 mμ) and β absorption for b and c (530 mμ). The lack of peaks in the 600 to 625 mμ range and no cyanide inhibition seemed to indicate the lack of a cytochrome oxidase. Chance (1957) reduced cytochromes with carbon monoxide and demonstrated cytochromes a_1, a_3, and b in Aerobacter aerogenes. In Aerobacter pasteurianum, he found an a_1 peak at 427 mμ. He also reported cytochromes b and c in Rhodospirillum rubrum but no distinguishing bands for the type a. Work on Haemophilus parainfluenzae (White, 1962; White and Smith, 1962; and Smith and White, 1962) showed cytochromes b, c, o, a, and a_2 after reduction with sodium hydrosulfite. No peroxidase was found and very little

cytochromes. Dobrogosz (1962), using sodium hydrosulfite as the reductant, demonstrated no absorption peaks in the 400 to 700 mμ range for Streptococcus faecalis and Pediococcus but definite bands for "positive" controls; i.e., B. subtilis and P. fluorescens.

The presence of cytochromes not only is a characteristic of a particular organism but is also a function of age and cultural conditions. Smith (1954) found that the level of cytochromes present increases with age. Gary (1954) found that B. subtilis grown on complex media lacked cytochrome oxidase whereas cells produced on a simple medium contained a cytochrome oxidase.

EXPERIMENTAL METHODS

The following abbreviations will be used: OAR for oxygen absorption rate expressed as mmoles of oxygen absorbed per liter per min; ATP for adenosine triphosphate; DPNH for reduced and DPN for oxidized diphosphopyridine nucleotide; TPNH for reduced and TPN for oxidized triphosphopyridine nucleotide; CoA for coenzyme A; TPP for thiamine pyrophosphate; G-6-P for glucose-6-phosphate, KDPG for 2-keto-3-deoxy-6-phosphogluconate; R-5-P for ribose-5-phosphate; Ru-5-P for ribulose-5-phosphate; Xu-5-P for xyulose-5-phosphate; F-6-P for fructose-6-phosphate; F-1,6-P for fructose-1,6-diphospate; G-3-P for glyceraldehyde-3-phosphate; DHAP for dihydroxy-acetone phosphate; EMP for Embden-Meyerhof pathway; HMP for hexosemonophosphate pathway; ED for Entner-Doudoroff pathway; TCA for tricarboxylic acid pathway; and OD for optical density.

Optical densities at wavelengths in the visible range were measured using a Bausch and Lomb Spectronic 20 colorimeter (Bausch and Lomb Optical Co., Rochester, N.Y.) and those in the ultraviolet range were made using the Beckman Model DU spectrophotometer.

Cultures and Cultural Methods

Cultures used: All cultures used in this study were obtained from the Northern Utilization Research Division, U.S.D.A., Peoria, Illinois. Culture designations are those used by the U.S.D.A. Initial studies involving substrate utilization and acid accumulation in growth media were made with strain NRRL B-2043. Later, strain NRRL B-2309-P was used since the U.S.D.A. reported that this strain attained higher populations in vitro. All metabolic studies were made with the latter strain.

Cultural media and maintenance: Two media were used throughout this study. The first, designated as B-1, was suggested by Hall (1961) as a medium in which good cell yields of B. popilliae had been obtained. This medium was used in the studies conducted with B. popilliae B-2043. A later modification of this medium was made, again through a suggestion from Hall (1961), to obtain higher cell yields and to retain viability over a longer period of time. This medium, designated as B-4, was used principally for the purpose of producing cells for metabolic determinations. Both of these media were used at various times for maintenance of strains by adding 1.5% agar and preparing slants. The composition of these media is given in Table 1.

Table 1. Composition of media used for growing cells of B. popilliae.

Ingredients	Medium B-1	Medium B-4
Yeast extract (DIFCO)	15.0 g.	15.0 g.
Glucose	3.0 g.	2.0 g.*
K_2HPO_4	3.0 g.	6.0 g.
Tryptone	3.0 g.	---
Distilled water	1000 ml	1000 ml
pH	7.0 - 7.2	7.3 - 7.4

*Actually 2.5 g of glucose were added, but it was found by experimentation that 2.5 grams of glucose per liter of medium would assay as 2.0 grams per liter when 250 ml amounts were autoclaved for 15 min at 15 pounds pressure.

B. popilliae was maintained principally in B-4 broth. Cultures were transferred at 48 to 74 hr intervals by inoculating flasks containing 250 ml of medium with a 10 ml inoculum. They were incubated at 30 C on a rotary shaker. Cultures on agar slants were incubated in parallel to assure that the strain would not be lost. This method of maintenance was necessary since viability is easily lost if cultures are stored "in stock" as is the custom with most other species of bacteria.

Production of cells and cell free extracts: Cells for metabolic studies were obtained either by inoculating several flasks of media or a 10 liter carboy. Flasks were shaken at 200 RPM on a New Brunswick shaker[1] and cultures

[1]New Brunswick Scientific Co., New Brunswick, N.J.

in carboys were agitated by forced filtered air. All flask cultures were inoculated with 10 ml of a 24-hr broth culture and carboys were inoculated with four 250 ml 24-hr cultures (1 liter inoculum). After the desired growth had occurred, cells were removed from the medium by centrifugation, washed twice, and resuspended in distilled water.

Extracts were prepared by breaking cells with No. 100 glass beads[2] in a high speed Servall[3] omnimixer. Approximately 12 g (wet weight) of cells were suspended with 40 to 45 g of glass beads in 50 ml of distilled water. The mixture was chilled for 30 min in an ice bath and the blender cup maintained in the ice bath throughout the breaking period which was completed in 10 to 15 min. The extract was centrifuged at 1500 x g for 15 min to remove beads and whole cells. These preparations will be referred to as whole extracts.

Separation of the whole cell-free extracts into soluble and particulate fractions was accomplished by centrifugation at 110,000 x g for 3 hr in the Beckman Model L Preparative Ultracentrifuge.[4] Particles thus obtained were

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[2]Minnesota Mining & Manufacturing Co., St. Paul, Minn.

[3]Ivan Sorvall, Inc., Norwalk, Conn.

[4]Spinco Division, Beckman Instruments, Palo Alto, Calif.

resuspended in 0.2 M phosphate buffer, pH 7.4.

Protein Determination

The Folin-Ciocalteu test for protein determination (Lowry et al., 1951) was performed on cell extracts in order to quantitate activity in terms of protein content. This reagent reacts with phenol groups (tyrosine and phenylalanine) to give a color intensity which is correlated directly with the protein content of the extract. Bovine serum albumin was the standard.

OAR Determination

OAR's were determined according to the method of Coreman et al. (1957). These were run in dimpled 500 ml Erlenmeyer flasks with urethane stoppers containing 50 ml of 0.41 N sodium sulfite and 0.001 M copper sulfate. The flasks were shaken at various rates on a New Brunswick shaker. Five ml samples, taken initially and at various time intervals, were pipetted directly into tubes containing dry ice chips which served to stop the oxygenation as well as to agitate the solution during titration. The solutions were titrated with standardized iodine using starch as an indicator. Titration differences were used in the following formula to calculate the OAR:

$$OAR = \frac{\text{ml titration difference} \times \text{iodine normality}}{4} \times \frac{1000}{5} \times \frac{1}{\text{min}}$$

Manometric Studies

Oxygen consumption and CO_2 evolution were determined manometrically with the Warburg respirometer by the procedures outlined by Umbreit et al. (1957). Flask contents were examined chemically for initial and residual substrate as well as for metabolic products.

Isotope Studies

Glucose-1-C^{14} and -6-C^{14} and acetic acid-1-C^{14} were mixed with cold substrate and oxidized by resting cells in the Warburg respirometer. The activity of radioactive substrate did not exceed 0.1 μc per vessel except where otherwise noted. Durham tubes cut in half and containing 0.2 ml of 20% KOH were placed in the center well of the Warburg flasks to trap CO_2. After the reaction was stopped with 4 N H_2SO_4, the tube was removed with forceps and the contents transferred to a 20 ml sample vial with a bulb pipette. The tube and pipette were successively washed out with measured quantities of distilled water and added to the radioactive sample. Distilled water was added to 1.0 ml. Likewise, samples were taken from the residue and similarly made up to 1.0 ml volume in the sample vial.

Using the method of Goldman (1961), CO_2 was collected from a growing culture of B. popilliae during the oxidation of acetic acid-1-C^{14} in B-4 medium. Five hundred ml flasks containing 25 ml of medium and 0.5 µc of label were stoppered with rubber plugs. Each plug was outfitted with a bent glass rod to which a 1.2 x 3.5 cm tube was secured with a rubber band. The tube, suspended above the medium, contained 0.5 ml of 40% KOH to trap all CO_2 from oxidized acetate. Flasks were shaken at 200 RPM at 30 C on a New Brunswick shaker. Individual flasks were removed periodically to examine the KOH for the presence of the label.

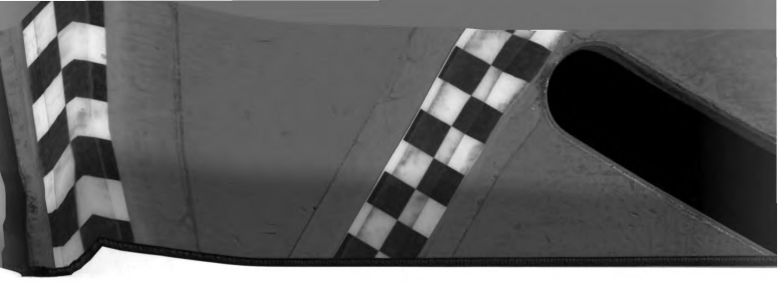
The composition of the "scintillator solution" (Baldwin, 1961) used for counting carbon-14 disintegrations is given in Table 2.

Table 2. Scintillator solution for counting carbon-14 disintegrations.

Ingredient	Amount*
xylene	192 ml
p-dioxane	192 ml
ethanol (absolute)	115 ml
PPO**	2.5 g
αNPO**	25 mg
naphthalene	40 g
Cabosil**	20 g

*The ingredients are combined in a Waring Blender and mixed at high speed for three min.

**Packard Instrument Company, LaGrange, Illinois.



Fifteen ml of the scintillator solution were transferred by means of a syringe into the 20 ml vial containing 1 ml of diluted sample. The vial was closed and vigorously shaken to mix the contents. The disintegrations were counted with Tri-carb Liquid Scintillation Spectrometer.[5] Benzoic acid-C^{14} was used as a counting standard.

Chemical Assays

Glucose: The colorimetric determination of reducing sugars as described by Neish (1952) was used for quantitatively determining glucose. As reducing sugars are heated in an alkaline curpic copper solution, the copper is reduced and precipitated as curpous oxide. The cuprous oxide, proportional to the reducing sugar, is determined colorimetrically by the formation of molybednum blue upon the addition of arseno-molybdate.

Acids: Total acids were determined by titration with standard NaOH using measured samples taken from growing cultures or Warburg flasks and centrifuged to remove cells.

A celite column described by Wiseman and Irvin (1957) containing celite and sucrose with alpha amine red as the internal indicator for acid detection were used for separation and identification of acids. Solvents consisting of mixtures

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[5]Packard Instrument Co., LaGrange, Illinois.

of Skelly Solve B and acetone are used to elute acids after introduction of the sample. Eluted acids were titrated with 0.0045 N $Ba(OH)_2$ standardized against potassium acid phthalate.

Volatile acids were separated from samples of media and Warburg flask contents by steam distillation in the presence of H_2SO_4 and magnesium sulfate and the distillates titrated with 0.1 N NaOH.

Lactic acid was determined by the method of Neish (1952). In the presence of concentrated sulfuric acid, lactate is oxidized quantitatively to acetaldehyde which is determined colorimetrically in the presence of copper sulfate and p-hydroxydiphenyl.

Ethanol: Ethanol was determined by the microdiffusion method described by Neish (1952). Using sealed Conway plates, ethanol diffused from the outer section into the center well thereby reducing the dichromate. Ethanol is calculated from the amount of dichromate reduced.

