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Microorganisms and Cyanide

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INTRODUCTION

The susceptibility of cytochrome oxidases to cyanide means that, although it is a valuable inhibitor for respiratory studies, cyanide is exceedingly toxic to living cells and, therefore, cyanide pollution causes great damage to microbial and other ecosystems.

Unfortunately, many industrial processes produce cyanide as a by-product and large quantities of it must be disposed of. Chemical methods of cyanide degradation are expensive; there have been many cases reported of cyanide pollution and the dangerous practice of "dumping" cyanide wastes. Moreover, industrial effluents are not the only sources of cyanide found in the environment. A remarkable number of plants, including many of agricultural importance, are cyanogenic, releasing much cyanide into the soil.

Thus, in addition to being of intrinsic scientific interest, an understanding of microbial mechanisms of cyanide resistance and utilization is of wider environmental, agricultural, and economic importance. It is the aim of this review to discuss cyanide production, utilization, degradation, and resistance by microorganisms.

PLANT CYANOGENESIS

To comprehend microbial cyanide production, it is necessary to have a knowledge of plant cyanogenesis. Luckily, there have been several recent reviews of plant cyanogenesis (35-37, 45, 151), and therefore it will be necessary only to briefly discuss this topic here.

Cyanophoric plants synthesize compounds (cyanogens) that, on hydrolysis, liberate hydrogen cyanide (cyanogenesis). At least 800 species

of higher plants of 70 to 80 families, including agriculturally important species such as cassava, flax, sorghum, alfalfa, peaches, almonds, and beans, are known to be cyanogenic (45). West African cassava flour contains sufficient quantities of cyanogens such that the human daily consumption of cyanide, when cassava is the staple part of the diet, is equivalent to about one-half the lethal dose, which is probably the reason for the widespread and chronic neurological disorders found in this area (36, 117).

Most of the known plant cyanogens are glucosidic derivatives of α-hydroxynitriles (cyanohydrins), although some plants form cyanolipids and other cyanogens (151). The glucosidic cyanogens are formed from several amino acids, e.g., lotaustralin from isoleucine, linamarin from valine, amygdalin and prunasin from phenylalanine, and dhurrin from tyrosine.

Experiments, involving feeding radiolabeled amino acids to cyanogenic plants, have shown that the α-carbon atom and the amino nitrogen of the amino acid are used to form the cyanide group of glucosidic cyanogens (Fig. 1), apparently without cleavage of the covalent CN linkage. The carboxyl group of the amino acid is lost, and the β-carbon atom is the center for glucosylation.

Although the pathway of glucosidic cyanogen formation is not yet clear, there is enough evidence to suggest that aldoximes are involved as intermediates (36, 101). The probable pathway is given in Fig. 1.

Catabolism of plant glucosidic cyanogens to release hydrogen cyanide occurs in two steps. A glucosidase (EC 3.2.1.21, β-D-glucose glucohydrolase) acts to remove glucose and release the aglycone, and an oxynitrilase then causes release of cyanide (Fig. 2).

Some plants can also metabolize cyanide (2, 15, 16, 48, 49, 69); this will be discussed in another section (page 661).

CYANIDE PRODUCTION BY FUNGI

Formation of hydrogen cyanide by a microorganism was first demonstrated by von Lösecke in 1871, when he observed it in the fungus *Marasmius oreades* (183).

Since then cyanide production has been observed in a wide range of fungi. Bach reviewed the early work on fungal cyanogenesis and listed 31 species of many genera that form cyanide, including several strains of *Clitocybe, Marasmius, Pholiota, Polyporus,* and *Tricholoma* (9). Locquin claimed that 300 species of basidiomycetes from 52 genera and several ascomycetes are cyanogenic (92). Singer (156) and Hutchinson (76) have more recently briefly reviewed fungal cyanogenesis. There has, to my knowledge, been only one report of cyanogenesis in eukaryotic microorganisms other than the fungi: the alga *Chlorella vulgaris* is able to produce low concentrations of cyanide (57).

The antibiotic activity of several cyanogenic fungi against other fungi and bacteria is related to their production of cyanide (92, 103, 140), and cyanide produced by *Fomes scutellatus* inhibits germination of lettuce seeds and growth of seedlings (103).

Cyanide-Linked Diseases

Snow mold disease. Winter crown rot or snow mold disease, so called because it develops under snow cover, is a disease of alfalfa (*Medicago sativa*) and other forage plants. It is

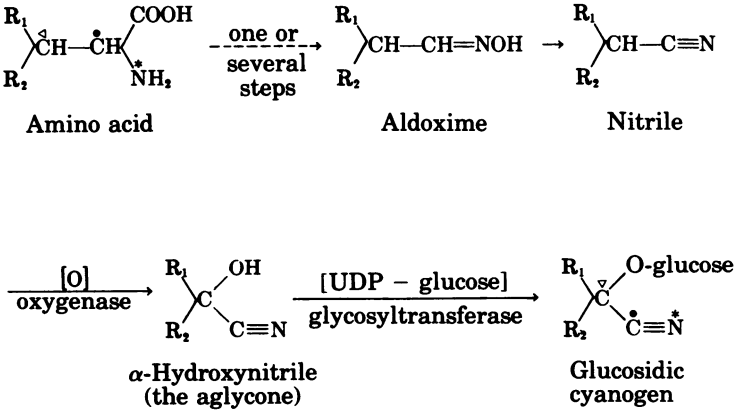


FIG. 1. *Probable pathway of formation of plant glucosidic cyanogens. The symbols show the fates of α- and β-carbon atoms and the amino-nitrogen atom of amino acids on conversion to cyanogens. UDP, Uridine 5-diphosphate.*

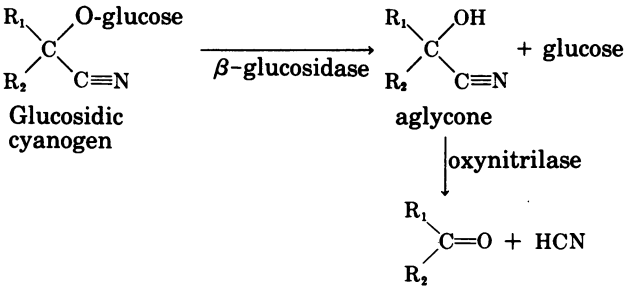


FIG. 2. *Release of cyanide from plant glucosidic cyanogens.*

caused by a psychrophilic, cyanide-producing basidiomycete which is particularly prevalent in western Canada (87, 88). This fungus has a wide range of hosts, damaging forage legumes, winter wheat, turf grass, and perennials (38).

Under artificial conditions maximal infection and death occurs when the soil, infected by the fungus, is kept at 2 to 4°C and insulated from the atmosphere by a layer of expanded mica or other insulant, mimicking the effect of snow cover (87, 88). The fungus must become associated with the host in the fall or early winter if the disease is to develop, and, after infection, the winter-dormant plants become increasingly susceptible (38). Damage is most severe when there is a slow thaw. The disease is able to develop when the crown bud tissues are infected (38, 87, 88).

From crown buds and shoots infected with the fungus and kept at winter soil temperatures (2 to 4°C), invasion of the tissues does not begin for about 30 days (87, 88), at which time lethal doses of cyanide have been produced in the crown tissues (88). Mass fungal invasion, both intracellular and intercellular, occurs after 45 to 60 days; the infected tissues then contain massive doses of cyanide many-fold greater than those found in the surrounding soil-fungus mat (87).

That cyanide production in the crown buds probably precedes invasion by the fungus is indicated also by experiments in the field (86). Here, analyses of infected alfalfa showed that in diseased plants damage to the host was proportional to its cyanide content. The crown buds of plants were infected by the basidiomycete in October, before winter dormancy commenced, and cyanide was observed to be present in the crown tissues from January to April, being at its maximum at the end of March and disappearing by the end of April, when the spring thaw started. The basidiomycete could be seen attached to the outer portions of the crown during the period up to the start of February but penetration did not occur until March, when there was extensive mycelial invasion and disintegration of the buds.

Additional evidence for the role of cyanide in pathogenesis of the disease is provided by a correlation between the concentration of cyanide formed in the plant tissues and the severity of infection of highly sensitive and less sensitive plant species. Thus, cold-hardy grasses (*Bromus inermis, Poa pratensis*) and alfalfa (*Medicago falcata*) are more resistant to the pathogen than cold-sensitive alfalfa (*M. sativa*) and grass (*Agrostis tennis*), and in the sensitive species the concentration of cyanide is much greater than in resistant species (85).

Although certain types of the basidiomycete can themselves produce cyanide (see next section), it seems probable that all or at least the major part of the cyanide found in the infected alfalfa is derived from host plant glucosidic cyanogens. Pathogenic types of the basidiomycete have been shown to produce an extracellular β-glucosidase that releases cyanide from the glucosidic cyanogen amygdalin, as well as from homogenates of crown bud tissues of the sensitive strain of alfalfa, *M. sativa*, but not the relatively resistant *M. falcata* (34). Cyanide is released by the reaction:

$$\text{plant cyanogen} \xrightarrow[\beta\text{-glucosidase}]{\text{fungal}} \text{aglycone} + \text{glucose}$$

$$\xrightarrow[\text{or}\ \text{oxynitrilase}\ \text{(plant or fungal?)}]{\text{non-enzymic}} \text{HCN} + \text{R}\cdot\text{CHO}$$

Three types of snow mold fungus have been obtained (186); their properties are summarized in Table 1. Type C do not produce cyanide and are not pathogenic. They are obtained in the spring from superficial mycelia on plants and are saprophytic. Type A cause production of high levels of cyanide in crown tissues and are very virulent; type B are less virulent and there is less cyanide formation in the host plant. Pathogenicity is therefore approximately pro-

TABLE 1. *Characteristics of three types of isolates of a psychrophilic basidiomycete*[a]

Characteristic	Isolate		
	Type A	Type B	Type C
Colony characteristics	Relatively slowly growing, mycelium appressed to substratum; abundant stroma-like bodies	Fairly rapidly growing, abundant fluffy aerial mycelium; stromata not usually seen	Heterogenous group; very rapidly growing; few aerial mycelia
Cyanide formation in culture	None	Prolific	None
Cyanide formation on alfalfa (*Medicago sativa*) in the field	Prolific (600–655 μg/ml in crown buds)	Moderate (175–240 μg/ml in crown buds)	None
Pathogenicity to *M. sativa* in the field	(a) 3–18% survival (b) 0–3% survival	(a) 85–95% survival (b) 47–60% survival	(a) 100% survival (b) 100% survival
Pathogenicity to *M. sativa* in a controlled environment	28–38% survival	68–81% survival	98% survival
Pathogenicity to a grass (*Dactylis glomerata*) in the field	62–70% survival	33–42% survival	100% survival
Cyanide tolerance in culture	Low (up to 50 μg of HCN per ml)	High (up to 250 μg of HCN per ml)	Low (up to 50 μg of HCN per ml)

[a] Data summarized from Ward et al. (186) through the courtesy of The National Research Council of Canada. All three types of isolates grow at 0 to 25°C (optimally 12 to 17.5°C) and at pH values 4.0 to 9.0 (optimally at pH 6.0 to 7.0).

portional to cyanide production in the host. Contrary to their effects on alfalfa, type B are more virulent then type A in a grass (*Dactylis glomerata*); unfortunately, the relationship to cyanide concentration has not been reported.

