THE GLUCONIC ACID OXIDIZING SYSTEM

OF Pseudomonas aeruginosa

by

THEKKEPAT RAMAKRISHNAN

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ABSTRACT

Earlier work has shown that <u>Pseudomonas</u> <u>aeruginosa</u>
<u>9027</u> can oxidize glucose to carbon dioxide and water by way of
gluconic, 2-ketogluconic and pyruvic acids. However, it has
been found that closely related organisms can phosphorylate
gluconic acid. The object of the present work was to isolate
the gluconate oxidizing enzyme, to solubilize it, purify it,
determine the co-factor requirements and ascertain whether or
not any energy was gained or lost by the system during the
reaction.

Cells harvested from a gluconic acid medium were
disintegrated in a 10 kc. Raytheon sonic oscillator. The
enzyme which was still attached to the cell particles was
solubilized with sodium glycocholate and remaining particles
were removed by the addition of 0.30 saturation ammonium
sulphate. Nucleoproteins were then removed by the addition
of protamine sulphate. Further fractionation with acid and
alkaline ammonium sulphate purified the enzyme 200 fold.
Finally the enzyme was absorbed on tricalcium phosphate and
eluted with M/5 phosphate buffer of pH 7.0. The pH optimum
of the purified enzyme was found to be 5.6 while in the whole
cells the maximum activity was at pH 7.0. A hydrogen acceptor
was necessary for linking the system to atmospheric oxygen;
2,6-dichlorophenolindophenol and pyocyanine were found to be
the most efficient acceptors. Ferricyanide poisoned the
system, while brilliant cresyl blue was inactive as a hydrogen

acceptor. Reaction with methylene blue was slow. Diphospho-
pyridine nucleotide, triphosphopyridine nucleotide, flavin
mononucleotide, flavin adenine dinucleotide, cytochrome c,
adenosine diphosphate and adenosine triphosphate had no influence
on the enzyme activity. Sodium fluoride, 2,4-dinitrophenol,
azide, iodoacetate, arsenite or 8-hydroxyquinoline did not act
as inhibitors. Cyanide, glutathione and cysteine activated
the enzyme slightly.

The enzyme is specific for gluconic acid. Glucose,
glucuronic acid, 2-ketogluconic acid, pyruvic acid, saccharic
acid, ribonic acid, arabonic acid, fructose, mannose, ribose-
5-phosphate, glucose-6-phosphate or 6-phosphogluconic acid were
not oxidized by the enzyme. No carbon dioxide was evolved
during the oxidation of gluconic acid by the enzyme. The
product on chromatographic analysis, was found to be 2-keto-
gluconic acid.

The enzyme was routinely stored at -10°C in M/10
tris buffer, pH 7.0. Under these conditions it was stable
for several weeks. At 4°C, under the same conditions, the
enzyme may be kept for three to four days without any appreci-
able loss of activity. When dialyzed against distilled water,
there was a gradual loss of activity after eight to ten hours,
accompanied by precipitation. Dialysis against neutral buffers
for as long as 24 hours in the cold produced no loss in
activity.

Instead of sodium glycocholate, "Cutscum" can be

used for solubilizing the enzyme. Purification can also be effected from the sonicate through the use of the ultracentrifuge. The supernatant left after one hour of centrifugation at 105,000 x G oxidized gluconic acid in the presence of pyocyanine and showed two peaks in the electrophoretic apparatus, one of which is believed to be due to protamine sulphate.

Though no phosphorylation of the substrate was demonstrable as evidenced by the lack of activation by ATP and the lack of inhibition by fluoride, the problem was further investigated in the sonic extracts. No increase in acid was found either aerobically or anaerobically in P. aeruginosa as tested by the method of Colowick and Kalckar. Moreover, sonic extracts failed to reduce TPN in the presence of gluconate and an excess of phosphogluconic dehydrogenase isolated from Brewer's yeast. In contrast to these data, it was found that by either of the last two mentioned criteria, P. fluorescens A. 312 did phosphorylate gluconate. P. fluorescens thus possesses an additional phosphorylated pathway for dissimilating glucose and this is absent in P. aeruginosa.

No energy was found to be produced in the initial stages of glucose oxidation. The system could not be coupled to the "zwischenferment" reaction of glucose which requires ATP. Chromatographic analysis failed to show any ATP formed during the oxidation of gluconic acid.

The significance of these findings in the light of the glucose metabolism by P. aeruginosa is discussed.

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THEKKEPAT RAMAKRISHNAN

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IN ROOM 102, AGRICULTURE BUILDING

COMMITTEE IN CHARGE:

Dean H.F. Angus - Chairman

B.A. Eagles   J.J.R. Campbell
D.G. Laird   W.J. Polglase
D.C.B. Duff   C.E. Dolman
S.H. Zbarsky   E. Morrison

External Examiner - P.G. Scholefield
McGill University

THESIS

THE GLUCONIC OXIDISING SYSTEM OF PSEUDOMONAS AERUGINOSA

Abstract

The enzyme which oxidises gluconic acid to 2 keto-gluconic acid in P. aeruginosa has been extracted in a soluble form and purified 300 fold. A hydrogen acceptor was necessary for linking the system to atmospheric oxygen; 2:6 dichlorophenol indophenol and pyocyanine were found to be the most efficient acceptors. The enzyme has a pH optimum of 5.6. DPN, TPN, FMN, FAD, cytochrome c, ADP and ATP had no influence on the enzyme activity. Sodium fluoride did not act as inhibitor indicating that phosphorylation of the substrate was not involved. DNP, azide, iodoacetate or 8 hydroxyquinoline also did not inhibit the activity.

The lack of phosphorylation of the substrate was confirmed with the sonic extract of the organism by measuring the increase in acidity produced when phosphate transfer took place. No increase in acidity was observed with P. aeruginosa while a related organism which was reported to phosphorylate gluconic acid prior to oxidation was shown to produce increased acidity under the same conditions.

No energy was found to be produced when gluconic acid was oxidised by P. aeruginosa as indicated by the lack of ATP production. Thus the oxidation system in this organism is different from that in other organisms in that in the initial stages no usable energy is either utilized or produced.

GRADUATE STUDIES

Field of Study - Agricultural Microbiology

Soil Bacteriology   D.G. Laird
Lactic Acid Bacteria   J.J.R. Campbell
Laboratory Methods
    and Procedures   J.J.R. Campbell
Dairy Mycology   N. Neilson

Other Studies:

General Biochemistry   S.H. Zbarsky
Enzyme Kinetics   W.J. Polglase
Molecular Structure and
    Biological Activity W.J. Polglase
Current Literature   J.J.R. Campbell

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HISTORICAL INTRODUCTION

Prior to 1948 our knowledge of the intermediate metabolism of carbohydrates was restricted almost entirely to the reactions of the Embden-Meyerhof pathway and the Kreb's tricarboxylic acid cycle. The importance attached to the glycolytic pathway was without doubt partly due to its almost universal demonstration in higher plants and animals as well as in yeasts, molds and bacteria. The fact that the enzymes of this system were water soluble and therefore capable of being isolated and purified greatly increased the scientific interest in this pathway. The result was that many of the greatest biochemists of this period turned their attention to this problem, perhaps causing the average worker to attach too great a significance to the pathway. The observation that animal tissue had an Embden-Meyerhof system and in addition, could oxidize carbohydrate, led to the suggestion that carbohydrate was fermented to the pyruvate stage by this glycolytic pathway and then oxidized by way of the tricarboxylic acid cycle to CO_2 and H_2O. This viewpoint was strengthened when the investigations were extended to bacteria. The bacteria studied were facultative types such as Escherichia coli and Aerobacter aerogenes and the importance of the Embden-Meyerhof pathway and its participation in the oxidation of carbohydrate were confirmed. As late as 1951, workers such as Umbreit (1949) and Colowick and Kaplan (1951) assumed that this glycolytic pathway was the only important pathway for the break-

down of hexoses. This was in spite of the fact that by this
time a great deal of evidence on the occurrence of alternate
routes of carbohydrate metabolism had accumulated.

Warburg and Christian (1931) found that hemolysate
required glucose-6-phosphate and would not use glucose. How-
ever, these workers concluded that this pathway was of no
importance in whole cells and could not compete for glucose-
6-phosphate with the Embden-Meyerhof system. The first schema-
tic presentation of an alternate pathway of glucose metabolism
was brought forward in 1936 by Dickens. In this preliminary
note he outlined a scheme of oxidation of carbohydrate in which
the first stages were the esterification of the hexose to
hexosemonophosphate; oxidation of this first to the phospho-
hexonate; then to 2-ketophosphohexonate; decarboxylation to a
pentose phosphoric acid and a continuation of these processes
eventually producing pyruvic acid, which would then be com-
pletely combusted.

In the same year Lipmann (1936) found that the
oxidation of one mole of phosphogluconic acid by ground yeast
resulted in the consumption of one atom of oxygen and the
production of one to 1.5 moles of CO_2. The amount of CO_2 was
reduced to about one mole in the presence of monobromoacetate.
Lipmann then advanced the theory that 2-keto,6-phosphogluconate
was first formed ($\frac{1}{2}$ O_2 consumed), this was then decarboxylated
(1 CO_2 liberated) and the further process consisted of the
fermentation of the theoretically expected d-arabinose phos-
phate. Some evidence tending to show keto-acid formation was

provided by experiments in which HCN appeared to act as a ketone fixative. In other experiments, addition of carboxylase to a carboxylase-free enzyme preparation caused a further oxidation and decarboxylation of phosphohexonate. Warburg and Christian (1936) meanwhile purified glucose-6-phosphate dehydrogenase and with it showed that the end-point in Lipmann's experiment was not valid and was, in reality, due to a gradual destruction of enzyme activity. Lipmann's experiments with HCN and carboxylase, apparently were too indirect and could not be rigidly interpreted.

Later Dickens (1938) established conclusively that pentose phosphoric acids arose from the oxidation and decarboxylation of hexose phosphoric acids. Subsequently, it was shown that pentose phosphate was readily fermented by yeast enzymes in the presence of coenzyme I and inorganic phosphate. The important point which arose during this work was that the pentose phosphoric acid most readily dissimilated both oxidatively and by fermentation was not arabinose-5-phosphate which would be expected to arise from simple oxidation and decarboxylation of carbon-1 of glucose but was ribose-5-phosphate. Ribose itself was not fermented either by yeast cells or yeast extract, nor was it oxidized by brain slices or by yeast extract. Dickens advanced the theory that ribose-5-phosphate originated from hexoses by a process of phosphorylation, oxidation and decarboxylation.

Shorr et al. (1938) observed that respiration in

yeast and mammalian tissue continued even after fermentation
has been inhibited by iodoacetate. This suggested that fer-
mentation and respiration proceeded by two different pathways
and indicated that the iodoacetate-insensitive system might
be of some importance.

Although Dicken's work gave the field of aerobic
metabolism a tremendous impetus, it remained for Horecker,
Smyrniotis and Seegmiller to carry this line of thought to
its logical conclusion. Horecker and Smyrniotis (1950) puri-
fied, from yeast, the enzyme which catalyzed the oxidation of
6-phosphogluconate. They found that pentose phosphate was
produced quantitatively according to the equation:

$$6\text{-phosphogluconate} + TPN \longrightarrow \text{pentose phosphate} + CO_2 +$$
$$TPNH + H^+$$
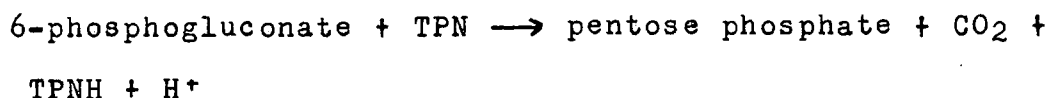
In addition, 85 per cent of the pentose phosphate, on a paper
chromatogram, corresponded to ribose-5-phosphate. Various
other procedures such as the enzymic removal of phosphate and
paper chromatography with four solvent systems confirmed the
presence of ribose. Their results placed ribose-5-phosphate
in the direct pathway of phosphogluconate oxidation.

This alternative pathway has been studied intensively
by these workers in recent years using a purified yeast pre-
paration. Glucose was shown to be oxidized by way of glucose-
6-phosphate and 6-phosphogluconate. The latter was then
oxidized with the formation of ribulose-5-phosphate (Cohen
and Scott, 1950; Horecker and Smyrniotis, 1951; Horecker,

Smyrniotis and Seegmiller, 1951). In order to account for
this reaction a hypothetical intermediate, 3-keto,6-phospho-
gluconate was postulated and this has now been isolated as
the crystalline calcium salt by MacGee and Doudoroff (1954).
It was shown that ribulose-5-phosphate was in equilibrium
with ribose-5-phosphate.

Independent work by Seegmiller and Horecker (1952)
on an enzyme preparation from rabbit liver, confirmed the
observation that 6-phosphogluconate is oxidized to pentose
phosphate and carbon dioxide. The keto-pentose, D-ribulose-
5-phosphate was identified as the product. The enzyme prepara-
tions contain also a phosphopentose isomerase which catalyzes
the reversible conversion of D-ribulose-5-phosphate to D-
ribose-5-phosphate reaching an equilibrium ratio of approxi-
mately one to three. An enzyme preparation from rabbit bone
marrow catalyzed the formation of glucose-6-phosphate from 6-
phosphogluconate, thereby demonstrating a reversal of the
direct oxidative scheme.

A variation of this scheme has been elucidated by
Entner and Doudoroff (1952) in Pseudomonas saccharophilia.
Instead of being oxidized, the 6-phosphogluconate is split
into one mole of 3 phosphoglyceraldehyde and one of pyruvate,
and these are metabolized by the usual pathways.

These phosphorylated schemes have been generally
accepted as being almost universally distributed in nature.
But, of late, still another pathway of carbohydrate metabolism

not involving the formation of any phosphorylated intermediates in the earlier stages of oxidation has assumed some importance in the biochemical field. As early as 1928 Müller made the observation that the enzyme Notatin, obtained from young cultures of A. niger and other molds, oxidized glucose to gluconic acid. Keilin and Hartree (1948) established that flavin adenine dinucleotide was the prosthetic group of this enzyme and that the enzyme system was specific for glucose. Further work by Bentley and Neuberger (1949) established that Notatin was a dehydrogenase and that the product of glucose oxidation was Δgluconolactone.

The first observation that a somewhat similar enzyme was present in other tissues was made by Harrison in 1931 when he noted that glucose could be converted to gluconic acid in liver. He isolated the glucose dehydrogenase catalyzing this reaction in crude form. However, biochemists viewed the importance of these findings with some skepticism. Since no energy transfer could be demonstrated in the conversion, the reaction was considered to be physiologically unimportant. There were other reasons why this scheme was not brought into a scheme of metabolism. Since the reaction was studied as an isolated system, and since no effort was made to determine whether or not gluconate was an intermediate in glucose oxidation, the gluconate was pictured as an end-product rather than an intermediate. Moreover, it is now known that glucose is not oxidized by way of this pathway in liver. Strecker and

Korkes (1952) have recently isolated this glucose dehydrogenase
in a fairly pure form and showed that it oxidizes beta-glucose
preferentially with Δ gluconolactone as a probable product.
Eichel and Wainio (1948) found that DPN, cytochrome c and cyto-
chrome oxidase were involved in the oxidation of glucose by
this enzyme and Renvall (1950) reported that diaphorase was
the link between the dehydrogenase and cytochrome c.

The formation of the lactone as the first step in
the oxidation of glucose has been stressed by Brodie and
Lipmann (1954). They isolated two enzymes from <u>Azotobacter</u>
<u>vinelandii</u>, one which oxidizes glucose to D-glucono-Δ-lactone
and the other which hydrolyzed this lactone to the corres-
ponding acid. Previously the reaction involved in the overall
oxidation of glucose to gluconic acid or glucose-6-phosphate
to 6-phosphogluconate has been assumed to involve a non-
enzymatic hydrolysis of the lactone to the acid. Lactone
formation has also been demonstrated by Cori and Lipmann (1952)
during the oxidation of glucose-6-phosphate by using hydroxy-
lamine as a trapping agent.

Many observations were recorded which indicated that
the direct oxidation of glucose might be carried out by a
variety of bacteria. In 1929 Bernhauer and his associates
showed the production of gluconic acid by <u>Bacterium xylinum</u>
and a few years later demonstrated the formation of gluconic
and 2-ketogluconic acids by other pseudomonads. In 1935
Bernhauer and Knoblock reported that when <u>Acetobacter</u>

suboxydans was grown on a glucose medium containing calcium
carbonate, 2-ketogluconate and 5-ketogluconate accumulated.
These data were criticized on the grounds that an impure cul-
ture must have been employed, since no known organism produces
both intermediates. Bernhauer and Gorlich (1940) also noted
the formation of 2-ketogluconic acid during glucose dissimila-
tion by Pseudomonas gluconicum.

On this continent Lockwood (1940) reported that
large quantities of gluconic and 2-ketogluconic acids accumula-
ted during the growth of P. fluorescens on a glucose medium.
Moreover, Barron and Friedman (1941), using whole cells of P.
aeruginosa, could not find any inhibition of glucose oxidation
by sodium fluoride and therefore concluded that the reaction
did not involve phosphorylation. However, with the exception
of Barron and Friedman, workers were interested only in the
commercial production of organic acids and were not concerned
with pathways of metabolism. They therefore made no effort
to determine whether or not the compounds in question were
intermediates in the direct pathway of carbohydrate oxidation.

Norris and Campbell (1949) found detectable amounts
of gluconic and 2-ketogluconic acids in 16 and 24 hour cultures
of P. aeruginosa when glucose was used as the growth substrate.
Since the resting cells harvested at these same periods of
time were shown to have strong systems for the oxidation of
these two acids it was concluded that gluconate and 2-keto-
gluconate were being continuously formed and continuously

oxidized and thus were intermediates in a pathway of glucose
oxidation. These workers also found that the oxidation of
glucose by cell extracts of this organism was not inhibited
by sodium fluoride, thus indicating that phosphorylated inter-
mediates were not involved. These data agree with Barron's
conclusion that whole cells of P. aeruginosa oxidize glucose
without phosphorylating it. Additional information against
phosphorylation was that under anaerobic conditions, resting
cell suspensions failed to remove any glucose or fructose or
to take up phosphate. Moreover, glucose-1-phosphate, glucose-
6-phosphate and fructose-6-phosphate were found to be absent
during glucose degradation. It was also found that dried cell
preparations oxidized glucose or gluconate with quantitative
accumulation of 2-ketogluconate, thus indicating that no other
pathway was active and that gluconate is an intermediate in
the pathway. All these results led Campbell and Norris (1950)
to conclude that a conventional scheme was not operative in
P. aeruginosa. Hill (1952) compared the enzymic pattern of
the cells grown on glucose medium with that of cells grown on
2-ketogluconate. He found that allowing for normal variations
between runs, the two types of cells showed no difference in
their enzymic makeup as indicated by their ability to attack
glucose, gluconic, 2-ketogluconic, pyruvic, α-ketoglutaric,
fumaric, succinic, citric and acetic acids. These data con-
firmed the conclusion that 2-ketogluconic acid was in the
direct pathway of glucose oxidation.

Warburton, Eagles and Campbell (1941) determined pyruvate at 16, 28 and 40 hours in a culture of P. aeruginosa when growing in a glucose medium. Throughout this interval the organism was shown to possess an enzyme capable of rapidly oxidizing pyruvate. They, therefore, concluded that pyruvate was a normal intermediate in glucose oxidation. Campbell and Stokes (1951) found that resting cells of the same organism, harvested from a growth medium containing acetate as the sole source of carbon, had no ability to oxidize citrate, cis-aconitate, iso-citrate, ∝-ketoglutarate, succinate or fumarate without a period of adaptation. However, when these fresh resting cells were dried, it was found that they then had the ability to oxidize these substrates immediately and also to oxidize malate, acetate, oxalacetate and pyruvate. They concluded that the organism oxidized glucose by way of Krebs' citric acid cycle.

