A PROPOSED BACTERIAL EVOLUTIONARY SCHEME BASED ON PHYSIOLOGICAL DEVELOPMENT

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Michael Paul Adamo

August 1976

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Abstract

An evolutionary scheme is proposed for bacteria which is based on physiological development of bioenergetically important electron transport systems. It is proposed, as has been done by others, that the "most primitive" procaryotes are the obligately anaerobic heterotrophs, whose energy for growth is obtained directly by substrate level phosphorylations, via pre-glycolytic pathways. The origin of this bacterium is unknown, but one can predict its physiological characteristics if the environment can be chemically defined at the time of its origin, and if the physiological types of today are representative of those that existed on primitive earth. As the environmental conditions on earth changed, this physiological-type of organism underwent evolutionary changes, developing more complex physiological processes, which continued until electron-transport phosphorylation processes evolved. The electron transport process, particularly those that are membrane-associated *are mandatory* for both anoxygenic and oxygenic photosynthesis, as well as for oxidative phosphorylation. Such highly developed electron transport systems contain multiple oxidation-reduction carriers, and components that are integrated into the photosynthetic or respiratory electron transport chain, which then allows for efficient energy conservation. As organic nutrients became depleted, and the atmosphere became less reduced, and then oxygenated, those life forms having efficient electron transport phosphorylation systems had more selective and adaptive advantages. How such electron transport systems evolved is discussed, the basic assumptions being that as bacteria evolved, the physiological processes became more complex, and more efficient bioenergetically. This development continued, until the ultimate was reached which today represents the obligate

aerobic bacteria having highly evolved, and very active multiple terminal oxidases. The specific physiological types of bacteria for each of the presumed evolutionary stages, are named, and the developmental scheme assumes that organisms today represent survivor types that once were prevelant when different environmental conditions existed on earth. The atmosphere on earth played a major selective role, particularly the appearance of oxygen, in determining the physiological type that evolved. An attempt was also made to show how the proposed evolutionary scheme would fit for Gram-positive and negative bacteria.

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Introduction

A scheme for bacterial evolution, based on the sequential development of complex physiological systems is proposed. It assumes that as bacteria evolved on earth, the simpler metabolic systems were subjected to genetic alterations, and that as these changes occurred, new organisms were selected out for that now had more complex physiological systems, because they were concomitantly more efficient bioenergetically. A classic example would be the metabolic "mechanistic" change from the anaerobic fermentative metabolism to the more complex, but bioenergetically efficient aerobic respiratory system. The scheme proposed is consistent with present day data and theory that concern geochemical evolution. For purposes of simplification the chemical history of the earth is arbitrarily divided into three biogeochemical phases: Phase I, Phase II and Phase III. How bacteria evolved during the various phases is discussed in detail.

Phase I is the era of anaerobic chemoorganotrophic (heterotrophic) metabolism. Solely anaerobic heterotrophic bacteria are initially found, which live on the organic compounds synthesized abiotically during Phase I. The atmosphere is highly reduced, consisting of H_2, CH_4, H_2S, NH_3, H_2O, and CO_2. Abiotic synthesis of Phase I is responsible for synthesizing organic substrates directly used for growth and multiplication as well as the manufacturing of new chemical precursors which can be assimilated, as is the case of porphyrin or heme for cytochrome biosynthesis. For bioenergetic purposes, the first "primitive" bacteria rely on two (or three) step pre-glycolytic fermentation pathways having no highly developed electron transport system. The abiotic synthesis of organic molecules, not physiologically active would tend to accumulate, like carbohydrates, and

now by selection, fermentative type bacteria appear. Such organisms would be the "true" fermenting bacteria which have a glycolytic pathway as the sole ATP generating mechanism. Subsequently, organisms would have evolved having the glyoxylate, and Krebs cycle, which from a bioenergetic viewpoint would be more efficient in that they now posssess an NAD(P)H generating system. It is proposed that the availability of an NAD(P)H generating system would now allow for the evolution of bacteria having complex electron transport systems. The anaerobic non-cytochrome dependent type electron transport systems probably appeared next. These consisted primarily of ferredoxin-flavoprotein type components which could utilize organic compounds, like fumarate, as terminal electron acceptors. Then bacteria would have evolved having cytochrome components as part of their electron transport system, with the first resemblance of a respiratory chain, also containing the other redox components like flavo-proteins. Initially the heme moeity, for incorporation into cytochrome systems, may have been manufactured abiotically, and this may have allowed for its incorporation into the cytochrome components needed for the cytochrome dependent anaerobic electron transport chains. This would have then allowed for ATP synthesis via a route, other than substrate phosphorylation, namely electron transport phosphorylation. The change from substrate phosphorylations to electron transport phosphorylation may have played a selection role as it would have been more efficient from a bioenergetic viewpoint. Then bacteria appeared which contained the multiple cytochrome respiratory chains but were still anaerobic; cytochromes b, c, and a appearing late in the era of Phase I.

Phase II represents the era in which bacteria adapted to the environmental pressure of nutrient depletion. The organic nutrients are no longer being synthesized at the same rate abiotically and those available

are being depleted because of bacterial assimilation. A major switch in bacterial type may have occurred from the anaerobic heterotrophic metabolism to anaerobic phototrophic metabolism. The atmosphere, due to the escape of H_2 gas is now "neutral" and consists primarily of CO_2, CO, $SO_4^=$, NO_3^-, N_2 and H_2O. For photosynthetic bacteria to have evolved, a complex electron transport system is needed. ATP synthesis via anaerobic or aerobic (oxygenic) photosynthesis does require a multicomponent electron transport system, therefore the anaerobic-type respiratory electron transport chains would have had to evolve prior to appearance of photosynthetic bacteria. This adaptation may have been directly due to the depletion of organic nutrients and culminated with the development of the procarotic blue-green bacteria (algae). These bacteria have the "simplest known" inorganic nutritional requirements of any bacteria (N_2 serves as nitrogen source, and CO_2 as carbon source, H_2O as the hydrogen source and sunlight as the energy source). It is here that the oxygenic photosynthetic capability evolved which eventually introduced oxygen (O_2) into the earth's atmosphere. However the bacteria in this phase which might have developed prior to oxygen evolution in a neutral or partially reduced atmosphere, are the obligately anaerobic sulfate and nitrate respires. The sulfate respirers may have evolved directly via a non-fermentative path from the pre-glycolytic bacteria. They require an electron transport mechanism, but do not ferment carbohydrates. The obligately anaerobic nitrate respirers, on the other hand, may have evolved along the main fermentative pathway. The nitrogenase system, which probably first appeared very early in the primitive pre-glycolytic bacteria, originally functioned soley to detoxify cyanides and cyanogens, two chemicals known to exist in the abiotic atomosphere. Now the nitrogenase system,

because of selective pressures (lack of organic nitrogen compounds) begins to serve as a means of synthesizing intracellular NH_3; thus, now the nitrogen fixation process becomes less important for detoxification but more important for bacterial growth. Also, at this time the photosystems begin to function to energize, via electron transport systems, the reduction of CO_2 to glucose, whereas previously CO_2 reduction directly formed the intermediate compounds of the Krebs cycle using this cycle in the "reverse" direction.

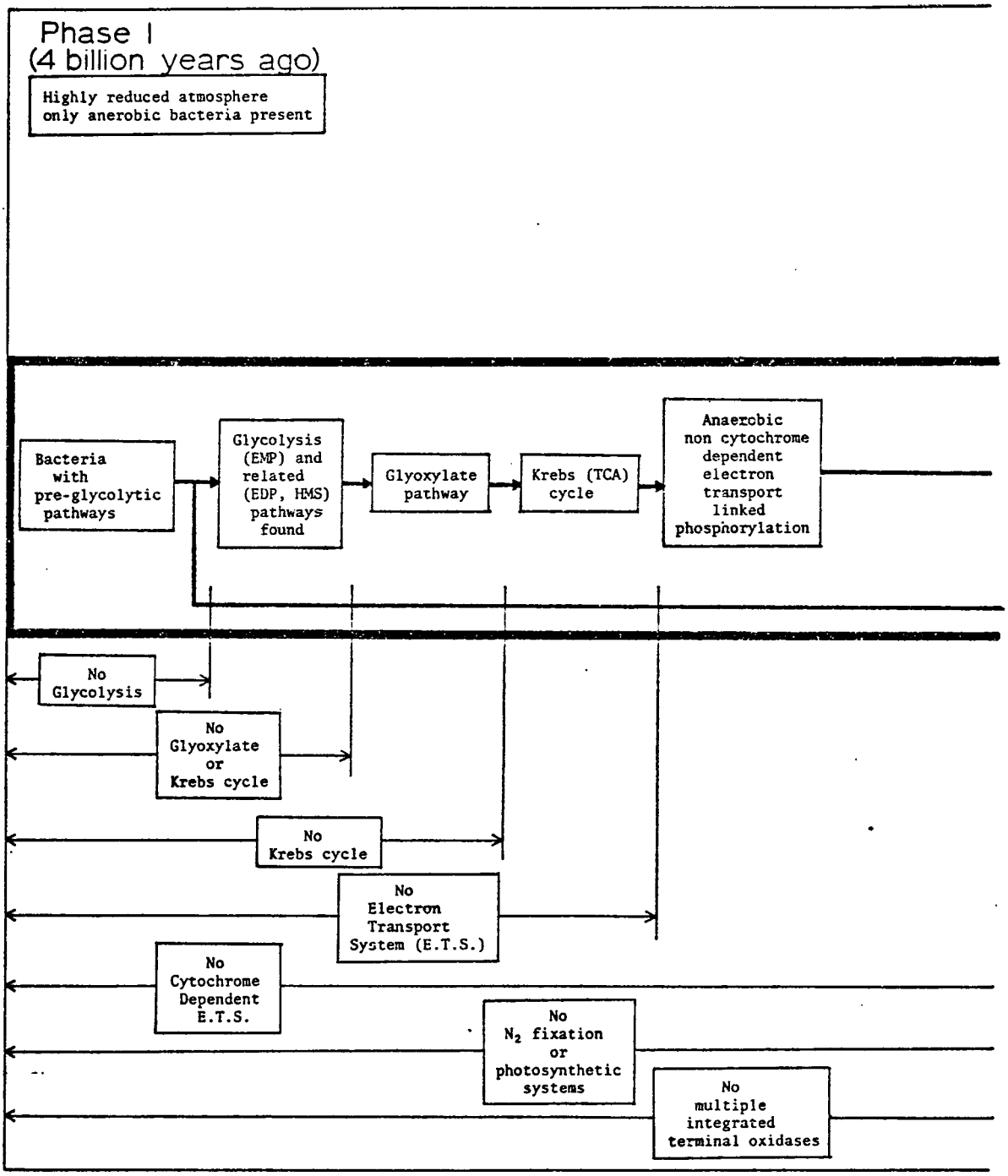
Phase III is the highly oxidative era, with the atmosphere consisting of N_2, O_2, CO_2, CO, NO_3^-, $SO_4^=$, and H_2O. In Phase III the atmosphere is oxygenated. This is the direct result of the oxygen evolution carried out photosynthetically by the blue-green bacteria. As oxygen accumulated in the atmosphere, the abiotic synthesis of organic compounds ceased entirely. An electron transport system can now evolve having a-type cytochromes as terminal oxidases, allowing oxygen to be utilized as terminal electron acceptor. It is proposed that the obligately aerobic chemoautotrophs and methylotrophs could not have existed until oxygen became available, and these may have developed directly from the phototrophic bacteria, through regressive evolution, by loss of the photosynthetic apparatus. The facultatively anaerobic bacteria are proposed to be among the first organisms to utilize the aerobic respiratory mechanism; they did not have to rely completely on oxidative phosphorylation, only if conditions permitted. They are the intermediate-type organisms which bridge the early strictly anaerobic fermentative and/or nitrate respiring and the highly evolved obligately aerobic bacteria. The obligately aerobic, oxidase negative, chemoheterotrophs evolved first from the facultative anaerobes. Such organisms obligately require oxygen for purposes of oxidative phosphorylation but do not have highly active (or highly evolved) cytochrome oxidases, which are the terminal

redox components of highly developed electron transport systems. Both the facultative anaerobes, and the obligate, oxidase negative, aerobes have in common a low TMPD oxidation (or cytochrome oxidase) capability, and are thus oxidase negative species. Such bacteria either lack c-type cytochromes or the c-type cytochrome, if present, is not integrated functionally with the terminal oxidase of the electron transport chain. The last, and most highly evolved bacteria to appear are the obligately aerobic, oxidase positive, chemoheterotrophs. These bacteria show high TMPD oxidation capability, indicating that cytochrome c is present and is functionally integrated with the terminal oxidase. There are indications that cytochrome oxidase activity may have evolved separately in some facultative anaerobes. These would be the fermenters which exhibit a high TMPD oxidation capability that might have evolved directly from the facultative anaerobic bacteria.

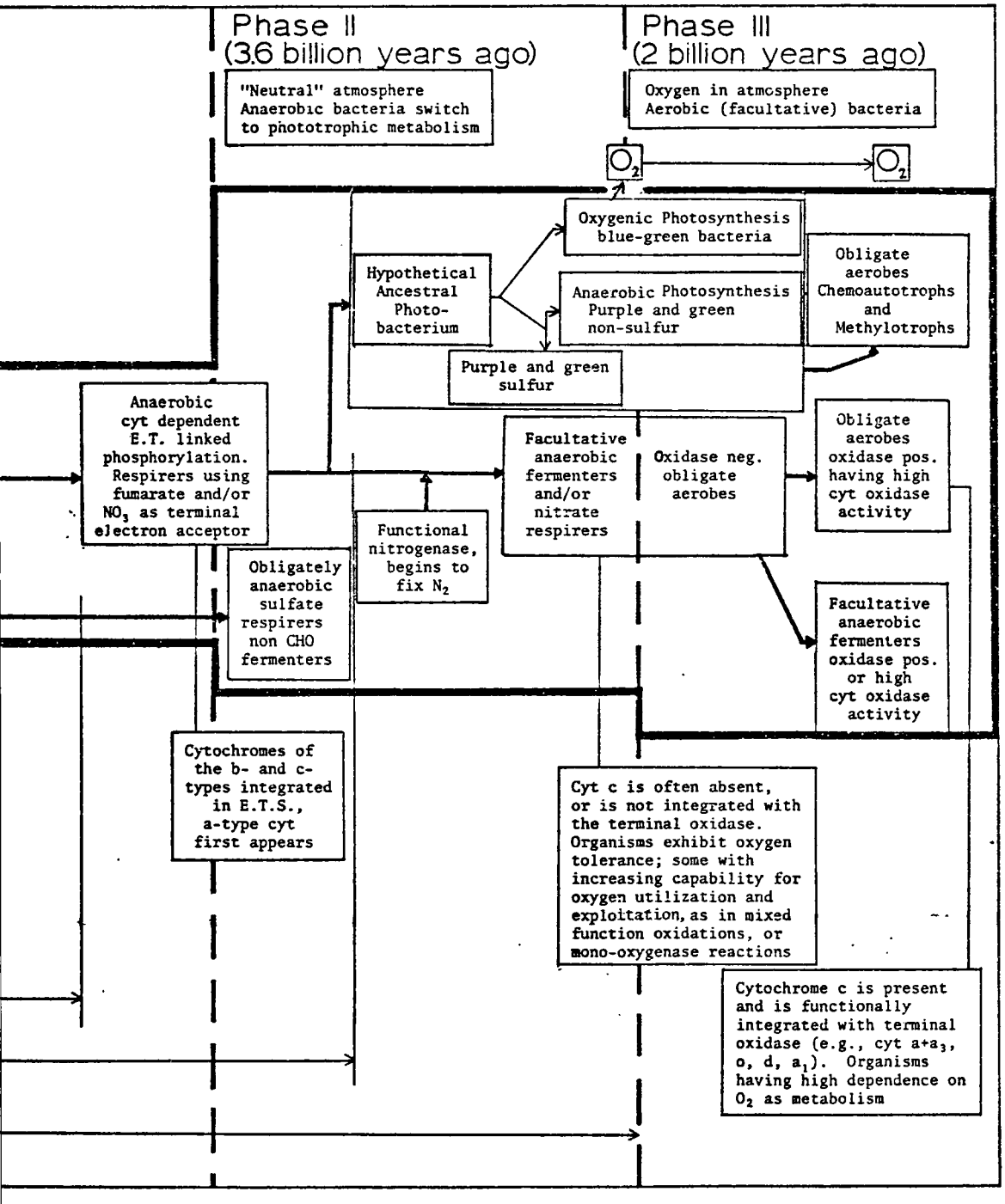
The key principles underlying this bacterial evolutionary proposal involve the formation and development of the bacterial electron transport systems. The rudiments of such a redox system had to be present in the first organism. Then, depending on both adaptive pressures, and the availability of metabolites changes would have occurred which allowed for the selection of the most efficient bioenergetic types that could survive in the present environment, and in which the atmosphere would play a major role. As the environmental changes occurred, bacteria began to evolve having first primitive, and then advanced type electron transport systems, the most complex type being those that can use molecular oxygen as a terminal electron acceptor, and can carry out ATP synthesis via electron transport phosphorylations.

A scheme showing the development of the bacterial evolutionary process proposed, is shown in Fig. 1. In this figure, the phases are defined:

Figure 1 A Scheme for



Bacterial Evolution



(a) in terms of chronological age, (b) the chemical nature of the atmosphere and (c) the physiological processes gained in the course of evolution, that relate to the bioenergetic mechanisms.

Chemical Evolution and Theories on the Origin of Life

The early experiments of Pasteur and of Tyndall dispelled the idea that living matter was generated from non-living matter (*i.e.*, spontaneous generation). Of course, this conclusion *did not* preclude the possibility that given the correct environmental conditions and eons of time that life forms could arise from aggregates of biomolecules. Present evolutionary theory requires some sort of continuity between the molecular (pre-biotic) and the biotic condition.

One of the earliest observations concerning bacterial evolution was made by the microbiologist Sigurd Orla-Jensen. In an article that appeared in the *Centralblatt fur Bakteriologie*, Section 2, XXII (1909), 305-346 and was entitled "Die Hauptlinien des Naturlichen Bakterien-Systems", the following statement was made{(cited by R.N. Doetsch (53)}:

> It is evident that the first bacteria on earth must have been autotrophic, but it is difficult to determine which autotrophic type is most primitive, since energy sources such as hydrogen, methane, hydrogen sulfide, carbon monoxide, and ammonia are products of volcanic action, and therefore all were present in quantity early in the history of the earth. We possess one clue, however, if we make the assumption that the easier a synthesis proceeds, the earlier it must have begun.

Although present evidence and theory disagree with Orla-Jensen that autotrophs were the first bacteria on earth, this statement represents the first hypothesis on bacterial evolution. Orla-Jensen would have an interest in bacterial evolution because historically, in 1908, he proposed a 'natural' classification of bacteria based on physiological characteristics, rather than morphological and stated that " a preliminary classification based upon a natural principle is better than no order whatsoever". In later years Orla-Jensen's hypothesis had been supported

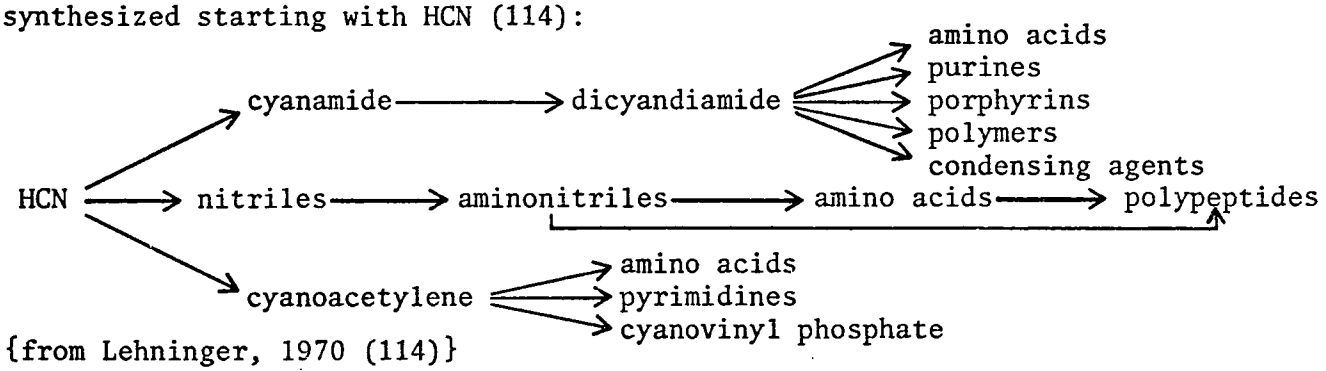
by A. Lwoff in 1944 (115), and by B.C.J.G. Knight in 1945 (106).

Any sound theory of bacterial evolution had to await geochemical evidence regarding the chemistry of the primitive atmosphere and oceans. Such an explanation, that is now widely accepted, was proposed independently in 1924 by A.I. Oparin (134) in the Soviet Union, and in 1929 by J.B.S. Haldane (67) in England. These two men suggested that biological evolution was preceded by an era of chemical, pre-biotic evolution. This period of chemical evolution lasted for approximately 2 billion years. During this period of chemical evolution the inorganic components of the atmosphere (H_2O, NH_3, CH_4, H_2S, H_2, N_2, CO_2) were exposed to ultraviolet light, electric discharges (lightning) and volcanic heat, the end result being the synthesis of a variety of simple organic compounds. Among these were the amino acids, purines, pyrimidines, hydrocarbons, and sugars. Hydrogen cyanide (HCN) was a common intermediate in the synthesis of many of the nitrogen containing simple organic compounds. Hydrogen cyanide itself is easily synthesized by the by the following type reactions (114).

$$2\ CH_4 + N_2 \longrightarrow 2\ HCN + 3\ H_2$$

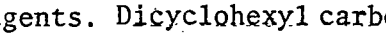
$$CO + NH_3 \longrightarrow HCN + H_2O$$

In experiments that simulated primitive earth conditions, it is believed that over fifty different organic compounds (12) could have been synthesized starting with HCN (114):



{from Lehninger, 1970 (114)}

Once the simple organic molecules were abiotically synthesized, and were dissolved in the primitive ocean, then,by condensation reactions involving both chemical and physical agents, H_2O was removed from monomeric compounds, forming polymers. Polymers of nucleotides, sugars, amino acids and fatty acids were thus produced.

It has been postulated that the dehydration reaction forming the biopolymers occured either under near anhydrous conditions and/or with the aid of chemical condensing agents. Dicyclohexyl carbodiimide () is an example of a chemical condensing agent, which is used to condense amino acids into polypeptides. There are some other carbodiimide derivatives which catalyze the formation of phosphate and acetate esters from alcohols, of polyphosphates from orthophosphate, and of ADP from AMP. Inorganic phosphate condensation into polymers also can occur on the surface of certain minerals such as clay and apatite. Polyphosphates or their esters, in the presence of heat, catalyze the condensation of amino acids into peptides, of mononucleotides into polynucleotides, and of glucose into glucose polymers (114).

All of the above mentioned condensing reactions have been carried out under simulated conditions postulated for primitive earth.

Many of the most interesting and eventful experiments in the field of chemical evolution and the origin of life have been performed in the laboratory of Fox. Fox and his co-workers have synthesized small nonrandom peptides which they call proteinoids. The proteinoids were formed by heating amino acid mixtures in the presence of polyphosphates at 50 to 60 C for long periods of time. Acidic, neutral and basic proteinoids have been synthesized in this manner. Heme proteinoids have been prepared by mixing heme with the amino acid mixture before heating (114). These thermally

formed proteinoids have been found to contain limited cayalytic activity. Hydrolysis of esters, decarboxylation of α-keto acids and various deamination reactions have been "rate-enhanced" with some of these proteinoids (55, 66, 82, 153). Zinc-containing proteinoids have been found to have limited ATP-ase activity (55). Dose and Zaki have also found that hemoproteinoids have peroxidase activity (55). The hydrolytic reaction using *p*-nitrophenyl acetate as substrate and a particular proteinoid as the catalyst, shows an optimum pH, exhibits Michaelis-Menten kinetics, is destroyed by heat, and has a histidine containing active site (152).

The synthesis of purine and pyrimidine bases, nucleosides and nucleotides under simulated primitive earth experiments has been well documented in several reviews (146). The template systems characteristic of life forms today could have been formed prebiotically. A given polynucleotide chain can act as a template which, through hydrogen bonds, can order in free complementary mononucleotides. Through the action of condensing agents the free mononucleotides can be covalently linked to one another forming a double helix of polynucleotides that are held together by hydrogen bonds, as is the case with DNA in contemporary life forms. Orgel has demonstrated template-directed reactions of mononucleotides using polyuridylic acid and AMP mononucleotide units (184).

The first forms of life must have arisen through the complex association of the prebiotically formed polymers (68, 134). The specific manner in which the biopolymers associated to form a unit of "life" has been, and still remains a mystery and a highly debated subject.

Oparin has proposed a theory based on the physical phenomenon called coacervation. This theory is commonly referred to as the protobiont theory (135). This theory states that the first unit of life could have arisen

from association of a group of proteinoids or other polymers into a highly concentrated coacervate droplet (135). Among the polymers in this coacervate droplet were various catalytic proteinoids able to conduct only one or possibly a few metabolic reactions. Oparin and his co-workers were able to synthesize coacervate droplets and, when, the appropriate enzymes were present, these droplets could incorporate enzymes whose particular metabolic activity could be demonstrated (135). When coacervate droplets containing glycogen phosphorylase and amylase were synthesized, maltose could be derived from glucose-1-phosphate (135). A coacervate droplet containing NADH dehydrogenase accepts reducing power in the form of NADH, and transfers the electrons from NADH to a dye which is released in the reduced form (135). A chlorophyll containing coacervate droplet has been shown to transfer electrons from ascorbic acid via chlorophyll to a dye, upon illumination.

An alternate theory on the prototype of the first unit of life has been proposed by Fox (69). Fox and his co-workers have demonstrated spontaneous formation of proteinoid microspheres when thermally formed proteinoids are allowed to cool under the correct conditions of controlled pH and ionic strength (64). These proteinoid microspheres are approximately 2.0 μM in diameter; and, under the appropriate conditions their boundary has double-layer structure (64). The microspheres are stable and have a semipermeable membrane surrounding an inner compartment of salts and various proteinoids (64). The membrane contains no lipid.

The proteinoid microspheres can be induced to divide by cleavage when exposed to a given pH or when exposed to $MgCl_2$ (66). Microspheres also demonstrate a budding phenomenon (67). The buds can separate from the parent microsphere, and the buds can then be demonstrated to grow (67).

A third hypothesis, originally stated by Muller in 1929, and developed more extensively later by Crick and Orgel, states that the primitive life form was based primarily on nucleotide chemistry rather than proteinoid chemistry (136). The basis of this theory is that the gene preceded the proteinoid; and the evidence supporting the theory is the presence of viruses and their mode of self-replication and molecular organization.

