STUDIES ON THE MICROBIAL PRODUCTION OF ACRYLIC ACID

by

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M.Sc., University of Bombay
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ABSTRACT

In response to present and future fossil resource depletion, chemicals production from an alternate raw materials base must be considered. The biologically catalysed degradation of photosynthetically stored energy, such as starch and ligno-cellulosics, is an important approach for chemicals production.

The objective of this study is to increase the productivity of acrylate from propionate, using resting cell suspensions of the obligate anaerobe Clostridium propionicum to catalyse the bioconversion, and to produce acrylate from propionate using metabolic mutants of the facultative aerobe Escherichia coli W. These two organisms were chosen from amongst many hypothesized to possess acrylyl-CoA as a metabolic intermediate.

Clostridium propionicum is a unique anaerobe fermenting L- and B-alanine to propionate, acetate and carbon dioxide by a direct reductive pathway in which acrylyl-CoA occurs as a terminal electron acceptor.

Preliminary studies conducted to standardize the bioconversion system revealed that cells harvested in late exponential phase of growth were optimally active in the capability to produce acrylate. In experiments designed to determine the factors affecting the duration of acrylate production, the effect of product inhibition, oxygen and alternate electron accepting dye toxicity were examined. 34 mmol/l acrylate was found to totally inhibit the synthesis of acrylate from 200 mmol/l propionate in the presence of air. Under anoxic conditions, the acrylate uptake rate showed first order reaction kinetics; 250 mmol/l acrylate is consumed at an initial specific rate of 4.05 mol/g wet weight cells-h and was found not to inhibit further acrylate uptake or production from propionate.
Suspending the cells anoxically for 3 h in 12 mmol/l methylene blue rendered them incapable of producing acrylate from propionate. Similar loss of acrylate production capability was found to occur following less than 180 min exposure of cells to air.

As a result of these studies, it was proposed that to achieve longer acrylate production ability, the bioconversion be conducted anoxically with constant regeneration of the reduced dye. Preliminary results show this to be feasible. Greater life of acrylate production capability is achieved at the expense of the reaction rate.

Anaerobic metabolism of lactate or acrylate prior to bioconversion, enhances acrylate productivity. The enhancement increases with the concentration of lactate or acrylate added, the duration of preincubation and is maximal with cells harvested in the mid-exponential or early stationary growth phases.

Using the bioconversion system devised for acrylate production, 20 mmol/l crotonate was produced from 500 mmol/l butyrate. Similarly, 6 mmol/l methacrylate was produced from 500 mmol/l isobutyrate.

Finally, the experimental strategy for isolating an acrylate producing mutant of Escherichia coli W was attempted. E. coli metabolizes propionate to lactate via acrylyl-CoA. Five mutants capable of growing on lactate, but not on propionate were isolated. However, no acrylate accumulation was observed by attempting bioconversion of propionate by resting cell suspensions of these mutants.

Of the numerous soil isolates, the yeast is especially interesting for it grows aerobically on up to 8 g/l acrylate.

Thesis Supervisor: Dr. Charles L. Cooney
Associate Professor of Biochemical Engineering
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And, finally, thank you Dan G. for helping me put the thesis together and thank you Ruth A., the product of whose unflagging effort is every typed page of this thesis.

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I. INTRODUCTION

The production of chemicals from photosynthetically stored solar energy resources such as starch and ligno-cellulosics has for many years been the subject of serious effort. Recent events concerning depletion of proven petroleum resources have served to emphasize the potential of alternate technologies for chemicals synthesis, such as the biologically catalysed degradation of the renewable raw materials resource - biomass. In addition, new developments in genetics and biochemistry have made available to the biochemical engineer powerful tools with which to increase the efficiency of existing processes and develop new methods by which to produce chemicals of industrial importance.

The objective of this thesis is to investigate the potential for the microbial production of acrylate from carbohydrate starting materials. The prospective substrates, lactate and propionate, are fermentation products of starch, hemicellulose and cellulose.

Acrylyl-CoA is hypothesized to occur as a metabolic intermediate in several microorganisms, both aerobic and anaerobic, as well as in plant and animal tissue.

Two organisms, the obligate anaerobe Clostridium propionicum and the facultative aerobe, Escherichia coli have been chosen for further study.
In this thesis research, it is sought to increase the productivity of acrylate bioconversion from propionate using Clostridium propionicum. It is anticipated that such experimentation will lead to a better understanding of the microbial processes involved during acrylate synthesis by resting cells, and of anaerobic resting cell bioconversions in general. Further investigation with the resting cell system will be extended to the study of the bioconversion of other 2,3-unsaturated fatty acids from short-chain fatty acids.

Experiments have been designed leading to mutation of the propionate oxidizing aerobe Escherichia coli W and selection of a mutant that might accumulate acrylate from propionate.

The first mutation would render the organism homofermentative, oxidizing propionate to lactate. The second lesion preventing acrylyl-CoA hydration to lactyl-CoA, it is reasoned, might cause acrylate accumulation from propionate.

In the following chapters will be presented the rationale for systems chosen and experimental design. The methodology and results of the experiments will be presented and discussed. From these experiments, it is planned that more information will be made available; contributing to the understanding of the proposed biological process for the manufacture of acrylic acid and of anaerobic resting cell bioconversions in general.
II. LITERATURE REVIEW

A. Chemicals from Biomass

The raw materials base for organic chemicals production is coal, petroleum and natural gas. Utilization of such fossil material constitutes spending the accumulated capital of natural resources. They are non-renewable, finite and unevenly distributed geographically. The unmitigated use of these resources also poses a potential environmental threat. With the future availability of petroleum in the decline (Wilson, 1977) it becomes necessary to examine the potential of turning to globally distributed renewable resources to serve as the raw materials base for chemicals and fuels production (Lipinsky, 1978).

Photosynthetically stored solar energy - biomass, available at a rate of $1.6 \times 10^{11}$ metric tons of dry organic matter per year (corresponding to $1 \times 10^8$ megawatts) is ten times the 1975 global energy requirement (Whittaker and Likens, 1973; Ehrlich et al., 1978) and one of the most promising long term alternatives to petroleum. The commercialization of chemicals production from biomass is predicted to be a viable proposition in the early 21st century (Krieger and Worthy, 1978). An important approach to the eventual solution is the anaerobic, low pressure and temperature, biologically catalysed conversion of biomass components cellulose, hemicellulose and lignin to organic
chemicals that can be used within the existing chemical economy.

Of the numerous petrochemicals used by industry, a large fraction can be produced by microbial fermentation of biomass (Table 1). As a result of new technological approaches and rising petroleum prices, some microbially synthesized chemicals are likely to become competitive with petrochemicals. In addition to increasing the efficiency of existing biologically catalysed processes, it is imperative to discover new methods by which to produce these chemicals.

The development of new microbial systems which can be used to synthesize industrial feedstock chemicals from ligno-cellulosic biomass remains a challenge.

Economically useful fermentations result in the production of useful cell mass or products of microbial metabolism. Microbes produce such chemicals either during the course of normal fermentation or as a result of manipulation of normal metabolism and its regulation. Anaerobic fermentations are particularly suited for such processes, owing to the fact that the anaerobic conversion of substrate to product involves only a small change in free energy when compared to the total free energy change associated with complete aerobic oxidation of the substrate. Most of the energy is conserved in the final product. The cells subsequently metabolize large amounts of the substrate to obtain energy sufficient for growth. Large
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Current Price (US $)</th>
<th>Annual Demand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>0.42/kg</td>
<td>1.27 x 10^9 kg</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.48/kg</td>
<td>9.64 x 10^8 kg</td>
</tr>
<tr>
<td>Butadiene</td>
<td>0.59/kg</td>
<td></td>
</tr>
<tr>
<td>2,3-Butylene glycol</td>
<td>1.12/kg</td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td>0.57/kg</td>
<td>2.9 x 10^8 kg</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>0.7/kg</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.33/liter</td>
<td>5.78 x 10^8 kg</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.1/kg</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>0.36/liter</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>1.01/kg</td>
<td></td>
</tr>
<tr>
<td>Malic Acid</td>
<td>1.36/kg</td>
<td></td>
</tr>
<tr>
<td>Methyl Ethyl Ketone</td>
<td>0.62/kg</td>
<td>2.86 x 10^9 kg</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>0.45/kg</td>
<td></td>
</tr>
</tbody>
</table>

(1) From Chemical Marketing Reporter (1979)
### TABLE 2

PRICES OF ACRYLIC ACID AND SOME DERIVATIVES

<table>
<thead>
<tr>
<th>Chemical</th>
<th>COST (US $) (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylic Acid</td>
<td>0.88/kg</td>
</tr>
<tr>
<td>Methyl Acrylate</td>
<td>0.86/kg</td>
</tr>
<tr>
<td>Ethyl Acrylate</td>
<td>0.75/kg</td>
</tr>
<tr>
<td>Butyl Acrylate</td>
<td>0.88/kg</td>
</tr>
<tr>
<td>Isobutyl Acrylate</td>
<td>0.88/kg</td>
</tr>
<tr>
<td>Methacrylic Acid</td>
<td>1.5 /kg</td>
</tr>
<tr>
<td>Methyl Methacrylate</td>
<td>1.01/kg</td>
</tr>
<tr>
<td>Butyl Methacrylate</td>
<td>1.41/kg</td>
</tr>
<tr>
<td>Crotonic Acid</td>
<td>1.89/kg</td>
</tr>
</tbody>
</table>

(1) Chemical Marketing Reporter, December, 1979
quantities of chemical products are hence accumulated by a relatively small population of growing cells.

Genetic, biochemical and engineering principles have been used for the purpose of increasing volumetric and specific metabolic abilities to the cells (Wang et al., 1979). Specific techniques and their applications are reviewed in another section.

The production of acrylic acid from biomass using these techniques is the objective of the project. The fermentation routes such a process may take are shown in Figure 1.

B. Acrylic Acid

Acrylic acid \( (\text{CH}_2 = \text{CH} - \text{CO}_2\text{H}) \) is an unsaturated three carbon carboxylic acid of considerable industrial importance. The annual production of the monomer and its esters in the U.S. during 1978 reached nearly \( 3.65 \times 10^8 \) kg. It is synthesized wholly from petrochemicals requiring both high temperature and pressure. The common starting materials for its synthesis are acetylene, ethylene, acetic acid and propylene (Figure 2). The principal synthetic sequence employs propylene, which is oxidized and dehydrated to yield acrylic acid (Kirk-Othmer, 1978). Acrylonitrile, a major monomer in modacrylic fibers is also an intermediate of acrylic acid synthesis from ethylene and propylene. Arnaud et al. (1976) and Commeyres et al. (1976) have used Brevibacteria and other bacterial nitrilases to catalyse
Figure 1 - Fermentation Routes to Acrylic Acid from Carbohydrates

Starch → Hexoses → Lactate → Propionate → ACRYLATE

Cellulose → Pentoses
Figure 2- Chemical Synthetic Routes to Acrylic acid, Methacrylic acid, their Esters and Crotonic acid.

ACETYLENE + CARBON MONOXIDE → ACETIC ACID → β-PROPIOLACTONE

ETHYLENE → PROPIONIC ACID

PROPIONIC ACID → ETHYLENE OXIDE → ACRYLONITRILE

ACRYLONITRILE → ACETALDEHYDE → β-HYDROXY PROPIOLACTONE

PROPYLENE → LACTIC ACID → ACRYLONITRILE

ACETONE → β-ALKOXY PROPIONATES

ACROLEIN → ACETONE CYANHYDRIN

ISOBUTYlene → β-HYDROXY PROPIONIC ACID

LACTIC ACID + ACETIC ACID → METHYL ACETOXY PROPIONATE

PROPIONIC ACID + FORMALDEHYDE → ACETALDEHYDE → ACETALDOL → CROTONALDEHYDE → CROTONIC ACID

ACRYLIC ACID & ESTERS

METHACRYLIC ACID & ESTERS

CROTONIC ACID
pH dependent hydrolysis of acrylonitrite and methacrylonitrile forming their amides and free acids.

Methacrylic acid (CH$_2$ = C(CH$_3$) CO$_2$H) is also a valuable monomer. In 1977, 6.8 X 10$^8$ kg of the compound and its esters were synthesized in the U.S. Growth in demand for the chemical is projected at a rate of 6 to 8% per year. Smith et al. (1942), Rehberg et al. (1944) and Filacione and Fisher (1944) demonstrated that methacrylic acid and its esters can be synthesized from fermentatively manufactured lactic and acetic acids. However, methacrylic acid is commercially manufactured entirely from petrochemicals isobutylene, acetone cyano-hydrin, propionic acid and formaldehyde (Kirk-Othmer, 1967).

Crotonic acid (CH$_3$-CH$_2$ = CH - CO$_2$H) is prepared commercially by the oxidation of crotonaldehyde with oxygen or organic solvents such as benzene, acetone, methyl ethyl ketone (Kirk-Othmer, 1978). Crotonaldehyde in turn is synthesized following aldol condensation of acetaldehyde, or oxidation of 1,3 butadiene amongst other methods.

Applications of acrylic acid, methacrylic acid, crotonic acid, their esters and other derivatives are found extensively in fibers, adhesives, films, surface coatings, resins and in rubber industries. Their properties of elasticity, colorless transparency, adhesive qualities, resistance to light and weathering are used to advantage in polymers and copolymers. Some of these polymers are sold under trade names such as
lucite, perspex, orlon, acrilon, plexiglass, etc. Derivatives of crotonic acid are also used in the preparation of DL-threonine and as a fungicide - Karathene.

C. Metabolic Pathways Involving Acrylic Acid

1. Introduction

In most of the literature reviewed, the evidence for acrylic acid or its coenzyme A thioester as an intermediate of metabolism is indirect. The only direct evidence obtained prior to the investigation conducted in this laboratory with Clostridium propionicum are examples of α-oxidation of three carbon β unsaturated compounds.

Cernaglia et al. (1976) demonstrated that Mycobacterium convolutum could oxidize propylene to acrylic acid. Jackson (1973) found acrylate accumulating from alkyl alcohol in mycelial suspensions of Trichoderma viride, preceeding which Jensen (1961) observed alkyl alcohol oxidation to acrylic acid, catalysed by Pseudomonas fluorescens and Nocardia corallina. Pseudomonas aeruginosa has been reported by Vander Linden and Thijjs (1965) to catalyse propylene oxidation to propionate.

2. Lactate Dehydration

Formation of acrylate could also be anticipated to occur as a result of β-oxidation. Though lactate is an end product of anaerobic metabolism in numerous bacteria, it can be further reduced to propionate. Two pathways facilitating these reactions are known to exist:
(a) The succinate or dicarboxylic acid pathway functions in most propionate producing organisms such as the propionibacteria (Johns, 1951; Wood and Leaver, 1953; Delwiche, 1956; Wood et al., 1956) and micrococcus (Johns, 1951; Delwiche et al., 1956).

\[
\text{Lactate + NADH + ADP + P}_i \rightarrow \text{Propionate + NAD + ATP}
\]

Lactate is oxidised to pyruvate, requiring a flavoprotein as electron acceptor. Oxalacetic acid is formed in a transcarboxylation reaction involving biotin as the CO\textsubscript{2} carrier. Succinyl-CoA is produced in a reaction sequence similar to a reversal of the TCA cycle. Methylmalonyl-CoA is then formed in a B\textsubscript{12} mediated rearrangement, following which it is decarboxylated and deacylated to yield propionate. Both the coenzyme A and carbon dioxide are recycled between intermediates. Acrylate, however, is not an intermediate in this pathway.

(b) The second pathway resulting in the direct sequential hydration and reduction of lactate to propionate is of greater interest. Cardon and Barker (1947) speculated that in Clostridium propionicum, acrylate resulted from lactate dehydration. The occurrence of not acrylate, but its thiol ester acrylyl-CoA has been substantiated by Stadtman and Vagelos (1958) in C. propionicum and a Pseudomonad; by Elsdon (1956), Ladd and Walker (1965) and Baldwin et al. (1965) working with Megasphaera elsdenii and by Wallnofer and Baldwin (1967) and Wallnofer et al. (1966) in Bacteroides ruminicola.
Figure 3 - Pathway of L-alanine fermentation by Clostridium propionicum
3. Propionate Oxidation

Most organisms are confronted with the need to degrade propionate or propionyl-CoA, occurring as an end product of the amino acids L-valine and L-leucine metabolisms. Some marine organisms confront this situation during oxidation of odd-number carbon chain fatty acids (Lehninger, 1975).

Such organisms, capable of oxidizing propionate to carbon dioxide and water, are aerobic. Anaerobic oxidation of propionate is accompanied with a large free energy value, and is therefore unfavorable. However, there are published reports demonstrating butyrate uptake by a mixed culture of anaerobes (Chyanoweth and Mah, 1971). Stadtman and Barker (1951) reported both butyrate and propionate uptake by *Methanobacterium suboxydans* and *Methanobacterium propionicum* respectively. They found that this unusual reaction involved decarboxylation of propionate to acetate and carbon dioxide. Though acrylate was not an intermediate in *M. propionicum*, examples of organisms in which propionate oxidation occurs via acrylyl-CoA are given below.

There are two principal aerobic pathways of propionate oxidation, the succinate and acrylate pathways. In Figure 4 is shown various routes of propionate oxidation.

The "Succinate" or "Dicarboxylate Pathway" of propionate oxidation operates in a reverse sequence of that in lactate metabolism. Flavin et al. (1957) and Kaziro and Ochoa (1964) have identified this as the principal pathway of propionate
oxidation in mammalian tissue. It also functions in numerous microorganisms; the Propionibacteria (Stadtman et al., 1960), Micrococcus (Smith and Kornberg, 1967), Rhizobium (DeHertogh et al., 1964), the protozoan Ochramonas (Arsstein and White, 1962), and the photosynthetic bacterium Rhodospirillium rubrum (Knight, 1962).

An "Acrylate Pathway" in animal tissue analogous to the reversal of the acrylate pathway of lactate metabolism was first proposed by Mahler and Huennekens (1953). Since then, a large body of evidence has been published, supportive of a similar pathway. Amongst these are the results of Stadtman and Vagelos (1958) demonstrating the pathway in Pseudomonas sp. and peanut cotyledons; Sokatch (1966) with Pseudomonas aeruginosa; Wegner et al. (1967, 1968) and Kay (1971) with Escherichia coli and Hodgson and McGarry (1967a; 1967b) with Moraxella lwoffii.

Propionate dehydrogenation by this pathway, it is believed, leads to the formation of acrylate or its thiolester, acrylyl-CoA, as an intermediate. Subsequent hydration could then occur in either the α position resulting in lactate, or in the β position, resulting in β-hydroxy propionate formation.

Some of the strongest evidence for an "α-hydration pathway" of lactate metabolism comes from the work of Wegner et al. (1967a; 1967b) who investigated E. coli strain E-26. They reported successful demonstration of the activity of enzyme reactions associated with acrylyl-CoA metabolism.
To further determine which of the numerous pathways is operative, a simple but powerful radiorespirometric technique has been used (DeHertogh et al., 1964; Wegner et al., 1967b). This technique has been particularly useful in differentiating between the occurrence of $\alpha$ and $\beta$ hydration of acrylyl-CoA. The rationale employed is as follows.

The radiorespirometric pattern of labelled carbon dioxide evolution from position labelled propionate is unique for each pathway. This is evident upon perusal of Figure 4, from which it can be seen that the sequence in which the carbon atoms are decarboxylated is significantly different in each pathway. A comparison of the expected profiles for labelled carbon dioxide evolution in each pathway is presented in Table 3.

When resting cells of *E. coli* E-26 were provided with position labelled propionate - $1^{14}$C, $2^{14}$C, or $3^{14}$C, the rate and total amount of carbon dioxide evolution was greatest from propionate - $1^{14}$C, less from $2^{14}$C and least from $3^{14}$C propionate. These results are consistent with what is anticipated if propionate is oxidized to lactate via acrylyl-CoA (Wegener et al., 1967b).

Hodgson and McGarry (1967a, 1967b) found that propionyl-CoA labelled in position 2 or 3 was converted to $14^{14}$C-acetate, whereas the radioactive carbon from propionyl-CoA $1^{14}$C ended mostly as labelled carbon dioxide. Viewed from the metabolic pathways of Figure 3, these results are consistent only with the oxidative pathway of propionate to lactate via acrylyl-CoA.
Figure 4 - Metabolic Routes of Propionate Oxidation

- OOC-CH$_2$-CH$_2$-COO$^-$ (Succinate) 
  \[ \xrightarrow{\text{CO}_2} \]

- OOC-CHO (Glyoxylic acid) 
  \[ \xrightarrow{\text{CO}_2} \]

- OCC-CHOH-CH$_2$-CH$_2$-COO$^-$ (a-Hydroxyglutarate) 
  \[ \xrightarrow{\text{CO}_2} \]

- OCC-CH$_2$-CH$_2$-COO$^-$ (Succinate) 
  \[ \xrightarrow{\text{CO}_2} \]

- CH$_3$-CH$_2$COCoA (Propionyl-CoA) 
  \[ \xrightarrow{\text{CO}_2} \]

- CH$_3$=CH-COCoA (Acryllyl-CoA) 
  \[ \xrightarrow{\text{CO}_2} \]

- CH$_2$OH-CH$_2$-COCoA (E-Hydroxypropionyl-CoA) 
  \[ \xrightarrow{\text{CO}_2} \]

- CH$_2$OH-CH$_2$-COO$^-$ (E-Hydroxypropionate) 
  \[ \xrightarrow{\text{CO}_2} \]

- OHC-CH$_2$-COCoA (Malonic semialdehyde-CoA) 
  \[ \xrightarrow{\text{CO}_2} \]

- CoA-CO-CH$_2$-COO$^-$ (Malonyl-CoA) 
  \[ \xrightarrow{\text{CO}_2} \]

- CoA-CO-CH$_3$ (Acetyl-CoA) 
  \[ \xrightarrow{\text{CO}_2} \]

- CH$_3$-COCoA (Acetyl-CoA) 
  \[ \xrightarrow{\text{CO}_2} \]

- CH$_3$-CO-COO$^-$ (Pyruvate) 
  \[ \xrightarrow{\text{CO}_2} \]

- CH$_3$-CO$^-$ (Acetate) 
  \[ \xrightarrow{\text{CO}_2} \]
### TABLE 3

**EXPECTED RADIORESPIROMETRIC PATTERNS OF $^{14}$CO$_2$ EVOLUTION DURING OXIDATION OF POSITION LABELLED PROPIONATE BY DIFFERENT PATHWAYS (1)**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Comparative Expected $^{14}$CO$_2$ Evolution Pattern (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylate Pathway, via Lactate</td>
<td>1 &gt; 2 &gt; 3</td>
</tr>
<tr>
<td>Acrylate Pathway, via Malonic Semi-aldehyde</td>
<td>1 &gt; 3 &gt; 2</td>
</tr>
<tr>
<td>Acrylate Pathway, via Malonic Semi-aldehyde-CoA</td>
<td>3 &gt; 1 &gt; 2</td>
</tr>
<tr>
<td>Succinate</td>
<td>1 &gt; 3 = 2</td>
</tr>
<tr>
<td>$\alpha$-Hydroxyglutarate</td>
<td>1 &gt; 3 = 2</td>
</tr>
</tbody>
</table>

(1) Adapted from Wegener et al. (1967b, 1968a)

(2) Numbers refer to the $^{14}$C labelled position of propionate supplied as substrate.
Using another isotope labelling method while investigating L-valine oxidation in *Pseudomonas aeruginosa*, Sokatch (1966) showed that position labelled $4,4'$-$^{14}$C-valine was converted to $1,3-^{14}$C-alanine. The following pathway was hypothesized to occur:

\[
\begin{align*}
^{14}\text{CH}_3 & \\
\text{CH-CH-CO}_2^- & + {^{14}\text{CH}_3-\text{CH}_2}^{14}\text{CO-CoA} & \rightarrow & (^{14}\text{CH}_3=\text{CH}^{14}\text{CO}_2\text{-CoA}) \\
{^{14}\text{CH}_3} & & & \\
\text{valine} & & & \text{propionyl-CoA} & & \text{acrylyl-CoA} & \\
\text{+} & & & & & & \\
{^{14}\text{CH}_3-\text{CO}^-} & + {^{14}\text{CH}_3-\text{CH-CO}}^- & \rightarrow & {^{14}\text{CH}_3-\text{CH-CO}}^- \\
\text{pyruvate} & & & & & & \text{alanine} & \\
\text{NH}_3 & & & & & & \\
\end{align*}
\]

in which the isopropyl carbons of valine act as precursors of pyruvate and alanine by a pathway not requiring randomization of the isotope. This, as well as other tracer evidence, strongly supports the work of Stadtman and Vagelos (1957) and Vagelos *et al.* (1959) who also hypothesized the occurrence of the "acrylate pathway" partly on the basis of the catalytic conversion of acrylyl-pantetheine by extracts of a *Pseudomonal* isolate and reduced safranine dye, respectively.