Katznelson, Tanenbaum and Tatum (1953) found that aged cell suspensions or cell-free extracts of Acetobacter melanogenum quantitatively oxidized glucose, gluconic or 2-ketogluconic acids to a common intermediate, thus supporting the view that 2-ketogluconate is part of a major pathway of glucose oxidation in many microorganisms. They claimed that 2,5-diketogluconic acid was the intermediate while Foda and Vaughn (1953) reported that this organism dissimilates maltose by way of glucose, gluconic acid and 5-ketogluconic acid, 2-ketogluconic acid not being an intermediate. Results in

agreement with Foda and Vaughn have been obtained by Khesghi et al. (1954) working with A. suboxydans. However, other workers obtained data that led them to conclude that this direct oxidative pathway for the degradation of glucose either is a shunt mechanism or does not exist. Using E. coli which had been adapted to gluconate, Cohen and Scott (1950) extracted an enzyme, gluconokinase, which catalyzed the phosphorylation of gluconate to 6-phosphogluconate. However, they could not obtain evidence for the existence of this enzyme in the un-adapted cells. When E. coli was adapted to gluconate, the cells were not simultaneously adapted to D-arabinose, ara-bonate or 2-ketogluconate. The phosphogluconate was appar-ently degraded exclusively via the oxidative pathway through pentose phosphate and presumably no non-phosphorylative oxida-tion of gluconate occurred.

Entner and Stanier (1951), studied glucose and gluconate oxidation by P. fluorescens after growth on media containing either asparagine or glucose. The asparagine grown cells were unadapted to gluconic and 2-ketogluconic acids but were immediately able to oxidize glucose. One atom of oxygen was taken up per mole of glucose before the lag period set in, showing that glucose was converted to gluconic acid but that the enzyme to oxidize gluconic acid was absent. Under con-ditions which prevented subsequent adaptation to gluconate and 2-ketogluconate, it was found that glucose grown cells oxidized glucose and gluconate immediately, whereas 2-keto-

gluconate was initially oxidized slowly with the rate in-
creasing slightly thereafter. These observations were inter-
preted as showing that glucose dissimilation proceeds largely
via gluconic acid. The possibility of glycolysis was not
excluded. Although 2-ketogluconate accumulated during the
oxidation of glucose or gluconate, the manometric data were
interpreted to show that the oxidation of these substances
could not proceed via 2-ketogluconate. However, the data
could also be interpreted as indicating only that the parti-
cular organism studied has an unusually weak system beyond 2-
ketogluconate.

A major criticism of the nonphosphorylated pathway
was answered by the work of Wood and Schwerdt (1953,1954).
Previously it was often suggested that gluconic and 2-keto-
gluconic acids occurred merely as dephosphorylated products
of the active compounds and were not on the pathway of metabol-
ism. Wood and Schwerdt were able to demonstrate that the sonic
extract of P. fluorescens had the ability to oxidize glucose-
6-phosphate, 6-phosphogluconate, ribose-5-phosphate, glucose
and gluconic acid. Using ammonium sulphate fractionation they
were able to separate the glucose-gluconic oxidizing system
from the soluble enzyme system which oxidized the phosphory-
lated compounds. The nonphosphorylated glucose-gluconate
system was not a flavoprotein oxidase and did not involve a
DPN or TPN specific glucose dehydrogenase. Cytochrome
carriers apparently are involved and so this glucose dehydro-

genase differs from Notatin or from that studied by Harrison (1931). The soluble enzyme for the phosphorylated compounds appears to be similar to the one reported by Entner and Doudoroff (1952) for P. saccharophilia but no hexokinase could be demonstrated. The presence of the system for oxidizing glucose-6-phosphate as well as the nonphosphorylated system for glucose oxidation in P. fluorescens A. 312 probably serves a necessary function in the intermediate metabolism of the organism. Apparently P. aeruginosa 9027 is similar in this respect (Wood, 1954). The differences between these pseudomonads and the usual hexokinase containing organisms may be considered to have a counterpart in the strains of A. aerogenes studied by Magasanik, Brooke and Karibian (1953). These workers found that a capsulated strain dehydrogenated glycerol without prior phosphorylation whereas the noncapsulated strain formed L-α-glycerophosphate before dehydrogenation.

In contradiction to all other studies on the subject, Claridge and Werkman (1954) presented data to indicate that P. aeruginosa 9027 possesses two glucose dissimilating mechanisms. Cell-free extracts were reported to phosphorylate glucose and the presence of triose phosphate dehydrogenase and aldolase was demonstrated. The aldolase had only weak activity and had its point of optimum activity at pH 8.3. This would indicate that glycolysis is not a major pathway in this organism. This is the only report indicating that this organism contains hexokinase. Experiments conducted both in our laboratory and in Wood's laboratory, however, show no phosphorylation of the

substrate in the early stages. The oxidation of glucose and gluconic acid without phosphorylation casts some doubt on the efficiency of the system in yielding energy. However, it is conceivable that the oxidation of these substrates is similar to the oxidation of the reduced form of DPN. Friedkin and Lehninger (1948) have shown that the hydrogen transport between DPNH and oxygen can be coupled with phosphorylation of a second system. By a system such as this, glucose and gluconic acid may be oxidized to 2-ketogluconic acid while an entirely different compound was phosphorylated during the oxidation. Since P. aeruginosa has been shown to possess a tricarboxylic acid cycle (1950) phosphorylation does take place in later stages. Also, Claridge and Werkman (1954) have shown that the aerobic dissimilation of 2-ketogluconate produces members of the citric acid cycle.

Moreover, recent data indicate that phosphorylation does occur at the 2-ketogluconate level. The metabolism of P. fluorescens has been shown to be different from that of P. saccharophilia in later stages, and is apparently similar to the system postulated by DeLey (1953). Narrod and Wood (1954) found that the former contained a magnesium-dependent 2-ketogluconate phosphorylating enzyme. They used the formation of acid-stable (ester) phosphate and acid formation as the criteria for phosphorylation. With crude preparations they found that a 2,4-dinitrophenylhydrazine reactive keto-acid was formed in the presence of 2-ketogluconate and ATP but not with 2keto-gluconate alone.

Although growth studies or experiments with resting cell suspension may greatly aid in the elucidation of problems relating to metabolic pathways, nevertheless, more intensive treatment of these problems almost invariably leads to the use of some type of cell preparation.

These preparations have the advantage that the semi-permeability of the cell membrane has been destroyed thus allowing all substrates to freely come into contact with the enzymes of the cell. This change in permeability also puts inhibitor studies on a sounder basis. In addition, the cells will have lost their ability to adapt to new substrates or to synthesize any cell constituents, thus removing the ability of the cell material to carry out oxidative assimilation. This, then, allows one to assume that the gas exchange values obtained with cell preparation can be given a rigid quantitative interpretation.

Moreover, often some enzymes are destroyed during the treatment of the cell suspension thus allowing the accumulation of an intermediate product. This, of course, is a mixed advantage and eventually techniques of securing a cell preparation with this enzyme intact must be found.

Certain grinding and extraction techniques produce, in quite isolated form, the required activity as long as a specific tissue source is employed. However, other very similar techniques will produce a complex of activities. Unfortunately cell preparations are still empirically produced and it is impossible to predict, with any degree of certainty

which activities can be extracted by any given procedure. A description of some of the general techniques available are presented here.

1. _Drying_: The simplest method for making cell preparations of bacteria is to spread a suspension thinly and dry _in vacuo_ over a drying agent. Doudoroff _et al_. (1943) working on phosphorolysis and synthesis of sucrose by _P. saccharophilia_ dried the cell paste over P_2O_5. Before use the cells were powdered and resuspended. Fosdick and Dodds (1945) spread the cells of _Aerobacter aerogenes_ thinly on a porous plate, dried them _in vacuo_ and then ground the cells lightly when dry. These cells were used in studies on glucose degradation. Sleeper _et al_. (1950) studying the oxidation of aromatic compounds, spread a centrifuge paste in a thin layer and dried slowly _in vacuo_ at room temperature. Dried cells were finely ground and stored in the freezing compartment of the refrigerator. They found that the endogenous respiration of such preparations increased for four to five days, thereafter dropping to a low level. Cells dried too rapidly had the undesirable characteristics of fresh cells. This method probably results in increased permeability of the cells, and during prolonged drying some lysis will occur. Most of the cells are dead after this treatment. Stadtman and Barker (1949), in their studies of the metabolism of fatty acid synthesis by _Clostridium kluyveri_ dried the cells _in vacuo_ over calcium chloride and extracted the enzymes which convert ethanol and acetate to butyrate and caproate with dilute sodium

sulphide solution at pH 7.0. Hughes and Williamson (1952)
prepared the glutaminase of _Clostridium_ _welchii_ by drying the
frozen cells _in_ _vacuo_ over phosphorus pentoxide followed by
extraction of the dried powder with Na_2HPO_4/KCl solution for
15 hours by rotating at $2^{\circ}C$ in a stoppered conical flask con-
taining 5 mm. diameter glass beads.

2. _Acetone_ _drying_: The method consists of mixing
the tissue mince or bacterial cells with cold acetone, fil-
tering and drying _in_ _vacuo_. According to the original method
of Bernheim (1928), 300 ml. of acetone were used for 1 lb. of
liver mince to obtain citric acid dehydrase and the procedure
repeated four times before drying _in_ _vacuo_. Waldvogel and
Schlenk (1949) in their studies on the enzymic conversion of
ribose to hexosemonophosphate, recommend an excess of acetone.
Wood _et_ _al._ (1947) washed the _E._ _coli_ cells with ether to ex-
tract tryptophanase while Epps (1944) used acetone-ether mix-
ture on _Streptococcus_ _faecalis_ to obtain tryosine decarboxylase.
Hochster and Quastel (1951) employed the acetone drying techni-
ques in their studies on the effect of nicotinamide on ferment-
ations by preparations of Baker's yeast. Acetone treatment
apparently does not destroy the cell structure but dehydrates
it rapidly and increases the permeability of the cell membrane.
Some enzyme systems are sensitive to this treatment. All the
enzymes which we have studied in _Pseudomonas_ _aeruginosa_, for
instance, are inactivated by acetone treatment.

3. _Grinding_ _technique_: Grinding is a very widely
used method of making cell preparations. The techniques of

grinding vary in their complexity from simple grinding in a
mortar to grinding in a machine such as the Booth-Green mill.
Formerly a meat grinder with a fine cutter was used. Claude
(1944) used this method to extract the succinate-fumarate
system from beef heart. Now it is used only as a preliminary
to more thorough homogenization in another apparatus. Colo-
wick et al. (1947) ground muscle in a mortar with water to
extract hexokinase; Claude (1944) used 0.85 per cent saline
for studies on nucleic acids in rat tumour extracts; Banga
et al. (1939) extracted brain tissue with 0.9 per cent KCl
to obtain pyruvate oxidase; and Boyer and Lardy (1943) ground
muscle with Ringer's solution to study the phosphorylation of
the adenylic system. Grinding in a mortar is not particularly
effective since few cells are disintegrated and little activity
is freed.

Greater maceration and increased release of activity
result if sand is used in the mortar along with the water or
extractant. Boyer and Lardy (1942), for instance, used this
method for studying the phosphorylation of creatine in muscle.
Diatomaceous earth (Müller, 1926) can also be used. The degree
of maceration can be judged by the increase in fluidity of the
mixture. This method is fine for animal tissue cells since
they are fairly large, but is of little use for bacterial cells
since they are too small and refractory to be broken easily.
The method of Wiggert et al. (1940) is widely used for bac-
terial cells. In this method a bacterial paste (3 gms.) is
mixed with 25 gms. of powdered glass and 7 ml. of pH 7.0 M/15

phosphate. The paste is ground vigorously in a mortar for not
longer than five minutes. Kalnitsky and Werkman (1943) studied
the anaerobic dissimilation of pyruvate by E. coli by this
method. Lee et al. (1942) used the same method to obtain hydro-
genase from Azotobacter but used one part of cells to two parts
of powdered glass and sufficient buffer to make a thick batter.
McIlwain (1948) found a slow cutting, polishing alumina was
superior to glass. He used 2.5 times the cell weight of alumina
in a chilled tube with a chilled glass rod. The material is
rubbed with maximum hand pressure for 30 seconds, fragmentation
being evidenced by an increase in fluidity and darkening.
Extracts were prepared from streptococci in this way, which
were capable of glycolysis, the deamination of adenosine di-
phosphate and the production of ammonia from arginine. The
mass is washed from the tube with extractant and centrifuged.
The enzyme activity is found in the clear supernatant. Barnard
and Hewlett (1911) devised a mill for grinding bacterial and
other cells. The mill was a phosphor-bronze cup containing
five hardened steel balls. The inside diameter of the cup was
slightly less than the sum of the diameters of three of the
balls. A central conical steel shaft fitted into the centre
kept the balls at the periphery and acted as a rotor. The
shaft could be forced down to bring close contact between the
shaft and the balls. Grinding was carried out at 1500 r.p.m.,
the chamber being cooled by a stream of CO_2 from liquid CO_2
or by being placed in an ice-salt bath. Ogston and Green
(1935) used a mill of similar structure for preparing the

lactic, succinic, α-glycerophosphoric, glucose and malic dehydrogenases from yeast.

Gunsalus and Umbreit (1945) devised a glass ball mill in which anaerobic grinding can be done. They used it to extract glycerol oxidizing enzymes from <u>Streptococcus faecalis</u>. The round bottom flask with a ground glass stopper is fitted with a tube having a stopcock at one end. Glass beads of varying sizes are added to the bottom of the flask and the material to be ground is added. The flask can then be evacuated and the stopcock closed. It may be, if desired, filled with nitrogen or other gas. The neck is then attached to a slowly rotating motor and the flask supported on a funnel filled with light oil. After eight hours or overnight grinding, 90 to 95 per cent of the cells were destroyed.

Heden (1951) designed a micro ball mill for bacterial cells, consisting of pyrex tubes bent into a V-shape and containing glass beads approximately of the same diameter as the internal diameter of the tubing. The tubes and beads may be etched with fluoride. The series of tubes is placed in a Woods metal holder and clamped down with a rod covered with rubber hose. The whole is mounted on the stage of a 50 c.p.s. vibrator in the cold. Bacterial cells are broken in four hours. This rate may be accelerated by addition of glass powder.

Paege and Schlenk (1950) have used the grinding apparatus of Utter and Werkman to extract cytosine nucleoside deaminase from <u>E</u>. <u>coli</u>. The mill consists of an inner glass

cone connected to a motor and filled with ice-salt, which
fits closely in a fixed outer cone at the end of which is a
chamber for the grinding mixture. The mixture of 1 gm. of
cell paste to 2 gm. ground glass is fed to the cones by a
plunger. The ground material is caught in a beaker which is
kept closed. The glass particles used average two microns in
diameter. Carborundum is a less efficient grinding agent.
One of the more widely used grinding mills is the Potter-
Elvejhem homogenizer. It is cheaply and easily made and
quickly and efficiently disintegrates large cells. A motor-
driven pestle made from a capillary tube whose end has been
sealed, is blown into a 20 mm. bulb and ground to fit a 150 x
16 mm. pyrex test tube. The pestle has 12 or so beads fused
onto the bottom edge and is driven at 1,100 to 1,200 r.p.m.
by a cone motor. The degree of disintegration of tissue can
be governed by the tightness of the fit of the pestle in the
tube. Grinding agents such as glass have been used, as for
instance, by Chantrenne and Lipmann (1950) in their studies of
the pyruvate-formate exchange system of E. coli. Nachmansohn
and John (1945) used silica instead of glass to extract choline
acetylase from brain. Different grinding fluids can be used.
Lipmann (1945) used phosphate to study the acetylation of
sulphanilamide by liver. Cohen and Hayano (1946) used Ringer's
solution for the enzymes converting citrulline to arginine in
mammalian liver. Ames (1947) in his studies of the succinic
oxidase system of rat muscle, used ice water, while Elliott
and Kalnitsky (1950) used saline to investigate the oxidation

of acetate by rabbit-kidney cortex. Ratner and Pappas (1949)
used a loose pestle apparatus to obtain active liver homo-
genates capable of converting citrulline to arginine.

The Booth-Green mill (1938) consists of three races
of roller bearings with a central tapered shaft forced into
the centre to cause zero clearance between the roller bearings.
The races are held in heavy hosing. Paper-thin bakelite
spacers are placed between the rollers to prevent excessive
wear. The shaft is driven at 6,000 r.p.m. at which speed
yeasts are fragmented in 10 minutes and the most resistant
microorganisms in two hours. A heavy cell suspension is
poured into the top of the mill and drops through the rollers,
collects in a pump and is recirculated through a cooling bath
to the top of the machine. Wirth and Nord (1940) used the
mill on Fusaria to study alcoholic fermentation. Still (1941)
used a bacterial paste diluted with an equal volume of water
in their investigation of pyruvic dehydrogenase of E. coli.
The mill has not been as widely used as the Potter homogenizer
since it is much more complicated and expensive, and it is
doubtful if it is any more effective. Its chief advantage is
its large capacity.

Muys (1949) described an automatic bacterial mill
in which cells could be crushed anaerobically if necessary.
A rounded cylinder of glass is made to revolve in a horizontal
plane inside a thick-walled glass-tube. The revolving cylin-
der is arranged to touch at its end the rounded end of the
tube, and it is across this point of contact that the bacteria

are forced by a gravity feed before they fall into a receiver.

Hughes (1951) has described a press which has the special feature of combining low temperatures and a short period of mechanical treatment. The cell suspension, together with appropriate abrasive, is placed in a cylinder hollowed in a stainless steel block previously cooled. An accurately machined piston is driven by means of a fly press at a force of 12 to 15 tons per square inch, on to the cells, and the latter are forced from the cylinder cut in the block. At temperatures from -20°C to -35°C, abrasives could be dispensed with, ice crystals being presumed to perform this function. Ghiretti and Barron (1954) have studied the glucose dehydrogenase in Corynebacterium creatinovorans by this method; Pollock (1953) investigated penicillinase adaptation by Bacillus cereus by crushing the bacterial spores in the cylinder; Bealing and Bacon (1953) disrupted mold mycelium by this method to study the invertase in the system.

4. Shaking techniques: Shaking cells with sand or other abrasives has been used to effect cellular fragmentation (Curran and Evans, 1942), and while the method is effective, the chief difficulties involved are that a great deal of heat results from the abrading of the particles, and the procedure is fairly lengthy. Either the equipment must be equipped with a cooling system or the procedure carried out in a cold room.

Mickle (1948) designed an apparatus for the rapid mechanical shaking of bacterial or yeast cells with glass

beads. A description of this apparatus is given by Hugo
(1954). Studies on the conditions for optimum breaking by this
method have been made for E. coli (Furness, 1952) and Staphylo-
coccus aureus (Cooper, 1953).

5. Sonic disintegration: One of the newer methods
of cellular fragmentation is by the use of sonic and ultra-
sonic vibrations which may be produced either by means of a
nickel rod or by means of crystals. Stumpf and Green (1944)
exposed a 1:2 dilution of a washed centrifuge paste of Proteus
vulgaris in a 200 ml. volumetric flask to a three inch quartz
generator operating at 1,000 volts and 500 watts for 20 minutes.
They were studying the L-amino acid oxidase in this organism.
The oil in the bath was cooled by circulation through an ice-
bath which kept the flask contents below 38°C. Stumpf (1945)
in a study of the pyruvic oxidase of Proteus vulgaris disinte-
grated cells by exposure in a 50 ml. erlenmeyer flask which
was lowered into the oil bath to the critical distance from the
crystal. The critical distance was found to be the point at
which the cone of fluid in the flask was highest. A thin flat-
bottomed flask was found essential for proper fragmentation.
A thin (25 mg. dry wt./ml.) suspension is more effective than
a heavy one. A heavy suspension causes the energy to be
dissipated in heat and little fragmentation takes place. Under
these conditions a 10 minute irradiation was found sufficient.
The vibrations were generated by a cross-cut quartz crystal
one to five inches in diameter and ground to 600 kc. The
generator ran at 200 volts and 700 watts. The frequency of

the waves was found to be of little importance but the ampli-
tude (intensity) was critical. Pappenheimer and Hendee (1949)
fragmented a saline suspension of Corynebacterium diphtheriae
by a 30 minute treatment in a 9 kc. oscillator manufactured by
the Raytheon Corporation to study the succinoxidase system.
Paege and Schlenk (1950) irradiated resuspended acetone treated
cells of E. coli for 20 minutes in the same apparatus to achieve
fragmentation and isolate cytosine nucleoside deaminase.
Kallio (1951) prepared an active disulphydrase from Pseudomonas
morganii by subjecting the previously dried cells to sonic os-
cillation in 0.05M phosphate buffer. Slade (1953) obtained an
arginine dihydrolase from Streptococcus faecalis by treating a
3 per cent (dry wt.) suspension in water to sonic oscillations
in a Raytheon 9 kc. oscillator for three hours.