It is obvious that the precise nature of the first form of life is unknown, and is the subject of much speculation. The relation of the prebiotic life forms to the most primitive bacteria is completely unknown and remains only speculative.

The object of this review is to deal with the evolution of bacteria and not orgin of life. This paper will concern itself with the proposal of a scheme of bacterial evolution based on physiological principles. It assumes that bacteria did originate from some unknown source and once found on primitive earth they evolved into other physiological types, based on environmental and chemical factors that arose from prebiotic chemical evolution.

Phase I

Possible Pre-Glycolytic Pathways

From a metabolic viewpoint, the earliest bacteria were probably precambrian cells that were anaerobic heterotrophs (118, 134, 135). Among contemporary bacteria, glycolysis is thought to be both a ubiquitous and primitive process. In fact, it has been assumed by others to be the most ancient metabolic process by which a cell obtains energy (56, 134, 135).

However, a number of bacteria contain enzyme systems which can be considered to be more primitive than those containing a solely glycolytic fermentative pathway. These would obtain energy from anaerobic redox reactions involving simple organic compounds less complex than glucose (11, 46, 137). These bacteria contain two or three step bioenergetic reaction processes, which do not require preliminary phosphorylation of the substrate at the expense of ATP (137).

H. A. Barker was the first investigator to list the metabolically peculiar reactions among such anaerobic bacteria, particularly those of the *Clostridium* species (11). DeLey later divided *Clostridium* into four fermentative groups: (i) the proteolytic types; (ii) the purinolytic types; (iii) the saccharolytic types—which carry out glycolysis; and (iv) the incompletely saccharolytic clostridia (46). The proteolytic *Clostridium* are those which hydrolyze proteins and decompose the amino acids in order to obtain energy. Most of these species are unable to ferment carbohydrates (11, 46). Two well studied examples are *C. tetani* and *C. histolyticum* (11, 46). Species of the anaerobic genus *Peptococcus* also use proteins as their sole energy source (29). The common feature in all of these bacteria is that they contain enzymes which catabolize amino acids. There are at least

fifteen species of *Clostridium* which carry out the fermentation of pairs of amino acids by a process known as the Stickland reaction (131). In the Stickland reaction one amino acid serves as the hydrogen donor and another as the hydrogen acceptor and energy is obtained via ATP synthesis. A typical reaction sequence for the Stickland reaction, involving alanine and glycine, is as follows (142):

$$\text{alanine} + NAD^+ \longrightarrow \text{pyruvate} + NADH + NH_3$$

$$\text{pyruvate} + NAD^+ + CoA \longrightarrow \text{acetyl-CoA} + CO_2 + NADH$$

$$\text{acetyl-CoA} + \text{phosphate} + ADP \longrightarrow \text{acetate} + ATP + CoA$$

$$2\ \text{glycine} + 2\ NADH + 2\ ADP \longrightarrow 2\ \text{acetate} + 2\ NAD + 2\ ATP + 2\ NH_3$$

$$\text{Sum: alanine} + 2\ \text{glycine} + 3\ ADP \longrightarrow 3\ \text{acetate} + 3\ NH_3 + 3\ ATP + CO_2$$

There are, in addition, several *Clostridium* species that are able to ferment single amino acids from which energy can be obtained (11). *Clostridium tetanomorphus* ferments either glutamate or histidine (11, 46). The overall fermentation equation for glutamate in *C. tetanomorphus* is (45):

$$5\ \text{glutamate} + 6\ H_2O \longrightarrow 6\ \text{acetate} + 2\ \text{butyrate} + 5\ CO_2 + 5\ NH_3 + H_2$$

C. propionicum ferments either alanine or threonine as follows:

$$3\ \text{alanine} + 2\ H_2O \longrightarrow 2\ \text{propionate} + \text{acetate} + 3\ NH_3 + CO_2$$

or in the case of threonine:

$$3\ \text{threonine} + 2\ H_2O \longrightarrow 2\ \text{propionate} + \text{butyrate} + 3\ NH_3 + 2\ CO_2$$

C. propionicum lacks a glycolytic scheme (11, 137) and these fermentations are two of the major energy producing mechanisms in this species (137).

Thus, some proteolytic bacteria obtain energy from reaction mechanisms

that could very well have been pre-glycolytic. Pantskhava has compiled a list of possible pre-glycolytic energy acquiring reactions that appear in some strict anaerobes (137). Other reaction mechanisms that Pantskhava considers as pre-glycolytic are (137):

(a) fermentation of an ethyl alcohol—acetic acid system by *C. kluyveri* {De Ley classifies this reaction system as an incompletely saccharolytic type as found in some *Clostridium*(46)}

(b) fermentation of lactic acid and pyruvic acid by *C. propionicum*

(c) fermentation of ethyl alcohol by *Methanobacillus omelianskii* (strain S) forming acetate and hydrogen.

(d) fermentation of purines {De Ley classifies these as the purinolytic bacteria (46)}.

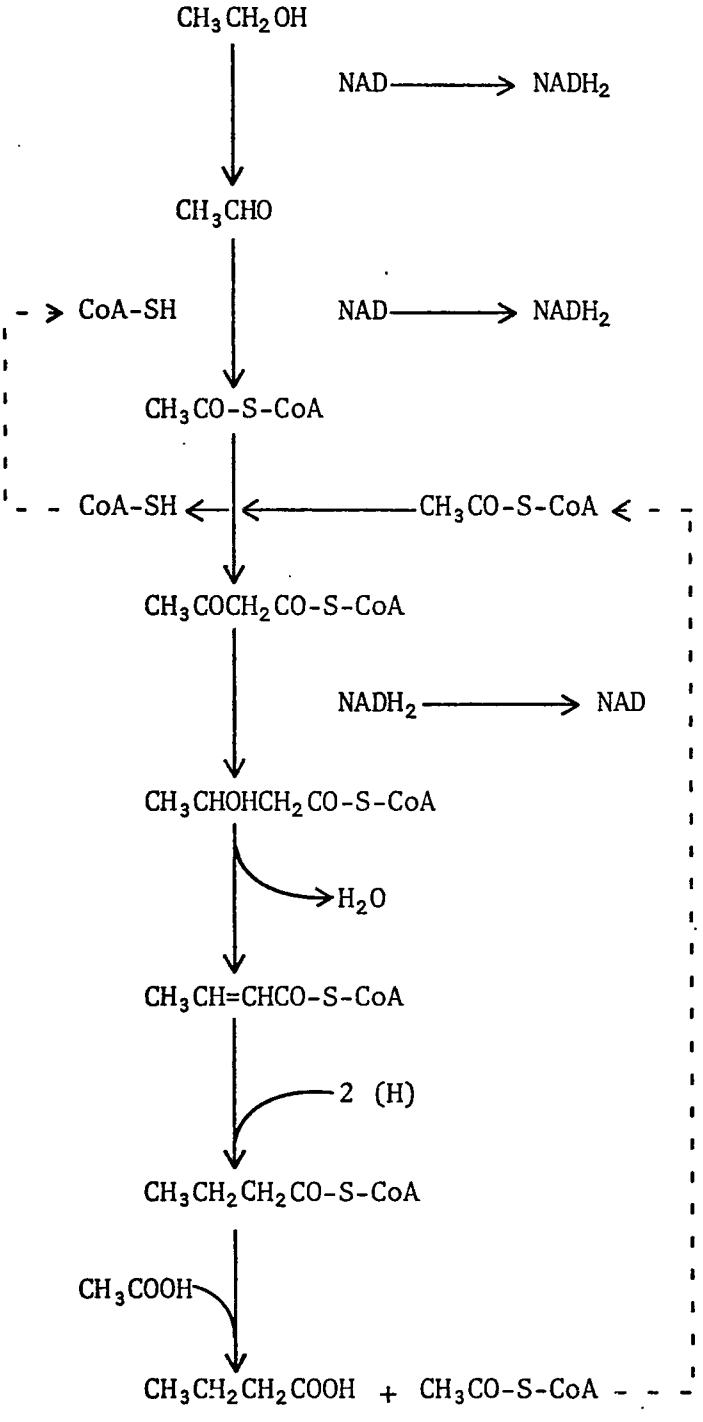
In *C. kluyveri* the principal mechanism providing the necessary energy is the fermentation of an ethanol and acetate mixutre to butyric acid (137). *C. kluyveri* is enzymically not equipped to ferment sugars or pyruvate (11, 46). Oxidation of ethyl alcohol, via acetyl coenzyme A produces energy rich thioether bonds which contribute indirectly to the formation of ATP (137). The mechanism of the fermentation of ethanol and acetate by *C. kluyveri* is shown on the following page.

Shuster and Gunsalus have shown that, in *C. kluyveri*, reduction of crotonyl—CoA by molecular hydrogen (H_2) or $NADH_2$ is coupled with the phosphorylation of ADP to ATP, as shown below (165):

$$\text{crotonyl—CoA} + H_2\ (NADH + H^+) + ADP + Pi \longrightarrow \text{butyryl—CoA} + ATP + NAD^+$$

In *C. kluyveri*, the enzyme catalyzing this reaction, butyryl—CoA dehydrogenase, is known to be a flavoprotein enzyme (54). Thus, this reaction conforms to a coupled phosphorylation reaction which involves a flavoprotein (54). It is important to note the appearance of ATP synthesis via a phosphorylation reaction other than substrate level one.

Mechanism of Fermentation of Ethanol and Acetate by *C. kluyveri*



Such reactions, which are present in these primitive bacteria, represent the type from which the more complex electron transport phosphorylation reactions evolved.

Burton and Stadtman demostrated that *C. kluyveri* has another enzyme system that will synthesize ATP at the expense of acetaldehyde oxidation with formation of acetate (31). The reaction sequence proceeds as follows:

(a) acetaldehyde + NAD + CoA $\longrightarrow$ acetyl—CoA + $NADH_2$

(b) acetyl—CoA + Pi $\longrightarrow$ acetylphosphate + CoA

(c) acetylphosphate + Pi + ADP $\longrightarrow$ acetate + ATP

Therefore, there are at least two processes resulting in the formation of ATP in *C. kluyveri* that are not strictly speaking substrate level phosphorylations.

Another class of possible pre-glycolytic pathways is fermentation of ethyl alcohol forming acetate and hydrogen. This reaction is found in *M. omelianskii* (strain S) (26). Ferredoxin and NAD participate directly in this reaction (22), while thioethers do not (137):

ethanol + X $\longrightarrow$ acetaldehyde + H_2X

acetaldehyde + Fd_{ox} $\longrightarrow$ Fd_{red} + acetate

Fd_{red} + NAD $\longrightarrow$ Fd_{ox} + $NADH_2$

$NADH_2$ + ADP + Pi $\longrightarrow$ ATP + NAD^+ + AH_2

The above process is, of course, an alternate source of ATP and does not involve the acetyl— CoA intermediate as does the process in *C. kluyveri*.

The fermentations of lactic acid and pyruvic acid by *C. propionicum* are other energy yielding mechanisms which Pantskhava has suggested as possible pre-glycolytic pathways (137). *C. propionicum* does not contain

a glycolytic scheme and must rely on the fermentation of lactate, pyruvate or alanine for energy production (137). The fermentation of alanine has already been discussed. Pantshkava has proposed a scheme for the generation of ATP from pyruvate (137):

pyruvic acid $\longrightarrow$ acetaldehyde + CO_2

acetaldehyde + CoA $\longrightarrow$ acetyl– CoA

acetyl– CoA + Pi $\longrightarrow$ acetylphosphate + CoA

acetylphosphate + ADP $\longrightarrow$ ATP + acetate

In lactate fermentation, lactate is converted into pyruvate by lactate dehydrogenase (199). Pyruvate is further degraded by the set of reactions just outlined above (137).

Another class of possible pre-glycolytic reactions in anaerobic bacteria, which do not ferment carbohydrates is the fermentation of heterocyclic compounds (12, 46, 137). These heterocyclic compounds were probably available in greater concentrations than the more oxidized carbohydrates, during this early biological era. It is interesting to note that some of the comtemporary representatives of this fermentative class of bacteria have survived by inhabiting the highly anaerobic bovine rumen (94). Both De Ley and Pantskhava have discussed aspects of the fermentation of purines among the *Clostridium* (46,137). The anaerobic dissimilation of heterocyclic compounds (purines and pyrimidines) can also be considered as an example of an energy generating reactions found in pre-glycolytic bacteria.

Purines are fermented by *C. acidiurici*, *Peptococcus aerogenes* (*Micrococcus aerogenes*), *P. asaccharolyticus*, *P. activus*, and *Veillonella alcalesens* (*M. lactilyticus*) (12, 29, 46, 137). Two species, *C. cylindrosporum* *C. uracilicum*, also have been reported to ferment purines (10, 32), but the

latter two organisms are not classified in the latest (8th) edition of *Bergey's Manual of Determinative Bacteriology* (29). *C. acidiurici* does not ferment carbohydrates or amino acids, and requires uric acid (or other purines) as a source of carbon and energy (29). All three species of the genus *Peptococcus* which ferment purines are additionally able to ferment a few amino acids (29).

Pyrimidine fermentation has been reported in two anaerobic bacteria, *C. oroticum* (*Zymobacterium oroticum*) and *P. aerogenes* (*M. aerogenes*) (12, 29). Of these two bacteria, only *C. oroticum* utilizes a pyrimidine as a major energy source (12).

The enzymic steps in the fermentation of xanthine have been investigated using cell-free extracts of *C. acidiurici* (21) and *C. cylindrosporum* (150, 151). In *C. cylindrosporum*, xanthine fermentation is believed to occur through ten successive reaction steps also resulting in ATP synthesis (12, 137, 150, 151):

xanthine ⟶ 4-ureido-5-imidazolecarboxylic acid ⟶

4-amino-5-imidazolecarboxylic acid ⟶ 4-aminoimidazole + NH_3 ⟶

4-imidazolone ⟶ formiminoglycine + THF* ⟶

glycine + 5-formimino– THF ⟶ 5,10-methenyl– THF ⟶

10-formyl– THF + ADP + Pi ⟶ ATP + HCOOH + THF

*THF = tetrahydrofolate

Thus, it appears that in some anaerobic bacteria, energy (ATP) is generated by processes which are "less complex" than glycolysis. In some of these organisms a glycolytic pathway is absent, and, therefore, these proposed pre-glycolytic reactions are the only energy generating mechanisms available to the bacterium. The substrates which are fermented are

simpler organic compounds, and are less oxidized than the hexoses which are fermented by the glycolytic or the equivalent type system. These proposed pre-glycolytic reactions require compounds such as NAD, coenzyme A, ferredoxin, and TPP (137). It is noteworthy that all of these compounds are products of molecular (pre-biotic) and early biotic evolution, and all belong to the most ancient biochemical catalyzers (137). Hydrogenases, pyridine nucleotide reductases, and ferredoxin would also be present and functional in bacteria having pre-glycolytic pathways (192).

Pantskhava (137) has listed the reactions which are common to both glycolysis and to the proposed pre-glycolytic reactions:

(a) decarboxylation of pyruvate

(b) formation of acetly– CoA or acetaldehyde

(c) oxidation of acetyl– CoA or acetaldehyde to acetate with formation of ATP

The study of possible pre-glycolytic energy yielding processes as outlined above, is an attempt to describe a group of reactions that could represent the first type of fermentation reaction that would have been found in the earliest anaerobic bacteria on earth. Organisms having a glycolytic pathway would have evolved next and glycolysis is viewed as the end product of fermentative evolution by Pantskhava (137). Glycolysis might have been preceded by a more simpler type of fermentation, one that did not require preliminary energization (phosphorylation) of the substrate at the expense of ATP (the hexokinase reaction).

In any case, these pre-glycolytic reactions are fermentative energy-yielding processes which require "more simpler" substrates than hexoses, and their conversion to other end products only requires a relatively few reaction steps.

The Methanogenic Bacteria

The methanogenic bacteria are a group of obligately anaerobic organisms, Gram variable, which reduce CO_2 to methane (CH_4). These bacteria do not ferment carbohydrates, lactate, or amino acids. The "methane" bacteria are divided into four genera based on morphology; and, their universal substrates are $H_2 + CO_2$ (181). Some of these bacteria are able to utilize formate, and one species utilizes methanol and acetate as growth substrates (181). The table below lists the four genera of methanogenic bacteria and their respective growth substrates (181):

Genus	Micromorphology	Motility	Substrates for Methane Formation
Methanobacterium (4 species)	rods, sometimes curved	variable	$H_2 + CO_2$; formate in most species
Methanococcus (2 species)	cocci	variable	$H_2 + CO_2$; formate
Methanosarcina (1 species)	cocci in cubical packets	absent	$H_2 + CO_2$; formate; acetate
Methanospirillum (1 species)	helical	polar flagella	$H_2 + CO_2$; formate

Most of the biochemical studies on methane bacteria have been conducted on only one species, *Methanobacillus omelianskii* (strain H). Originally, these bacteria were thought to be the anaerobic counterparts of the strictly aerobic hydrogen bacteria which can only use O_2 as the terminal electron acceptor, whereas the strictly anaerobic methane bacteria can only use CO_2 as the ultimate acceptor of reducing power. However, closer analysis has revealed that the methanogenic bacteria do not assimilate CO_2 through the Calvin cycle, and they apparently do <u>not</u> possess a cytochrome-containing

electron transport chain (181). Many aspects of the biochemistry and physiology of the methanogenic bacteria remain obscure, and it is only recently that microbiologists have begun to classify these bacteria into one physiological group. Because so little is known about the mechanism of ATP synthesis in methane bacteria, it is very difficult to place these organisms into an evolutionary niche. Hall (80) included these bacteria among the anaerobic respirers, suggesting that CO_2 was the terminal electron acceptor in a respiratory chain analogous to the sulfate and nitrate respirers. In light of the information presented above, this proposal seems untenable. It seems more likely that this group of bacteria belong to the class of organisms having the pre-glycolytic energy yielding pathways (43).

The basic physiological characteristics of the methanogenic bacteria which suggest that they possess the pre-glycolytic type metabolism are (181):

(a) strictly anaerobic metabolism

(b) lack of a carbohydrate fermentation mechanism

(c) use of simple organic and inorganic compounds as growth substrates

(d) although CO_2 is reduced to CH_4, this process does not require a complex type of electron transport system as one would expect of a CO_2 respirer

Development of Glycolysis and Related Pathways

The next catabolic reaction mechanism to evolve most probably would have been the glycolytic type pathway (24, 80, 114). The glycolytic scheme or the Embden– Meyerhof– Parnas pathway, the Entner– Doudoroff pathway, and the pentose phosphate pathway (hexose monophosphate shunt), also referred to as the Warburg– Dickens– Lipmann scheme, are the three basic

reaction pathways by which bacteria oxidize sugars to pyruvate. These three pathways are common to both respiratory and fermentative bacteria (181), and the enzymes which carry out these interconversions are completely soluble. The fermentation of sugars by any of the above pathways is considered to be relatively inefficient, the energy yielding capability being low.

Glycolysis {the Embden– Meyerhof– Parnas (or EMP) scheme} is the major energy generating pathway for many anaerobic fermenters. The glycolytic pathway is used by some of the saccharolytic *Clostridium* species, which ferment sugars to acetic and butyric acids (butyric acid bacteria); the lactic acid bacteria, which either ferment carbohydrates exclusively to lactate (homofermenters), or to lactate, ethanol, and CO_2 (heterofermenters); by some species of *Peptococcus* and *Ruminococcus*. The lactic acid bacteria are basically aerotolerant, indicating that they may have concomitantly evolved mechanisms to detoxify oxygen e.g., possessing the superoxide dismutase, enabling them to grow even in the presence of air. However, the lactic acid bacteria (unlike *Propionibacteria* which synthesize multiple a- and b-type cytochromes and are also aerotolerant fermenters) do not synthesize any cytochrome components (181). Aspects of the origin and evolution of the *Propionibacteria* will be discussed in a later section in this review.

The metabolic end-products of the EMP pathway are pyruvate, ATP and NADH. Once the glycolytic pathway evolved, the development of reaction mechanisms for the further oxidation of pyruvate became necessary. The development of an enzyme system which converts pyruvate to acetyl-CoA was a critical step in biochemical evolution, since acetyl-CoA is the substrate for both the glyoxylate cycle and the Krebs cycle.

Development of the Glyoxylate and the Krebs Cycle

As stated by Klein and Cronquist (104), it is very difficult to determine whether the glyoxylate cycle and the Krebs cycle arose simultaneously or sequentially. The glyoxylate cycle is present in many *Clostridium* species which do no contain an intact Krebs cycle. The glyoxylate cycle is also present in other "primitive type" bacteria that do not contain a complete Krebs cycle (104). Thus, Klein and Cronquist speculate that the glyoxylate cycle probably evolved first prior to the Krebs cycle (104). Horowitz gives a scheme of enzyme evolution (85), which notes that component enzymes of the glyoxylate cycle also are present in the Krebs cycle.

Krebs cycle {tricarboxylic acid (TCA) cycle}, which generates NADH, at the expense of pyruvate decarboxylation, also generates certain intermediates which are required in biosynthetic pathways (181). Therefore, most bacteria, including the most obligate anaerobes, possess some of the enzymes of this cycle (181). The only step lacking in some anaerobes (*Clostridium* species), which do not contain the intact cyclic process, is the conversion of α-ketoglutaric acid to succinic acid (181), which is carried out by α-ketoglutarte dehydrogenase. The appearance of the glyoxylate and Krebs cycles also now will allow for the development of some sort of primitive electron transport system which can trap and conserve the energy available from the oxidation of NAD(P)H which can be generated in large amounts, particularly by the Krebs cycle.

Anaerobic Electron Transport Systems Utilizing Organic Compounds as Terminal Electron Acceptors

It is now known that all organisms, even those considered to be primitive types,do carry out transport reactions that involve two or possibly three

redox type components that were undoubtedly present in the earliest anaerobic chemoorganotrophic bacteria (80). These primitive electron transport systems would have as active components, ferredoxin(s), flavoproteins, possibly non-heme iron, and in the more advanced organisms, cytochromes. The electron donor naturally was $NADH_2$ (or $NAD(P)H_2$), and the earliest electron acceptors would have been simple organic compounds, an example of which would be fumarate.

These primitive electron transport reactions would have carried out the redox reactions that resulted in the direct phosphorylation of ADP (to ATP) coupled to electron transport. It is still unknown when this process of electron transport linked phosphorylation first appeared in the evolution of bacteria. A number of anaerobic bacteria appear to have the capability of obtaining energy (synthesizing ATP) through the process of electron transport linked phosphorylation (80).

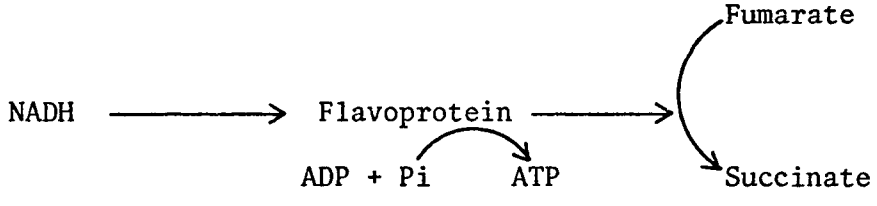
As in the case with aerobic electron transfer systems, the first reaction for this type of anaerobic phosphorylation reaction would be the electron transfer from $NADH+H^+$ to flavoprotein (114), and the ranges for the oxidation reduction potentials would have been within that proposed for all "biological electron transport", i.e., from the hydrogen electrode to the oxygen electrode (9).

With regard to specific examples, Barker in 1956, described the probable generation of ATP by an electron transport reaction involving the oxidation of NADH by flavoprotein in *C. kluyveri* (11). Barker proposed that NADH reduced flavoprotein and the flavoprotein ultimately reduced crotonyl-CoA; somewhere in the electron transport process ATP was generated. Stadtman showed that the free energy change for this reaction (-18.0 kcal/mol) was sufficient to drive phosphorylation of ADP to ATP (175). Recent studies

have demonstrated, however, that the high ATP levels in *C. kluyveri* can be accounted for only by the substrate-level phosphorylation reactions (187).

The reductive deamination of glycine by *C. sticklandii* (177) and *C. lentoputrescens* (176), is perhaps another example of a possible phosphorylation reaction coupled to electron transport. In this reductive deamination reaction, glycine is converted to acetate and ammonia, with NADH serving as the electron donor. Acetylphosphate has been ruled out as a possible intermediate, and the one ATP produced in this reaction must be from some coupled reaction, most likely an electron transport reaction (176, 177).

Another interesting example of a "primitive" electron transport linked phosphorylation reaction appears in *Streptococcus faecalis* strain 10 C1. This particular species (as all streptococci) lacks cytochromes. Cytochromes are not found in any of the anaerobic bacteria discussed so far, which are capable of carrying out the "primitive" type electron transport linked phosphorylation reaction. Studies have shown that *S. faecalis* also contains a cytochrome-independent electron transport system which is localized in the soluble fraction of the cell (45, 60). The electron transport reaction carried out is mediated by a flavoprotein enzyme complex and fumarate serves as the terminal electron acceptor (45, 60, 89). The following represents the reaction sequence for the "primitive" electron transport system in *S. faecalis*:



The above reaction showing that phosphorylation, coupled to NADH oxidation via a soluble flavoprotein-containing electron transport system with an organic compound like fumarate serving as the terminal electron acceptor, is

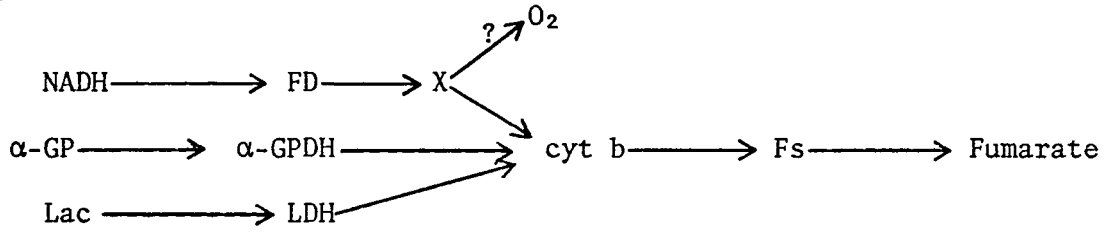
found in some fermentative bacteria (80). This same reaction can also be carried out by membrane bound enzymes in bacteria and thus, this fumarate reductase reaction represents the first electron transport system that probably evolved to the contemporary aerobic type now commonly found for most aerobic bacteria. Hall suggests that this primitive type electron transport reaction could not have been utilized to any great extent due to the shortage of suitable organic electron acceptors like fumarate (80).