Fairy ring disease. Fairy ring disease is caused by fungi of the order *Agaricales*, particularly *Marasmius oreades*. *M. oreades* is destructive of grasslands, including lawns, pastures, and parks (89). Pathogenesis is initiated by a toxic excretion from *M. oreades* that damages the root tops of the host plant, weakening it and permitting mass invasion by the fungus (13, 46, 47).

This toxic excretion could be a glucosidase that acts to promote cyanide production in the plant, causing initial damage before fungal invasion. Thus, although considerable work is needed to confirm it, it is possible that the etiology of fairy ring disease is related to snow mold disease and copperspot disease (see next section). Unlike the snow mold basidiomycete (38), *M. oreades* grows poorly below 15°C, indicating that it has little activity in the cooler sections of the year (89).

That cyanide can cause damage to plants sensitive to *M. oreades* has been shown by suspending root systems of three different grasses above cyanide-evolving cultures of the fungus; root discoloration and damage occurred (47).

Copperspot disease. *Stemphylium loti* causes copperspot disease of birdsfoot trefoil (*Lotus corniculatus*) (58). Stem cankers, red or brown leaf spots, and defoliation of affected leaflets are the principal symptoms of the disease.

Drake showed that *S. loti* grows intercellularly on trefoil, apparently producing a substance that damages the plant before mass intracellular invasion and collapse of the plant cells (43).

Millar and Higgins have shown that the toxic excretion from *S. loti* is a β-glucosidase that causes release of cyanide from the cyanogenic glucosides (linamarin and lotaustralin) of the plant; the released cyanide is lethal to the plant (107). Two types of trefoil were used; both formed cyanogenic glucosides, but only one type (HCN^+) released cyanide. The other type (HCN^-) was unable to form cyanide because it did not contain a β-glucosidase. *S. loti* was pathogenic to both HCN^+ and HCN^- plants,

causing cyanide release from shoots of the plants and excised leaflets: the β-glucosidase must therefore have originated from the fungus. Release of cyanide was accompanied by depletion of the plant cyanogens, showing that these, and not cyanogenic compounds excreted by *S. loti*, were the source of cyanide.

Several other fungi, not pathogenic to trefoil, were shown to cause cyanide release from trefoil substrate when they had been grown *in vitro* on autoclaved trefoil shoots (107). *S. loti* is pathogenic because it is able to adapt to growth in the presence of cyanide, forming a cyanide-tolerant respiratory system (51, 52) and synthesizing cyanide hydratase (53) for detoxication of cyanide to formamide.

Cyanide Production in Culture

In the early papers (cf. Bach, reference 9) on fungal cyanogenesis, there was considerable confusion as to when cyanide formation occurs during the growth cycle. There was general agreement that fruiting bodies form cyanide but conflicting evidence for its formation by mycelia. Bach (9) showed that cyanide was produced in young, fresh fruiting bodies of *Pholiota aurea* only when they had been damaged. Older fruiting bodies and old mycelia also formed cyanide. Cyanide production was related to availability of oxygen.

Lebeau and Dickson (87, 88) were the first to study fungal cyanide production in liquid culture. They showed that the snow mold basidiomycete grew as mycelia with clamp connections in stationary liquid culture in a basal salts medium, in soybean meal soil, or ground alfalfa crown tissues. For both growth and cyanide production the optimum temperature was 12°C. Cyanide production was found to occur during active growth on all media, but especially on the complex media.

The isolation of three types of the snow mold fungus (186) has already been mentioned. Only type B produce cyanide in culture, whereas type A are more virulent in alfalfa (*M. sativa*) and cause more cyanide production in the host plant (Table 1). It is therefore possible that type A and B isolates differ in their abilities to form the enzymes for cyanide production and to produce the extracellular β-glucosidase that causes release of cyanide from the host plant cyanogens.

In shake flasks a B-type isolate of the snow mold fungus, growing on complex and synthetic media, produced cyanide, but only during the autolytic phase, several days after growth had reached a maximum (185). This result would seem to contradict the observations on cyanide production during active growth of mycelial surface colonies (87, 88). However, in mycelia, the growing outer edge is followed by an inner area of autolyzing organisms; the latter fraction of the colony could be responsible for cyanide production.

Inclusion of glycine in the growth medium resulted in a stimulation of cyanide production by a B-type isolate of the fungus growing in shake flasks on a glucose-basal salts medium (188). Unlike the response to it by bacteria (27, 104, 122, 195), glycine here appeared to stimulate cyanide production during growth rather than in the stationary phase. However, this could have been due to cyanogen formation (see next section) in the logarithmic phase (184), since assay of cyanide was by steam distillation of the culture: the cyanide observed was the total of free cyanide plus heat-labile cyanogen.

Cyanide is produced directly from glycine, with the cyanide carbon derived from the glycine methylene group; addition of [1-^{14}C]- or [2-^{14}C]glycine to growing cultures of the snow mold fungus resulted in formation of H^{14}CN only when the label was in the [2-^{14}C]-position of the added glycine (188). A lower level of cyanide was produced from serine, presumably due to the conversion of serine to glycine via serine hydroxymethyl transferase (EC 2.1.2.1) or other enzymes.

Despite earlier reports to the contrary (110, but see reference 9) mycelia of *M. oreades*, as well as fruiting bodies, produce cyanide (89). Cyanide is produced when the organism is growing under natural conditions as fairy rings (46, 47), as well as in shake flask cultures during the autolytic phase of the mycelial growth cycle (89).

Formation of Cyanogens

Ward (184) has shown that a B-type isolate of the snow mold basidiomycete produces an unstable, probably not glucosidic, cyanogen in shake cultures. There was no free cyanide in the cultures until the start of the stationary phase, but an increasing rate of synthesis of the cyanogen occurred during active growth; at all times the concentration of the cyanogen was much greater than that of the free cyanide. Unfortunately, the reasons for release of cyanide from the cyanogen during autolysis were not studied. Perhaps induction of an oxynitrilase, causing breakdown of the cyanogen, occurred during this phase. Alternatively, on autolysis, the enzyme may become accessible to the cyanogen, or an increase in pH may occur, destabilizing the cyanogen.

Free cyanide was released slowly from the cyanogen by a non-enzymatic process, not affected by a β-glucosidase formed by the fungus

or by a commercial emulsin preparation. The partially purified cyanogen was unstable at temperatures above 40°C and at pH values higher than 6.0 (184).

Tapper and MacDonald (174) have shown that the major cyanogen present in extracts of the snow mold fungus is glyoxylic acid cyanohydrin. Lower concentrations of pyruvic acid cyanohydrin are also formed. Glyoxylic acid cyanohydrin has pH and temperature characteristics similar to those of the cyanogen isolated by Ward (184) and is presumably the same compound.

Stevens and Strobel (168) measured the effectiveness of various amino acids as substrates for cyanogenesis by the snow mold and concluded that valine and isoleucine were the best precursors of hydrogen cyanide. Valine and isoleucine are the precursors of linamarin and lotaustralin, respectively, in higher plants (23, 24); Stevens and Strobel (168) claim to have detected small quantities of these glycosidic cyanogens in the snow mold fungus. However, their suggestion that valine and isoleucine act as the preferred precursors of the cyanogen(s) in the fungus has been severely criticized by Ward et al. (189), who also confirmed that glycine is the best precursor of cyanide. Furthermore, Tapper and MacDonald (174) have been unable to detect linamarin or lotaustralin in the fungus.

Stevens and Strobel (168) also showed that the snow mold formed an oxynitrilase and two β-glucosidases of differing substrate specificities. They contend that formation of these enzymes supports their hypothesis that linamarin and lotaustralin are the precursors of cyanide. However, the β-glucosidases are probably responsible for the extracellular glucosidic activity noted by others (34, 184) to cause cyanide release from host plant cyanogenic glucosides. The oxynitrilase, since it is of broad specificity to aliphatic nitriles, could be used for release of cyanide from the fungal glyoxylic acid cyanohydrin (174) or breakdown of the aglycones of host plant cyanogens (34).

Plantlike cyanogenic glucosides have not been detected in cultures of *M. oreades* (59, 110, 183), but this fungus forms a cyanogen (187) of seemingly similar properties to that of the snow mold basidiomycete (184, 189). This cyanogen is therefore probably the cyanohydrin of glyoxylic acid (174).

CYANIDE PRODUCTION BY BACTERIA

Species Producing Cyanide

Although cyanide production is widespread in fungi and cyanide is probably a normal fungal metabolite, certain pseudomonads and *Chromobacterium violaceum* are the only bacterial species known to produce cyanide (Table 2). Conflicting reports on the types of pseudomonads producing cyanide could be the result of either the conditions of culture tested, since significant cyanide production occurs only under specific growth conditions (see next section), or the assay procedures. In the earlier studies insensitive techniques were used (smell and picric acid-Na_2CO_3 indicator papers), whereas in recent studies more sensitive methods have been used (colorimetric tests and gas chromatography-mass spectroscopy), enabling detection of much lower levels of cyanide.