     6. Lysis: This technique has not been too widely
used since during lysis any sensitive enzymes will be des-
troyed. Some of the earlier workers used the method quite
satisfactorily before other techniques were available. Stick-
land (1929) allowed E. coli cells to lyse in order to study
the decomposition of formic acid. Due to mild proteolysis
during lysing, insoluble enzymes will be free from particulate
matter. Incidental lysis may be responsible for the success
of some of the other techniques for freeing enzyme activity.
The simplest method of lysis is to place the cells in distilled
water after washing in an isotonic solution. Harvey (1949) in-
vestigated the carbohydrate metabolism of Trypanosoma hippicum
by this method, which, however, is valid only for very fragile

cells. Autolysis by incubation of cells in buffer solutions
is another simple method of freeing enzymes. Neuberg and
Lustig (1948) lysed dried yeasts by incubating in diammonium
phosphate with shaking at 37°C. Stephenson (1928) lysed E.
coli and liberated lactic dehydrogenase by suspending the washed
cells in M/2 phosphate for five to six days. Umbreit and Gun-
salus (1945) autolysed dried cells of E. coli for two hours at
37°C in 0.15M borate at pH 8.2. Bovarnick (1941) lysed E. coli
communior for 16 days after acetone drying. A more drastic
treatment makes use of crude enzymes found in egg white and
saliva. In order to extract oxalacetate decarboxylase Krampitz
and Werkman (1941) lysed a 10 per cent acetone cell suspension
of Micrococcus lysodeikticus with lysozyme in 1/10 volume of
saliva by incubating for 60 minutes at 36°C. Utter et al.
(1946) in an investigation of acetyl phosphate oxidation,
lysed acetone preparations of the same organism by adding 3 ml.
of a 1:20 dilution of egg white per gm. of cell preparation.
In their studies of the production of peroxide by pneumococci,
Avery and Neill (1924) added 0.5 ml. of sterile ox-bile to 15
ml. of a bacterial suspension and incubated anaerobically at
37°C for 12 hours. In order to obtain formic dehydrogenase
Stickland (1929) lysed a washed aerated suspension of E. coli
with 5 ml. of liquor pancreaticus per 100 ml. of suspension in
a solution buffered at pH 7.6. Fluoride (0.1 per cent) was
sometimes added to the lysing solution to inhibit growth.
Sher and Mallette (1953) obtained extracts of E. coli by lysing
the cells with the specific bacteriophage. It was found that

the lysine and arginine decarboxylases in the cell-free extract had five times the activity of the whole cells.

7. _Freezing and thawing_: Koepsell and Johnson (1942) prepared pyruvic acid oxidizing enzymes from Clostridium butyricum by freezing a Sharples paste for 12 days. The freezing ruptured the cells and on thawing the debris was removed by centrifuging. Koepsell et al. (1944) froze a cell paste of the same organisms for 14 days and ground after thawing. Nason et al. (1951) froze Neurospora mats at -18°C for one to three hours to rupture the cells. Wood and Gunsalus (1947) froze and thawed a preparation of E. coli and subsequently let it autolyze to obtain tryptophanase. Loomis (1950) stabilized mitochondrial activity for oxidation and phosphorylation by freezing at -100°C and storing in dry ice while still frozen. Black (1951) prepared an aldehyde dehydrogenase from Baker's yeast by freezing the cells in liquid nitrogen and extracting at pH 8.3 at 4-5°C for five days.

METHODS

Bacteriological: The organism used throughout the experimental work was Pseudomonas aeruginosa ATCC 9027. In some comparative experiments Pseudomonas fluorescens A. 312 obtained from Dr. R. Y. Stanier was used. Stock cultures were incubated at 30°C for 48 hours and then removed to the refrigerator for storage. A fresh culture was taken from stock once a month. The stock medium had the following composition:

	Per cent
Tryptone	1.0
K_2HPO_4	0.5
Glucose	0.1
Glycerol	0.5
Yeast extract	0.1
Agar	0.5
Gelatin	2.0
pH 7.2	

The medium used for securing active resting cells with a low rate of endogenous metabolism was that of Campbell et al. (1949) but with gluconate replacing glucose as the carbon source. This medium has the following composition:

	Per cent
$NH_4H_2PO_4$	0.3
K_2HPO_4	0.3
Sodium gluconate	0.3
$MgSO_4 \cdot 7H_2O$	0.1
Fe as $FeCl_3$	0.5 p.p.m.
pH 7.0	

For the growth of large quantities of cells the medium was dispensed in 100 ml. quantities into Roux flasks. Inoculum was prepared from a stock culture by transferring to a glucose agar slant. At least three daily transfers were made before the culture was used for inoculating a larger volume of medium. Normally one Roux flask containing the gluconate medium was inoculated and incubated at 30ºC for 18 to 24 hours. This

then served as the source of inoculum for the large volume of medium. Inoculum at the rate of 1.0 per cent was used.

Production of active cell preparations: The cells were harvested in a Sharples centrifuge and washed twice with M/30 pH 7.0 phosphate buffer. The resulting cell paste was resuspended in a similar buffer at a rate of 200 milligrams per ml. of distilled water. The cell suspension was subjected to sonic oscillation for 20 minutes in a Raytheon 10 kc. oscillator. The resultant mixture (hereafter called the sonicate) was placed in chilled plastic centrifuge cups and carried in an ice bath to a Servall SS-1 centrifuge maintained at approximately $-10°C$. The sonicate was then centrifuged for five minutes at 25,000 times gravity and the particulate matter discarded. The supernatant could be stored in the deep freeze until needed for manometric or fractionation studies.

Chemical: Metabolic gas exchanges were measured in the Warburg respirometer according to the standard procedure outlined by Umbreit et al. (1949).

Two-ketogluconic acid was detected by paper chromatography with an ethanol-methanol-water (45:45:10) solvent system and 0.1N $AgNO_3$ in 5N NH_4OH as described by Norris and Campbell (1949). Phosphate esters were looked for by chromatography with an isopropanol-4 per cent ammonium sulphate (60: 35) solvent system and 4 per cent ammonium molybdate in 60 per cent perchloric acid (Hanes and Isherwood, 1949; Bandurski and Axelrod, 1951) and observing the paper under a source of ultraviolet light.

When chemical analysis of the cup contents was desired the reaction was carried out in a 125 ml. Warburg vessel containing the following solutions:

	ml.
Glucose (25 uM/ml.)	2.0
KH_2PO_4 (M/15, pH 5.6)	15.0
Water	10.0
Enzyme	1.0
Pyocyanine (3.75 mg./ml.)	2.0

The rate of oxidation was followed with a conventional Warburg system containing one-tenth of the above constituents and with KOH in the centre well. When the reaction was complete, the large cup was taken out and the reaction mixture was concentrated to 2 ml. by lyophilization. This concentrate was then used for chromatographic purposes.

In the initial stages of purification protein was estimated by a modification of the method of Hiller et al. (1948) with casein as the standard. Five ml. of 10 per cent trichloroacetic acid were added to 0.5 ml. of the sonicate and the resulting precipitate separated by centrifugation at 3,000 r.p.m. The precipitate was dissolved in freshly prepared 3.0 per cent NaOH and then 0.6 ml. of a 20 per cent $CuSO_4 \cdot 5H_2O$ solution was added for the biuret colour reaction. The solution was rapidly brought to a final volume of 25 ml. with 3 per cent NaOH and shaken vigorously for one minute; allowed to stand for 10 minutes, and centrifuged again at 3,000 r.p.m. for 10 minutes. The supernatant was then read

at 560 millimicrons in a Beckman DU spectrophotometer. When
the protein solution was coloured as when sodium glycocholate
was present the colour was compensated for by using as a
blank a duplicate sample which had received a similar treatment
with the exception that the copper sulphate stage was omitted.

After the bulk of the nucleoprotein had been re-
moved the protein was determined by the 280/260 ratio method
of Warburg (1944). This also gave the amount of nucleoprotein
present in the sample. The Beckman DU spectrophotometer was
used for this estimation with hydrogen lamp as the light
source. All the ammonium sulphate fractions are reported as
the per cent of saturation; a saturated solution of $(NH_4)_2SO_4$
containing 70.6 gm. of the solid in 100 ml. of water at 0°C.
The fractions were obtained by adding solid $(NH_4)_2SO_4$ slowly
with stirring to the protein solution. After five minutes
the solution was centrifuged at 25,000 x G for 10 minutes.
The temperature was kept below 10°C to lessen the danger of
denaturation. After separation, the fractions were dialyzed,
with stirring, against ice-cold distilled water for one hour.
For alkaline ammonium sulphate fractionation a saturated
solution of ammonium sulphate was prepared in distilled water
and ammonium hydroxide added until a 1:5 dilution of the
solution showed a pH of 7.5.

The protamine sulphate solution was prepared accord-
ing to the method of Lindstrom (1953) in which the protamine
sulphate is dissolved in pH 5.2 buffer with a final concen-
tration of 20 mg. per ml. The cold protamine sulphate was

added slowly beneath the surface of a stirred protein solution

containing 10-15 mg. of protein per ml. The protamine sul-

phate presumably removes nucleoprotein from solution on a mole

for mole basis. Therefore, there should be no protamine sul-

phate left in solution. Excess protamine sulphate also carries

down some of the active enzyme and to prevent this it was

added in small portions, centrifuged after each addition and

a protein determination carried out on the supernatant by the

280/260 ratio method. This was continued until the nucleo-

protein content was reduced to about 5 per cent (ratio 0:90).

The bile salt, sodium glycocholate, obtained from

City Chemical Corporation, New York, was added directly to the

sonicate and homogenized in a van Potter homogenizer. The

homogenate was centrifuged for 10 minutes at 25,000 x G and

then dialyzed against distilled water. This method is a modi-

fication of the procedure followed by Williams and Sreenivasan

(1953).

The tricalcium phosphate for adsorption experiments

was prepared by the method of Keilin and Hartree (1938), 150

ml. of $CaCl_2$ solution (88.5 g. $CaCl_2.2H_2O$ per litre) was diluted

to about 1600 cc. with tapwater and shaken with 150 ml. tri-

sodium phosphate solution (152 g. $Na_3PO_4.12H_2O$ per litre).

The mixture was brought to pH 7.4 with dilute acetic acid and

the precipitate washed with large volumes of distilled water

in a tall jar until the precipitate was free of chloride ions.

The gel was kept moist and a dry weight determination carried

out after which more distilled water was added until the sus-

pension contained 25 mg. per ml. For adsorption work, the

enzyme was dialyzed against M/100 tris (hydroxymethyl) amino

methane buffer of pH 7.0. Varying amounts of the tricalcium

phosphate were added to the enzyme solution kept in test

tubes in an ice bath. A typical protocol is given below.

Tube	1	2	3	4	5
Enzyme (ml.)	1.0	1.0	1.0	1.0	1.0
Calcium phosphate suspension (ml.)	0.0	0.05	0.10	0.25	0.50
Water (ml.)	1.0	0.95	0.90	0.75	0.50

A stirring rod was placed in each tube and the gel and enzyme

were mixed for five minutes. At the end of this time the mix-

ture was centrifuged and the protein content and enzyme

activity of the supernatant determined. Elution of the enzyme

from the gel was accomplished by adding 1.0 ml. of M/50 pH

7.0 phosphate buffer to the centrifuged gel and extracting

the iced mixture for 15 minutes. If the enzyme was not eluted

by this method, increasing concentrations of buffer were used.

The inhibitors, whose final concentration in the

reaction mixtures is reported here, were added directly to the

Warburg cup. The substrate was tipped in within 20 minutes

of the addition of the inhibitor. The centre wells of the

cups used for cyanide inhibition contained 0.2 ml. of 4N NaCN

in 10 per cent KOH (Eisenberg, 1953).

Final Concentration of Inhibitors

Sodium fluoride	$2.5 \times 10^{-2}M$
Sodium azide	$3 \times 10^{-3}M$
8-hydroxyquinoline	$1 \times 10^{-4}M$
Sodium Iodoacetate	$1 \times 10^{-3}M$
2:4 dinitrophenol	$2 \times 10^{-3}M$
Sodium arsenite	$1 \times 10^{-3}M$
Potassium cyanide	$1 \times 10^{-3}M$

The coenzymes were prepared according to the Merck Index (1952) and added directly to the Warburg cup. Their concentrations in the reaction mixtures are given below.

Final Amounts of Coenzymes Per Warburg Vessel

Riboflavin phosphate (FMN)	5.0 micromoles
Flavin adenine dinucleotide (FAD)	5.0 micromoles
Diphosphopyridine nucleotide (DPN)	5.0 micromoles
Triphosphopyridine nucleotide (TPN)	5.0 micromoles
Adenosine triphosphate (ATP)	100 micromoles
Cytochrome C	520 micromoles
Magnesium ($MgSO_4 \cdot 7H_2O$)	$2.5 \times 10^{-3}M$

The adenosine triphosphate was neutralized with sodium hydroxide before use.

Cysteine or glutathione was used in a final concentration of $1 \times 10^{-3}M$. The hydrogen acceptors were added in the same manner as the inhibitors. The final concentration in the reaction mixture is reported here.

Final Concentration of Hydrogen Acceptor

Methylene blue	$1 \times 10^{-4}M$
Brilliant cresyl blue	$1 \times 10^{-4}M$
2:6 dichlorophenolindophenol	$1 \times 10^{-3}M$
Potassium ferricyanide	$1 \times 10^{-3}M$
Pyocyanine	$1 \times 10^{-3}M$

EXPERIMENTAL

Entner and Stanier (1951) have reported that the glucose oxidizing enzyme of P. fluorescens is constitutive whereas the gluconic enzyme is adaptive in nature. To check on whether or not the same situation prevailed in P. aeruginosa a culture was grown in the medium of Campbell et al. with acetate replacing glucose as the carbon source. The cells were harvested, washed, made to a concentration of 20 times growth and tested for their ability to oxidize glucose and gluconic acid. As shown in Figure 1, adaptation to gluconate occurred after one hour while glucose was oxidized immediately. However, it can be seen that adaptation to gluconate was a prerequisite to further glucose oxidization thus confirming that gluconate is an intermediate in glucose oxidation in this organism.

It was felt that growth of the culture on gluconate medium might induce the formation of a higher concentration of gluconate oxidizing enzyme. This was indeed found to be the case. The weight of cells grown on gluconic acid was, however, the same as that on glucose.

A series of experiments were carried out in an
effort to determine the optimum pH of gluconate oxidation
by the whole cells. Veronal buffer of twice the ordinary
concentration was used as there was a tendency, on the part
of the solution, to attain neutrality.

> 9.714 gms. sodium acetate) dissolved in 250 ml.
> 14.714 gms. sodium veronal)
>
> 5 ml. of this stock solution in a total volume
> of 25 ml. of buffer.

A pH of 7.0 was found to be the most satisfactory (Fig. 2).
The culture was, therefore, grown in the gluconate medium at
pH 7.0.

Methods for disrupting cells: Various methods were
employed in order to disrupt the cells and solubilize the
enzyme. Cells were ground in a van Potter homogenizer with
and without powdered glass and extracted with cold saline. In
other experiments they were ground either with sand or with
two and one-half times their weight of abrasive alumina (Nor-
ton Company) and extracted as before with cold saline. In
neither case was the supernatant active, indicating that the
enzyme was not released from the cells. A technique which
looked very promising because of the low temperatures used
was also tried. The gluconic acid grown cells were harvested
after 18 hours and washed once with distilled water. They
were then suspended in distilled water at a concentration of
200 mg./ml. slowly added to a steel cylinder which had been
cooled in the deep freeze, and finally placed in a bath of
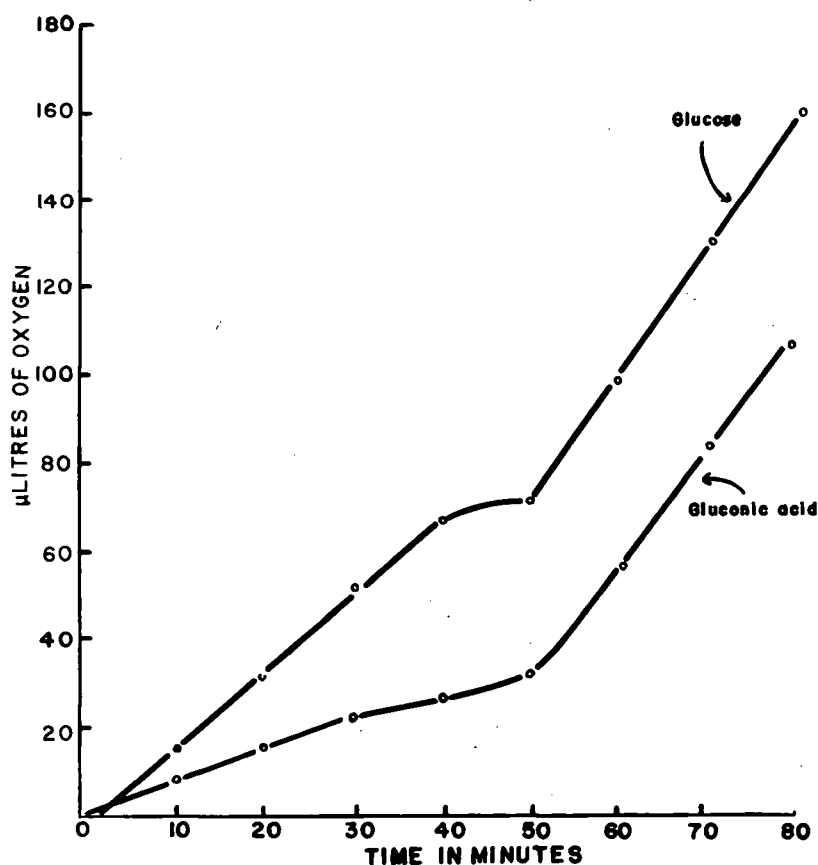dry ice-alcohol. After the cells were frozen solid a cold

FIGURE 1:
        Constitutive nature of the glucose enzyme and the
adaptive nature of the gluconate enzyme in whole cells har-
vested from the acetate medium.

        The reaction was carried out at 30.5°C. The Warburg
cups contained 1.0 ml. of cell suspension; 1.5 ml. of M/15
phosphate buffer of pH 7.0; 0.2 ml. of substrate (5 uM); 0.15
ml. of 20 per cent KOH; water to 3.15 ml.

        Endogenous has been subtracted.

steel piston was inserted into the cylinder and 12,000 pounds
pressure from a Carver Laboratory press was applied. Within
a minute or two the frozen mass melted and the cells were
ruptured in the process. After centrifuging at 5,000 r.p.m.
at 4°C the preparation was tested at the rate of 1 ml./cup
in manometric studies. Though the preparation was active to-
wards gluconic acid, there were a few live cells present and
the reaction was not completed even after three hours. The
results are also inconsistent and not reproducable - probably
because whole uncrushed cells adhered to the gummy superna-
tant material and resisted centrifuging.

The use of a ball mill for crushing the cells was
also investigated. The washed cells were dried under vacuum
in a desiccator and ground with dry washed alumina in the
ratio 3/7.5 in a ball mill _in vacuo_ for 20 hours. The ground
cells were extracted with M/30 phosphate buffer of pH 7.2.
In none of these cases, however, was the supernatant found to
be active against gluconic acid showing that the enzyme had
not been solubilized by any of these methods.