Although it has long been assumed that *most* anaerobic bacteria of the Order *Eubacteriales* do not contain cytochromes (this was particularly true for *Streptococcus* and *Clostridium* species), evidence to the contrary has recently been published. Cytochrome b , and sometimes cytochromes of the a, d, or o types recently have been observed in some Eubacteria or true bacteria (120). The presence of these cytochromes is, in most cases, dependent on the presence of exogenous heme in the growth medium (120).

A strict anaerobe, *Eubacterium lentum*, recently has been shown to contain cytochromes of the a, b, c and o types. Cytochromes b and c were reduced by formate and reoxidized by nitrate, and cytochrome a was partially oxidized by nitrate (174). The significance of this finding will be discussed later in this review.

Although no cytochromes are believed to be directly involved in the primitive electron transport reactions of *S. faecalis*, *C. kluyveri*, *C. sticklandii* and *C. lentoputresens*, there is evidence that cytochromes can be utilized by some organisms with fumarate serving as the terminal electron acceptor (50, 173, 195). Species of propionic acid bacteria, a group of anaerobic—aerotolerant fermenters,contain cytochromes b, a, d, and a carbon monoxide-binding pigment (possibly an o-type cytochrome)(50). Experiments with HOQNO, a known inhibitor of bacterial cytochrome b function

have indicated that in four species of propionic acid bacteria (*P. freudenreichii*, *P. shermanii*, *P. rubrum*, and *P. pentosaceum*) cytochrome b functions directly in anaerobic electron transport reactions involving the oxidation of lactate by fumarate (50). In a study on the oxidoreductases found in *P. arabinosum*, membrane fractions were obtained which contained NADH, α-glycerol phosphate and lactate dehydrogenases, a succinate dehydrogenase or a fumarate reductase-type enzyme, a b-type cytochrome as well as flavoprotein and non-heme iron components (173). All of these components are undoubtedly associated with a type of an electron transfer system where the succinate degydrogenase probably acts as fumarate reductase, while the NADH, lactate and α-glycerol phosphate oxidations are coupled to fumarate reduction. These observations, together with the studies on the effect of various electron inhibitors, suggest that the following type of electron transport pathway is functional in *P. arabinosum* (173):



FD = NADH dehydrogenase
Fs = succinate dehydrogenase (or fumarate reductase)
LDH = lactate dehydrogenase
α-GPDH = α-glycerophosphate dehydrogenase

In some ways the above scheme resembles those reported for many aerobic bacterial respiratory chains, although it lacks the multiple cytochrome components which link the electron transport system to oxygen (173). Spectrophotometric analysis suggests that the presence of cytochrome a or o is questionable in the membranes of *P. arabinosum*. Although, the propionic acid bacteria do unquestionably contain the anaerobic respiratory systems

with fumarate serving as the terminal electron acceptor, their physiological behavior and biochemical characteristics indicate that they are better adapted to aerobic life than are most other fermentative bacteria (173). For example, Sone has demonstrated active oxygen uptake in whole cells of *P. arabinosum*. However, this oxygen uptake is not sensitive to cyanide and azide suggesting that the usual type of terminal cytochrome oxidase is not involved in this reaction (173). A fumarate respiration system, similar to the one just discussed in *Propionibacterium*, is also operative in *B. ruminicola* (195). In both of the above cases hemin was added to the media as a growth requirement (173, 195).

Other recent studies indicate that many other anaerobic heterotrophs can synthesize cytochrome components when hemin is added to the growth media (51, 75, 90, 116). *Bacteroides fragilis* var. *fragilis* is a strict anaerobic heterotroph that when grown in the absence of hemin, does not synthesize the cytochrome components of the fumarate reductase enzyme complex. In the absence of hemin, this bacterium ferments glucose to fumarate, lactate, and acetate (116). In the presence of hemin, *B. fragilis* synthesizes both b-type and c-type cytochromes, as well as a very active fumarate reductase; glucose is now fermented to succinate, propionate, and acetate (116). The generation time for *B. fragilis*, in the presence of heme, was reduced from 8 to 2 hours; the molar growth yield increasing proportionately (116). Macy, *et al.* conclude that the enormous increase in the growth yield observed may have occurred because of the increased ATP production resulting directly from the reduction of fumarate to succinate via the anaerobic electron transport system (116). Thereby an inducible electron transport chain involving cytochromes linked to phosphorylation of ADP to ATP is implicated (116).

The old assumption that all *Clostridium* spp. do *not* contain cytochromes

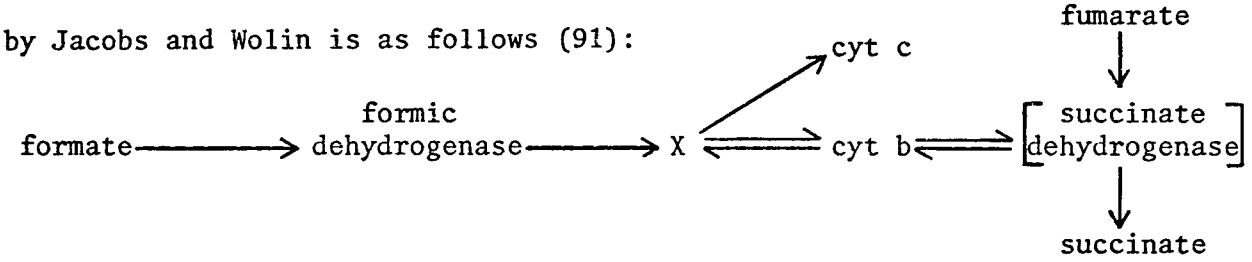
also is incorrect. A report of the presence of cytochromes in *Clostridium* has been published (75). Cytochrome b and menaquinone have been demonstrated as being present in the homoacetate fermentating *Clostridium formicoaceticum* and *C. thermoaceticum* (75). Fumarate reductase activity also is present in *C. formicoaceticum*. The presence of cytochrome b and the fumarate reductase enzyme complex suggests the presence of a primitive electron transport system in *C. formicoaceticum* (75).

C. thermoaceticum apparently does not exhibit fumarate reductase activity (75); the presence of cytochrome b and menaquinone in this organism is more difficult to explain. The presence of these two electron carriers may be rationalized by the suggestion that *C. thermoaceticum* may carry out some unknown electron transport phosphorylation step needed for CO_2 reduction to acetate (3).

Cytochrome b has also been discovered in other obligate anaerobes among which are *Selenomonas ruminantium*, *Anaerovibrio lipolytica* and *Veillonella alcalescens* (51). It has been established that in these bacterial species, cytochrome b functions in anaerobic electron transport systems in which fumarate acts as the terminal electron acceptor (51). In *S. ruminantium* and *V. alcalescens* cytochrome b is also involved in the anaerobic electron transport dependent reduction of nitrate (51). Small amounts of cytochrome a as well as a carbon monoxide binding pigment have also been observed in these three species (51). Electron transport linked phosphorylation is again implicated as the source of the increased amounts of ATP synthesized concomitant to the high molar growth yields obtained for these anaerobic microorganisms (51).

Cytochromes of the b and c type also were observed in *Vibrio succinogenes* cells grown with formate as an energy source, and either fumarate or nitrate serving as an electron acceptor (90). The electron transport scheme proposed

by Jacobs and Wolin is as follows (91):



Pyridine nucleotides are not involved in any of these reactions (90); and, the succinic dehydrogenase can be thought of as a functional fumarate reductase.

Enoch and Lester have recently reported isolating and characterizing the membrane bound formate dehydrogenase in anaerobically grown *E. coli* (58). The enzyme is a b-type cytochrome that is completely reduced by formate (58). The enzyme did *not* contain any FAD or FMN. The same procedure used to purify the formate dehydrogenase yields a nitrate reductase which also contained significant amounts of heme (57). It would be of interest to determine whether or not the formate dehydrogenase of *Vibrio succinogenes* also consists solely of a b-type cytochrome.

It can be summarizied that the anaerobic heterotrophs described herein, containing a primitive type of electron transport system, have the capability for the synthesis of components such as cytochrome b, flavoproteins and the fumarate reductases. The cytochromes of the c-type and a-types (the latter being looked upon as terminal oxidases) are very seldom found in such "primitive type" species. The appearance of a more "neutral" (less reduced) atmosphere and eventually an oxygenated one may have resulted in evolutionary changes in bacteria which allowed for the selection of those organisms synthesizing c and a-type cytochromes. These bacteria had the electron transport systems that were more advanced, bioenergetically efficient in ATP synthesis, and capable of using molecular O_2 as a terminal electron acceptor. Today the most highly evolved types would be considered those obligate aerobes having very active cytochrome oxidase activity. The

use of fumarate and to some extent NO_3^- as terminal electron acceptors is consistent with the concept that these anaerobic heterotrophic bacteria evolved from the primitive fermentative types at a time when organic compounds were becoming depleted on earth, and the atmosphere was changing from a highly reduced form to a "neutral" type.

The important conclusion of this section of the review is that electron transport linked phosphorylation probably appeared first in some of the more primitive bacteria. Most bacterial evolution studies assume that phosphorylation coupled to electron transport appeared somewhat later in evolution, usually after the development of photosynthetic bacteria. The early evolutionary appearance of electron transport-linked phosphorylation is really a prerequisite for the development of further physiological complexity. The photosynthetic bacteria, and to some extent, the nitrogen fixing organisms could not have successfully evolved until such anaerobic electron transport linked phosphorylation systems were fully evolved. Hall states (80):

> The significance of the invention of this first step in electron transport-linked phosphorylation lay more in the possibilities it opened for the future development than in the advantages it conferred on its immediate possessors.

Phase II

In Phase I of this bacterial evolution scheme, simple organic compounds were used both as substrates and as terminal electron acceptors and eventually the anaerobic primitive type electron transport systems evolved because it was more efficient bioenergetically. The atmosphere, when the first bacteria appeared, was very reduced, with the partial pressure of ammonia (NH_3) being such that at equilibrium, the ocean contained an ammonium ion (NH_4+) concentration of about 10^{-3}M (5). Urey has stated that the primitive atmosphere contained highly reduced forms of carbon and nitrogen, *i.e.*, methane (CH_4) and ammonia (NH_3) (191). The spectroscopic data on the heavy planets appears to substantiate this view (167).

Recently, however, new geochemical evidence suggests that because of the continual escape of hydrogen gas from the atmosphere, a "neutral" atmosphere replaced the reductive one (25, 38, 39, 121, 155). This neutral atmosphere, in contrast to the previous reductive type, would contain more carbon in the form of CO_2 (and CO) instead of methane (CH_4) and more nitrogen in the form of N_2 rather than ammonia (NH_3) (25, 39, 121, 155). The evidence given for the formation of a neutral atmosphere which replaced the reductive one, is listed below:

(a) volcanic gases are rich in N_2 and CO_2, NH_3 and CH_4 are absent (39).

(b) the gas phase of meteorites contains N_2 and CO_2, not NH_3 and CH_4 (155).

(c) CO_2, with very little CH_4, is released from pyrolized moon samples (1).

(d) photodissociation would reduce the NH_3 concentration in the primitive atmosphere (158).

(e) traces of oxygen (O_2) in the primitive atmosphere would oxidize the NH_3 and CH_4 present (103).

Thus, it is probable that most of the physiological processes which first appear in Phase II of this scheme, evolved in an atmosphere that was less reducing and more neutral, and it is at the end of this era that oxygen started to accumulate, being formed via oxygenic photosynthesis of the procaryotic blue-greens.

During the Phase II geochemical era, there was very little reduced nitrogen (NH_3) or carbon (CH_4) in the atmosphere. As a result of bacterial growth and proliferation in the primitive sea, there occurred a gradual depletion of organic carbon and nitrogen compounds on earth (114, 134, 146, 156). This was particularly true if other"lytic" types of bacteria had not yet evolved that were capable of hydrolyzing the biopolymers which represented the cells of all dead bacteria that had evolved to that point. The absence of simple organic compounds would have led to the evolution of bacteria capable of utilizing the less reduced inorganic nitrogen and carbon compounds like N_2 and CO_2. Thus, bacteria with nitrogen-fixing and photosynthetic capabilities first appeared. Photosynthesis and nitrogen fixation then, appear as specific metabolic processes which now can carry out the same synthetic reactions that had previously been performed abiotically during the natural phases of chemical evolution.

Obligately Anaerobic Sulfate and Nitrate Respiration

One of the physiological peculiarities of bacteria one can note for the second biogeochemical era is the utilization of inorganic electron acceptors rather than of organic acceptors. This process now allows bacteria to obtain more energy from their organic substrates; the end products formed are no longer at the same oxidation-reduction level (80). The atmosphere of this second biological era is still reducing; with no oxygen being present to serve

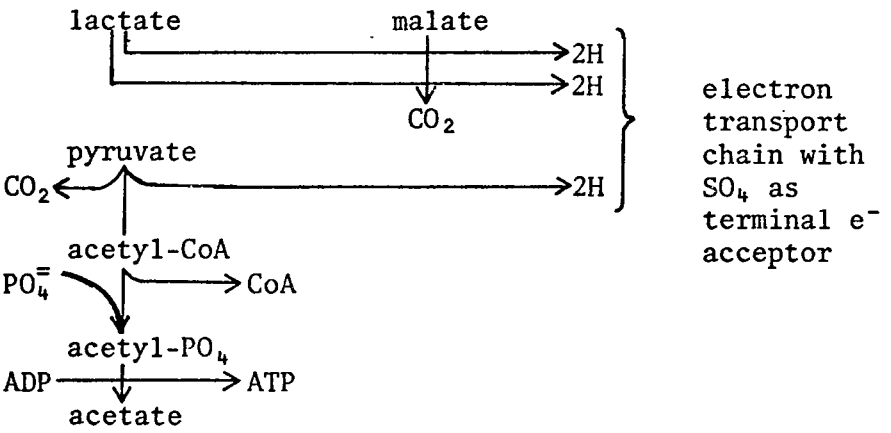
as an electron acceptor for either chemical or biological reactions. Organic compounds were still somewhat available for use as substrates, but inorganic molecules replaced the organic compounds as terminal electron acceptors.

Two major groups of bacteria exist today that are known to couple the oxidation of organic substances to the reduction of inorganic compounds, other than O_2: these are the sulfate and nitrate respirers (80).

The sulfate respirers use either sulfate, sulfite, bisulfite, trithionate, or thiosulfate as terminal electron acceptors (113). The nitrate respirers use either nitrate or nitrite as terminal electron acceptors (80). Contemporary sulfate respirers are strict anaerobes, while nitrate respirers are mostly facultative types. Some obligate anaerobes have been reported having a nitrate respiration mechanism (37, 100, 174). Although both sulfate and nitrate respirers contain a cytochrome-dependent electron transport system, there are fundamental differences in their physiology which suggest that they evolved along different pathways.

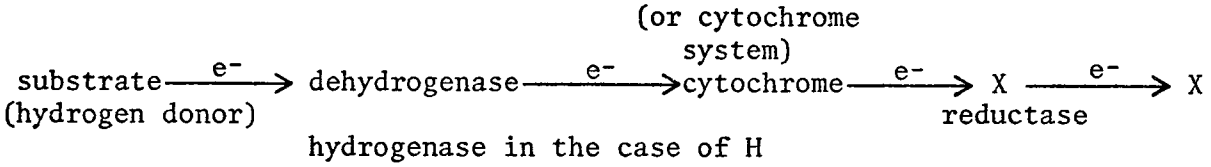
The Sulfate Respirers

The sulfate respirers do not ferment carbohydrates, but instead utilize lactate, malate, pyruvate and ethanol as growth substrates (181). The pathways of lactate and malate oxidation coupled to sulfate reduction are shown below (181):



These metabolic pathways are quite similar to those proposed pre-glyoclytic pathways present in many primitive *Clostridia* species. Therefore, it is probable that the sulfate respirers evolved along a nonfermentive pathway directly from organisms with pre-glycolytic pathways.

On the other hand, the nitrate respirers all appear to contain carbohydrate fermenting mechanisms and probably developed along the main glycolytic pathway. Anaerobic respiration can be schematically represented by the following general equation (56):



X = trithionate, thiosulfate, bisulfite, sulfate, sulfite, nitrate, or nitrite.

The sulfate respirers fall into two distinct genera. There are five species of *Desulfovibrio* and three species of the genera *Desulfotomaculum*. The species of *Desulfotomaculum* are spore formers and are very easily confused with *Clostridium* species (113). *Desulfovibrio* species are non-spore forming organisms containing cytochrome c_3, and a pigment desulphoviridin (113). The three spore forming species of *Desulfotomaculum* are rod shaped organisms which contain a b-type cytochrome and have no desulphoviridin (113). Some species of both genera can grow anaerobically in the absence of sulfate by fermenting compounds such as pyruvate, fumarate, or choline (113).

A variety of electron carriers have been isolated from the sulfate respirers. Multiple c-type cytochromes, b- and d-type cytochromes, non-heme iron proteins, flavoproteins and some quinones have all been implicated in sulfate respiration which is concomitant with sulfate reducing electron transport chains (113, 143). The roles of ferredoxin, flavodoxin, quinones, and rubredoxin in the electron transport chain of the sulfate respirers is as yet unknown (113). The amino acid sequence data of ferredoxins from various

species has allowed the construction of a phylogenetic tree for ferredoxins (143). This phylogenetic tree indicates that ferredoxins of *Desulfovibrio gigas* are more highly evolved than ferredoxins from various species of *Clostridium*(143). This data supports the placement of sulfate respirers in this era of bacterial evolution. The fact that the sulfate respirers have the lowest energy yield from their respiratory chain, coupled with the fact that the electron carriers are of very low redox potential, places the sulfate respirers among the most primitive organisms of Phase II (80, 104, 130, 161).

Sulfate respiration in *D. gigas* proceeds with ferredoxin as an electron carrier rather than a cytochrome (2). *D. vulgaris* has cytochrome c_3 as an electron carrier component. The electron carrier might have changed from ferredoxin to cytochrome c_3 in sulfate respiration during the evolution of the respiratory chain (199). There are several amino acid sequences common to both the cytochrome c_3 of *D. vulgaris* and the ferredoxin of *D. gigas* (199). The histidine-cysteine sequence seems to be necessary for a heme group to combine with a protein to form a cytochrome of the c-type (199). The histidine-cysteine sequence is absent in *D. gigas* ferredoxin (188). Thus, the appearance of a protein-heme complex, such as cytochrome c, had to await the evolution of an amino acid sequence where histidine is located next to cysteine.

The electrode potential of the cytochrome c_3 found in *D. desulfuricans* is -205 mV., which is one of the lowest electrode potentials ever recorded for a cytochrome (80). A cytochrome of such a low redox potential would be expected to be an electron carrier of a primitive anaerobic respirer, since the atmosphere of this era was still very reducing. A low redox level cytochrome is also consistent with recent theory and data concerning the evolution of the components of electron transport systems (9).

The sulfite reductase of *D. vulgaris* is a cytochrome with a prosthetic group which is a derivative of chlorin (111). Chlorin is a compound that resembles heme d in structure; which suggests that the sulfite reductase of *D. vulgaris* is a cytochrome of the d-type (199).

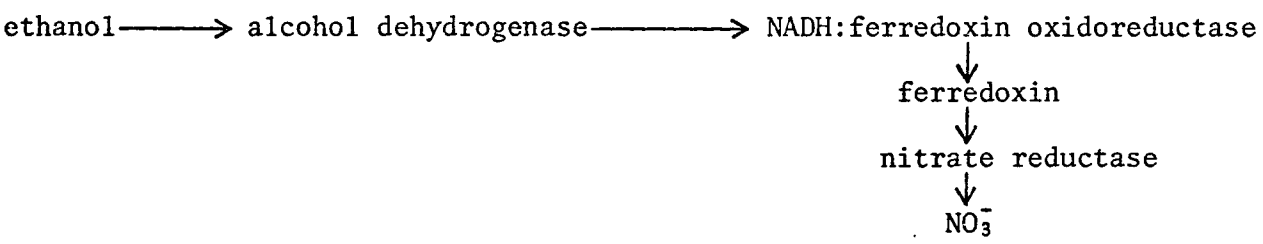
Sulfate is not in the free form when it is used as the terminal electron acceptor. The form in which sulfate is used as the terminal electron acceptor is the molecule adenosine 5'-phosphosulfate (APS) (139). The APS molecule is synthesized in the organism from ATP (adenosine triphosphate), and sulfate ($SO_4^{=}$) by the enzyme ATP sulfurylase (143). Extracts of *D. desulfuricans* reduce APS to AMP and sulfite in the presence of molecular hydrogen and an intact, naturally occurring electron transport chain (138). The enzyme catalyzing the reduction of APS is the APS reductase, an iron-containing flavoprotein, with a molecular weight of 220,000 (140).

The Nitrate Respirers

The inclusion of nitrate respiration as part of the metabolic processes of Phase II is very controversial. Broda asserts that nitrate respiration must have evolved rather late, because nitrate itself would not have been available until free oxygen appeared in the biosphere (24). Broda also argues that these nitrate respirers must have appeared late in bacterial evolution since they can also use oxygen respiration under aerobic conditions. The scheme for bacterial evolution that Broda proposes places nitrate and sulfate respirers after photosynthetic bacteria, i.e., in the oxygenic biochemical era (24). The fact that nitrate respirers have energy yields comparable to aerobic respirers also supports the theory that nitrate respiration appeared after oxygen became available in the atmosphere.

Evidence, however, also has accumulated which supports the placement of

nitrate respirers in early Phase II of this bacterial evolution scheme. It has been reported (37, 100) that the strict anaerobe *C. perfringens* reduces nitrate to nitrite, during the fermentation of glucose, ethanol, glycerol and some other substrates. Egami refers to the nitrate reduction of *C. perfringens* as "nitrate fermentation" (56); and the following scheme has been proposed as the electron transfer chain system for *C. perfringens* nitrate reduction (37):



The nitrate reductase in *C. perfringens* is an inducible enzyme (100). Hall has suggested that in *C. perfringens* nitrate reduction may be used only to reoxidize the reduced pyridine nucleotide for recycling during the fermentation reactions (80). With regard to this possibility, it would be interesting to establish whether or not *C. perfringens* is able to carry out an electron transport-linked phosphorylation reaction.

Recently, another report of an obligate anaerobe reducing nitrate has appeared (174). *Eubacterium lentum* contains cytochromes a, b, and c as well as an o-type, and cytochrome a being detected only when the cells were analyzed after growth in 50 mM nitrate. Cytochrome a appeared to be partially oxidized by nitrate and completely oxidized by air (174). When *E. lentum* was grown anaerobically either with or without nitrate, cytochromes b and c were reduced by formate and oxidized by nitrate (174).

These reports suggest that the early (or primitive) obligate anaerobes already had genes specific for nitrate reductases, and further suggests

that nitrate accumulated before the atmosphere became oxygenated. Hall concludes that ultraviolet light could excite reduced nitrogen compounds, arising from volcanic effluents, which would probably react with the little free oxygen that was available, to produce nitrogen oxides (81). The observation that many anaerobes contain assimilatory nitrate reduction processes is further evidence to support the view that nitrate was available in the Phase II biochemical era to serve as a metabolite for anaerobic nitrate respiration.

Therefore, two obligate anaerobes, *C. perfringens* and *E. lentum*, appear to carry out the process of nitrate respiration (dissimilatory nitrate reduction). Electron transport-linked phosphorylation, however, has not yet been demonstrated in either of these two species. In addition, some of the obligate anaerobic bacteria with a fumarate reducing electron transport system, i.e., *C. thermoaceticum, Selenomonas ruminantium, Anaerovibrio lipolytica, Veillonella alcalescens* also are capable of using nitrate as a terminal electron acceptor (see Phase I, page 32).

Since the energy yields from nitrate respiration approach those of aerobic metabolism, nitrate respiration appears to be a bioenergetically efficient process and undoubtedly was maintained from an evolutionary viewpoint. This might explain how nitrate respiration is now present and appears as a physiological peculiarity among many aerobes. On the other hand, sulfate respiration, in sharp contrast, is not as efficient and has remained a physiological process distributed exclusively among the obligate anaerobes.

ATP synthesis, however, in the case of both nitrate and sulfate reducers, involves the direct coupling of electron transport to the phosphorylation of ADP (80, 141, 142). The question as to whether or not the cytochrome linked phosphorylation of the nitrate and sulfate respirers has the same

type of reaction mechanism, as demonstrated for the flavoprotein-linked phosphorylation of Phase I, remains speculative, since very little is known about the mechanisms of either of these reactions (80).

The first demonstration of electron transport-linked phosphorylation among sulfate respirers was with a cell-free extract of *D. gigas* (141). ATP was produced during the reduction of sulfite by hydrogen (H_2). The phosphorylation was shown to be uncoupled by 2,4– dinitrophenol, pentachlorophenol and gramicidin.

Electron transport-linked phosphorylation also has been demonstrated in cell free extracts of *D. gigas* when fumarate is used as the terminal electron acceptor (14). The electron donor in this case can be either molecular hydrogen or L(+)-lactate (14). An ATPase enzyme has been demonstrated in soluble and particulate fractions of *D. gigas* (78).

Vosjan has demonstrated ATP generation by electron transport in *D. desulfuricans* (194). A phosphorylation yield of less than 0.5 was estimated for electron transport-linked phosphorylation in *D. desulfuricans* (194).

Electron transport-linked phosphorylation has not yet been observed or reported in the two obligately anaerobic species of bacteria that have been shown to be nitrate respirers.

Origin and Evolution of Nitrogen Fixation

The ability of organisms to fix molecular nitrogen (N_2) into organic nitrogen usually is regarded as a "primitive" or early metabolic process (25, 47, 114, 148). The fact that the early atmosphere contained ammonia, and that the most primitive oceans were rich in NH_4^+ (5), makes the appearance of the nitrogen fixation process unnecessary in primitive organisms (167).

However, it has become apparent that an atmosphere, chemically evolving from a highly reduced state (containing CH_4 and NH_3) to a "neutral" state (rich in N_2 and CO_2) (25, 38, 39, 121, 155), would be depleted of NH_3 and a selective pressure would be exerted for bacteria to evolve a mechanism to fix atmospheric nitrogen (N_2) (114). This neutral state would have occurred during Phase II (2-4 aeons ago) of the earth's geological history (167). The residual ammonia would have completely disappeared due to the growth and proliferation of bacteria in the ocean and all organic nitrogen was now in the form of stable biopolymers in dead cells.
Thus, the inavailability of simple reduced nitrogen compounds, in the primitive sea might have created the selective pressure that was needed for the nitrogen fixation process to be evolved and it can be viewed as being a necessary survival mechanism for bacteria, at this point in time.