Conditions of Cyanide Production

Lorck was the first to show clearly that optimal cyanide production by bacteria is dependent on the inclusion of glycine in the growth medium (93). Growth of *Pseudomonas aeruginosa* on nutrient broth resulted in the formation of a high level of cyanide, but in a glycerol-synthetic medium cyanide evolution only approached that found for growth on nutrient broth if glycine was included as a nitrogen source. Others have since confirmed the dependence of cyanide production by bacteria on

TABLE 2. *Cyanide-producing bacteria*

Organism	Comments	Reference
C. violaceum	Mesophilic *C. violaceum* species were cyanide producing; *C. lividum* species did not form cyanide	162–164
C. violaceum	4 out of 9 strains tested were cyanide producing; *C. lividum* was found not to form cyanide	104
C. violaceum		122
P. chlorophis	2 strains formed cyanide	
P. aureofaciens	2 strains formed cyanide	104
Pseudomonas sp.		22, 195, 196
P. aeruginosa	74 out of 110 strains formed cyanide	27
P. fluorescens	1 out of 5 strains formed cyanide	
P. polycolor (= *P. aeruginosa*)	2 out of 4 strains formed cyanide	
P. fluorescens		50
B. pyocyaneus (= *P. aeruginosa*)	9 strains formed cyanide	126
B. pyocyaneus		33

the inclusion of glycine in the growth medium (19, 20, 27, 104, 197).

Michaels and Corpe showed (104) that with *C. violaceum*, which grew well on peptone or on a minimal salts medium containing L-glutamate, DL-alanine, L-histidine, or glucose plus ammonia cyanide production was much greater on peptone than on the other substrates. However, addition of guanine, alanine, glycine, or methionine, but not any of 45 other amino acids or nitrogenous compounds, to cells grown to the stationary phase on glutamate caused a stimulation of cyanide production without further growth. A mixture of glycine and methionine caused a much greater stimulation of cyanide production, to a level similar to that found for growth on peptone. Glycine could be replaced by several related compounds, and glycine methyl ester gave an even greater cyanide yield.

Cyanide was also formed when glycine and methionine were added to harvested, washed cells suspended in basal salts, which had been first grown to the stationary phase on glutamate-basal salts medium; addition of glycine and methionine to the growth medium 2 h before harvesting substantially increased the cyanide yield (104). Addition of succinate (or malate or fumarate) to the washed cell suspension in basal salts containing glycine and methionine doubled the yield of cyanide without further growth. The stimulatory effect of succinate was inhibited by both azide and dinitrophenol, suggesting that it acts as a respiratory energy source, although it could also be a precursor for extra glycine or another substrate.

Glycine is thus the direct substrate for cyanide production. However, the role of methionine is less clear. It can be replaced by betaine, dimethyl glycine, and choline and could therefore act as a methyl donor (104).

Both cyanide yield and rate of synthesis of cyanide in bacterial growth media are maximal at the start of the stationary phase (27, 104, 122, 195). Castric noted three phases of cyanide formation during aerobic growth of *P. aeruginosa:* no cyanide was formed during lag and logarithmic growth phases, active cyanogenesis occurred during the transition from logarithmic to stationary phases, and limited cyanogenesis occurred in the stationary phase (27). The data of Michaels and Corpe (104) and Niven et al. (122) show that there is a similar situation in *C. violaceum*. All these workers used growth conditions of relatively low efficiency of oxygen transfer, and therefore the phase of decelerating growth could be due to oxygen limitation, indicating that cyanide formation is maximal under conditions of low aeration or anaerobiosis. However, growth of *P. aeruginosa* under conditions of very low aeration (stationary culture) led to a drastic decrease in cyanide production (27). Anaerobic growth in the presence of nitrate, which permits anaerobic respiration, also caused a decrease in cyanide production, suggesting a requirement for molecular oxygen rather than a respiratory-driven removal of reducing equivalents or a supply of energy. Wissing, using a *Pseudomonas* species, elegantly demonstrated (196) that cyanide production in cells suspended in phosphate buffer plus glycine was low under anaerobic conditions and not only oxygen, but also artificial electron acceptors (phenazine methosulfate, methylene blue, 2,6-dichlorophenol indophenol, and ferricyanide), could greatly stimulate cyanide formation. Some inhibitors of flavin-linked enzymes, although not amytal or rotenone, caused partial inhibition of oxygen-driven cyanide formation. Other respiratory inhibitors, uncoupling agents, or ionophores were not tested, and it is not possible to tell whether the flavin-associated step is respiratory linked, peroxidative, an oxygenase, etc.

Cyanide production does not itself inhibit growth of the bacteria themselves or cause loss of viability, because addition of cyanide in the lag or logarithmic phases of cultures of *C. violaceum* has no effect on the final growth yield (104).

Cyanide production in growing *P. aeruginosa* has a narrower temperature range than growth; it also requires the presence of iron and is inhibited by high phosphate concentrations (27). Addition of chloramphenicol to late-logarithmic-phase *P. aeruginosa* inhibits cyanide formation, showing a requirement for protein synthesis.

There have been no reports of the formation of any cyanogenic compounds by bacteria.

Metabolic Precursors of Cyanide

Michaels et al. grew *C. violaceum* on a glutamate-basal salts medium and incorporated methionine and glycine into the medium 2 h before harvesting, to adapt the cells for cyanide production (105). Cells were harvested, washed, and resuspended in basal salts containing succinate, glycine, and methionine. In different experiments, [1-^{14}C]- or [2-^{14}C]glycine or [1-*methyl*-^{14}C]methionine were labeled. Cyanide and CO_2 formation in the non-proliferating cells were followed for 6 h, and the distributions of the radioactivity was measured. About 7 and 2% of the [2-^{14}C]- and [1-^{14}C]glycine, respectively, were taken up by the cells, mainly into the protein fraction. About 10% of the [1-^{14}C]glycine (the carboxyl carbon) was converted

to CO_2, and 11% of the [2-^{14}C]glycine (the methylene carbon) was converted to cyanide. The specific activity of cyanide formed from [2-^{14}C]glycine was 94% of the specific activity of the substrate, whereas when [1-^{14}C]glycine or [1-*methyl*-^{14}C]methionine were the substrates the specific activity of cyanide was less than 0.1% of the substrate's specific activity.

The carbon of the cyanide formed must therefore have originated from the methylene group of the glycine, and the CO_2 presumably stemmed from the carboxyl group:

$$NH_2 \cdot \mathring{C}H_2 \cdot \dot{C}OOH \rightarrow \rightarrow \rightarrow H\mathring{C}N + \dot{C}O_2 + 4\,[H]$$

Brysk et al (20) grew *C. violaceum* on glutamate-basal salts and then supplemented the medium with methionine plus [2-^{14}C^{15}N]glycine and continued growth for 18 h. Of the glycine consumed, 25% was incorporated into free cyanide and 16% into β-cyanoalanine. β-Cyanoalanine is formed from cyanide and serine and is the first step of cyanide utilization by non-proliferating cells of *C. violaceum* (cf. reference 19 and page 661). However, radioactivity in β-cyanoalanine could have arisen during metabolism of glycine to serine or by other pathways, rather than via cyanide formation. Analysis of the carbon distribution of radioactivity in β-cyanoalanine showed that 44% of the activity was incorporated into the cyano carbon (i.e., 7% of the total glycine consumed). Therefore, at least 25 + 7 = 32% of the glycine utilized was converted to cyanide. This figure is an underestimate because it does not take into account the further metabolism of β-cyanoalanine. Thus, synthesis of cyanide is an important, possibly major, pathway of glycine metabolism under these conditions. The ^{15}N/^{14}C ratios of the administered glycine, unconsumed glycine, and HCN and β-cyanoalanine formed were essentially the same, indicating that the methylene carbon of glycine was converted to cyanide without cleavage of the carbon-nitrogen bond. Reversible transamination and oxidative deamination of glycine therefore did not occur.

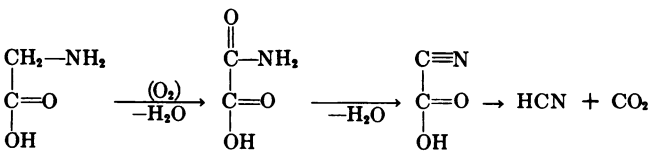
Wissing (196) has measured the molar ratio of cyanide produced to total glycine consumed by a *Pseudomonas* species suspended in phosphate buffer (in the absence of methionine or other methyl donors and without succinate or other energy sources). A ratio of 1.0:1.1 was found. Therefore, no further metabolism of either cyanide or glycine (e.g., to β-cyanoalanine) occurred, and future studies of the metabolic pathway of cyanogenesis from glycine should be facilitated by use of these conditions. Cyanide production by the *Pseudomonas* species was low unless oxygen or an artificial electron acceptor was present, and the process was inhibited by several flavin inhibitors, indicating that it is probably linked to a flavoprotein oxidase, oxygenase, or respiratory dehydrogenase (196, 197).

Recently (196), glycine-driven cyanide production has been obtained in a cell-free extract of the *Pseudomonas* species (derived by sonication), which will enable studies of the enzymology of the system. Optimal cyanide-producing activity was obtained when the cell-free extract was prepared and activity was assayed in the presence of phenazine methosulfate and flavin adenine dinucleotide, which presumably act as an oxidizing agent or electron carrier and coenzyme, respectively. Sucrose density gradient fractionation of cell-free extracts shows that activity is associated with particulate rather than soluble material. Interestingly, this organism contains internal membrane structures reminiscent of those found in type II methylotrophic bacteria (133), although *C. violaceum* has internal membranes of the mesosomal type (143).