Glycerol: Upon oxidation by periodate, 1 mole of glycerol forms 2 moles of formaldehyde and one of formic acid. The method for periodate oxidation outlined by Neish (1952) was followed. The formaldehyde formed in this oxidation was quantitated by a method described by Nash (1953). Glycerol was calculated from the formaldehyde.

Acetaldehyde: Acetaldehyde was estimated by the bisulfite binding method detailed by Neish (1952). Bisulfite, quantitatively bound by acetaldehyde is released upon the addition of sodium bicarbonate. The released bisulfite is titrated with iodine.

Acetoin: The method of oxidizing acetoin by alkaline iodine as described by Neish (1952) was employed. After acidification, the excess iodine is measured by thiosulfate titration. The iodine reduced by reaction with acetoin is calculated.

Pyruvate: The colorimetric method described by Umbreit et al. (1957) was used for pyruvate determinations. Pyruvate is reacted with 2,4-dinitrophenylhydrazine and extracted successively with ethyl acetate and socium carbonate. The color, which is directly proportional to the pyruvate content is measured at 520 mμ.

Enzyme Assays

Glucose-6-phosphate dehydrogenase: G-6-P dehydrogenase was assayed according to the method of Kornberg and Horecker (1955). With G-6-P as the substrate, TPN reduction was followed by an increase in OD at 340 mμ. Magnesium chloride as well as TPN were supplied as cofactors in a pH 7.5 glycylglycine buffer.

Hexose monophosphate pathway (HMP) enzymes: The presence of the complete HMP enzyme system was assayed by supplying R-5-P as the substrate and TPP, $MgCl_2$, and TPN as cofactors. Where the complete HMP system is present, TPN is reduced as evidenced by an increase in OD at 340 mμ. This enzyme system generates F-6-P and in the absence of ATP, G-6-P will be formed through the action of phosphohexoisomerase. As the G-6-P is generated it is oxidized by the G-6-P dehydrogenase, and TPN is reduced. This method was reported by Sutton and Star (1960).

DPNH oxidase, TPNH oxidase, and TPNH-DPN transfer enzyme: DPNH oxidase was assayed by measuring the decrease in OD at 340 mμ in the presence of the extract. Likewise, TPNH oxidase was assayed by replacing DPNH with TPNH. The TPNH-DPN transfer enzyme was assayed by placing these two cofactors in the presence of the extract and observing OD readings at 340 mμ. Since no TPNH oxidase was present, a decrease in OD would occur if this enzyme were present.

KDPG aldolase: KDPG aldolase (Heath et al. 1958) was assayed by supplying KDPG, 0.5 μmole; DPNH, 1 μmole; imidazole buffer, pH 8.0, 10 μmole; 0.0002 ml of extract and an excess of lactic dehydrogenase in a total volume of 0.15 ml. If this enzyme is present, pyruvate is generated and DPNH oxidized at a rate in excess of the DPNH oxidase alone.

Phosphoketolase: Xu-5-P was used as the substrate for this enzyme assay. Cuvettes contained Xu-5-P, 0.2 μmole; phosphate buffer pH 6.5, 50 μmole; DPNH, 1 μmole; $MgCl_2$, 0.5 μmole; TPP, 0.1 μmole; glutathione, 10 μmoles, extract, 0.0002 ml; and α-glycerophosphate dehydrogenase and triose-phosphate isomerase in excess. The total volume was 0.15 ml. This enzyme generated G-3-P which, in the presence of supplied enzymes, forms α-glycerophosphate with the oxidation of DPNH. As with the KDPG aldolase assay, the oxidation of DPNH would be in excess of the DPNH oxidase activity. This method was described by Kovachevich and Wood (1955).

Aldolase, triosephosphate isomerase, and phosphofructo-kinase: Aldolase was assayed by the method of Sibley and Lehninger (1949). The action of aldolase converts one mole of F-1,6-P into one mole each of G-3-P and DHAP. The trioses are trapped by hydrazine in a 1:1 proportion as they are formed. The hydrazones are treated with alkali and colori-metrically assayed at 540 mμ after the addition of NaOH and 2,4-dinitrophenylhydrazine.

Triosephosphate isomerase was assayed by a modifi-cation of the aldolase assay. Hydrazine added initially traps G-3-P and DHAP as they are formed in approximately a 1:1 proportion. By adding the hydrazine after incubation, the triosephosphate isomerase is allowed to act which

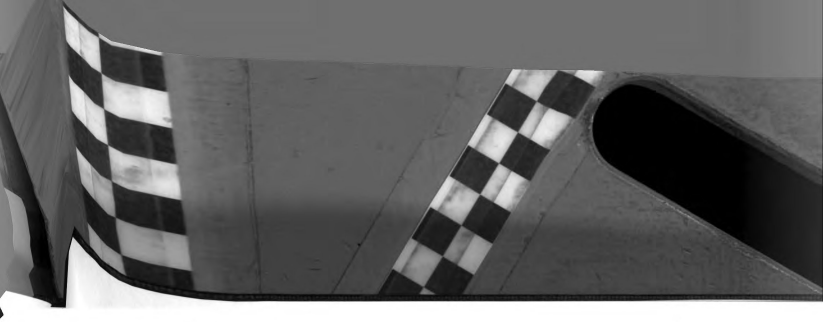
results in a G-3-P:DHAP ratio of 4:96. Since DHAP reacts more strongly with dinitrophenyl hydrazine than does G-3-P, the enzyme is demonstrated by the color differential.

Phosphofructokinase was determined by the aldolase assay method with F-6-P and ATP substituted for F-1,6-P.

Acetokinase: Acetokinase was determined by a colorimetric procedure described by Rose (1955) in which acetyl phosphate, formed from acetate and ATP, is converted to the hydroxamic acid derivative. The reaction of the latter compound with iron ($FeCl_3$) gives a colored compound which is measured at a wavelength of 540 mμ.

Phosphotransacetylase and the condensing enzyme: The method of Ochoa (1955) was employed for assaying phosphotransacetylase and the condensing enzyme at the same time. Acetyl phosphate disappearance proportional to extract concentration in the presence of CoA and oxalacetate was used as evidence for the presence of these two enzymes. Controls in which CoA and oxalacetate were omitted separately were used to demonstrate specificity. Acetyl phosphate was determined by the method of Lipmann and Tuttle (1945).

Glucose oxidase and glucose dehydrogenase: The manometric determination of glucose oxidase described by Bentley (1955) was used. The direct oxidation of glucose by cell extracts is measured by respiratory oxygen consumption.



An alteration of the method of Strecker (1955) for glucose dehydrogenase was made. Instead of observing an increase in OD at 340 mμ caused by the reduction of DPN in the presence of glucose and the enzyme, these elements were supplied and oxygen consumption was measured. If the enzyme were present, DPN would be reduced to DPNH and DPNH would be oxidized by the DPNH oxidase known to be present resulting in oxygen uptake.

Catalase: Catalase activity was assayed by three methods: viz., (a) dropping H_2O_2 on agar colonies of B. popilliae, (b) tipping H_2O_2 into buffered cell free extracts and manometrically observing oxygen release, and (c) by the iodometric titration method of Herbert (1955) in which residual H_2O_2 is determined after various periods of time in the presence of the extract.

Peroxidase: A method described by Dolin (1957) for assaying a flavin linked peroxidase was used to establish the presence or absence of this enzyme. Dolin used this method for establishing peroxidase activity in Streptococcus faecalis and Walker (1963) used it with lactobacilli. Briefly, DPNH oxidase activity was assayed with a decrease in OD at 340 mμ. The same test was then run with peroxide added. A faster rate of OD decrease indicates the presence of this enzyme. The assay was run at pH 5.4.

Hydrogen peroxide production: Whole cell free extracts as well as soluble and particulate fractions separated by ultracentrifugation were used to oxidize DPNH in the Warburg respirometer. When oxygen consumption ceased, catalase was tipped and oxygen return was measured. Net return of oxygen (less endogenous) indicates the presence of hydrogen peroxide.

Cytochromes: Suspensions of extract particles (110,000 x g) were reduced by a few crystals of sodium hydrosulfite as described by Dobrogosz et al. (1962). A difference spectrum between the oxidized and reduced particles was obtained by the split beam method employing the use of the Cary Model 15 Spectrophotometer.[6] Absorbancy differences at wavelengths varying from 400 to 700 mμ were determined.

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[6]Applied Physics Corp., Monrovia, Calif.

RESULTS

Initial studies with B. popilliae involved establishing growth curves from liquid shake cultures. Shaking 50 ml of medium in 500 ml flasks at 200 RPM resulted in growth approximating 2 to 3 x 10^8 cells per ml in 24 to 36 hr. The viability dropped approximately 99.9% between 48 and 72 hr.

Growing cultures were examined for pH changes. The steady drop in pH indicated a continuous accumulation of acid. When acid production was compared with glucose utilization, it was found that the glucose oxidation products were quantitatively accumulating as acid intermediates rather than proceeding through to terminal oxidation (Fig. 1).

Since one of the overall objectives of this project was the achievement of high populations of cells with prolonged viability, the effects of oxygen and pH on growth and cell viability were studied. Efforts were made to determine whether a specific level of oxygen was necessary for high populations. Furthermore, the rapid drop in pH was studied as a possible inhibitor of increased growth as well as a factor which affects overall viability. The results (Table 3)

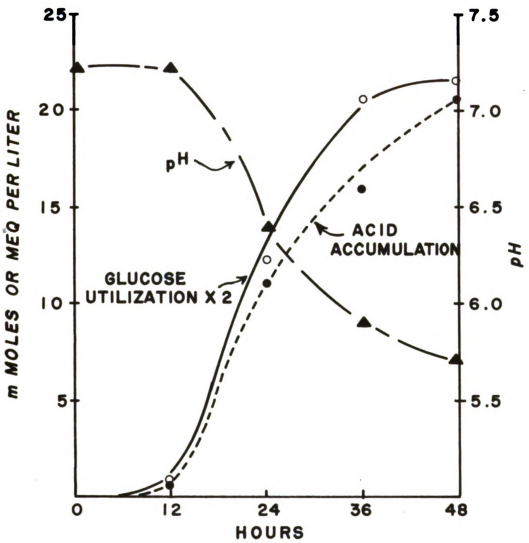
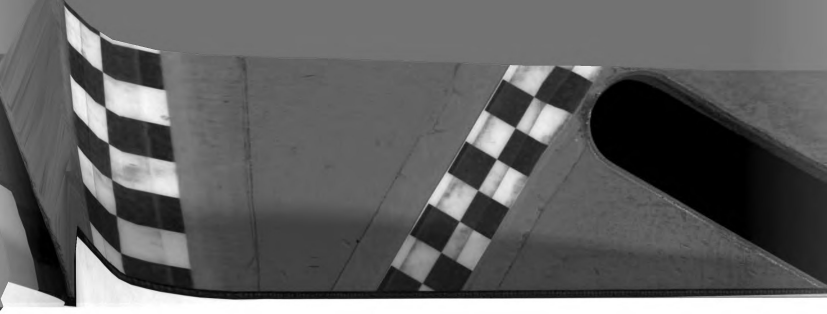


Figure 1. Glucose utilization, acid accumulation, and pH changes in a broth culture of B. popilliae. Fifty ml of B-1 medium contained in a polystyrene plugged 500 ml dimpled flask were inoculated with 0.1 ml of a 22 hr broth culture. Flasks were incubated at 30 C and shaken at 200 RPM.



show that growth responded up to an OAR of about 1.0; increased oxygenation appeared to be in excess. The neutralization of acids also resulted in higher final populations. However, neither oxygen level nor pH control increased the cell yield more than 2X, nor was there any significant increased retention of cell viability. There are obviously other factors which limit growth and viability.

Table 3. Effect of oxygen availability and pH control upon the growth of B. popilliae.

	OD[b] (670 mμ)			
	Unneutralized[c]		Neutralized[d]	
O.A.R.[a]	24 Hr	48 Hr	24 Hr	48 Hr
0.262	0.075	0.14	0.03	0.175
0.865	0.14	0.25	0.135	0.28
1.12	0.23	0.28	0.24	0.38
2.91	0.23	0.26	0.23	0.36

[a]O.A.R. oxygen absorption rate rexpresed as millimoles of oxygen absorbed/liter/minute.