The biochemical characteristics of organisms able to fix nitrogen are supportive of placing nitrogen fixation in this phase of bacterial evolution. Nitrogen fixation is distributed exclusively among procaryotic organisms and the enzymes needed for nitrogen reduction are of the most primitive type (148). Enzymes such as hydrogenase, ferredoxins, methane-producing and pyruvic phosphoroclastic systems are all associated with nitrogen fixation requirements (114, 148). The nitrogenase proteins are irreversibly inactivated by exposure to oxygen (30, 148). The nitrogen fixation process operates at low redox potential levels (114, 124), which is consistent with the reducing atmosphere of this geological era. Nitrogen fixation seems to be more predominant among anaerobic than aerobic heterotrophic bacteria (148). The evolution of physiological and morphological mechanisms that were required to protect the nitrogenase from oxygen, and the ultimate appearance of symbiotic systems of eucaryotic cells, are other indications

of the primitive nature of the nitrogen fixation process.

It should be emphasized that not all investigators agree that nitrogen fixation is a primitive process (148, 167). Silver and Postgate have pointed out that since the atmosphere would have contained at least some ammonia during this era, that the development of a nitrogen fixing system would be unnecessary and uneconomic (167).

A more recent development of nitrogen fixation is consistent with the cross-reactivity, and biochemical uniformity of the two component proteins of nitrogenase (148, 167). The nitrogenases from aerobic, anaerobic or symbiotic bacteria are remarkably similar in subunit weight, response to elution from a DEAE cellulose column, substrate specificity and metal content (167). Cross-reactivity (artifical combination of nitrogenase subunit of one species with the complementary subunit from another species to form an active nitrogenase) has been tested for over thirty combinations (48, 102, 148, 167, 171). These studies indicate a lack of evolutionary divergence among the component proteins of nitrogenase from various organisms (148), again arguing against its early appearance in primitive bacteria.

Also, because of the scattered distribution of nitrogen fixation among many genera of bacteria, especially *Klebsiellae*, the possibility exists that the nitrogen fixation gene cluster (*nif*) exists as a plasmid (148). This would again substantiate the proposal that nitrogen fixation appeared late in the evolution of bacteria, since it would be expected that the *nif* gene would have become stable and universal by this time (148).

It is obvious from the evidence presented that the problem of whether nitrogen fixation is a primitive process in bacteria or one that appeared recently remains unresolved. Silver and Postgate have suggested that compounds other than molecular nitrogen might have been the original

substrates for the nitrogenase system (167). The discovery in 1966 that nitrogenase can also catalyze the reduction of acetylene to ethylene (52, 160), led to the realization that many small, triply bonded molecules can be reduced by the nitrogenase system. It is curious that many of the molecules which act as substrates for the nitrogenase system are known biological poisons (*i.e.*, cyanides and cyanogen) (148, 167). Many of these toxic compounds were believed to be constituents of the primitive environment, and were likely to have interfered with metabolism of bacteria, probably reacting with non-heme iron enzyme systems (167). Thus, it appears that the original function of the nitrogenase system might well have been to detoxify many poisonous compounds that were harmful to bacteria (167), thus being a survival enzyme process. Some of the molecules that can be reduced by the nitrogenase enzyme complex (other than molecular nitrogen) are acetylene, hydrogen cyanide, acylonitrile, methyl isocyanide, vinyl isocyanide, cyanogen, hydrogen azide, nitrous oxide, hydrogen thiocyanate, methyl isothiocyanate, and hydrogen ion (148, 167).

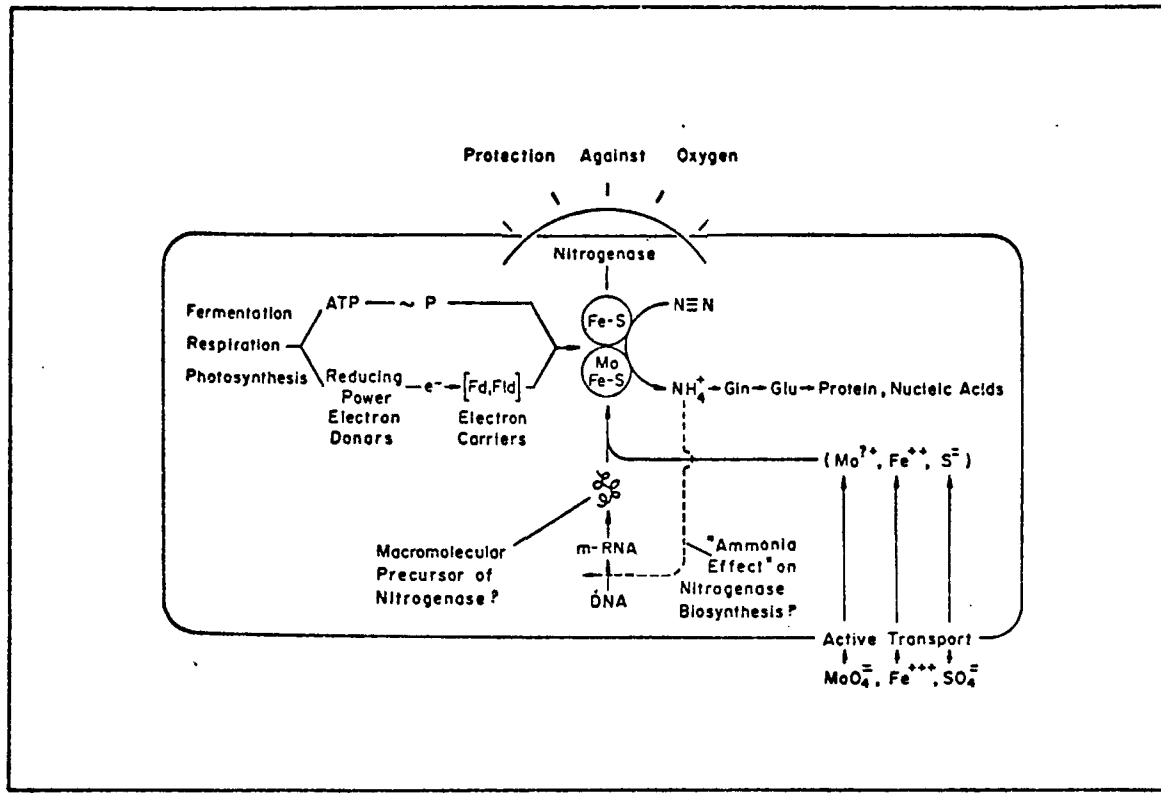
Postgate, a prominant investigator, feels that nitrogen fixation appeared very recently (148). Postgate states that nitrogen fixation appeared when eucaryotes were so aerobic that they could not provide a suitable environment for the enzymes involved in the nitrogen fixing process. Thus, the *nif* gene cluster was "received" by procaryotes which contained the low redox potential electron carriers (ferredoxins and flavodoxins) required for the nitrogen fixing process (148). Postgate also points out that the procaryotes are more susceptible to genetic modification (conjugation, transduction, and transformation) than are the eucaryotes; and thus, were more likely to accept the foreign (*nif*) DNA (148).

For the bacterial evolution scheme proposed herein, it is suggested that

the nitrogenase system (whatever its original role) began to function as a nitrogen fixing mechanism for bacterial growth in the era of Phase II. More detailed justification for this opinion and a brief review of the mechanisms of the nitrogenase system will follow.

There are two ecological types of nitrogen fixing microorgamisms: (a) the symbiotic type (114, 183), and (b) the non-symbiotic type (114, 183). Non-symbiotic nitrogen fixation is found in the blue-green bacteria, the aerobic soil bacterium *Azotobacter*, the facultative bacteria of the *Klebsiella* and *Achromobacter* types, and among the species of the strictly anaerobic *Clostridium* (114, 148, 183). It is also believed that all photosynthetic bacteria can fix nitrogen. Many species of the sulfate-reducing bacteria are known to be able to fix molecular nitrogen (148). Symbiotic nitrogen-fixation is distributed among leguminous plants, and among two-humdred fifty non-leguminous plants (114, 148). Symbiotic associations are varied. Algal, leaf nodule, leaf, root, animal, as well as the familiar root nodule association, are all commonly encountered symbiotic systems (148). The most intensely studied symbiotic system is that of the root nodule type. In the root nodule association, micoorganisms (usually species of the genus *Rhizobium*) invade the roots of leguminous plants. The bacteria invade the parenchyma of the root and a nodule is formed. The nodule is in direct contact with the plants vascular system (114). In all cases of symbiotic associations, the enzymes of nitrogen fixation are located in the bacteria (usually referred to as bacteroids) while the plant provides both supportive components and a relatively anaerobic environment (114, 148).

The detailed mechanism of the nitrogen fixation process has yet to be solved completely. An overall concept of biological nitrogen fixation is schematically represented as follows (183):



The three sources of reducing power (electrons) for nitrogen fixation are themselves a sort of evidence for the early appearance, and subsequent evolution of a nitrogenase system. For example, the anaerobic bacteria (*i.e., Clostridium*) use pyruvate, molecular hydrogen or formate as electron donors to the nitrogenase (17, 35, 36, 63, 127). These three electron donors are not effective for nitrogen reduction in aerobic bacterial systems (183). In *C. pasteurianum*, a ferredoxin-linked phosphoroclastic reaction supplies reducing power and ATP for nitrogenase function (17, 129, 183). The pyruvate which is the substrate for the phosphoroclastic reaction, is the end-product of sugar fermentation. ATP can be produced by other processes in addition to the phosphoroclastic reaction (183). Pyruvate will supply both reducing power and ATP for active nitrogen fixation in cell extracts of *C. pasteurianum*, (126), *Bacillus polymyxa* (163), *Klebsiella pneumoniae* (63), *Chromatium* (18),

and in a few blue-green algae (170).

Organisms which use formate to support nitrogen fixation contain a ferredoxin-linked formate dehydrogenase (183). Formate can be an electron donor in such species as *C. pasteurianum* (127), *K. pneumoniae* (17), and *B. polymyxa* (63).

In the aerobic bacteria (*i.e., Azotobacter vinelandii*), pyridine nucleotides ($NADPH_2$) supply electrons to the nitrogenase system (16, 183). Benemann, *et. al.*, showed that $NADPH_2$ could support nitrogen fixation, because the actual redox potential of $NADPH_2$ in the cell was such that thermodynamically it would be able to reduce ferredoxin (16). In *A. vinelandii*, metabolites which supply reduced pyridine neucleotides to the cell are isocitrate (13), malate, glucose-6-phosphate (16), ∝-ketoglutarate (183), and possibly others. It has been proposed that isocitrate specifically may furnish the reducing equivalents, via the isocitrate (NADP) dehydrogenase, required for energizing the *A. vinelandii* nitrogenase system (13). $NADPH_2$ (or $NADH_2$) can support nitrogen fixation in extracts of aerobic (16), facultative (17), fermentative (49), and photosynthetic bacteria (170).

The electron donors for nitrogen fixation, in the photosynthetic bacteria, have yet to be conclusively established (183). In the green bacteria *Chloropseudomonas ethylicum*, the photosynthetic apparatus has been shown to reduce the nitrogenase (59), in a ferredoxin-mediated reaction. The report that this culture contains two or more bacteria has cast doubt on the validity of this finding (183).

The fact that three processes are known to provide reducing power and ATP to the nitrogenase system indicates that the nitrogenase system has evolved simultaneously with the evolution of energy acquiring mechanisms.

The electron carriers among the nitrogen fixing organisms are proteins which are strongly suspected as being of very ancient origins (9, 79, 148). Bacterial ferredoxin was originally isolated in extracts of the nitrogen fixing organism *C. pasteurianum* (125). Flavodoxin was originally isolated as a protein capable of replacing ferredoxin in the phosphoroclastic reaction of *C. pasteurianum* (107).

Ferredoxins derived from the various nitrogen fixing bacteria differ in chemical, physical, and biological properties (183). Hall, *et al.* (79), have proposed that the 8 Fe(4Fe +4 labile S)ferredoxins from *Clostridium* may have been amongst the earliest proteins formed during the origin of life. The ferredoxins from five species of *Clostridium* studied have striking homology (42, 79). With only fifty-five amino acid units, these ferredoxins have molecular weights of approximately 6,000 (79, 183). Some of the primitive characteristics of these *Clostridium* ferredoxins are:

(1) these *Clostridium* ferredoxins contain only nine common simple amino acids, six of which have been detected in the Murchison meteorite (79).

(2) The cysteine residues are invariant in position, and, the molecules consist of two similar halves, indicative of gene duplication (9, 79, 166).

(3) the oxidation-reduction potential at neutral pH of these ferredoxins is about -420 mV, which is the same as the hydrogen electrode (9, 79, 183). This is consistent with the reduced environment of this period.

A. vinelandii ferredoxin, on the other hand, has a molecular weight of 14,500, which is higher than any other ferredoxin studied to date (200). This ferredoxin has low activity in the clostridial phosphoroclastic reaction (183). Together, this evidence indicates an evolutionary pattern among ferredoxins. Within, the nitrogen-fixing organisms, the ferredoxins appear to have evolved from simple, low molecular weight proteins of the strict anaerobes, to larger and more advanced types in the aerobic species like

Azotobacter.

The ferredoxin isolated from *Rhizobium japonicum* has 2 (Fe-S) complexes, and has a molecular weight of 9,400 (108).

The ferredoxins isolated from the green photosynthetic bacteria of the *Chlorobium* species have similar physical properties and amino acid sequences to the *Clostridium* type ferredoxins (28).

Flavodoxins have been isolated, and identified as electron carriers in a number of nitrogen fixing bacteria (183, 201). In *A. vinelandii*, the flavodoxin which functions in nitrogen fixation is termed azotoflavin (15). Azotoflavin is not known to participate in any other process except nitrogen fixation (183). Flavodoxins contain the prosthetic group FMN, but do not contain any metals or labile sulfur (183). Flavodoxins, like ferredoxins, operate at low redox potentials, and contain an acidic protein backbone (183). Baltscheffsky (9), has suggested that flavoproteins in general might have evolved from ferredoxins. The average molecular weight of flavodoxins is is approximately 15,000, while azotoflavin is considerably larger having a molecular weight of 23,000 (183).

The nitrogenase enzyme is a complex of two protein components (114, 183). One of the components is a molybdenum-iron-sulfur protein, with a molecular weight of 220,000 (114, 183). The other component is an iron protein, having a molecular weight of 60,000 (114, 183). Neither of the components are active alone, and they are functional in a ratio of one Mo-Fe protein to two Fe proteins (183).

The observation that various anatomical and phsyiological devices have evolved to protect the nitrogenase from oxygen is additional evidence that the nitrogenase enzyme system appeared early in evolution (during anaerobic conditions).

Among the physiological protective devices, respiratory protection is the best understood. Respiratory protection refers to a mechanism whereby an organism creates an anaerobic intracellular environment by maintaining a high rate of respiration (148). *Klebsiella pneumoniae*, a facultative anaerobe, was regarded as only able to fix nitrogen anaerobically (148). Klucas has shown that *K. pneumoniae* will synthesize nitrogenase in air provided that its respiration rate is high enough to keep the dissolved oxygen concentration very low (105). Postgate has confirmed the findings of Klucas concerning *K. pneumoniae* (148). The role of respiration as a protective device in *Azotobacter* has been discussed by many authors (41, 92, 147). Postgate has accumulated four pieces of evidence that support the theory of respiratory protection in *Azotobacter* (148):

(1) Nitrogen fixation operates most efficiently when the oxygen concentration limits respiration.

(2) Oxygen stress lead to augmented respiration with no change in nitrogenase activity.

(3) The optimal pO_2 for nitrogenase activity is greater when the respiratory activity is highest for a population of *Azotobacter* cells although the absolute nitrogenase activity at the optimal pO_2 is unchanged.

(4) It is possible to overcome respiratory protection in *Azotobacter* by exposing populations to pure O_2, or by applying an oxygen stress to phosphate-limited populations.

Postgate has suggested that the *Azotobacter* species exhibit a highly evolved respiratory protective device (148), due to their high respiratory activity, and their ability to adjust respiration rate to oxygen concentration (112). The *Azotobacter* branched-chain electron transport pathway prevents the over-production of ATP during respiratory protection (92). The aerobe *Mycobacterium flavum* grows very well in air when supplied with exogenous ammonium ion, but behaves as a micro-aerophile when fixing nitrogen (148). Thus, this aerobe which has no respiratory protective device will only fix

nitrogen at low oxygen concentrations. The organism *Derxia gummosa* appears to contain a more primitive protective mechanism than *Azotobacter*, but more advanced than *Klebsiella* (83, 148). Thus, Postgate has proposed a scheme of physiological respiratory protection, starting with *Clostridium* which has no protective mechanism (148):

Clostridium ⟶ *Klebsiella* ⟶ *Mycobacterium flavum* ⟶ (*Derxia gummosa*?) ⟶ *Azotobacter*

Postgate has asserted that symbiotic association has evolved from casual commensalism due to partial or complete interdependence between plant and microbe (148). Among the root associations there appears to be a physiological evolution in the direction of increasing interdependence between the bacteria and plant (148):

Klebsiella ⟶ *Azotobacter paspali* ⟶ *Rhizobium* ⟶ *Frankia*

It is known, for example, that *Klebsiella* shows casual syntrophism, *A. paspali* shows host specificity, *Rhizobium* does not fix nitrogen except when it is in symbiotic association, and *Frankia* has not been cultured away from plant tissue (148).

In conclusion, the nitrogenase enzyme complex undoubtedly was found in the primitive bacteria of Phase I of this scheme: its original function being one of detoxification of the environmental poisons. It was not until ammonia depletion, or the replacement of NH_3 with atmospheric N_2 that the nitrogenase enzyme system began to function as an ammonia generating mechanism for bacterial growth. Therefore, nitrogen fixation as a physiological growth process, first appears in this middle Phase II of the proposed scheme of bacterial evolution. The fact that physiologically more primitive bacteria (*i.e., Clostridium pasteurianum*) contain the nitrogen fixation mechanism is

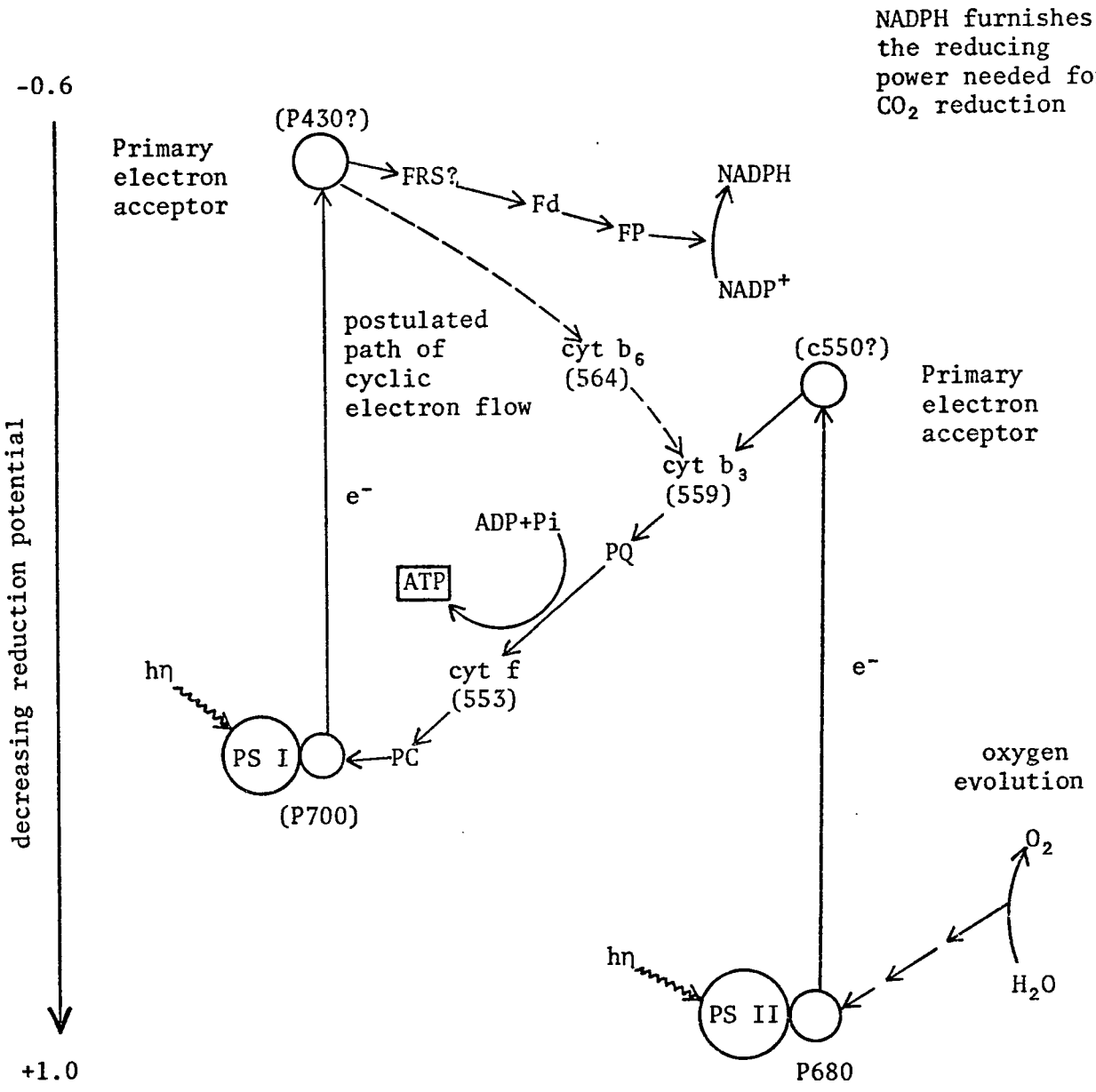
consistent with the view that the enzymes of nitrogen fixation originally functioned to detoxify the environment. In this organism the role of the nitrogenase enzyme complex changed from detoxifying poisonous compounds to an ammonia generating system, as the atmospheric environment changed chemically from a reduced state to a more oxidized one.

ORIGIN AND EVOLUTION OF PHOTOSYNTHESIS

Basic Physiology of Procaryotic Photosynthesis

All higher life forms on the earth today are nutritionally dependent on photosynthesis, the process by which the radiant energy of light is transduced to chemical energy via chlorophyll. Photosynthesis today is distributed widely among both eucaryotic and procaryotic organisms. The photosynthetic eucaryotes include the higher green plants, the green, brown, and red algae, euglenoids, dinoflagellates, and diatoms. Among the procaryotes, photosynthesis is found in the cyanobacteria (blue-green bacteria), the green bacteria, and the purple bacteria. There are two different types of photosynthesis: oxygenic photosynthesis, which is the type found in photosynthetic eukaryotes and the cyanobacteria; and anaerobic photosynthesis, which is limited to the green and purple bacteria.

Oxygenic photosynthesis, is mechanistically more complex than anaerobic photosynthesis; it requires two photoreactive centers (photosystem I and photosystem II) and H_2O, upon photolysis is used as the electron donor (hydrogen source) for the reduction of CO_2. The oxygen atom of H_2O is released as molecular oxygen, which is a by-product of the photolytic reaction, and thus the name oxygenic photosynthesis. This process which occurs in chromatophores for procaryotic cells, and in chloroplasts for eucaryotic cells can be schematically diagrammed as follows:



"Z scheme" for photosynthetic electron transport via photosystems I & II, modified from Lehninger (114).

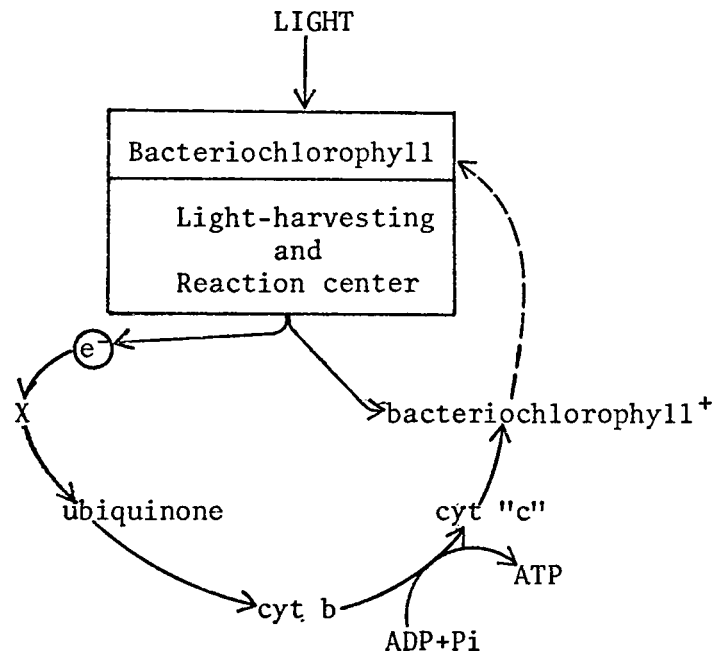
Oxygenic photosynthesis is non-cyclic; and ATP, molecular oxygen, and NAD(P)H are the end products of the light dependent reactions represented above. The ATP provides the energy, and the NAD(P)H the reducing power to the Calvin cycle. The Calvin cycle, or dark reaction of photosynthesis involves the utilization of the chemical energy of ATP and the reducing power

of NADPH to bring about the reduction of carbon dioxide to hexoses and other products. The Calvin cycle is, of course, intact in all photosynthetic organisms, whether oxygenic or anaerobic. The enzyme reactions of the Calvin cycle are, in large part, common to those of the oxidative pentose phosphate cycle (181). The Calvin cycle appears to be identical in all photosynthetic organisms, and thus can not be used to determine an evolutionary scheme among the photosynthetic procaryotes. Thus the heterogeneity of the light reactions, with regard to both their molecular components and mechanisms, gives the only clues as to how the photosynthetic apparatus evolved. Earlier it was stated that oxygenic photosynthesis is a non-cyclic process. While it is true that the non-cyclic mechanism is the major photoreactive process in oxygenic photosynthesis, it is also apparent that the non-cyclic mechanism cannot supply all the ATP needed for the assimilation of CO_2 (181). The extra ATP needed is produced by the reactions of cyclic photophosphorylation. Therefore, both cyclic and non-cyclic photochemical processes operate in oxygenic photosynthesis to permit the assimilation of CO_2 (114, 181).

The green and purple bacteria carry out another, quite different type of photosynthesis. These bacteria, neither produce nor use molecular oxygen; most being strict anaerobes and are either poisoned by oxygen or at best aerotolerant. This anoxygenic photosynthesis is characterized by: (a) the absence of photosystem II; (b) the use of H_2, H_2S (or other reduced inorganic sulfur compounds), or simple organic compounds like acetate and formate, as the electron donors (or hydrogen source). A much simpler photochemical mechanism is therefore present than for oxygenic photosynthesis (114, 181).