The above cited literature were instances in which the $\alpha$-hydration of acrylyl-CoA occurred. The $\beta$-hydration of acrylyl-CoA, resulting in the formation of $\beta$-hydroxypropionyl-CoA, is
analogous to the reaction catalysed by enoyl-CoA hydratase [EC 4.2.1.17] (Crotonase), during fatty acid oxidation. A glance at Figure 4 will help determine the possible fates of acrylyl-CoA by these pathways. In *Clostridium kluyveri* (Vagelos, 1960) the malonyl semialdehyde CoA pathway is operative, whereas in the colorless algae *Prototheca zopfii* (Callely and Lloyd, 1964) and in certain animal tissues (Rendida and Coon, 1957; Yamada and Jakoby, 1960).

These constitute the major known oxidative pathways of propionate metabolism. Another pathway, in which propionyl-CoA and glyoxylate condense to form α-hydroxy glutaryte CoA and then succinyl-CoA is crucial for the synthesis of four carbon compounds and occurs in conjunction with one or more of the earlier described pathways. Wegener *et al.* (1968c; 1969) described the aperlotic role of this pathway, and it is reviewed in greater detail later in this chapter. This condensation pathway also occurs in the fungus *Aspergillus glaucus* and possibly even in *Penicillium* sp. (Richards and Lloyd, 1966).

A unique condensation reaction occurs during propionate metabolism in *Candida lipolytica*. Tabuchi and Uchiyama (1975) provided enzymatic evidence for a cyclic pathway, analogous to the tricarboxylic acid cycle, for the partial oxidation of propionyl-CoA to pyruvate. Propionyl-CoA condenses with oxalacetate to produce methyl citrate. Acrylate is not an intermediate in this sequence of reactions.
4. Other Acrylate Pathways

Finally, propionate and lactate are not the only known precursors of acrylyl-CoA.

Though Statdman and Vagelos (1957) presented evidence for the amination of acrylyl-CoA to form β-alanyl-CoA, they were unable to demonstrate the reverse reaction in C. propionicum. Jacobsen and Wang (1968), however, did report the occurrence of the reverse reaction in Penicillium digitatum.

Interestingly, they found this organism able to synthesize ethylene from acrylate. One hypothesized pathway involves the sequential decarboxylation of fumarate and acrylate (Jacobsen and Wang, 1965).

Succinate (malate) → Fumarate → Acrylate → Ethylene

Working with plant tissue, Meheruick and Spencer (1967) and Shimokawa and Kasai (1970) also identified acrylate as a precursor of ethylene biosynthesis. The following reactions have been hypothesized.

\[
\begin{align*}
\text{Acetate} & \rightarrow \text{Malonate} \rightarrow \text{Malonic Semialdehyde} \\
& \downarrow \\
\text{propionate} & \rightarrow \text{β-hydroxy propionate} \rightarrow \text{β-alanine} \\
& \downarrow \\
\text{acrylate} & \downarrow \\
\text{ethylene} &
\end{align*}
\]
In summary, the purpose of this section entitled "Metabolic Pathways Involving Acrylate" was to cite references of pathways and organisms potentially applicable to the microbial production of acrylate. As can be seen from the large body of literature reviewed, there are numerous biochemical reactions involving acryloyl-CoA. Principal amongst these, but by no evidence the only useful examples, are the acrylate pathways of lactate and propionate metabolism occurring anaerobically and aerobically, respectively. The metabolism of acrylate in Clostridium propionicum and Escherichia coli, the organisms chosen for further study, are reviewed in separate sections.
D. Metabolic Pathways Involving Methacrylate and Crotonate

The metabolic pathways by which methacrylate and crotonate occur are very similar to those described for the direct pathway of acrylate metabolism.

1. Methacrylate

In a sequence of reactions similar to the acrylate pathway of propionate oxidation, methacrylyl-CoA is the dehydrogenation product of isobutyrate (Massey et al., 1976).

Rendida and Coon (1957) proposed the reaction sequence shown in Figure 5, with DL-Valine as the starting substrate. The investigation was conducted with animal tissues. Other investigators, Sokatch (1966), Marshall and Sokatch (1972) and Puukka (1973) demonstrated activity of the enzymes catalysing isobutyryl-CoA dehydrogenation and subsequent methacrylyl-CoA hydration to 3 hydroxy isobutyryl-CoA in different species of Pseudomonas. The organisms grew on valine, 2 ketoisovalerate and isobutyrate.

Studying the enzymes metabolizing isobutyryl-CoA in P. putida, Marshall and Sokatch (1972) found isobutyryl-CoA dehydrogenase activity constitutive. It has not been resolved, however, whether the enzyme specificity is intended for isobutyryl-CoA or butyryl-CoA. Enoyl-CoA hydratase from P. fluorescens UK-1 was found to be inducible. Again, its specificity is not conclusively known.
Figure 5 - Pathway of D,L-valine metabolism involving methacrylic-CoA as intermediate

\[
\begin{align*}
\text{D,L-VALINE} & \rightarrow \text{2-KETOISOVALERATE} \\
\text{CH}_3\text{CH}_3\text{CHCH(NH}_3^+\text{)}\text{CO}_2^- & \rightarrow \text{CH}_3\text{CH}_3\text{CHCOCO}_2^- \\
\text{2-Keto isovalerate dehydrogenase EC 1.2.4.4.} & \\
\text{ISOBUTYRYL-CoA} & \rightarrow \text{METHACRYLYL-CoA} \\
\text{CH}_3\text{CH(CH}_3\text{)}\text{CO-SCoA} & \rightarrow \text{CH}_2=\text{C(CH}_3\text{)}\text{CO-SCoA} \\
\text{Isobutyryl-CoA dehydrogenase EC 1.3.99.3 (?)} & \\
\text{METHACRYLYL-CoA} & \rightarrow \text{3-HYDROXY ISOBUTYRYL-CoA} \\
\text{CH}_2=\text{C(CH}_3\text{)}\text{CO-SCoA} & \rightarrow \text{CH}_2\text{OHCH(CH}_3\text{)}\text{CO-SCoA} \\
\text{3-hydroxy isobutyryl-CoA deacylase} & \\
\text{METHYL MALONYL SEMIALDEHYDE} & \rightarrow \text{PROPIONYL-CoA} \\
\text{CHOCH(CH}_3\text{)}\text{CO}_2^- & \rightarrow \text{CH}_3\text{CH}_2\text{CO-SCoA} \\
\text{Methyl malonate semialdehyde dehydrogenase} & 
\end{align*}
\]
**Clostridium propionicum**, among numerous other anaerobes (Holdeman *et al.*, 1977), produce isobutyrate. However, unlike propionate accumulation by the acrylate pathway, isobutyrate is a result of valine decarboxylation. Methacrylyl-CoA is not an intermediate.

2. Crotonate

Crotonyl-CoA occurs widely in nature, being the dehydration product of β-hydroxy butyryl-CoA (or ACP) during fatty acid oxidation and synthesis respectively.

Unlike both acrylyl-CoA and methacrylyl-CoA which are usually hydrated in the α-position, crotonase catalyses the sterospecific hydration in the β-position.

Crotonyl-CoA is also a commonly found intermediate in butyrate synthesizing anaerobes (Gottschalk, 1977). The butyrate and butanol synthesizing *Clostridia* sequentially dehydrate β-hydroxybutyryl-CoA and then reduce crotonyl-CoA to butyrate.

**Clostridium propionicum** metabolizes threonine, yielding butyrate, propionate and carbon dioxide (Barker and Wikens, 1948) by the following route:

\[
\text{Threonine} + \alpha\text{-Ketobutyrate} \rightarrow \alpha\text{-Hydroxybutyryl-CoA} \\
\downarrow \\
\text{Propionyl-CoA} \quad \text{Crotonyl-CoA} \\
\downarrow \\
\text{Butyryl-CoA}
\]
The scheme is similar to the metabolism of L-alanine, and hence strategies applicable to acrylate production could conceivably be applied towards crotonate production.

E. *Clostridium Propionicum*

1. General Metabolism

*Clostridium propionicum*, an obligate anaerobe, was isolated by Cardon and Barker (1946) from black mud of the San Francisco Bay. The organism occupies a unique environmental niche metabolizing mostly three carbon compounds - lactate, pyruvate, acrylate, L-alanine, ß-alanine, serine, and cysteine. The cells do not metabolize succinate, malate or fumurate, which along with other isotopic tracer data obtained by Johns (1952) and Ladd and Walker (1959) eliminates succinate as an intermediate, and suggests the involvement of the "direct pathway." Cardon and Barker (1946; 1947) investigated the metabolism of this organism, and the subsequent results provided the preliminary evidence on which a "direct pathway" was postulated.

The fermentation products as shown in Table 4 do not include hydrogen, but are typically carbon dioxide, acetate and propionate appearing in ratios which are shown in Table 5. Ammonia formation is a product of amino acid deamination. Catalysed by L-amino acid transferase [EC 2.6.1.11], the amino group is transferred to α-ketoglutarate yielding glutamate, while alanine itself is oxidized to pyruvate. The glutamate in turn undergoes oxidative deamination.
### TABLE 4

**SUBSTRATE FERMENTATION AND PRODUCT FORMATION IN CLOSTRIDIUM PROPIONICUM**

<table>
<thead>
<tr>
<th>Product*</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
<th>Serine</th>
<th>Acrylate</th>
<th>Threonine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>100.0</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Carbon Dioxide</td>
<td>34.0</td>
<td>61.6</td>
<td>36.5</td>
<td>65.9</td>
<td>30.6</td>
<td>74.8</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>32.6</td>
<td>66.7</td>
<td>33.0</td>
<td>66.2</td>
<td>29.1</td>
<td>-</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>66.4</td>
<td>33.3</td>
<td>66.0</td>
<td>33.1</td>
<td>58.2</td>
<td>67.4</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>33.7</td>
</tr>
</tbody>
</table>

* mmol/l Product/100 mmol/l Substrate Consumed

**DEAMINATION REACTIONS**

\[ \text{ALANINE} : \text{CH}_3-\text{CHNH}_3 + \text{CO}_2^- + \text{NAD} \rightarrow \text{alanine transaminase} \rightarrow \text{NH}_3 + \text{NADH}^+ + \text{H}^+ + \text{CH}_3\text{CO}_2^- \]

\[ \text{SERINE} : \text{CH}_2\text{OH} - \text{CHNH}_3 + \text{CO}_2^- \rightarrow \text{serine dehydrase} \rightarrow \text{NH}_3 + \text{CH}_3\text{CO}_2^- \]
### TABLE 5

**ELECTRON BALANCE AROUND SUBSTRATE UPTAKE AND PRODUCT FORMATION IN C. PROPIONICUM**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>mol</th>
<th>Electron Pairs Produced</th>
<th>Electron Pairs Accepted</th>
<th>Product Accumulated (MOLS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-ala → pyr.</td>
<td>pyruvate → acetate</td>
<td>lactate → pyruvate</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Lactate</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Serine and cysteine are also converted to pyruvate and ammonia (Cardon and Barker, 1947). The deamination is proceeded by dehydration of serine by serine dehydratase or in an analogous reaction with cysteine, by the removal of sulfur as hydrogen sulfide catalysed by cysteine desulfurase.

Threonine similarly yields α-ketobutyrate and ammonia (Barker and Wiken, 1948). The final products are, interestingly, propionate and butyrate, just as the products of three-carbon compound metabolism are acetate and propionate. A similar sequence of reactions is strongly suggested. Threonine metabolism has been reviewed in the section titled "Pathways Involving Methacrylate and Crotonate."

All the succeeding enzymes of the three-carbon pathway have been demonstrated by Baldwin et al. (1962; 1965) in Megasphaera elsdenii, the most intensively studied organism with the direct pathway.

Pyruvate is reduced to lactate by an NAD independent D-lactate dehydrogenase (Brockman and Wood, 1975b), and decarboxylated by the pyruvate dehydrogenase complex to acetate and carbon dioxide (Peel, 1960). Either acetyl-CoA is produced, or as a result of substrate level phosphorylation, ATP and acetate are produced.

D and L lactate are racemized by lactate racemase [EC 5.1.2.1] (Schneider and Wood, 1969), following which lactyl activation to lactyl-CoA occurs, catalyzed by a coenzyme A transferase. The enzyme has been purified and characterized by Tung
and Wood (1975). They demonstrated that a small priming quantity of acetyl-CoA served to generate acrylyl-CoA, which by hydration, generated β-hydroxypropionyl-CoA. This product then served to generate more acrylyl-CoA in a cyclical manner. A propionyl-CoA transferase [EC 2.8.3.1] described by Stadtman (1953) uses propionyl-CoA also, transferring the coenzyme to lactate. In this fashion, both lactate and acrylate can be activated without a net expenditure of ATP. These transferases facilitate the use of resting cell suspensions to catalyse biochemical reactions, by undermining the requirement for ATP, in CoA activation reactions. In the absence of CoA transferase activity, coenzyme thiolesters of acetate, propionate and acrylate can be catalysed by a acetyl-CoA synthetase [EC 6.2.1.1] (Eisenberg, 1955).

Numerous investigations all suggest that the intermediates of the reversible conversion of lactate to propionate are coenzyme A intermediates (Stadtman and Vagelos, 1958; Ladd and Walker, 1959; Baldwin et al., 1965).

Once activated, lactoyl-CoA dehydratase [EC 4.2.1.54] is believed to catalyse acrylyl-CoA formation, though the dehydration has not been demonstrated in either M. elsdenii or C. propionicum. However, the reverse reaction has been shown to occur in Pseudomonas extracts (Vagelos et al., 1959). The resulting 2,3-unsaturated compound serves as a terminal electron acceptor from the Electron Transferring Flavoprotein (ETF),
catalysed by propionyl-CoA dehydrogenase (or butyryl-CoA dehydrogenase [EC 1.3.99.2], and consequently reduced to propionyl-CoA (Stadtman and Vagelos, 1958). Baldwin and Milligan (1964) also have purified an acyl-CoA dehydrogenase active on acrylyl-, crotonyl- and other acyl-CoA compounds.

Many of the above reactions, and those in the next section on electron transfer, have been elucidated from investigations with M. elsdenii and extrapolated to C. propionicum. This is not entirely arbitrary; the patchwork of data available on C. propionicum strongly supports this sequence of reactions.

2. Electron Transfer and Balances

The electron balance in C. propionicum during anaerobic metabolism is maintained by intramolecular electron transfer. Intermolecular electron transfer systems capable of disposing reducing equivalents occur in organisms possessing electron transferring systems - such as the hydrogenase system; in the electron transport chain of aerobes and by way of the Stickland reaction in which coupled oxidation-reduction occurs between pairs of amino acids (Decker et al., 1970).

Cardon and Barker (1946) studied the metabolism of three-carbon compounds and threonine by C. propionicum and measured product formation (Table 4). By closing the electron balance around the metabolism of a particular substrate, it can be determined that the product ratio is governed by the
Figure 6 - Hypothesized electron transport system in Clostridium propionicum

D,L-LACTATE $\rightarrow$ LACTYL-CoA $\rightarrow$ ACRYLYL-CoA

\[ (E'_0 = -185 \text{ mV}) \]

Lactate dehydrogenase

\[ (E'_0 = -185 \text{ mV}) \]

Propionate dehydrogenase

\[ (E'_0 = -186 \text{ mV}) \]

PYRUVATE $\rightarrow$ Electron Transferring Favoprotein (ETF) $\rightarrow$ PROPIONYL-CoA

Pyruvate dehydrogenase

\[ (E'_0 = -300 \text{ mV}) \]

Ferredoxin

\[ (E'_0 = -413 \text{ mV}) \]

ACETATE $\rightarrow$ NADH $\rightarrow$ PROPIONATE

\[ (E'_0 = 11 \text{ mV}) \]

\[ (E'_0 = 816 \text{ mV}) \]

ALTERNATE ELECTRON ACCEPTORS

\( (2H^+ + 2e) + \text{METHYLENE BLUE} \text{ (ox)} \rightarrow \text{METHYLENE BLUE} \text{ (red)} \)
need for internal compensation of reducing equivalents. Calculations for L-alanine, pyruvate and lactate as substrate are shown in Table 5.

One advantage of working with C. propionicum is that the product ratios are not complicated by the formation of larger than three-carbon compounds, as they are in M. elsdenii (Retamal Marchant, 1978).

Electron pairs are produced stoichiometrically during L-alanine transamination, lactate dehydrogenation and pyruvate decarboxylation. Depending upon the substrate, electrons are accepted by pyruvate and acrylyl-CoA, resulting in lactate and propionyl-CoA respectively (Figure 2). Not having a hydrogenase system, acrylyl-CoA serves as the only electron acceptor during lactate metabolism in C. propionicum.

Baldwin and Milligan (1964), as well as Brockman and Wood (1975a) made detailed studies of the intramolecular electron transfer reactions occurring in the acrylate pathway of M. elsdenii. Using this system as a model, attempts have been made to determine the system of electron transfer in C. Propionicum.

As a result of the oxidative deamination of L-alanine, mediated by glutamate, NADH is produced. This electron pair is balanced by the reduction of pyruvate to lactate. Electrons produced during pyruvate decarboxylation reduce ferredoxin.
Apparently, rubredoxin and flavodoxin, isolated from *M. elsdenii* by Mayhew and Peel (1966) and Mayhew and Massey (1969) can function in place of ferredoxin, serine, cysteine and threonine deamination does not result in the production of a reducing equivalent.

Electrons from reduced lactate dehydrogenase-FAD and reduced ferredoxin destined for transfer to propionyl-CoA dehydrogenase, are first transferred to the electron transferring flavoprotein (ETF) in a unique inter-flavin electron transfer reaction. The ETF, containing a 6-hydroxy-FAD group, carries the electrons to an FAD linked propionyl-CoA dehydrogenase which catalyses reduction of acrylyl-CoA to propionyl-CoA, in a sequence similar to that documented by Engel and Massey (1971) with butyryl-CoA dehydrogenase [EC 1.3.99.2]. Electrons in the form of NADH, for anabolic reactions, are believed to be made available from ferredoxin.

These principal electron transfer reactions are summarized in Figure 6.

3. β-alanine Metabolism

*C. propionicum* metabolizes not only L-alanine but also β-alanine as a major source of carbon and energy for growth. Both α and β-alanine metabolism result in the formation of propionate, acetate and carbon dioxide. Though β-alanine is not an intermediate or precursor of the L-alanine fermentation, the
Figure 7 - Pathway for β-alanine metabolism in Clostridium propionicum

\[ \text{NH}_3^+ \text{CH}_2-\text{CH}_2\text{COO}^- \]
\[ \beta\text{-ALANINE} \]

\[ \text{NH}_3 \]
\[ \downarrow \]
\[ \text{CHO-CH}_2-\text{COO}^- \]
\[ \text{MALONATE SEMIALDEHYDE} \]

\[ \text{CH}_2\text{OH-CH}_2-\text{COO}^- \]
\[ \beta\text{-HYDROXY PROPIONATE} \]

\[ \text{CH}_2\text{OH-CH}_2-\text{CO-SCoA} \]
\[ \beta\text{-HYDROXY PROPIONYL CoA} \]

\[ \text{CH}_2=\text{CH-CO-SCoA} \]
\[ \text{ACRYLYL-CoA} \]

\[ \downarrow \]
\[ \text{CH}_3-\text{CH}_2-\text{CO-SCoA} \]
\[ \text{PROPIONYL-CoA} \]

\[ \downarrow \]
\[ \text{CH}_3-\text{CH}_2-\text{COO}^- \]
\[ \text{PROPIONATE} \]
pathway as shown in Figure 5 does, however, involve the formation of acrylyl-CoA. The metabolism of \( \beta \)-alanine hence provides two additional routes to acrylate production in \textit{C. propionicum}. One route is via malonate semialdehyde. The other is the result of \( \beta \)-alanyl-CoA Ammonia lyase [EC 4.3.1.6] which (Stadtman, 1955) catalyses the amination of acrylyl-pantetheine. It has been purified by Vagelos et al. (1959a). They found that the equilibrium constant of the reaction favors production of \( \beta \)-alanine. Except for the example of \textit{Penicillium} (Jacobsen and Wang, 1968) such a pathway is not known to be present in other organisms with the acrylate pathway.

In an interesting experiment, Stadtman (1956) was able to convert propionate to \( \beta \)-alanine with extracts of \textit{C. propionicum} in the presence of an alternate electron acceptor.

4. Theoretical Biological Conversion Yields of Substrate to Acrylic Acid

The stoichiometry for the conversion of glucose to acrylate is

\[
C_6H_{12}O_6 + 2 \text{CH}_2 = \text{CH CO}_2^- + 2\text{H}_2\text{O}
\]

A molar yield of 2 can be obtained by the conversion of glucose to acrylate. This corresponds to a maximum yield of 0.84 g acrylate per g glucose. Calculated from a cellulose monomer unit, the yield is 0.94 g acrylate per g cellulose.
\((C_6H_{11}O_5)_n + 2n \text{ CH}_2 = \text{ CHCO}_2^- + 2n \text{ H}_2\text{O}\)

Substrate utilization during the course of fermentation is not, however, stoichiometric with product formation because a fraction is converted to cell material and used as maintenance energy. Hence, the theoretically maximum yield from glucose must be less than 0.84.