The object of the investigation was to obtain the
gluconate oxidizing enzyme in a soluble form, and from this
point of view, the only successful method of disintegration
of the cells was that of subjecting the cells to sonic waves
in a Raytheon oscillator for 20 minutes at a concentration of
200 mg. per ml. Higher concentrations gave erratic oxygen
uptake. The suspension of the cells can be made either in
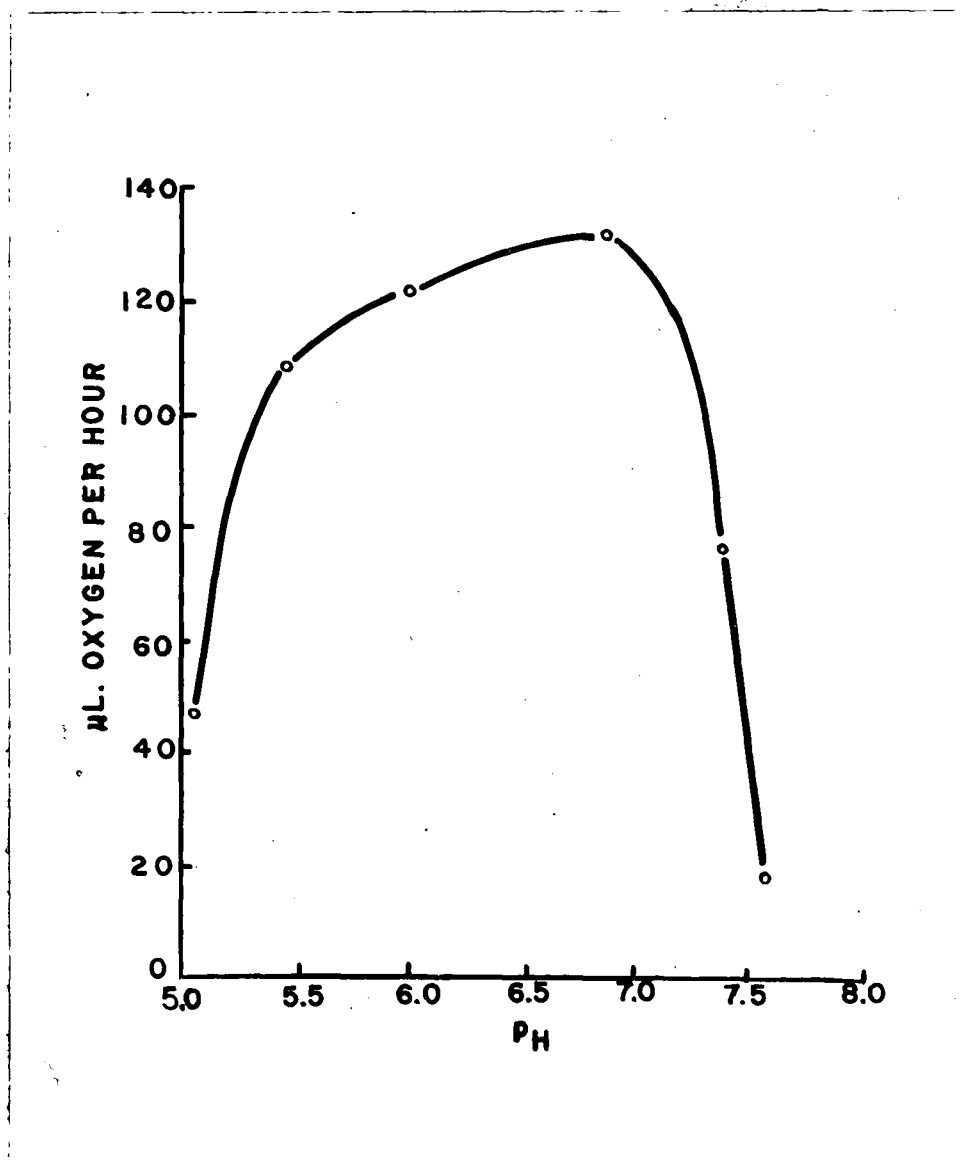M/30 phosphate buffer of pH 7.0 or in distilled water. The

FIGURE 2:
        Rate of oxidation of gluconate by whole cells as a function of pH.

        The Warburg cups contained 0.5 ml. of cell suspension; 0.2 ml. gluconate (5 uM); 2.0 ml. of M/15 veronal buffer; 0.15 ml. of 20 per cent KOH; water to 3.15 ml.

        Endogenous has been subtracted.

sonicate took up 2 atoms of oxygen per mole of glucose and 1 atom for gluconate, indicating that both glucose and gluconate were oxidized to 2-ketogluconate (Fig. 3).

Attempts to fractionate the sonicate: Experiments were conducted with a view to separating the glucose oxidizing enzyme from the gluconic enzyme. The addition of 20 per cent solid ammonium sulphate brought both the enzymes down in the precipitate and further fractionation of the precipitate was not possible. Alumina CY and calcium phosphate adsorbed the enzymes, but elution could not be effected from these adsorbants. Similarly, Amberlite ion exchange resin IRC-50 adsorbed both enzymes, but it was not possible to elute either of them. These results indicated that the enzymes were associated with particulate matter and that fractionation could be effected only after the enzymes had been converted to a soluble form.

Solubilization of the enzyme: Attempts were made to bring the enzymes into solution by the use of surface active agents. Sodium glycocholate was found to effect this. One gm. of the salt was added to 10 ml. of the sonicate which was homogenized in a Potter homogenizer for one minute and centrifuged at 25,000 x G for 30 minutes in the cold. The supernatant was separated from the residue, but no enzymic activity was found in either of the fractions. The presence of 0.2 ml. of 2:6 dichlorophenol indophenol activated the supernatant towards gluconic acid and to some extent to glucose, while the same added to the precipitate activated
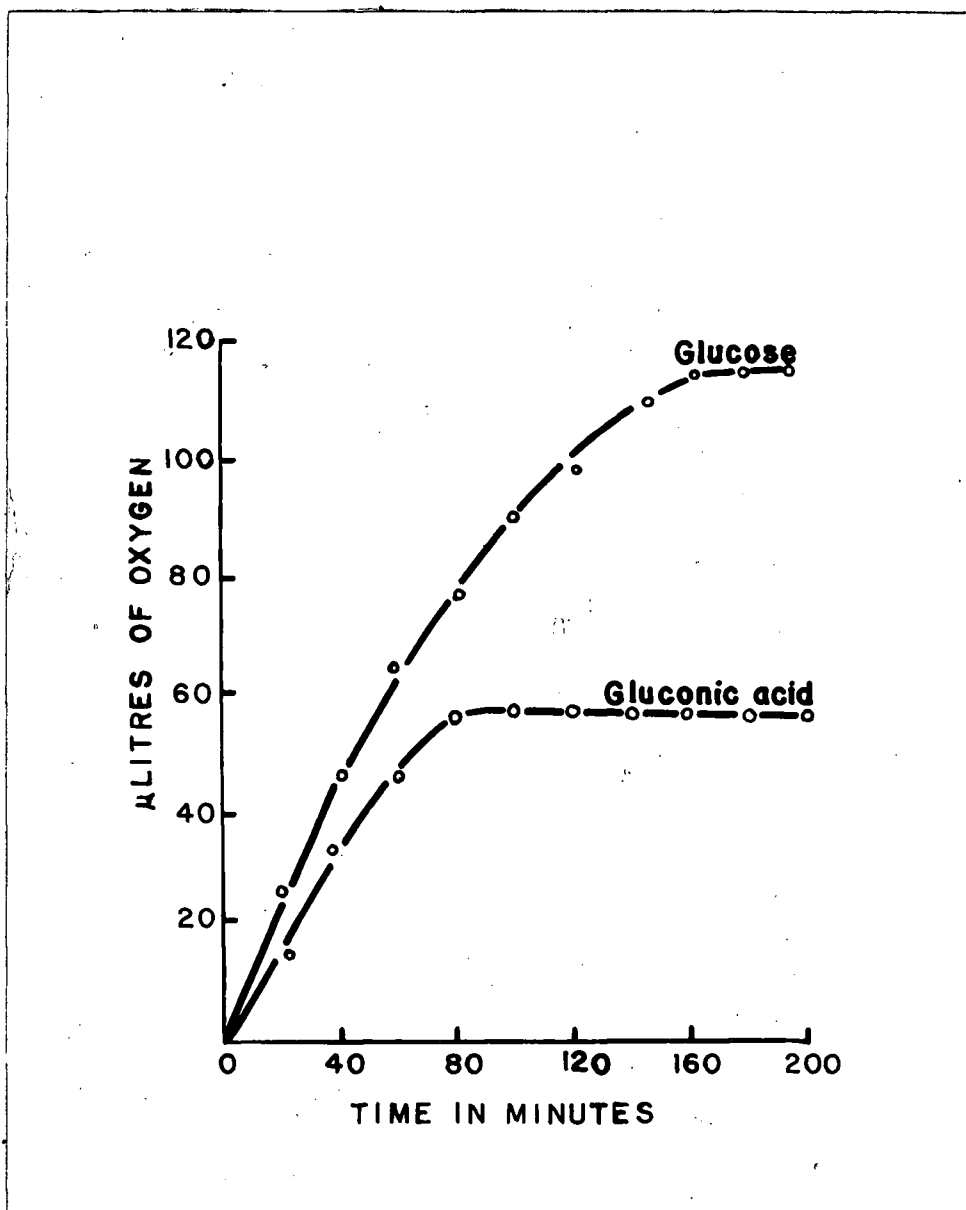
FIGURE 3:
Activity of sonic disintegrated cell suspension on glucose and gluconate.

The reaction was carried out at 30.5°C. Each cup contained; 1.0 ml. of soniced cell suspension; 1.5 ml. of M/15 phosphate buffer of pH 6.0; 0.2 ml. of substrate (5 uM); 0.15 ml. of 20 per cent KOH; water to 3.15 ml.

mainly the oxidation of glucose. The dye presumably completes the hydrogen transport system. Neither methylene blue nor ferricyanide acted as hydrogen acceptors but pyocyanine was found to give a faster rate than even indophenol. Indophenol was decolorized in the presence of gluconic acid and was not reoxidized by oxygen whereas pyocyanine was reoxidized and quantitative oxygen uptake resulted. It was found that a correlation between the rate of reduction of 2:6 dichlorophenol indophenol and the rate of oxygen uptake in the presence of pyocyanine could be established. 2:6 dichlorophenol indophenol has a maximum absorption at 600 mu and the rate of reaction can be followed spectrophotometrically by measuring the reduction in optical density at this wavelength and converted to oxygen uptake. The reduction was found to be over in eight minutes whereas 1 atom of oxygen was taken up in 90 minutes with gluconic acid as substrate. The corresponding amount of oxygen uptake can be calculated from the reduction of 2:6 dichlorophenol indophenol:

Amount of dye used $= 0.26$ uM

.26 uM dye can transfer .26 uM (H_2)

$= .26$ u Atoms O_2 in eight minutes.

$\therefore$ In 90 minutes this corresponds to an uptake of 2.9 u Atoms of oxygen.

For the dye reduction 0.25 ml. of the enzyme was used while for the manometric experiment 0.50 ml. of the enzyme was used.

. . In 90 minutes with 0.5 ml. enzyme dichlorophenol
indophenol the corresponding uptake would be 5.8
u Atoms of oxygen.

In the same period of time pyocyanine reduces 5 micro
atoms of oxygen.

Dichlorophenol indophenol is thus a more efficient
hydrogen acceptor than pyocyanine, but since the former is not
autooxidizable, pyocyanine had to be used as the hydrogen
acceptor in manometric experiments.

Ammonium sulphate fractionation: The enzyme extract
which had been "solubilized" by glycocholate treatment was
chilled to 5°C. Solid ammonium sulphate was then added slowly
with constant stirring to bring the mixture to 25 per cent of
saturation. The resulting precipitate was removed by centri-
fugation and resuspended in the original volume of M/30 phos-
phate buffer, pH 6.0. In the presence of pyocyanine the re-
suspended precipitate oxidized glucose at a good rate while its
action on gluconate was weak. The supernatant, on the other
hand, oxidized gluconate at a good rate and had only very weak
activity towards glucose (Fig. 4). It would, therefore,
appear that this step has largely separated the two enzymes.
From the curves it can be seen that although gluconic acid
is quantitatively converted to 2-ketogluconic acid, glucose
oxidation stops after approximately 1 atom of oxygen has been
taken up. This can be explained on the assumption that two
enzymes are necessary for the conversion of glucose to

gluconic acid, one of which is missing. This lends weight
to the finding of Brodie and Lipmann (1954) that glucono-Δ-
lactone is the primary oxidation product of glucose and it is
enzymatically hydrolyzed to gluconic acid. The hydrolyzing
enzyme is probably absent in the 25 per cent ammonium sulphate
fraction.

Solid ammonium sulphate was again added to the
chilled supernatant slowly, with constant stirring, until 90
per cent of saturation was reached. The resulting precipi-
tate was separated by centrifuging; dissolved in the original
volume of M/30 phosphate buffer of pH 6.0 and tested for its
activity against gluconic acid. It contained all the activity
of the original solution. The presence of ammonium sulphate
did not affect the measurement of enzymic activity.

Nucleoprotein removal: The 25-90 fraction was
dialyzed against cold distilled water for one hour with con-
stant, vigorous mechanical stirring. The dialyzed protein was
treated with protamine sulphate to remove nucleoproteins by
the method of Lindstrom (1953). The dialysis was found to be
necessary for obtaining a heavy precipitate. The most favour-
able conditions for the precipitation were a pH of 6.0 and a
protein concentration of about 10 mg. per ml. Sufficient
protamine sulphate solution was added to bring the nucleic
acid content of the supernatant to about 5 per cent. The
precipitate was centrifuged off at 25,000 x G for 20 minutes
and discarded. The supernatant which was clear and nearly
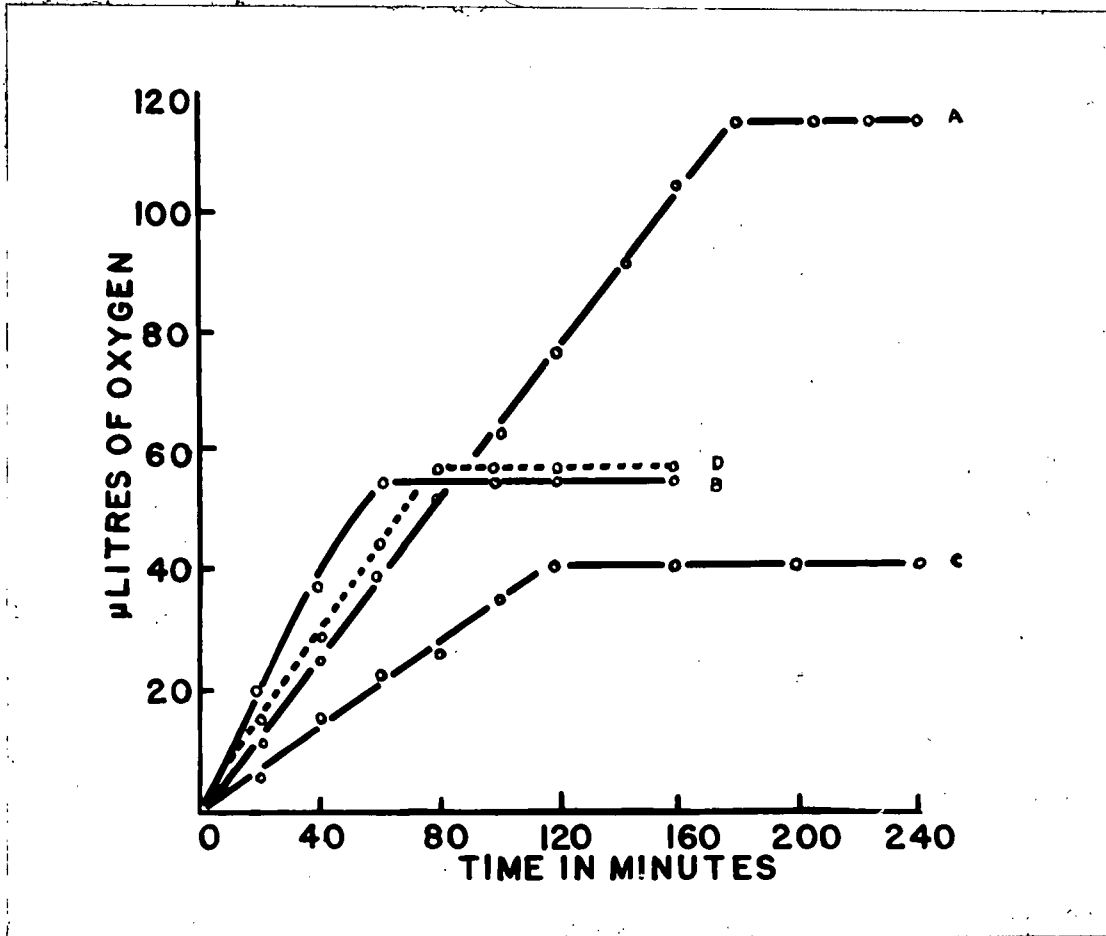colourless, was dialyzed against M/100 tris (hydroxymethyl)

FIGURE 4:
       Activity of cell extracts solubilized with sodium
glycocholate against glucose and gluconate.

       The reaction was carried out at 30.5°C. Each cup
contained; 0.5 ml. of cell extract; 1.5 ml. of M/15 phosphate
buffer of pH 6.0; 0.2 ml. of pyocyanine (10-3M); 0.2 ml. of
substrate (5 uM); 0.15 ml. of 20 per cent KOH; water to 3.15
ml. Endogenous has been subtracted.

       A = sodium glycocholate treated extract + glucose.
       B = sodium glycocholate treated extract + gluconate.
       C = supernatant from 25 per cent ammonium sulphate
              precipitation of solubilized extract + glucose.
       D = supernatant from 25 per cent ammonium sulphate
              precipitation of solubilized extract + gluconate.

aminomethane buffer pH 7.0 to precipitate any nucleoprotein which might remain dissolved due to the presence of traces of ammonium sulphate. If a precipitate formed, it too was centrifuged off and discarded. The supernatant retained the activity of the original solution against gluconic acid (Fig. 5).

Final fractionation with ammonium sulphate: The solution was now divided into four portions by fractional precipitation with ammonium sulphate. The fractions were 0-25, 25-42, 42-59 and 59-90 per cent of saturation. The activity of these various fractions (made up to the original volume) against gluconic acid was determined manometrically. The activity was found to be in the 0-25 and 25-42 fractions:

Saturation of Ammonium Sulphate	Activity of Enzyme (Oxygen uptake in ul per hr./ml.)	Total Volume	Total Activity
0-25	75.3	30 ml.	2159
25-42	105.9	30 ml.	3177
42-59	0	30 ml.	0
59-90	0	30 ml.	0

Another experiment using 0-20, 20-35 and 35-42 saturations with ammonium sulphate resulted in the major activity being concentrated in the 20-35 fraction. This fraction had no activity against glucose:

Saturation of Ammonium Sulphate	Activity of Enzyme (Oxygen uptake in ul per hr./ml.)	Total Volume	Total Activity
0-20	26.4	30 ml.	792
20-35	138.0	30 ml.	4140
35-42	2.8	30 ml.	84
42-90	0	30 ml.	0

This last named fraction was dissolved in the original volume of M/30 phosphate buffer and was further fractionated with saturated alkaline ammonium sulphate of pH 7.5. The gluconic dehydrogenase was found to be in the 20-30 cut.