Although photosystem II is not present in anoxygenic photosynthesis, photosystem I can operate either in a cyclic or non-cyclic fashion. Since

some of the electron donors used by the green and purple bacteria (*e.g.*, H_2) can serve directly for the generation of NAD(P)H by non-light dependent reactions; a photochemical reaction for the production of reducing power is not necessary (114, 181). Thus, it is probable that non-cyclic photophosphorylation is much less important in anoxygenic photosynthesis than in oxygenic photosynthesis (181). The primary function of the photochemical mechanism in anoxygenic photosynthesis is to provide ATP through cyclic photophosphorlyation (181). The following scheme is a general outline for cyclic photophosphorylation among purple bacteria (74):



Fuller and Nugent have suggested that the "x" in the above scheme may be a low redox potential pteridine (71). The cyclic photophosphorylation complex is located in the cytoplasmic membrane and its extensive invaginations (189). If the cell membrane is fragmented using procedures such as sonication, the fragments will spontaneously seal to yield spherical vesicles (chromatophores). These vesicles will phosphorylate ADP through

cyclic photophosphorylation (74). Baltscheffsky *et al.* demonstrated that in the absence of ADP, inorganic pyrophosphate (Pi-Pi) is formed from the interaction of inorganic phosphate (Pi) through the mechanism of cyclic photophosphorylation (6, 8). This same worker using chromatophores, derived from the purple non-sulfur photosynthetic bacterium, *Rhodospirillum rubrum*, has also shown (7):

(1) the presence of a Mg^{++} stimulated inorganic pyrophosphatase reaction.

(2) an inorganic pyrophosphate induced reversed electron transport at the cytochrome level.

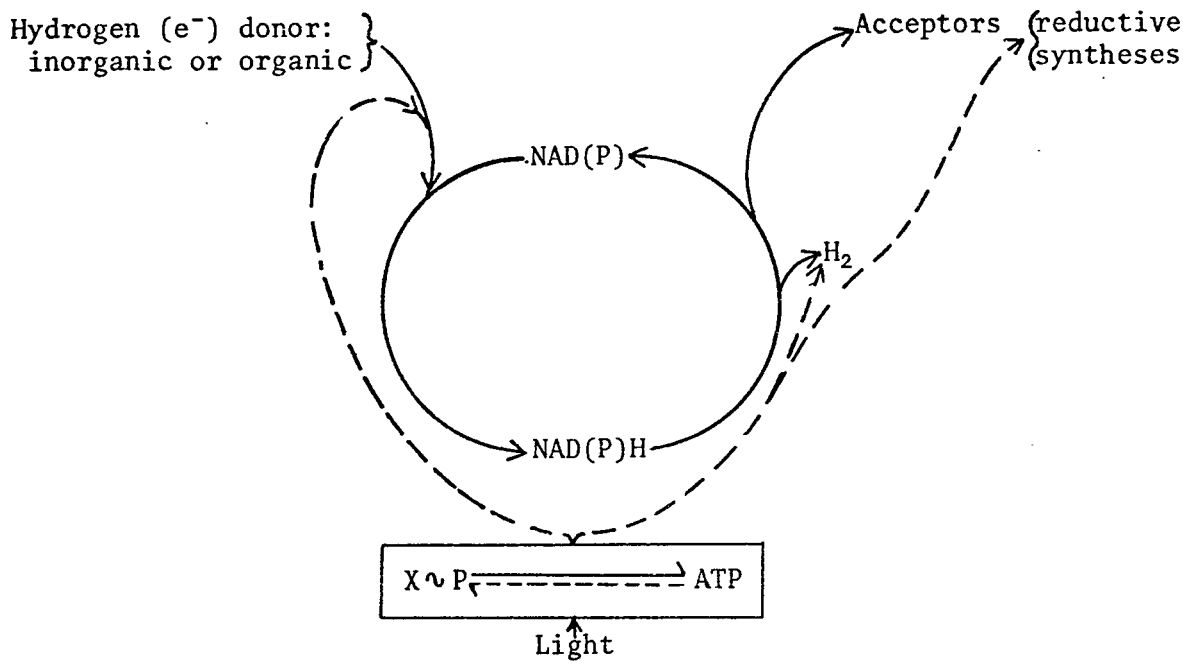
(3) an inorganic pyrophosphate induced carotenoid absorption band shift.

(4) an inorganic pyrophosphate induced energization of the chromatophore membrane as measured by fluorescent probe.

In light of the data obtained, Baltscheffsky has proposed that "inorganic phosphates preceded adenosine phosphates as energy carriers in both substrate level and electron transport level phosphorylation systems" (7). Thus, it is apparent that research with photosynthetic bacteria has produced data which might answer fundamental questions about biochemical evolution.

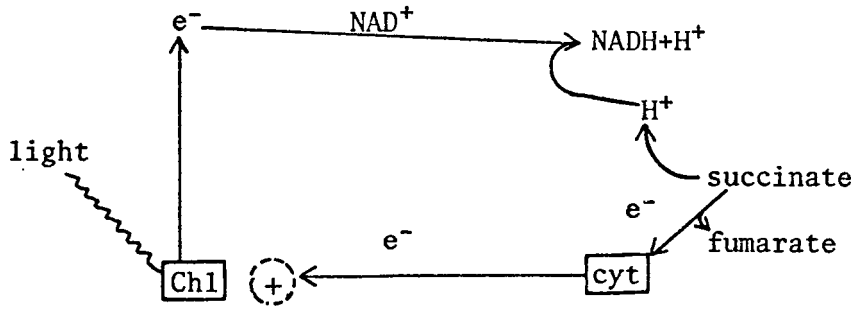
Although anoxygenic photosynthesis lacks photosystem II, and has no mechanism for the photolysis of water, a *noncyclic photo-phosphorylation* utilizing photosystem I can occur in purple and green bacteria (181). The electrons in this noncyclic process are used for the reduction of pyridine nucleotides and, the bacteriochlorophyll is reduced by electrons from an exogenous source (reduced inorganic sulfur compound, H_2, or a simple organic compound). The actual existence of a noncyclic light-induced electron flow in purple and green bacteria has been vigorously debated because of the presence of a mechanism for production of NAD(P)H that does not require a noncyclic process (73, 179) For example, Gest has suggested the following

noncyclic mechanism for the net production of NAD(P)H (72):

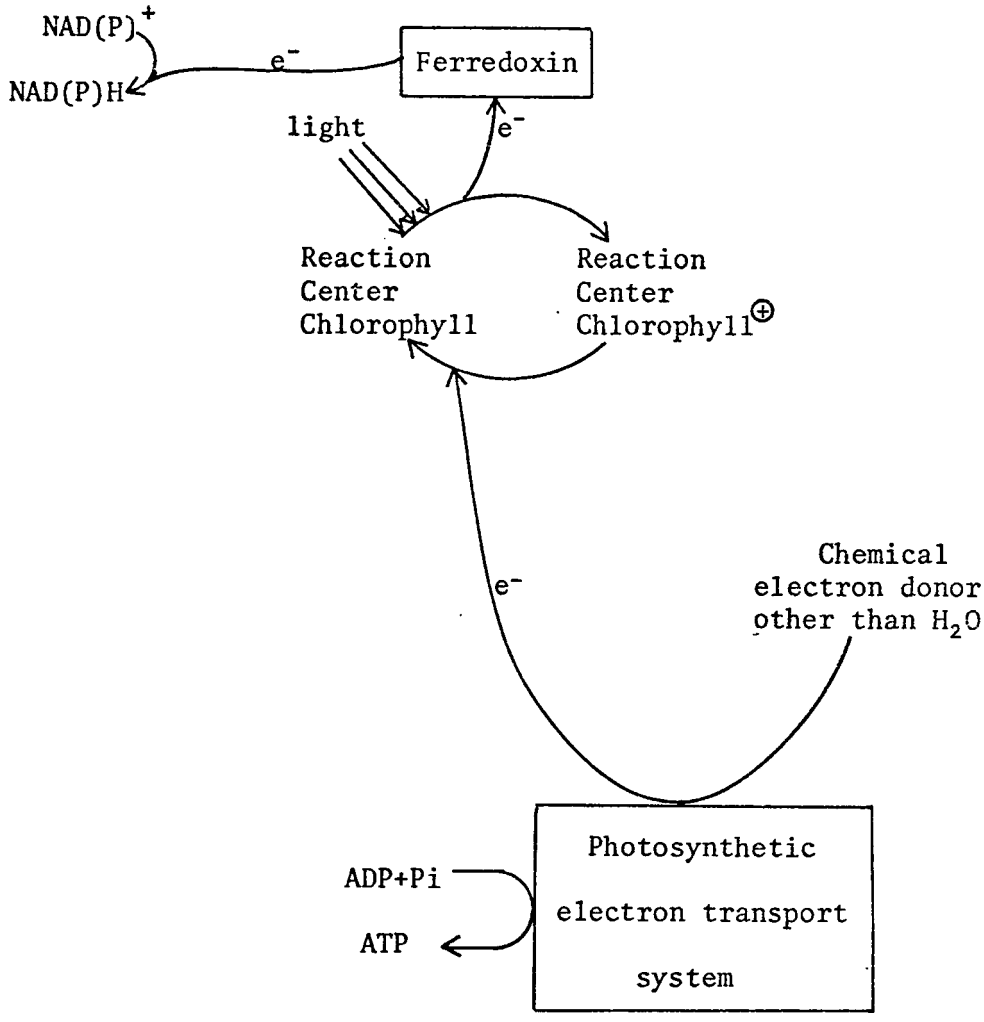


It is important to note that the only role attributed to light is ATP synthesis through cyclic photophosphorylation (179).

However, when a simple organic compound like succinate is used as an electron donor, a problem arises. Unlike electron donors such as H_2S and H_2, electrons from succinate cannot serve for the direct reduction of NAD(P) (179). Frenkel has shown that bacterial chromatophores are capable of performing a light dependent reduction of NAD(P), coupled with the oxidation of succinate (70). This reaction occurs independently of cyclic photophosphorylation (70). A noncyclic mechanism for the generation of reducing power in *Rhodospirillum rubrum* has been postulated by Stanier (179):



A more general scheme for non-cyclic photophosphorylation can be represented as follows (181):



(Scheme for Anoxygenic noncyclic photophosphorylation producing NAD(P)H and ATP. From Stanier, *et al.* (181).

Both anoxygenic and oxygenic photosynthesis are represented in species of bacteria. Purple and green bacteria perform the anoxygenic type; while blue-green (algae) bacteria (cyanobacteria) carry out the "plant", oxygenic type of photosynthesis (29, 181). The table below lists

some of the physiological characteristics of the groups and subgroups of the phototrophic bacteria:

Phototrophic bacteria (all are Gram negative)

I. Purple bacteria

II. Green bacteria

III. Blue-green bacteria (Cyanobacteria)

Anoxygenic Photosynthesizers

I. Purple bacteria:

Rods, cocci, or spirilla
Motile by means of flagella
About 30 species, which are genetically diverse
Unicellular, and reproduce by binary fission, or in a few species by budding.
Many species can fix nitrogen
There are two physiological subgroups:

A. Purple sulfur bacteria:

Growth is predominantly through photoautotrophic metabolism
Strict anaerobes
All species can also grow photoheterotrophically

B. Purple non-sulfur bacteria:

Predominantly photoheterotrophs (photoorganotrophs)
Many strains can grow aerobically, in the dark, and, thus possess an aerobic electron transport chain.
A few strains can grow by fermentation of pyruvate or sugars
All species can grow photoautotrophically with H_2, and sometimes reduced inorganic sulfur compounds as electron source.
Require B vitamins for growth

II. Green bacteria:

Most are small, immotile, rod-shaped organisms
There are only nine species placed in five genera
They are genetically not diverse
Many species fix nitrogen
Some species require Vitamin B
There are two physiological subgroups:

A. Green sulfur bacteria:

Anaerobic photoautotrophs
They use H_2S, H_2, or other reduced inorganic sulfur compounds as electron donors
Cannot grow photoheterotrophically

B. Green non-sulfur bacteria (the *Chloroflexus* group)

Filamentous, gliding bacteria
A typical photoheterotroph, and facultative chemoheterotroph

Although these bacteria differ from green sulfur bacteria in their structure, nutrition, metabolism, and ecology, they are green bacteria because they contain chlorobium vesicles, and because they contain bacteriochlorophylls c and a as the major and minor chlorophyllous pigments, respectively.

Chloroflexus can perform anoxygenic photosynthesis

Oxygenic Photosynthesizers

III. Blue-green bacteria (cyanobacteria):

They are structurally and genetically diverse
Their mean DNA base composition has a span as wide as that for procaryotes in general
All are photoautotrophs; assimilation of CO_2 occurs through the Calvin cycle
They all lack a functional TCA cycle due to the absence of the enzyme α-ketoglutarate dehydrogenase
Some are immotile, unicellular rods or cocci which reproduce by binary fission. Some reproduce by multiple fission
Many are filamentous, and reproduce by multiple fission
Some species can grow in dark, but growth is very limited
They are the only oxygenic photosynthesizers that can also fix nitrogen

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The information in the table above was obtained from Stanier, *et al.* (181).

The tables below list the genera of the purple and green bacteria (181):

The genera of purple sulfur bacteria

Genus:

1. *Thiospirillum*
2. *Ectothiorhodospira*
3. *Chromatium*
4. *Thiosarcina*
5. *Thiocapsa*
6. *Lamprocystis*
7. *Thiodictyon*
8. *Thiopedia*
9. *Amoebacter*

The genera of purple nonsulfur bacteria:

Genus:

1. *Rhodospirillum*
2. *Rhodopseudomonas*
3. *Rhodomicrobium*

The genera of green sulfur bacteria:

Genus:

1. *Chlorobium*
2. *Prosthecochloris*
3. *Pelodictyon*
4. *Clathrochloris*

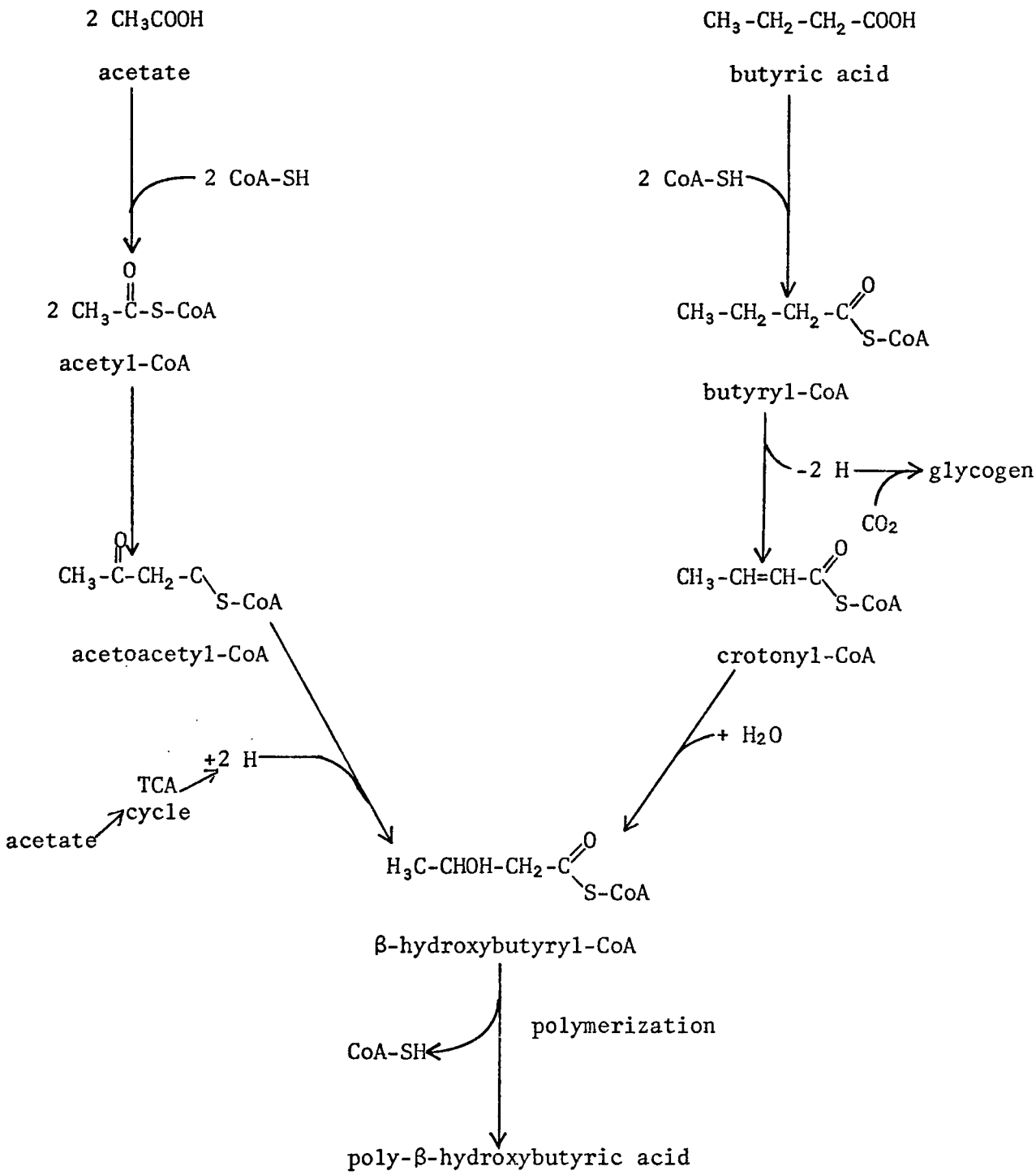
The genera of green nonsulfur bacteria:

1. *Chloroflexus*

The information included in the tables above was obtained from Stanier *et al.* (181).

Theories on the Origin of Photosynthesis In Bacteria

There are two different theories that have been proposed for the origin of photosynthesis in bacteria. The first theory assumes that much of the photochemical system already was present in the earliest heterotrophic fermenters (133). This theory proposes that the photosystem originally functioned as an ATP generating system to supply energy for the assimilation of the abiotically formed exogenous organic nutrients. The proposed ancestral photobacterium would have possessed a photoreaction quite similar to the cyclic photosystem of contemporary anoxygenic photosynthesis (133). Modern examples of this type of photometabolism are found among the purple non-sulfur bacteria (*Athiorhodaceae*), the green non-sulfur bacteria (*Chloroflexus*), and a number of algae adapted to heterotrophic conditions (133, 181). This type of metabolism is known as photoorganotrophy (or photoheterotrophy); and under these circumstances all material is derived largely from the organic substrate (181). An example of photoassimilation by non-sulfur bacteria is shown in the scheme below (181).



(The light driven assimilation of acetate and butyrate to poly-β-hydroxybutyric acid by non-sulfur bacteria, from Stanier *et al.* (181)

In non-sulfur purple bacteria the photoassimilation of acetate and butyrate is very efficient, since some 90% of the organic substrate is converted to cellular reserve material (181). The ATP, necessary for the activation of acetate to acetyl-CoA, is furnished by the process of cyclic photophosphorylation (181). The photoassimilation of acetate would probably best represent the primitive process in a hypothetical ancestral photobacterium, since it would occur in the absence of CO_2. Photoassimilation of butyrate occurs anaerobically only if the Calvin cycle is present to accept the hydrogens produced (181).

The blue-green algae (bacteria) *Chlorogloea fritschii* photoassimilates sucrose in the absence of CO_2, after adaptation (61). The green algae *Chlamydomonas mundana* (157), and *Chlamydobotrys* (*Pyrobotrys*) *stella* (76), photoassimilate acetate with no CO_2 fixation or oxygen evolution (133). Both *Chlorella* and *Scenedesmus* will photoassimilate glucose in the absence of CO_2 or oxygen (133). In all of the above cases ATP drives the assimilation; and, the ATP is derived from cyclic photophosphorylation (133).

It is rather difficult to imagine how the complex components of the photosystem, (cytochromes, quinone, chlorophylls, and accessory pigments), spatially organized within a membrane-bound complex, could have appeared simultaneously, *de novo*, in a primitive heterotroph. However, the recent report of the presence of cytochromes in some *Clostridium* species (75), gives some support to aspects of this theory. This theory becomes more credible if one assumes that contemporary phototrophic bacteria are the culmination of a long series of phototrophic forms in which all of the primitive species have become extinct, or have yet to be isolated (80).

A second, alternative theory for the origin of photosynthesis, is the theory proposed by Hall (80). Hall suggests that the mechanism and apparatus

of photosynthesis in bacteria developed from the already existing respiratory electron transport system of the sulfate or nitrate reducers (80). The cytochromes and chlorophylls both contain functional heme moieties; and, the biosynthetic pathway leading to protoporphyin, for both the cytochromes and the chlorophylls, is the same (93). This theory speculates that one of the cytochromes of either the sulfate or nitrate respirers might have been altered through mutation, so that it could produce an excited electron by absorbing light (80). The excited electron, it is proposed, would naturally flow along the respiratory chain. If this mutation occurred on a duplicated gene, there would have been no interference with the normal metabolism of the organism. Thus, the light reaction would originally function only to supplement respiratory energy production; and, only after selection provided more efficient light trapping apparatus, and the advantages of photosynthesis became apparent, would the bacteria become truly photosynthetic (80). This theory avoids the problem of proposing a sudden, *de novo* appearance of the complex system of photosynthesis. This theory is, in principle, somewhat easier to imagine, and, is consistent with the scheme of evolution presented in this paper.

It is interesting that both of these theories assume that the "primitive" photoreaction had the sole function of generating ATP. The two proposals disagree only on when the first light reaction appeared in the chronological evolution of bacteria.

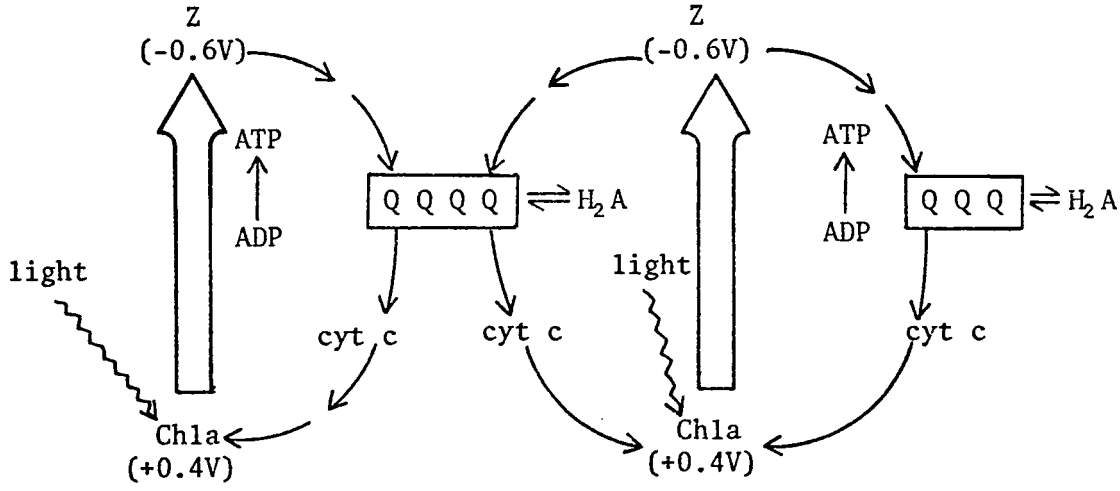
The Evolution of Photosynthesis among the Procaryotes

All of the speculation and data that has been proposed to date, agrees that "true" photosynthesis (photoreduction of CO_2 to hexose) did not appear until the shift from heterotrophic to autotrophic growth was required (80, 114, 133).

The primary light reaction, already functional in some bacteria, could then be modified to produce the reducing power needed for reduction of CO_2.

The subject of evolution among the procaryotes has, in the past, been given little, if any, consideration. Olson (133) has suggested a scheme of evolution among the procaryotes which remains the only paper entirely devoted to the subject. In Olson's opinion, the photosynthetic bacteria (oxygenic and anoxygenic) share a common anaerobic photoheterotrophic ancestor (133). This speculative ancestral photobacterium would have utilized a light driven electron transport system which was the precursor of both photosystem I of anoxygenic photosynthesis, and photosystems I and II of oxygenic photosynthesis (133). If a light driven phosphorylation did develop from the sulfate or nitrate respirers, it is reasonable to assume that it functioned to produce ATP for the assimilation of the little organic material still available, before photoreduction of CO_2 became necessary for survival. Olson proposes a light driven cyclic electron transport system for the hypothetical ancestral bacterium (133):

H_2A = exogenous electron donor (not H_2O)
Q = Quinone
Z = Unknown primary electron acceptor



{from Olson (133)}

In the above scheme, a pool of quinone is shared by more than one electron transport chain; which, according to Olson, was a fundamental requirement for the development of two photochemical systems in series. It will be noted, also, that chlorophyll a is the only pigment for this proposed photosystem (133). The intermediates in the synthesis of both chlorophylls and bacteriochlorophyll a are derived from chlorophyllide a in photosynthetic bacteria (20). This evidence seems to indicate that chlorophyll a evolved before bacteriochlorophyll a (133).

It appears that this ancestral light reaction evolved along two simultaneous, but quite divergent lines (133). One line terminated in the contemporary anoxygenic photosynthesizers (purple and green bacteria). The other line terminated in oxygenic photosynthesis demonstrated by the blue green bacteria, algae and the green plants. Here again, very little speculation has been attempted regarding the details of each of these different paths of evolution. The major evidence in support of the divergent theory of photosynthesis is both the structure of the photosynthetic apparatus, and the mechanisms of photosynthesis among procaryotes. The table below demonstrates very well the structural and functional gap between anoxygenic photosynthesis (purple and green bacteria), and oxygenic photosynthesis (blue-green bacteria) (181):

	Procaryotes with Chromatophores			Eucaryotes with Chloroplasts	
	Purple bacteria	Green bacteria	Blue-green bacteria	Red Algae	Green Algae and Plants
Cell structure containing photosynthetic apparatus	Cell membrane	Chlorobium vesicles	Thylacoids & phyco-bilisomes	Thylacoids & phyco-bilisomes	Thylacoids
Photosystems					
I	+	+	+	+	+
II	-	-	+	+	+
Reductants used for CO_2 assimilation	H_2S, H_2, or organic compounds	H_2S, H_2	H_2O	H_2O	H_2O
Principal photosynthetic carbon source	CO_2 or organic compounds	CO_2 or organic compounds	CO_2	CO_2	CO_2

{modified from Stanier *et al.* (181)}

It is obvious from the above table that the two major variations on the theme of photosynthesis first arose and evolved in the context of the procaryotic cell (180).