[b]An OD of 0.1 = approximately 1 x 10^8 cells/ml.

[c]In unneutralized cultures, the pH dropped to about 5.7.

[d]pH controlled between pH 6.5 and 7.2 with sterile NaOH.

Products of Glucose Metabolism

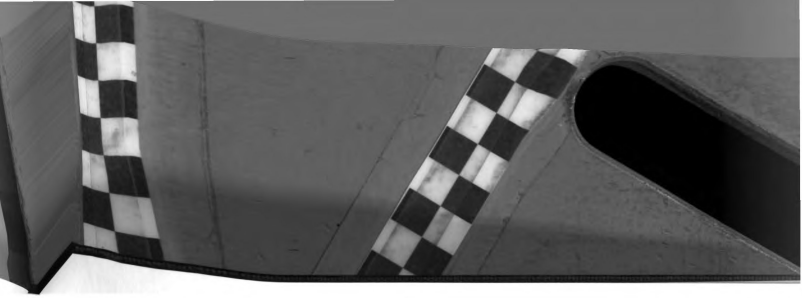
Acid production: The principal objective of this research was to characterize the catabolism of glucose by

B. popilliae. The obvious place to begin this investigation was to establish the nature of the metabolic products accumulating during growth. Since these were obviously mostly acids, the method selected for initial characterization was the separation of the acids by column chromatography.

Samples of a 24-hr broth culture, and Warburg flask contents containing resting cell oxidation products from glucose were introduced into the celite column described by Wiseman and Irvin (1957). The solvents, mixtures of Skelly Solve B and acetone in various proportions, are designed to elute the following acids in the order listed: butyric, propionic, acetic, formic, lactic, and succinic. Only two bands descended the column; one corresponding to acetic acid and the other to lactic acid. When broth culture samples were passed through the column acetic acid was either the principle or only acid appearing. Warburg flask contents produced both bands, sometimes in equivalent titratable amounts.

Of the two acids which are produced and accumulated in quantity by B. popilliae, one is volatile (acetic) and the other non-volatile (lactic). This served as a second method for separating and identifying these acids.

A broth culture was examined after 24 hours and 48 hours for total acid production (titratable acidity), for



lactic acid (chemical assay), and for volatile acid content. The results (Table 4) show that no lactate was produced and all of the acid was volatile. This corresponds with the results obtained with column chromatography where broth culture samples passed through the column produced only the acetic acid band.

Table 4. Acids produced by a growing culture of B. popilliae.

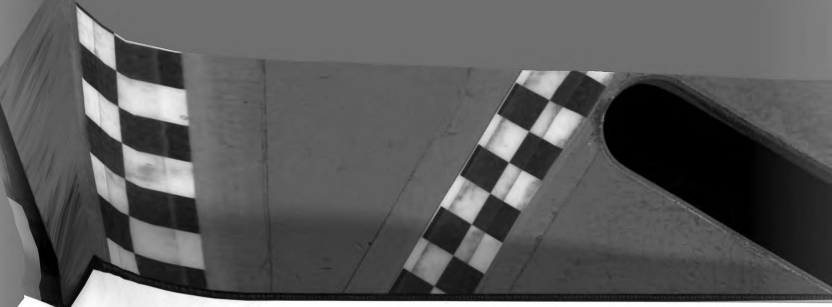
Hours Incubation	Acids Produced (meq per liter)		
	Total[a]	Volatile[b]	Non-volatile[c]
24	12.2	13.4	0
48	14.6	14.56	0

[a]Titratable acidity.

[b]Steam distilled acids.

[c]Difference confirmed by direct tests for lactic acid.

Resting cells of B. popilliae were allowed to oxidize 3.9 μmoles of glucose including approximately 10 μc of glucose-1-c^{14} in Warburg vessels. Lactic acid was determined directly by chemical assay. A sample of the reaction mixture was distilled, and the volatile acid collected in alkali, concentrated, and counted for radioactivity. The results (Table 5) show that of the 3.9 μmoles of glucose oxidized (7.8 μmoles of C_3 units), exactly one half appeared as lactate



and the radioactivity data show approximately one half of the products to be volatile acid. These data correspond to the chromatography results which show lactate and acetate appearing in approximately equal quantities in Warburg vessels.

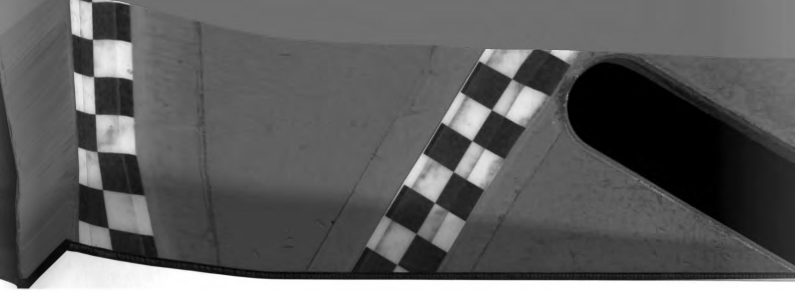
Table 5. Separation of glucose oxidation products into volatile and non-volatile acids.

Type of test		Results
I. Radioactive counts		
Initial count of vessel contents		9.9×10^6 cpm*
Counts after steam distillation		
Volatile Acid	5.1×10^6 cpm	
Residue	4.5×10^6 cpm	9.6×10^6 cpm
II. Chemical assay		
Glucose oxidized x 2		7.8 µmoles
Lactic acid produced		3.9 µmoles

*cpm = counts per minute

3.9 µmoles of glucose containing approximately 10 µc of glucose 1-C^{14} were oxidized by B. popilliae resting cells in a Warburg vessel in an atmosphere of air. A sample was steam distilled for volatile acids. Radioactive counts were made on initial levels of radioactivity, the volatile acid content, and the residue. The counts were calculated back to the total flask contents. Chemical assays were made for reducing sugar and lactic acid.

Carbon balance of glucose products: Glucose was oxidized by B. popilliae resting cells in Warburg vessels containing an atmosphere of air and the amount oxidized determined as the difference between initial and residual



levels. Oxygen consumption and CO_2 evolution were measured manometrically. Total acids were measured by titration, lactate was determined chemically, and acetate estimated as the mole difference. Tests for glycerol, ethanol, acetoin, and acetaldehyde were made as described in the section on methods. Lactate, acetate, and CO_2 appeared as the principal products and ethanol, glycerol, acetaldehyde, and acetoin as minor products (Table 6). With the approximately 97% carbon recovery, it is evident that all principal products were recovered. The CO_2 calculated to observed ratio of 0.7 could result from some HMP and TCA cycle participation.

Ratio of lactate to acetate: Upon repeating the above carbon balance several times, it was observed that the ratio of lactate to acetate varied considerably while the total acid production remained substantially the same. This variation was found to be correlated with the concentration of cells per vessel. Where the concentration of cells was high, lactate predominated, and acetate and CO_2 production and oxygen consumption were low. Where the cell concentration was low, the reverse was true. This was verified by using a single cell harvest in a concentrated (51 mg dry wt per vessel) and a diluted form (17 mg dry wt per vessel) to oxidize glucose simultaneously (Table 7).

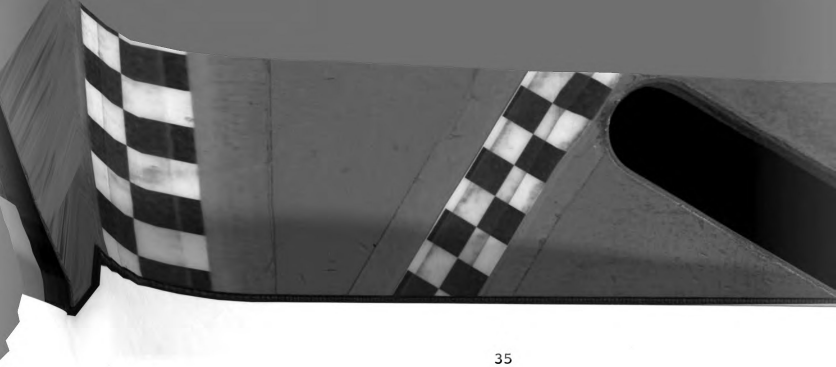


Table 6. Carbon balance of glucose oxidation products.

Material	µg	% of utilized glucose	µmole per 100 µmoles of C_3 utilized	µmole of carbon	Calc. CO_2 µmole
Glucose added	7200				
Glucose not oxid.	70				
Glucose oxidized	7130		50	300	
Lactate	3960	55.54	55.40	166.0	
Acetate	1190	16.69	25.00	50.0	25.00
CO_2	1670	23.42	47.90	47.9	
Ethanol	311	4.36	8.46	16.9	8.46
Glycerol	68	0.54	2.80	8.4	
Acetoin	trace				
Acetaldehyde	trace				
	7199	100.55	139.56	289.2	33.46

Carbon recovery: $\frac{289.2}{300} = 96.4\%$

$$\frac{CO_2 \text{ Calc.}}{CO_2 \text{ Obs.}} = 0.7$$

Glucose was oxidized by B. popilliae resting cells in the presence of air. The flask contents were buffered at pH 7.0.

Table 7. Acid production by concentrated and diluted cells of the same harvest.

Acid production per 50 µmoles glucose oxidized	Cells per vessel 51 mg	17 mg
Total acid, µeq.	76.5	77.2
Lactic acid, µeq.	43.7	23.1
Acetic acid, µeq.	32.8	54.1

Total acids were determined by direct titration, lactic acid by chemical tests, and acetic acid estimated as the mole difference.

Using the values obtained from several experimental runs, the molar ratio of lactate to acetate was plotted against the cell concentration (mg dry wt.). These results are shown in Fig. 2.

Assuming that these results might be due to the availability of molecular oxygen per cell, a single cell concentration was used to oxidize glucose in atmospheres containing 0, 3, 7, 20, 60, and 100% O_2. Although the level of acid remained constant, the results shown in Fig. 3 demonstrate the relationship between the type of acid produced and the atmosphere present. Where no oxygen was present in the atmosphere, no utilization of the substrate took place; i.e., all initial glucose was recovered and no acid was produced.

Dissimilation of pyruvate: Pyruvate was dissimilated both aerobically and anaerobically. Under anaerobic conditions, approximately one half of the substrate was reduced to lactate. The hydrogen ions necessary for this reduction were obtained by oxidizing the remainder of the pyruvate, presumably to acetate. Decarboxylation of one-half of the pyruvate was measured by the appearance of CO_2 in the amount of one-half of the molar concentration of the initial pyruvate. The following reaction probably represents the anaerobic dissimilation of pyruvate:

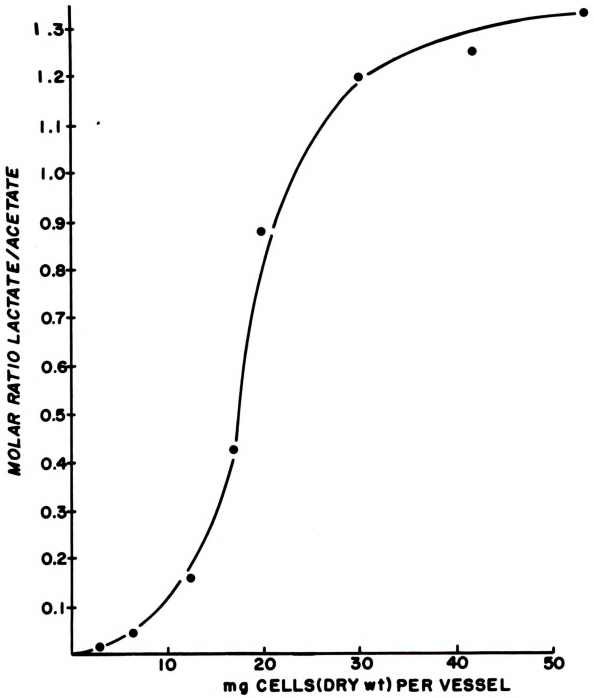


Figure 2. Effect of cell concentration upon the molar ratio of lactate to acetate. The points were obtained from several experiments with the Warburg respirometer. Reaction vessels contained cells, 0.5 ml of 0.2 M phosphate buffer, pH 7.0; 0.5 ml of 0.0667 M glucose; 0.2 ml of 2 N H_2SO_4 in a side arm and 0.2 ml of 20% KOH in the center well. Total volume was brought to 3.0 ml with water. The gas phase was air.