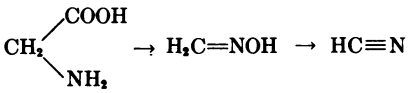
SPECULATIONS ON THE METABOLIC PATHWAY OF CYANIDE PRODUCTION BY MICROORGANISMS

Glycine is the metabolic precursor of cyanide, with the carbon derived from the methylene carbon of glycine, in both bacteria and fungi (20, 105, 188). It seems reasonable, therefore, to suggest that the mechanism of cyanide production is similar in all the fungi and bacteria so far studied.

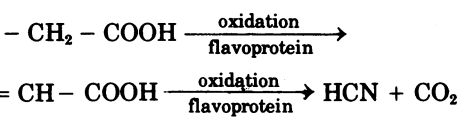
On the basis of their studies with *C. violaceum*, Michaels et al. (105) proposed the following scheme for cyanide formation, although without evidence for formation of the intermediates or assay of enzyme activities:



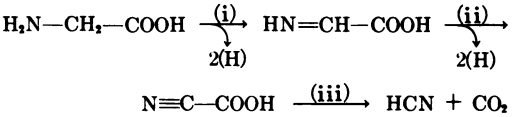
Conn has suggested (36) that cyanide may be formed by microorganisms via a pathway analogous to that for formation of plant cyanogenic glucosides (Fig. 1). A relatively simple reaction sequence from glycine would be involved, with formaldoxime as an intermediate:



Wissing (196) has more recently proposed a pathway of bacterial cyanide production from glycine, which could also be applicable to fungal cyanogenesis. Two flavoprotein steps are involved:



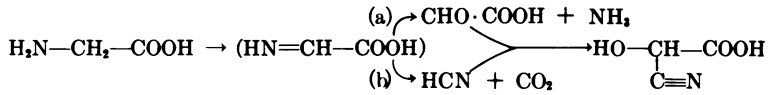
This is a simplification and, if this scheme is operative, it is probable that there are two oxidative steps:



There are at least two enzymes known for glycine oxidation: (a) an amino acid oxidase (glycine:oxygen oxidoreductase [deaminating]), which is flavin linked and peroxidative (121, 137); and (b) glycine dehydrogenase (glycine:nicotinamide adenine dinucleotide [NAD^+]oxidoreductase [deaminating], EC 1.4.1.10). Since cyanogenic oxidation of glycine is flavin linked (196), it is possible that step (i) is peroxidative and flavin linked and that the enzyme is related to glycine oxidase. On the other hand, there are obvious energetic advantages to the organism if the enzymes catalyzing steps (i) and (ii) are classical dehydrogenases associated with the respiratory system. It should also be noted that the unstable imine (HN = CH.COOH), proposed as an intermediate of cyanide formation, is almost certainly an intermediate in glycine oxidase (62).

Release of cyanide from the aglycones of plant cyanogenic glucosides occurs by the action of an oxynitrilase. Plant oxynitrilases either have flavin adenine dinucleotide as a prosthetic group (14, 150) or are devoid of flavin (150, 17). The requirement for flavin in the scheme given above could therefore be for oxidation of the unstable nitrile via step (iii) rather than for the oxidative steps. It will be interesting to see whether the oxynitrilase present in the snow mold fungus (168) requires flavin for activity. The nitrile (N ≡ C—COOH) is likely to be highly unstable and step (iii) could also be non-enzymatic.

Cyanide is a highly reactive group and easily reacts (non-enzymatically) with keto groups to form cyanohydrins (136). The cyanohydrins of glyoxylic acid and pyruvic acid found in the snow mold fungus (174) could therefore arise from direct chemical interaction of cyanide with the acids. Glyoxylic acid is obtained from glycine via glycine oxidase, and the cyanohydrin would then be formed by:



In the presence of significant intracellular levels of glyoxylic acid (or other keto compounds such as pyruvic acid or α-oxoglutaric acid), there is not likely to be much free cyanide present. Oxynitrilase would then act to release hydrogen cyanide from the cyanohydrins and might be expected to have a broad specificity.

I would like to emphasize the speculative nature of this hypothetical pathway: it is proposed in the hope that it will stimulate research in this area. In particular, the properties and substrate specificity of the fungal oxynitrilase need to be further examined, and a search needs to be made for glyoxylic acid cyanohydrin and other cyanogens in bacteria, as well as evidence for or against the formation of the intermediates of the proposed pathway.

CYANIDE ASSIMILATION BY CYANOGENIC MICROORGANISMS

As will be discussed below, metabolic pathways of cyanide assimilation have so far been studied mainly in species that are also able to produce cyanide, but, as far as is known, are unable to grow on it as a carbon and/or nitrogen source. In these cases cyanide may be a normal intracellular metabolite, possibly present as glyoxylic acid cyanohydrin or other cyanogens. Therefore, formation of high concentrations of cyanide could be due to excretion of an abnormal excess of it, or as the end product for removal of some other metabolite such as glycine that has been overproduced. It is noteworthy that production of appreciable concentrations of cyanide only occurs on certain media, under specific growth conditions, and in the stationary phase of growth (27, 104, 185).

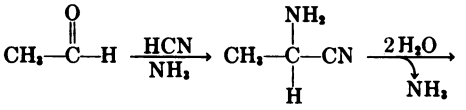
Alanine Formation

Strobel first showed that the cyanide-producing snow mold basidiomycete could assimilate cyanide (169). He incubated mycelia with $H^{14}CN$ for 20 h and then harvested, disrupted, and fractionated the cells. Alanine and, to a lesser extent, glutamate were the only labeled amino acids. In shorter-term experiments, alanine was the only labeled amino acid. Control

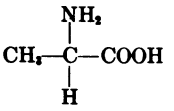
studies with $^{14}CO_2$ showed that cyanide was not converted to CO_2 before assimilation.

Cyanide assimilation into several other fungi has been tested (4). *M. oreades, Pholiota adiposa, Pholiota aurivella, Pholiota praecox,* and *Rhizopus nigricans* incorporated $H^{14}CN$ into alanine and, to a lesser extent, into other amino acids, whereas *Fusarium nivale* incorporated cyanide into asparagine only. No radiolabeled amino acids were detected in *Clitocybe illudens. Rhizoctonia solani, Aspergillus flavus,* and *Fusarium solani.*

Strobel has shown that mycelia of the snow mold basidiomycete incubated with $H^{14}CN$ formed labeled α-aminopropionitrile and L-alanine (170). The label appeared in the α-aminopropionitrile during the first 12 h of incubation and then decreased, whereas the quantity of radioactive alanine increased steadily for 24 h. When $H^{14}C^{15}N$ was used as the substrate, carbon labeling appeared exclusively in the C1 position of both products and nitrogen labeling appeared in the cyano group of α-aminopropionitrile. These results are consistent with the following pathway:



acetaldehyde α-aminopropionitrile

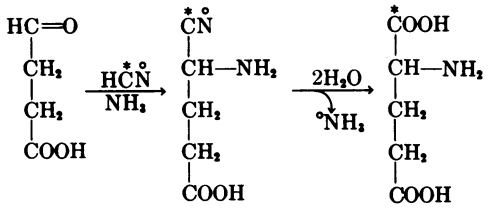


L-alanine

In support of this, incubation of a crude enzyme extract from the fungus with KCN, $(NH_4)_2HPO_4$, and acetaldehyde resulted in formation of α-aminopropionitrile. The conversion presumably occurs via the intermediary formation of a cyanohydrin, with subsequent replacement of the hydroxyl group by an amino group; one or both steps could be enzymatically driven. Intracellularly, acetaldehyde could arise from oxidation of ethanol by alcohol dehydrogenase (EC 1.1.1.1., alcohol:NAD^+ oxidoreductase) or other sources. The breakdown of the nitrile to alanine could occur either via the formation of α-aminopropionamide (cf. reference 53) or in one stage by a nitrilase (EC 3.5.5.1, nitrile aminohydrolase; 141, 176).

Glutamic Acid Formation

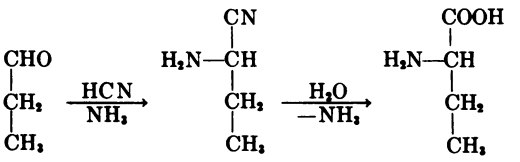
The earlier demonstration that labeled glutamate is formed when the snow mold fungus is fed $H^{14}CN$ (169) has been confirmed (172). In a pathway analogous to that resulting in alanine formation, glutamate is derived from cyanide and succinic semialdehyde via 4-amino-4-cyanobutyric acid:



In extracts, aminocyanobutyrate nitrilase activity was observed, but there was little aminocyanobutyrate synthase activity (172). Glutamate decarboxylase, succinate semialdehyde dehydrogenase, and 3-aminobutyrate-glutamate transaminase activities were also present. A cyclic conversion of cyanide to CO_2 was proposed (Fig. 3). If this is the case, there would be no assimilation of cyanide-carbon by the organism. The pathway would serve to detoxify the cyanide, possibly with assimilation of the cyanide-nitrogen.

α-Aminobutyric Acid Formation

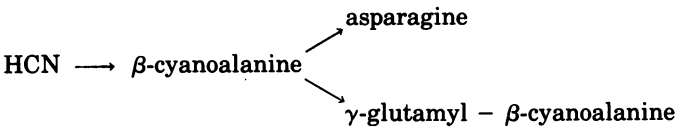
The fungus *Rhizoctonia solani,* which has been reported not to produce cyanide (4), has a pathway of cyanide incorporation into propinonaldehyde to give α-aminobutyronitrile and α-aminobutyric acid (118):



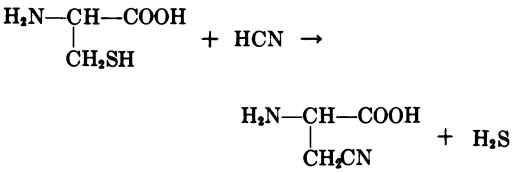
The enzymology of this pathway is presumably similar to the acetaldehyde and succinate semialdehyde pathways mentioned above (170, 172). In cultures fed ammonia, cyanide, and propionaldehyde, the formation of the nitrile preceded formation of α-aminobutyrate. α-Aminobutyronitrilase activity was detected (118).