Other evidence supporting a divergent theory for the evolution of photosynthesis is the pigment and lipid components of the anoxygenic, and oxygenic photosynthesizers (180, 181):

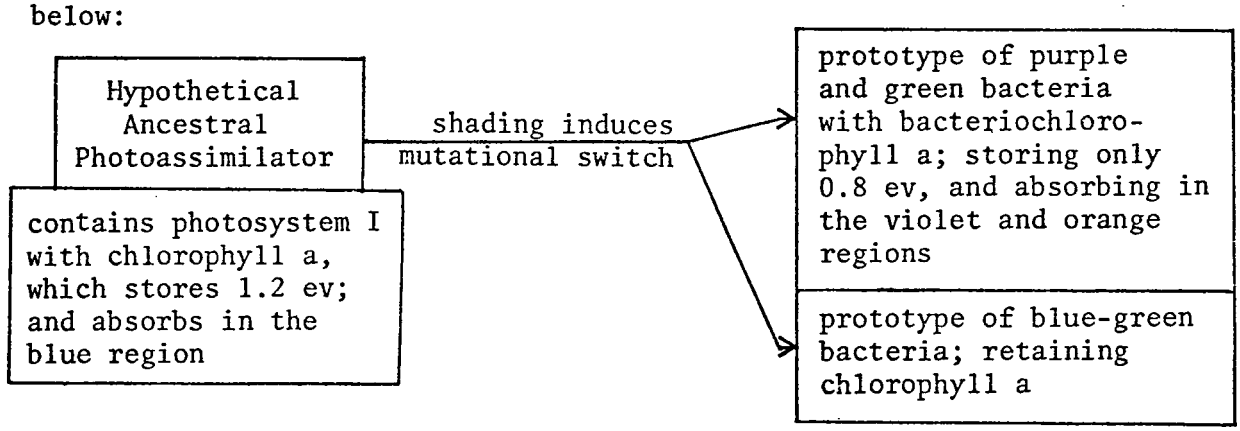
	Procaryotes with Chromatophores			Eucaryotes with Chloroplasts	
	Purple bacteria	Green bacteria	Blue-green bacteria	Red Algae	Green Algae and Plants
Chlorophyll Pigments	Bacteria a or bacteria b	minor: bacteria a major: bacteria c, d or e	chlorophyll a	chlorophyll a	chlorophyll a and b
Predominant carotenoids					
Alicyclic (β-carotene & related oxy carotenoids)	-	-	+	+	+
Aryl carotenoids, aliphatic, bearing methoxyl groups	+ -	+	-	-	-
Phycobiliproteins					
Allophycocyanin	-	-	+	+	-
Phycocyanin	-	-	+	+	-
Phycoerythrin	-	-	+ or -	+ or -	-
Lipids					
monogalactosyl-diglyceride	-	+	+	+	+
digalactosyl-diglyceride	-	-	+	+	+
polyunsaturated fatty acids	-	-	+ or -	+	+

{modified from Stanier *et al.* (181)}

The data presented in the above table confirms the existence of sequential complexities of patterns that are reflected in the mechanism and apparatus of photosynthesizing cells. There is, without question, both a structural and functional gap among the photosynthetic procaryotes. As clearly indicated, no contemporary biological organisms contain either apparatus or photosystems that can be interpreted as intermediate between anoxygenic and oxygenic photosynthesis.

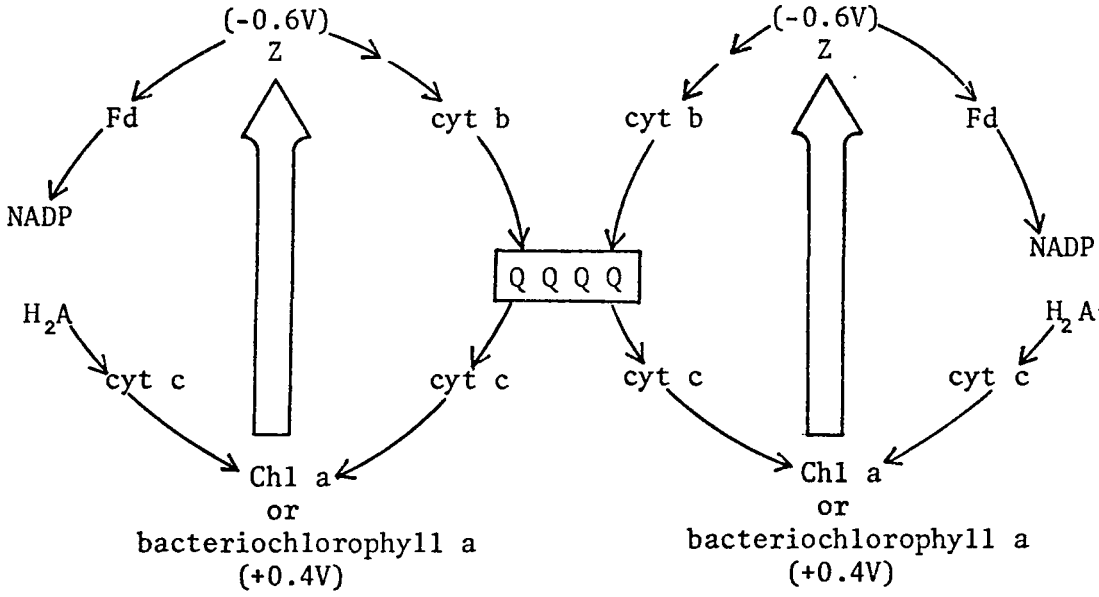
A detailed examination of the mechanisms operating to cause the divergent evolution of photosynthesis from the hypothetical ancestral photobacterium has been attempted only once (133). Central to the development of two different themes of photosynthesis was the type of chlorophyll present in the photoreactive center of the bacteria. Ross and Calvin have estimated that the maximum free energy stored in chlorophyll a is 1.2 electron volts (ev); while that for bacteriochlorophyll a is only 0.8 ev (154). Olson has noted that because of mutual shading among the phototrophic bacteria, competition for the available light in the blue region of the spectrum occurred (133). A selective advantage for some phototrophic bacteria to undergo a mutational switch from chlorophyll (absorbing near 440 nm), to bacteriochlorophyll (absorbing in the violet and orange regions, 370 nm and 590 nm) would have occurred (133). This mutational switch resulted in the loss of -0.4 ev in the free energy stored in the primary photoact; and prevented the bacteria containing bacteriochlorophyll from ever developing the capacity for the photolysis of water (133).

A schematic representation of the theory of divergent evolution is presented below:



Evolution of Photosystems I and II in Procaryotes

Simultaneously with the development of the photosynthetic pigment systems that absorb in different regions of the light spectrum, the cyclic photosystems of the phototrophs were being modified by mutations to a non-cyclic form, which in addition to producing ATP, also generated reducing power (NADPH).



{from Olson (133)}

The photosystems, due to the presence of two different light absorbing pigments, also evolved along divergent paths (133). One line of development culminated with two photosystems (PS I and PS II); one of which (PS II) operates at a redox potential such that water can be oxidized, to provide the electrons for reduction of NADP (133). The other line of evolution resulted in anoxygenic photosynthesis, with the cyclic photosystem being retained; and, possibly a non-cyclic process, using an alternate pigment center, being developed.

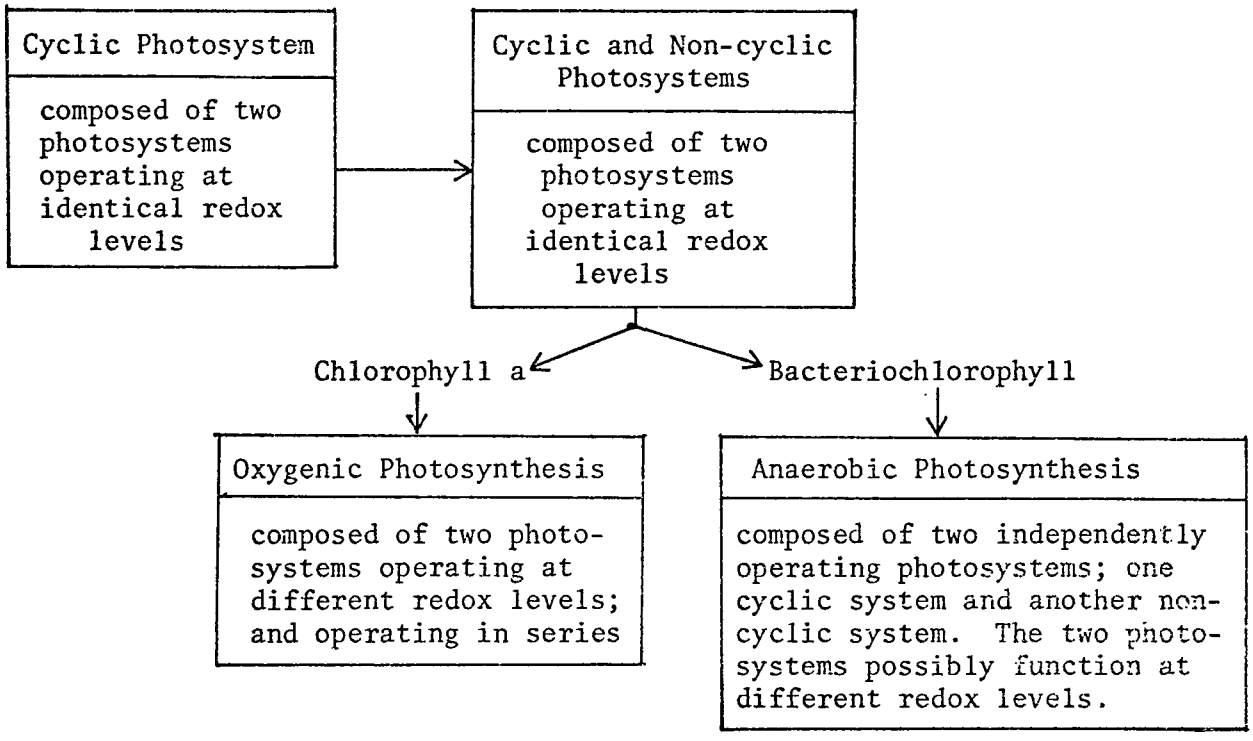
The presence of a second photosystem (PS II) in the purple and green bacteria is still a matter of debate among microbiologists. It has been reported that at least two species of *Chromatium* contain two independently operating photosystems; one cyclic system generating ATP, and one non-cyclic system producing NADPH (84, 99, 123). Also, two separate photosystems have been reported in *Rhodospirillum rubrum* (185). However, Gest suggests that the non-cyclic mechanism for light driven production of reducing power has not been proven to exist in anoxygenic photosynthesizers (74).

The first electron donors utilized by the prototype of modern blue-green bacteria may have been hydrozine and/or hydroxylamine (87, 133). Eventually, however, some of these blue-green prototypes found themselves in environments where these powerful reductant electron donors were used up (133). The evolutionary pressure for the utilization of ever weaker electron donors gradually resulted in the selection for one of the photoreaction centers being functional at the higher redox potential in order to extract electrons from these weaker reductants (133). Thus, through a series of small mutations, photosystem II evolved from photosystem I (133). This gradual movement of photosystem II toward higher redox potentials resulted in the ability of the blue-green bacteria to extract electrons from water. The two photosystems

connected in a series, using water as the electron donor, was the culmination of one line of evolutionary development among the procaryotic photosynthesizers. This represented from a bioenergetic viewpoint an ideal mechanism for energy production giving such organisms a tremendous environmental advantage.

The alternate path of development was the result of a different type of light absorbing pigment in the photoreactive center. Those bacteria which contained bacteriochlorophyll a in their reaction center stored 0.4 ev less energy than the prototype of the blue-green bacteria, which contained chlorophyll a (154). This required the bacteriochlorophyll containing procaryotes to utilize powerful reductants (i.e., H_2, H_2S, organic coupounds) as the electron donors. These procaryotes were the ancestors of the contemporary purple and green bacteria; and their growth was forever limited to highly reducing, anaerobic environments (133). As was noted earlier in this chapter, the debate as to whether or not a non-cyclic photosystem is operative in purple and green bacteria has not been resolved. Therefore, it is very difficult to speculate on the evolution of the photosystems of anoxygenic photosynthesis. We do know that cyclic photophosphorylation was retained in the purple and green bacteria. If a non-cyclic photosystem does exist in anaerobic photosynthesis, it could operate by utilizing the same pigment system utilized by the cyclic process (40). Another possibility, that has recently been suggested, is that a second pigment system, operating at a more negative redox potential than the cyclic process, functions in a non-cyclic manner in anaerobic photosynthesis (84, 99, 123, 185). The cyclic and non-cyclic photosystems would operate independently in anaerobic photosynthesis.

The divergent theory for the evolution of the photosystems in bacteria can be represented as follows:



The Origin of Oxygen in the Atmosphere

The atmosphere of the earth remained relatively free of molecular oxygen until the advent of oxygenic photosynthesis that was carried out by the blue green bacteria (24, 80, 133). Nothing in the entire course of evolution had a greater impact on the subsequent development of life on the earth! Both the geology of the surface of the earth, and the metabolic possibilities available to living organisms was radically altered due to the appearance of free oxygen (80). The evolution of oxygen through oxygenic photosynthesis was probably only predated by the abiotic photolysis of water, however, the production of oxygen by this process never resulted in an appreciable amount of free oxygen (24). Oxygen became generally abundant in the atmosphere only after the appearance of life forms, (i.e., the blue-greens)

which could carry out oxygenic photosynthesis (24, 133). According to the Berkner-Marshall theory for the origin of atmospheric oxygen, the present amount of oxygen in the air is solely due to the photosynthetic process carried out first by the cyanobacteria and then the green plants, for the past 2 billion years (19).

The accumulation of oxygen in the atmosphere enhanced the production of the ozone layer, which effectively absorbed much of the ultraviolet light (80). Thus, the abiotic synthesis of organic compounds via ultraviolet light ended.

As a result of this, life became dependent on photosynthetic bacteria for a source of organic carbon (80); and, on nitrogen fixing bacteria for a source of fixed nitrogen. The blue-green bacteria (cyanobacteria) represent the culmination of the adjustment to autotrophic growth (80). The blue-green bacteria, through the process of evolution (mutation plus selection), were freed from dependence on exogenous organic nutrients (80). The cyanobacteria have the simplest known nutritional requirements. These organisms can grow in a mineral medium, in the light, with CO_2 as a carbon source, and N_2 as a nitrogen source (181). In essence all they need today is the ocean for the soluble mineral components and the atmosphere for CO_2, N_2; and oxygen for respiration. Another result of the elimination of the toxic ultraviolet radiation on the surface of the earth was that bacteria were now able to inhabit surface waters and the land (80).

With the accumulation of molecular oxygen in the atmosphere, the abiotic synthesis of biomolecules ceased (114, 121, 134), and organic compounds became vulnerable to abiotic oxidative degradation. This would have been devastating to life had it not been for the development of mechanisms for rendering oxygen harmless (24), as is the case with the superoxide dismutase

enzyme complex.

The evolution of oxygenic photosynthesis in the blue-green bacteria, with the resultant production of an oxidative atmosphere, ushered in a new biogeochemical phase, the aerobic phase.

Phase III - The Aerobic Phase of Bacterial Evolution

Since free oxygen is "chemically aggressive", organisms exposed to it undoubtedly had to develop reaction mechanisms for rendering it harmless. One of the best ways of exploiting free oxygen (O_2) is to use it as terminal electron acceptor in a respiratory electron transport chain (24). By this one step the toxic effects of oxygen are eliminated, H_2O is resynthesized and the net free energy obtained from complete oxidation of glucose is increased from that obtained when nitrate is used as terminal electron acceptor (422 kcal/mol for nitrate, and 674 kcal/mol for O_2) (186). In many anaerobic respirers, cytochromes of the b- and c-types were already integrated in electron transport systems (*i.e.*, the sulfate respirers, and the anerobic phototrophs). Cytochromes of the a-type had already developed in such obligate anaerobes as the sulfate respirer, *Desulfovibrio vulgaris* (199), and the bacterium *Eubacterium lentum* (174).

However, with the advent of free oxygen in the atmosphere a new set of enzymes appeared, those capable of activating and utilizing molecular oxygen. There are three major classes of oxygen activating enzymes (119):

(a) enzymes catalyzing the incorporation of O_2 into substrates, called oxygen transferases or oxygenases, or dioxygenases.

(b) enzymes catalyzing the simultaneous 2-equivalent reduction and incorporation of molecular oxygen, called mixed function oxidases, or hydroxylases.

(c) enzymes catalyzing the reduction of both atoms of molecular oxygen from donors of one or two reducing equivalents. These would be the "true" oxidases and cytochrome oxidase falls into this last class and it functions specifically as the terminal acceptor site for aerobic electron transport systems.

As Mason points out in "Homology Among Oxidases" (119), the oxidases possess active sites that contain non-heme iron, copper, heme and flavins as components. All of these would have already evolved to meet the needs of anaerobic bacterial metabolism *i.e.*, the post-primitive bacteria in Phase II. Thus, the appearance of oxidases can be considered the result of continuing genetic modifications of the electron transport chain components in the previously existing anaerobic respirers. The development of multiple terminal oxidases among bacteria is the event that is most relevant to this section of the review, and therefore, the discussion of the distribution and evolution of oxidases will be limited to terminal oxidases, and no attempt is made to consider the bacterial monooxygenases and dioxygenases.

Among contemporary bacteria, four different terminal oxidases are known to exist, namely, cytochromes a_1, a_2(or d), ($a+a_3$), and o (95). The following table summarizes the distribution of the terminal oxidases among the various bacteria found today (95). It is apparent from the table that some bacteria may have only one functional terminal oxidase, whereas others contain two or three. It is also known that the content or type of cytochrome oxidase present may vary, depending both on the phase of the growth curve cycle, as well as the nutritional and physical environment (95).

Microorganism	Cytochrome Oxidase Component			
	a_1	$d(a_2)$	$a+a_3$	o
Acetobacter pasteurianum	+			
Acetobacter peroxydans	+			
Acetobacter suboxydans				+
Achromobacter strain D	+	+		+
Aerobacter aerogenes				+
(Log phase)				+
(Stationary phase)		+		+
Agromyces rhamnosus			+	

Microorganism	a_1	$d(a_2)$	$a+a_3$	o
Alcaligenes faecalis		+		
Azot :acter chroococcum	+	+		+
Azotobacter vinelandii	+	+		+
Bacillus brevis			+	+
Bacillus cereus	+?		+	
Bacillus licheniformis	+			
Bacillus megaterium			+	+
Bacillus stearothermophilus			+	
Bacillus subtilis			+	+
Beneckea natriegens	+	+		+
Corynebacterium diphtheria			+	
Desulfovibrio africanus		+		
Escherichia coli	+	+		+
(Log phase)				+
(Stationary phase)		+		+
Halobacterium cutirubrum			+	+
Halobacterium halobium				+
Halobacterium salinarium			+	+
Hemophilus parainfluenzae	+	+		+
Hydrogenomonas eutropa			+	+
Klebsiella aerogenes	+	+		+
Leptospira	+?		+?	+
Leucothrix mucor				+
Micrococcus denitrificans		+	+	+
Micrcoccus luteus			+	
Micrococcus lysodeikticus			+	
Moraxella lwoffi		+?		

Microorganism	a_1	$d(a_2)$	$a+a_3$	o
Mycobacterium flavum			+	+
Mycobacterium phlei			+	+
Mycobacterium tuberculosis			+	
Neisseria catarrhalis	+			+
Neisseria flava	+			+
Nitrobacter agilis	+			
Nitrobacter winogradskii	+			
Nitrosomonas europaea	+			
Propionibacterium freudenreichii	+	+	+?	
Propionibacterium shermanii	+	+	+?	
Propionibacterium rubrum	+	+	+?	
Propionibacterium pentosaceum	+	+	+?	
Proteus rettgeri	+	+		+
Proteus vulgaris (Log phase)				+
(Stationary phase)	+	+		+
Pseudomonas aeruginosa	+	+		
Pseudomonas oleovorans				+
Pseudomonas ovalis				+
Pseudomonas putida				+
Pseudomonas saccharophila			+	+
Pseudomonas stutzeri		+		+
Rhizobium japonicum			+	+
Rhodopseudomonas palustris			+	+
Rhodopseudomonas spheroides			+	+
Rhodospirillum rubrum				+
Saprospira grandis			+	+

Microorganism	a_1	$d(a_2)$	$a+a_3$	c
Salmonella typhimurium	+	+		
Spirochaeta aurantia				+
Staphylococcus albus			+	+
Staphylococcus aureus				+
Staphylococcus epidermidis				+
Streptococcus lactis		+		
Thiobacillus concretivorus	+	+		
Thiobacillus denitrificans			+	+
Thiobacillus neapolitanus			+	+
Thiobacillus novellus			+	+
Vitreoscilla spp.				+

From Jurtshuk *et al.* (95).

A known,defined,aerobic respiratory electron transport system, which contains multiple terminal oxidases, is found for the following physiological groups of bacteria:

(a) the chemoautotrophs and methylotrophs

(b) the phototrophic blue-green bacteria

(c) the phototrophic purple and green nonsulfur bacteria

(d) the aerobic heterotrophs which are facultative fermenters (many of the *Enterobacteriaceae*, and related organisms)

(e) the obligate aerobes which are oxidase negative

(f) the obligate aerobes which are oxidase positive

Aspects of the origin and evolution of each of the above groups of aerobic bacteria with the exception of the phototrophs, which previously have been discussed,will be the subject of the remainder of this review.

The chemoautotrophic and methylotrophic bacteria are obligately aerobic, Gram negative organisms. The chemoautotrophs derive energy from the oxidation of inorganic compounds, while the methylotrophs derive both the energy and the carbon required for growth from methane (CH_4), and other one carbon organic compounds (181). The cytochrome pattern of the respiratory electron transport systems of these autotrophs is the c-type cytochrome, together with either a, $a+a_3$, o, or in some cases cytochrome b (120). The table below indicates the physiological groups of chemoautotrophs:

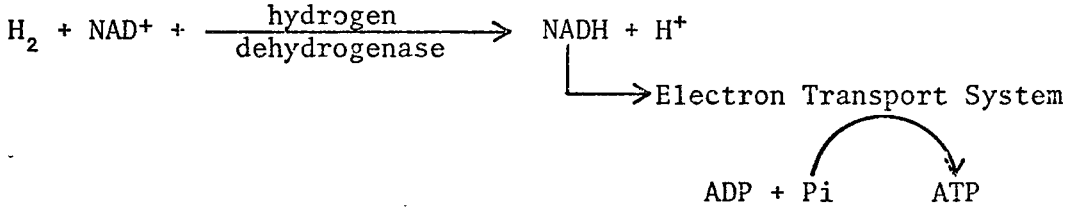
Group	Oxidizable Substrate	Oxidized Product	Terminal Electron Acceptor	Representative Data
nitrifying bacteria: NH_3 oxidizers	NH_3	NO_2^-	O_2	*Nitrosomonas* *Nitrospira* *Nitrosococcus*
nitrifying bacteria: NO_2^- oxidizers	NO_2^-	NO_2^-	O_2	*Nitrosodobus* *Nitrobacter* *Nitrospina* *Nitrococcus*
sulfur oxidizers	H_2S, S $S_2O_3^{2-}$	SO_4^{2-}	O_2; sometimes NO_3^-	*Thiobacillus* *Thiomicrospira* *Sulfolobus*
iron bacteria	Fe^{2+}	Fe^{3+}	O_2	*Gallionella* *Sphaerotilus*
hydrogen bacteria	H_2	H_2O	O_2; sometimes NO_3^-	*Pseudomonas* *Alcaligenes* *Paracoccus* *Nocardia*

From Stainer, R.Y., *et al.* (181).

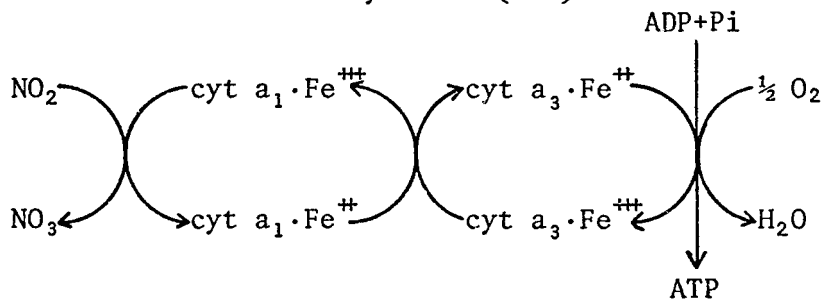
The nitrifying bacteria are mostly obligate autotrophs, although some *Nitrobacter* strains have been shown to use acetate as an energy source (181, 101). The sulfur, iron, and hydrogen bacteria are facultative autotrophs, and can readily be grown chemoheterotrophically (181). When the chemoautotrophs are grown on inorganic synthetic media, high concentration levels of two enzymes are found (carboxydismutase, phosphoribulokinase), both

characteristic of the Calvin cycle (181). In addition, many obligately autotrophic thiobacilli and nitrifying bacteria possess carboxysomes (specialized procaryotic organelles which contain carboxydismutase) (181). The presence of these enzymes supports the idea that in chemoautotrophs CO_2 is assimilated through the Calvin cycle (181).

The chemoautotrophs generate ATP by oxidative phosphorylation (142). In the hydrogen bacteria, ATP is generated through an integrated electron transport system by the reaction sequence indicated below:



Since hydrogen bacteria also oxidize a wide range of organic compounds, in addition to their capability to grow autotrophically, their electron transport chain is highly developed. Electron transport particle fractions of cell free extracts from *Nitrobacter*, also mediate the oxidation of nitrite (NO_2^-) by O_2 (181). Cytochromes a_1 and a_3 are the components in an electron transport chain as shown schematically below (181):



The terminal oxidase components of the respiratory chain are specifically involved in nitrite oxidation. The oxidation of ammonia NH_3 and H_2S by chemoautotrophs, as exerogonic reactions, has not been studied in such detail as has the oxidation of nitrite (181). However, it is believed that the first

step in NH_3 oxidation is its conversion to hydroxylamine (NH_2OH), which is mediated by an oxygenase (181). Because so little is known on the structure and function of the chemoautotrophic electron transport chain, the precise mechanism for the oxidation of inorganic substrates remains speculative.

The methylotrophs fall into two respiratory subgroups (181):

(a) obligate methylotrophs (*Methylosinus, Methylocystis, Methylomonas, Methylobacter, Methylococcus*)

(b) facultative methylotrophs (*Hyphomicrobium*)

The obligate methylotrophs possess complex membrane systems, which are of two types: type I (stacks of flattened disks), and type II (pairs of membranes, running parallel to the cytoplasmic membrane). It has been noted that among the obligate methylotrophs, the organisms with type II membrane structure contain enzymatic machinery which resembles that of the facultative methylotrophs. Those obligate methylotrophs with type I membrane systems have enzymatic machinery resembling many obligate chemoautotrophs (181).

Because of the simplicity of the substrates required for their growth, both chemoautotrophs and methylotrophs were once considered to be the first physiological group of bacteria to inhabit the earth (181). In light of the Oparin-Haldane hypothesis, and subsequent supportive geochemical evidence concerning the nature of the primitive atmosphere, the chemoautotrophs and methylotrophs must now be viewed as a more advanced group of bacteria (181, 24, 80, 101). Both their biochemical machinery as well as their structural organization are as complex as those of the aerobic chemoheterotrophs (181).