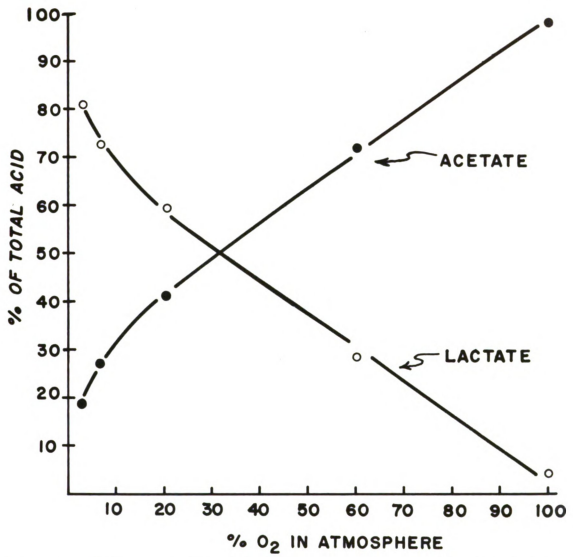


Figure 3. Effect of various oxygen levels upon the type of acid produced. Reaction vessels contained 48.8 mg dry wt. of 24 hr harvested cells; 0.5 ml of 0.3 M phosphate buffer, pH 7.0; 0.5 ml of 0.0667 M glucose. The center well held 0.2 ml of 20% KOH and a side arm contained 0.2 ml H_2SO_4 to stop the reaction except in vessels where the contents were used to determine titratable acidity. Water was added to 3.0 ml and the gas phase contained mixtures of oxygen and helium to give desired oxygen concentrations.

$$CH_3COCOOH + H_2O \longrightarrow CH_3COOH + CO_2 + 2H^+$$

$$CH_3COCOOH + 2H^+ \longrightarrow CH_3CHOHCOOH$$

$$2\ CH_3COCOOH + H_2O \longrightarrow CH_3COOH + CH_3CHOHCOOH + CO_2$$

Under aerobic conditions, molecular oxygen can serve as the hydrogen acceptor, and there is much less lactate produced per mole of substrate dissimilated.

Pathways of Glucose Catabolism

With the products from glucose established, efforts were made to determine the metabolic routes for glucose catabolism. The first approach involved the use of metabolic inhibitors. Although not definitive, inhibitors give strong presumptive evidence of metabolic pathways involved. Next, estimation of respirative $C^{14}O_2$ resulting from equal amounts of glucose-1-C^{14} and -6-C^{14} gives strong indications of the various pathways. Little or no radioactive CO_2 signifies purely EMP whereas the appearance of the label from glucose-1-C^{14} but not from -6-C^{14} in the CO_2 indicates HMP or a heterolactic pathway. Large amounts of isotope from glucose-6-C^{14} in the CO_2 would indicate terminal oxidation. The third method involved the direct assays for individual enzymes. With essential agreement of evidence gathered from all three approaches, the establishment of metabolic routes for

the organism can be made with confidence.

Inhibition of glucose oxidation: Two inhibitors, iodoacetate and fluoride, were used to gain preliminary information concerning pathway participation. Iodoacetate inhibits G-3-P dehydrogenase and fluoride inhibits enolase. Inhibition of glucose oxidation was measured by decrease in oxygen consumption as compared with the non-inhibited control. Iodoacetate (0.01 M) inhibited glucose oxidation 100%, 0.01 M fluoride inhibited approximately 50%, and 0.1 M fluoride inhibited 100%. Fig. 4 shows the effects of various levels of fluoride on glucose oxidation.

These data indicate that the ED and the phosphoketolase pathways are unlikely routes for glucose oxidation in B. popilliae since they should both be insensitive to iodoacetate and low level of fluoride. Both the EMP and the HMP systems would be greatly affected by both of these inhibitors.

Distribution of isotope from glucose-1-C^{14} and -6-C^{14}: Equal amounts of glucose-1-C^{14} and -6-C^{14} were catabolized by B. popilliae resting cells and CO_2 was collected for radioactivity counting. Relatively little label from the 6th carbon was obtained. However, when the incubation period was extended, increasing amounts of CO_2 appearing from carbon 6 were observed. Lowering the pH to about 6.0 essentially stopped the formation of all $C^{14}O_2$ from glucose

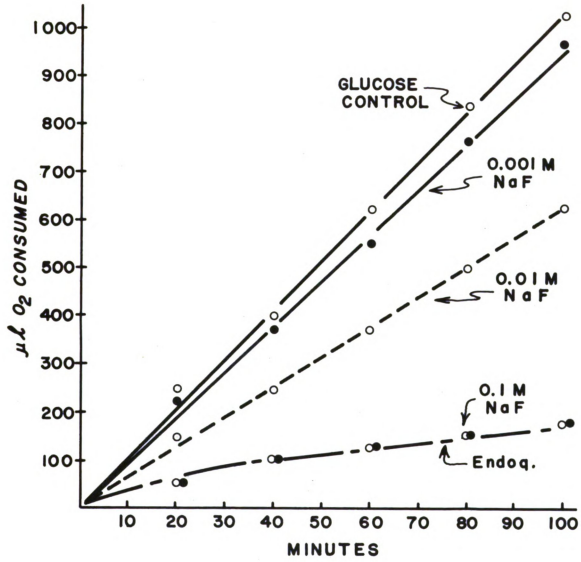


Figure 4. Effect of fluoride upon glucose oxidation. Reaction vessels contained 32.25 mg dry wt. of cells; 0.5 ml of 0.3 M phosphate buffer, pH 7.0; 0.5 ml of 0.0667M glucose; and fluoride as indicated. One side arm held 0.2 ml of 2 M H_2SO_4 and the center well contained 0.2 ml of 20% KOH. Water was added to 3.0 ml. The gas phase was air.

6-C^{14}. A significant amount of label appeared in the CO_2 from glucose-1-C^{14}, and the relative quantity of CO_2 from the first carbon remained the same during extended incubation.

The method of Wang et al. (1958) was used to estimate glucose participation in phosphogluconate decarboxylation. Calculations for this participation were made according to the following formula:

$$G_p = \frac{G_1 - G_6}{G_t}$$

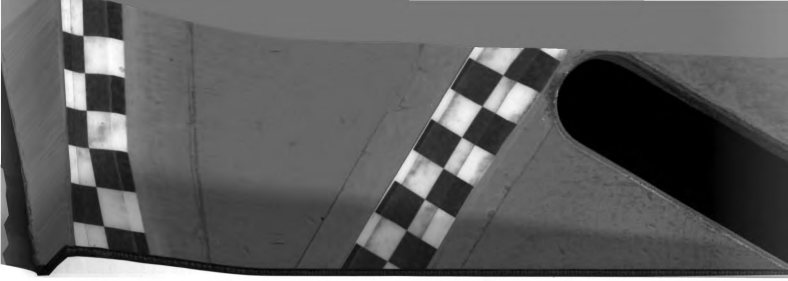
where

G_p = the fraction of glucose participating in the phosphogluconate decarboxylation

G_1 and G_6 = total activity of respiratory CO_2 utilizing equal amounts of C_1 and C_6 labeled glucose

G_t = total activity of administered substrate

The participation of phosphogluconate decarboxylation was quite small (2.1%) when the vessel contained 60 mg (dry wt) of cells and an atmosphere of air. However, this could be altered by varying the cell concentration and/or the atmosphere. The effect of the atmosphere upon the participation of the HMP enzymes in oxidizing glucose was determined by allowing cells (48 mg dry wt per vessel) to oxidize glucose in the presence of air and pure oxygen. The percent participation was 9.05% in air and 39.05% in oxygen.



Thus, the availability of oxygen obviously controls the pathway used to a great degree.

Enzymes of catabolic pathways: A key enzyme in HMP system is the G-6-P dehydrogenase. The presence of this enzyme was established as evidenced by the reduction of TPN with G-6-P as substrate (Fig. 5). The soluble nature of the enzyme was demonstrated when an extract was separated into its soluble and particulate fractions by ultracentrifugation (110,000 x g for 3 hr). The activity was concentrated in the supernatant.

The possibility of direct oxidation of glucose to gluconate was investigated by manometric tests for both glucose oxidase and glucose dehydrogenase as described in the section on methods. Negative results were obtained for each. Evidently, glucose has to be phosphorylated by this organism before it can be further oxidized.

Since G-6-P oxidation can lead to one of three pathways, the possibility of each was examined. The presence of the ED pathway was tested by assaying for one of its key enzymes, KDPG aldolase. No increase in DPNH oxidation occurred beyond that of DPNH oxidase alone. Likewise, the extract was examined for the presence of phosphoketolase and this was also found missing. However, when R-5-P

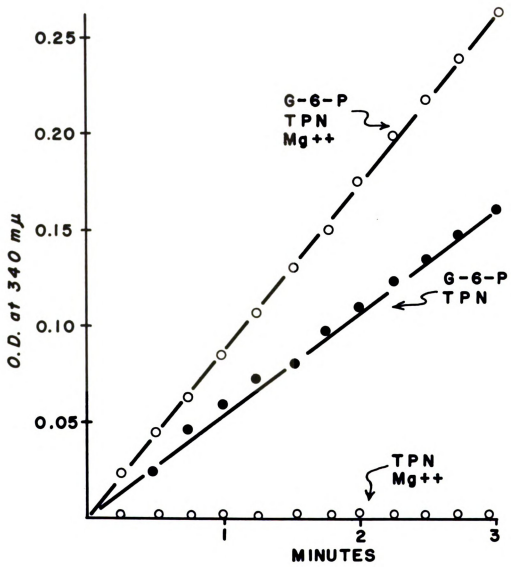
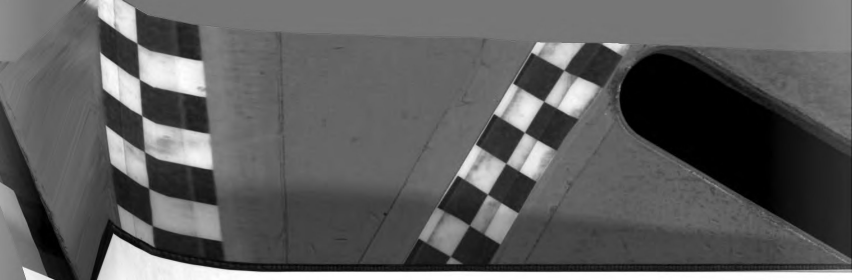


Figure 5. Glucose-6-phosphate dehydrogenase activity in cell free extracts. All vessels contained dialized extract, 1.0 ml of 0.04 M glycyl glycine buffer, pH 7.4. Glucose-6-phosphate, 2 μmoles; TPN, 0.9 μmole; and $MgCl_2$, 20 μmoles were added as indicated. Water was added to 3.0 ml.



was used as the substrate in the presence of the proper co-factors, TPN was reduced to TPNH indicating the presence of the HMP enzymes (phosphoriboisomerase, phosphoketopentoepimerase, transketolase, and transaldolase) and of phosphohexoisomerase.

Efforts to demonstrate the presence of the upper half of the EMP pathway were made by assaying for each enzyme. The presence of aldolase was demonstrated by the colorimetric procedure of Sibley and Lehninger (1949). By omitting the hydrazine until after the incubation period, a more intense reaction was obtained, thus demonstrating the presence of triosephosphate isomerase. With the substitution of F-6-P and ATP for F-1,6-P, phosphofructokinase was shown to be present in the extract. The presence of phosphohexoisomerase has already been referred to in the establishment of the HMP system. No attempts were made to assay for the enzymes of the lower half of the EMP system since they are required for both this system and the HMP, and all of the data indicate the participation of both pathways.