The conversion of glucose to lactate by homofermentative lactic acid bacteria results in the production of 2 moles of ATP per mole glucose.

\[
glucose \rightarrow 2\text{-glyceraldehyde-3-phosphate} + 2\text{ATP} + 2\text{ADP}
\]

\[
\rightarrow 2\text{pyruvate} + 2\text{lactate}
\]

\[
4\text{ADP} \rightarrow 4\text{ATP}
\]

Growth yield estimates from theoretical ATP yields are possible by the method of Bauchop and Elsden (1960). They empirically arrived at a cell yield of 10.5 g cell mass per mole ATP generated. Hence, 2 moles ATP per mole glucose corresponds to a maximum yield of 0.12 g cell mass per g glucose. Allowing for 10% cell mass not having its origin in glucose (minerals, nitrogen), of one gram glucose metabolized, only (1-0.11) gram will be available for acrylate production. Hence, 0.73 becomes the
maximum yield when cell mass is concurrently produced. The yield of acrylate would correspondingly increase as a result of decreased cell yield, or for example as a result of non-growth associated product formation - for instance, as one would obtain with resting and immobilized cells.

In the above calculations, it has been assumed that acrylate will be produced from lactate by the same organism, or a resting cell suspension of another organism, requiring no additional energy for growth. However, numerous other schemes are possible in which the production of lactate and its subsequent conversion to acrylate is conducted by separate organisms, and in which may produce undesirable products. Such systems will necessarily have lower yields.

F. The Acrylate Pathway in Escherichia coli

The pathways and regulation of short-chain fatty acid metabolism have been reviewed in two excellent papers by Kornberg (1966) and Wegener et al. (1968). The oxidation of propionate in \textit{E. coli} has been shown to proceed by a variety of routes, one of which is the acrylate pathway. The literature reviewed in this section provided the basis for both, choosing a strain of \textit{E. coli} and the strategy for using it to produce acrylate.
This is the "Acrylate Pathway" of propionate formation (Gottschalk, 1979) and is represented in Figure 3.

1.5 Glucose (or 3 lactate)
   \[ \rightarrow 2 \text{ propionate} + \text{acetate} + \text{carbon dioxide} \]

The reactions of the acrylate pathway are not unlike those known to occur during butyrate production, as a result of the dehydration and reduction of \( \beta \)-hydroxy butyryl-CoA and crotonyl-CoA respectively.

Lactate is just one example of a three-carbon compound from which acrylate could be produced by fermentation. Propionate is another such substrate. It too is readily accumulated as a product of soluble carbohydrate fermentation by the propionibacteria (Wood et al., 1956) and from cellulose by mixed culture fermentation using Bacteroides and Selenomonas (Sheifinger and Wolin, 1973). Even glycerol is convertible to propionate by Veillonella sp. (Mahler and Cordes, 1971).
1. Propionate Oxidation Pathways

Propionate metabolism to acetate can occur by any of the five pathways shown in Figure 4. Different metabolic routes are used by the various strains and change in a particular strain in response to altered culture conditions. Wegener et al. (1967b) and Kay (1971) employed radiorespirometric techniques to confirm the functioning of the acrylate pathway in strains E-26 and K12 respectively. The rationale of this method was reviewed in the section titled "Metabolic Pathways Involving Acrylate."

Their results with *E. coli*, that the evolution rate of $^{14}\text{CO}_2$ from position labelled propionate $-1^{14}\text{C}$ is greater than $-2^{14}\text{C}$ which is greater than $-3^{14}\text{C}$ are consistent with the preliminary but unsubstantiated results of Wegener et al. (1967a) that the necessary enzymes, propionyl-CoA synthetase, propionyl-CoA dehydrogenase (similar to butyryl-CoA dehydrogenase [EC 1.3.99.2] and lactyl-CoA dehydratase [EC 4.2.1.54] were present in extracts of *E. coli* E-26 grown on propionate. This evidence suggests that propionate must be metabolized via acrylate and lactate. The intermediates of the aerobic "acrylate pathway," like its anaerobic counterpart are coenzyme A derivatives. Hodgson and McGarry (1968b) demonstrated *Moraxella lwoffii* specificity for propionyl-CoA. The operation of an active CoA transferase activity may conserve energy spent in activation.
Figure 8 - Pathways of propionate metabolism in *Escherichia coli*.
Vanderwinkel et al. (1968) found *E. coli* growth on fatty acids to be accompanied with such transferase activity.

The condensation of propionyl-CoA with glyoxylate forming α-hydroxy glutarate is yet another route for propionate metabolism. However, this reaction is part of a central regulatory scheme that is reviewed in the next section.

The methyl malonyl/succinate pathway presumably does not operate owing to the absence of B12 synthetic ability in *E. coli*.

2. Operation of Aneplerotic Pathways

Aerobic organisms growing on propionate and other three-carbon compounds must generate four-carbon compounds, regenerate spent tricarboxylic acid cycle intermediates, by anaplerotic reactions. Propionate cannot be used as the sole source of carbon without such a provision. Wegener et al. (1968c) and Kolodziej et al. (1968) demonstrated that it is this capacity that some other pathways of propionate oxidation are employed.

There are three mechanisms to achieve this end in *E. coli*, each having different energy requirements and functioning in different strains. Two of them are glyoxylate dependent mechanisms for the formation of four-carbon acids from acetyl and propionyl-CoA.

Acetyl-CoA is the product of propionate metabolism by the acrylate pathway. Kornberg and Krebs (1957) concluded that organisms growing aerobically on acetate as the sole carbon
and energy source, required the functioning of the isocitrate lyase [EC 4.1.3.1] and malate synthease [EC 4.1.3.2] to condense acetyl-CoA and glyoxylate to four-carbon compounds by the glyoxylate shunt. Glyoxylate also participates in a condensation reaction with propionyl-CoA catalysed by α-hydroxyglutarate synthase [EC 4.1.3.9] forming α-hydroxyglutarate and its decarboxylated product, succinate.

Finally, in the third anaplerotic mechanism, carboxylation reactions are a significant source of four-carbon compounds for E. coli cultured on propionate. Pyruvate produced from lactate is the substrate for phosphoenol pyruvate synthase [EC 2.7.9.2]. Phospho-enol pyruvate in turn is carboxylated by PEP carboxylase [EC 4.1.1.3] to oxalacetate - a tricarboxylate cycle intermediate.

The differences in regulating the levels of enzymes in these three anaplerotic pathways, as well as the enzymes for short-chain fatty acid oxidation and transport account for differences observed in the growth behavior, on propionate, amongst E. coli W, K12 and E-26.

3. Regulation of Propionate Oxidation

Short-chain fatty acids are not readily metabolized by E. coli, as the sole source of carbon and energy. The enzymes that actively oxidize fatty acids do not oxidize the short-chain compounds (Weeks et al., 1969; Salanitro and Wegener, 1971).
The exponential growth of *E. coli* W and E-26 on propionate minimal medium is preceded by a 60-90 h lag (Wegener *et al.*, 1968b). The transition from lag to exponential growth phase is believed to occur following "adaptation." If the propionate-minimal medium is supplemented with glucose, succinate or even lactate, the lag is reduced to between 24 and 48 hours. The "adaptation" period therefore appears to be the period required for the cells to generate adequate pools of four-carbon compounds.

From studying the enzymes and growth characteristics of E-26 metabolic mutants, Wegener *et al.* (1969) hypothesized the occurrence of a change in metabolism, from carboxylation reactions to isocitrate lyase catalysed condensation reactions for the synthesis of four-carbon compounds, in response to environmental changes.

The shift is finely regulated. The carboxylation of phosphoenol pyruvate occurs during early growth phases and when the cells are growing on low concentrations (5-20 mmol/l) propionate. During the late exponential growth phase and on greater than 25 mmol/l propionate concentration, isocitrate lyase synthesis is derepressed, and anaplerotic reactions such as those of the glyoxylate shunt and α-hydroxyglutarate synthesis, are active.

A mechanism for this shift is suggested from the finding by Ashworth and Kornberg (1963) that phospho-enol pyruvate
is a potent non-competitive inhibitor of isocitrate lyase. Phospho-enol pyruvate synthase [EC 2.7.9.2] is itself inhibited by aspartate and other four-carbon compounds (Kornberg, 1966a; 1955b). Hence, phospho-enol pyruvate represses isocitrate lyase activity until sufficient accumulation of four-carbon compounds occur causing phospho-enol pyruvate synthase inactivation and subsequent utilization of isocitrate lyase dependent anaplerotic reactions. Hence, a mutant without this enzyme would not be expected to grow readily. Ashworth and Kornberg (1964) were able to isolate such a mutant, unable to grow on acetate as the only carbon source but, nevertheless, able to grow on 5-10 mmol/l propionate at rates comparable to that of the wild type. Though this is a seemingly contradictory observation, it is possible since strain W primarily utilizes the carboxylation anaplerotic reactions. Acetate negative, but propionate and lactate positive revertants of acetate and propionate negative, but lactate positive mutants have been isolated (Wegener et al., 1969), thereby further demonstrating the redundancy of isocitrate lyase dependent pathways in strain W, during propionate metabolism.

The above review is of particular importance in attempting to produce acrylate from propionate; all branch reactions such as that involving condensation with glyoxylate, are best eliminated. It is therefore valuable to know that of all E. coli strains, W can grow normally without isocitrate lyase.
Using a mutant M-18 of strain W (Ashworth and Kornberg, 1964), Wegener et al. (1969) were able to obtain a lesion in one of the enzymes of the direct pathway of propionate oxidation. Since such a mutant is useful for acrylate production, it is an encouraging result.

Through strains W and E-26 grow on propionate following only adaptation, mutation is required prior to growth on valerate and butyrate (Wegener, 1968a). Kay (1971) found strain K12 cells able to grow on propionate following spontaneous mutation at a frequency of $1 \times 10^{-7}$, following incubation for 1-2 weeks in propionate minimal medium.

Such mutants were found derepressed for both the α-hydroxyglutarate pathway, as well as the glyoxylate shunt. Unlike strain W, isocitrate lyase negative mutants do not grow on propionate, demonstrating the relative importance of the glyoxylate shunt in K12. However, imparting isocitrate lyase constitutively to the organism is not in itself sufficient; to grow on propionate it requires another mutation.

Long and intermediate chain fatty acid β-oxidative enzymes are not active on propionate; the mutation must impart this specificity. This requirement for propionate specificity is illustrated by the propionate oxidizing mutant that could not oxidize four-carbon compounds (Salanitro and Wegener, 1971).

There is no concise summary for the propionate oxidation regulatory mechanisms in E. coli. Suffice it to note that
the components of the system are the β-oxidative enzymes and the anaplerotic enzymes. The details of how they are regulated during growth on propionate vary sharply amongst strains of *E. coli*.

G. Energy Conservation

Conservation of energy during chemotrophic anaerobic metabolism occurs principally by substrate level phosphorylation. In some anaerobes, however, ATP is generated by electron transport mediated phosphorylation (Thauer *et al.*, 1977). Although theoretically possible, it has not been demonstrated conclusively in either *C. propionicum* or *M. elsdenii* (Anderson and Wood, 1969).

Free energy calculations (made from the free energy of formation values) predict the availability of sufficient energy for electron transport at the level of acrylyl-CoA metabolism.

\[
\text{Crotonyl-CoA} + 2H^+ \rightarrow \text{Butyryl-CoA}
\]

\[\Delta G^{\circ'} = 18.6 \text{ kcal/mole}\]

Electron transport from pyridine and flavin nucleotide cofactors to electron transferring flavoproteins occurs in *C. propionicum* much as it occurs in aerobes.

In this organism, the following are the electron generating reactions.
Pyruvate $+ 2H^+ + 2e^- \rightarrow$ Lactate ($\Delta G^{\circ} = -10.3$ kcal/mole)

$E^1_o = -185$ mV

Pyruvate $\rightarrow$ Acetate $+ \text{CO}_2 + 2H^+ + 2e^-$

$G^1 = -9.4$ kcal/mole

whereas the electron accepting part of the couple via the electron transferring flavoprotein (ETF) is

Butyryl-CoA dehydrogenase-FAD $+ 2H^+ + 2e^-$

$\rightarrow$ Butyryl-CoA dehydrogenase-FADH$_2$

$E^1_o = 187$ mV

Acrylate $+ 2H^+ + 2e^- \rightarrow$ Propionate

$E^1_o = -15$ mV

The electron potential ($\Delta E$) of the couple ($\Delta E = E^1_o(\text{acceptor}) - E^1_o(\text{donor})$) is sufficiently positive to accept electrons made available from the oxidation of lactate ($E^1_o = -185$ mV), NADH $+ M^+$ ($E^1_o = -320$ mV), FADH$_2$ ($E^1_o = -180$ mV), or ferredoxin ($E^1_o = -413$ mV).

Decker et al. (1970) calculated the free energy required to generate ATP as equal to which is made available by a standard electrode potential drop of 250 mV. This requirement is satisfied in the above couples.

Conclusions can be made from these calculations, about the number of moles of ATP produced per mole substrate consumed in C. propionicum.

However, calculations can also be made from empirical growth yield data and thermodynamic efficiency considerations. The ATP
growth yield for Clostridium sp calculated by Stadtman (1966) was 10 g cell mass per mole ATP formed, and that for C. kluyveri grown on crotonate was 9.1 (Thauer et al., 1968).

From such calculations, it is possible to determine whether one or two moles of ATP are synthesized per mole substrate and conclude whether the reactions of the acrylate reduction support phosphorylation.

H. Alternate Electron Acceptors

Acrylyl-CoA is the usual terminal electron acceptor in C. propionicum. However, in the event that an alternate electron acceptor with an electrode potential more positive than that in the reduction of acrylyl-CoA is available to the cells, there is evidence that intermolecular electron transfer will occur with the alternate electron acceptor becoming reduced instead.

O'Brien and Morris (1971) exposed a growing culture of C. acetobutylicum to oxygen. Butanol accumulation ceased and butyric acid accumulated. This, it was reasoned, was evidence that oxygen had preferentially drained reducing equivalents from NADH otherwise destined to reduce butyryl-CoA.

The transfer of electrons to the alternate electron acceptor oxygen is mediated, in certain anaerobes, by electron transport flavoproteins which function as NADH oxidases (Dolin, 1961a, 1961b). Diaphorase, a flavin bound enzyme from C. kluyveri mediates the transfer of electrons from NADH and NADPH to elec-
tron accepting dyes such as 2,6-dichlorophenol indophenol (Kaplan et al., 1969; Brower and Woodbridge, 1970).

_C. propionicum_ dehydrogenases that oxidize amino acids valine, leucine, phenylalanine and others, reduce methylene blue (Cardon and Barker, 1946). Hauge (1956) used indophenol to accept electrons resulting from propionate dehydrogenation, and could drive the reaction to completion. Stadtman and Vagelos (1958) catalysed the reverse reaction using reduced safranin dye. Methylene blue has also been used to transfer electrons from an _E. coli_ hydrogenase system (Mizuguchi et al., 1964). Other examples of the use of dyes and electro chemical systems to regenerate reduced cofactors is reviewed by Wang and King (1979).

I. Acrylic Acid Toxicity

The development of a microbiially catalysed process for acrylate production is confronted with the fact that acrylate is toxic to biological systems.

Acrylate has been found an inhibitor of fatty acid oxidation. It couples with coenzyme A in the cell to form acrylyl-CoA, thereby depleting the cellular pool of the coenzyme (Thijssse, 1964).

It is also an inhibitor of aerobic and anaerobic glycolysis in _E. coli_. Heyser and Glombitza (1973) found that in older cells, acrylate was a competitive inhibition of succinate dehydrogenase, inhibiting the tricarboxylic acid cycle.
The normal metabolism of *Micrococcus lactilyticus* is altered by acrylate; though it remains unmetabolized, it prevents pyruvate reduction to propionate (Withley and Ordal, 1957). Other instances of acrylate toxicity have been reported by Sullivan and Ikawa (1972) and Glombitza (1972).

Acrylate is known to be an alkylating agent and could conceivably alkylate cellular proteins. In addition, it polymerizes in the presence of air. Antipolymerizing agents such as p-methoxyphenol, hydroquinone, etc. can be made.

In mammals, the lethal dose, LD$_{50}$, is 2.5 mg/kg. From the standpoint of a microbial process, the question of toxicity can be dealt with by using resting cells, anaerobic bioconversions and other techniques.

J. Oxygen Toxicity

The genus clostridia mostly consists of organisms which are obligately anaerobic, although some are aerotolerant and will grow in the presence of oxygen: *Clostridium tertium*, in pO$_2$ = 0.21 (Morris, 1970); *C. haemolyticum*, in pO$_2$ = 0.007; *C. novyi* type A, in pO$_2$ = .02 (Loesche, 1969); *C. carnis* and *C. listolyticum* in air on solid medium (Laskin and Lechewelier, 1971). There are others still that have the capacity to grow normally following short exposures to high oxygen concentrations.

There are two extant hypotheses for oxygen toxicity. In the first, molecular oxygen acting as a powerful oxidant raises...
the $E_h$ acts as a preferred terminal electron acceptor, consequently draining the cell of reducing ability ($O_2 + 4H^+ + 4e \rightarrow 2H_2O$); at pH 7, $E'_o = 815 mV$) and oxidises essential, labile SH compounds, iron sulfur proteins, tetrahydropteridines, flavoproteins, etc. In the second hypothesis, toxicity to the cells results from the products of the interaction between molecular oxygen and cellular compounds. Superoxide free radicals, hydrogen peroxide, single oxygen, hydroxyl ion, formaldehyde are formed in cells growing in the presence of oxygen. Their influence is believed to be primarily on the cellular membrane and related proteins.

The best studied examples of detoxification of oxygen related toxic compounds are the superoxide ion and hydrogen peroxide detoxifying systems. Superoxide dismutase catalyses the dismutation of $O_2^-$ at 10 times the spontaneous rate, producing hydrogen peroxide and oxygen (Fridovich, 1974). Hydrogen peroxide is enzymatically removed by catalase or peroxidase. The superoxide theory of anaerobiosis attempts to distinguish between obligate anaerobes and aerobes. Here, the superoxide radical, hydrogen peroxide and their products - the singlet oxygen and hydroxyl ions are held responsible for toxicity. Hence, organisms with a detoxifying system could survive in oxygen. McCord et al. (1971) show that aerobic organisms have high levels of these detoxifying enzymes, whereas anaerobes do not (Table 6). Aerotolerant organisms were found to have low
TABLE 6
LEVELS OF OXYGEN DETOXIFYING ENZYMES, SUPEROXIDE DISMUTASE
AND CATALASE IN A VARIETY OF ORGANISMS (1)

<table>
<thead>
<tr>
<th></th>
<th>Superoxide Dismutase</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg</td>
<td>units/mg</td>
</tr>
</tbody>
</table>

AEROBES

<table>
<thead>
<tr>
<th>Organism</th>
<th>Superoxide Dismutase</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>1.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>1.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Halobacterium salonarium</td>
<td>2.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Rhizobium japonicum</td>
<td>2.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Micrococcus radiodurans</td>
<td>7.0</td>
<td>280.0</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>3.7</td>
<td>13.5</td>
</tr>
<tr>
<td>Mycobacterium sp.</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>2.0</td>
<td>22.5</td>
</tr>
</tbody>
</table>

STRICT ANAEROBES

<table>
<thead>
<tr>
<th>Organism</th>
<th>Superoxide Dismutase</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veillonella alcalescens</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clostridium pasteurinum,</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sticklandii, barkeri,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lentoputrscens, cellubioparum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium acetobutylicum</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium sp (strain M.C.)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Butyrivibrio fibrisolvens</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>N2C3 (2)</td>
<td>0</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

AEROTOLERANT ANAEROBES

<table>
<thead>
<tr>
<th>Organism</th>
<th>Superoxide Dismutase</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyribacterium rettgeri</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus bovis</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus mitis</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus lactis</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>Zymobacterium oroticum</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>Lactobacillus planterum</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(1) From McCord et al. (1971)

(2) N2C3 is an unclassified cellulytic Gram+ve rod isolated from the rumen of an African zebu steer.
levels of superoxide dismutase (SOD). Aerobic organisms induce the synthesis of the enzymes in response to elevated oxygen partial pressures (Gregory and Fridovich, 1973a). Aerotolerant organisms also respond to higher oxygen pressures by synthesizing more superoxide dismutase. Exceptions exist and the correlation between SOD and aerotolerance is not clear: aerotolerant \textit{C. tertium} capable of growing in 1 atmosphere of air has less SOD than \textit{C. perfringens} type A, an obligate anaerobe that is unable to grow in 2\% pO\textsubscript{2} (Hewitt and Morris, 1975). Other aero-intolerant species, \textit{C. pasteurinum} and \textit{C. perfringens}, have significant levels of the enzyme (Morris, 1970).

Most clostridia have very little if any catalase or peroxidase (Bergey's, 1974), but peroxidase was found in \textit{C. perfringens} 19P, a strain in which aerotolerant was imparted following mutation (Zavadova et al., 1974).

K. \textbf{Resting Cells and Immobilized Cells}

Bioconversions are most often conducted with actively growing cells (Sebek and Kieslich, 1978), and in some cases where the reaction is growth associated, exclusively by growing cells. The use of microbial resting cell suspensions is a versatile technique for the conversion of substrate to product, uncoupled from the growth process. This capacity of non-growth associated product formation can be likened to that of cells whose metabolism is uncoupled from growth by the absence of a key nutrient
(Cooney et al., 1976; Henderson et al., 1969). The cells continue to metabolize the substrate without increase in cell mass thereby increasing the theoretical product yield.

A dense cell suspension, up to 10% w/v on a dry cell basis can be used (Pirt, 1975), thereby increasing the volumetric productivity. Cell recycle in fermentors is used for the same purpose. The technique is particularly suitable for anaerobic processes where oxygen transfer is not a limiting step. In resting cell suspensions, the recovery of products is also simplified since complex growth requirements can be excluded from the reaction mixture.

Resting cells offer the unique advantage of performing conversions involving cofactor and ATP regeneration, thus permitting redox reactions to be catalysed without the complexities of immobilized cofactor continuous regeneration. As catalysts, resting cells are more stable than free enzymes.

Immobilization of whole cells has been employed in a variety of situations to accomplish selective biotransformations. Both the methods and applications of cell immobilization have been reviewed by Jack and Zajic (1977), Durand and Navarro (1978) and Abbott (1976).

Following immobilization, cellular activity is found to be lower than that in free cells, although there are some exceptions reported in the literature (Kennedy, 1976). The half life of the cells, however, usually shows marked im-
Yamamoto et al. (1974) reported a half life of 120 days for citrulline production by polyacrylamide entrapped *P. putida*.

In addition to increasing the stability, immobilization imparts resting cells with greater mechanical support, particle density and cell loading. These advantages permit greater flexibility in reactor design; better flow characterization, exclusion of the cells from the product stream, efficient cell recovery and separation from the reaction mixture, higher cell concentrations in the reactor, etc.

Following immobilization, alterations in the metabolic ability of the cells occur; *Clostridium butyricum* was observed by Karube et al. (1976) to produce only hydrogen from glucose.

Cellular viability is also sometimes maintained after prolonged catalytic activity and subsequent inactivation of a particular catalytic activity. Reactivation of such cells by suspending the gels in growth media (Klein et al., 1976) is possible.

The majority of procedures used to immobilize whole cells are (1) entrapment in polyacrylamide and related gels; (2) physical and ionic adsorption; (3) covalent attachment to surfaces; and (4) entrapment in natural fibres and gels. The model investigations for entrapment in polyacrylamide polymers were conducted by Chibata et al. (1974) and Tosa et al. (1974) with *E. coli* for the production of aspartate.
III. MATERIALS AND METHODS

A. Microorganisms

*Clostridium propionicum* ATCC 25522 (NCIB 10656) originally isolated by Cardon and Barker (1946) and *Escherichia coli* ATCC 9637-2 which is the wild type strain of *E. coli* W, isolated by Waksman (1950), were obtained as lyophilized cultures from the American Type Culture Collection (Rockville, MD).