Any speculation on the origin of the chemoautotrophs and methylotrophs cannot be divorced from a consideration of the atmospheric constituents, and

of any selective pressure operating at the time of their proposed origin. The placement of the chemoautotrophs in the highly oxidative phase III is consistent with their aerobic respiratory metabolic pattern, and also with their development due to the selective pressure toward "autotrophy" at the end of phase II. This later point can be explained by the depletion of organic nutrients whose abiotic synthesis ceased as a result of the development of a neutral oxygenating atmosphere. As a result of bacterial photosynthesis, the organic carbon supply on earth was slowly being replenished. However, it is reasonable to assume that at the end of phase II, and beginning of phase III, some of the earliest obligate aerobes would have had a survival advantage by possessing the capability to oxidize the abundant inorganic or simple one carbon organic compounds(i.e., CH_4) present.

It has been suggested that aerobic chemoautotrophs and methylotrophs arose from photosynthetic bacteria (either oxygenic or anaerobic) (4, 24, 181, 23). The assumption inherent in this suggestion is that the contemporary chemoautotrophs and methylotrophs lost their photosynthetic capability (181). This assumption seems tenable in light of the structural and biochemical properties shared by some members of both phototrophic bacteria and the chemoautotrophs and methylotrophs. The following are the common morphological and physiological features:

(1) several elaborate, distinctive types of internal membrane ultrastructure.

(2) the use of the pentose phosphate cycle as the major pathway for glucose oxidation for both blue-green bacteria and chemoautotrophs.

(3) presence of the Calvin cycle or its analogue, the pentose phosphate cycle.

(4) location of a key enzyme of the Calvin cycle (carboxydismutase) in carboxysomes.

In this proposed scheme of bacterial evolution, the chemoautotrophs and methylotrophs are the only major group of organisms believed to have developed through loss of physiological function that was present in their immediate ancestors. This regressive evolution resulted in the development of a new type of physiological line which today is known as autotrophy. Its development could only have occurred in an oxygenated atmosphere.

The other line of physiological development occurred among the anaerobic heterotrophic bacteria, which became facultatively anaerobic, and subsequently obligately aerobic chemoheterotrophs (4). Today these facultatively anaerobic and obligately aerobic bacteria are among the best studied organisms in microbiology. Their physiology is consistent with the thesis that they developed along a main line which originated directly from the anaerobic respirers (obligately anaerobic bacteria using organic compounds and/or nitrate as terminal electron acceptors). These obligately anaerobic respirers had already highly developed electron transport systems and the facultatively anaerobic chemoheterotrophs probably serve as the bridge between the obligate anaerobes and the obligate aerobes (110, 43, 4). Representative organisms are mostly found in a single family: the *Enterobacteriaceae*. A list of genera would include *Escherichia, Salmonella, Shigella, Yersinia, Proteus, Ewinia, Enterobacter,* and *Serratia*. These bacteria when grown anaerobically produce energy through the fermentation of carbohydrates, and when grown aerobically a wide range of organic compounds serve as substrates for respiratory metabolism (181). In addition to oxygen, many of these bacteria alternately use nitrate as the terminal electron acceptor and carry out "anaerobic respiration" (186). Pichinoty has reported that the respiratory nitrate reductase, in many facultative anaerobes, is induced by nitrate and repressed by air (145). The nitrate

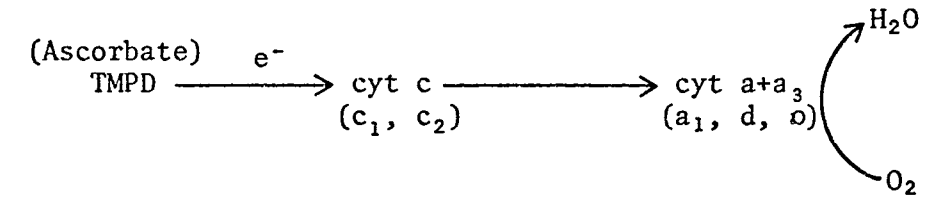
reductase in most bacteria is associated with a b-type cytochrome (159).

Nitrate respiration (an anaerobic process) produces energy yields which approach those of aerobic respiration (80). The complete oxidation of glucose to carbon dioxide with nitrate being reduced to nitrite is 422 kcal/mol; whereas, the complete oxidation of glucose to carbon dioxide with oxygen being reduced to H_2O is 674 kcal/mol (186). The nitrate respiration of *Escherichia coli* generates two ATP's per electron pair transferred, while the aerobic respiration of *E. coli* generates three ATP's per electron pair transferred (196).

In light of the information presented above it is highly probable that nitrate respiration preceded aerobic respiration, and that the anaerobic electron transport chain was genetically modified so that oxygen could now replace nitrate as the terminal acceptor of electrons (80, 199).

Additional evidence supporting the proposal that facultative anaerobes predated obligate aerobes is the data obtained from the N, N, N', N' - tetramethyl-*p*-phenylenediamine (TMPD) oxidation study which was used as a Kovacs oxidase test (96, 97, 98)

The TMPD quantitative oxidase test is a manometric assay system employing ascorbate and N, N, N', N'-tetramethyl-*p*-phenylenediamine (TMPD) to quantitate terminal oxidase activity in bacterial non-proliferating whole cells (96, 97, 98). This test enables quantitation of the hitherto qualitative Kovacs oxidase test. According to Jacobs (88), TMPD acts to mediate electrons from ascorbate to cytochrome c in the electron transport chain, as indicated in the scheme below:



Cytochrome oxidase itself does not react with TMPD, unless there are catalytic amounts of cytochrome c present.

Recently, the results of a survey study of the TMPD oxidation rates of 79 bacterial strains (representing 34 genera) was published (97, 98). Resting-cell suspensions of physiologically diverse bacteria were examined for their capabilities of oxidizing TMPD. For organisms having this capability, it was possible to calculate the conventional TMPD oxidase Q (O_2) value (microliters of O_2 consumed per hour per milligram dry weight). All cultures were grown heterotrophically at 30C, under identical nutritional conditions, and were harvested at the late-logarithmic growth phase (98). The TMPD oxidase Q (O_2) values showed perfect correlation with Kovacs oxidase test (97,98). In fact, the increased sensitivity of this quantitative assay allowed for further reclassification within the two major divisions of Kovacs oxidase-positive and -negative groups (97,98). The table below lists the organisms studied, and their Kovacs oxidase reaction (97):

Organism	Kovacs oxidase reaction	Fig. 2 abbreviation
Group I		
Achromobacter xerosis(14780)	+	Ax
Aeromonas liquefaciens(14715)	+	Al
Agrobacterium tumefaciens 15955)	+	Agt
Alcaligenes faecalis(8750)	+	Alf
Azotobacter vinelandii O(12518)	+	Av
Azotomonas insolita(12412)	+	Ai
Branhamella catarrhalis(25238)	+	Nc
Branhamella catarrhalis Gp4	+	Nc
Branhamella catarrhalis NC31	+	Nc
Moraxella osloensis	+	Mo
Neisseria elongata(25295)	+	Ne
Neisseria flava(14221)	+	Nf
Neisseria mucosa	+	Nm
Neisseria sicca	+	Ns
Rhizobium meliloti F-28	+	Rm
Rhizobium meliloti 3DOa1	+	Rm
Pseudomonas acidovorans(15668)	+	Psac
Pseudomonas aeruginosa(15422)	+	Psa
Pseudomonas bathycetes C_2M_2	+	Psb
Pseudomonas fluorescens(13525)	+	Psf
Pseudomonas stutzeri 320	+	Pss

Organism	Kovacs oxidase reaction	Fig. 2 abbreviation
Spirillum itersonii (12639)	+	Si
Sporosarcina ureae	+	Su
Group II		
Aeromonas hydrophila (9071)	+	Ah
Flavobacterium capsulatum (14666)	+	Fc
Micrococcus luteus	+	Sl
Micrococcus luteus	+	Sf
Vibrio parahaemolyticus biotype *alginolyticus* 156-70	+	Va
Vibrio parahaemolyticus FC1011	+	Vp
Vibrio parahaemolyticus SAK 3	+	Vp
Group III		
Acinetobacter calcoaceticus (19606)	-	Ac
Acinetobacter calcoaceticus 208	-	Ac
Arthrobacter globiformis (8010)	-	Ag
Bacillus cereus	-	Bc
Bacillus firmus (14575)	-	Bf
Bacillus megaterium	-	Bm
Bacillus subtilis W-23	-	Bs
Corynebacterium 7E1C (19067)	-	7E1C
Corynebacterium diphtheriae (11913)	-	Cd
Enterobacter aerogenes	-	Ea
Escherichia coli B	-	Ec
Escherichia coli B	-	Ec
Escherichia coli B/r	-	Ec
Escherichia coli C	-	Ec
Escherichia coli Crookes	-	Ec
Escherichia coli K-12	-	Ec
Escherichia coli K-12S	-	Ec
Escherichia coli K-12S	-	Ec
Escherichia coli F	-	Ec
Escherichia coli $0111a_1$	-	Ec
Klebsiella pneumoniae M5a1	-	Kp
Mycobacterium fortuitum	-	Mf
Mycobacterium phlei	-	Mp
Mycobacterium smegmatis	-	Ms
Micrococcus luteus (4698)	-	Ml
Nocardia asteroides (3308)	-	Na
Proteus morganii	-	Pm
Proteus vulgaris	-	Pv
Pseudomonas maltophilia (13637)	-	Psm
Staphylococcus aureus (6538)	-	Sau
Staphylococcus aureus	-	Sau
Streptomyces griseus (10137)	-	Sg
Vibrio cholerae biotype *proteus*	-	VM
Group IV		
Bacillus pumilus 70	-	Bp
Bacillus subtilis	-	Bv
Gaffkya tetragena (10875)	-	Gt
Gaffkya tetragena	-	Gt
Klebsiella pneumoniae (13883)	-	Kp
Lactobacillus casei (7469)	-	Lc

Organism	Kovacs oxidase reaction	Fig. 2 abbreviation
Pediococcus cerevisiae (8081)	-	Pc
Salmonella typhimurium (6444)	-	St
Serratia marcescens	-	Sm
Staphylococcus aureus	-	S
Streptococcus faecalis (8043)	-	Sf
Streptococcus faecalis (9790)	-	Sf
Streptococcus pneumoniae (6303)	-	Spn
Streptococcus pyogenes (10389)	-	Spy
Vitreoscilla stercoraria (15218)	-	Vs
Xanthomonas phaseoli (9563)	-	Xp

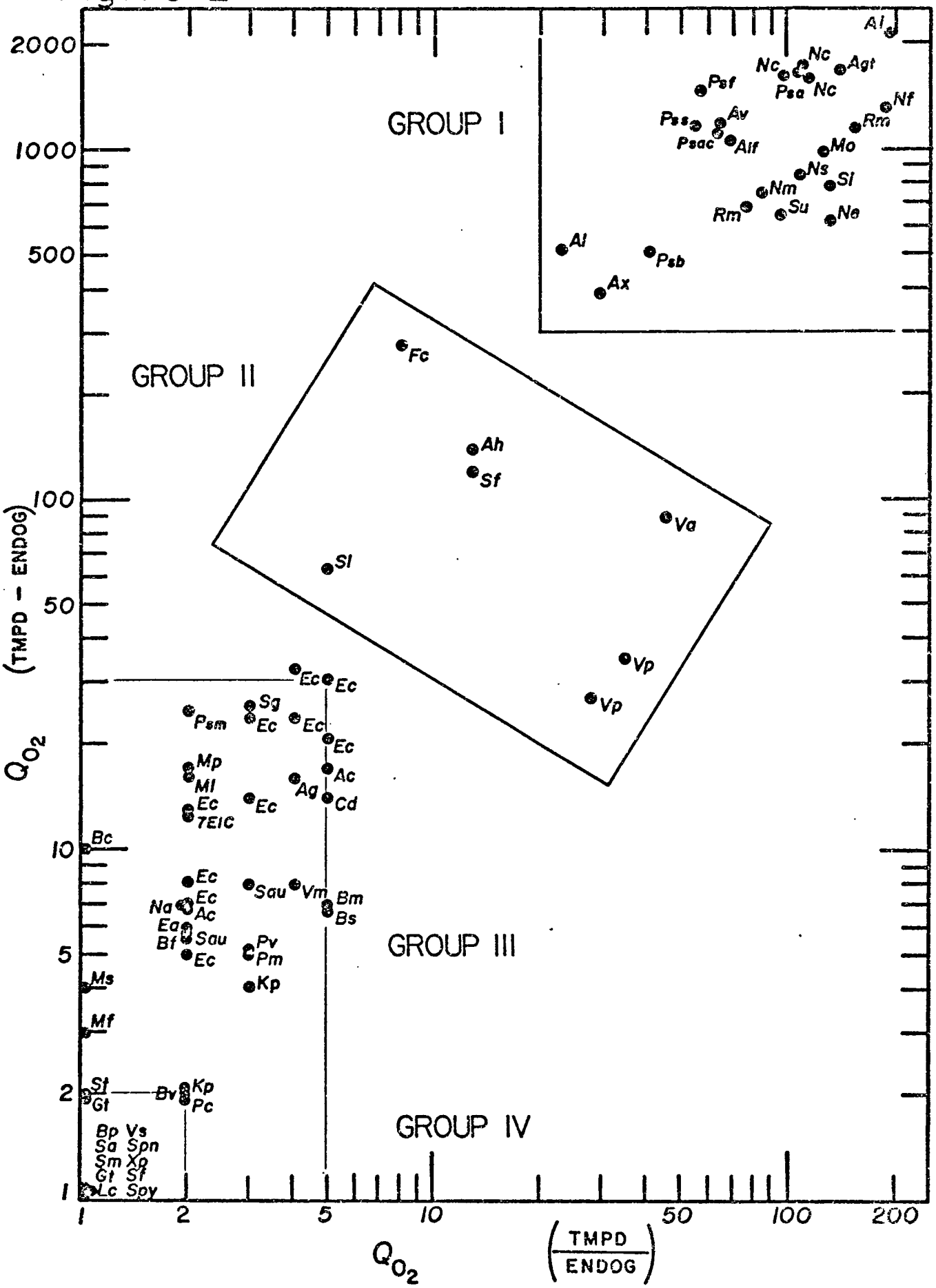
Figure 2 indicates the further reclassification within the two major divisions of Kovacs oxidase-positive and negative groups (97). In the diagram, Groups I and II contain all of the oxidase positive microorganisms and the bacteria listed in group I had the highest TMPD oxidase rates, the $Q(O_2)$ values (microliters of O_2 consumed per hour per milligram dry weight at 30 C) ranging from 393 to 2,164. The organisms listed in group II have moderately high TMPD oxidase activity, the $Q(O_2)$ values ranging from 27 to 280. All oxidase-negative bacteria fall into groups III and IV. Bacteria in group III have low but still measurable TMPD oxidase rates, the $Q(O_2)$ values ranging from 3 to 33, whereas the bacteria found in group IV are inert and unable to oxidize TMPD (97). From this diagram one is able to resolve that point which separates oxidase-positive from oxidase-negative bacteria (97).

This point, for non-proliferating cells, was found to be an absolute TMPD oxidation $Q(O_2)$ value of 33 (after correcting for the endogenous rate by subtraction) and a $Q(O_2)$ TMPD/endogenous ratio of 5; the latter parameter indicated that the uncorrected TMPD oxidation $Q(O_2)$ value had to be five times greater than the rate for endogenous respiration (97). All Kovacs oxidase-positive organisms were found to have TMPD oxidase $Q(O_2)$ values greater than these two metabolic parameters, whereas all Kovacs oxidase-negative organisms had lower values (97).

SURVEY ANALYSIS

TMPD-OXIDASE REACTION

Figure 2



In general, bacteria that exhibit a respiratory mechanism have high TMPD oxidase values, whereas fermentative organisms have low TMPD oxidase activity (98). The exceptions will be discussed later. This quantitative study also demonstrates that organisms that (i) lack a c-type cytochrome, or (ii) lack a cytochrome-containing electron transport system, like the lactic acid bacteria, exhibit low or negligible TMPD oxidase $Q(O_2)$ values (98). Through studies with *Azotobacter vinelandii* whole cells, it has been found that modified growth conditions can lower the TMPD oxidase rates by either (i) altering the nature of the intracellular terminal oxidase formed (or induced), or (ii) altering surface permeability (96). From the 79 bacterial species (36 genera) examined, it appears that this quantitative oxidase test has taxonomic value that can differentiate the oxidative relationships between bacteria at the subspecies, species, and genera levels (98).

As was stated earlier, the results of the TMPD quantitative oxidase test support the proposal that the facultatively anaerobic bacteria preceded the obligate aerobes in evolutionary development. Most of the facultatively anaerobic bacteria show low or no TMPD oxidation capability (97, 98). These oxidase negative bacteria have a cytochrome pattern consisting of types a, d, o, and b (120). Cytochrome c is often absent in these bacteria (120). Since the c-type cytochrome is believed to be required for TMPD oxidation, the low TMPD oxidation data would be expected for facultatively anaerobic bacteria. The data on TMPD oxidation rates can be extended further to enable one to propose a general scheme for the evolution of aerobic bacteria. The overall scheme proposed in this review consists of two divisions: (i) the oxidase negative bacteria, and

(ii) the oxidase positive bacteria. The oxidase negative bacteria are, in turn subdivided into organisms unable to oxidize TMPD (Group IV), and organisms having low but still measurable TMPD oxidase rates (Group III). The oxidase negative bacteria, with low cytochrome oxidase activity, lack cytochrome c entirely, or if cytochrome c is present it is not integrated with the terminal oxidase (120, 97). These bacteria represent the primitive aerobic bacteria, which lack an integrated terminal or cytochrome oxidase enzyme complex (97). The most ancient oxidase negative bacteria, most probably, are those bacteria unable to oxidize TMPD (Group IV). The inability of group IV bacteria to oxidize TMPD can be attributed to the absence of a c-type cytochrome and/or the lack of a functional terminal oxidase, *i.e.*, cytochrome o, $a+a_3$, etc., or to both (97). Many of the bacteria listed in group IV have previously been shown to lack cytochrome components (*i.e.*, the lactic acid bacteria) (44); others only possess the flavo-protein type electron transport systems that are completely devoid of cytochrome components (54). A list of mostly facultatively anaerobic bacteria falling in group IV, having no TMPD oxidation capability, include *Bacillus pumilus, Bacillus subtilis, Gaffkya tetragena, Staphylococcus aureus (S. albus), Xanthomonas phaseoli, Vitreoscilla stercoraria,* some enterobacteria, *i.e., Klebseilla pneumonia, Salmonella typhimurium, Serratia marcescens* (97, 98). The *Lactobacillus, Pediococcus,* and *Streptococcus* species of group IV are the aerotolerant obligate fermenters, and thus are not considered in this section on aerobic evolution.

The more highly evolved oxidase negative bacteria are those organisms with low, but still measurable TMPD oxidation capability. Most of the family *Enterobacteriaceae* are included in this group III. Of interest, however, are organisms of the genera *Mycobacterium (Mycobacterium fortuitum,*

Mycobacterium phlei, Mycobacterium smegmatis), Nocardia (Nocardia asteroides, Nocardia rhodochrous), Streptomyces (Streptomyces griseus), and some Bacillus species *(B. cereus, B. firmus, B. megaterium, B. subtilis* W-23) which are obligately aerobic yet lack significant TMPD oxidation capability and are in essence Kovacs oxidase negative (97). Other examples of obligate aerobes which have low TMPD oxidation rates (low cytochrome oxidase activity) are the genera *Micrococcus, Xanthomonas, Acinetobacter, Acetobacter,* some species of the *Bacillus* and *Corynebacterium* genera (97, 98). This would imply that, in such oxidase-negative obligate aerobes, there must be a type of terminal oxidase that allows for some residual TMPD oxidation, or that perhaps a permeability problem exists which prevents TMPD from entering the cell and thus does not allow this electron donor to be oxidized at the high rates observed for most oxidase-positive organisms (97). The possibility that the mycobacterial-nocardial-type bacterial cells are impermeable to TMPD is supported by studies which indicate that by merely sonicating resting-cell suspensions of *M. smegmatis* one can increase the uncorrected TMPD oxidase $Q(O_2)$ value fivefold (98). In any case these obligately aerobic oxidase negative bacteria must be considered highly evolved organisms due to their dependence on oxygen as a metabolite. Their position in this scheme of evolution is intermediate, between facultative anaerobic oxidase negative and obligate aerobic oxidase positive bacteria. That is, even though these obligate aerobes have evolved a high degree of capability for utilization and exploitation of oxygen, an integrated terminal oxidase complex consisting of cytochrome c, integrated with the terminal oxidase, has not developed in these bacteria. Meyer and Jones (120), have reported that the c-type cytochrome is often absent in both the facultative anaerobes and the oxidase negative obligate aerobes.

The most highly evolved group of bacteria, from both a physiological and biochemical consideration, are the obligately aerobic, oxidase positive, organisms (23, 4, 43, 133, 80, 117). These are the organisms with high TMPD oxidation capabilities, having a high dependence on oxygen as a metabolite. Representative organisms are the bacteria of groups I and II in the TMPD oxidase survey study (see page 91). The bacteria found in these groups I and II possess an integrated terminal or cytochrome oxidase enzyme complex consisting of both a c-type cytochrome and a terminal oxidase component such as cytochrome o and/or $a+a_3$, possibly in combination with cytochrome a, or a_2 (97). This later point has been thoroughly discussed in a recent review article (95). The Kovacs oxidase-positive bacteria listed in group I have high corrected TMPD $Q(O_2)$ values ranging from 393 to 2,164 (97). In this group one finds the *Achromobacter*, *Azotobacter*, *Neisseria*, *Pseudomonas*, and *Spirillum* species (97). The oxidase-positive bacteria listed in group II exhibited intermediate but still relatively high TMPD oxidation rates, the $Q(O_2)$ values ranging from 27 to 280 (97). Organisms in this group included members of *Aeromonas*, *Flavobacterium*, *Micrococcus*, and most *Vibrio* species (97). It should be noted that a few facultative anaerobes (which are basically fermenters) demonstrate high cytochrome oxidase activity. Their high TMPD oxidation rates can be attributed to a highly evolved and integrated cytochrome oxidase complex. These facultative anaerobes can be viewed as highly evolved aerobes which retained the fermentative capability. Representative bacteria are *Aeromonas hydrophila*, *A. liquefaciens*, *Vibrio cholerae*, and *V. parahaemolyticus*.

In conclusion, a scheme for the evolution of aerobic heterotrophic bacteria is proposed on the basis of:

(a) TMPD oxidation capability, which measures the extent to which

oxygen can be used by the bacterial cytochrome oxidase reaction.

(b) physiological growth considerations (whether the bacteria can grow facultatively anaerobic or obligately aerobic), and

(c) the cytochrome content found for the electron transport systems of aerobic bacteria.

The facultatively anaerobic bacteria, which exhibit low or no TMPD oxidation capability would be considered the earliest aerobic bacteria. The obligate aerobes, with low TMPD oxidation capability, possibly due to the lack of a c-type cytochrome integrated in a cytochrome oxidase complex, would represent organisms of an intermediate level of evolutionary development. The obligate aerobic bacteria, with high TMPD oxidation capability, due to an integrated terminal oxidase complex, are proposed to be the highest evolved aerobic bacteria.

Discussion

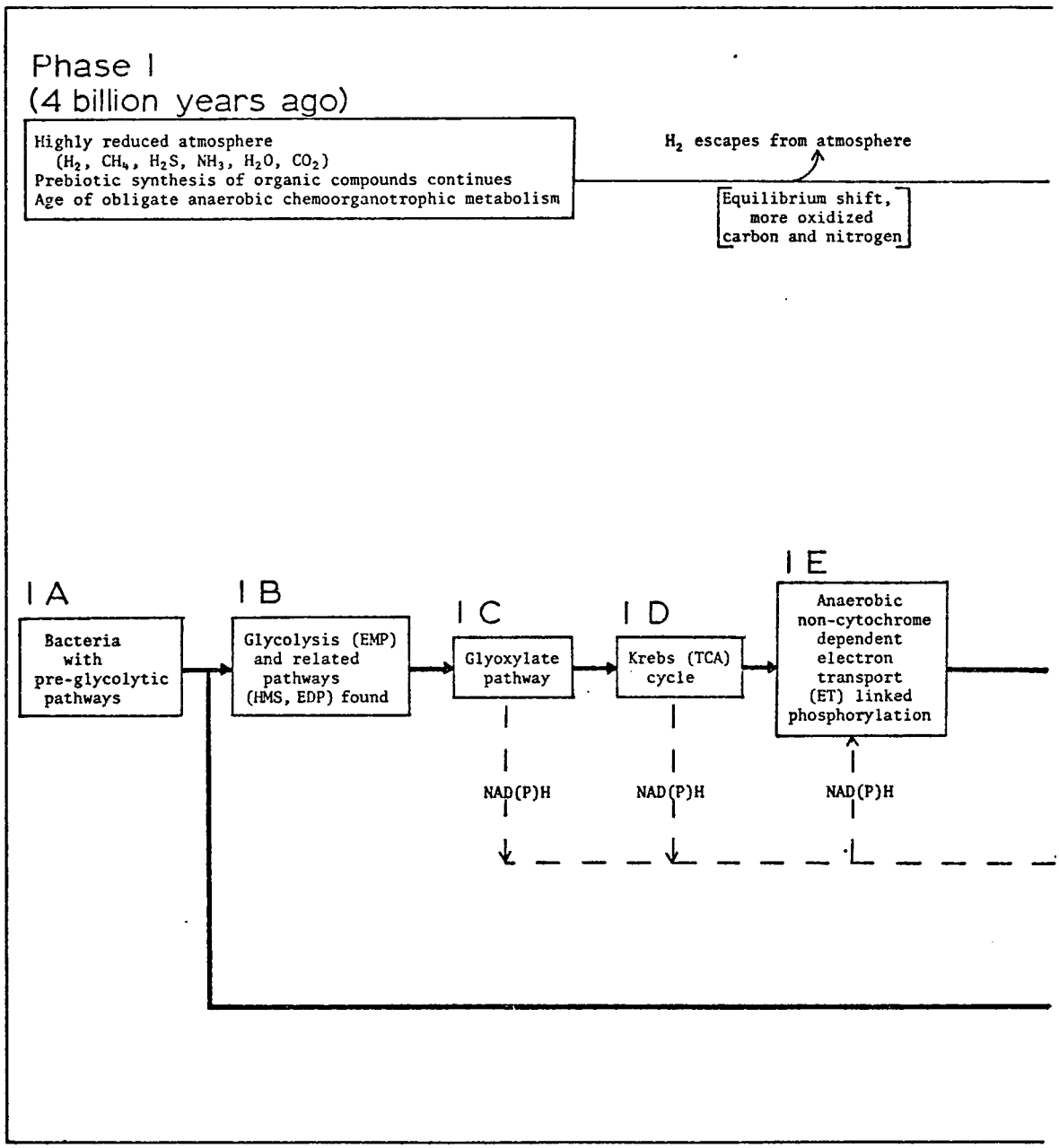
The subject of bacterial evolution in the past decade has become an increasingly thought prevoking topic for both the evolutionists and the microbiologist. It is recognized that bacteria represent the most "primitive" life forms that exist on earth today. The microbiologist, who is familiar with the physiological diversity of bacteria, can predict with a certain degree of confidence, the metabolic types that could have existed under defined environmental conditions. All life forms, bacteria included, are reflections of the immediate or past environment. Those bacteria that survive today represent the best adapted life forms, and they have evolved through all changes in environmental pressure that accompanied the chemical evolution of our planet, Earth. In essence *all life* is an expression of the continued physiological solution(s) to environmental changes and pressures. Those life forms, including the bacteria, who most successfully adapted, and transmitted this physiological solution to their progeny (via genes) are those that survive today. The present review is a proposal for a bacterial evolutionary scheme, based on physiological development. Others have proposed similar type schemes for bacterial evolution (23,24,25,43,80,117,118) each one emphasizing the author's personal views on which biochemical, physiological and/or morphological characteristics best represent the most realistic path of development. For example, Broda proposes a scheme which asserts that phototrophic bacteria evolved *directly* from fermentative bacteria. The phototrophic bacteria were, then, the immediate ancestors of all other physiological types of bacteria. Broda speculates that the sulfate and nitrate respiring bacteria could not have evolved before the atmosphere

became aerobic, since these oxidized forms of sulfur and nitrogen were not present in the anaerobic atmosphere. Also, according to the author, the aerotolerant fermenters (lactic acid and propionic acid bacteria) evolved from aerobic respirers through loss (regressive evolution) of the respiratory chain (23,24,25).