Terminal Oxidation of Acetate

Initial attempts to oxidize acetate with resting cells of B. popilliae failed. Later, it was found that an alkaline pH was necessary rather than an acid one. Furthermore, cells harvested during the logarithmic phase of growth

oxidized acetate very poorly whereas cells harvested in the stationary phase oxidized acetate more rapidly. Incorporation of acetate in the growth medium failed to alter this characteristic of log phase cells. Fig. 6 shows the difference in acetate oxidation by similar concentrations of cells harvested after 9 hr and 24 hr incubation.

During the course of investigating acetate oxidation by B. popilliae (designated as the "A" strain), another culture of this strain (designated as "D") was obtained from the Northern Utilization Research Division, U.S.D.A., Peoria, Illinois. The "D" strain was found not to have the ability to oxidize acetate, at least under the same conditions as the "A" strain; i.e., at an alkaline pH using stationary phase harvested cells. Both of these strains oxidized glucose in the Warburg respirometer (Fig. 7) with two obvious differences; viz., strain "A" had a Q_{O_2} of 13.2 µl O_2 per hr per mg cells and continued to utilize oxygen after the glucose had disappeared, while strain "D" had a Q_{O_2} of 21.5 µl O_2 per hr per mg cells and abruptly stopped consuming oxygen with the exhaustion of the glucose.

Apparently, a strain with the acetate oxidizing characteristic was selected by the method of maintaining the cultures. The "A" strain was maintained on B-4 broth, and transferred every 48 - 72 hr with continuous incubation.

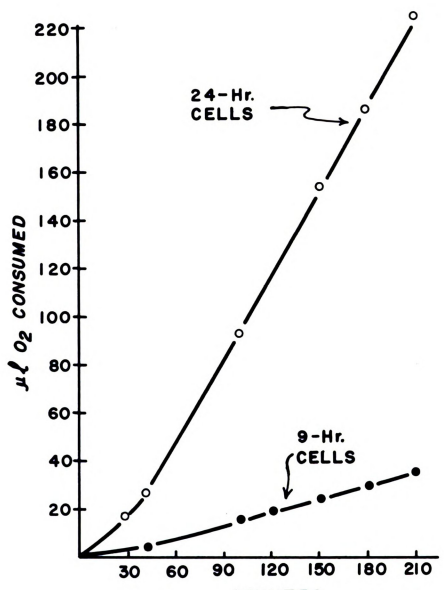


Figure 6. Ability of log phase and stationary phase harvested cells to oxidize acetate. Reaction vessels contained 9 hr harvested cells (35.2 mg dry wt.) or 24 hr harvested cells (32 mg dry wt.); 0.5 ml of 0.3 M phosphate buffer, pH 7.4; 120 μmoles of acetate. 0.2 ml of 2 M H_2SO_4 was contained in a side arm and 0.2 ml of 20% KOH in the center well. Water was added to 3.0 ml. The gas phase was 100% O_2.

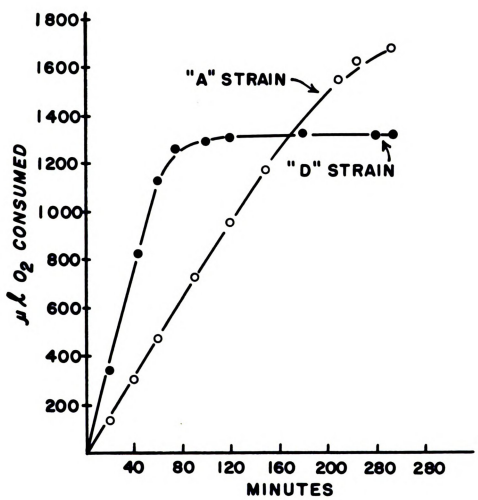


Figure 7. Glucose oxidation characteristics of acetate oxidizing (A) and acetate non-oxidizing (D) varieties of B. popilliae NRRL B-2309P. Reaction vessels contained 38 mg ("A" variety) and 35.3 mg ("D" variety) of cells; 1.1 ml of a 0.3 M phosphate buffer, pH 7.4; and 0.5 ml of 0.0667 M glucose. 0.2 ml of 2 N H_2SO_4 was contained in a side arm and 0.2 ml of 20% KOH in the center well. Water was added to 3.0 ml. The gas phase was 100% O_2.

During most of this time, acetate was present in substantial amounts with the result that any mutant with the ability to oxidize acetate would have a selective advantage. The "D" strain, on the other hand, had been maintained on agar slants. Metabolic products in this situation would more easily diffuse away from the immediate vicinity of the cells. The "A" strain is apparently a mutant since it retained most of the growth characteristic of the original culture and it was checked by the U.S.D.A. for its pathogenicity and was found able to cause the "milky disease" in the Japanese beetle larvae. The "D" strain was not used for any other experiments during these investigations.

To determine the rate of oxidation during growth, acetate-1-C^{14} was incorporated in the B-4 growth medium. The CO_2 was trapped in alkali and periodically examined for radioactivity. No significant amount of label was detected in the CO_2 through the first 12 hr of incubation. Only after the stationary phase had begun was there any accumulation of $C^{14}O_2$ (Table 8). Resting cells of B. popilliae were induced to oxidize glucose primarily to acetate by supplying a pure oxygen atmosphere. Fig. 8 shows the relationship between oxygen consumption, glucose oxidation, and acetate accumulation. The acetate was oxidized after the glucose had disappeared as evidenced by the drop in

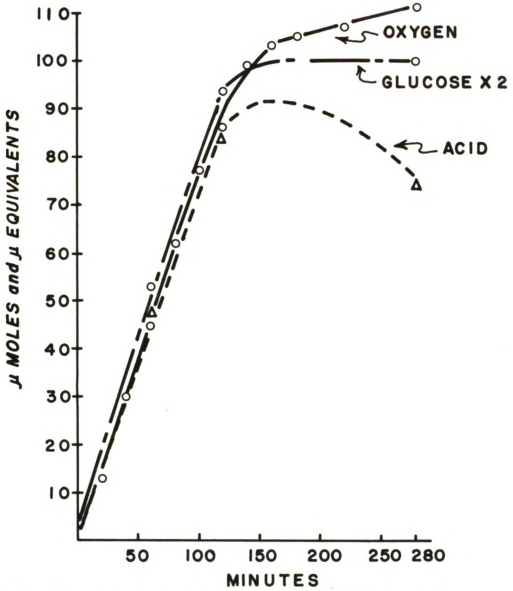


Figure 8. Comparison of glucose oxidation with oxygen consumption and titratable acidity. Reaction vessels contained 46.2 mg dry wt. of cells, 1.0 ml of 0.3 M phosphate buffer, pH 7.1; and 0.5 ml of 0.0667 M glucose. 0.2 ml of 2.0 N H_2SO_4 was contained in side arms except for those vessels which were used to determine titratable acidity. The well contained 0.2 ml of 20% KOH and water was added to 3.0 ml. The atmosphere was 100% O_2.

titratable acidity with a continued oxygen consumption.

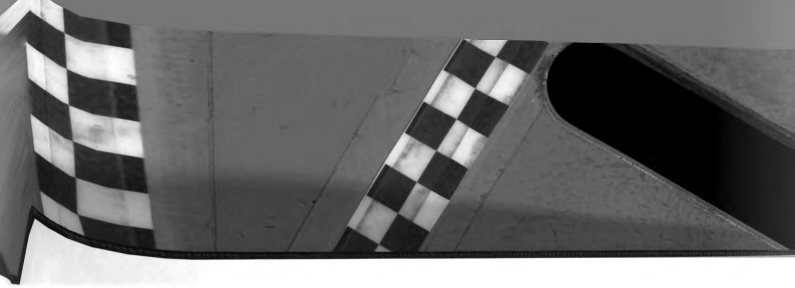
Table 8. Oxidation of acetate-1-C^{14} by a growing culture of B. popilliae.

Incubation Time	% of Label in CO_2
6 hr	0.053
12 "	0.103
24 "	2.25
36 "	8.70
48 "	16.00
120 "	31.00

0.05 µmole of acetic acid 1-C^{14} (0.5 µc) was incorporated into B-4 broth. The flasks were stoppered with rubber plugs and the CO_2 was collected in 40% KOH. Individual flasks were removed periodically to examine the KOH for radioactive label. The percent label contained in the CO_2 was based on the total amount initially present in the medium.

When glucose was oxidized at pH 6.0, oxygen consumption ceased at the time when glucose was depleted. Likewise, no significant amount of $C^{14}O_2$ appeared from glucose-6-C^{14}. This is further evidence of the lack of terminal oxidation of the accumulated acetate at this pH.

A number of observations were made which strongly indicate the presence of an operative TCA cycle in B. popilliae. First, during the oxidation of acetate, oxygen and CO_2 were liberated in a 1:1 ratio which is the theoretical relationship for the TCA cycle. Arsenite (0.01 M) inhibited oxidation of acetate 100%. Since this inhibitor blocks the



decarboxylation of α-keto acids, the point of inhibition is most likely the decarboxylation of α-ketoglutarate. The complete inhibition is evidence against a glyoxylate shunt. Finally, three TCA intermediates were oxidized by whole cells. The three, succinate, malate, and fumarate were oxidized at both pH 6.1 and 7.6. However, most rapid oxidation took place at pH 6.1, probably because the acids are less dissociated at this pH; and, thus, the cell is more permeable to them. Fig. 9 shows the oxidation of these three acids compared with the oxidation of acetate at both pH values. It will be seen that only acetate responded more favorably in the alkaline range.

More direct evidence for the TCA cycle was obtained when enzymes linking acetate to the production of citrate were demonstrated. Using the method of Rose (1955), acetokinase was demonstrated in a cell free extract of B. popilliae. In the presence of this enzyme, acetyl phosphate is formed from acetate and ATP, and this is converted into its hydroxamic acid by reacting with neutral hydroxylamine and the color is developed by complexing with $FeCl_3$. The development of color, read at 540 mμ, was directly proportional to enzyme concentration (Table 9).

Evidence for both phosphotransacetylase and the condensing enzyme was obtained by measuring the disappearance