Saturation of Ammonium Sulphate	Activity of Enzyme (Oxygen uptake in ul per hr./ml.)	Total Volume	Total Activity
0-20	0	20 ml.	0
20-30	272.0	20 ml.	5240
30-40	4.4	20 ml.	88
40-50	0	20 ml.	0

Purification by calcium phosphate gel: The fraction which was brought down between 20-30 per cent saturation of alkaline ammonium sulphate was dialyzed against M/100 "tris" buffer of pH 7.0 overnight as adsorption by calcium phosphate is best effected in a solution of low ionic concentration. The investigations were only of a preliminary nature to establish conditions for calcium phosphate adsorption. To 1.0 ml. of the enzyme various amounts of tricalcium phosphate suspension containing 25 mg. solid per ml. were added:
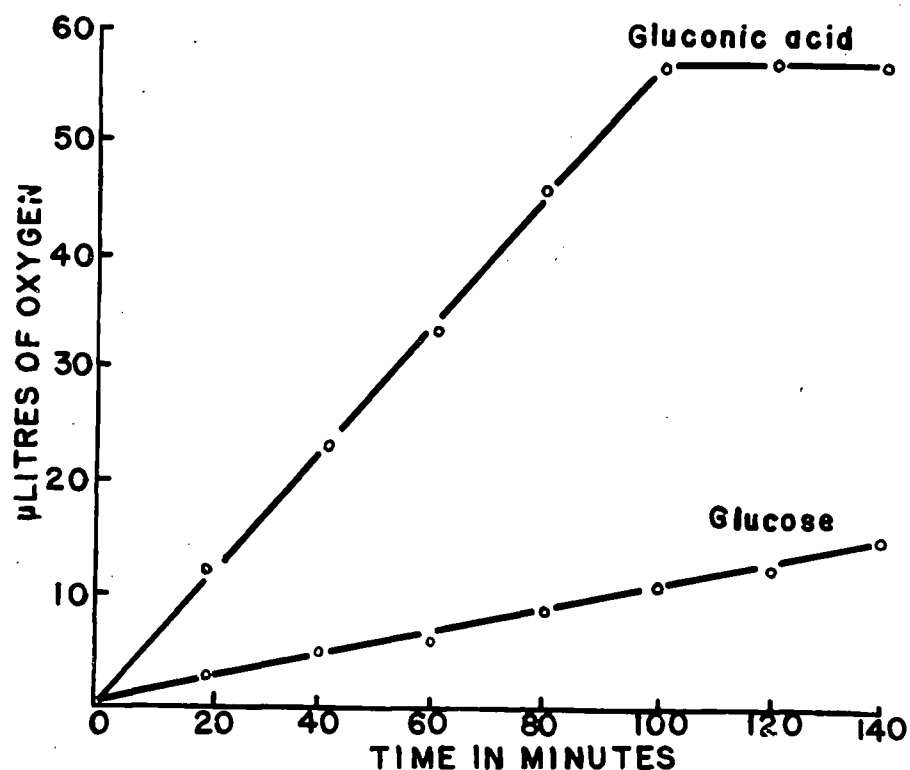
FIGURE 5:
        Activity of protamine treated enzyme solution on glucose and gluconate.

        Each cup contained; 0.5 ml. of enzyme solution; 1.5 ml. of M/15 phosphate buffer of pH 6.0; 0.2 ml. of pyocyanine (10-3M); 0.2 ml. of substrate (5 uM); 0.15 ml. of 20 per cent KOH; water to 3.15 ml.

Enzyme (ml.)	1.0	1.0	1.0	1.0	1.0
Calcium phosphate (ml.)	0	0.05	0.10	0.25	0.50
Water (ml.)	1.0	0.95	0.90	0.75	0.50

The mixtures were kept in an ice bath with stirring rod in each and stirred for five minutes after which they were centrifuged and enzymic activity and protein determined on the supernatant. For protein determination by the 280/260 method, M/100 "tris" buffer shaken with the same amount of tricalcium phosphate was used as the blank.

It was found that 0.25 ml. of calcium phosphate suspension did not take up any significant amount of enzyme but that 0.5 ml. adsorbed all of it.

Calcium Phosphate Suspension	Total Volume	Protein Concen. in Supernatant	Enzyme Activity per mg. Protein (ul oxygen taken up per hour	Total Activity
0 ml.	2.0 ml.	1.82 mg./ml.	420	1528.8
0.05 ml.	2.0 ml.	1.82 mg./ml.	420	1528.8
0.1 ml.	2.0 ml.	1.54 mg./ml.	420	1293.6
0.25 ml.	2.0 ml.	0.93 mg./ml.	337	628.8
0.5 ml.	2.0 ml.	0.52 mg./ml.	0	0

A second series of adsorption experiments showed that 0.35 ml. was an appropriate amount of tricalcium phosphate in that a large proportion of the enzyme was adsorbed.

Calcium Phosphate Suspension	Total Volume	Protein Concen. in Supernatant	Enzyme Activity per mg. Protein (ul oxygen taken up per hour)	Total Activity
0 ml.	2.0 ml.	1.82 mg./ml.	420	1528.8
0.3 ml.	2.0 ml.	0.90 mg./ml.	315	467.0
0.35 ml.	2.0 ml.	0.75 mg./ml.	21	31.6
0.4 ml.	2.0 ml.	0.67 mg./ml.	0	0

Next the .35 fraction of calcium phosphate was mixed with 2.0 ml. of M/30 phosphate buffer of pH 7.0, agitated for 15 minutes in the cold and centrifuged. The gel was then mixed with M/10 buffer of the same pH and centrifuged in the same manner. The process was repeated with M/5 and M/1 phosphate buffer.

Concen. of Buffer	Protein Concen. In Elute	Enzyme Activity per mg. Protein (ul Oxygen Uptake per hr.)	Total Volume	Total Activity
M/30	0.35 mg./ml.	0	2.0 ml.	0
M/10	0.53 mg./ml.	0	2.0 ml.	0
M/5	0.71 mg./ml.	632	2.0 ml.	897.6
M/1	0.77 mg./ml.	632	2.0 ml.	973.2

From the table it can be seen that M/5 phosphate buffer of pH 7.0 is able to selectively elute the enzyme from the calcium phosphate. Sixty per cent of the original protein was recovered but there was only a loss of 10 per cent of the activity.

The various stages of purification are summarized in the following table:

Stage of Purification	Volume ml.	Protein per ml.	Activity /hr./ml.	Total Protein	Activity /hr./mg. Protein	Total Activity /hr.
Sonic extract	10	56.00	118	560.0	2.1	1176.0
Glycocholate solution	10	29.30	960	293.0	32.8	9610.4
25-90(NH$_4$)$_2$SO$_4$ fraction	20	6.93	444	138.0	64.0	8832.0
Protamine SO$_4$ treated extract	30	2.78	300	83.4	107.8	8989.5
20-35(NH$_4$)$_2$SO$_4$ fraction	30	2.25	300	67.5	133.3	8997.5
Alk.(NH$_4$)$_2$SO$_4$ 21-30 fraction	20	1.28	540	25.6	421.5	10790.4
Calcium phosphate elute	20	0.71	485	15.4	631.5	9724.1

There is thus a 300 fold purification.

Properties of the Enzyme

Stability of the enzyme: The enzyme was routinely stored at -10°C in M/10 "tris" buffer, pH 7.0. Under these conditions it was stable for several weeks. At 4°C, under the same conditions, the enzyme could be kept for three to four days without any appreciable loss of activity. When dialyzed against distilled water, there was a gradual loss of activity after eight to 10 hours, accompanied by precipitation. Dialysis against neutral phosphate or "tris" buffers for as long as 24 hours in the cold produced no loss of activity.

Activity of electron acceptors: In the presence of methylene blue, 2:6 dichlorophenol indophenol or pyocyanine, gluconic dehydrogenase catalyzed the oxidation of gluconic acid. Ferricyanide appeared to poison the system and brilliant cresyl blue was inactive. Reaction with methylene blue was slow while that with indophenol blue could not be used for manometric work since the dye is not reoxidized by oxygen.

Hydrogen Acceptor	Oxygen taken up/hr. ul.
Methylene blue	26.0
Pyocyanine	78.0
2:6 dichlorophenol indophenol	0*
Brilliant cresyl blue	0
Ferricyanide	0

*Dye reduced in eight minutes as measured in spectro-photometer.

Reaction with pyocyanine was rapid and the rate increased
with increasing concentrations of pyocyanine until an optimum
concentration of 680γ of pyocyanine per 3 ml. was reached.
Beyond that there was no increase in rate (Fig. 6).

Cofactors: No cofactors other than the hydrogen
acceptor were found to be necessary for the activity of the
enzyme. The addition of DPN, TPN, ATP, FMN, FAD, cytochrome
c, Mg, Mn or Fe++ did not increase the activity. DPN or TPN
was not reduced as measured by increase in optical density of
the reaction mixture at 340 mu. The reaction mixture contained
0.1 ml. of the purified enzyme, 0.2 ml. of gluconic acid, 0.1
ml. of .005M DPN or TPN, 1.5 ml. of pH 5.6 buffer and distilled
water to make up to 3.0 ml. Treatment with charcoal to remove
any bound DPN (Taylor et al. 1948) did not reduce the enzymic
activity. The enzyme solution in this case was treated in
the cold with 1.3 mg. of norit per ml. and after gentle agita-
tion for three minutes the norit was removed by centrifuga-
tion and filtration. The absence of metal requirement for
the enzyme was confirmed by dialyzing against versene according
to the method of Racker (1953). The solution was dialyzed
against 1000 volumes of 0.6 per cent versene in 0.02M phosphate
of pH 7.4 for 20 hours and then against 1000 volumes of 0.6
per cent versene in 0.9 per cent KCl for another 20 hours.

Effect of inhibitors: Iodoacetate, sodium azide,
2:4 dinitrophenol, sodium arsenite and sodium fluoride did
not inhibit the action of the enzyme. The absence of fluoride
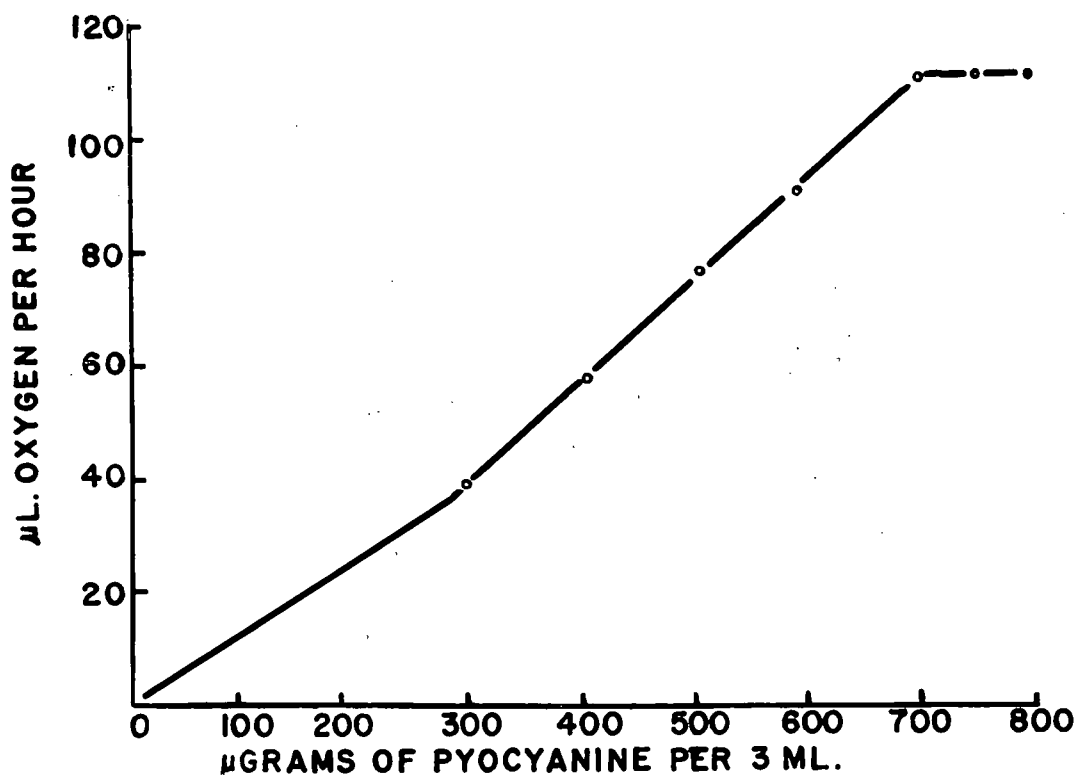inhibition indicates that phosphorylation of substrate is not

FIGURE 6:
        Effect of pyocyanine concentration on velocity of oxidation by gluconic dehydrogenase.

        Oxygen uptake is shown using 5 micromoles of substrate and varying concentrations of pyocyanine. The reaction was carried out at 30.5°C and pH 5.6.

involved. Cyanide, glutathione and cysteine increased the rate of oxidation (Fig. 7).

Substrate specificity: Glucose, glucuronic acid, 2-ketogluconic acid, fructose, glucose-6-phosphate, 6-phosphogluconate and mannose, were not oxidized by the enzyme system. The purified enzyme appears, therefore, to be quite specific for gluconic acid.

Product of the enzymic activity: The product of the oxidation of gluconic acid has been reported to be 2-ketogluconic acid. In this reaction no CO_2 evolution takes place.

$$C_6H_{12}O_7 + \tfrac{1}{2}O_2 \longrightarrow C_6H_{10}O_7 + H_2O$$

Data for this were obtained by the manometric technique of Dixon (1943). Manometric experiments were conducted with (1) enzyme and substrate and with KOH in the centre well; (2) with no KOH but with 0.3 ml. of 3N HCl in the side-arm. At the end of the reaction (where $\tfrac{1}{2}$ uM of oxygen per uM of gluconate was taken up), the HCl was tipped into the reaction chamber in the third cup. There was no evidence of carbon dioxide evolution. There was also no difference between the apparent oxygen uptake in the cup containing KOH and that where KOH had been omitted, showing that no CO_2 was evolved during the oxidation.

For identification of the product the chromatographic procedure described under "Methods" was followed. With ammoniacal-silver nitrate reagent the product from gluconic acid oxidation gave a spot identical in appearance with 2-
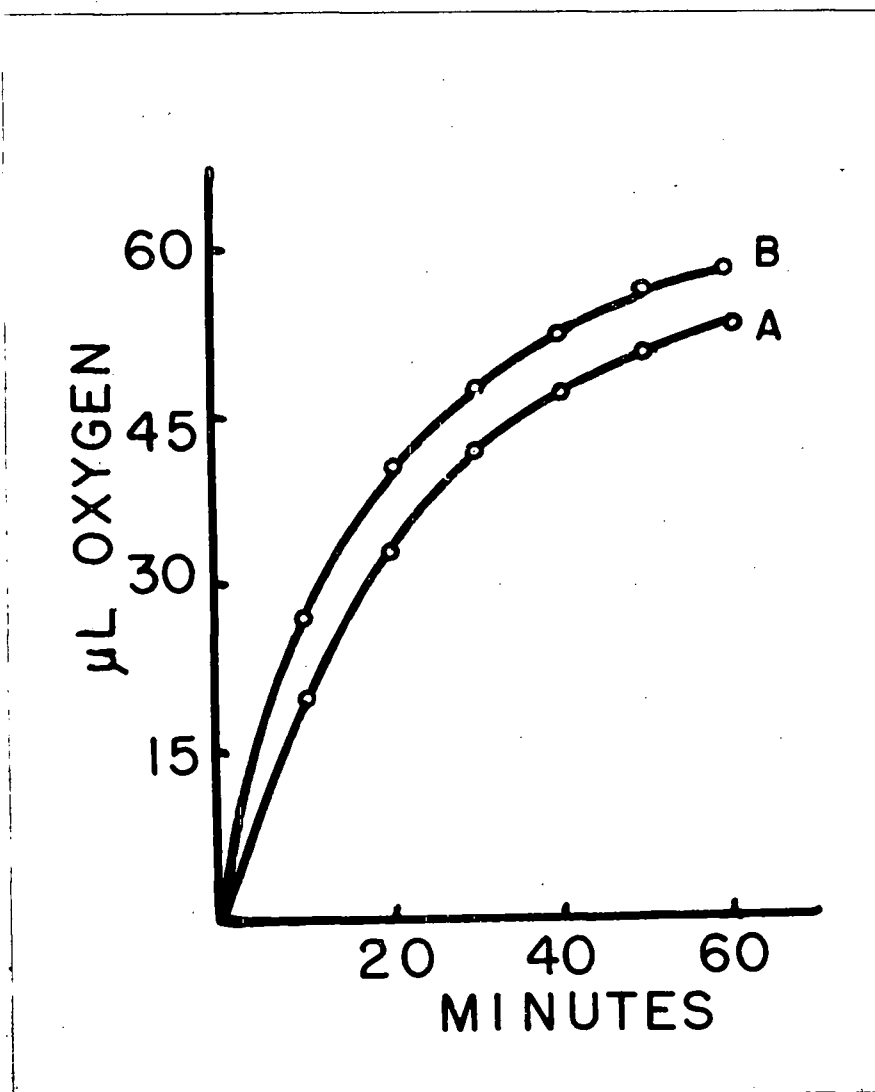
FIGURE 7:
Influence of reducing agents on the oxidation of gluconate.

Each cup contained; 0.5 ml. of purified enzyme; 1.5 ml. of M/15 phosphate buffer of pH 5.6; 0.2 ml. of gluconate (5 uM); 0.3 ml. of KCN (10^{-3}M) with 4N KCN in 20 per cent KOH in centre well; (or 0.3 ml. of glutathione or cysteine 10^{-3}M); 0.2 ml. of pyocyanine.

A = gluconate alone
B = gluconate + KCN (or glutathione or cysteine).

ketogluconic acid. The R_f values for the chromatogram further substantiated the finding.

				R_f
Height of solvent front	=	19.25 ins.		--
Height of glucose spot	=	10.00 ins.		0.52
Height of gluconic acid spot	=	5.75 ins.		0.29
Height of 2-ketogluconic acid spot	=	7.00 ins.		0.36
Height of oxidation product	=	7.00 ins.		0.36

Since 5-ketogluconic acid cannot be oxidized by either whole cells or cell extracts of P. aeruginosa and since earlier work (Norris and Campbell, 1949) conclusively eliminated it as a possible product of the reactions being studied, it was not included as a standard in these chromatograms.

With Hane's reagent no phosphate compounds could be detected on a paper chromatogram.

Michaelis constant: Experiments were conducted with the purified enzyme to determine the concentration of substrate required to saturate the enzyme. 0.2 ml. of the enzyme solution containing 0.364 mg. of protein was allowed to react in the Warburg respirometer with amounts of gluconic acid varying from 2.5 to 15.0 uM. The rate of reaction in each case was plotted against the substrate concentration (Fig. 8). The substrate concentration expressed in moles of substrate per litre at half the limiting velocity gave the Michaelis constant (Km).
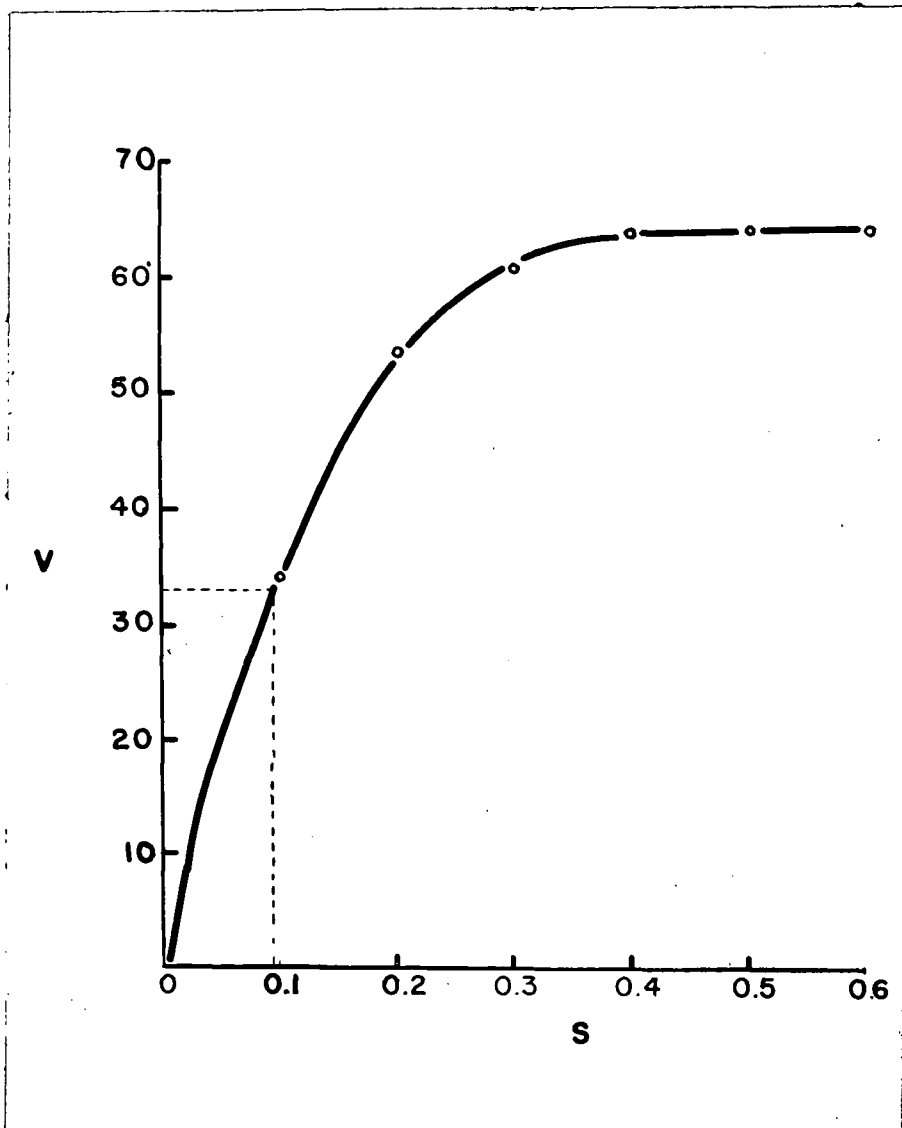
FIGURE 8:

Michaelis constant of gluconic dehydrogenase.