β-Cyanoalanine Formation

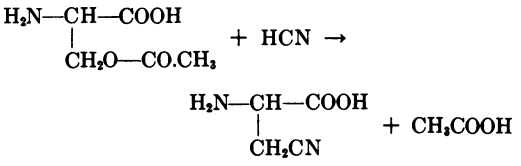
Many plants are able to assimilate cyanide into asparagine (15) or the dipeptide γ-glutamyl-β-cyanoalanine (49). The latter compound is a normal constituent of *Vicia* species but only occurs in other plants when they are fed cyanide (139). In plants fed $H^{14}CN$, the label enters the amide carbon of asparagine (15). Formation of both the dipeptide and asparagine occurs via β-cyanoalanine:



Initially, it was thought that β-cyanoalanine was synthesized by plants from serine and cyanide (15, 48). However, in extracts of *Lotus tennis* (birdsfoot trefoil), the rate of formation of β-cyanoalanine is 50-fold greater when cyanide and cysteine are the substrates than when HCN and serine are supplied (48). β-Cyanoalanine synthase (EC 4.4.1.9, L-cysteine-hydrogen sulfide-lyase [adding HCN]) has been purified from mitochondria of *Lipinus angustifolia* (blue lupine). It catalyzes β-cyanoalanine formation from cysteine and HCN (2, 16, 69):



Serine is not a substrate, but the enzyme is also able to utilize *O*-acetyl-L-serine (about 20 times less effectively than cysteine):



Blue lupine also contains (2, 69) a soluble *O*-acetyl-L-serine sulfhydrase (EC 4.2.99.8, *O*-acetyl-L-serine acetate lyase [adding H_2S]). This enzyme catalyzes formation of cysteine from sulfide and *O*-acetyl-L-serine but is also able to use *O*-acetyl-L-serine and cyanide, at a low rate, to form β-cyanoalanine.

When *Chlorella pyrenoidosa* is fed cyanide, it forms β-cyanoalanine and γ-glutamyl-β-cyanoalanine (49), and *Escherichia coli* is able to convert cyanide to β-cyanoalanine. Dunnill and Fowden (44) suggested that the *E. coli* β-cyanoalanine synthase activity represents a function of an indispensible but nonspecific enzyme, possibly serine sulfhydrase (EC 4.2.1.22, L-serine hydro-lyase). The primary function is:

$$\text{serine} + H_2S \rightarrow \text{cysteine}$$

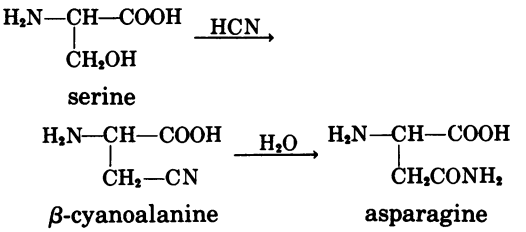
The other possible reactions are:

$$\text{serine} + HCN \rightarrow \beta\text{-cyanoalanine}$$

$$\text{cysteine} + HCN \rightarrow \beta\text{-cyanoalanine} + H_2S$$

Catalysis of these reactions by *E. coli* extracts is stimulated fourfold by 10 mM adenosine 5′-triphosphate.

Brysk et al. have shown that cyanide produced by *C. violaceum* (strain 9) can be assimilated into β-cyanoalanine and then into the amide carbon of asparagine (19):



When washed suspensions of *C. violaceum*, grown on glutamate-minimal salts medium, were incubated with glycine, methionine, and succinate, β-cyanoalanine accumulated in the medium. Maximal concentrations of β-cyanoalanine occurred with 5 h of incubation; longer periods of incubation resulted in its disappearance.

Incubation of washed cells with serine, K^{14}CN, and succinate resulted in incorporation of radioactivity into cells and formation of β-cyanoalanine. Replacement of serine by glycine, and glycine plus *N*,*N*-dimethyl glycine or methionine, gave less, but still significant, formation of β-cyanoalanine and incorporation of radioactivity into the cells. Radioactive incorporation from K^{14}CN was into the C4 position of β-cyanoalanine and asparagine. When [2-^{14}C]glycine was used (plus KCN and succinate), radioactivity was found mainly in the C2, C3, and C4 atoms of β-cyanoalanine. With labeled succinate as substrate, there was extensive incorporation of radioactivity into the cells but not into β-cyanoalanine: succinate was probably acting as an energy donor for adenosine 5′-triphosphate formation. However, with [^{14}C]formaldehyde, but not [^{14}C]formate, as substrate (plus KCN and succinate), the β-cyanoalanine formed was radioactive.

Since the label from radioactive glycine was incorporated into β-cyanoalanine, it was proposed that glycine can act not only as a precursor of cyanide, but also to supply serine (19). Figure 4 shows a possible pathway of glycine and cyanide metabolism by *C. violaceum* strain 9. In this scheme, utilization of three molecules of glycine is required for formation of β-cyanoalanine. One molecule is converted to cyanide and CO_2, one to a C-I unit (and presumably ammonia and carbon dioxide), and one

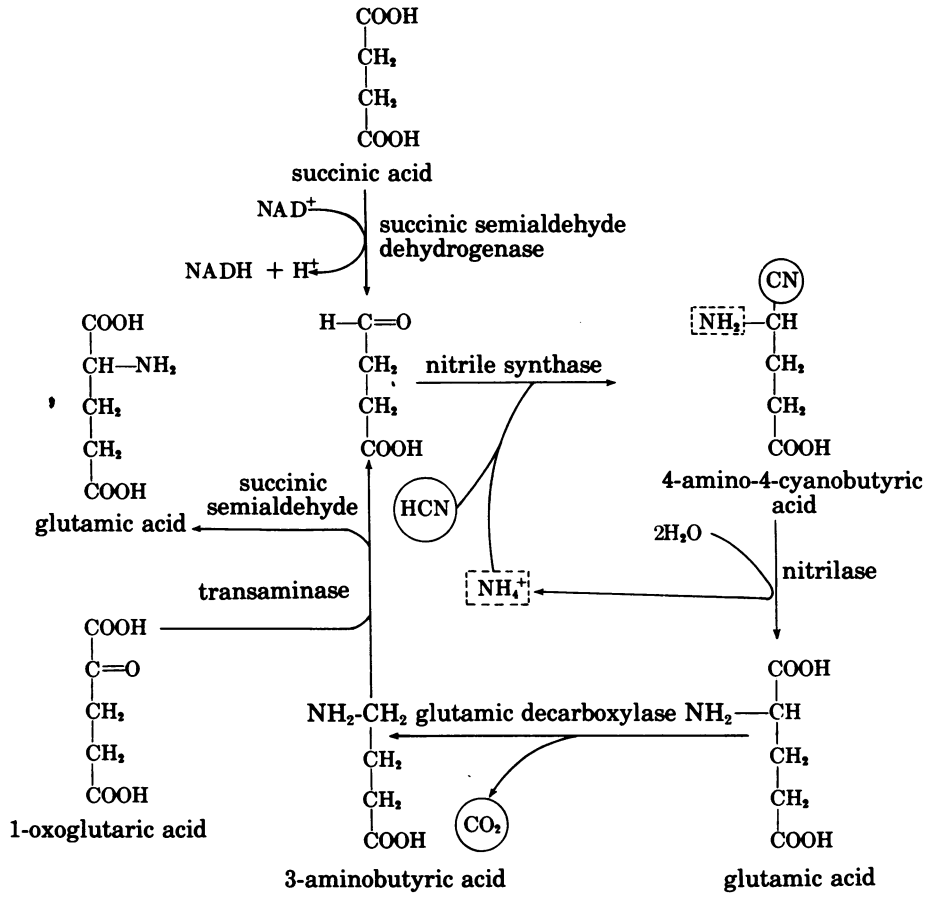


FIG. 3. *Possible pathway for detoxication of cyanide by the snow mold basidiomycete, involving formation of 4-amino-4-cyanobutyric acid. Redrawn from Strobel (170) by courtesy of the Jorunal of Biological Chemistry.*

links up with the C-I unit to form serine, which then combines with cyanide to form β-cyanoalanine. An alternative possibility, not discussed by the authors (19) but consistent with their data, is that two molecules of glycine are converted to cyanide. One of the cyanide molecules could be converted to formamide and then formate (53), which acts as a supply of C-I units for addition to the third molecule of glycine, converting it to serine.

In view of the possibility that β-cyanoalanine is synthesized in *E. coli* by a nonspecific function of serine sulfhydrase (44) and that in plants cysteine is the donor for its formation (69), it is a pity that the properties of the β-cyanoalanine synthase enzyme of *C. violaceum* have not been studied. Does addition of cysteine plus KCN to washed cells of *C. violaceum* also cause stimulation of β-cyanoalanine formation?

Castric and Strobel isolated a cyanide-resistant and -metabolizing strain of *Bacillus megaterium* from Fargo clay that had been planted with the cyanogenic plant, flax, for many years (28). The organism was grown on Trypticase soy broth containing 1 mM cyanide: growth coincided with cyanide disappearance from the medium. When $K^{14}CN$ was added to the medium, the label was incorporated into CO_2, the amide carbon of asparagine, and the C4 carboxyl of aspartate. Washed cells fed serine converted it to β-cyanoalanine and asparagine, and cells fed β-cyanoalanine converted it to asparagine, but β-cyanoalanine was not detected during conversion of serine to asparagine by cell-free extracts: possibly nitrilase activity for conversion to β-cyanoalanine to asparagine is more active than β-cyanoalanine synthase activity under the conditions used in the latter experiment. Cysteine did not act as a substrate for β-cyanoalanine or asparagine synthesis in extracts or in washed cell suspensions. It was proposed that β-cyanoalanine was synthesized from serine and cyanide, which was converted to asparagine and then aspartate.