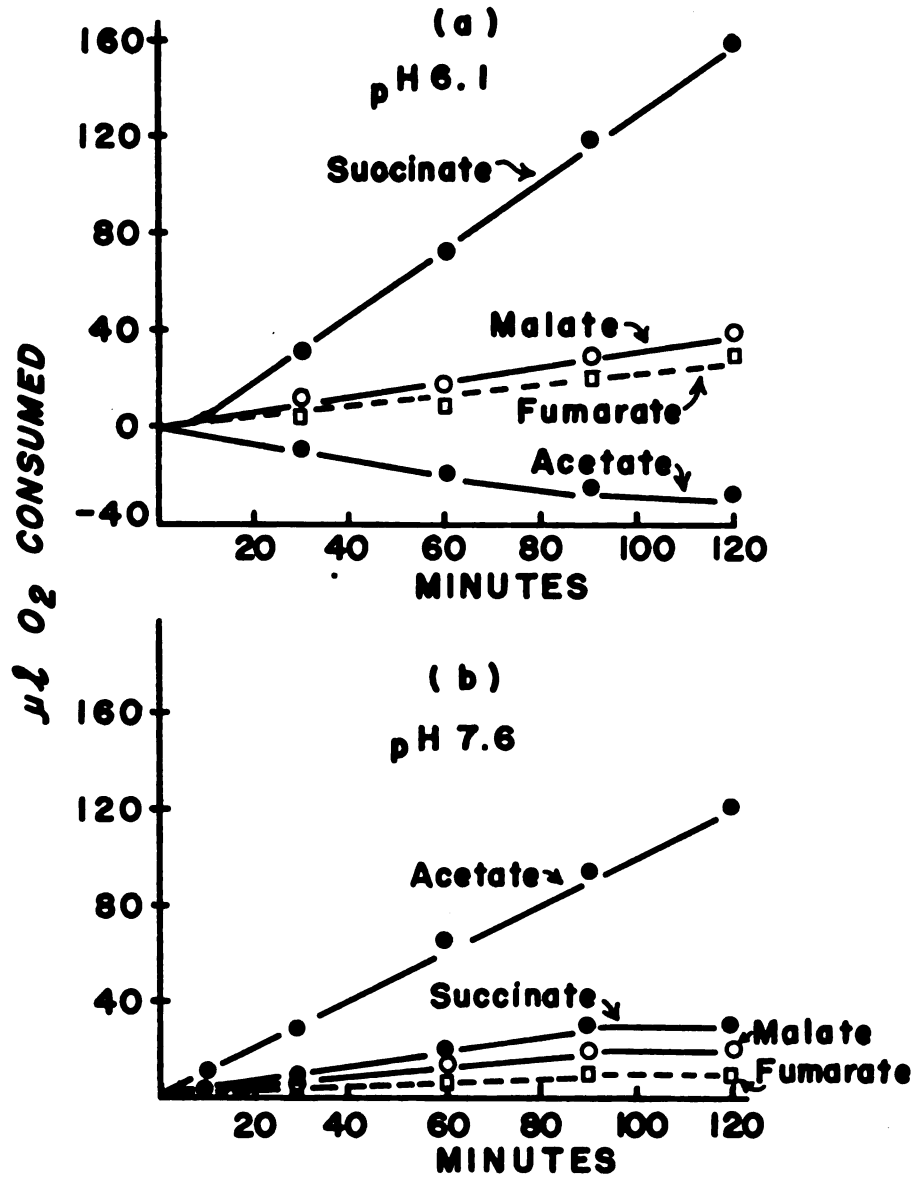
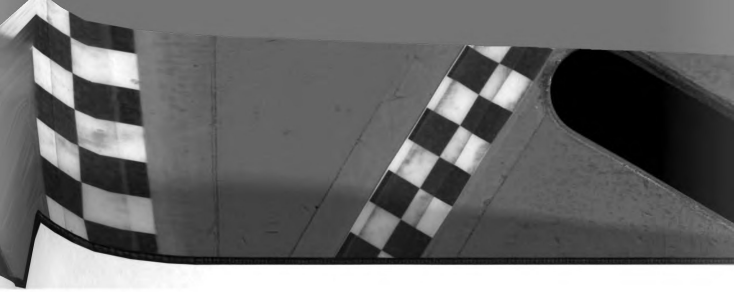


Figure 9. Effect of pH upon the oxidation of acetate, succinate, fumarate, and malate by resting cells of B. popilliae. Reaction vessels contained 41.4 mg dry wt. of cells (48 hr harvest), 0.5 ml of 0.2 M phosphate buffer (final pH shown above); and 120 μmoles of substrate. 0.2 ml of 2 M H_2SO_4 was contained in the side arm and 0.2 ml of 20% KOH in the center well. Water was added to 3.0 ml. The atmosphere was 100% O_2.



of acetyl phosphate in the presence of the extract, CoA, and oxalacetate (Table 10). Where either CoA or oxalacetate were omitted, the level of acetyl phosphate remained essentially the same. The reaction was proportional to the concentration of the extract. Assaying for acetyl phosphate was performed by the method of Lipman and Tuttle (1945).

Table 9. Acetokinase activity in extracts of B. popilliae.

Extract Concentration	OD at 540 mμ
None (control)	0
3 mg protein	0.34
6 mg protein	0.70

The reaction mixture contained 0.3 ml of stock substrate (3.2 M potassium acetate; 1.0 M tris buffer, pH 7.4; and 1.0 M $MgCl_2$ in a volume ratio of 25:5:1), 0.35 ml of neutral hydroxylamine, 0.1 ml 0.1 M ATP, and water to 1.0 ml. The mixture was incubated at 29 C for 2 min in a water bath and stopped by the addition of trichloroacetic acid. Color was developed by the addition of 4.0 ml of $FeCl_3$ (5% in 0.1 N HCl).

Electron Transport System

Methods used to characterize the electron transport system involved enzyme assays, inhibitors, and direct spectrophotometric absorption patterns. Initially, assays were made for oxidases of reduced pyridine nucleotides.

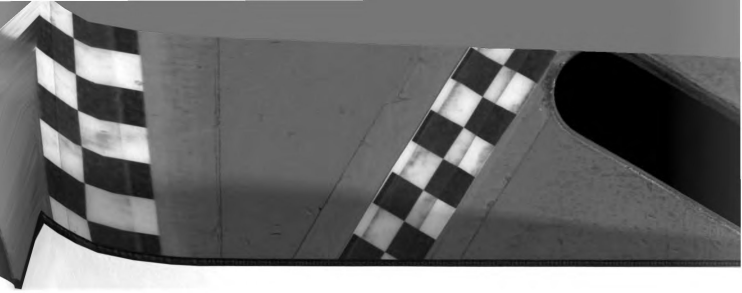


Table 10. Phosphotransacetylase and condensing enzyme activities in a B. popilliae extract.

Extract Concentration	OD at 540 mμ
28 mg protein, no oxalacetate	0.54
28 mg protein, no CoA	0.58
14 mg protein	0.43
28 mg protein	0.35

The reaction mixture contained 25 μmole phosphate buffer, pH 7.4; 10 μmole of cysteine; 20 μmole oxalacetate; 10 μmole acetyl phosphate, and extract. Water was added to 10 ml. Omissions were made as indicated. After incubation at 25 C for 10 min, trichloroacetic acid was added and acetyl phosphate was determined by the method of Lipmann and Tuttle (1945).

Examinations for a DPNH oxidase, TPNH oxidase, and a TPNH-DPN transfer enzyme were made. An active DPNH oxidase was readily demonstrated but neither of the latter two was found. DPNH was oxidized by both the particulate and soluble fractions of the cell extracts. The activity was highest in the particulate fraction (Fig. 10).

Assays for the presence of catalase were made by dropping 3% H_2O_2 on agar colonies, tipping hydrogen peroxide into Warburg vessels containing cell extract, and by iodometric titration. No bubbles appeared when H_2O_2 was dropped on B. popilliae colonies nor was there any oxygen release measured when 0.5 ml of 1.5% H_2O_2 was tipped into a Warburg vessel containing cell extract. Neither was there any significant evidence of catalase as

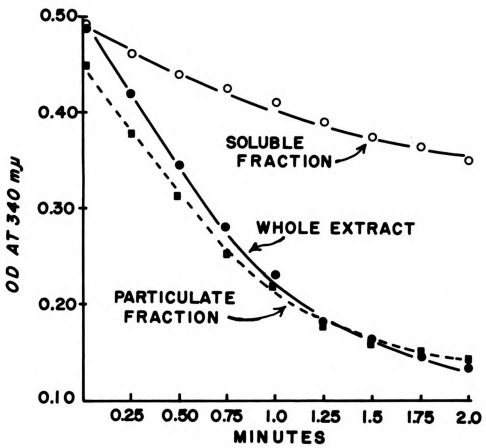


Figure 10. DPNH oxidase activity in whole cell free extracts and in the soluble and particulate fractions. The following extract concentrations were used: whole extract, 0.5 mg protein; soluble fraction, 1.6 mg protein; and particulate (110,000 x g) fraction, 0.35 mg protein. Cuvettes also contained 0.5 μmole DPNH and 0.5 ml of 0.2 M phosphate buffer, pH 7.0. Water was added to 3.0 ml.

determined by iodometric titration (Table 11).

Table 11. Assay for catalase in a B. popilliae extract.

Minutes contact between H_2O_2 and cell extract	ml 0.0172 N thiosulfate to titrate
0.00	0.76
0.25	0.74
0.50	0.74
0.75	0.74
1.00	0.72

The reaction mixture contained 5.0 ml of H_2O_2, in 0.01 M phosphate buffer, pH 6.8, and 1.0 ml of extract. The reaction was run at 25 C and stopped with 2.0 ml of 1.0 N H_2SO_4. The H_2O_2 was determined by adding 0.5 ml of 10% KI and one drop of 1% ammonium molybdate and titrating with sodium thiosulfate.

Since catalase was apparently missing, it was particularly important to determine if hydrogen peroxide was produced. DPNH was oxidized by the whole extract as well as by the particulate and soluble fractions in the Warburg respirometer; and the production of H_2O_2 determined by oxygen release upon the addition of catalase. It will be seen (Fig. 11) that H_2O_2 was produced in the presence of the soluble but not the particulate fraction. On tipping in catalase, there was only 2% oxygen return with the whole extract, 36.5% with the soluble fraction, and none with the particulate fraction. If all O_2 consumed was converted into H_2O_2, a 50% return would be theoretical. However,

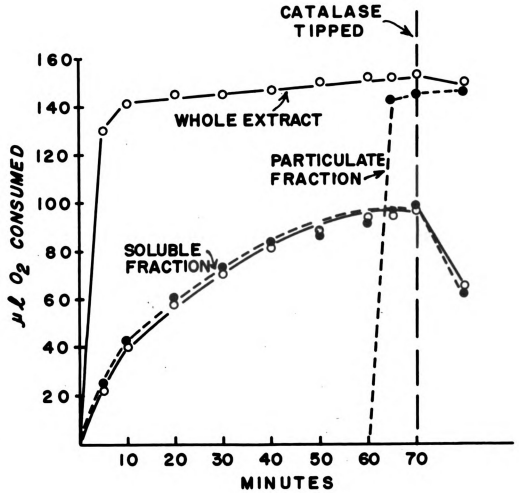


Figure 11. Oxidation of DPNH in the Warburg respirometer by a whole cell free extract and by the soluble and particulate fractions. The following concentrations were used: whole extract, 15 mg protein; soluble fraction, 1.2 mg protein; and particulate (110,000 x g) fraction, 21 mg protein. Reaction vessels also contained 20 μmoles of DPNH and 0.5 ml of 0.2 M phosphate buffer, pH 7.6. A side arm contained 0.5 ml of catalase and the center well 0.2 ml of 20% KOH. Water was added to 3.0 ml. The atmosphere was 100% O_2. No substrate was tipped into particulate fraction until 10 min before catalase was tipped.

there is always a problem with substrate inactivation of catalase; and, thus, the percentage of oxygen going to peroxide cannot be estimated from these data.

With the absence of catalase and the obvious production of hydrogen peroxide, a peroxidase would be of much importance to the cell. The assay described by Dolin (1957) has been used extensively for assaying flavin linked bacterial peroxidases. The activity of the DPNH oxidase is established and a net difference observed when peroxidase is present. Fig. 12 shows that peroxidase activity was present in a Streptococcus faecalis extract but no activity was found in the B. popilliae extract.

Cyanide (0.01 M) and azide (0.01 M) inhibited completely the oxygen consumption by the particulate extract fraction (Fig. 13a). However, where the same inhibitors were used with the soluble fraction, no inhibition of oxygen uptake occurred (Fig. 13b). Upon tipping catalase, about 30% oxygen return was observed with the control and the cyanide containing flask. No return occurred in the azide flask. However, azide may have inactivated the supplied catalase.

Extract particles were treated with carbon monoxide and were compared with non-treated particles for their ability to oxidize DPNH. The carbon monoxide inhibited this

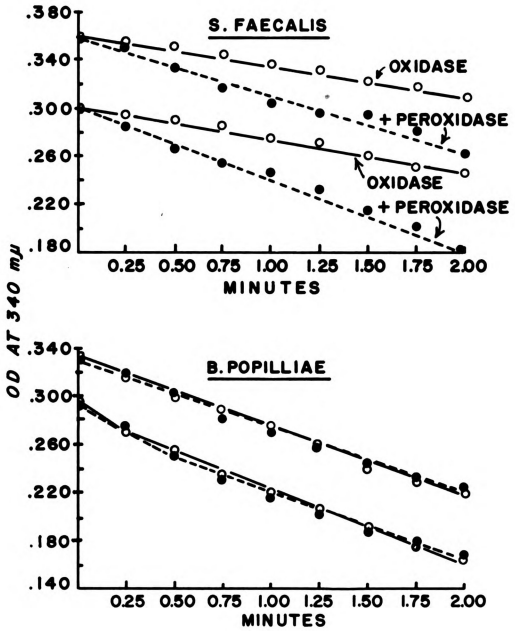


Figure 12. Peroxidase activity in extracts of Streptococcus faecalis and B. popilliae. To assay for DPNH oxidase, cuvettes contained 1.0 ml of 0.1 M acetate buffer, pH 5.4, 0.5 μM DPNH, and 0.5 mg protein. To assay for peroxidase, 4 μmoles of hydrogen peroxide were present in addition. Water was added to 3 ml.