Oxygen uptake is shown using varying concentrations of gluconate. Each cup contained; 0.2 ml. of purified enzyme; 1.5 ml. of M/15 phosphate buffer pH 5.6; 0.2 ml. of pyocyanine; gluconate; 0.15 ml. of 20 per cent KOH; water to 3.15 ml.

Maximum velocity $= 64$ uM O_2 per hour

Substrate concentration)
)
Corresponding to 32 uM O_2 per hour) $= 0.9$ ml. $= 2.25$ uM

$$Km = \frac{2.25 \times 1000 \; M}{3 \times 1000 \times 1000} = \underline{.00075 \; M}$$

Properties of the Enzyme System

Influence of pH: Using the crude sonicate as the source of enzyme, experiments were conducted over the ranges of pH 3.2-7.5 in veronal buffer. The optimum pH was found to be 5.6 (Fig. 9). While the oxidation proceeds there is a strong tendency for the reaction mixture to become basic and so the veronal buffer had to be prepared with double the concentration of the reagents. If any precipitation occurred, the solution was warmed to above 30°C and then used for the experiment.

When the purified enzyme plus pyocyanine were used in place of the sonicate for these experiments, there was the suggestion of a second maximum at pH 4.6 (Fig. 10). Since decarboxylation was possible at this low pH, manometric experiments for carbon dioxide determination by Dixon's method were repeated. However, no carbon dioxide was evolved. The product was chromatographically analyzed as before, but 2-ketogluconic acid was the only product. It may be concluded from these experiments that at this pH it was the hydrogen acceptor and not the enzyme which was influenced.

As in the case of many other enzymes, the gluconic
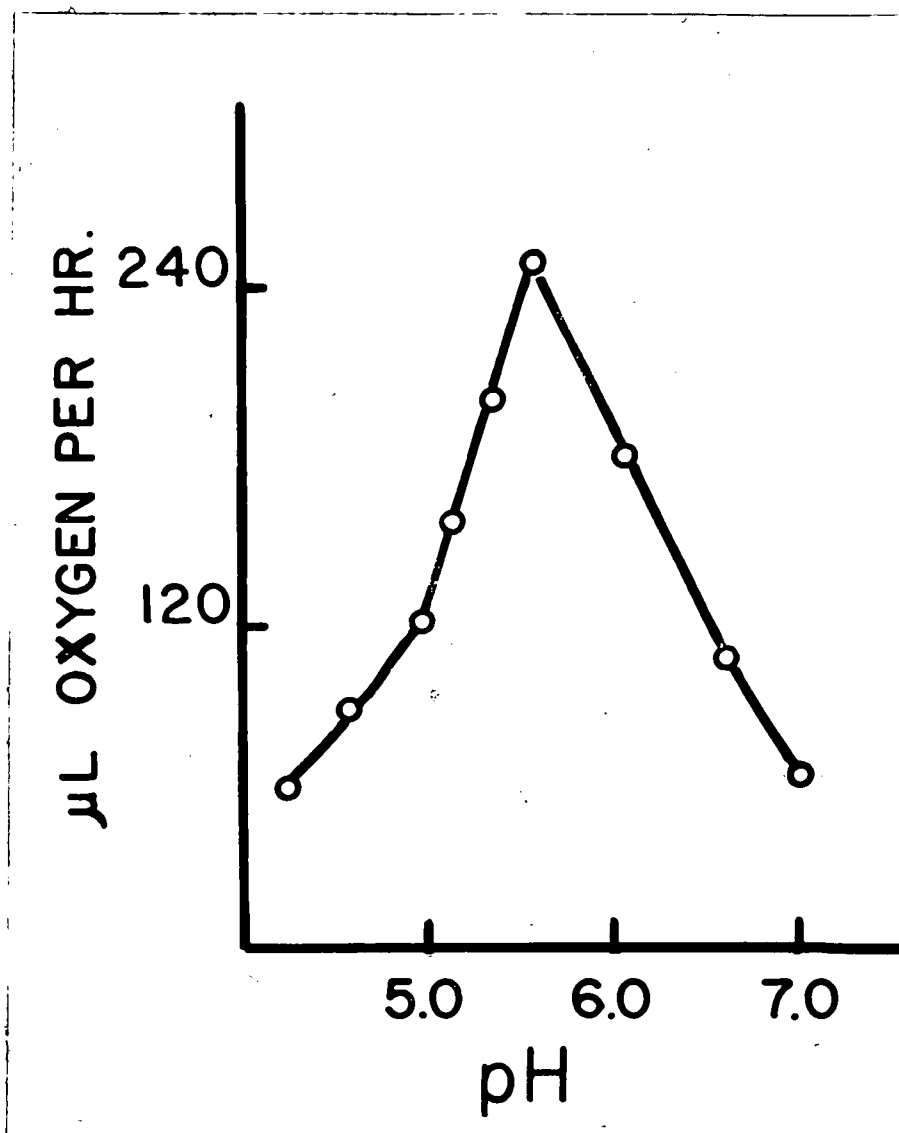
FIGURE 9:
Rate of oxidation of gluconate by soniced cells as a function of pH.

Each cup contained; 0.5 ml. of sonicate; 2.0 ml. of M/15 veronal buffer; 0.2 ml. of gluconate (5 uM); 0.15 ml. of 20 per cent KOH; water to 3.15 ml.

dehydrogenase isolated from the cell has a pH optimum (5.6)
different from that of the whole cells (7.0). Although this
has been used in arguing that working with isolated enzymes
is making use of an artificial environment, the advantages
accruing from the study of an isolated enzyme are many-fold.

Phosphorylation: The lack of inhibition of the
enzyme by sodium fluoride indicated that phosphorylation of
the substrate was not involved in its oxidation. Another
proof of this was the fact that neither ATP nor AMP activated
the enzyme. However, Narrod and Wood (1954) reported that
P. fluorescens phosphorylates gluconate prior to oxidation
and that the phosphorylated intermediate is 6-phosphogluconate.
In view of this observation and the fact that an enzyme capable
of dehydrogenation of gluconate had never been isolated, a
series of experiments were conducted to determine whether or
not P. aeruginosa phosphorylated gluconate prior to oxidation.

(a) Production of acid: The production of
acid is the method followed by Colowick and Kalckar (1943)
to detect phosphorylation. In this procedure, the substrate
is reacted on by the enzyme in the presence of ATP. The
transfer of phosphate from ATP results in the liberation of
1 mole of acid per mole of phosphate. In the presence of
bicarbonate, the rate of phosphate transfer can be followed
manometrically by observing the evolution of carbon dioxide.
The phosphate transfer is thus proportional to acidification
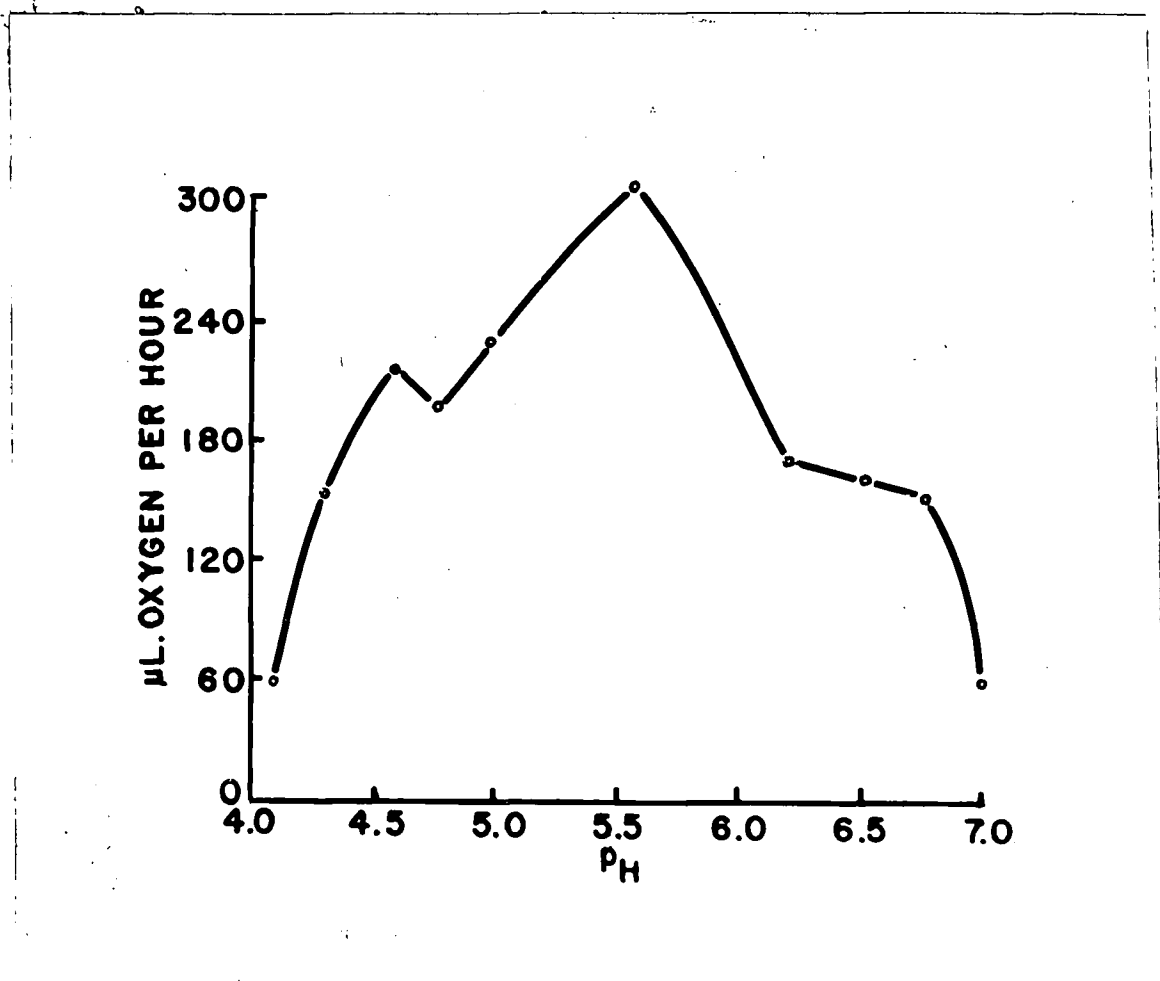and is measured manometrically as carbon dioxide liberated

FIGURE 10:
Rate of oxidation of gluconate by purified gluconic dehydrogenase as a function of pH.

Each cup contained; 0.2 ml. of enzyme solution; 2.0 ml. of M/15 veronal buffer; 0.2 ml. of gluconate (5 uM); 0.15 ml. of 20 per cent KOH; water to 3.15 ml.

from the bicarbonate. If no carbon dioxide is evolved it can be concluded that phosphorylation is absent in the system. This experiment was carried out with extracts of P. aeruginosa and P. fluorescens under aerobic and anaerobic conditions. Warburg flasks fitted with gas vents were used and the following solutions added; 0.5 ml. of the sonic extract, 50 uM ATP; .005 M iodoacetate (to inhibit oxidative processes and to permit accumulation of primary esters formed); M/18 sodium fluoride (to inhibit decomposition of the phosphate esters) and 0.05 M sodium bicarbonate to react with the acid formed during phosphate transfer thus releasing CO_2. Gluconic acid was used as the substrate. The CO_2-air mixture was circulated through the cups for 15 minutes and the stopcocks closed off. After gluconic acid was tipped in, there was no carbon dioxide evolution, showing absence of phosphorylation. The experiment was repeated using nitrogen instead of air, but still phosphorylation was not shown. To eliminate the possibility that phosphorylation is not evident because of the high pH used in the experiments, another anaerobic experiment was run at pH 6.0 (in the presence of 6.6×10^{-4}M sodium bicarbonate). Still no CO_2 was evolved.

The above experiments were repeated with a sonic extract of P. fluorescens A. 312 under identical conditions. Here though aerobically no phosphorylation could be detected, anaerobically, carbon dioxide was evolved (156 ul of CO_2 per 5 uM of gluconate) showing phosphorylation. It is thus clear that there is no phosphorylation in the system in P. aeruginosa
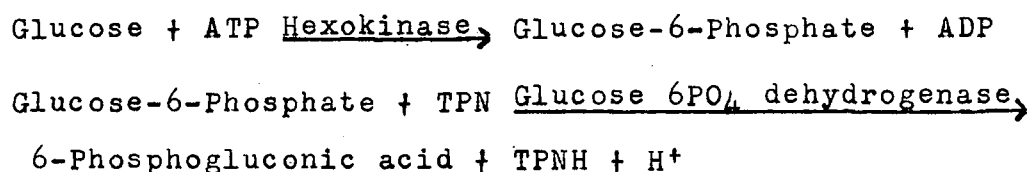
while it is present in P. fluorescens. The anaerobic experiment is much more useful for it stops the reaction prior to the oxidative step and favors the accumulation of a phosphorylated intermediate.

(b) Reduction of TPN: If gluconic acid is phosphorylated to phosphogluconic acid, phosphogluconic dehydrogenase should be able to oxidize it in the presence of TPN. The measurement of TPN reduction should then indicate whether the initial phosphorylation has taken place.

Phosphogluconic dehydrogenase was isolated from Brewer's yeast by the method of Horecker and Smyrniotis (1951). The enzyme was first tested for its activity against phosphogluconic acid and when it was found active tried against the sonic extracts of P. aeruginosa 9027 and P. fluorescens A. 312. The cuvettes contained 0.1 ml. sonic extract, 0.1 ml. TPN (3.0 uM per ml.), 0.5 ml. phosphogluconic dehydrogenase, 0.04 ml. gluconic acid (50 uM per ml.), 0.05 ml. of ATP (200 uM per ml.), 0.02 ml. of $MgCl_2$ (0.1 M) and water to 3.0 ml. were added and the reduction of TPN, if any, measured at 340 mu every minute for 10 minutes, (Fig. 11). As the figure shows, TPN reduction occurred only with P. fluorescens, thus showing that only in this organism was 6-phosphogluconate being formed.

(c) Formation of high energy bond: Although one might expect that ATP would be needed to initiate the reaction with gluconic acid, nevertheless, it would be usual for the overall oxidation to result in the accumulation of high energy bonds. Lehninger (1951) calculated that for every atom

of oxygen taken up by a biological reaction, three high energy
bonds are produced. It has already been shown that no ATP
could be detected chromatographically as the product of glu-
conate oxidation. Another method is to substitute the oxida-
tion of glucose or gluconate for ATP where the latter is
necessary for initiating a reaction. Such a reaction is the
formation of phosphogluconic acid from glucose.

Glucose + ATP <u>Hexokinase</u>⟶ Glucose-6-Phosphate + ADP

Glucose-6-Phosphate + TPN <u>Glucose 6PO$_4$ dehydrogenase</u>⟶

6-Phosphogluconic acid + TPNH + H⁺

ATP is necessary for the initial phosphorylation of glucose
and the presence of ATP may be followed by measuring the re-
duction of TPN at 340 mu by the method of Kornberg (1950).
The components of the test were; glucose (5 uM) 0.2 ml., MgCl$_2$
(15 uM) 0.1 ml., lyophilized hexokinase (10 mg. per ml.) 0.1
ml., sonicate of <u>P</u>. <u>aeruginosa</u> 0.05 ml., AMP (50 uM per ml.)
0.1 ml., and water to a final volume of 3.0 ml. The activity
of the enzymes was tested with 5 uM ATP in place of the
<u>Pseudomonas</u> sonicate. The blank adsorption cell contained
water in the place of TPN. Initial readings were obtained and
glucose-6-phosphate dehydrogenase was added to start the re-
action. Readings were taken every minute, (Fig. 12). As the
figure shows, the oxidation of glucose by <u>P</u>. <u>aeruginosa</u> extract
does not produce ATP which indicates that the reaction is
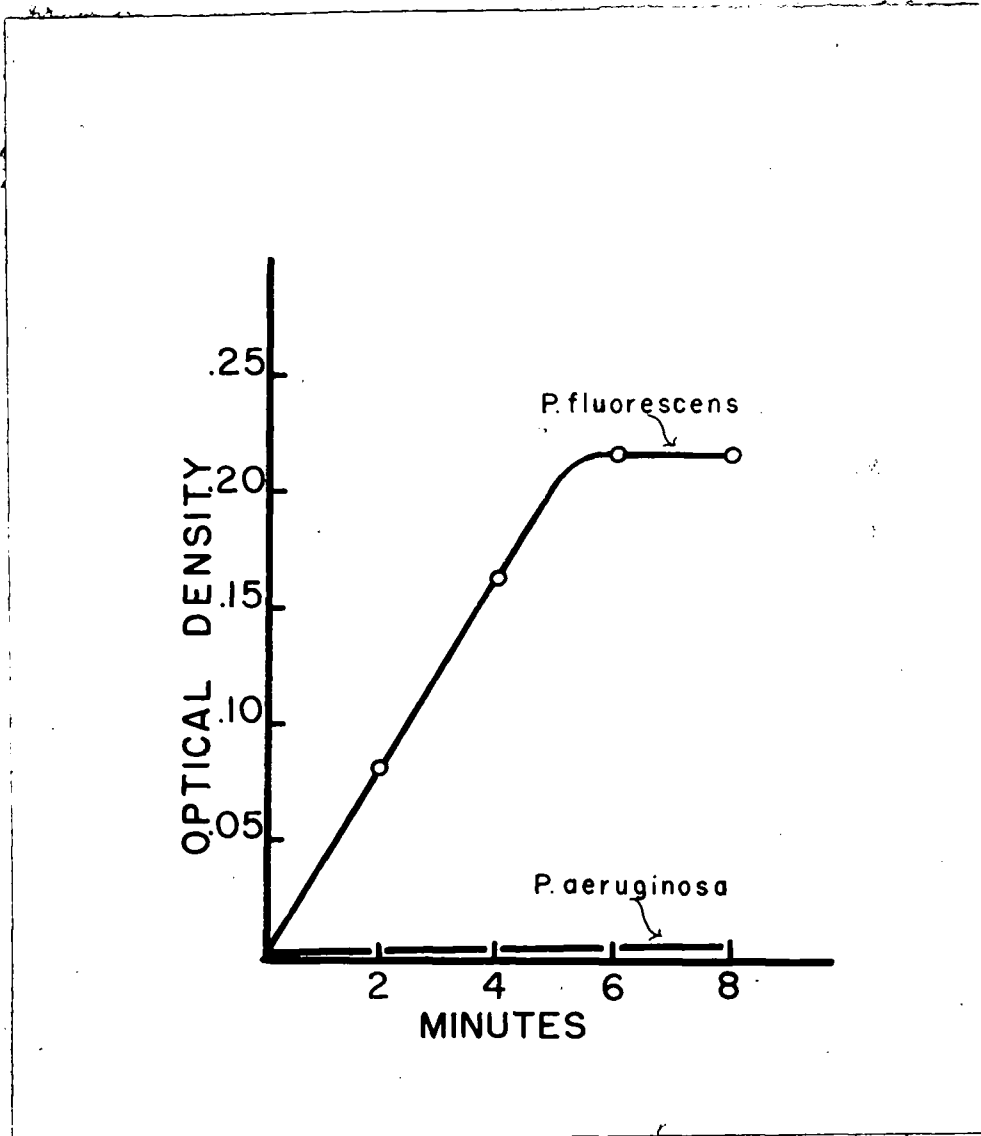different from most which have previously been studied.