B. Culture Media

*Clostridium propionicum* was cultured in complex nutrient medium slightly modified, as noted in Table 7, from that originally employed by Cardon and Barker (1946) and recommended by ATCC. Supplemented with 2% Difco Agar, it was used in plates and slants.

Resazurin was used as the redox indicator ($E'_o = -51$ mV). It is colorless when reduced (dihydroresorufin) and turns pink (resorufin) and then violet (resazurin) on exposure to oxygen. Cysteine hydrochloride is used to maintain a low redox potential.

*E. coli* was grown in M9 minimal synthetic medium containing mineral salts and varying carbon sources. The components of the medium are listed in Table 8. 2% agar plates and slants were made with the same medium.
Table 7 - L-ALANINE COMPLEX MEDIUM FOR GROWTH OF CLOSTRIDIUM PROPIONICUM

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>CONCENTRATION, G/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine (Sigma)</td>
<td>8.0¹</td>
</tr>
<tr>
<td>Bacto Peptone (Difco)</td>
<td>3.0</td>
</tr>
<tr>
<td>Bacto Yeast Extract (Difco)</td>
<td>4.0</td>
</tr>
<tr>
<td>Cysteine Hydrochloride Hydrate (Sigma/Eastman)</td>
<td>0.3¹</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.05</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>0.01</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.68 (5 mmol)</td>
</tr>
<tr>
<td>CaSO$_4$ (Saturated)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Resazurin</td>
<td>0.002</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>to 1000.0 ml</td>
</tr>
</tbody>
</table>

Adjust to pH 7.1 with 3 mol/l NaOH

¹ Modified from the medium recommended by ATCC and used by Cardon and Barker (1946).
Table 8 -
MINIMAL MEDIUM FOR GROWTH OF ESCHERICHIA COLI

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>CONCENTRATION, G/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Mineral Salts$^1$</td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>$5 \times 10^{-5}$</td>
</tr>
<tr>
<td>MnSO$_4$.H$_2$O</td>
<td>$3 \times 10^{-5}$</td>
</tr>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
<td>$4 \times 10^{-5}$</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>$4 \times 10^{-5}$</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>$3 \times 10^{-5}$</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>$4 \times 10^{-5}$</td>
</tr>
<tr>
<td>b. M9</td>
<td></td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>6.0</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O (1 mol/l)</td>
<td>1.0 ml$^2$</td>
</tr>
<tr>
<td>CaCl$_2$ (10 mmol/l)</td>
<td>10.0 ml$^2$</td>
</tr>
<tr>
<td>c. Carbon Source</td>
<td></td>
</tr>
<tr>
<td>Glucose, acetate, propionate$^3$ or lactate</td>
<td>variable</td>
</tr>
</tbody>
</table>

$^1$ Mineral salts prepared separately (X 100 strength) and 10 ml added per liter medium. Prepared at pH 5-6.

$^2$ Autoclaved and added separately.

$^3$ Propionate is supplemented with trace amounts of yeast extract, succinate or glucose to help initiate growth. Details are given alongside the experiment.
C. Cell Culture Techniques

Pure cultures were grown from single colonies of *C. propionicum* isolated on agar plates of the complex medium. Colonies were grown on plates using a Gaspak anaerobic system (BBL, Cockeysville, MD) in a carbon dioxide atmosphere. In a Gaspak jar at room temperature, colonies remained viable for over six months. Isolated colonies were used to inoculate stock cultures grown in liquid medium using Hungate tubes. Following 36 h growth at 37°C, they were stored at 0°C. Stock cultures from grown Hungate tubes were not used to inoculate fresh stock cultures; each stock culture was grown separately from an individual colony. 100 ml starter cultures, inoculated from Hungate tubes were grown in 500 ml anaerobic flasks (Daniels and Zeikus, 1975). Larger batches of cells, 2 to 4 liters, were inoculated with 24 h old starter cultures, and were grown at 37°C.

*C. propionicum* is able to grow even after brief exposure to air. Therefore, procedures such as plating and transfer of colonies to stock cultures, were conducted in air atmosphere at 25°C. Other inoculations, sterilizations and incubations – both during growth and storage – were performed in a nitrogen environment. The growth medium was prereduced prior to inoculation by addition of cysteine-HCl, autoclaving and nitrogen sparging. Techniques for maintenance of anoxic conditions were followed
according to the Anaerobic Laboratory Manual (Holdeman et al., 1977). The purity of the culture was determined by microscopic examination and by testing the unique metabolic capacity of the organism. It grows on L-α- and β-alanine, but not on a very wide range of other carbon substrates (Appendix I). The profile of volatile fatty acids produced during growth on peptone-yeast extract medium make the organism readily distinguishable from other clostridia (Holdeman et al., 1977), and was useful in confirming that the organism cultured was in fact *C. propionicum*.

*E. coli* was revived from the lyophilized culture by growth in 5% trypticase soy broth (Difco). Colonies were isolated and maintained on nutrient agar plates and slants. The purity of cultures was determined by microscopic examination and differential media techniques. *E. coli* is indole, methyl red and eosin-methylene blue positive, but Simmons citrate negative.

Pure cultures were maintained on slants containing the minimal medium shown in Table 8 with 20 mmol/l of sodium propionate at 0°C.

Cells were grown in 250 ml or 500 ml Erlenmeyer flasks at 37°C and aerated by agitation on a rotary shaker at 250 rpm.

D. **Dry Cell Weight**

Duplicate samples consisting of 30 ml of the culture were centrifuged (10,000 xg for 10 min at -5 to 0°C). The pellets
were washed twice with distilled water and transferred into a preweighed aluminum dish. They were dried at 70°C till constant weight (typically for 36 h).

E. Culture Turbidity

The optical density of the cell culture was measured with either a Klett-Sommerson colorimeter with a red filter or a Gilford 240 Spectrophotometer at 660 nm wavelength. To operate within the linear response to light scattering of the instrument, bacterial cultures were diluted to between 10 and 100 Klett units, or 0 and 0.5 O.D. units. Appropriate blanks were employed where necessary; for example, the color interference caused by oxidized resazurin was compensated by using a blank containing oxygenated fresh medium. To permit direct O.D. measurement in side arm flasks, O.D. readings above 100 Klett units were corrected by a standard curve (Appendix II) correlating inaccurate, higher than 100 values with diluted values.

A linear correlation between Dry Cell Weight and turbidity was also obtained. A factor of 190 Klett units/gm dry cell wt or 2.3 O.D. units/g dry cell wt was used to correlate optical measurements and dry cell weight with *E. coli*. 
F. Protein Determination

The method of Lowry et al. (1951) was modified for whole-cell protein analysis. Bovine serum albumin (Grade V, Sigma) was used as the standard. 0.5 ml of washed and resuspended cells (1 to 4 mg wet weight/ml) were added to an equal volume of 1 mol/l NaOH and heated for 10 min at 100°C; 1 ml of water was added to the cooled solution of cells, and then 5 ml of reagent containing 0.1% Cu$_2$(SO$_4$)$_3$ and 0.2% NaK tartarate in 2% Na$_2$CO$_3$. Following 10 min incubation at room temperature, 5.0 ml freshly prepared (by dilution of 1:1 with water) phenol solution (Folin Reagent) was mixed in and allowed to stand for 30 min. Absorbance was read at 660 nm, or with a #66 Klett filter, against a reagent blank. The calibration curve is shown in Appendix II.

G. Viable Cell Enumeration

The viable plate count method was used to determine the number of viable *E. coli* cells. Measured aliquots of a serially diluted culture were spread evenly on the surface of nutrient agar plates. After 24 h incubation at 37°C, the colonies were counted from which the number of viable cells per culture volume was determined.

The most probable number (MPN) technique was used to enumerate *C. propionicum* viable cells. Strict anoxic conditions
are easily maintained with this method, making it suitable for the enumeration of anaerobes. The viable cell count per culture volume is obtained within 95% confidence limits by multiple dilution to extinction (dilution of 5 identical samples sequentially to extinction in 5 sets of tubes containing anaerobic growth medium) and subsequent determination of the most probable number of bacteria by statistical evaluation of distribution of tubes in which growth occurs (Mayou, 1977).

H. Short Chain Fatty Acid Analysis

Volatile and non-volatile short chain fatty acids (two to six carbon) were analyzed quantitatively by gas-liquid chromatography. Two separation columns used were: (1) a 10% DEGS-PS (diethyleneglycol succinate - phosphoric acid) on Chromosorb W AW 80/100 mesh (Supelco, Bellefonte, PA) and (2) a 5% Thermon 1000 with 5% phosphoric acid in Chromosorb W 80/100 mesh, washed with DMCS (Shimadzu Manuf. Co., Japan). Both were packed in 1.85 m teflon tubing (Altech, Arlington Hghts., IL) with an outer diameter 3.18 mm. A Hewlett-Packard 5830 A gas-liquid chromatograph with a computerized integrator-recorder and an automatic sample injector, model 7671A, was used in this work.

The DEGS liquid phase would not accept aqueous samples, nor would it separate acrylate from isovalerate, or propionate from isobutyrate. However, despite these drawbacks, the DEGS column was useful for rapid isothermal assays.
Figure 9 - Protocol for the Quantitative Analysis of Volatile Short Chain Fatty Acids

100 μl reaction mixture

Acidify with 20 μl 50% H₂SO₄

Stored refrigerated (-10°C)

250 μl cold diethyl ether containing 1 g/l valeric acid and 1 g/l formic acid

Mix ether and aqueous phases by shaking vigorously. Keep cool in an ice bath

Centrifuge for 2 min at 25°C at 500 xg to break the emulsion

Pipette ether fraction into 100 μl glass vials with crimp-on teflon seals

Inject 3 μl ether samples automatically into GLC for separation by either Therm-on or DEGS columns

Column conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Temperature</td>
<td>200°C</td>
</tr>
<tr>
<td>Oven Temperature</td>
<td>100°C-155°C at 15°C/min</td>
</tr>
<tr>
<td>Flame Ionization Detector Temperature</td>
<td>300°C</td>
</tr>
<tr>
<td>Carrier Gas</td>
<td>Helium</td>
</tr>
<tr>
<td>Carrier Gas Flow Rate</td>
<td>30 ml/min</td>
</tr>
<tr>
<td>Internal Standard</td>
<td>Valeric acid</td>
</tr>
</tbody>
</table>

1 Described in Section L of this Chapter.
2 Rate 8°C/min for separation in Figure 6.
Figure 10 - Chromatogram showing Separation of Acetic, Propionic, Acrylic and Valeric acids on a "Thermon" Column.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Retention Time</th>
<th>Substance</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Standard</td>
<td>5.83 min</td>
<td>Acetic acid</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3.11 min</td>
<td>Propionic acid</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>3.69 min</td>
<td>Acrylic acid</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>4.61 min</td>
<td>Valeric acid</td>
<td>1</td>
</tr>
</tbody>
</table>

At concentrations typical of a bioconversion, the protocol of which is described in Section L of this Chapter.
Figure 11 - Chromatogram showing Standard Separation of Isobutyric, Butyric, Isovaleric, Methacrylic, Valeric and Crotonic Acids on a "Thermon" Column.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Retention Time</th>
<th>Substance</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Standard</td>
<td>7.80</td>
<td>Valeric acid</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>5.53</td>
<td>Isobutyric acid</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>6.30</td>
<td>Butyric acid</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>6.83</td>
<td>Isovaleric acid</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>7.10</td>
<td>Methacrylic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>8.52</td>
<td>Crotonic acid</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 12 - Standard calibration curve for propionic acid quantitation in ether extracts by gas-liquid chromatography.
Figure 13 - Standard calibration curve for acrylic acid quantification in ether extracts by gas-liquid chromatography
Figure 14 - Standard calibration curve for acetic acid quantitation in ether extracts by gas-liquid chromatography.
Figure 15 - Protocol for the Quantitative Analysis of Non-Volatile Short Chain Fatty Acids

100 µl reaction mixture

Acidify with 20 µl 50% H₂SO₄

Store refrigerated (-10°C)

Add 200 µl methanol containing 1 g/l oxalic acid, then 40 µl 50% (v/v) H₂SO₄

Stopper and heat at 60°C for 30 min

Add 100 µl water and 200 µl chloroform

Extract methyl esters into chloroform phase by vigorous shaking; cool in an ice bath

Centrifuge for 2 min at 25°C at 500 xg to break emulsion

Pipette chloroform fraction into 100 µl glass vials with crimp-on teflon seals

Inject 3 µl samples automatically into GLC for separation either by DEGS or Thermon columns

Column conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Temperature</td>
<td>200°C</td>
</tr>
<tr>
<td>Oven Temperature</td>
<td>125°C–155°C at 8°C/min</td>
</tr>
<tr>
<td>Flame Ionization Detector Temperature</td>
<td>300°C</td>
</tr>
<tr>
<td>Carrier Gas</td>
<td>Helium</td>
</tr>
<tr>
<td>Carrier Gas Flow Rate</td>
<td>50 ml/min</td>
</tr>
<tr>
<td>Internal Standard</td>
<td>Oxalic acid</td>
</tr>
</tbody>
</table>
Figure 16 - Chromatogram showing Standard Separation of Pyruvic, Lactic, Oxalacetic, Oxalic and Succinic Methyl Esters on a Thermon Column.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Retention Time</th>
<th>Compound</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Standard</td>
<td>3.69 min</td>
<td>Methyl oxalate</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1.67 min</td>
<td>Methyl pyruvate</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>2.47 min</td>
<td>Methyl lactate</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>3.27 min</td>
<td>Methyl oxalactate</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>7.43 min</td>
<td>Methyl ß-hydroxy butyrate</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 17 - Standard calibration curve for quantification of lactic acid methyl ester in chloroform extracts by gas-liquid chromatography.
The standard curves and separation profiles for the Thermon column are shown in Figures 9 through 17. The protocol for volatile fatty acid analysis is shown in Figure 9. The DEGS column was used for experiments with *E. coli*, whereas with *C. propionicum*, the Thermon column was used. When using DEGS, it was necessary to dry the ether samples by addition of MgSO₄ and 0.1% formic acid was added to each sample to help elute acetic acid from the column.

The chromatogram for the separation of acetic, propionic, acrylic and valeric acid is shown in Figure 10 and calibration curves are shown in Figures 12 to 14. Separation of acetic, isobutyric, butyric, isovaleric, methacrylic, valeric and crotonic acids on a Thermon column is shown in Figure 11.

Non-volatile fatty acids were assayed as their methyl esters extracted in chloroform. The protocol for methylation, extraction and separation is summarized in Figure 15.

I. Mass spectra

Gas chromatographic-mass spectrometric analysis of acrylic, methacrylic and crotonic acids produced from the dehydrogenation of propionic, isobutyric and n-butyric acids was conducted with a Perkin-Elmer 990 Gas Chromatograph coupled to a Hitachi RMU 6L Mass Spectrometer and an IBM 1800 data acquisition and control system. Every 5-6 seconds, the GC separated flow stream
was scanned and mass spectra were taken. Total ionization plots, mass spectra for each scan (spectrum index number), as well as the distribution of each m/e value over the entire scan were plotted. The data analysis system has been described by Hills and Biemann (1968). A 70 eV ionization beam was used.

Acrylic acid containing 200 ppm p-methoxyphenol (Eastman), Methacrylic acid (Eastman) and 98% Crotonic acid were used as standards.

The analyses were performed by Dr. Cathy Costello and Dr. Steve Carter at the Mass Spectrometry facility in the Department of Chemistry, MIT.

J. Isocitrate Lyase Assay

Isocitrate lyase (E.C. No. 4.1.3.1) was assayed spectrophotometrically at 324 nm according to the method of Dixon and Kornberg (1959). To 1 mg cell free extract protein obtained by sonic disruption of \textit{E. coli} using a Branson S125 sonicator with a microtip (Heat Systems), 20 µl phenyl hydrazine, 5 µl glutathione, 200 µl MgCl₂, at pH 8.0 were added to 10 µl isocitrate to make a final volume of 3 ml. Glyoxylate phenyl hydrazine (extinction coefficient = 1.7 X 10⁴) formation was measured. Activity is expressed as µmol glyoxylate produced/mg protein/h.
Figure 18 - Protocol for mutagenesis of *Escherichia coli* with ethylmethane sulfonate

Grow cells on 20 mmol/l propionate minimal medium and harvest in mid-exponential growth phase. Resuspend in minimal medium without carbon source.

**Method of Osborn et al. (1967)**

Add 1 mol/l TRIS buffer pH 7.4 to prepare a 9.6 ml suspension containing 0.5 to 1.2 x 10⁹ cells/ml

Dissolve mutagen with vigorous shaking for 5 min at 37°C

Centrifuge, wash out mutagen and resuspend in 100 ml 15 mmol/l glucose minimal medium

Grow overnight to segregate genetic lesions

**Method of Lin et al. (1962)**

Add 200 mmol/l TRIS buffer pH 7.5 to prepare a 9.85 ml suspension containing 5 x 10⁸ cells/ml

Shake vigorously to dissolve mutagen, then aerate for 2 h at 37°C

Dilute 10 fold with 15 mmol/l glucose in minimal medium

Dilute an aliquot 10 fold in glucose minimal medium and grow overnight to further genetic segregation
K. Mutagenesis, Enrichment and Selection: E. coli mutants

Ethyl methane sulfonate (EMS, Sigma) was used to mutagenize wild type E. coli W cells. Two methods were used, that of Osborn et al. (1967) in which 0.5 to 1.2 x 10^9 cells/ml were treated with 4% EMS for 5 min at pH 7.4 and that of Lin et al. (1962) involving the exposure of 5 x 10^8 cells/ml to 1.5% EMS for 2 h followed by a 10-fold dilution and overnight growth. The protocols are summarized in Figure 18.

Mutagenized cells were enriched by an adaptation of the method originally devised by Gorini and Kaufman (1960) using penicillin G. By obtaining exponentially growing cells before penicillin is added and minimizing the duration of treatment, this method decreases the opportunity for cross feeding of mutants by lysed cells. The time required to reach exponential growth phase served to starve the mutants of residual endogenous metabolites. To aerate the cells without agitation, 10 ml bacterial culture was resuspended in 250 ml Erlenmeyer flask with surface aeration. Cell lysis was observed by decreasing optical density of the culture suspension in a side-arm flask (Appendix II).

Ampicillin (20 µg/ml) and D-cycloserine (2 x 10^{-3} mol/l for 5 x 10^9 cells/l) were used to prevent enrichment of penicillin resistant mutants (Miller, 1972). The protocol for penicillin enrichment is presented in Figure 19.
Figure 19 - Protocol for Penicillin Enrichment of Mutants

- Harvest 5 ml of mutagenized cells grown overnight \(10^8\) cells/ml. Wash with 100 mmol/l phosphate buffer pH 7.0.
- Add 50 ml minimal medium with 20 mmol/l acetate or propionate.
- Grow at 37°C with aeration til 2-3 fold increase in O.D.
- Add 3000 units penicillin-G/ml.
- Incubate 10 ml culture at 37°C in 250 ml Erlenmeyer flask without agitation for 120 min till cells lyse.
- Centrifuge at 10,000 xg at 0°C for 15 min or milipore filter to remove penicillin. Wash cells with phosphate buffer, pH 7.0.
- Resuspend in 100 ml 0.2% glucose minimal medium.
- Grow cells overnight.
- Repeat entire cycle twice, once with penicillin G and finally with ampicillin or D-cycloserine.
- Select mutants on agar plates.
Mutants were selected on 1.2% Noble Agar (Difco) plates with minimal medium and varying carbon sources.

Isocitrate lyase negative (aceA) mutants were scored as cells unable to grow on acetate but which grew on acetate supplemented with glyoxylate. Three methods were used to score such colonies. Using differential media plates containing 0.1% acetate and 0.01% glycerol or succinate, pinpoint colonies were scored and tested for growth on acetate and glyoxylate. Alternatively, following growth on 0.5% acetate, 0.1% glyoxylate was sprayed on each plate. Freshly appearing colonies were scored. Finally, the replica plating technique using sterile velvet pads was employed; cells growing on acetate and glyoxylate, but not on acetate alone were scored.

To select propionate negative, lactate positive mutants of isocitrate lyase deficient cells, differential plates containing 0.2% propionate and 0.01% lactate were used. Alternatively, the spraying technique and replica plating were resorted to. Cells were grown at 37°C.

L. Resting Cell Bioconversion System

Resting, not growing cells have been exclusively used to catalyse propionate dehydrogenation to acrylate. After harvesting during a growth phase in which they were optimally active for the bioconversion, a dense suspension (10-20% wet
weight) of washed cells was made in buffer and without growth media. The suspension, pipetted into a glass test tube, could now be treated with different reagents, substrates and environmental factors. Samples could be withdrawn at any time and assayed. To maintain anoxic conditions the tubes were placed inside a nitrogen hood; when aeration was required, a 1 ml reaction mixture in a 13 x 100 mm glass test tube was shaken in air with a Wrist Action Shaker (Burrell Corp.). Figure 20 shows the protocol for a typical bioconversion, that of propionate to acrylate. \textit{C. propionicum}, though obligately anaerobic, tolerates short exposures to oxygen; all procedures therefore, from harvest to bioconversion, were performed in an air atmosphere.
Figure 20 - Protocol for Bioconversions of Propionate to Acrylate with Resting Cell Suspensions of C. propionicum

Grow cells in L-alanine complex medium as described in sections B and C

Centrifuge for 5 min. at 10,000 g at 0°C when in late exponential growth phase (roughly 170 Klett units)

Wash cells 3 times with 0.03% Na₂S in buffer

Resuspend pellet in buffer¹ to a concentration of 100-200 mg wet wt/ml

Pipette 250 µl into a 13 x 100 mm disposable test tube and make up to 500 µl with special components² and buffer

Start reaction by addition of 500 µl propionate reaction mixture (PRM) containing twice strength 200 mmol/l Na-propionate, 25 mmol/l Na-lactate and 5.2 mmol/l methylene blue in buffer

Agitate mechanically at 32°C in air or nitrogen atmosphere

Withdraw 100 µl samples and acidify to stop reaction with 20 µl 50% (v/v) sulfuric acid. Store samples frozen at -10°C.

¹ The buffer is 50 mmol/l TRIS at pH 8.5.
² Special components such as activators, inhibitors, etc.
IV. RESULTS

The results are presented in two sections, A and B. In the former, results are presented on experimentation with Clostridium propionicum. The latter section describes results obtained with Escherichia coli and propionate utilizing isolates.

A. Acrylate Production by Clostridium propionicum

1. Introduction

Experiments were conducted with resting cells except when studying changes in cellular activity during different growth phases. The majority of experiments are therefore organized under the titles "Fermentation by Resting Cells" and "Limitations on Acrylic Acid Production." The remainder of the experiments, such as "Anaerobic Production of Acrylate," "Immobilization" and "General Applicability of the System" were a consequence of the first two groups of experiments.