An alternate scheme for bacterial evolution has been proposed independently by Hall and Margulis which also is based on the development of increasing physiological complexity (43,80,117,118). This scheme is the one that is most consistent with the overall scheme presented herein. However, the scheme proposed in this review is more detailed and considers all physiological aspects prior to placing a group of bacteria in a particular evolutionary niche.

In this review, some parts of each of the previously proposed schemes have been incorporated, other proposed ideas were completely excluded as being inconsistent with a physiological development starting from simple bioenergetic systems that have evolved to more complex ones. This scheme of bacterial evolution, as are all others, is both presumptive and speculative in nature, and attempts to show how evolution might have occurred along the pathway of progressively increasing metabolic complexity amony procaryotes. The following chart (Fig. 3, and the related tables that follow) summarizes the highlights of the proposed scheme for bacterial evolution.

Figure 3 A Scheme for Bacterial Evolution



Based on Physiological Development

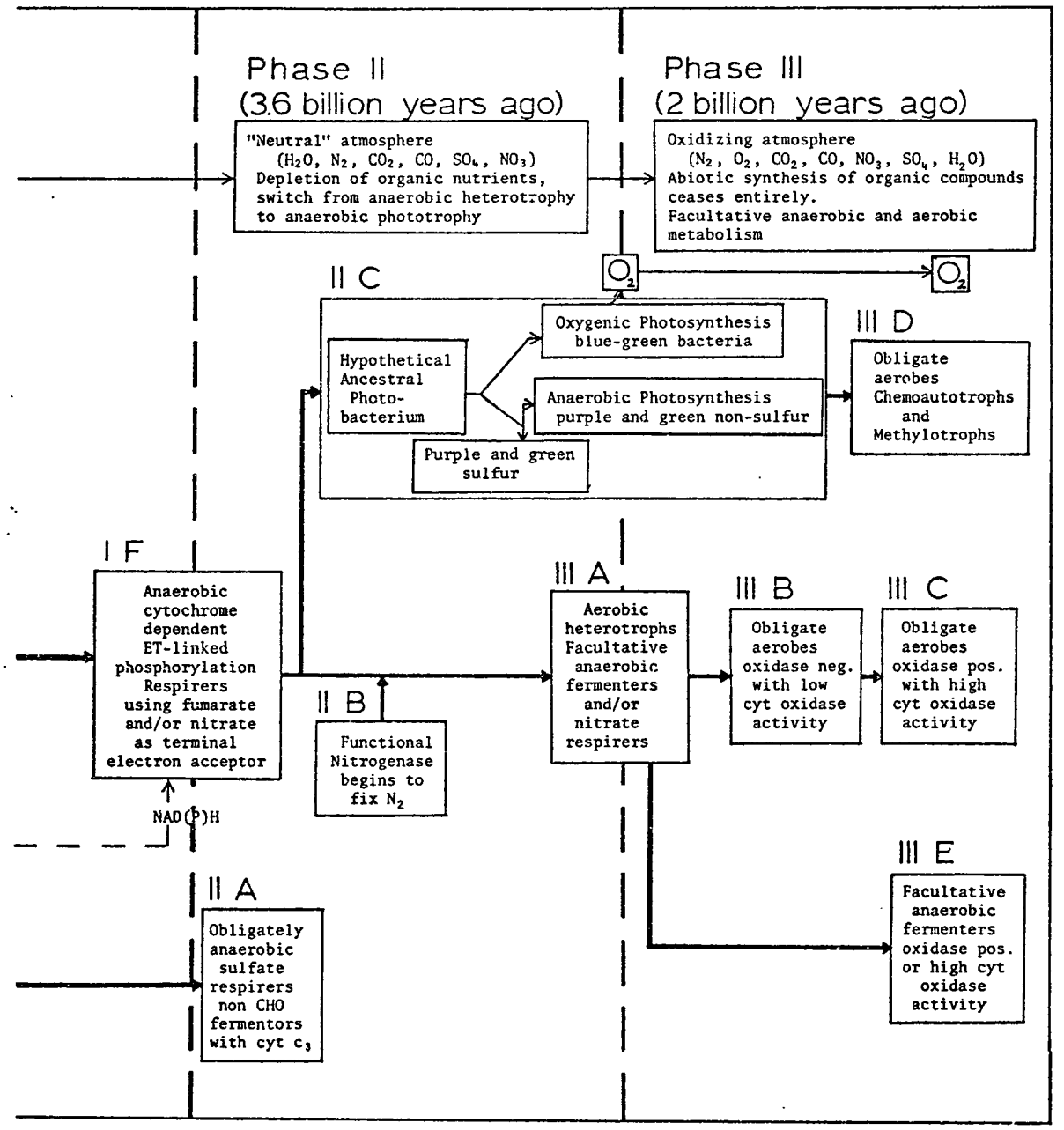


Table 1

The Physiological Characteristics of Bacteria Associated with Different Phases of Evolutionary Development

Phase I (highly reduced atmosphere)

I A

Bacteria with pre-glycolytic pathways

- cytochromes absent
- NAD, CoA, ferredoxins, and TPP are the coenzymes of these pathways.
- no electron transport systems (ETS) are present
- species do not contain a glycolytic pathway, and no intact Krebs cycle is found.
- 5 classes of energy yielding reaction...
  1) Stickland reaction as found today in 15 species of clostridia, e.g., *C. tetani, C. histolyticum, C. tetanomorphus, C. propionicum;* and many species of the genus *Peptococcus.*
  2) fermentation of ethanol and/or acetic acid as exhibited by *C. kluyveri* and *Methanobacillus omelianskii* strain S.
  3) fermentation of lactate and pyruvate by clastic reactions, i.e., *C. propionicum.*
  4) fermentation of purines and pyrimidines by some *Clostridium* and *Peptococcus* species, and *Veillonella alcalescens*
  5) the methanogenic bacteria (no respiratory chain or Calvin cycle) using CO_2 as the hydrogen acceptor

I B

Glycolysis (EMP) and related pathways (HMS, EDP)

- cytochromes absent
- flavodoxins and ferredoxins present
- no electron transport systems (ETS) are present
- no intact Krebs cycle; one or more of the Krebs cycle reactions found
- surviving types are the:
  1) the butyric acid *Clostridia*
  2) the lactic acid bacteria (this specific group are today aerotolerant (oxygen tolerant), indicating that they adapted well to aerobic environments)
  3) some species of *Peptococcus* and *Ruminococcus*

Table 1 (continued)

I C

Glyoxylate pathways

- the glyoxylate cycle is found in many *Clostridium* which do not contain an intact Krebs cycle

I D

Krebs (TCA) cycle

- even though most anaerobic chemoheterotrophs do not contain the intact Krebs cycle, most of the enzymes of the cycle are present in all bacteria, since many of the intermediates of the Krebs cycle are required in biosynthetic pathways

I E

Anaerobic non-cytochrome dependent electron transport-linked phosphorylation

- cytochromes absent
- primitive, flavoprotein mediated, electron transport systems present.
- Organic compounds used as terminal electron acceptor.
- Examples:
  1) NADH $\longrightarrow$ flavoprotein $\xrightarrow[\text{ADP+Pi}]{?}$ $\xrightarrow[\text{ATP}]{}$ crotonyl-CoA in *C. kluyveri*
  2) the reductive deamination of glycine by *C. sticklandii* and *C. lentoputrescens*
  3) $NADH_2$ $\longrightarrow$ flavoprotein $\xrightarrow[\text{ADP+Pi}]{}$ $\xrightarrow[\text{ATP}]{}$ fumarate in *Streptococcus faecalis* 10CL
- the shortage of suitable organic conpounds as electron acceptors, *e.g.*, fumarate, prevented the extensive utilization of this process

I F

Anaerobic cytochrome dependent electron transport-linked phosphorylation. Respirers using fumarate and/or nitrate as terminal electron acceptors.

- first cytochromes evolve and are utilized
- cytochromes of the b- and c-type are components in the electron transport systems
- multiple a-type cytochromes first appear, but are not extinsively utilized in integrated electron transport systems
- organisms having carbohydrate fermentation mechanisms, and an intact Krebs (TCA) cycle
- Examples:
  1) cytochromes b and c, in an electron transport system with fumarate as terminal electron acceptor, (*Bacteroides fragilis*)

Table 1 (continued)

2) cytochrome b functions in electron transport system utilizing fumarate or nitrate as terminal electron acceptors, (*C. thermoaceticum, Selenomonas ruminantium, Anaerovibrio lipolytica, Veillonella alcalescens, Vibrio succinogenes*)
3) obligate anaerobes utilizing only nitrate as terminal electron acceptors as in *C. perfringens* or *Eubacterium lentum* contains cytochrome a, b, c, and o.
electron transport-linked phosphorylation has yet to be demonstrated
4) aerotolerant anaerobic fermenters having cytochromes of b-type utilized in electron transport systems with fumarate as terminal electron acceptor. Organisms with multiple a-type cytochromes. These organisms are well adapted for survival in aerobic environments, and in some instances can possibly use O_2 as terminal electron acceptor (propionic acid bacteria)

Table 1 (continued)

Phase II (neutral atmosphere)

II A

Obligately anaerobic sulfate respirers (non-carbohydrate fermenters)

- multiple c-type (c_3), b-type, and d(a_2) -type cytochromes are present
- non-heme iron proteins, flavoproteins, and quinones are also present
- electron transport systems, utilizing SO_4 as terminal electron acceptor are present
- carbohydrates are not fermented
- organic acids used as growth substrates
- organic acids are fermented through reaction mechanisms similar to the pre-glycolytic pathways
- the sulfate reducing bacteria are proposed to have developed along a non-glycolytic path from the earliest pre-glycolytic bacteria
- Examples:
  1) 5 species of *Desulfovibrio*
  2) 3 species of *Desulfotomaculum*

II B

Functional Nitrogenase utilized for growth

- the nitrogenase system probably appeared first in the more primitive organisms of Phase IA; its function originally was to detoxify cyanides and cyanogens
- organisms now fix N_2 because of a selective pressure caused by the depletion of organic nitrogen compounds
- primitive electron transport systems are now required for N_2 reduction, with components such as hydrogenases, ferredoxins, and mechanisms such as the pyruvate phosphoroclastic reactions; the nitrogenase system operationally requires low redox potentials
- the nitrogenase system is distributed more prominantly among the anaerobic heterotrophs rather than the aerobic heterotrophs
- N_2 fixation is distributed among:
  1) *C. pasteurianum*, *C. butyricum* and many other butyric acid clostridia
  2) some phototrophic bacteria (*i.e.*, *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, the genus *Rhodomicrobium*, the blue-green bacteria)
  3) many sulfate reducing bacteria (sulfate respirers)
  4) some facultative anaerobes (*i.e.*, *Klebsiella pneumoniae*, *K. rubiacearum*)
  5) obligate aerobic bacteria (*i.e.*, some *Bacillus* species, the *Azotobacteraceae* family).

Table 1 (continued)

II C

Development of photosynthesis and aerobiosis

- light first functioned to generate ATP through a cyclic photosystem, much like the cyclic system of contemporary purple and green bacteria
- the ATP, so produced, energized the assimilation of exogenous organic nutrients
- once a depletion of organic nutrients occurred, a selective pressure for the modification of the cyclic system to a non-cyclic photosystem which generates reducing power to enable the fixation of CO_2
- because of shading, two different pigment centers appeared:
    1) bacteriochlorophyll a (not storing enough energy to ever be capable of the oxidation of H_2O, i.e., the anoxygenic photosynthesizers)
    2) chlorophyll a (capable of the photolysis of H_2O producing O_2 and hydrogen for reducing power
- the purple and green non-sulfur bacteria also can grow aerobically, among the first to use a respiratory mechanism
- representative species:
    1) the oxygenic photosynthesizers (i.e., the blue-green bacteria)
    2) the anoxygenic photosynthesizers (i.e., purple and green nonsulfur, and purple and green sulfur bacteria). (see text for a complete listing of these genera)

Table 1 (continued)

Phase III (Oxygen in atmosphere)

III A

Aerobic heterotrophs, facultative anaerobic fermenters and/or nitrate respirers

- cytochrome patterns: a, d, o, b, and sometimes c are present
- cytochrome c is often absent, or is non-integrated with the terminal oxidase
- these organisms show either no or low TMPD oxidation capability
- these bacteria can grow fermentatively in an anaerobic environment, or through aerobic respiration in an aerobic environment
- many of these bacteria can replace nitrate for oxygen, as terminal electron acceptor
- representative genera include: *Escherichia, Salmonella, Shigella, Proteus, Serratia,* and the species *Vibrio metschnikovii*

III B

Obligate aerobes, oxidase negative, with low cytochrome oxidase activity

- cytochrome pattern: a, d, o, b, and sometimes c are present
- cytochrome c is often absent, or is non-integrated with the terminal oxidase
- these organisms show low TMPD oxidation capability
- these bacteria grow exclusively aerobically using a respiratory mechanism
- representative genera include: *Bacillus, Micrococcus, Xanthomonas, Acinetobacter, Acetobacter, Corynebacterium, Mycobacterium, Nocardia*

III C

Obligate aerobes, oxidase positive, with high cytochrome oxidase activity

- cytochrome pattern: a, d, o, b, and c.
- cytochrome c is present and is functionally integrated with the terminal oxidase, *e.g.*, cyt $a+a_3$, o, d, a_1
- these organisms show high TMPD oxidation capability
- these bacteria grow exclusively aerobically using a respiratory mechanism
- representative genera include: *Achromobacter, Aeromonas, Azotobacter, Neisseria, Rhizobium, Pseudomonas,* and the species *Spirillum itersonii.*

Table 1 (continued)

III D

Obligately aerobic, chemoautotrophs and methylotrophs

- cytochrome pattern: a, $a+a_3$, o, b, and c
- these bacteria oxidize inorganic compounds (chemoautotrophs), or one carbon organic compounds (methylotrophs), via an aerobic respiratory chain
- these organisms reductively assimilate CO_2 to glucose through the Calvin cycle
- it is proposed that these bacteria evolved from the phototrophic bacteria through loss of the photosynthetic apparatus (regressive evolution)
- representative organisms include:

  Chemoautotrophs
  1) nitrifying bacteria *Nitrosomonas, Nitrosospira, Nitrosococcus*
  2) sulfur oxidizers *Thiobacillus, Thiomicrospira, Sulfolobus*
  3) iron bacteria *Sphaerotilus, Gallionella*

  Methylotrophs
  Methylomonas and *Methylococcus*

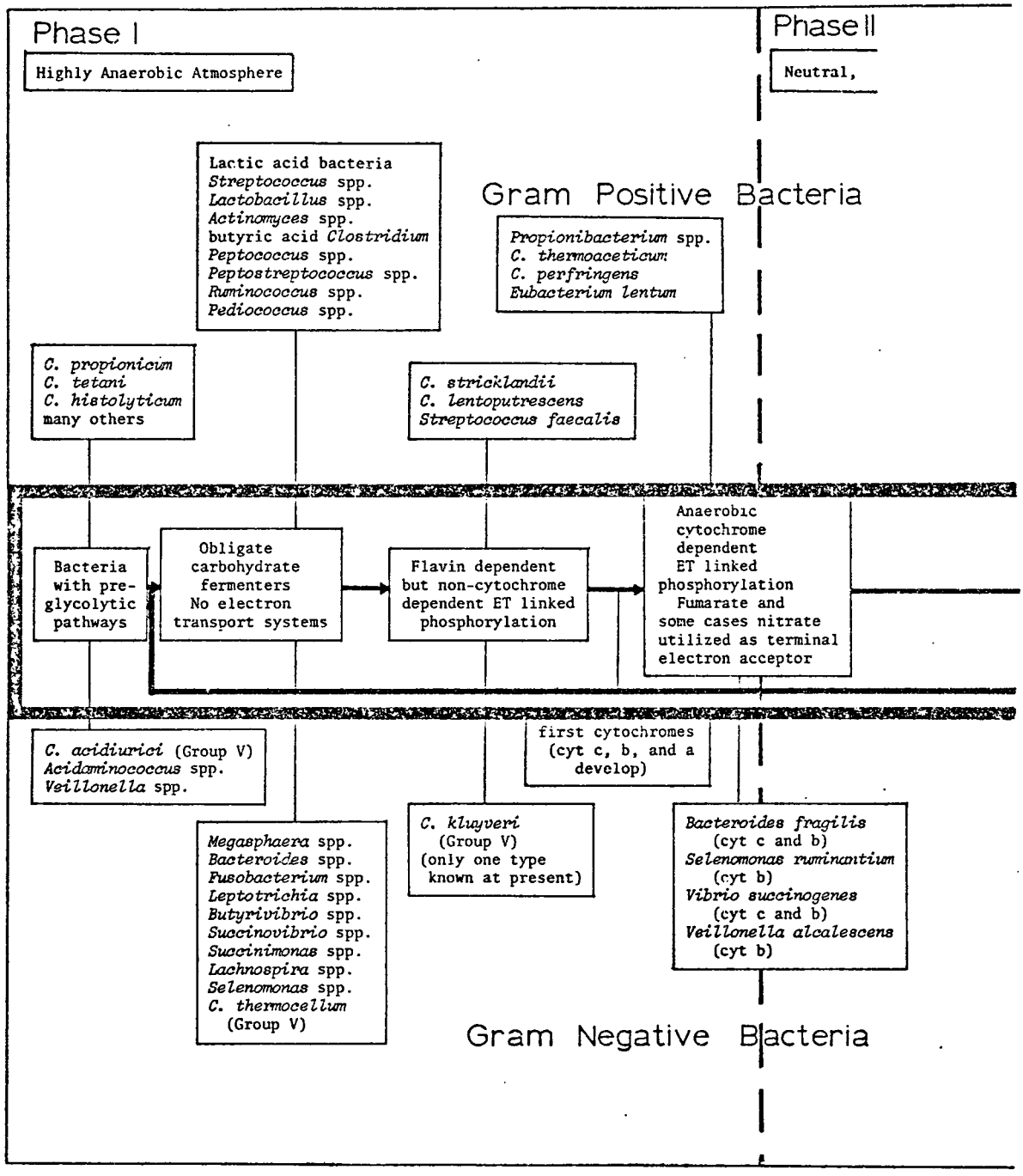
III E

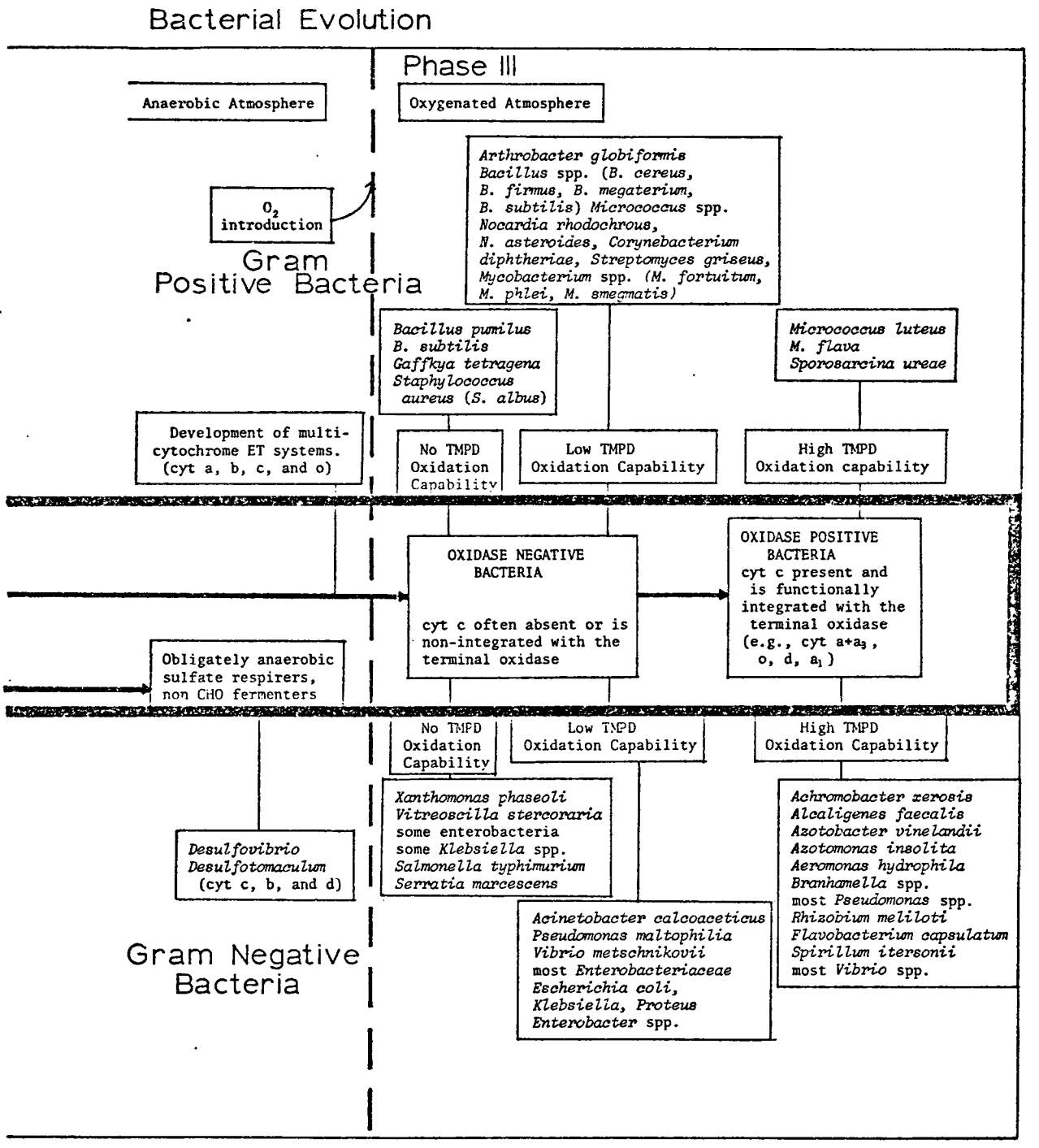
Facultative anaerobic fermenters, oxidase positive, with high cytochrome oxidase activity

- cytochrome pattern: a, d, o, b, and c present
- cytochrome c is present, and is functionally integrated with the terminal oxidase
- these organisms show high TMPD oxidation capability
- these bacteria can grow fermentatively in an anaerobic environment, or use a respiratory mechanism in an aerobic environment
- representative species: *Aeromonas hydrophila, Vibrio cholerae, V. parahaemolyticus*

In the course of developing the ideas of this evolutionary scheme, it became apparent that another related scheme could be proposed which combined both the physiological developmental pathway, with a breakdown of bacteria, according to their Gram reaction. Thus, another scheme is presented (Fig. 4), which takes into account the Gram reactions of the specific genera and species that represent the present day survivors, at the different physiological levels previously proposed.

Figure 4 A Scheme for




Bacterial Evolution
Phase III
Anaerobic Atmosphere
Oxygenated Atmosphere
O_2 introduction
Gram Positive Bacteria
Arthrobacter globiformis
Bacillus spp. (B. cereus, B. firmus, B. megaterium, B. subtilis) Micrococcus spp.
Nocardia rhodochrous, N. asteroides, Corynebacterium diphtheriae, Streptomyces griseus, Mycobacterium spp. (M. fortuitum, M. phlei, M. smegmatis)
Bacillus pumilus
B. subtilis
Gaffkya tetragena
Staphylococcus aureus (S. albus)
Micrococcus luteus
M. flava
Sporosarcina ureae
Development of multi-cytochrome ET systems. (cyt a, b, c, and o)
No TMPD Oxidation Capability
Low TMPD Oxidation Capability
High TMPD Oxidation capability
OXIDASE NEGATIVE BACTERIA
cyt c often absent or is non-integrated with the terminal oxidase
OXIDASE POSITIVE BACTERIA
cyt c present and is functionally integrated with the terminal oxidase (e.g., cyt a+a3, o, d, a1)
Obligately anaerobic sulfate respirers, non CHO fermenters
No TMPD Oxidation Capability
Low TMPD Oxidation Capability
High TMPD Oxidation Capability
Xanthomonas phaseoli
Vitreoscilla stercoraria
some enterobacteria
some Klebsiella spp.
Salmonella typhimurium
Serratia marcescens
Achromobacter xerosis
Alcaligenes faecalis
Azotobacter vinelandii
Azotomonas insolita
Aeromonas hydrophila
Branhamella spp.
most Pseudomonas spp.
Rhizobium meliloti
Flavobacterium capsulatum
Spirillum itersonii
most Vibrio spp.
Desulfovibrio
Desulfotomaculum (cyt c, b, and d)
Acinetobacter calcoaceticus
Pseudomonas maltophilia
Vibrio metschnikovii
most Enterobacteriaceae
Escherichia coli,
Klebsiella, Proteus
Enterobacter spp.
Gram Negative Bacteria

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