FIGURE 11:

Gluconate phosphorylation by P. fluorescens as measured by TPN reduction in the presence of excess phosphogluconic dehydrogenase. Extracts of P. aeruginosa 9027 gave no reduction of TPN under these conditions.

The cuvette contained; 0.1 ml. of sonic extract; 0.2 ml. of gluconate (0.5 uM); $MgCl_2$ (0.15 uM) 0.1 ml.; 0.5 ml. of phosphogluconic dehydrogenase; 0.1 ml. of TPN (2 mg. per ml); 0.05 ml. of ATP (10 uM); water to a final volume of 3.0 ml. Measurement of O.D. at 340 mu.

Alternative Methods of Enzyme Purification

(1) Use of Cutscum as solubilizer: Cotzias et al.,
(1954) have reported the use of isooctylphenoxypolyethoxy-
ethanol.for solubilizing enzymes. This reagent is an active
ingredient of the detergent Cutscum. Experiments were con-
ducted to replace sodium glycocholate by Cutscum since the
latter does not leave any colour in solution. Cutscum was
added slowly to the cold sonicate to a total concentration of
5 per cent by volume, and homogenized in a van Potter homo-
genizer. Precipitation with 30 per cent ammonium sulphate
showed enzymic activity in the supernatant. However, the
detergent had to be dialyzed away completely before treatment
with ammonium sulphate in order to avoid the formation of a
difficultly separable scum. The activity of the treated pre-
paration was the same as with sodium glycocholate.

(2) Use of the ultracentrifuge: The addition of
a foreign agent such as sodium glycocholate or Cutscum
produces complexes with the lipids present in the sonicate.
These complexes were found to interfere in electrophoretic
analysis. It was, therefore, decided to try to separate the
enzyme mechanically from the cell surface without the use
of Cutscum and through the use of the ultracentrifuge.

The washed cells as usual, were subjected to sonic
oscillation for 20 minutes and centrifuged for 10 minutes at
25,000 x G. The supernatant was centrifuged at 105,000 x G
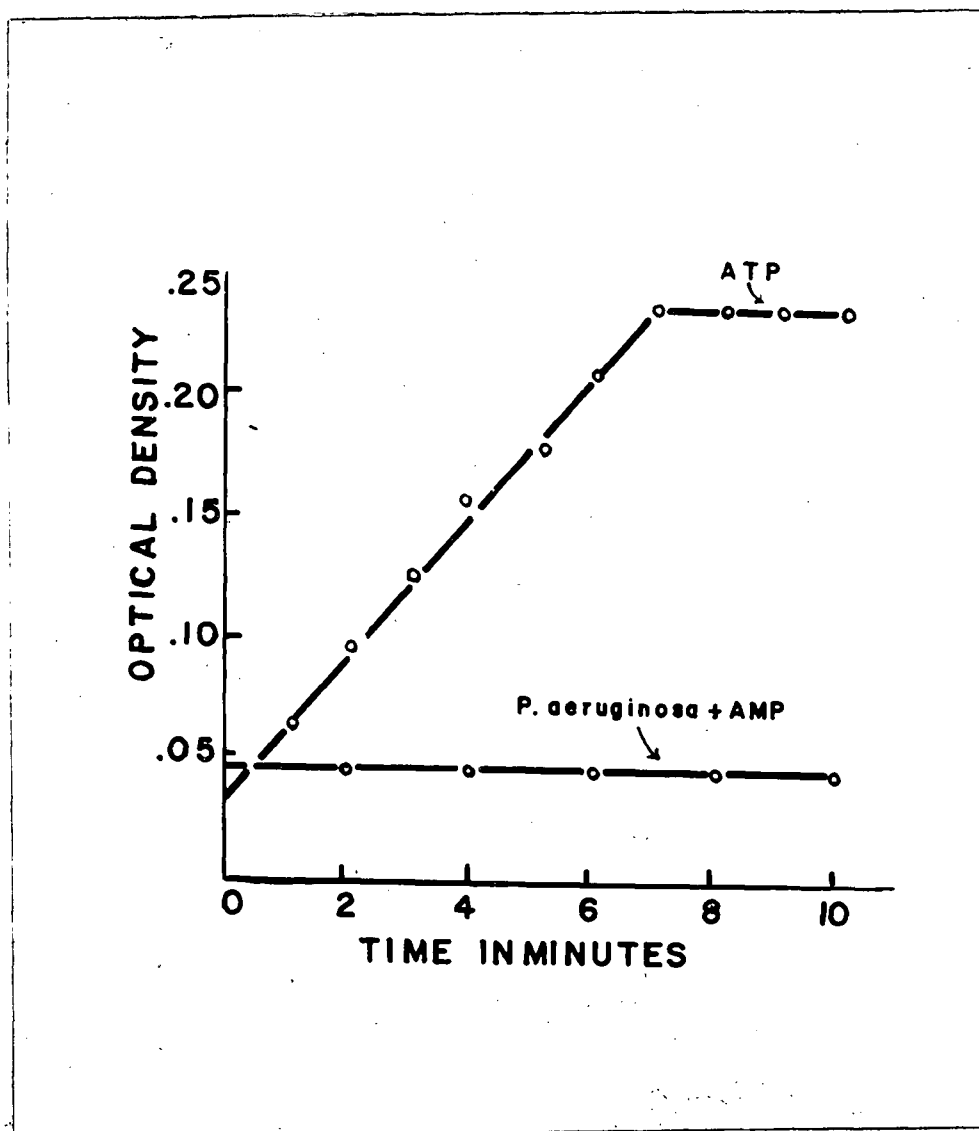for 60 minutes in an ultra-centrifuge at 4°C. A clear liquid

FIGURE 12:

       Production of high energy phosphate during glucose oxidation by P. aeruginosa 9027 as measured by TPN reduction in the presence of hexokinase and glucose-6-phosphate dehydrogenase.

       The first cuvette contained 0.2 ml. of glucose (5 uM); 0.1 ml. of MgCl$_2$ (15 uM); 0.1 ml. of hexokinase (10 mg. per ml.); 0.1 ml. of glucose-6-phosphate dehydrogenase (3 mg. per ml.); 0.1 ml. of TPN (2 mg. per ml.); 0.1 ml. of ATP (10 uM); 1.5 ml. of M/15 phosphate buffer pH 7.0 and water to 3.0 ml.

       In the second cuvette 0.1 ml. of sonic extract of P. aeruginosa and 0.1 ml. of AMP (10 uM) replaced ATP.

separated from a reddish-brown precipitate which may be cyto-
chromes. The supernatant by itself had only poor activity
against gluconic acid but was stimulated three-fold in the
presence of pyocyanine. The precipitate suspended in dis-
tilled water, also had some activity against gluconic acid.

Ammonium sulphate to 30 per cent of saturation was
added to the supernatant and the precipitate formed was centri-
fuged off. Both the precipitate and supernatant had activity
against gluconic acid showing that part of the enzyme had been
solubilized. The supernatant was dialyzed and treated with
protamine sulphate as usual and the nucleoproteins centrifuged
off. The solution was lyophilized and dissolved in the minimum
amount of M/10 phosphate buffer of pH 6.8 after which it was
dialyzed against the same buffer overnight and made up to
one-third the original volume. The solution was clear and
colourless.

The final product was subjected to analysis in the
electrophoretic apparatus. The following are the relevant
data for the analysis:

Current	10 m.a.
Duration	2 hours, photographs taken each hour.
Temperature	1°C
Slit	40°
Filter	Red
Position	3 cm.
Buffer	0.1 ionic strength of phosphate buffer of pH 6.83

Coulombs carried by left electrode:

754.00 cathode

778.00 anode

The photograph shows two peaks, one of which because of its high mobility, appears to be protamine sulphate. The other may be the enzyme.

Further work to corroborate these finding could not be carried out because of lack of facilities.

DISCUSSION

In the past the insolubility of oxidative enzymes has been a barrier preventing the elucidation of the mechanism of metabolic pathways. The preparation of gluconic dehydrogenase in a soluble form in a state of purity where it is specific for the substrate has made it possible to study the cofactors necessary for its activity and to find out whether energy was utilized or produced during oxidation. As Ghiretti and Barron (1954) have pointed out a study of the pathways of metabolism must be made with as many techniques as possible. Some of these techniques are detection in the medium of different intermediates; the detection of the enzyme in cell-free extracts and the use of specific inhibitors on the activity of the enzyme. "Use of only one of these methods may give erroneous answers as shown in the contradicting conclusions reached by a number of investigators who used only a single approach".

Earlier work from this laboratory indicated a lack of phosphorylation during the oxidation of glucose by P. aeruginosa at least as far as the 2-ketogluconic stage. The absence of inhibition by fluoride indicates that no phosphorylation is involved in the oxidation of gluconic acid by the enzyme. This is confirmed by the fact that ATP does not activate the enzyme. The absence of increased ionization as determined by the technique of Colowick and Kalckar and the failure to reduce TPN in the presence of phosphogluconic dehydrogenase are added confirmation that no phosphorylation is involved in the oxidation of gluconic acid by extracts of this organism. The additional phosphorylated system for gluconic acid oxidation reported by Wood and Schwerdt to be present in P. fluorescens is, therefore, absent from the purified enzyme obtained from P. aeruginosa 9027.

There is also evidence from experiments with the purified enzyme that no energy in the form of ATP is produced during the oxidation of gluconic acid. No increase in activity of the enzyme was noticed in the presence of AMP and inorganic phosphate. When the product of oxidation of gluconic acid was analyzed chromatographically there was no evidence of ATP. It has been already reported from this laboratory (Campbell et al. 1954) that no energy is produced during the oxidation of gluconic acid as evidenced by the fact that the same amount of growth of P. aeruginosa occurs with equimolecular amounts of glucose, gluconic or 2-ketogluconic acids. This was confirmed by the experiment in which the oxidation of glucose by

P. aeruginosa did not provide energy for the phosphorylation
of glucose by hexokinase. This finding shows that oxidation
by P. aeruginosa is different from that by other tissues where,
according to Hunter, Ochoa, Lehninger and other workers, 1
atom of oxygen is taken up with the production of 3 moles of
ATP.

The production of 3 molecules of high energy during
the oxidation of the substrate is associated with the pyridine
nucleotides, flavoproteins and the cytochrome system. Lipmann
(1946) illustrates this with the following diagram:
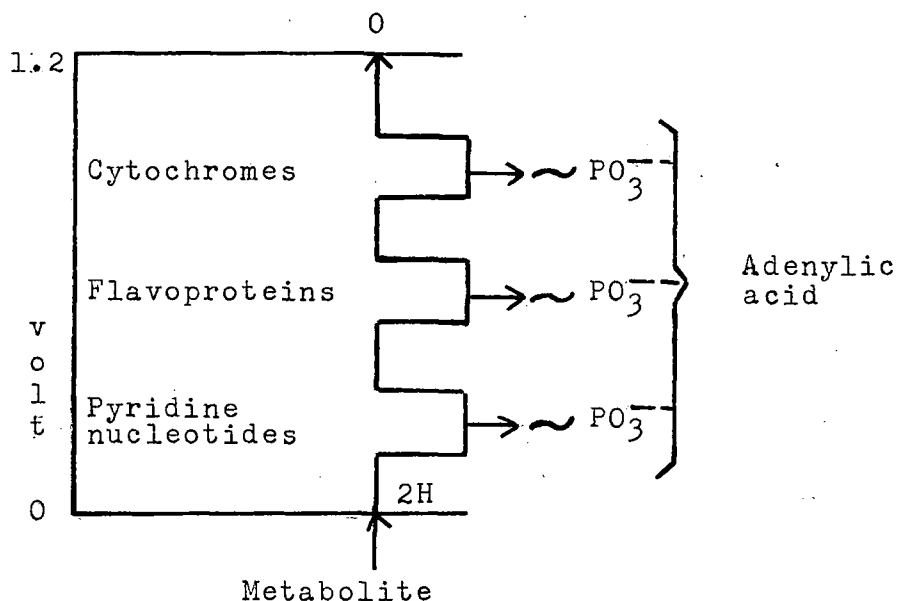
In the case of gluconic dehydrogenase DPN or TPN was not
reduced by the enzyme nor was the system activated by flavin
mononucleotide or flavin adenine dinucleotide. The system,
therefore, does not seem to be linked through the pyridine
nucleotide system or the flavoproteins. Pyocyanine acted as
a rapid hydrogen acceptor in the system and it is possible
that this pigment functions in a similar manner in growing

cells.

Friedheim (1931) found that pyocyanine catalysis
does not have an indiscriminate effect in all oxidations, but
only in the oxidation of certain substances closely connected
with the bacterial body like glucose, asparagine and pyruvic
acid. It is, therefore, logical that gluconic acid oxidation
can be similarly affected. Pyocyanine has been found to in-
crease the respiration of living cells to a great degree and
the reversibility of its oxidation and reduction is respon-
sible for this. Friedheim and Michaelis (1931) have found
that at pH's less than 6, it behaves entirely as a reversible
dye of quinonoid structure and that the slope of the titration
curve indicates that 1 molecule of the dye combines with 2
hydrogen atoms simultaneously. This gives an indication of the
kinetics of gluconate oxidation which is most efficient at pH
optimum of 5.6. Most dehydrogenases have a high pH optima
and Asnis and Brodie (1953) explain this by the fact that in
the oxidation-reductions of the type in which hydrogen ions
participate;

Substrate-H_2 + DPN$^+$ $\rightleftharpoons$ substrate + DPNH + H$^+$
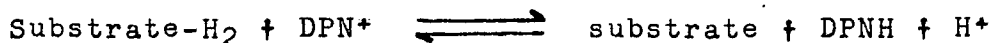
a high hydrogen ion concentration would favour the equilibrium
to the left. Where, on the other hand, 2 hydrogen atoms can
be accepted simultaneously, low pH cannot affect the oxidation
adversely while, at the same time, there is no competition for
the hydrogen acceptor from the other enzyme systems. This
appears to be what is taking place in gluconate oxidation with

P. aeruginosa.

Pyocyanine has been found to catalyze the oxygen
uptake of the system as well as of bringing about the synthesis
of high energy phosphate compounds. In this respect pyocyanine
is similar to methylene blue in the first instance and the
pyridine nucleotides in the second instance. Runnstrom and
Michaelis (1935) found that in a system consisting of haemol-
yzed blood, hexosemonophosphate and methylene blue, addition
of cozymase from yeast cells had two effects; it increased
oxidation and mediated in the synthesis of inorganic phosphates
to phosphate esters. These two effects went hand in hand;
when respiration was suppressed by omitting methylene blue,
phosphorylation did not occur either. On the contrary, the
amount of inorganic phosphorus increased with time, showing
that phosphate esters could, in such a system, either be syn-
thesized or broken down according to whether or not respira-
tion took place. When methylene blue was replaced with pyo-
cyanine, however, respiration was coupled with phosphate ester
synthesis and the addition of cozymase was not necessary.

Since it has been shown that phosphorylation does
not occur when gluconic acid is oxidized in the presence of
pyocyanine, it is possible that in the intact cells of P.
aeruginosa the cytochrome system is acting as the electron
transport system. Though cytochrome c has not been found ex-
perimentally to act in this capacity, it is possible that along
with other components of the system like cytochrome a, it acts

as a link between the substrate and molecular oxygen.

It has been generally accepted that a bivalent
oxidation in homogeneous solution proceeds in two successive
univalent steps. Since carbon is quadrivalent, the product
of a univalent oxidation is unstable as it has a free radical
with one of its atomic valences unoccupied. This instability
implies a relatively high energy content, to overcome which
the substrate is activated by the enzyme.

Most of the biological oxidations are linked through
the pyridinoprotein system which accepts 1 H ion and 2 electrons
at a time and passes them on to the flavoproteins. The flavo-
proteins have the capacity to pass on 2 hydrogen ions and 2
electrons at a time to oxygen. Under these conditions, per-
oxide is formed. In aerobic microorganisms catalase is
present in the system to destroy peroxide.

In the gluconic dehydrogenase system both pyridino-
protein and flavoprotein seem to be absent as hydrogen accep-
tors and their place may be taken by pyocyanine or 2,6-di-
chlorophenol indophenol, both of which accept 2 hydrogen atoms
at a time. Ferricyanide, methylene blue or brilliant cresyl
blue, which can accept only 1 hydrogen atom at a time, were
not effective in this system.

Dickens and McIlwain (1938) determined the activity
of the phenazines and some nonphenazine dyestuffs as carriers
in the hexosemonophosphate system. They found that the
phenazine derivatives (e.g., pyocyanine) were more active than

nonphenazine derivatives like brilliant cresyl blue and meth-
ylene blue. Methyl Capri blue, though in the same range of
potential as the active phenazine derivatives was found to be
inactive. They, therefore, concluded that structure is more
important than E_h in determining the activity of carriers for
their system and the peculiar activity of phenazines in the
system may be due to their ability to form semiquinones.
Dickens and McIllwain also found a poisoning effect on the
system by brilliant cresyl blue and methylene blue when pres-
ent in high concentrations.

The activity of the hydrogen acceptor in gluconic
dehydrogenase seems to follow this pattern:

Hydrogen Acceptor	Structure	E_h, pH 7	Activity
2, 6-dichlorophenol indophenol	Semiquinone	+0.217	Good
Pyocyanine (4-keto-N-methyl-phenazine)	Semiquinone	-0.011	Good
Methylene blue	Non-semiquinone	+0.011	Feeble
Brilliant cresyl blue	Non-semiquinone	+0.045	Nil
Ferricyanide	Non-semiquinone	+0.360	Nil

2, 6-dichlorophenol indophenol can accept 2 hydrogen atoms and
be reduced.
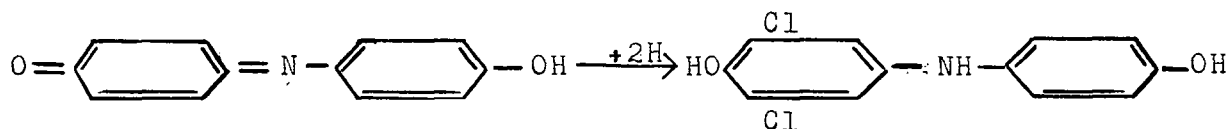
Pyocyanine can accept 2 hydrogen atoms and an electron to form a semiquinone radical. It is an example of a cationic semi-quinone. Friedheim and Michaelis (1931) have shown that in ranges of pH greater than 6.0 it behaved entirely as a reversible dye of a quinonoid structure. The slope of the titration curve indicated that 1 molecule of the dye combined with 2 hydrogen atoms simultaneously. At pH ranges greater than 6.0 the titration curves showed a different shape which was interpreted by the assumption that the 2 H atoms were accepted in two separate steps. With gluconic dehydrogenase there is a definite drop in activity at pH 6.0 even in crude cell preparations which seems to indicate the participation of pyocyanine in the living cells also. Weil-Malherbe (1937) found a dehydrogenase in animal tissues which oxidized l(—)α-hydroxyglutaric acid to α-ketoglutaric acid. The properties of this enzyme are comparable to those of the gluconic dehydrogenase. Its action did not depend on any coenzyme. A carrier was necessary for the reaction with molecular oxygen; pyocyanine was active whereas cytochrome c was inactive. Brilliant cresyl blue and methylene blue were much less efficient than pyocyanine. The structure of methylene blue indicates that only 1 hydrogen atom can be accepted at a time while apparently this dehydrogenase and gluconic dehydrogenase both require simultaneous transfer of 2 hydrogen atoms.

$$\left[(CH_3)_2 N \underset{S}{\overset{N}{\diagdown}} N(CH_3)_2 \right]^+ Cl^-$$
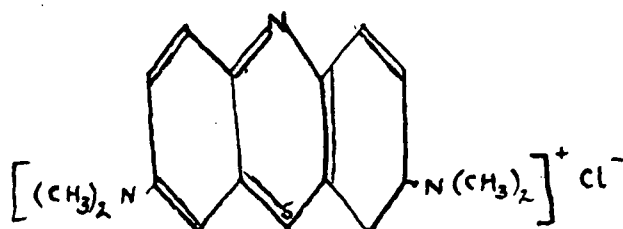
The action of cyanide in stimulating the enzyme may be either in forming a cyanohydrin derivative with the product or in reducing the potential of the system. According to the first alternative, 2-ketogluconic acid, formed by oxidation of gluconic acid may be acting as an inhibitor of the reaction and cyanide removes it by forming the cyanohydrin derivative. However, semicarbazide did not stimulate the enzyme nor did increasing concentrations of 2-ketogluconic acid inhibit the activity of the enzyme. The second alternative seems to be the more acceptable one, since both glutathione and cysteine activated the enzyme to the same degree as cyanide.