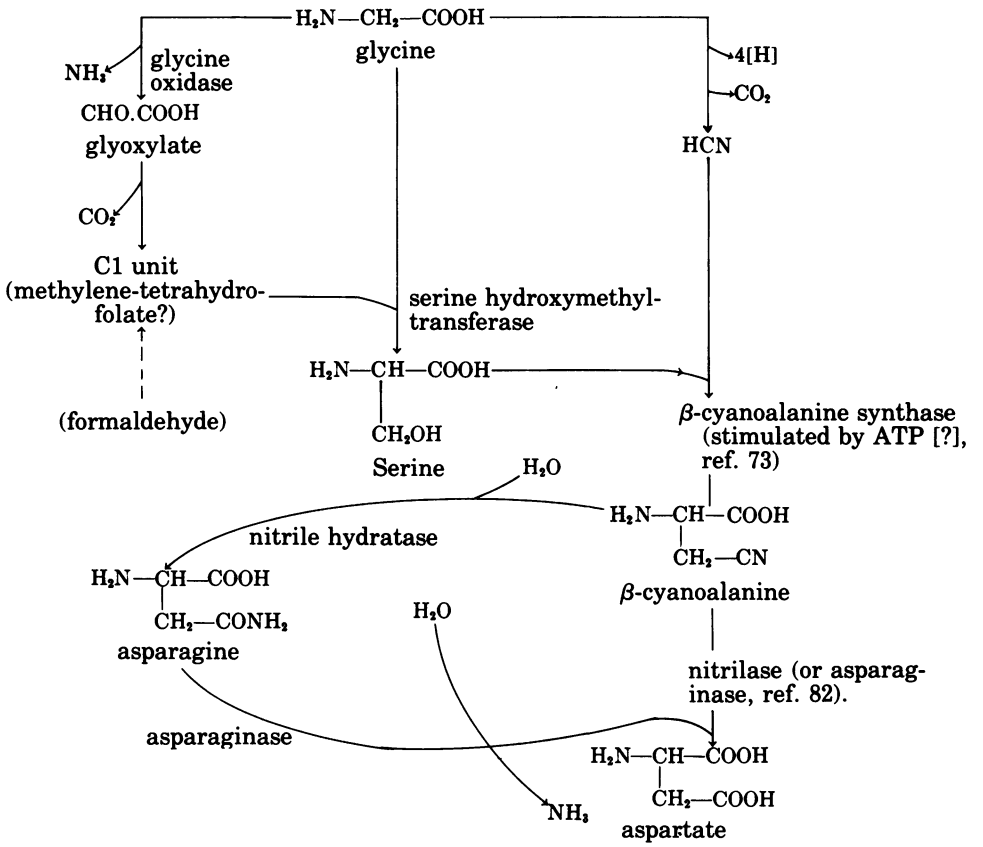


FIG. 4. *Incorporation of cyanide and glycine into β-cyanoalanine and asparagine by C. violaceum strain 9. Adapted from the data of Brysk et al. (19) by courtesy of the Journal of Bacteriology. For details of C1 conversions involving tetrahydrofolate, see reference 39. ATP, Adenosine 5'-triphosphate.*

Castric and Conn have more recently shown formation of β-cyanoalanine in extracts of the cyanide-metabolizing strain of *B. megaterium* (29). Whereas serine (plus cyanide) could act as a substrate for β-cyanoalanine synthesis, cysteine and *O*-acetyl-serine were 17 to 18 times more effective as substrates. This suggests that one of the enzymes of cysteine biosynthesis is able to catalyze β-cyanoalanine formation. It was reasoned that if β-cyanoalanine synthesis from cysteine and cyanide is of physiological significance, then this activity should be inducible by inclusion of cyanide in the medium: this did not occur. If, on the other hand, the enzyme that catalyzes β-cyanoalanine synthesis is an enzyme involved in cysteine biosynthesis, and the β-cyanoalanine synthase activity is only a secondary function of it, then the enzyme should be repressed by inclusion of cysteine in the growth medium. Activities of β-cyanoalanine (from cyanide and cysteine) and cysteine (from *O*-acetyl-serine and sulfide) synthases were equally repressed by growth with cysteine as the sulfur source rather than sulfate or djenkolic acid. Furthermore, *O*-acetyl-serine sylfhydrase was purified 30-fold and the activity of β-cyanoalanine synthesis exactly paralleled the purification (but at a 4×10^{-4}-fold lower activity).

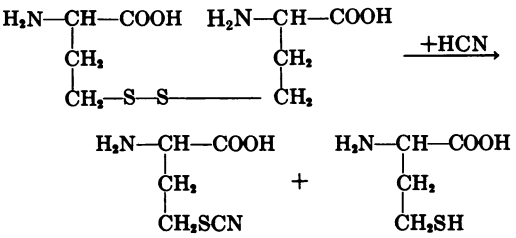
This data (29) and the earlier report (28) that serine stimulates β-cyanoalanine and asparagine synthesis in *B. megaterium* raise the question as to how the conversion from serine occurs in this organism (and possibly also in *C. violaceum;* see reference 19 and above). Serine can (i) act as a direct substrate for β-cyanoalanine formation by a serine-linked β-cyanoalanine synthase, (ii) be converted to cysteine by serine sulfhydrase, which is then converted to β-cyanoalanine by a cysteine-linked β-cyanoalanine synthase or by a secondary function of *O*-acetyl-serine sulfhydrase, or (iii) be converted to *O*-acetyl-serine by serine acetyltransferase (EC 2.3.1.30., acetyl-coenzyme A:L-serine *O*-acetyl-transferase) and then to β-cyanoalanine by *O*-acetyl-serine sulfhydrase. In *B. megaterium,* pathway (ii) probably occurs, and if serine transferase is present, possibly by (iii),

whereas the serine-linked β-cyanoalanine synthase (i) does not appear to be formed.

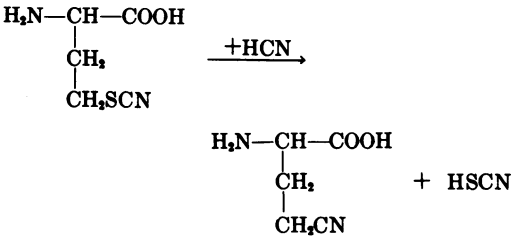
γ-Cyano-α-Aminobutyric Acid Formation

Brysk and Ressler have reported that strain D341 of *C. violaceum* is able to form γ-cyano-α-L-aminobutyric acid (21). This compound was not detected during growth on a glutamate-minimal salts medium, when cyanide and β-cyanoalanine were formed, but when washed, early logarithmic-phase cells were incubated with cyanide plus glutamate, serine, or threonine there was accumulation of both β-cyanoalanine and γ-cyano-α-aminobutyric acid. When cyanide and aspartate or asparagine were used, only γ-cyano-α-aminobutyric acid accumulated. Aspartate could have been acting as an end product repressor of the β-cyanoalanine pathway, hence preventing β-cyanoalanine synthesis (21). Aldehydes can act as metabolic precursors of nitrile formation (118, 170, 172, and above), and it is possible that aspartate semialdehyde acts as the precursor of γ-cyano-α-amino-butyric acid in *C. violaceum* (21). However, incubation of the organism with cyanide and aspartate semialdehyde did not result in its formation, although this could have been due to permeability problems for entry of the semialdehyde into the cells. Labeling experiments showed that the cyano group of γ-cyano-α-butyric acid stemmed from cyanide and the C1 to C4 atoms from aspartate. Thus, either aspartate acted as a direct precursor of the nitrile or a substance derivable from it participated without degradation of the carbon chain. There was no evidence in in vivo experiments for further metabolism of γ-cyano-α-aminobutyric acid, and in sonic extracts no nitrilase activity was detected, suggesting that the nitrile is a detoxication product of cyanide rather than the first metabolite of a pathway of cyanide assimilation (21).

Ressler and her co-workers have since purified an enzyme from *C. violaceum* strain D341 that catalyzes synthesis of γ-cyano-α-aminobutyric acid from the unusual amino acid homocystine and from cyanide (138). The first step is non-enzymatic formation of γ-thiocyano-α-aminobutyric acid and homocysteine from cyanide and homocystine:



The enzyme γ-thiocyano-α-aminobutyric acid thiocyano-lyase (adding CN; EC 4.4.1.) catalyzes the formation of γ-cyano-α-aminobutyric acid and thiocyanate:



The enzyme requires pyridoxal phosphate as a cofactor. β-Cyano-α-aminobutyric acid synthase activity does not appear to be a secondary function of the enzyme. In particular, *O*-acetylhomoserine is only a poor substrate.

Washed cell suspensions of *C. violaceum* therefore probably convert aspartate to homocystine before addition of cyanide and formation of γ-thiocyano-α-aminobutyric acid and γ-cyano-α-aminobutyric acid. Ressler et al. have also shown that incubation of *C. violaceum* with γ-thiocyano-α-aminobutyric acid and cyanide results in a greater formation of γ-cyano-α-butyric acid than incubation with aspartate and cyanide (138).

GROWTH OF MICROORGANISMS ON CYANIDE

In view of the large quantities of cyanide effluents produced by industry and the formation of cyanide by many plants, it is remarkable how few studies there have been of the growth of microorganisms on cyanide as a carbon and/or nitrogen source. Furthermore, cyanide is a one-carbon compound, and the utilization of other C1 compounds by microorganisms has been extensively studied because of their scientific (133) and industrial (81) importance.

Pettet and Ware (128) and Ware and Painter (190) isolated a cyanide-utilizing bacterium. A small percolating filter was seeded with cyanide-acclimatized sewage sludge and percolated with up to 100 μg of KCN per ml in tap water for several weeks. Cyanide-utilizing organisms could not be obtained by plating the cyanide-free effluent onto nutrient agar, but small colonies were isolated when silica gel plates containing cyanide in tap water were inoculated with some of the crust lining the coke particles of the percolating filter. The colonies were mixed cultures of a gram-negative bacillus and a gram-positive filamentous organism. The former was removed by plating in the presence of dihydrostreptomycin sulfate.