2. Fermentation by Resting Cells

(a) Influence of the Growth Phase on Acrylate Production Capability

The activity of the resting cells on the formation of acrylate had been observed empirically to vary greatly with the growth phase from which the organisms were harvested. An
experiment was conducted in which acrylate production was correlated with culture harvest time. Between 2.0 and 3.0 g cells wet weight/l, corresponding to 260 klett units were produced after 24 h growth on L-alanine, complex medium, when inoculated with 5% of a 36 h grown starter culture. Cells were harvested at different times during the growth cycle, washed immediately and used in the bioconversion reaction system according to the protocol described in Figure 20, using 200 mg wet weight cells/ml.

As seen in Figure 22, cells harvested in the late exponential phase, corresponding to between 150 and 200 klett units, are most active in the conversion of propionate to acrylate. Both the initial rate and total amount of acrylate produced reached a maximum with cells harvested in this phase of growth.

The extracellular accumulation of volatile fatty acids during the course of growth is shown in Figure 23; 70 mmol/l propionate and 37 mmol/l acetate are produced after 24 h of fermentation. Additionally, isobutyrate, isovalerate, and butyrate are produced early in the fermentation following which they remain at constant concentration.

(b) Endogenous Metabolism

To examine endogenous metabolism, cells harvested by centrifugation were resuspended in pH 8.0 buffer at 32°C
Correlation of harvest time and acrylate production capabilities of Clostridium propionicum.

Figure 22:

- O- INITIAL RATE OF ACRYLIC ACID PRODUCED, mmol / 1-hr
- TOTAL ACRYLIC ACID PRODUCED, mmol / l

TIME (HOURS)

CELL MASS (KITT UNITS)

RESTING CELLS
Figure 23 - Accumulation of volatile fatty acids during the growth cycle of *Clostridium propionicum* grown on L-alanine, complex medium.
without a carbon or nitrogen source in a nitrogen atmosphere. The aliquots of the culture were removed at different times and assayed for volatile fatty acids. The results are shown in Figure 24a. Propionate and acetate were produced in the ratio of 2:1. Other volatile, short-chain fatty acids produced were butyrate, isobutyrate and isovalerate.

Aerobic endogenous metabolism of the resting cell suspension in buffer resulted in less than 0.5 mmol/l butyrate and less than 0.05 mmol/l isobutyrate and isovalerate. As shown in Figure 24b, propionate formation did not occur and only 11 mmol/l acetate was produced compared to 16 mmol/l produced anaerobically. In both experiments on endogenous metabolism, 200 mg wet weight cells/ml were used.

(c) Metabolism of Lactate and Acrylate by Resting Cells

Varying concentrations of acrylate, from 0 to 250 mmol/l, were added to resting cells and its uptake measured under anaerobic conditions buffered at pH 8.5 by 50 mmol/l TRIS. The relation between rate of acrylate consumption and initial concentration follows first order reaction kinetics, as can be seen in Figure 25a. The highest concentration studied, 18.5 g/l (250 mmol/l), is consumed at a rate of 60 g/l-h (0.81 mol/l-h). The specific rate is 4.05 mole/gm wet weight cells-h.

The mass balance for metabolism of 125 mmol/l acrylate is shown in Figure 25b. The acrylate is converted
Figure 24a - Endogenous anaerobic accumulation of short chain volatile fatty acids by <em>C. propionicum</em> resting cells
Figure 24b - Endogenous aerobic accumulation of short chain volatile fatty acids by *C. propionicum* resting cells
Figure 25a - Kinetics of acrylate uptake by resting cells of Clostridium propionicum under anaerobic conditions
Figure 25b - Mass balance for 125 mmol/l acrylate following anaerobic consumption by C. propionicum resting cells.

- Acrylate Uptake
- Total Volatile Acids Produced
- Propionate Produced
- Acetate Produced
- Lactate Produced
- β-Hydroxy propionate Produced
entirely to 40 mmol/l propionate and 23 mmol/l acetate, 65 mmol/l lactate and 4 mmol/l β-hydroxypropionate within 40 min. The stoichiometry may be written as

6 acrylate → acetate + 2 propionate + 3 lactate

The stoichiometry, however, is not always the same. 50 mmol/l acrylate is converted entirely to 33 mmol/l propionate and 19 mmol/l acetate. When incubated in an aerobic environment, acrylate remains unfermented and is entirely recoverable as the monomer after 5 h of incubation.

Lactate metabolism by anaerobic, resting cells showed that 50 mmol/l lactate is fermented at a rate of 10 mmol/l-h; this is roughly 15% of the rate at which an equal concentration of acrylate is metabolized. Acetate and propionate are the only products.

Aerobic consumption of lactate also occurs, but without propionate production.

3. Propionate Dehydrogenation by Resting Cells

(a) Activation by Lactate

It was observed that the addition of 20 to 30 mmol/l lactate to a reaction mixture, containing 200 mmol/l propionate and other bioconversion reaction mixture components, stimulated the rate of conversion of acrylate from propionate.
The results in Figure 26a show that 25 mmol/l L-alanine or pyruvate can be used to substitute for lactate with nearly the same stimulation of acrylate productivities. Addition of 25 mmol/l β-hydroxy-propionate or β-alanine has no effect. The amount of acrylate produced is equal to the amount produced in the absence of lactate, pyruvate or L-alanine.

The amount of lactate consumed was equal to the amount of acetate produced which was calculated as the total amount of acetate measured, minus the acetate produced endogenously by the cells. The results are shown in Figure 26b.

(b) **Optimum Propionate Concentration**

A study to determine the optimum concentration of substrate with respect to both maximum acrylate productivity and maximum yield was conducted. Using cells harvested from the mid log phase of growth (see section 2),

the greatest total amount of acrylate produced after 3 h of bioconversion was 40 mmol/l. This could be achieved with propionate concentrations of 200 mmol/l and above. However, as pointed out, not every batch of cells harvested during the mid log phase is capable of producing as high as 40 mmol/l acrylate from 200 mmol/l propionate, for reasons which have yet to be determined. Such cells are referred to as being "not optimally active." With one such batch of cells, only 6 mmol/l acrylate was produced from 200 mmol/l propionate.
Figure 26a - Effect of substituting 25 mmolar lactate with equimolar L-alanine, pyruvate or \( \beta \)-alanine on acrylate production

![Graph showing the effect of substituting lactate with various compounds on acrylate production](image-url)
Figure 26b - Uptake of 25 mmolar lactate and acetate production during a normal bioconversion of propionate to acrylate
In Figure 27a, this amount of acrylate is given the value 1, and other quantities of acrylate produced are plotted relative to it. As can be seen in Figure 27a, by increasing the concentration of the substrate, "non-optimally active" cells continue to produce greater quantities of acrylate. The correlation between substrate concentration and acrylate is linear, at least up to 800 mmol/l propionate, the highest concentration tested. The open and closed circle points in Figure 27a are data from separate experiments.

Other data shows that the initial rate of acrylate production also increases with increasing substrate concentration. At 30 mmol/l propionate concentration the initial rate of acrylate production was 20 mmol/g wet weight cells-h; at 100 mmol/l propionate the rate was 22 mmol/g wet weight cells-h; at 300 mmol/l - 25 mmol/g wet weight cells-h; at 400 mmol/l - 60 mmol/g wet weight cells-h and at 800 mmol/l propionate the initial rate was 420 mmol/g wet weight cells-h. As can be seen, there is a substantial increase in initial rates at propionate concentrations above 300 mmol/l.

In another experiment, the results of which are presented in Figure 27b, the conversion of propionate was determined. The cell batch used produced 40 mmol/l acrylate from 200 mmol/l propionate, a conversion of 25%.

The yield of acrylate from propionate is speculated to be 100%.
Figure 27a - Total acrylate production as a function of propionate concentration
Figure 27b - Conversion of propionate to acrylate
Figure 28 - Kinetic profile of acrylate production from propionate, using the optimized bioconversion system.
4. Factors Limiting Acrylate Production

It had been continually observed that acrylate production ceased within 180 minutes of initiation of the bioconversion. Furthermore, a maximum of 400 mmol/g wet cell weight is produced. Figure 28 is presented by way of illustrating the maximum productivity of acrylate obtained, and the observed ceasure of acrylate production. Possible factors limiting the productivity of acrylate are examined in this section. Product inhibition, biocompatibility of redox dyes, oxygen toxicity, polymerization of acrylate, etc. are amongst factors investigated.

(a) Product Inhibition

The effect of product concentration on the initial rates of the bioconversion was examined. Acrylate (0-34 mmol/l) was added at the start of the bioconversion to the reaction mixture, as a potential inhibitor. The reaction mixture was sampled periodically to estimate initial rates of acrylate production from propionate. Michaelis-Menten type saturation kinetics were not observed with substrate concentrations ranging between 0 and 300 mmol/l propionate. A linear relationship, as shown in Figure 29a, was observed.

Increasing the concentration of acrylate added to the reaction mixture at the start of the bioconversion, has the effect of decreasing the conversion of propionate to acrylate.
Figure 29a - Inhibition of the bioconversion by acrylate
Figure 29b - Double reciprocal plot of the acrylate inhibition kinetics

-17 mM ACRYLATE
-13.5 mM
-5.5 mM
-NO ACRYLATE

1/S (1/mmolar propionate)

1/V (hr/mmolar)
34 mmol/l (2.5 g/l) acrylate added to the reaction mixture containing 200 mmol/l propionate totally prevents the bioconversion from occurring. This concentration of acrylate, it should be noted, is close to the maximum amount of acrylate accumulation normally observed.

A double reciprocal plot of the data in Figure 29a is presented in Figure 29b. A decrease in $V_{\text{max}}$ and an increase in $K_m$ are clearly observed.

(b) Irreversible inhibition of cellular proteins by Acrylate

Irreversible inhibition of cellular proteins by acrylate could not be ruled out, especially since acrylate is a known alkylating agent. Non-site specific irreversible inactivation of enzymes was studied by addition of 0-10 mg protein (bovine serum albumin) per ml, to the reaction mixture described in Figure 42, and containing 200 mg wet weight cells per ml. Addition of protein to a constant limiting concentration of acrylate with cells in the bioconversion reaction mixture would be observed as an increased activity of acrylate production capability owing to the lower irreversible inactivator to protein ratio. However, as can be seen by comparison with the control experiment in Figure 30, no such effect was observed even on the addition of 10 mg/ml protein, a doubling of protein in the reaction mixture.
Figure 30 - Effect of supplementing the bioconversion medium with protein, antipolymerizing agent or nutrients on acrylate productivity
Dimerization and polymerization of acrylate is known to occur in the presence of oxygen. It was also observed that following bioconversion the physical appearance of the resting cells had changed to a viscous gellified sediment. In order to prevent the potential occurrence of polymerization, a final concentration of 200 ppm paramethoxyphenol was maintained in the bioconversion reaction mixture. Again, as is seen in Figure 30, no improvement in acrylate productivity was observed.

Though it had previously been observed that acrylate productivity increased linearly with increasing concentration of cell suspension between 0 and 5 mg protein/ml, other constituents being kept constant (Figure 42), the possibility of nutrient limitation was examined. The addition of 10 mg/ml yeast extract, 20 mmol/l lactate, 50 mmol/l phosphate and MgCl₂ did not increase acrylate productivity either but, on the contrary, decreased with the addition of phosphate and MgCl₂. This data is also shown in Figure 30.

(c) Effect of Methylene Blue on Acrylate Production

Owing to the uncertain biocompatibility of chemical dyes, the effect of methylene blue on acrylate production capability was examined. A 200 mg/ml wet cell suspension in TRIS pH 8.5 at 32°C in a nitrogen atmosphere was incubated with varying concentrations of methylene blue (0-12 mmol/l)
Figure 31 - Inactivation of acrylate production capabilities in the presence of methylene blue
for 3 hours following which the suspensions were reconstituted to similar cell and methylene blue concentrations by dilution or addition of the dye. Bioconversion reaction components were added such that final concentrations of all components were as shown in Figure 42. As can be seen from the data in Figure 31, a clear correlation between the concentration of methylene blue used and a decreasing acrylate production capability was observed. Other electron acceptors have been used, in particular 2,6 dichlorophenol indophenol, and quinhydrone. Though in neither instance was increased acrylate productivity observed, in the latter, acrylate production and lactate uptake were inconsistently but often observed to occur with attenuated acetate production. This data, because of its preliminary and inconclusive nature, is not presented.

5. Oxygen Inactivation

(a) Having determined that acrylate inhibits the bioconversion of propionate to acrylate in the presence, but not in the absence of air (Figures 25 and 29a), experiments were conducted to determine the contribution of air in inactivating acrylate production capabilities of the cells. In order to determine a correlation between duration of exposure of cells to air and the subsequent acrylate production capability of the cells, samples of a 200 mg wet cell suspension in 50 mmol/l TRIS buffer pH 8.5 at 32°C were agitated in air atmos-
Figure 32- Inactivation of acrylate production capability by oxygen

(Cells incubated in N$_2$ or Air prior to Bioconversion)
phere by a wrist action shaker for between 0-3 hours. At intervals, a sample of cell suspension was withdrawn, reconstituted with propionate, lactate, methylene blue, etc. to final concentrations as shown in Figure 42. The acrylate production activity was determined as described in Figure 20.

The data is unambiguous in that cellular capacity to produce acrylate decreases with increasing duration of exposure of cells to air. A linear decrease in activity with time is shown in Figure 32.

In a parallel experimental control, samples of the cell suspension were exposed to a nitrogen, not air environment. The result, interestingly, was an increase in acrylate production capabilities with duration of incubation. Data is presented in Figure 32.

(b) If oxygen inactivates acrylate production capability, it must be eliminated and replaced by a more suitable electron acceptor, or at least be decreased to tolerable concentrations.

The results obtained by excluding oxygen from the reaction mixture, keeping all other components, and environmental variables constant, is shown in Figure 33. Anoxic conditions were maintained in a nitrogen atmosphere. Production of 4.5 mmol/l acrylate is followed by its rapid disappearance. The disappearance begins following the reduction of methylene blue, observed as a change in color from blue to colorless.
Figure 33- Anaerobic bioconversion of propionate to acrylate in nitrogen atmosphere

Graph showing the bioconversion of propionate to acrylate with two different cell protein concentrations: 2 mg/ml and 0.4 mg/ml. The graph illustrates the total reduction of redox dye over time (hours).
The concentration of methylene blue was found to be limiting to the bioconversion. In order to determine the maximum duration for which acrylate can be produced anaerobically (compared to 3 hours during aerobic bioconversion), the methylene blue concentration had to be made non-limiting. Since the data in Figure 31 already determined that a loss in acrylate production capability would follow the use of higher methylene blue concentrations, this was achieved by decreasing cell concentration to 0.4 mg protein/ml.

The results, Figure 33a, were encouraging; acrylate production under anoxic conditions proceeded beyond 3 hours. The increased duration of acrylate production was, however, quite expectedly, achieved at the expense of production rate.

5. Stability and Regeneration of Cells

(a) It was of interest to know the meaning of "oxygen inactivation", translated into observable changes in metabolism, other than the cessation of acrylate production capabilities.

As are shown in Figures 34b and 34c, respectively, it results in the cellular inability to reduce acrylate and in an altered propionate to acetate production ratio following lactate uptake.

In Figure 34b, data was obtained by transferring a suspension of cells producing acrylate (as seen by open
Figure 34a- Acrylate production with cells preincubated with lactate or acrylate
ircles) from air atmosphere to nitrogen atmosphere at times marked by arrows. The fate of acrylate following nitrogen sparging and incubation of the sample is shown by closed circles. Evidently, cells are still able to reduce acrylate following 1.5, but not 3 hours of air exposure and acrylate production. The 1-2 hour lag and slight increase in acrylate, prior to which the cells reduce acrylate is probably a result of the oxidized methylene blue remaining in the reaction mixture.

In Figure 34c, data is presented on the effect of air exposure on cellular ability to metabolize lactate. Following varying durations of exposure to air as a result of agitation by a wrist action shaker at 32°C, samples of 200 mg/ml wet weight cell suspension were sparged with nitrogen and placed in a nitrogen atmosphere. 100 mmol/l lactate was added and 3 hours later the amount of acetate and propionate accumulation were quantified. The bar chart shows data that without air exposure, both propionate and acetate are produced in a ratio anticipated by intramolecular electron balance. Following 3 hours of air exposure, equimolar quantities of the two are produced; following 6 hours exposure lactate metabolism to propionate ceases and less than 5 mmol/l acetate is produced. The product ratios are correlated with time of air exposure in the accompanying graph in Figure 34c.
Figure 34b— Effect of air exposure on cellular ability to reduce acrylate

- IN AIR ATMOSPHERE
- IN NITROGEN ATMOSPHERE
Figure 34c - Effect of air exposure on cellular ability to metabolize lactate anaerobically.

**Diagram Description:**
- **Y-axis:** Lactate, Acetate, Propionate concentration (mmol/l)
- **X-axis:** Time (Hours)
- **Graph 1:**
  - Lactate added (100 mmol/l)
  - Acetate produced (80 mmol/l)
  - Propionate produced (40 mmol/l)
- **Graph 2:**
  - Molar ratio of propionate to acetate produced from lactate anaerobically.
  - Time (Hours): 0, 3, 6, 9
  - Molar ratio: 3, 2, 1, 0
(b) The effect of air exposure on C. propionicum resting cell suspensions is to at least prevent acrylate uptake and propionate production. However, in contrast, anoxic incubation has the effect of increasing the cellular ability to produce acrylate (Figure 32). This effect is enhanced considerably when anoxic incubation in a nitrogen atmosphere of C. propionicum resting cell suspension prior to the bioconversion (called "preincubation" hereafter) is performed with either lactate or acrylate added to the reaction mixture. Seen in Figure 34a, the enhancement in acrylate producing capability is a function of lactate or acrylate concentration added during preincubation. The enhancement is also increased with longer durations of preincubation, at least up to 6 hours. In addition, the enhancement never caused the cells to accumulate greater than 40 mmol/l acrylate, though often it would result in a lower acrylate productivity (Figure 34a).

Other interesting data, though not presented graphically, is that the enhancement in acrylate production capability following preincubation is dependent upon the time of the harvest. Cells harvested in the early, mid-exponential or early stationary phase, possessing lesser capability to produce acrylate from propionate (Figure 23), than cells harvested in the late exponential growth phase, are maximally enhanced following preincubation. Typically, cells harvested in mid-exponential growth phase producing 5-15 mmol/l acrylate from
propionate are able to produce 30-40 mmol/l acrylate from propionate following preincubation. Cells harvested in the late exponential growth phase and producing 35-40 mmol/l acrylate from the bioconversion of propionate do not accumulate greater concentrations of acrylate following preincubation. The rate of acrylate accumulation was observed to increase following preincubation of cells harvested in mid-exponential growth phase; it was not measured in late exponential growth phase cells.

(c) Cells which are inactivated following air exposure are inactivated with respect to their ability to produce acrylate from propionate are not reactivated upon lowering of the redox potential by addition of cysteine or thioglycollate (1 mg/ml) nutrients such as yeast extract (1 mg/ml). However, addition of L-alanine, singly or in conjunction with cysteine to such cells (as described above) results in renewed acrylate production capability; 5-10% of the total amount of acrylate produced by freshly harvested cells can be produced by reactivated cells.

6. Production of Crotonate and Methacrylate

(a) Crotonate

L-Threonine is metabolized by a sequence of reactions in C. propionicum, that are similar to the sequence
of L-alanine metabolism, as discussed in Section II.D.2. The products, however, are propionate and butyrate. It follows then that just as propionate is dehydrogenated to yield acrylate, butyrate may be dehydrogenated to yield crotonate.

Using the conditions standardized for acrylate production from propionate, 500 mmol/l butyrate yielded 20 mmol/l crotonate. The peak observed on the Thermon column was identified by gas chromatography-mass spectrometry techniques (see Sections III.H and I). The chromatogram itself is shown in Figure 36a. The mass spectrum of a standard sample of crotonic acid (MW = 86) in diethyl ether could be matched with the mass spectrum of the peak shown in Figure 36a, corresponding to crotonic acid and appearing as a result of butyrate bioconversion by *C. propionicum*. This data is shown in Figure 36b. Additionally, the spectrum index number of the compound - allegedly crotonic acid, obtained from the total ionization plot, could be matched with the spectrum index number of a standard crotonic acid spectrum index number.

In Figure 36b, 2 spectrum index plots are presented (0-100 and 100-200). Every 5-6 sec a scan is made of the ionization fragment. The distribution of MW=86 compounds is marked as MW=86 on the figure. Superimposed on the total ionization plot, it can be seen that the peak - allegedly crotonic acid - and the distribution of MW = 86 coincide. These were accepted as sufficient evidence that a product of butyrate bioconversion was crotonate.
Figure 36a - Gas-liquid chromatogram of products from the aerobic bioconversion of butyrate by C. propionicum resting cells
Figure 36b - Gas chromatography-mass spectrometry identification of crotonic acid

Mass Spectrum of Crotonic Acid (M.W.=36)

Mass Spectrum of Butyrate Bioconversion Product

M/E = 86 Distribution Superimposed on a Total Ionization Plot

Spectrum Index Number (= Retention Time)
Figure 36c - pH profile for the bioconversion of butyrate to acrylate using conditions otherwise standardized for acrylate production from propionate.
The kinetics for the anaerobic uptake of butyrate are classical Michaelis-Menten type (Figure 36c), unlike the first order reaction kinetics of acrylate uptake. Butyrate uptake was measured also as crotonate appearance, and both values are plotted.

Since the preliminary bioconversion had been conducted with conditions standardized for acrylate production, it was anticipated that further optimization may lead to greater crotonate productivity. Only the pH profile has been determined yet. As seen in Figure 36d, it is different from that of acrylate.

(b) Methacrylate

Unlike crotonate, methacrylate is not known to be a metabolic intermediate in C. propionicum. Nevertheless, 400 mmol/l isobutyrate, when used to substitute either propionate or butyrate in the bioconversion system, a GC peak was observed with a retention time coinciding with the retention time of standard methacrylate, as shown in Figure 37a. This peak elutes on the tail of isobutyrate, making GC-MS analysis problematic. Nevertheless, repeated GC analysis, comparison with standards and the use of appropriate controls, it is strongly suggested that the peak is methacrylate occurring as a result of the bioconversion of isobutyrate, catalysed by C. propionicum. As much as 6 mmol/l are produced from 500 mmol/l isobutyrate.
Figure 36d - Kinetics of crotonate uptake by resting cells of Clostridium propionicum under anaerobic conditions
Figure 37a - Gas-liquid chromatogram of products from the aerobic bioconversion of isobutyrate by C. propionicum resting cells.
Figure 37b - Correlation of methacrylate production with varying pH and lactate concentrations, by C. propionicum resting cells
Figure 37c - Kinetics of methacrylate uptake by resting cells of Clostridium propionicum under anaerobic conditions
The bioconversion is also sensitive to pH and lactate concentration. However, since the quantities produced are so small, there is considerable variation in results obtained with GC analysis. Results are shown in Figure 37b.

The anoxic uptake of methacrylate shows saturation kinetics, but a very low $K_m$, Figure 37c.

Having observed differences in the rate of acrylic, crotonic and methacrylic uptake, and their different $K_m$ values it was suggested that methacrylate may competitively bind to the propionyl-CoA(butyryl-CoA?) dehydrogenases, decreasing acrylate uptake. The data of numerous experiments measuring the rate of uptake of one of the three above mentioned compounds in the presence of the other showed there was no rate inhibition of one by the other.