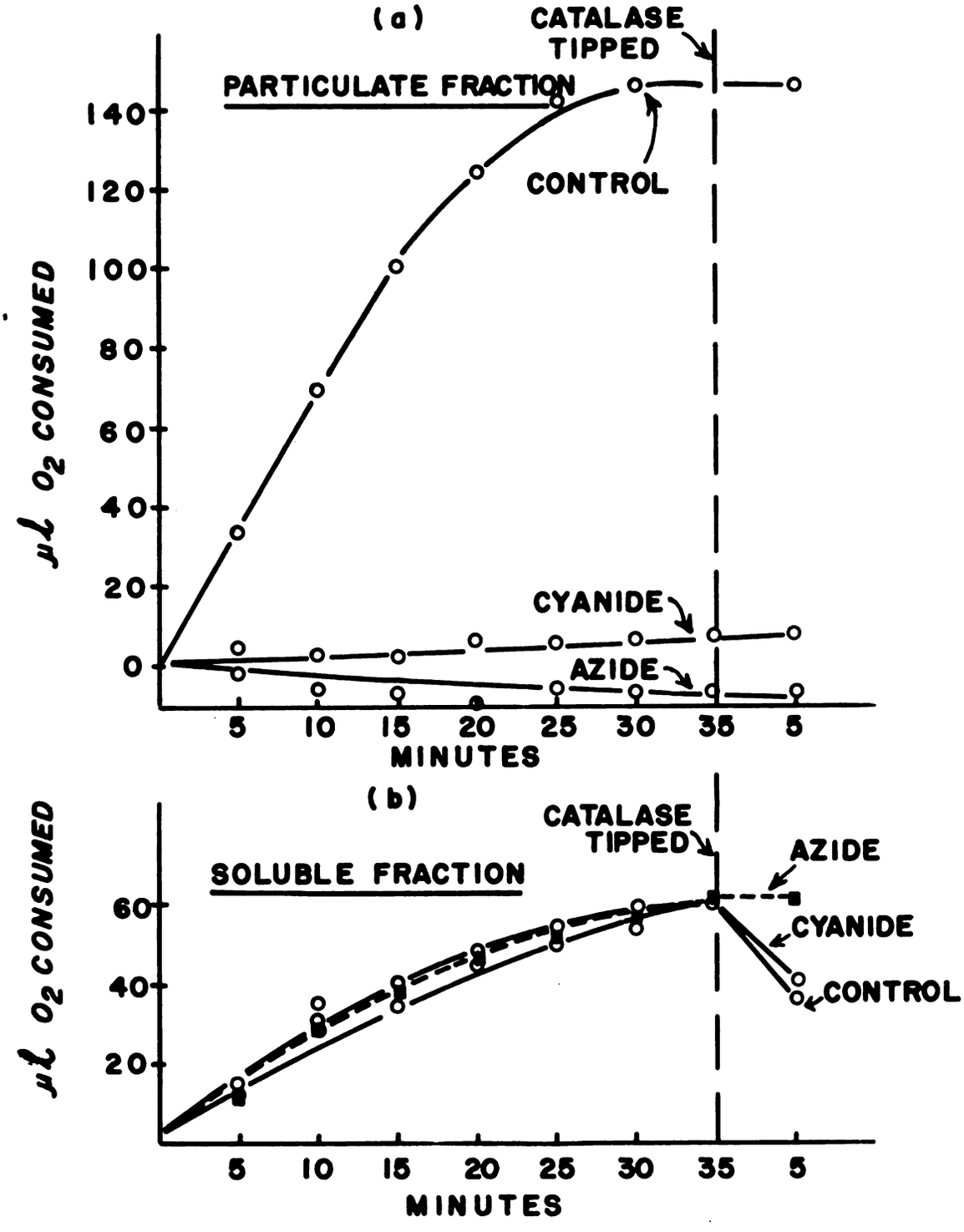
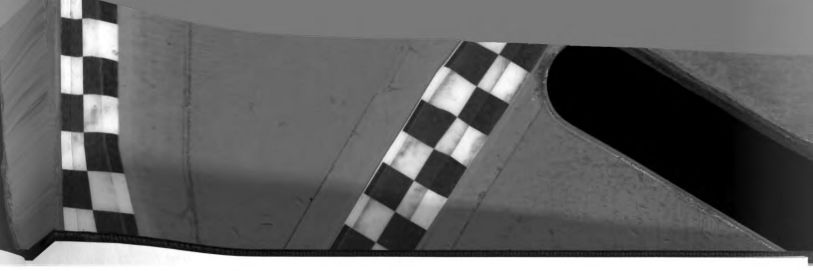


Figure 13. Effect of cyanide and azide on DPNH oxidation by soluble and particulate fractions of a cell free extract as measured by oxygen consumption. Reaction vessels contained extract protein (soluble, 1.2 mg; particulate 4.2 mg), 20 μmoles of DPNH and 0.5 ml of 0.2 M phosphate buffer, pH 7.6. A side arm contained catalase and the center well 0.2 ml of 20% KOH. Water was added to 3.0 ml. The atmosphere was 100% O_2. Cyanide (30 μmoles) and azide (30 μmoles) were added as shown above.



reaction nearly 100% (Fig. 14).

A difference spectrum was obtained with the Cary Model 15 Spectrophotometer by the split beam method. One cuvette contained particulate extract reduced with sodium hydrosulfite and the other cuvette contained oxidized extract. The results (Fig. 15) give positive evidence for the presence of cytochromes. The peaks at 562 mμ and 530 mμ correspond to the α and β peaks of cytochrome b; the peaks at 602 mμ and at 426 mμ are probably due to cytochrome a_1. The trough at 452 to 462 mμ is likely indicative of flavoprotein. The 555 mμ α peak for cytochrome c is missing. However, cytochrome components differ between organisms and even within an organism depending upon cultural conditions and age. It is important, however, to note positive evidence for this mode of electron transport.

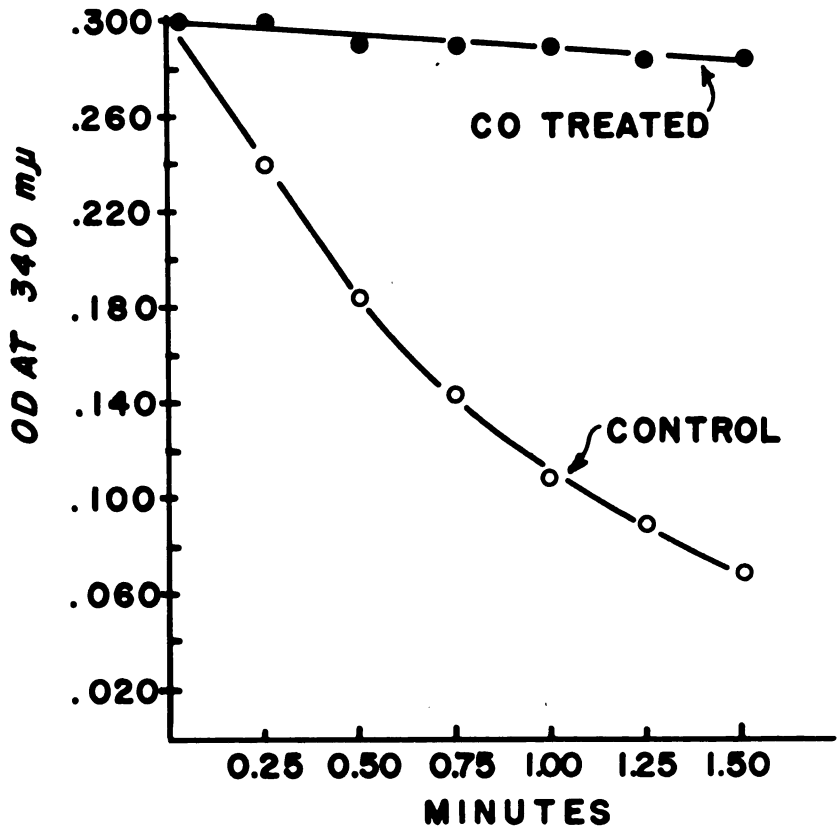


Figure 14. Effect of carbon monoxide upon DPNH oxidation by the particulate fraction of a cell extract. Cuvettes contained 0.5 μmole DPNH, 0.7 mg particulate extract protein, 0.5 ml of 0.2 M phosphate buffer, pH 7.0 and water to 3.0 ml. CO was bubbled through the treated reaction mixture for 1 min before adding substrate.

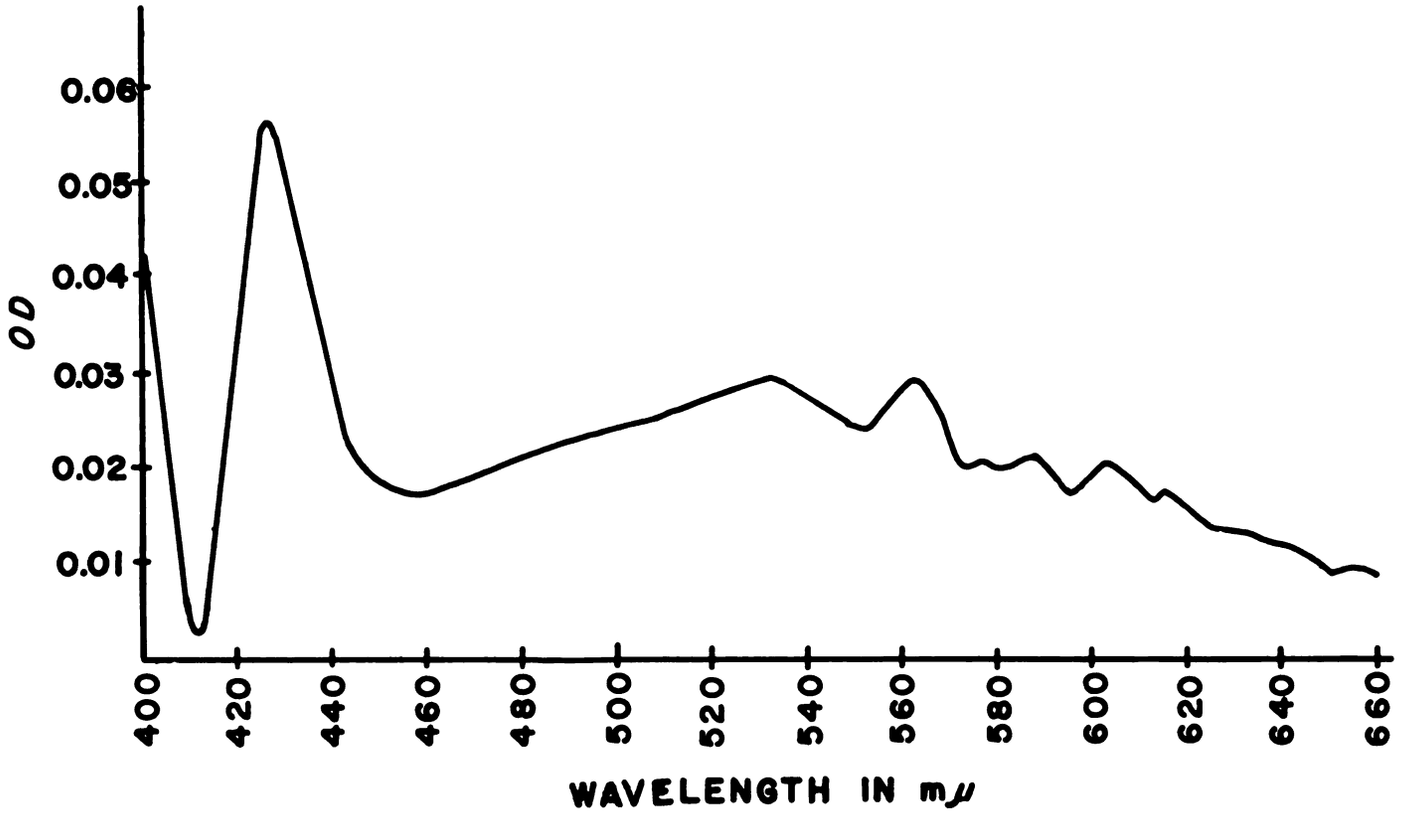
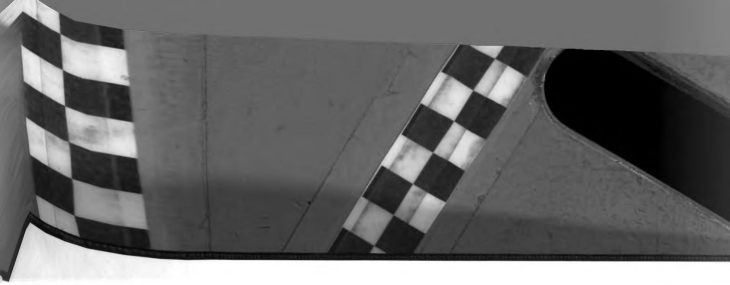


Figure 15. Difference spectrum of an oxidized vs. reduced particulate fraction of a cell extract. Cytochromes were reduced with $Na_2S_2O_4$ and its spectrum measured against the oxidized extract with the Cary Model 15 Spectrophotometer.