The gram-positive filamentous bacterium is

probably an actinomycete (128). On silica gel plates it forms white colonies with segmented hyphae. Aerial hyphae that bear conidia are produced, but the organism is not resistant to drying. Growth occurs at pH 7.5 to 9.0, and the optimum temperature is "below 30°C." It is a strict autotroph; growth is slow and sparse and inhibited by agar or peptone. The optimal cyanide concentration for growth is about 1.5 mM, but up to 6 mM cyanide can be utilized. Cyanide is converted to ammonia.

Winter (194) utilized a sample of the cyanide-acclimatized sludge of Pettet and Ware to seed coke-filled percolating filters, and isolated from them two organisms capable of growing on cyanide as a carbon and nitrogen source. These organisms are facultative autotrophs, able to grow rapidly and profusely on cyanide and a variety of organic nutrients. Both organisms grow as filamentous, gram-positive rods with sparse aerial hyphae. Coccoid, spore-like cells form, which germinate to form fresh mycelia. They are catalase positive and have an optimum temperature range of 25 to 30°C. They are both actinomycetes, probably of the genus *Nocardia*.

Skowronski and Strobel (157) have isolated a strain of *Bacillus pumilus* from samples of Fargo clay that had been planted with flax. The organism was isolated in media containing 2.5 M cyanide, and it degrades cyanide to carbon dioxide and ammonia when grown in Trypticase soy broth containing 0.1 M cyanide. In cyanide-containing media, the organism grew in a filamentous form, which did not revert to the rod form when regrown in cyanide-free media. In a basal salts medium containing cyanide as the sole carbon and nitrogen source, a 10-fold increase in cell numbers was noted.

Sakurai (144) (quoted by Mikami and Misono [106]) has isolated a cyanide-decomposing bacterium, and Howe (73) cites the isolation of a cyanide-utilizing strain of *Arthrobacter* by Aaslestad.

There have also been several reports of microorganisms able to use cyanide as a nitrogen source. Rangaswami and Balasubramanian (134, 135) and Iwanoff and Zwetkoff (77) claim to have isolated strains of *Aspergillus niger* that utilize cyanide as the sole source of nitrogen, and Furuki and co-workers (54) have studied the conditions of optimum growth of a bacterium that utilizes cyanide as a nitrogen source. The latter organism grows best at pH 10.2 in the presence of 400 to 500 μg of cyanide per ml and with glucose, fructose, mannose, or galactose as carbon sources.

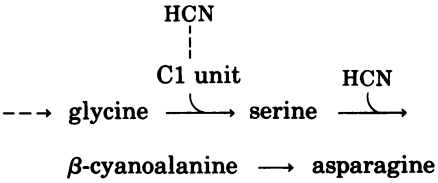
Trelawny et al. have isolated a soil bacterium that can utilize cyanoacetate as the sole source of nitrogen and carbon (178). It is unable to utilize cyanide as a carbon and nitrogen source, even though free cyanide is formed during metabolism of cyanoacetate, but in the presence of succinate it utilizes cyanide, thiocyanate, and ferri- or ferrocyanide as nitrogen sources.

There have been several reports of autotrophic bacteria able to grow on thiocyanate as a carbon and nitrogen or a nitrogen source (64, 65, 75, 132, 167, 199). Putilina reported that a thiocyanate utilizer (claimed to be *Pseudomonas nonliquefaciens*) was also able to oxidize cyanide as a carbon and nitrogen source (132).

There have been no reports of the metabolic pathways of cyanide assimilation by organisms able to utilize it as a carbon and/or nitrogen source. However, cyanide is a relatively simple molecule, and it is possible to predict some of the pathways of incorporation.

Formamide hydrolyase (cyanide hydratase), purified from the fungus *S. loti*, degrades cyanide to formamide (53). Many methylotrophic bacteria can grow on formamide as a carbon and nitrogen source (133), and it is therefore possible that some methylotrophs or closely related organisms are able to utilize cyanide.

Cyanide could also be incorporated into microorganisms by the pathways of assimilation utilized by cyanogenic organisms (page 660). For example, in the case of the β-cyanoalanine pathway, a variant of the methylotrophic serine pathway (133) can be envisaged:



Howe has suggested several different pathways of cyanide incorporation, including pathways involving thiocyanate and cyanate as intermediates (73, 74).

UTILIZATION OF CYANIDE BY MICROBIAL ECOSYSTEMS

Soil Ecosystems

Despite the large number of reports of plant cyanogenesis, there has been little research on the effect of cyanide release into the soil on the neighboring microbial ecosystems.

Rangaswami and Balasubramanian (134, 135) analyzed the cyanide content of the roots of six varieties of sorghum: in each case the cyanide content was high in seedlings but reduced somewhat with age. As with non-cyanogenic plants, there was a distinct rhizosphere effect;

i.e., there were more microorganisms in the root rhizosphere than in the surrounding soil, presumably due to exudation of nutrients by the plants. No clear relationship was established between microbial population and cyanide content of the roots, although there were more gram-negative and fewer gram-positive and sporeforming bacteria in the rhizospheres than in the surrounding soil. No strains of the plant pathogens *Helminthosporium* and *Fusarium* could be detected in the rhizospheres.

When a volatile exudate of the roots of sorghum plants, containing cyanide, was passed through a suspension of the rhizosphere microflora of sorghum, the bacterial population increased fivefold, the fungal population decreased fivefold, and the population of the actinomycetes was unaffected (135). Germination and growth of the pathogens *Helminthosporium turcicum* and *Fusarium monoliforme* were delayed by 1 to 2 days by the sorghum exudate but were not further inhibited, nor was growth of the fungi when they were added to soils containing growing sorghum plants.

Strobel has shown that cyanide can be metabolized by various soil ecosystems (171). Fargo clay that had been planted with flax for many years was especially able to utilize cyanide. Double-labeled cyanide ($H^{14}C^{15}N$) was metabolized by Fargo clay to $^{14}CO_2$, $^{15}NH_3$, and other products. Measurements of the ratio of ^{14}C-^{15}N in the administered HCN to the ratio in the evolved CO_2-NH_3 (other than NH_3 itself and hydrolizable nitriles) suggested that more cyanide nitrogen than carbon was retained by the soil.

Allen and Strobel have made the intriguing proposal that a "cyanide microcycle" operates in the soil (4). The occurrence of both cyanide-evolving and -utilizing plants, fungi, and bacteria means that in the microatmosphere of the soil molecules of HCN could be directly transferred from plants to microorganisms, and vice versa, as sources of nitrogen and/or carbon without prior conversion to CO_2 and NH_3.

Disposal of Cyanide Wastes by Sewage Systems

The electroplating, steel, carbonization, and other important industries produce large quantities of cyanide wastes. To prevent damage to natural ecosystems, the cyanide content of these effluents must be reduced to essentially zero before discharge into the environment. Even for discharge into a sewer there must be a drastic initial reduction to no more than 1 to 2 μg of cyanide per ml (128). Conventional chemical methods of disposal of cyanide wastes are wasteful of resources, because other chemicals, such as chlorine, are required for detoxication of the cyanide (72, 161). In addition, chemical degradation is relatively expensive and often requires further disposal of the products (73).

It was long thought, due to the toxicity of cyanide and its affinity for cytochrome oxidase, that biological disposal of cyanide wastes would be impractical. Furthermore, cyanide wastes usually contain other extremely toxic chemicals such as phenol, organic nitriles, cyanate, thiocyanate, and, in electroplating wastes, heavy metals (Cd, Cr, Cu, Fe, Ni, and Zn). Shock treatment of sewage systems does, in fact, lead to loss of viability, but it became apparent about 20 years ago that gradual acclimatization of sewage systems enables them to degrade moderate concentrations of cyanide with a high degree of efficiency. More recently, several patents (e.g., U.S. patents 3,145,166 and 3,165,166) have been taken out for biological methods of decomposition of cyanide wastes and some commercial sewage plants have been constructed. High initial capital costs are involved in the construction of suitable sewage systems, but running costs are much lower than for chemical methods of cyanide disposal; biological treatment is therefore particularly suitable for regular disposal of large quantities of wastes of moderate cyanide concentration (up to about 100 μg/ml).

Howe has reviewed the literature on cyanide decomposition by sewage systems (72–74). Some of the information cited is of greater interest to pollution control engineers than microbiologists, and only the papers of more direct microbial relevance will be discussed here; the reader should refer to Howe for other information and references (see also 106, 152–154).

Pettet and Mills investigated the effects of feeding settled domestic sewage containing KCN and the cyanide complexes of Zn, Cd, Cu, Ni, and Fe to small percolating filters filled with metallurgical coke (127). Zn, Cd, and Ni were present as $K_2M(CN)_4$ (M = metal), Cu was present as $K_3Cu(CN)_4$, and Fe was present as $K_4Fe(CN)_6$: stabilities of the complexes increase in the order Cd, Zn, Cu, Ni, Fe.

The filters were gradually acclimatized to increasing concentrations of the metal cyanides. Exposure to 1 to 4 μg of the cyanides per ml caused initial disturbances in biological oxygen demand and nitrification, but recovery occurred within a few days and up to 150 μg of cyanide per ml could be tolerated. Very little cyanide was detected in the effluents from filters fed 1 to 100 μg of K, Zn, and Cd cyanides per ml, but on filters fed 100 μg of Cu, Ni, and Fe cyanides per ml the cyanide contents of the effluents were 2.7, 15, and 38 μg/ml, respec-

tively. This result was probably due to the inhibitory effects of the more stable Cu and Ni cyanides and the inactivity of the sewage system towards ferrocyanide.

Filters acclimatized at 100 μg of the metal cyanides per ml in sewage were subsequently treated by the cyanides containing gradually decreasing concentrations of sewage, until eventually the metal cyanides in tap water were used as the feedstuffs. The K, Zn, and Cd cyanides were totally degraded, and most of the nitrogen was recovered as nitrate and nitrite. The Cu and Ni complexes were 75 to 80% degraded and nitrogen was recovered as ammonia, nitrate, and nitrite, but ferrocyanide was attacked only slightly. Thus, sewage microorganisms not only degrade cyanide, but some of them at least are also able to subsist on cyanide.