B. Studies on Acrylate Production by Escherichia coli

1. Introduction

It has been observed (Section II.F) that in the aerobic oxidation of propionate, acrylyl-CoA was, in all probability, an intermediate (Figure 8). It was reasoned that in such an organism, genetic lesions could be made which would cause the cells to produce and accumulate acrylate from propionate. As seen from Figure 8, such a mutant, unable to metabolize propionate
beyond acrylyl-CoA, might cause the latter compound to deacetyl-ate and accumulate as free acrylate. In *E. coli* W, it was hypothesized, two enzyme lesions were required, one to prevent hydration of acrylyl-CoA and the other to prevent the branch reaction of propionate metabolism, catalysed by 2-hydroxyglutarate synthase [EC 4.1.3.9].

Resting cells of the above mutant could conceivably be used to effect the bioconversion of acrylate to propionate, since such a double mutant would not grow on propionate.

The results obtained with *E. coli* are summarized in this section. Though the major effort was in obtaining mutants, data are presented on growth kinetics, resting cell suspensions, and cell free extracts.

Additionally, prior to recognizing certain advantages of working with *E. coli* W, numerous soil isolates were tested for their ability to produce acrylate. These results are presented briefly under the sub-heading of Isolates.

2. Isolates

Microorganisms were obtained from soil by incubating 500 mg soil samples in 100 ml of mineral salts medium at pH 7.0 and containing 2 g/l Na-propionate as the sole source of carbon, supplemented with 0.15 g/l yeast extract and incubated aerobically at 30°C. Organisms from growing cultures were isolated on agar plates containing 2 g/l propionate and subsequently examined for growth on acrylate.
Several organisms were isolated by this method; they included eubacteria, mycelial organisms and yeast. Some representative isolates are described below; 530-10, 1-2 mm diameter circular yellow colonies with entire margin, stained gram positive cocci in pairs and clusters; 530-20, less than 1 mm circular gray colored colonies with entire margins, stained gram positive dumbell shaped rods in pairs, 530-40, 2 mm circular/rhizoid form, white colonies with circular margin, oval budding cells which stained gram positive (on extended storage circular form becomes rhizoid with growth of mycelia like structures); 66-01, 0.5 to 2 mm filamentous form, greenish-brown colonies with umbonate elevation; 66-02, filamentous white mycelia growing evenly over the surface of the plate; 530-60, 1 mm filamentous form, white colonies with mycelia growing from erose margin.

Results obtained with two isolates, shown in Figure 39a, are representative of the group of organisms which grew on propionate, but not on acrylate. In the presence of 4 g/l propionate alone, growth is rapid ($\mu = 0.35 \text{ h}^{-1}$), and maximum cell density occurs between 12 and 24 hours after inoculation with a 2 to 5% inoculum. With both 2 g/l acrylate and 2 g/l propionate present as substrate, the cell density is less than that expected from the growth on 2 g/l propionate alone. Addition of 2 g/l acrylate has the effect of lowering the yield on propionate by slightly more than half with isolate 530-10 and
Figure 39a - Aerobic growth of soil isolates on propionate and propionate plus acrylate as the sole carbon sources.
Figure 39b - Aerobic growth of a yeast isolate (530-40) on propionate and acrylate
to nearly zero with isolate 530-20. One set of organisms is totally unable to grow in the presence of acrylate, whereas the other set is partially inhibited. In Figure 39b, is shown the growth of yeast isolate 530-40, which is an example of an isolate capable of growing on both substrates, acrylate and propionate. The biphasic growth is apparently caused by depletion of yeast extract components.

Acrylate was, however, not detected in the supernatant of propionate growing cells. A 10% resting cell suspension of washed exponentially growing 530-40 cells in 30 mmol/l phosphate buffer, pH 7.0 at 30°C was able to oxidize both 5 g/l propionate and acrylate. The reaction mixture however did not contain either acetate or other volatile fatty acids as measured on the DEGS column. Acrylate accumulation did not occur even following 120 min incubation.

The cells were able to grow on glucose and β-alanine in the presence of 0.15 g/l yeast extract and most interestingly on up to 8 g/l acrylate.

3. **Growth Kinetics of E. coli W**

The growth kinetics of *E. coli* W measured on media containing 2 g/l propionate (26 mmol/l), acrylate (27 mmol/l), glucose (11 mmol/l), glucose (11 mmol/l) plus 1 g/l acrylate (13.5 mmol/l), propionate (26 mmol/l) plus trace amounts of glucose (0.5 mmol/l), propionate (26 mmol/l) plus 1 g/l acrylate
Figure 40 - Growth kinetics of *E. coli* W on propionate and other carbon sources
(13.5 mmol/l), and acetate (33 mmol/l). The results are presented in Figure 40. The inoculum were exponentially growing cells cultured in media containing 26 mmol/l propionate.

Growth on propionate minimal medium was preceded by a lag of 48 to 72 hours prior to exponential growth when inoculated from stationary phase cultures. This lag could be reduced by inoculating with exponentially growing cultures or by adding trace amounts of glucose (0.5 mmol/l) or yeast extract (0.1 g/l) to inoculum in the stationary phase. In the presence of trace quantities of glucose or yeast extract, di-auxie was observed. The doubling time of E. coli W was 510 min on propionate and 90 min on glucose.

Acrylate did not support growth, but neither did it totally inhibit its growth on glucose or propionate. Addition of 2 g/l (27 mmol/l) acrylate increases the doubling time of the organism on 2 g/l (11 mmol/l) glucose from 90 to 360 min. Further experiments were conducted with strains isolated from cultures which grew well on glucose in the presence of acrylate.

The profile of substrate usability is also shown in Table 9.

4. Resting Cell Metabolism

In the next set of experiments, the metabolism of 2 g/l propionate by resting cell suspensions was investigated. Resting cell metabolism was of particular significance since the
mutant in question, unable to grow on propionate, would necessarily have to catalyse the bioconversion as a resting cell suspension.

A 10% wet weight resting cell suspension of the wild type strain was prepared after growth on 2 g/l propionate at 37°C and pH 7.0 by harvesting during the exponential growth phase. The data in Table 9 show that propionate is oxidized at 0.3 g/l-h. The uptake is completely inhibited in the presence of 2 g/l lactate and 1 g/l propionate; 1 g/l acrylate added to the resting cells was not metabolized. It did, however, weakly inhibit propionate uptake.

Numerous analogs of acrylate were used in anticipation that the activity of lactoyl-CoA dehydratase might be inhibited. The results obtained with their use are tabulated in Table 9. Propionate was effective in preventing propionate uptake. In the presence of acrylate, the rate of propionate uptake decreased from 0.3 g/l-h to nearly 0.2 g/l-h. None of the analogs resulted in the detection of acrylate accumulation.

5 g/l of Na-lactate was also studied as a substrate. A major product of lactate uptake was an unidentified non-volatile fatty acid, whose retention time on the DEGS column coincided with oxalacetic acid. In the presence of acrylic acid, despite the uptake of 2.85 g/l of lactate, interestingly, this product was not formed.
### Table 9 - Oxidation of propionate by resting cells of Escherichia coli in the presence of substrate analogs and enzyme inhibitors

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc. g/l</th>
<th>Time (min)</th>
<th>Residual Propionate (g/l)</th>
<th>Residual Lactate (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>30</td>
<td>90</td>
</tr>
<tr>
<td>Propionate</td>
<td></td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Propionate + Glyoxylate</td>
<td>2.0</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Propionate Anaerobically</td>
<td>2.0</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Propionate + (NH₄)₂SO₄ 0.1%</td>
<td>2.0</td>
<td>2.0</td>
<td>1.83</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.99</td>
<td>1.05</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td></td>
<td>1.04</td>
<td>-</td>
</tr>
<tr>
<td>Propionate + HCO₃⁻ 0.1%</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.0</td>
<td>1.97</td>
<td>-</td>
</tr>
<tr>
<td>Propionate + Lactate 0.2%</td>
<td>2.0</td>
<td>2.0</td>
<td>1.72</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.72</td>
<td>1.36</td>
<td>-</td>
</tr>
<tr>
<td>Propionate + B hydroxy-</td>
<td>2.0</td>
<td>2.0</td>
<td>1.85</td>
<td>1.78</td>
</tr>
<tr>
<td>propionate 0.1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate + acrylamide 0.1%</td>
<td>2.0</td>
<td>2.0</td>
<td>1.85</td>
<td>1.78</td>
</tr>
<tr>
<td>Propionate + acrylate 0.1%</td>
<td>2.0</td>
<td>2.0</td>
<td>1.85</td>
<td>1.78</td>
</tr>
<tr>
<td>Propionate + cyclopropane</td>
<td>2.0</td>
<td>2.0</td>
<td>1.84</td>
<td>1.72</td>
</tr>
<tr>
<td>Carboxylate 0.1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2.0</td>
<td>1.66</td>
<td>1.62</td>
</tr>
<tr>
<td>Propionate + oxalate 0.1%</td>
<td>2.0</td>
<td>2.0</td>
<td>1.66</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>5.0</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ propionate 0.2%</td>
<td>5.0</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ oxamate 0.2%</td>
<td>5.0</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ acrylate 0.1%</td>
<td>5.0</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ arsenite</td>
<td>5.0</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
5. **Mutation, Enrichment and Selection**

In order to obtain a mutant which cannot oxidize propionate beyond acrylyl-CoA, a series of mutation, enrichment and selection experiments were conducted with *E. coli* W.

Mutation conducted with EMS using the method of Olson *et al.* (1962) described in Figure 18, resulted in roughly 70% kill of viable cells. To determine the extent of mutagenesis, a control was performed in which the creation of auxotrophs was enumerated. Mutagenized cells were recovered in trypticase soy broth and plated on both trypticase soy and glucose-mineral salts agar. 10 to 20% of the cells could not grow in glucose mineral salts medium without the addition of amino acids. This figure gave an indication of the extent of mutagenesis occurring.

To enrich acetate negative cells, the culture recovered in glucose was transferred to 2 g/l acetate mineral salts medium. *E. coli* W grew on acetate with a specific growth rate of 0.15 h⁻¹. Therefore, following two doublings of the culture on acetate (the turbidity was measured using a side arm flask and linearized as described in Materials and Methods), penicillin was added for a duration of between 260 and 480 min. Antibiotic enrichment of the mutants was performed in both hypertonic and normal media with no difference in results. In the latter case, the method was also modified to centrifuge or
milipore filter the cells every 2 hours to minimize cross feeding of mutants by lysing cells. Following centrifugation or filtration, cells were resuspended in acetate and permitted to enter exponential growth prior to repeating antiobiotic enrichment. During enrichment, a turbidity decrease of 60 to 70% was observed and the number of viable cells decreased $1 \times 10^{-5}$. An 80% turbidity decrease occurred within the first doubling period after antibiotic addition, the remaining 20% over the next 2 to 3 doubling times. Surviving cells were mostly antiobiotic sensitive.

The maximum enrichment of acetate negative mutants was 0.005%, obtained with ampicillin enrichment.

Propionate negative organisms were enriched similarly, with 2 g/l propionate replacing the acetate. The doubling time on propionate is 510 min, the antibiotic was added following entry of the cells into the exponential growth phase, for a duration of 8 to 15 hours.

Maximum enrichment of propionate negative organisms was between $0.1 \times 10^{-4}$.

From amongst the acetate negative mutants isolated by differential plating with acetate and glycerol to select for pinpoint colonies unable to grow on acetate, two of nearly one hundred negative mutants were scored as isocitrate lyase negative ($ace^A$) owing to their inability to grow on acetate alone within 72 hours, but being able to do so on acetate and
glyoxylate together. Such mutants were also able to grow on propionate. However, neither was stable and both reverted to acetate positive following extended growth on acetate or propionate. Five propionate negative mutants were selected by scoring for growth after spraying lactate on agar plates as described in Section III.K.

These mutants were examined for their ability to accumulate acrylate from propionate. They were grown in 2 g/l lactate minimal medium, harvested during the exponential growth phase and subjected to resting cell bioconversion at pH 7.0 and 37°C as described with the wild type under the section Resting Cell Suspensions. However, no acrylate was found to accumulate; in fact, the 5 g/l propionate remained unoxidized.
V. DISCUSSION

This chapter is divided into three sections: (A) A perspective of approaches taken toward acrylate production and preliminary findings; (B) Acrylate production by *Clostridium propionicum*; and (C) Studies on acrylate production by *Escherichia coli* W.

A. A Perspective of Approaches taken and Preliminary Findings

1. Selection of Organisms

The purpose of this section is to summarize the preliminary work directed towards the microbial production of acrylate. At the onset, it was necessary to choose organisms appropriate for the study, e.g. having the correct biochemical pathways, since an organism with the ability to produce acrylate from cellulose was not described in the literature. Therefore, organisms were chosen in which acrylate or its coenzyme A thiol-ester was hypothesized to exist as an intermediate.

As cited in the Literature Review, it has been suggested that the anaerobes *Megasphaera elsdenii* and *Clostridium propionicum* possess these intermediates when metabolizing lactate. From amongst the aerobes, *E. coli* was selected on the basis of published evidence suggesting that propionate metabolism was via acrylyl-CoA. Additionally, numerous organisms
were isolated from soil which could grow on both acrylate and propionate. These isolates are described later in this chapter.

2. Preliminary Findings with Anaerobes

Neither growing nor resting cells of *Megasphaera elsdenii* were found to accumulate acrylic acid (Retamal, 1978). Lactate and glucose when used as the substrate are metabolized to acetate, propionate, butyrate and other larger fatty acids. In view of the complex nature of metabolism in this organism, work with it was discontinued. Attempts to identify acrylic acid as a product of *C. propionicum* growth on L-alanine or β-alanine were unsuccessful. Since lactate and acrylate were inhibitory to the growth of this organism (50% inhibition of the growth rate was caused by 70 mmol/l propionate, 20 mmol/l lactate, 200 mmol/l acetate or less than 5 mmol/l acrylate), fermentation of these and other compounds was studied using resting cells.

Initially, β-alanine grown resting cells of *C. propionicum* were found to transiently accumulate trace quantities of acrylate anaerobically from β-alanine (Akedo et al., 1978). The identification as acrylate was confirmed by GC-MS analysis. Acrylate accumulation also resulted from aerobic propionate oxidation. Under aerobic conditions, acrylate accumulation was stable whereas in an anaerobic environment, acrylate
Table 10 - Aerobic and anaerobic fermentation by *C. propionicium* cells grown on L- and β-alanine

<table>
<thead>
<tr>
<th>SUBSTRATE FOR RESTING CELLS</th>
<th>PRODUCTS OF RESTING CELL METABOLISM mmol/l</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells Grown on L-Alanine</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>Propionate</td>
</tr>
<tr>
<td>ANAEROBIC METABOLISM</td>
<td>Substrate mmol/l</td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Lactate</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>Acrylate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Propionate</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>AEROBIC METABOLISM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactate</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Acrylate</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Propionate</td>
<td>55</td>
<td>2</td>
</tr>
</tbody>
</table>

(1) Akedo (1979)
was rapidly metabolized to acetate and propionate. These results are summarized in Table 10. It is also seen that the metabolic products are in the ratio predicted by electron balances. Interestingly, cells grown on L-alanine were found not to metabolize \( \beta \)-alanine, but the reverse did occur.

3. Optimization of Substrate Concentrations and Environmental Factors for the Production of Acrylate from Propionate

From an economic standpoint, \( \beta \)-alanine is a poor choice as a substrate for acrylate production; it is more valuable than the product. As a consequence, propionate, the only other substrate from which acrylate has been obtained, was the obvious choice for further work. The results in Table 11 summarize the optimal conditions for the propionate to acrylate bioconversion.

Of particular significance is the requirement for 20-30 mmol/l lactate, in addition to propionate. Lactate is believed to facilitate the activation of propionate to propionyl-CoA through the production of acetyl-CoA or ATP. Since the uptake of lactate is not stoichiometric with acrylate produced, it is hypothesized that acetyl-CoA not ATP activates propionate via the action of a transferase operating on acetyl-, lactate-, acrylyl- and propionyl-CoA. From observations made with cell free extracts of *C. propionicum*, it has been possible to conclude that propionate activation occurs by acetyl-CoA or in the presence of coenzyme A and high energy compounds such as ATP and acetyl-phosphate.
Table 11 - Optimization of the conditions for the bioconversion of propionate to acrylate

<table>
<thead>
<tr>
<th>SUBSTRATES (mmol/l)</th>
<th>pH</th>
<th>t (°C)</th>
<th>CELLS g wet wt/ml</th>
<th>ACRYLATE PRODUCED Specific mmol/gm-hr</th>
<th>Initial Rate mmol/l-h</th>
<th>Total mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) -</td>
<td>7.5 (1)</td>
<td>37</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(2) Lactate, 0-100</td>
<td>7.5</td>
<td>37</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(3) Propionate 25</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>0.8</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>0.91</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>1.25</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>(4) Propionate 200</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>2.2</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>+ Lactate 0</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>3.2</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>+ Lactate 10</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>3.3</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>+ Lactate 20</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>3.4</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>+ Lactate 50</td>
<td>7.5</td>
<td>37</td>
<td>0.053</td>
<td>3.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>(5) Propionate 200</td>
<td>6.0 (2)</td>
<td>37</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>+ Lactate 25</td>
<td>7.0</td>
<td>37</td>
<td>0.1</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>37</td>
<td>0.1</td>
<td>3.1</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0 (3)</td>
<td>37</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>(6) Propionate 200</td>
<td>8.5</td>
<td>37</td>
<td>0.1</td>
<td>1.5</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>+ Methylene Blue 0</td>
<td>8.5</td>
<td>37</td>
<td>0.1</td>
<td>1.5</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>37</td>
<td>0.1</td>
<td>2.0</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>37</td>
<td>0.1</td>
<td>4.0</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>(7) Propionate 200</td>
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<td>37</td>
<td>0.1</td>
<td>5.0</td>
<td>3.5</td>
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<tr>
<td>+ Lactate 25</td>
<td>8.5</td>
<td>37</td>
<td>0.1</td>
<td>6.0</td>
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<tr>
<td>+ Methylene Blue</td>
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<td>0.1</td>
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<td>0.1</td>
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<tr>
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<td>.038</td>
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<tr>
<td>+ L-alanine 20</td>
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<tr>
<td>+ L-alanine 20</td>
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<td>8.5</td>
<td>32</td>
<td>.067</td>
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</table>

(1) Triethanolamine buffer, 50 mmol/l used except where stated.
(2) Citric acid - sodium phosphate dibasic buffer, 50 mmol/l.
(3) Sodium carbonate - sodium bicarbonate buffer, 50 mmol/l.
(4) 20 mmol/l L-alanine was found to have the same effect as 25 mmol/l lactate.
(5) Calculated from protein measurement; cell wet wt = (g protein × 100/15 × 2) g. Conversion used in all following cell wet wt/ml data.
The reactions involved in acrylate production from propionate in the presence of an electron acceptor.

\[
\begin{align*}
\text{CH}_3\text{-CHOH-} & \text{-SCoA} \\
\text{LACTYL-CoA} & \\
\text{H}_2\text{O} & \\
\text{CH}_2=\text{CH-C-SCoA} & \rightarrow \text{ACRYLATE} \\
\text{CH}_3\text{-CH}_2\text{-C-SCoA} & \\
\text{PROPIONYL-CoA} & \\
\end{align*}
\]
The reason for the range of lactate concentration between 20 and 30 mmol/l is not as yet clear, but may have something to do with the observation that above 30 mmol/l, lactate strongly inhibits the growth of the organism on L-alanine.

Alternate electron acceptors, oxygen and/or methylene blue can be used to drive the oxidation of propionate to acrylate. In the absence of oxygen, acrylate accumulation occurs only in the presence of methylene blue. At concentrations greater than 5.3 mmol/l methylene blue has the effect of decreasing total acrylate produced, though the rate of the reaction continues to increase.

Optimum conditions for the propionate to acrylate bioconversion may be summarized as follows: Na-propionate, 200 mmol/l; Na-lactate, 25 mmol/l; methylene blue, 5.3 mmol/l; pH 8.5 using triethanolamine buffer; 32°C temperature and cell concentration equal to 100-200 mg wet wt/ml. The entire reaction mixture is agitated in an air atmosphere.

A schematic of the relevant reactions in the bioconversion of propionate to acrylate is shown in Figure 41. With this as a basis and an understanding of the optimal conditions for the bioconversion, studies were performed with the objective of understanding the mechanism of the reaction and maximizing the rate of acrylate production.
Figure 42 - A schematic representation of the standardized bioconversion system employed for acrylate production from propionate

SUBSTRATE
Na-Propionate, 200 mmol/l

ACTIVATOR
Na-Lactate, 25 mmol/l

ELECTRON ACCEPTOR
Methylene blue (oxidized), 5.3 mmol/l

CELLS
10% Wet Cell Suspension in TRIS, pH 8.5 Buffer

BIOCONVERSION
One ml Reaction Mixture Agitated in Air at 32°C

GAS CHROMATOGRAPHIC ANALYSIS
B. Acrylate Production by Clostridium propionicum

The reaction mixture for acrylate production had, except for certain variables, been standardized. The experiments performed with the optimized bioconversion system were directed along lines to improve the productivity of acrylate, by maximizing cellular activity, by extending the life of catalytic activity, and by increasing both the rate and extent of the bioconversion.

1. Growth Phase and Activity

Variations in cellular activity were found to be associated with the growth phase in which the cells were harvested. It was shown that maximum activity occurred when the cells were harvested in the late exponential growth phase. The standardized bioconversion procedure used during much of this work, and referred to as the standardized bioconversion system, is shown schematically in Figure 42.

Growth of C. propionicum in batch culture on yeast extract and L-alanine follows kinetics typical of culture growing on complex medium; the specific growth rate decreases after the initial exponential growth; this is due to sequential exhaustion of available nutrients. No clear diauxie is apparent and L-alanine is believed to be metabolized throughout the growth phase. The profile of short chain volatile fatty acids
produced during growth compares favorably with the profile observed by Holdeman et al. (1977) and is shown in Appendix I. This profile is unique to *C. propionicum* and was used during this project as an indication of culture purity. 65 mmol/l propionate is produced at the end of the fermentation from 90 mmol/l (8.1 g/l) of L-alanine added initially. It is tempting to conclude that L-alanine is being metabolized entirely to propionate and acetate, in the predicted 2:1 ratio, producing ATP and reduced flavin nucleotides, with little cell mass formation. The specific propionate production rate remains roughly constant at 24 mmol/g wet cells-h during the brief exponential phase. The same production pattern occurs for acetate which is produced at a rate of 12 mmol/g cell mass-h.

Since isobutyrate and isovalerate are products of valine and leucine deamination and decarboxylation, the accumulation of these acids are indicators of yeast extract and peptone metabolism. It appears, therefore, that yeast extract and peptone are degraded early in the growth cycle.