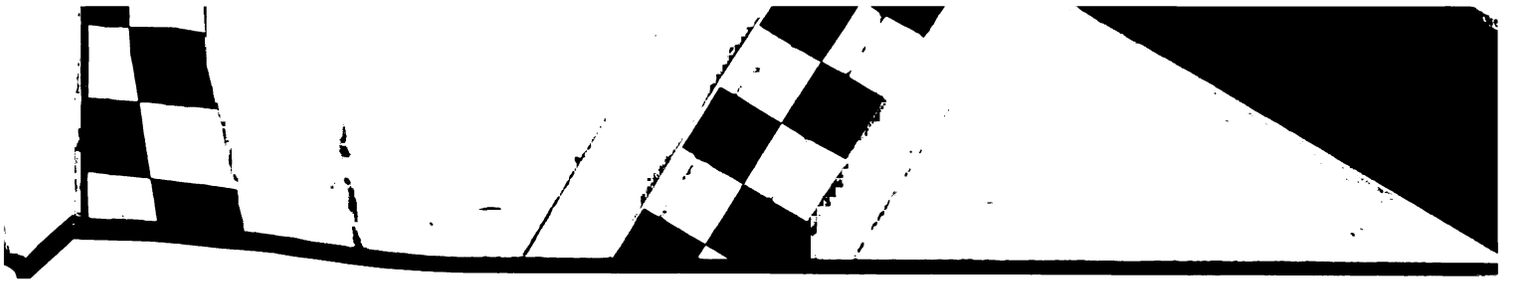
DISCUSSION

B. popilliae catabolizes glucose to pyruvate via the EMP and HMP routes. The evidence for this, provided by enzyme assays, isotopic studies, and inhibitor data, was in complete agreement. Thus, the pattern is the same as wi most aerobic species of bacteria studied to date. Wang et al. (1958), using isotopic techniques studied pathway participation by several aerobic and facultative microbial species. Of the nine species studied, seven catabolized glucose via both the EMP and HMP routes, one by the HMP and ED routes, and one by the ED route only. The HMP route was found to be used primarily during growth, and is undoubtedly useful in the production of TPNH for synthesis of lipids, etc. The extent of the participation of the HMP route is influenced in B. popilliae by the atmosphere provided. Increased oxygenation increases the percent participation. It is undoubtedly very active in shake cultures since it was shown that these conditions resulted in a purely respiration type of metabolism.

Although B. popilliae contains the essential enzyme for dissimilating glucose gy glycolysis, glucose is not fermented in the absence of air by resting cell suspensions

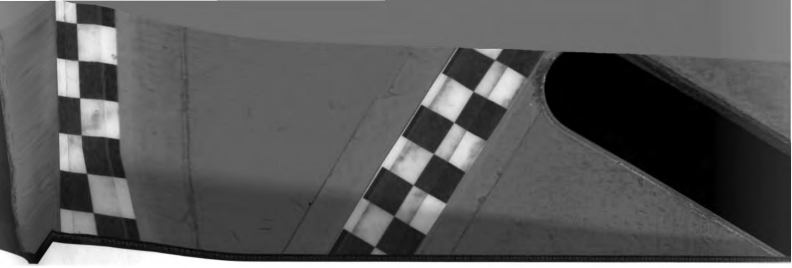
A small amount of oxygen is an absolute essential requirement for glucose to be dissimilated. Apparently some need is being supplied by the presence of oxygen that otherwise is not being met. It is possible that sufficient ATP is not generated by glycolysis and the presence of oxygen may provide enough through oxidative phosphorylation for the continued movement of glucose through glycolytic system. The organism may contain an active ATPase which is responsible for such a limitation.

Oxygen affects the type of acid produced by this organism. With increased oxygen, acetate is produced almost exclusively whereas with minimal oxygen present, lactate is preferentially produced. Although the lactate producing potential is present in an oxygen atmosphere, apparently oxygen is the preferential hydrogen acceptor over possible organic acceptors such as pyruvate. This is not an unusual phenomenon. Neish et al. (1945) found that the presence of air increased acetate and depressed lactate when comparing aerobic and anaerobic dissimilation of glucose by B. subtilis. Similarly, Ehrlich and Segel (1959) found that increased aeration depressed lactate production. Gary and Bard (1952) also found corresponding relationships between the type of acid produced and the presence of oxygen in the atmosphere when working with B. subtilis.



The B. popilliae strain used in this study was found to have the ability to oxidize acetate whereas a more recently acquired culture of the same strain was found not to have this ability. Both, however, were able to cause the "milky disease" of the Japanese beetle larvae and had similar cultural characteristics. The acetate oxidizing strain was maintained in liquid culture where acetate accumulates and thus an acetate oxidizing strain would have a selective advantage. It would be of considerable interest to study the mutation rate of this organism since it produces H_2O_2 and apparently lacks an enzyme to break it down. This is a known mutagen and the organism may be more susceptable to mutations than most.

Nakata and Halvorson (1960) reported that B. cereus accumulates acetate and pyruvate during the log phase of growth, dropping the pH of the growth medium to about 4.9. It apparently does not have the constitutive enzyme complement in vegetative cells at this stage to oxidize the acetate. However, when the growth phase is completed, acetate is oxidized and the pH rises. These workers found that the oxidation of acetate during this phase not only takes place simultaneously with sporulation, but that the presence of acetate is apparently necessary for this cellular change. The route for acetate oxidation was described by Hanson, Srinvasan, and

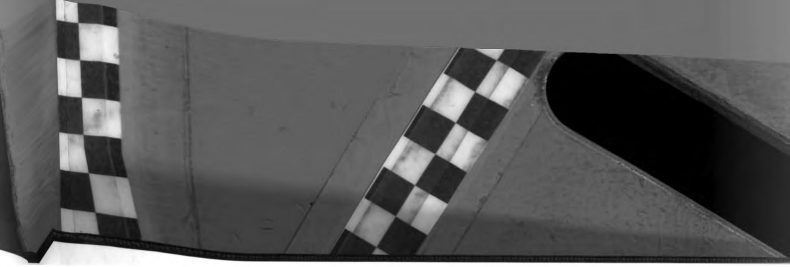


Halvorson (1962) as through acetate kinase and transacetylase.

B. popilliae sporulates readily in the Japanese beetle larva but very little success has been achieved in vitro. The oxidation of acetate appears to proceed by the same route as that used by B. cereus, i.e., via acetokinase and phosphotransacetylase. However, B. cereus oxidized acetate at pH 4.9 and B. popilliae will perform this operation only near neutrality or above. This seems to indicate that an active transport for acetate is involved since acetate is largely in the undissociated state at neutral pH levels, and thus, could not be likely to enter the cell by diffusion. This is in contrast with the oxidation of some TCA intermediates which were oxidized by whole cells best in the acid pH range, probably because these acids enter the cell by diffusion. Recent work by Costilow (1963) indicates that acetate oxidation in B. popilliae is related to sporulation.

Of course, it is possible that the cell is so permeable to acetate at low pH levels that the acid accumulates in the cell to a high extent. This could cause a pH change in the cytoplasm and result in an inhibition of the oxidizing enzymes.

There may be several reasons as to why growth and viability are limited in B. popilliae. The accumulation of



hydrogen peroxide and acids may be in part or wholly responsible for this. Although the cell has the potential to produce hydrogen peroxide, it produces no catalase or peroxidase. Consequently, this harmful product can accumulate in this organism whereas most other aerobes and facultative aerobes have mechanisms for its breakdown. Dutky (1963) has reported that he has achieved high populations of cells of this organism which retain their viability over a long period by culturing in deep broth cultures with added riboflavin. Here, where there is a low oxygen level, another final electron acceptor may be utilized circumventing the requirement for oxygen and eliminating the production of hydrogen peroxide. This may possibly simulate conditions present in the larvae. Certainly, studies should be conducted utilizing other potential electron acceptors.

The accumulation of acids may be detrimental to the cell. It is well known that acetate is more harmful to most bacteria than is lactate. As the pH is lowered, most cells are more permeable to undissociated acids and more harm is effected. Acetate obviously gets into the cells at alkaline pH values since it is oxidized by at least the one strain; and, as mentioned above, may be more readily taken up in acid pH ranges. Therefore, mere neutralization may not result in much retention of viability. Data were

presented which indicated that this indeed was the case. Little acetate is produced when oxygen is greatly limited. This might explain the prolonged viability observed by Dutky (1963) in deep broth cultures.

The apparent need of acetate for sporulation and its possible detrimental effect on the cell is an apparent paradox. It is possible that the larvae may supply acetate oxidation products to the bacterium which may be as active as acetate in stimulating and supplying nutrients necessary for sporulation; or the larvae may control the levels of acetate and the pH at just the proper level for sporulation to occur. Such an approach may well be a fruitful one *in vitro*.

SUMMARY

Initial studies with B. popilliae showed that glucose is catabolized primarily to acid intermediates. Because of the rapid pH drop in growing cultures, tests were performed to determine whether neutralization of acids with sterile NaOH would increase cell yields and improve viability. Shaking cultures at various speeds also provided a series of oxygen levels. Results showed that neutralizing acids and maintaining an OAR of 1.0 increased cell yields about 100% but no significant increase in viability was achieved.

Carbon balances demonstrated lactic acid, acetic acid, and CO_2 to be the major metabolic products from glucose while glycerol, ethanol, acetoin, and acetaldehyde comprised the minor products. Lactate to acetate ratios were controlled by adjusting the oxygen levels; lactate predominated while oxygen was limited and acetate when oxygen was in excess. The total acid production was unaffected by oxygen level changes, however. Glucose was not catabolized in the absence of oxygen while pyruvate was dissimilated both aerobically and anaerobically.

The action of inhibitors, radioactive isotope studies, and enzyme assays gave ample evidence for the catabolism of glucose via the EMP and HMP routes. The percent participation of the HMP system is very dependent upon oxygen availability. Approximately 40% of the glucose was catabolized by this route in an atmosphere of pure O_2; while only 2% participation of the HMP was observed in air with high cell concentrations.

Terminal oxidation of glucose and acetate oxidation takes place at neutral pH levels or above. Enzymes connecting acetate with the TCA cycle were shown to be operative in cell extracts. This evidence combined with the oxidation of succinate, malate, and fumarate by resting cells indicated participation in the TCA cycle.

Inhibition of the particulate extract fraction by azide, cyanide, and carbon monoxide as well as spectrophotometric data demonstrated electron transport through the cytochrome system. Hydrogen peroxide production was demonstrated but no catalase or peroxidase was found. It is believed that hydrogen peroxide production may be an important factor in limiting the in vitro growth of B. popilliae; and, also, in destroying the viability of vegetative cells.

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