The nine-fold increase in total activity during purification may be due to the removal either of toxic factors or competing reactions. However, the startling increase in total activity occurred at the solubilizing step and so probably represents an increase in enzyme surface.

Energy for synthesis in biological materials may be available commonly in the form of oxonium ion, sulfonium ion, quaternary nitrogen, thioester or high energy phosphate bond. Of these, the high energy phosphate bond is probably the most common and has been studied in detail.

The question of whether high energy bonds are formed during the oxidation of all substrates has been a highly controversial one. It is now accepted that the transfer of hydrogen from reduced DPN or TPN to oxygen is associated with the production of 3 high energy bonds and the oxidation of 3 phosphoglyceraldehyde with the production of 1 molecule of high energy phosphate bond. Ochoa (1947) working with pigeon breast muscle found that ATP-ase present in the tissue destroyed the ATP which was being produced. Even the addition of sodium fluoride which inhibits ATP-ase could only bring the destruction down to 45-50 per cent. To correct for this, he studied the oxidation of 3 phosphoglyceraldehyde in the presence of DPN and pyruvate and in the absence of oxygen, a reaction which was known to give 1 high energy bond per pair of hydrogens transferred. The oxidation of 3 phosphoglyceraldehyde in the muscle served as a means of measuring the amount of high energy bonds destroyed by the ATP-ase. Next, pyruvate was oxidized by muscle and the values corrected for the amount of ATP destroyed in the reaction. It was found that 3 moles of ATP were formed per atom of oxygen taken up.

$$2CH_3COOH + 2\tfrac{1}{2}O_2 \rightleftharpoons 3CO_2 + 2H_2O + 15 \sim ph.$$
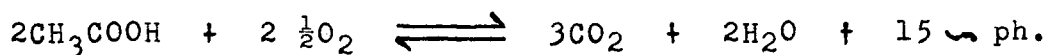
Ochoa also tentatively concluded that DPN- and TPN-linked oxidations were not coupled with phosphorylation. He found that massive concentrations of DPNH were readily oxidized by preparations of known activity towards substrates of the Kreb's cycle, but no phosphorylation occurred. Secondly,

the oxidation of isocitrate in similar systems poisoned by arsenite, to abolish further oxidation of α-ketoglutarate, likewise showed no phosphorylation.

Lehninger (1951) questioned the validity of Ochoa's findings. It was found that in his first experiment high concentrations of DPNH uncoupled phosphorylation from oxidation; in his second, arsenite uncoupled it. Using a labelled system Friedkin and Lehninger (1948) found that there was as much high energy phosphate formed during the oxidation of 1 mole of DPNH as of 1 mole of malate. They also found that in the case of mitochondria there was a difference between the activity of 'internal' and 'external' cytochrome c and DPN. Their experiments indicated that permeability and the inner structure of the mitochondria are of great importance in determining the P/O ratio. Thus 'internal' DPN is more heat-stable than externally added DPN; maximal P/O ratios were obtained only at the optimum osmotic concentration; 'internal' cytochrome c is available for the oxidation of DPNH; and the rate of oxidation of DPNH is three to five times as fast when the mitochondria are first exposed to hypotonic conditions. DPN or cytochrome c has also to be attached to the cell surface before phosphorylation can take place. He found that the oxidation of reduced DPN or TPN gives rise to 3 high energy bonds per mole.

Slater (1950) has presented data indicating that the P/O ratios during α-ketoglutarate oxidation by heart

muscle preparations are the same when using either oxygen or
cytochrome c as the ultimate electron acceptor. He blocked
cytochrome c with cyanide and got the same amount of high
energy bonds produced. He, therefore, concluded that no phos-
phorylation occurs between cytochrome c and oxygen. Lehninger
(1951) is prone to argue this contention since he feels that
the methods which Slater used are inadequate for measuring the
very small exchanges which might occur in the region of cyto-
chrome c to oxygen which makes up a very substantial portion
of the "E.M.F. span".

The results obtained with gluconic dehydrogenase
seem to corroborate Slater's findings. Though flavoproteins
and pyridinoproteins do not take part in the oxidation by this
enzyme, the lack of activity in the absence of an added hydro-
gen acceptor when the enzyme is solubilized shows that cyto-
chromes may be involved in the oxidation. Since no phosphory-
lation is involved during the span of oxidation, it seems rea-
sonable to conclude that no phosphorylation occurs in this case
also between cytochrome c and oxygen.

The oxidation of gluconic acid in some mammalian
tissues also seems to follow this pattern. Salmony and White-
head (1954) have reported that gluconic acid is oxidized by
kidney slices, but they could not demonstrate the product of
the reaction. They could not detect any phosphorylation of
gluconate by kidney homogenate and no oxidation of gluconate
was observed with kidney homogenates in the presence of coenzyme
and hydrogen carriers such as DPN, TPN or cytochrome c.

BIBLIOGRAPHY

1. Ames, S.R., J. Biol. Chem., <u>169</u>, 503 (1947).

2. Asnis, R.E., and Brodie, A.F., J. Biol. Chem., <u>203</u>, 153 (1953).

3. Avery, O.T., and Neill, J.M., J. Exp. Med., <u>39</u>, 543 (1924).

4. Bandurski, R.S., and Axelrod, B., J. Biol. Chem., <u>193</u>, 405 (1951).

5. Banga, I., Ochoa, S., and Peters, R.A., Biochem. J., <u>33</u>, 1980 (1939).

6. Barnard, J.E., and Hewlett, R.T., Proc. Roy. Soc. Lond., B<u>84</u>, 57, (1912).

7. Barron, E.S.G., and Friedman, T.E., J. Biol. Chem., <u>137</u>, 593 (1941).

8. Bealing, F.J., and Bacon, J.S.D., Biochem. J., <u>53</u>, 277, (1953).

9. Bentley, R., and Neuberger, A., Biochem. J., <u>45</u>, 584, (1949).

10. Bernhauer, K., and Gorlick, B., Biochem. J., <u>303</u>, 308, (1940).

11. Bernhauer, E., and Knoblock, H., Biochem. J., <u>280</u>, 367 (1935).

12. Bernhauer, K., and Schon, X., J. Physiol. Chem., <u>180</u>, 232 (1929).

13. Bernheim, S., Biochem. J., <u>22</u>, 1178 (1928).

14. Black, S., Arch. Biochem., <u>34</u>, 86 (1951).

15. Booth, V.H., and Green, D.E., Biochem. J., <u>32</u>, 855 (1938).

16. Bovarnick, M., Proc. Soc. Exp. Biol. Med., <u>47</u>, 191 (1941).

17. Boyer, P.D., and Lardy, H.A., J. Biol. Chem., <u>146</u>, 673 (1942).

18. Boyer, P.D., Lardy, H.A., and Phillips, P.A., J. Biol. Chem., <u>149</u>, 529 (1943).

19. Brodie, A.F., and Lipmann, F., Proc. Soc. Am. Bact., <u>107</u> (1954).

20. Campbell, J.J.R., Linnes, A.G., and Eagles, B.A., Trans. Roy. Soc. Can., <u>48</u>, 49 (1954).

21. Campbell, J.J.R., Norris, F.C., and Norris, M.E., Can. J. Res., C<u>27</u>, 165 (1949).

22. Campbell, J.J.R., and Norris, F.C., Can. J. Res., C28, 203 (1950).

23. Campbell, J.J.R., and Stokes, F.N., J. Biol. Chem., 190, 853 (1951).

24. Chantrenne, H., and Lipmann, F., J. Biol. Chem., 187, 757 (1950).

25. Claridge, C.A., and Werkman, C.H., J. Bact., 68, 77 (1954).

26. Claude, A., J. Exp. Med., 80, 19 (1944).

27. Cohen, P.P., and Hayano, M., J. Biol. Chem., 166, 239 (1946).

28. Cohen, S.S., and Scott, D.C.M., Nature, 166, 781, (1950).

29. Colowick, S.P., Cori, G.T., and Slein, M.W., J. Biol. Chem., 168, 583 (1947).

30. Colowick, S.P., and Kalckar, H.M., J. Biol. Chem., 148, 117 (1943).

31. Colowick, S.P., and Kaplan, N.O., Ann. Rev. Biochem., 20, 513 (1951).

32. Cooper, P.D., J. Gen. Microbiol., 9, 199 (1953).

33. Cori, O., and Lipmann, F., J. Biol. Chem., 194, 417 (1952).

34. Cotzias, G.C., Serlin, I., and Greenough, J.J., Science, 120, 144, (1954).

35. Curran, H.R., and Evans, F.R., J. Bact., 43, 125 (1942).

36. De Ley, J., Enzymologia, 16, 99 (1953).

37. Dickens, F., Nature, 138, 1057 (1936).

38. Dickens, F., Biochem. J., 32, 1626 (1938).

39. Dickens, F. and McIlwain, H., Biochem. J., 32, 1615 (1938).

40. Dixon, M., Manometric Methods, MacMillan, N.Y., (1943).

41. Doudoroff, M., Kaplan, N.O., and Hassid, W.Z., J. Biol. Chem. 148, 67 (1943).

42. Eichel, B., and Wainio, W.W., J. Biol. Chem., 175, 155 (1948).

43. Eisenberg, M.A., J. Biol. Chem., 203, 815 (1953).

44. Elliott, W.B., and Kalnitsky, G., J. Biol. Chem., 186, 477 (1950).

45. Entner, N., and Doudoroff, M., J. Biol. Chem., 196, 853 (1952).

46. Entner, N., and Stanier, R.Y., J. Bact., 62, 181 (1951).

47. Epps, H.M.R., Biochem. J., 38, 242 (1944).

48. Foda, O., and Vaughn, R.E., J. Bact., 65, 233 (1953).

49. Fosdick, L.S., and Dodds, A.F., Arch. Biochem., 6, 1, (1945).

50. Friedheim, E., J. Exp. Med., 54, 207 (1931).

51. Friedheim, E., and Michaelis, L., J. Biol. Chem., 91, 355, (1931).

52. Friedkin, M., and Lehninger, A.L., J. Biol. Chem., 174, 757 (1948).

53. Furness, G., J. Gen. Microbiol., 7, 335 (1952).

54. Ghiretti, F., and Barron, E.S.G., Biochem. et Biophys. Acta., 15, 445 (1954).

55. Gunsalus, I.C., and Umbreit, W.W., J. Bact., 49, 347 (1945).

56. Hanes, C.S., and Isherwood, F.A., Nature, 164, 1107 (1949).

57. Harrison, D.C., Biochem. J., 25, 1016 (1931).

58. Harvey, S.C., J. Biol. Chem., 179, 435 (1949).

59. Heden, C., Science, 113, 181 (1951).

60. Hill, R.W., Thesis, University of British Columbia, (1952).

61. Hiller, A., Grief, R.L., and Beckman, W., J. Biol. Chem., 176, 1421 (1948).

62. Hochster, R.M., and Quastel, J.H., Arch. Biochem., 31, 278 (1951).

63. Horecker, B.L., and Smyrniotis, P.Z., Proc. Am. Chem. Soc., 118th Meeting, 41 C (September 1950).

64. Horecker, B.L., and Smyrniotis, P.Z., J. Biol. Chem., 193, 371 (1951).

65. Horecker, B.L., Smyrniotis, P.Z., and Seegmiller, J.E., J. Biol. Chem., 193, 383 (1951).

66. Hughes, D.E., Brit. J. Exp. Path., 32, 97 (1951).

67. Hughes, D.E., and Williamson, D.H., Biochem. J., 51, 45 (1952).

68. Hugo, W.B., Bact. Revs., 18, 87 (1954).

69. Kallio, R.E., J. Biol. Chem., 192, 371 (1951).

70. Kalnitsky, G., and Werkman, C.H., Arch. Biochem., 2, 113 (1943).

71. Katznelson, H., Tanenbaum, S.W., and Tatum, E.L., J. Biol. Chem., 204, 43 (1953).

72. Keilin, D., and Hartree, E.F., Proc. Roy. Soc. Lond., B121, 399 (1938).

73. Keilin, D., and Hartree, E.F., Biochem. J., 42, 221 (1948).

74. Khesghi, S., Roberts, H.R., and Bucek, W., Appl. Microbiol., 2, 183 (1954).

75. Koepsell, H.J., and Johnson, M.J., J. Biol. Chem., 145, 379 (1942).

76. Koepsell, H.J., Johnson, M.J. and Meek, J.S., J. Biol. Chem., 154, 535 (1944).

77. Kornberg, A., J. Biol. Chem., 182, 779 (1950).

78. Krampitz, L.O. and Werkman, C.H., Biochem. J., 35, 595 (1941).

79. Lee, S.B., Wilson, J.B., and Wilson, P.W., J. Biol. Chem., 144, 273 (1942).

80. Lehninger, A.L., Phosphorus Metabolism, 1, 344 (1951).

81. Lindstrom, E.S., J. Bact., 65, 565 (1953).

82. Lipmann, F., Nature, 138, 588 (1936).

83. Lipmann, F., J. Biol. Chem., 160, 173 (1945).

84. Lipmann, F., Currents in Biochemical Research, Interscience, N.Y., (1946).

85. Lockwood, L.B., Tabenkin, B., and Wood, G.K., J. Bact., 42, 51 (1940).

86. Loomis, W.I., Arch. Biochem., 26, 355 (1950).

87. MacGee, J., and Doudoroff, M., J. Biol. Chem., 210, 617 (1954).

88. Magasanik, B., Brooke, M.S., and Karibian, D., J. Bact., <u>66</u>, 611 (1953).

89. McIlwain, H., J. Gen. Microbiol., <u>2</u>, 288 (1948).

90. Mickle, H., J. Roy. Microscop. Soc., <u>68</u>, 10 (1948).

91. Müller, D., Chem. Ztg., <u>50</u>, 101 (1926).

92. Müller, D., Biochem. J., <u>199</u>, 136 (1928).

93. Muys, G.T., Leeuwenhoek ned. Tijdschr., <u>15</u>, 203 (1949).

94. Nachmansohn, D., and John, H.M., J. Biol. Chem., <u>158</u>, 157 (1945).

95. Narrod, S.A., and Wood, W.A., Bact. Proc., <u>108</u> (1954).

96. Nason, A., Kaplan, N.O., and Colowick, S.P., J. Biol. Chem., <u>188</u>, 397 (1951).

97. Neuberg, C., and Lustig, H., Arch. Biochem., <u>19</u>, 191 (1948).

98. Norris, F.C., and Campbell, J.J.R., Can. J. Res., C<u>27</u>, 253 (1949).

99. Ochoa, S., Ann. N.Y. Acad. Sci., <u>47</u>, 835 (1947).

100. Ogston, F.J., and Green, D.E., Biochem. J. <u>29</u>, 1983 (1935).

101. Paege, L.M., and Schlenk, F., Arch. Biochem., <u>28</u>, 348 (1950).

102. Pappenheimer, A.M. and Hendee, E.D., J. Biol. Chem., <u>180</u>, 597 (1949).

103. Pollock, M.R., J. Gen. Microbiol., <u>8</u>, 186 (1953).

104. Racker, E., J. Am. Chem. Soc., <u>75</u>, 1010 (1953).

105. Ratner, S., and Pappas, A., J. Biol. Chem., <u>179</u>, 1199 (1949).

106. Renvall, S., Acta Chem. Scand., <u>4</u>, 738 (1950).

107. Runnstrom, J., and Michaelis, L., J. Gen. Physiol., <u>18</u>, 717 (1935).

108. Salmony, D., and Whitehead, J.K., Biochem. J., <u>58</u>, 408 (1954).

109. Seegmiller, J.E., and Horecker, B.C., J. Biol. Chem., <u>194</u>, 261 (1952).

110. Sher, I.H., and Mallette, M.F., J. Biol. Chem., 200, 257 (1953).

111. Shorr, E., Barker, S.B., and Malam, M., Science, 87, 168 (1937).

112. Slade, H. D., Arch. Biochem., 42, 204 (1953).

113. Slater, E.C., Nature, 166, 982 (1950).

114. Sleeper, B.P., Tsuchida, M., and Stanier, R.Y., J. Bact., 59, 129 (1950).

115. Stadtman, E.R., and Barker, H.A., J. Biol. Chem., 180, 1085 (1949).

116. Stephenson, M., Biochem. J., 22, 605 (1928).

117. Stickland, L.K., Biochem. J., 23, 1187 (1929).

118. Still, J.L., Biochem. J., 35, 380 (1941).

119. Strecker, H.J., and Korkes, S., J. Biol. Chem., 196, 769 (1952).

120. Stumpf, P.K., J. Biol. Chem., 159, 529 (1945).

121. Stumpf, P.K., and Green, D.E., J. Biol. Chem., 153, 387 (1944).

122. Taylor, J.F., Velick, S.F., Cori, G.T., Cori, C.F., and Slein, M.W., J. Biol. Chem., 173, 619 (1948).

123. Umbreit, W.W., and Gunsalus, I.C., J. Biol. Chem., 159, 333 (1945).

124. Umbreit, W.W., Burris, R.R., and Stauffer, J.F., Manometric Techniques and Tissue Metabolism, Burgess, Minn. (1949).

125. Utter, M.F., Krampitz, L.O., and Werkman, C.H., Arch. Biochem., 9, 285 (1946).

126. Waldvogel, M.J., and Schlenk, F., Arch. Biochem., 22, 185 (1949).

127. Warburg, O., and Christian, W., Biochem. J., 238, 131 (1931).

128. Warburg, O., and Christian, W., Biochem. J., 287, 302 (1936).

129. Warburg, O., and Christian, W., Biochem. J., 310, 384 (1942).

130. Warburton, R.H., Eagles, B.A., and Campbell, J.J.R., Can. J. Botany, 29, 143 (1951).

131. Weil-Malherbe, H., Biochem. J., <u>31</u>, 2080 (1937).

132. Wiggert, W.P., Silverman, M., Utter, M.F., and Werkman, C.H., Iowa State College J. Sci., <u>14</u>, 179 (1940).

133. Williams, J.N., and Sreenivasan, A., J. Biol. Chem., <u>203</u>, 899 (1953).

134. Wirth, J.C., and Nord, F.F., Science, <u>92</u>, 15 (1940).

135. Wood, W.A., Personal Communication (1954).

136. Wood, W.A., Gunsalus, I.C., and Umbreit, W.W., J. Biol. Chem., <u>170</u>, 313 (1947).

137. Wood, W.A., and Gunsalus, I.C., J. Biol. Chem., <u>181</u>, 171 (1949).

138. Wood, W.A., and Schwerdt, R.F., J. Biol. Chem., <u>201</u>, 50 (1953).

139. Wood W.A., and Schwerdt, R.F., J. Biol. Chem., <u>206</u>, 625 (1954).