The medium pH, though buffered by only 1 mmol/l phosphate and complex media components at pH 7.0, remains relatively constant, decreasing to only pH 6.7. Cardon and Barker (1946) showed that nitrogen from L-alanine, when fermented by resting cells is entirely recoverable as free ammonia. The dissolved ammonia could serve as a regulator of pH.
No attempt has been made to study the kinetics of substrate utilization or the influence of medium components and environmental factors on the expression of enzymes required for the bioconversion during growth. The medium composition is often crucial in the optimization of fermentations and can be expected to contribute to optimizing cellular activity for acrylate production. A chemically defined medium has been devised. Using this medium, with 5-10 g/l L-alanine, supplemented with 4 g/l ammonium chloride, the cell yield doubled. Such results indicate the potential to make the fermentation more efficient.

2. Fermentation by Resting Cells

Endogenous anaerobic metabolisms by resting cells resulted in the production of acids as was observed during growth. To confirm the source of isobutyrate and isovalerate, 2 g/l valine and leucine were added to the cell suspension. They were converted to 0.25 g/l isobutyrate and 1.3 g/l isovalerate respectively, probably by the following sequence of reactions:

\[
\begin{align*}
\text{Valine} & \quad \text{Isoketovaleter} \quad \text{Isobutyrate} \\
\text{CH}_3 & \quad \text{CH} \quad \text{CH-CO}_2^{-} \quad \text{NH}_3^{+} \quad \text{NH}_3 \quad \text{CH} \quad \text{C-CO}_2 \quad \text{CH}_3 \quad \text{CH-CO}_2^{-} \quad \text{CH}_3 \end{align*}
\]

\[
\begin{align*}
\text{Valine} & \quad \text{Isoketovaleter} \quad \text{Isobutyrate} \\
\text{CH}_3 & \quad \text{CH} \quad \text{CH-CO}_2^{-} \quad \text{NH}_3^{+} \quad \text{NH}_3 \quad \text{CH} \quad \text{C-CO}_2 \quad \text{CH}_3 \quad \text{CH-CO}_2^{-} \quad \text{CH}_3
\end{align*}
\]
Cardon and Barker (1946) demonstrated that amongst other amino acids, d,l-valine, d,l-leucine, l-cysteine and l-glutamic acid were not degraded to propionate, acetate and ammonia, but were acted upon by dehydrogenases which could transfer the electrons to methylene blue. During the fermentation of yeast extract no ammonia was found. However, all of the nitrogen from L-alanine, serine and threonine was recovered as ammonia during resting cell fermentations. This data suggests that the cells obtain nitrogen directly from some amino acids by enzyme mediated reactions and/or from dissolved ammonia.

In summary, *C. propionicum* is capable of degrading amino acids either by utilizing the l-alanine system, as in the case with serine and threonine, or by the action of decarboxylating α-keto acid dehydrogenases, possibly with the involvement of Stickland type intramolecular electron transfer and transamination reaction.
At this point, it will be useful to note that in C. propionicum, isobutyrate formation does not occur via \( \beta \)-hydroxyisobutyryl-CoA and \( \beta \)-unsaturated methacrylyl-CoA as a result of reactions analogous to butyrate formation from threonine, but as previously shown, from valine. In aerobes, however, as mentioned in the Literature Survey when discussing possible routes to methacrylic acid, isobutyrate is metabolized via the 2-unsaturated intermediate. Certain anaerobes such as Clostridium oceanicum (Holdeman et al., 1977) do produce disproportionately large amounts of isobutyrate and isovalerate, and deserve to be examined more closely.

Returning to the discussion of endogenous cellular metabolism, it is seen that air has the effect of selectively preventing propionate production by preferentially accepting reducing equivalents. Under aerobic condition, acetate production decreases either due to oxygen toxicity of alteration in the cellular redox balance.

While looking at the metabolism of lactate and acrylate by resting cells, the rapid uptake of acrylate by anaerobically metabolizing cell suspensions stood out as being a unique property of the system. Acrylate toxicity was not apparent from the first order kinetics which occurred up to at least 18.5 g/l (250 mmolar) acrylate. At this concentration, acrylate is metabolized at 60 g/l-h (81 mmol/g cell protein-h). In addition, the sequential uptake of two samples of 50 mmol/l acrylate
added in succession suggests the absence of interaction of acrylate with the cells, under these anoxic conditions, and for these limited durations of exposure.

The ability of the cells to tolerate acrylate and metabolize it at a high rate were encouraging observations. The equilibrium was found to favor acrylate hydration under anaerobic conditions

Acrylate is not, however, converted entirely to propionate or a mixture of propionate and acrylate; at concentrations above 100 mmol/l acrylate, nearly half is converted to lactate and traces of β-hydroxy propionate. Approximate calculations of ΔG°' from standard free energies of formation predict a positive free energy of 1.4 kcal per mole, accompanying the hydration. In the presence of an oxidized electron acceptor like methylene blue, acrylate is not metabolized. This suggests that the reactions leading to lactate may be driven by the very negative standard free energy associated with reduction of acrylyl-CoA to propionyl-CoA.

When compared to the rate of uptake of acrylate, lactate is consumed slowly. Using identical cell concentrations as in the study of acrylate uptake, 50 mmol/l lactate is consumed at the rate of 1 mmol/g cell protein-L to propionate and acetate in the now familiar 2:1 ratio. No measurable accumulation of acrylate occurred during lactate metabolism.
3. Propionate Dehydrogenation by Resting Cells

Although lactate consumption did not result in acrylate production, the critical requirement for between 20 and 30 mmol/l lactate has been recognized, as discussed earlier in this chapter. A mass balance on lactate during acrylate production revealed that lactate was metabolized to acetate. Production of more acrylate than lactate consumed gave evidence that acrylate was being produced from a compound other than lactate, probably propionate. This has now been confirmed with the aid of radioisotopes. Substituting lactate (for reasons facilitated by an observation which will shortly be discussed) with (1-\^3H-) L-alanine, the tritium label was not to be found in acrylate (Wang et al., 1979).

Evidence that this activation could be achieved when lactate was substituted with L-alanine or pyruvate, but could not be replaced with \(\beta\)-hydroxypropionate or \(\beta\)-alanine prompted the inference that lactate activation was linked to acetyl-CoA formation and possibly activation of an acyl-transferase through which propionate could be acylated. The importance of both regulatory and energetic factors are suggested. However, since the acrylate produced is not stoichiometric with the lactate added, a regulatory effect is more likely.

In the process of studying acrylate production kinetics, it was observed that acrylate production increased linearly
with increasing initial concentrations of the substrate, up to at least 800 mmol/l propionate, the highest concentration tested. The total amount of acrylate produced did not exceed 40 mmol/l, irrespective of the substrate concentration. The lowest substrate concentration at which 40 mmol/l could be produced was 200 mmol/l propionate.

The ability to produce 40 mmol/l acrylate from 200 mmol/l propionate varied with different cell batches; some less active batches required higher propionate concentrations.

Hence, 200 mmol/l propionate is not the saturating concentration for the bioconversion; there appear to be other factors involved in limiting total acrylate production to 40 mmol/l. If these factors can be identified and overcome, it should follow that greater productivities can be attained by increasing the propionate concentration.

Though linear regression gave a convenient plot for the data in Figure 27a, two separate correlations are apparent. Earlier studies mistook the tailing at around 200 mmol/l to be indicative of substrate saturation. It is useful to note that at concentrations above 200 mmol/l, the initial rate of acrylate production increases significantly.

The molar conversion yields of propionate to acrylate decreased with increasing concentration of propionate. The greatest molar yield, 40%, was obtained with only 30 mmol/l propionate as the substrate being converted to 12 mmol/l acrylate.
The trade-off is between conversion yield and productivity. One of the unstated objectives of this study is the complete conversion of propionate to acrylate. Owing to their very similar physical properties, propionate and acrylate are difficult to separate, thus complete conversion would be a major contribution to the development of a process.

4. Factors Limiting Acrylate Productivity

The notable issue at this point was that the bioconversion terminated within two hours of being initiated and did not result in the production of more than 40 mmol/l acrylate. It was not known whether these were related effects or independent of one another. Experiments were therefore performed to identify the factors responsible for the short life of cellular catalytic activity.

In attempting to determine the cause for the short life of the system, it was recognized that the product-acrylate, well known for its toxicity to other organisms, might intervene under aerobic conditions to inhibit its further production in C. propionicum. The inhibitory effect of acrylate was clearly observed. A double reciprocal Lineweaver-Burk plot of the kinetic data suggests linear mixed type inhibition of the activity associated with acrylate production. It should be noted that the kinetic data was obtained with a resting cell suspension and not with a cell-free enzyme system.
Some simplifications have also been allowed in the interpretation of this data. Principal amongst these was the speculation that the rate limiting step of a multienzyme system catalysing propionate to acrylate was the dehydrogenation of propionyl-CoA to acrylyl-CoA, measured as the rate of acrylate accumulation. These factors make the interpretation of $k_M$, $V_{\text{MAX}}$, $k_i$ less meaningful.

Since the inhibitor and the product are the same compound, the model proposes that acrylate, the product, once formed would bind at a site other than the active site, thereby altering the dissociation rate constant of the enzyme-substrate complex. Since the product and inhibitor are the same compound, it would be reasonable to suggest that inhibition is due to competition for the active site, especially in the light of high rates of anaerobic acrylate uptake seen in Figure 25a.

Acrylate is also known to alkylate proteins. However, the addition of up to 10 mg/ml bovine serum albumin, to 5 mg/ml of cellular protein in the reaction mixture did not decrease inhibition. This suggests that non-specific irreversible inhibition of proteins does not occur. The possibility of site specific inhibition has not yet been studied.

5. Oxygen Inactivation

In addition to the role of product accumulation in limiting acrylate productivity, the effect of oxygen during bio-
conversion on this obligate anaerobe was examined. Oxygen in air was used as the terminal electron acceptor for electrons produced during the oxidation of propionate. \textit{C. propionicum}, though obligately anaerobic, possesses a highly active NADH oxidase system (Stadtman and Vagelos, 1958) which it uses to "detoxify" oxygen when exposed to air for short periods during plating, etc. In these bioconversion experiments, the cells were exposed to nearly 0.3 mmol/l of oxygen (10 mg/l) for 2 hours. The data in Figure 32a show that within 100 min of exposure to air the cells completely lose their ability to catalyse the bioconversion. It was possible, therefore, to assign major responsibility for the short life of bioconversion activity to inactivation by oxygen.

The natural mechanisms by which microorganisms detoxify oxygen have been summarized in the Literature Review. The mechanism of toxicity in \textit{C. propionicum} has yet to be determined; in the event that it is caused by hydrogen peroxide, addition of extracellular catalase may be helpful. It may be reasonable to induce increased synthesis of the oxygen detoxifying system that already exists in the organism by exposing the culture to oxygen prior to harvesting or by selecting for a more aerotolerant strain on agar plates. These proposals and others are discussed in the chapter on Suggestions for Future Work.

Regeneration of oxygen inactivated cells was considered. In addition to losing the ability to produce acrylate, what
other metabolic functions were lost or altered following pro-
longed exposure to air? In addressing this question, it was
found that such cells had also lost the ability to reduce
acrylate, when returned to an anoxic environment. Cells that
had been exposed to air for up to 4 hours had not lost,
however, their viability, as determined by enumerating viable
cells by the most probable number (MPN) method. Such cells
were also found to be able to produce small amounts of ace-
tate, but no propionate from lactate under anaerobic condi-
tions. The change from being able to produce propionate and
acetate in the ratio of 2:1 to the situation described above
following prolonged exposure to air, is shown in Figure 34b.
The results suggest (although weakly) that air expo-
sure inactivates the enzyme propionyl-CoA dehydrogenase; the
reactions catalysed by this enzyme, in any event, did not occur.
It was reasoned that lactate, which now could not be metabol-
ized to propionate, might be dehydrated to acrylate. However,
acrylate accumulation could not be shown to occur from lactate
with oxygen inactivated cells.

Partial regeneration of inactivated cells was obtained
following incubation with L-alanine. The value of using re-
generated cells for increasing the productivity of acrylate
production is only slight. The process is long and the re-
generated activity only marginal.
6. **Choice of Electron Acceptor**

The attenuating effect of methylene blue on acrylate production is shown in Figure 31. This observation led to the use of other electron acceptors in this system. Clearly, methylene blue with a standard reduction potential \(E'_o\) of 11 mV is not sufficiently positive to accept electrons from propionyl-CoA dehydrogenase which has a standard reduction potential \(E'_o\) = 186 mV, except when in high concentrations. The \(E'_o\) for the reduction of propionyl-CoA to acrylyl-CoA though equal to 15 mV as calculated by Hauge (1956) is not used here since it is speculated that methylene blue will accept electrons from the enzyme and not from the substrate directly.

By calculating equilibrium constants using a derivative of the Nernst equation \(E'_o = \frac{2.3RT}{nF} \log k' eq\), it is evident that much lower concentrations of a primary electron acceptors such as 2,6 dichlorophenolindophenol \(E'_o = 217 \text{ mV}\) or p-quinone \(E'_o = 280 \text{ mV}\) could replace methylene blue with greater efficiency and possibly without creating toxic side effects due to high concentrations.

The use of these electron acceptors had some interesting affects on the cells, but increased acrylate productivity was not one of them. One wonders whether the frequent use of methylene blue with biological systems reported in the literature is coincidental or a result of some unique biocompatibility of the dye.
7. Anoxic Production of Acrylate

A fortuitous observation made while studying controls for product and oxygen related inhibition of activity was that anoxic incubation of the cells prior to the bioconversion step ("preincubation") had the effect of increasing both the rate and total amount of acrylate produced. Such an influence on acrylate productivity is even more pronounced when preincubation is conducted in the presence of lactate or acrylate. This was shown in Figure 34a.

The effect is most evident with cells (which without "preincubation" would have typically produced only 5-20 mmol/l acrylate) harvested in early exponential or stationary growth phases. Cells which would produce 30 to 40 mmol/l acrylate without preincubation were not found to produce more acrylate after preincubation. The point raised in this latter case is therefore whether no increase in acrylate production ability is observed despite de novo enzyme synthesis, or whether such protein synthesis does not occur during preincubation in cells harvested in the optimal growth phase. Unfortunately, the initial rates of acrylate production were not determined; hence, no inference can be drawn regarding enzyme levels. In the former case, however, experiments with chloramphenicol support the hypothesis that de novo synthesis of relevant enzymes occurs during preincubation.
Alternatively, the effect of preincubation with lactate or acrylate may be similar to that observed by the addition of an "activator"-lactate to the bioconversion reaction mixture. This suggestion is supported by the observation that "preincubation" with lactate preempts the need for addition of 20 to 30 mmol/l lactate during the bioconversion. This finding has significant importance if one should choose to conduct the bioconversion anoxically in the presence of a limited quantity of electron acceptor, as will be shortly discussed. Separation of the lactate "activation" step from the bioconversion means that fewer electrons need be removed by the electron acceptor during bioconversion.

Having recognized that a solution for improvement of both productivity of acrylate and consumption of propionate should address the short life of cellular catalytic activity caused by oxygen, product accumulation and methylene blue toxicity, it was sought to conduct the bioconversion anoxically using low concentrations of methylene blue with continuous regeneration of the reduced methylene blue and, if possible, product removal.

Initial results are encouraging, and as seen in Figure 33, in the absence of oxygen, termination of acrylate productivity is not found to occur after 2 hours. The rate of methylene blue reduction could be further decreased by conducting the bioconversion without lactate, since the "activation" was done separately prior to the bioconversion, during preincubation.
Such methods as continuous regeneration of reduced methylene blue by controlling the dissolved oxygen concentration and utilization of a platinum electrode, fuel cell as terminal electron acceptor, are being considered, and are discussed in the chapter on Suggestions for Future Work. Initial results with the electrolytic regeneration system have been encouraging, though the rapid rate of propionate dehydrogenation poses a problem.

8. General Applicability of the System

The lactate reduction system in \textit{C. propionicum} is in many respects similar to the metabolic sequence involving butyrate formation via the $\beta$-unsaturated intermediate crotonyl-CoA occurring in other clostridia. The flexibility of the lactate reduction system in \textit{C. propionicum} may permit its application to threonine metabolism, resulting in $\beta$-hydroxy butyrate reduction to butyrate, although a separate enzyme system may exist to metabolize threonine. Nevertheless, the promise of obtaining $\beta$-unsaturated derivatives of saturated fatty acids other than propionate prompted the effort to obtain crotonate from butyrate with the system previously used for acrylate production.

In excess of 20 mmol/l crotonate was produced under conditions optimized for acrylate production. It is anticipated that following optimization of other bioconversion parameters, greater productivity can be achieved.
Unlike acrylate (bp 140.0 °C) and propionate (bp 140.1 °C), the physical properties of butyrate (bp 163.5 °C) and crotonate (bp 185 °C, mp 71.6 °C) are sufficiently different as to make the separation of the two relatively easy.

The whole cell system used for the conversion of propionate to acrylate was further used to produce methacrylate from isobutyrate. Though unconfirmed by GC-MS analysis, evidence obtained with GC analysis suggests that the product obtained by replacing propionate with isobutyrate in the bioconversion reaction mixture is methacrylic acid. What is particularly noteworthy is that methacrylyl-CoA, unlike acrylyl and butyryl-CoA is not a metabolic intermediate in this organism. The reaction, it is speculated, must be catalysed by a non specific butyryl or propionyl dehydrogenase. Given the tertiary carbon bonds of the alpha carbon of isobutyrate, the reaction is particularly fascinating.

In addition to dehydrogenation reactions, the reverse reduction of 2,3-unsaturated fatty acids to their corresponding saturated fatty acids was demonstrated. The results for anoxic reduction of acrylate, crotonate and methacrylate are shown in figures 25a, 36d and 37c respectively. The specific rates of reduction are comparable with rates observed by other workers.

Using C.kluyveri resting cell suspensions in the presence of 1 atm. hydrogen, Simon et al. (1974) were able to conduct stereospecific hydrogenations of 2,3-unsaturated fatty
acids including acrylate and methacrylate. At 2.45 g/l initial acrylate concentration, the specific reduction rate was 11.8 mg/g wet cell weight-h. The initial specific rate of methacrylate reduction was 0.1 mmol/g wet cell weight-h. The rates observed with \textit{C. propionicum} were 25 mg/g wet cell weight-h and 0.15 mg/g wet cell weight-h respectively. The results are therefore quite comparable. However, quite unlike \textit{C. propionicum}, \textit{C. kluvyveri} converts acrylate entirely to propionate.

One final observation was that the kinetic constants of acrylate, crotonate and methacrylate anaerobic uptake were very different. Despite different $k_M$ values, any two compounds added together (0 to 30 mmol/l) did not influence the initial rate of reduction of one another. This data is suggestive of the absence of competition for an active site.

\textbf{C. Studies on Acrylate Production by Escherichia coli}

1. \textbf{Introduction}

It was hypothesized that a study of acrylate production from propionate would best be made with an organism in which (a) acrylyl-CoA or actylate had been strongly suggested to occur as an intermediate of propionate oxidation, (b) in which propionate could be metabolized solely via the acrylate
intermediate, thereby maximizing the conversion yield, and (c) in which genetic manipulation would be facilitated.

With such an organism, it was sought to effect acrylate accumulation by altering environmental factors, causing enzyme inhibition and as mentioned in the introduction to Section B of the Results chapter, by the creation of an enzymatic lesion in the metabolic step following acrylyl-CoA synthesis.

From amongst the various organisms implicated to oxidize propionate with acrylyl-CoA as an intermediate (see review in the second chapter) E. coli was chosen. It metabolizes propionate via the lactate pathway and 2-hydroxyglutarate as summarized in the Literature Review. Carboxylation of propionate resulting in methylmalonyl-CoA, though common in mammalian tissue and certain microorganisms, does not occur in this organism, probably owing to the absence of vitamin B_{12} synthetic enzymes.

2. Mutation Scheme

In E. coli, mutations were desired in (a) lactyl-CoA hydratase [EC 4.2.1.54] which would prevent acrylyl-CoA from being hydrated to lactyl-CoA, and in (b) isocitrate lyase [EC 4.1.3.1]. The second mutation would in effect behave like a 2-hydroxyglutarate synthase negative mutant. In the absence of isocitrate lyase, glyoxylate would not be produced and the condensation between propionate and glyoxylate resulting in 2-hydroxyglutarate would not occur. Propionate would hence be
metabolized solely via acrylyl-CoA, maximizing the conversion yield of acrylate from propionate. This scheme for preventing 2-hydroxyglutarate synthesis is necessitated by the difficulty involved in directly selecting a 2-hydroxyglutarate synthase [EC 4.1.3.9] deficient mutant. Such a mutant, as just described, it was argued could accumulate acrylate and should have a 100% conversion yield.

From preliminary results, it became clear that E. coli would not grow on acrylate, thereby making direct selection for the mutant difficult. An indirect method was therefore employed; inability to grow on propionate while retaining the ability to grow on lactate was the phenotype selected for on plates. The lesions resulting in such a phenotype could occur at one or more of several steps involving propionate transport, activation, dehydrogenation or acrylyl-CoA dehydration. Screening of individual colonies so isolated would be required to determine which ones were deficient in lactoyl-CoA hydratase [EC 4.2.1.54].

The procedure for selection of an isocitrate lyase deficient mutant (aceA) was relatively straightforward; such cells being unable to grow on acetate without glyoxylate. However, of the various strains of E. coli with which propionate metabolism had been studied, strains K-12 and E 26 aceA mutants were unable to grow on propionate. As mentioned in the Literature
Review, when growing on propionate they replenish spent four-carbon intermediates of the TCA cycle principally by the action of 2-hydroxyglutarate synthase [EC 4.1.3.9], followed by decarboxylation to succinate. The selection of lactoyl-CoA hydratase deficient mutants from amongst isocitrate lyase deficient (ace\textsuperscript{A}) mutants with these two strains therefore becomes extremely difficult.

Strain W on the other hand, not significantly dependent on 2-hydroxyglutarate synthesis for growth, grows without event even in the absence of isocitrate lyase [EC 4.1.3.1]. Strain W was therefore used during the course of this investigation.

Prior to the mutation work, preliminary experiments were conducted in order to become better acquainted with both strain W and propionate metabolism.

3. Growth Kinetic Profile

Initially, growth characteristics of the organism were observed. The anomalous growth kinetics, similar to that documented with other strains of E. coli, was observed to occur with strain W when growing on propionate. The long lag between 48 and 96 hours preceding exponential growth on propionate minimal medium, it has been suggested, enables synthesis of anaplerotic pathway intermediates. The observation that addition of such intermediates in the form of less than 1 mmolar succinate, glucose or yeast extract reduces the lag to less than 12 hours supports the suggestion.
The inability to grow on acrylate was an inconvenient property of the organism, making selection of the appropriate mutant more circuitous. What this meant was that the inability of the organism to grow on acrylate could not be employed as the phenotype for selection of lactyl-CoA hydratase [EC 4.2.1.54] deficient mutant. Attempts were made to select a mutant capable of growing on 1 g/l (13.5 mmolar) acrylate, but without avail.

It was encouraging however, that 2 g/l acrylate only decreased the rate and did not stop growth on propionate and glucose. The possibility that resting cells could be used to catalyse the bioconversion remained a possibility.

4. Resting Cells

Having identified the need to work with resting cells for a variety of reasons, it followed that the resting cell performance of wild type cells should be evaluated. There also lurked the possibility that addition of chemical analogs of acrylate and inhibitors of lactate dehydrogenase might affect acrylate accumulation.

Though propionate was detected as shown in Table 9, it was not accompanied by accumulation of acrylate or other short-chain volatile fatty acids. Though the absence of acrylate was anticipated, it was interesting to learn that propionate is
metabolized entirely to carbon dioxide or non-volatile fatty acids. The latter, unfortunately, were not assayed. Concentrations of propionate greater than 26 mmolar could have been used. It would be interesting to determine the products from metabolism of 100 to 200 mmolar propionate, especially since this range of propionate has been successful with C. propionicum. Though the inability of numerous analogs and inhibitors to cause acrylate accumulation was noted with regret, higher substrate concentrations should also have been tried.

Arsenite, unexpectedly, did not cause pyruvate accumulation despite lactate uptake. However, under the conditions of the experiment it is possible that pyruvate was metabolized to oxalacetate, which is probably the non-volatile compound that was detected by GC analysis. If lactate, in the absence of any inhibitor or analog is metabolized to oxalacetate, it is plausible evidence of the high activity of the phosphoenol pyruvate carboxylase system, which has been suggested to be the main route for four-carbon intermediate synthesis in this strain, as opposed to 2-hydroxyglutarate synthesis in strains K 12 and E 26 (Wegener et al., 1969).

5. Mutation Enrichment and Selection

Finally, having conducted the preliminary investigation, the mutation program was begun. Using acetate and glycerol differential media for selection of acetate negative mutants
(as described in Materials and Methods), the frequency of aceAns was low, only 0.005%. The use of roughly 1 mmolar glycerol was unsatisfactory for this purpose. Glycerol, in addition to supporting acetate negative mutants with marginal growth so that they can be scored as pin point colonies, also supplements acetate negative colonies with four-carbon intermediates to enable them to grow without the glyoxylate bypass on acetate and appear on the plate as large colonies. It, therefore, became clear that by this method of selection, lesions rendering the cells acetate negative owing to deficiencies other than those in the glyoxylate bypass and isocitrate lyase would be selected as pinpoint colonies. Additionally, it was observed that the size distribution of wild type colonies on agar included a large number of pinpoint colonies making selection of mutants by this method less specific.

Differential media technique was hence replaced by spray and replica plating methods; 100 μl of 20% glycerol (corresponding to a final concentration of 1 g/l in the agar) was sprayed. Selection was further tightened by spraying glyoxylate, thereby selecting for only aceAns mutants. To overcome acetate depletion in the agar by cells already grown, acetate was added to the glyoxylate. However, no significant change in the frequency of appearance of mutants was observed. In retrospect, it is noted that though the selection technique appears to be without flaw, the sample size studied with this improved selec-
The reaction procedure was very small. More cells should have been screened.

The two isocitrate lyase negative mutants eventually obtained from amongst nearly 100 acetate non-utilizing mutants reverted shortly thereafter and began to grow on acetate following incubation on acetate agar plates for periods greater than 5 days.

Though the inability to select stable ace<sup>A</sup> mutants was a disappointment, it became obvious that the selection of a propionate negative mutant need not be dependent upon the deficiency of isocitrate lyase [EC 4.1.3.1]. Owing to a lesion in a step between propionate and lactate, propionate—the sole carbon source would not be metabolized beyond lactate. Hence in the absence of glyoxylate production, 2-hydroxyglutarate would not be synthesized thereby preempting the need for removing this parallel mode of propionate metabolism.

Work with an ace<sup>A</sup> mutant, however, would be more satisfying, since utilization of propionate solely by the acrylate pathway would be guaranteed under all conditions.

Using the spray technique, 1 g/l lactate was sprayed on the plates in the form of 100 ul of 20% solution lactate, following 72 h of growth on 2 g/l propionate plates, 5 propionate negative, lactate positive mutants were obtained and tested for their ability to accumulate acrylate from propionate. The fact that neither acrylate accumulation nor propionate uptake occurred could be explained if all five mutants had lesions
in transport, activation or dehydrogenation steps. As shown in Table 12, Wegener et al. (1969) isolated an ace\textsuperscript{A}, propionate negative and lactate positive mutant called M-18. These are the same characteristics searched for during the course of this investigation. Using a propionate revertant of M-18, M-18P, they were able to show that the propionate negative lesion was independent of isocitrate lyase and in the acrylate pathway between propionate and lactate. Though not alluded to in the paper, it can be inferred that the inability of M-18 to grow on propionate alone or propionate and glyoxylate together must be due to lesions in transport or activation of propionate or due to a mutation in each branch of propionate metabolism; growth would otherwise be expected to occur. All the mutants, however, with the appropriate phenotype, apparently do not have the lesion in lactyl-CoA hydratase.

6. Isolates

Prior to initiating work with \textit{E. coli}, a sequence of experiments were conducted to select a suitable organism from the environment. "Suitable" was taken to mean one which would readily produce acrylate from propionate, aerobically. However, since direct selection of an organism with the stated abilities was difficult, it was decided that the organism should be one capable of metabolizing both acrylate and propionate. The ability to metabolize acrylate suggests the likelihood of acrylate or its CoA derivative being an intermediate in that organism.
### TABLE 12

**MUTANTS OF ESCHERICHIA COLI W DEFICIENT IN PROPIONATE OXIDATION RELATED METABOLISM**

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<td>+ Glyoxlate</td>
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<td>+ Glyoxlate</td>
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* Exponential growth preceded by 48-72 hour log phase
Hopefully, propionate and acrylate would be metabolized sequentially; thereafter, straightforward mutation and selection could be applied. Numerous such organisms, mostly mycelial, were obtained. The yeast 530-40 appeared to be particularly "suitable" for further study. However, with *Candida lipolytica* Tabuchi and Uchiyama (1975) showed the existence of a cyclic pathway of propionyl-CoA condensation with oxalacetate into methylcitrate, from which pyruvate was produced and oxalacetate regenerated. The nature of the pathway for acrylate metabolism and the question whether there exists a parallel pathway of propionate oxidation by the acrylate pathway in yeast remain undetermined. Nevertheless, the finding with *Candida* did not bid well our intention to ferment propionate to acrylate using the yeast isolate 530-40. Though investigation with this organism was terminated following preliminary growth and resting cell experiments as described in results, it remains an interesting and potentially useful isolate. Surprisingly, it grows on up to 8 g/l acrylate.

Subsequent to extensive search of the literature, no allusion was found to organisms capable of growing aerobically on acrylate. We have isolated several such mycelial organisms and a yeast.

As a consequence of the lack of success with *E. coli* and some aerobic isolates, it was decided to terminate this
approach and pursue acrylic acid production using Clostridium propionicum, with which initial success had been met.

While the results of the study with E. coli are consistent with the postulated pathway of acrylate as an intermediate, problems with mutant enrichment and selection, as well as potential acrylate toxicity to the cells are difficulties encountered with this approach.

In light of our experience with E. coli W, recommendations are made in Suggestions for Future Work, which could be incorporated in further investigations in this area.
VI. SUMMARY AND CONCLUSION

In response to conditions requiring a shift from dependency on fossil raw materials for chemicals synthesis, it was sought to produce acrylic acid - a petrochemical, from biomass - a renewable resource, using microbially catalysed reactions.

On the basis of an extensive survey of the literature and by pursuing the preliminary results obtained in this laboratory, two organisms were chosen for further study. *Clostridium propionicum*, an obligate anaerobe was studied for its ability to produce acrylate from propionate and, if possible, from lactate. In addition, mutants of the facultative aerobe, *Escherichia coli* W were sought with the rationale that certain lesions in metabolic enzymes of propionate oxidation could enable resting cells of the mutant to accumulate acrylate from propionate.

The major effort with *C. propionicum* was directed at increasing the specific productivity of acrylate from propionate by maximizing cellular activity, rates of conversion and by increasing the life of the acrylate production capability. The feasibility of increasing conversion of propionate to acrylate was also examined.

Finally, the capacity of the bioconversion to catalyse 2,3-dehydrogenation of saturated fatty acids other than propionate was examined.
With *E. coli* W, experiments were performed evaluating the growth ability of the organism on propionate and acrylate. Mutants were then sought, that would first metabolize propionate to lactate homofermentatively and then be unable to hydrate acrylyl-CoA to lactyl-CoA, thereby accumulating acrylate. Soil isolates were also examined for the ability to produce acrylate.

Results with *C. propionicum* show a maximum level of 40 mmol/l acrylate produced, corresponding to a specific productivity of 7 mmol/l g protein-h, at an initial rate of 3.8 mmol/l-h. The highest molar conversion of acrylate from propionate was 40%, when using 40 mmol/l propionate.

Continued standardization of the bioconversion system resulted in identifying that cells harvested in the late exponential growth phase are optimal for acrylate production. Acetate, propionate, butyrate, isobutyrate and isovalerate are products of growth on L-alanine, complex medium.

The bioconversion of propionate to acrylate does not proceed beyond three hours in the presence of air. Acrylate itself, air and methylene blue were shown to contribute to the occurrence of this phenomenon. 34 mmol/l acrylate added to the bioconversion reaction mixture containing 200 mmol/l propionate, completely inhibited production of acrylate. This concentration of acrylate is nearly equal to the maximum that is found to accumulate during bioconversion. Anoxically, however, acrylate
is not inhibitory. Acrylate was metabolized anaerobically at a rate of 250 mmol/l (4.05 mol/g-h). That of lactate uptake was only 15% of that observed with acrylate. Following 3 hours of anonic incubation with 12 mmol/l methylene blue, cells were unable to catalyse acrylate production. The concentration of the dye used in the bioconversion, for comparison, is only 5.3 mmol/l. Exposure of a 20% cell suspension to air for less than 180 min also had a similar effect; these cells could no longer produce acrylate. These three factors, collectively are held possible for the short life of the bioconversion system. Polymerization, non-site specific inhibition and nutrient limitation did not contribute significantly.

Anoxic "preincubation", on the contrary, resulted in enhanced acrylate production capabilities. This enhancement is even greater if preincubation is conducted with lactate or acrylate. It is a function of the concentration of lactate/acrylate, the duration of preincubation and the age of harvest of cells. The induction of relevant enzymes is believed to occur. An interesting observation was that "activation" of acrylate production by lactate could be conducted by treating cells with lactate prior to the bioconversion.

Anoxic bioconversion of propionate is feasible, though in preliminary experiments the increased life of acrylate production is compensated by lower rates of production. The inability to use very high concentrations of the electron acceptor limits anoxic acrylate productivity.
Using the standardized bioconversion system devised for the production of acrylate, other 2,3-unsaturated derivates of short-chain fatty acids have been obtained. The product of butyrate bioconversion by resting cells of \textit{C. propionicum} in the presence of alternate electron acceptors is crotonate, the identity of which has been confirmed by GC-MS analysis. 20 mmol/l crotonate is produced from 500 mmol/l butyrate. The optimum pH was 6.5. The kinetics of anaerobic uptake of butyrate show a classical Michaelis-Menten saturation curve. The $V_{\text{max}} = 240$ mmol/g wet weight cells-h and the $K_{\text{m}} = 20$ mmol/l.

Similarly, methacrylate has been produced from isobutyrate. Though its identity has not yet been confirmed by mass spectrometry methods, gas chromatography data is strongly suggestive that it is in fact methacrylate. 6 mmol/l accumulates from 500 mmol/l isobutyrate. The optimum pH range is 6.5-7.5 and the optimum lactate concentration for the bioconversion is between 30 and 35 mmol/l. Anoxic uptake of methacrylate, like crotonate, shows Michaelis-Menten type kinetics. The $V_{\text{max}} = 150$ mmol/g wet weight cells-h and $K_{\text{m}} = 10$ mmol/l.

\textit{E. coli} W was chosen since unlike other strains of the organism, an ace$^A$ mutation (isocitrate lyase negative) does not significantly affect its growth rate of propionate.

The organism does not grow on acrylate but will on glucose in the presence of 1 g/l acrylate. Growth on propionate is preceeded by a long lag unless traces of glucose, yeast extract
or four-carbon compounds are also provided in the medium. Resting cells metabolize propionate and lactate without accumulating volatile short chain fatty acids. Two isocitrate lyase negative mutants and five propionate negative, lactate positive mutants were isolated. Acrylate accumulation from propionate was not observed with the propionate negative mutants.

The non-mycelial soil isolates were divided into two groups according to their ability to grow on propionate in the presence of acrylate or not. An interesting and potentially useful isolate was the yeast 530-40, found to grow on up to 8 g/l acrylate.

Owing to lack of preliminary success with E. coli, it was decided to terminate this approach.

In conclusion, some factors limiting acrylate productivity in C. propionicum have been recognized and ways have been proposed to circumvent them. The potential of this bioconversion system for the catalysis of other dehydrogenation has also been realized.

Suggestions for pursuing some of the findings are made in the next chapter.
VII. SUGGESTIONS FOR FUTURE WORK

A. INTRODUCTION

The overall objective of the study is not only to produce acrylate, but to acquire a basic understanding of the metabolic processes involved during acrylate synthesis by resting cell systems.

The focus of this thesis was the synthesis of acrylate from propionate, with effort made to produce it from lactate. Though the goal in mind has been that the bioconversion may eventually have commercial value, the scope of this thesis did not include serious consideration of the economics of acrylate production.

Suggestions are made here for future work which is consis-tant with the stated objective. Suggestions are made to pursue specific experiments with which initial success has been met. These areas would contribute directly to improving the productivity and the conversion of propionate to acrylate. In addition, though a certain amount has been learned (during this study) about the stability of C. propionicum resting cells, suggestions are made for examining the stability of this system in greater depth. It is expected that such a study would result in obtaining clearer insight into anaerobic resting-cell catalysed bioconversions, in general. Finally, as has been noted, the bioconversion system used can be applied
to substances other than propionate. Suggestions are made to study this further.

B. Propionate to Acrylate

With the objective of increasing the productivity of acrylate fermentation, an anaerobic bioconversion with continuous regeneration of low concentration of an electron acceptor is suggested to replace the existing bioconversion using oxygen as the terminal electron acceptor.

(1) One strategy could be to regenerate the reduced dye with a controlled concentration of dissolved oxygen. The optimum concentration of oxygen would not result in decreased rates of the bioconversion, but would be low enough to increase the life of the cellular activity. The regeneration could be achieved in a 2-stage reactor, in which the reduced dye could be separated from the cells and regenerated.

(2) Some preliminary work using an anaerobic bioconversion system coupled to an electrochemical system for the regeneration of the reduced dye has been conducted. A platinum electrode was used as a terminal electron acceptor. Preliminary results demonstrate that acrylate can be produced by this method. Further work is necessary if the productivities are to match those already being achieved by the standard method.
(3) The need for seeking another terminal electron acceptor to replace oxygen is necessitated by oxygen toxicity to the cells. The role of oxygen detoxifying enzymes in determining oxygen lability of cells was reviewed in the chapter "Literature Review." Of particular note is the finding by Zavadova et al. (1974) that C. perfringens can be mutated to impart to it greater oxygen tolerance. Other investigators have shown that oxygen detoxifying enzymes are induced in response to greater oxygen partial pressures.

C. propionicum, though an obligate anaerobe, has been found to be relatively tolerant to oxygen, a property that has been used advantageously in microbiological techniques of plating, etc. However, to increase the productivity of acrylate in the presence of oxygen, even greater tolerance is needed.

Strategies for increasing the ability of C. propionicum to withstand oxygen could include mutation and selection for oxygen tolerant strains; the use of recombinant DNA techniques to provide C. propionicum the genetic information to synthesize superoxide dismutase; by induction of oxygen detoxifying enzymes such as NADH oxidase by growing cells in the presence of low dissolved oxygen concentrations. Prior to these approaches, catalase, peroxidase, superoxide dismutase levels should be evaluated to determine the cells natural defenses against oxygen. The sensitivity of the cells to hydrogen peroxide should also be determined. If in fact hydrogen
peroxide formation occurs, such techniques as the addition of catalase to cell free extracts can be considered.

C. Growth

In retrospect, it is noted that the medium employed to culture *C. propionicum*, is significantly identical to the complex medium first devised by Cardon and Barker (1946). The influence of medium composition on enzyme induction is well documented in other organisms, and it may well serve the purpose of future investigators to emphasize this aspect of the problem.

(1) DL-alanine has not been used as a substrate, although it is much cheaper than L-alanine, and an attempt to use it could be made.

(2) A wide variation in activity for acrylate production has been noted in different batches of cells despite harvest at identical culture turbidity: To enable greater productivity of the acrylate production capability of cells, other parameters may be used for determining the optimum growth phase of the cells. Such parameters are the profile and concentration of volatile fatty acids and the redox potential of the culture.
(3) Koplove (1977) suggested that an ideal organism for the study of transient behavior in continuous culture would be one in which acetate kinase is the sole phosphorylating enzyme. In such an organism, the effect of transience on substrate level phosphorylation could be studied by monitoring acetate kinase, and the data interpreted without the ambiguity inherent in studying organisms with parallel ATP-phosphorylating mechanisms. *Clostridium propionicum*, it is believed, synthesizes its ATP solely from acetyl phosphate.

(4) Induction of the acrylate pathway in batch cultures by causing transient growth following pulse environmental changes has also been suggested. With *C. propionicum*, such a perturbation could be achieved by exposing cells to a pulse of oxygen prior to harvest. It is hypothesized that this may induce oxygen detoxifying enzymes and NADH oxidases (O'Brien and Morris, 1971). It may also have an interesting effect on the levels of lactoyl-CoA dehydratase and propionyl-CoA dehydrogenase.

D. Resting Cell Systems

Anaerobic resting cell systems are potentially powerful catalysts for the production of chemicals. In their capacity as growth uncoupled organisms, their ability to perform multiple and complex reactions may require that the cells remain viable, or at least retain particular functions.
Clostridium propionicum is an attractive organism to use as a model system for the study of resting cells. The organism is believed to produce only one mole of ATP per mole substrate, catalysed by the action of acetate kinase. This step and the activity of an active transacetylase are crucial not only during growth, but especially during resting cell catalysed bioconversions which require activation of substrates. Hence, an understanding of conditions for the expression and stability of acyl-CoA transferase and acetate kinase will contribute towards the understanding of resting cell stability. Along with the study of relevant transport enzymes, such an investigation could lead to the better understanding of anaerobic resting cell systems in general.

E. Versatility of the Bioconversion System

The ability of the cells to produce methacrylate which, unlike acrylate and crotonate, is not a metabolic intermediate, is suggestive that there may be other dehydrogenations that the cell can catalyse.

(1) Other reactions that may be interesting and useful are the dehydrogenation of hydrocinnamic acid to cinnamic acid, hexanoic (caproic) and its isomers to their 2,3 unsaturated derivatives, pentanoic acid and its isomers to their 2,3 un-
saturated derivatives. Will the system dehydrogenate succinic acid to fumaric acid? While the imagination suggests it might be a possible reaction, only future work will tell.

Simon et al. (1974) used gaseous hydrogen to hydrogenate 2,3-unsaturated compounds. The reaction was catalysed by resting cells of Clostridium kluyveri. Performing the bioconversion in H(D/T)O, they were able to obtain chiral compounds owing to the stereospecific hydrogenations by H or T. Such chiral compounds could undoubtedly be synthesized by Clostridium propionicum, which has the benefit of higher hydrogenation rates. This would be a reasonable way to synthesize stereoisomeric methyl groups.

Finally, though a wide range of dehydrogenations are possible with C. propionicum, other organisms may be considered. C. oceanicicum produces relatively large amounts of isobutyrate from peptone yeast extract medium and may be a candidate for methacrylate production. Similarly, C. butyricum may be a more suitable organism for further study of crotonate production.

F. E. coli

Although the program for mutation and selection of acrylate producing mutants of E. coli was terminated owing to lack of success, a few observations are made here, in addition to those
made in the chapter titled "Discussion." These may be considered while attempting similar work.

(1) Considerable effort may be saved by selecting for a propionate negative, lactate positive mutant directly, without first seeking an isocitrate lyase mutant first, provided differential plating technique is replaced in favor of replica plating.

The isocitrate lyase negative mutation was necessitated in differential plating since glyoxylate, a product of lactate metabolism might condense with propionate by the α-hydroxyglutarate pathway thereby recording an organism with a lesion between propionate and lactate, as falsely propionate positive. This will not occur in replica plates.

(2) Propionate negative, lactate positive isolate resting cell suspensions were found not to oxidize propionate. This may not be an indication as previously suggested that the mutants are CoA-activation deficient. Following growth on lactate, induction of propionate oxidizing enzymes may require to be induced. Longer periods of resting cell bioconversion and addition of trace quantities of glucose are suggested.

(3) Other than E. coli there are numerous aerobes which possess acrylyl-CoA as a metabolic intermediate. Some like the yeast isolate (530-40) are far more tolerant of acrylate. This is also true of the mycelial isolates. These organisms deserve a second look.


Commeyeras, A. 1976. 
5th Int. Ferm. Symp. 


Klein, J. 1976

Knight, M. 1962. The photometabolism of propionate by Rhodo-

metabolism. IV. Significance of carboxylation reactions dur-

Koplove, H.M. 1977. The production of acetate kinase in con-
tinuous culture. Ph.D. Thesis. Massachusetts Institute of
Technology.

Kornberg, H.L. 1966a. "Anaplerotic sequences and their role

Kornberg, H.L. 1966b. The role and control of the glyoxylate

Kornberg, H.L. and H.A. Krebs. 1957. Synthesis of cell con-
stituents from C₂-units by a modified tricarboxylic cycle.
Nature 179: 988-991.

Applied to World Needs (CHEMRAWN) I faces up to raw materials

Ladd, J.N. and D.J. Walker. 1959. Fermentation of lactic acid
by the rumen microorganism Peptostreptococcus elsdenii. Ann.

Ladd, J.N. and D.J. Walker. 1965. Fermentation of lactic acid
by the rumen microorganism Peptostreptococcus elsdenii. N.Y.

LaRoche, H. 1971.
Z. Naturforchg. 26B:369.

LaRoche, H. 1971.

for isolating constitutive mutants for carbohydrate cataboliz-

Laskin, A.I. and M.A. Lechevelier 1977. "CRC Handbook of
Microbiology." (2nd Ed.), CRC Press.


APPENDIX I

Clostridium propionicum

Reference Strain: ATCC 25522, NCIB 10656

Isolation: Cardon and Barker (1946) from black mud of San Francisco bay

Microscopic Characteristics: Gram positive rods, (0.5-0.8) X (1.8-5.0) um. Non-motile. Oval terminal spores. Often in pairs.

Colony Characteristics: Circular, entire margin and green-gray in color.

Carbohydrate Fermentation Profile:

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Volatile Fatty Acid Fingerprint:

C. propionicum, ATCC 25522 (NCIB 10656)

PYG Peptone yeast extract glucose growth medium

a = acetate
P = propionate
ib = isobutyrate
b = butyrate
iv = isovalerate
APPENDIX IIa - Standard calibration curve for protein quantification (bovine serum albumen)

Figure 44 - APPENDIX IIa - Standard calibration curve for protein quantification (bovine serum albumen)
Figure 45 - APPENDIX IIb - Standard calibration curve correlating dry cell weight with absorbance (using Escherichia coli W)
Figure 46 - APPENDIX IIc - Standard calibration curve correlating undiluted and diluted Klett units (using E. coli W)
Figure 47 - APPENDIX IId - Standard calibration curve correlating dry cell weight and Klett units (using *E. coli* W)