Winter seeded laboratory-scale trickling filters with two cyanide-utilizing actinomycetes (page 666) and fed the filters KCN and cyanide plating wastes (194). The filters degraded over 90% of the cyanide when fed 25 μg of KCN per ml for several weeks, but with an influent containing 45 to 137 μg of KCN per ml, the efficiency of degradation was only 63%. Feeding $Cu(CN)_2$ or a 4:1 mixture of $Cu(CN)_2$-$Zn(CN)_2$ at up to 18 μg of metal per ml (plus KCN to give 50 to 100 μg of cyanide per ml) resulted in a 70 to 90% degradation of cyanide. When "grab samples" of cyanide plating wastes, containing 0 to 50 μg of Ni, Zn, Cu, Fe, Cr, and Al per ml (7 to 35 μg of cyanide per ml, total), were fed to the filter, cyanide degradation declined to almost zero but totally recovered when refed KCN. Maintaining the grab samples at pH 6.5 gave an efficiency of cyanide decomposition of only 40%, but when composite cyanide plating wastes, of greater uniformity of metal composition, were used the efficiency was 95 to 100% (at pH 8 and 20 to 25 μg of cyanide per ml).

Degradation of cyanide has also been studied in activated sludge systems. Nesbitt et al. (121) gradually acclimatized settled, primary sludge to cyanide and then fed 3-liter aerated, activated sludge systems with 60 or 120 mg of cyanide per day for several months. Cyanide, as the sole source of carbon and nitrogen, was completely metabolized, and there was no loss of suspended solids. About 98% of the cyanide-carbon was converted to CO_2 and 75 to 90% of the cyanide-nitrogen was converted to ammonia, nitrate, and nitrite.

Ludzack and Schaffer (94) studied degradation of cyanide, cyanate, and thiocyanate in a laboratory-scale, complete-mixing activated sludge unit. Utilization of cyanide was found to be improved by addition of dextrose to the influent. Up to 60 mg of cyanide per liter of influent was completely degraded, with no loss of suspended solids and with recovery of the cyanide-nitrogen as ammonia and nitrate plus nitrite. The periods of acclimatization to increasing concentrations of cyanide were found to be crucial, although acclimatized sludges readily accepted variable influent levels of cyanide. On the other hand, shock (slug) doses of cyanide were found to be metabolized by unacclimatized sludges, but it took the system several days to recover normal operating capacity: cyanide was probably bacteriostatic rather than bacteriocidal.

Activated sludge systems were also found to degrade thiocyanate and cyanate, although cyanate was more difficult to handle and cyanate sludges were unstable (94). It has been argued that cyanate and thiocyanate are interconverted (199) or converted to cyanide (72, 74) during their utilization. Therefore, sewage systems acclimatized to cyanate, thiocyanate, or cyanide might exhibit cross-acclimatization to the other chemicals. However, this was not found to be the case (94), and degradation of each of these chemicals probably occurs via pathways not involving the formation of the others as intermediates.

Aerated, activated sludge systems are able, like percolating filters, to degrade cyanide in the presence of significant concentrations of heavy metals. Cyanide-acclimatized activated sludges were shown by Mikami and Misono (106) to totally degrade 100 to 150 μg of cyanide per ml, and 85 to 95% of the cyanide-nitrogen was found in the effluent stream as ammonia, nitrate, and nitrite. Provided the sludge was gradually acclimatized to Cr(VI) (up to 15 μg/ml) or Cu(II) (up to 10 μg/ml) and the level of cyanide loading (at 150 μg of cyanide per ml) was suitably adjusted, total degradation of cyanide and the nitrogen balance were maintained. When Ni(II) was fed to the system mixed with the cyanide influent, it formed a nondegradable complex; the complex did not form when separate cyanide and Ni(II) (up to 3 μg/ml) feeds were used and cyanide degradation proceeded, but with enhanced nitrification.

Because cytochrome oxidases are usually highly cyanide sensitive, it might be expected that anaerobic digestion systems would have a greater capacity to degrade cyanide wastes than aerobic systems, particularly when other organic foodstuffs are present. A two-stage anaerobic digester has been developed (73) that uses a carefully controlled primary stage and has partical recycling of the sludge from the

second stage, and that is notably efficient in its ability to degrade relatively high concentrations of cyanide wastes at high loading rates.

MECHANISMS OF CYANIDE RESISTANCE AND DETOXICATION

It is a common myth that cyanide is a *specific* inhibitor of cytochrome oxidase. In fact, cyanide inhibits a wide range of enzymes, many of which, though by no means all, are hemoproteins or other metal-containing oxidases or oxygenases. Dixon and Webb have listed some of the enzymes known to be inhibited by cyanide (41). Cyanide is a very reactive molecule, and it is not surprising that it is catholic in its inhibitory tastes. It forms stable complexes with many metals, reacts with keto groups to form cyanohydrins, and reduces thiol groups (136). At concentrations of about 10^{-4} M or lower, cyanide is usually highly inhibitory to cytochrome oxidase but has little effect on other enzymes, which require 10^{-4} to 10^{-2} M cyanide for significant inhibition; there are, of course, many exceptions to this generalization. Therefore, treatment of cells with "low" concentrations of cyanide often results in an apparently specific inactivation of respiration.

How, then, do some microorganisms manage to adapt to growth in the presence of cyanide? They can either induce enzymes for degradation and detoxication of cyanide or form cyanide-resistant enzymes. Because cytochrome oxidase is sensitive to cyanide poisoning and respiration is central to functioning of the cell, the development of cyanide-resistant respiratory systems is of particular interest and, consequently, there have been several studies of cyanide-resistant respiration, but there has been, to my knowledge, no research on the adaptation of other cyanide-sensitive enzymes to cyanide resistance.

Differentiation between cyanide-sensitive and cyanide-resistant organisms has been used as a taxonomic test. Møller developed a cyanide-containing medium (116), which has been improved by inclusion of an indicator (56) and a higher concentration of cyanide (119). It has been shown that the *Enterobacteriaceae* can be subdivided into cyanide-sensitive (*Escherichia, Shigella, Edwardsiella, Salmonella, Arizona*) and cyanide-resistant (*Citrobacter, Klebsiella, Enterobacter, Serratia, Proteus, Providencia, Aeromonas*) species (79, 119).

Preliminary studies in my laboratory have shown that the active component of complex cyanide differentiation media is probably cysteine (N. Porter, D. E. F. Harrison, and C. J. Knowles, unpublished observations).

Cyanide-Resistant Respiration

There have been innumerable reports on the effects of cyanide on respiration by intact microorganisms, mitochondria, and cell-free extracts (10, 55, 68, 91, 160). Cyanide-resistant "alternate oxidases" (60, 69, 70, 77, 82, 91, 146, 148, 157), which are not cytochromes, have been found in various eukaryotic microorganisms; such oxidases can be induced by manipulating the growth conditions or by forming mutants (60, 70, 82, 124, 146). Seemingly, they are synthesized to counteract a depression in the level of cytochrome oxidase or other respiratory components. Branched respiratory systems also occur in bacteria, with one branch less cyanide sensitive than the other (1, 78, 192, 193).

It is not relevant to this review to discuss further the mechanism of inhibition of respiration by cyanide or cyanide-resistant alternate oxidases. Only the respiratory systems of cyanide-evolving microorganisms and adaptation of the respiratory systems of cyanide-resistant microorganisms to growth in the presence of cyanide will be discussed.

One method by which microorganisms could exhibit cyanide-resistant respiration depends on the spatial organization of the respiratory system across the cytoplasmic or mitochondrial membrane. For example, a bacterial cytochrome oxidase could be located on the cytoplasmic (inner) side of the plasma membrane, with the membrane impermeable to exogenously added cyanide. However, at physiological pH values, hydrogen cyanide (pKa = 9.1) is essentially undissociated, and as it is a small molecule it probably permeates membranes rapidly (3, 31). Using the turbidity change technique (3) with NH_4CN and KCN as substrates (plus ionophores, see reference 63), Wissing and Knowles (unpublished observations) have shown that a cyanide-evolving pseudomonad is readily permeated by cyanide. It is therefore improbable that crypticity of cytochrome oxidase to cyanide is an explanation for cyanide-resistant respiration.

Achromobacter. Arima and his colleagues (5, 111-115, 123) have studied the respiratory system of a strain of *Achromobacter* isolated from soil which grows rapidly in rich media containing 1 mM cyanide; cyanide is not degraded during growth (111).

Cytochromes *b* and *o* were formed during logarithmic growth under conditions of high aeration, but cytochromes *d* and a_1 were only induced in the stationary phase (5, 123). When 1 mM cyanide was included in the growth medium, the cytochrome *b* concentration of logarithmic-phase cells doubled, cytochrome *o* de-

creased by 40%, the cytochrome *d* content was 10-fold higher, and cytochrome a_1 was induced. Under conditions of limited aeration the cytochrome composition was similar to that found for aerobic growth in the presence of cyanide (5, 114).

A shift from aerobic growth in the presence of cyanide to aerobic growth in its absence caused cytochromes a_1 and *d* to disappear and the cytochrome *b* content to halve but had little effect on the cytochrome *o* content (5). Conversely, addition of 1 mM cyanide to aerobic cultures caused a massive induction of cytochromes a_1 and *d* and small decreases in the concentrations of cytochromes *b* and *o*.

Succinate oxidase activities of intact cells and sonic extracts and succinate dehydrogenase activity of extracts of cyanide-sensitive cells (aerobic growth) were 50 to 200% greater than the corresponding activities of cyanide-resistant cells (aerobic growth; 1 mM cyanide), whereas the activities of glucose oxidase in intact cells and NADH oxidase in extracts were both 50% greater in the resistant cells (5). Furthermore, resistant cells required 200-fold more cyanide for inhibition of respiration than sensitive cells (2×10^{-3} M and 10^{-5} M, respectively) (123). Adaptation of cyanide-resistant cells to cyanide sensitivity and vice versa resulted in the appropriate changes of cyanide sensitivity of the oxidases (5, 112).

To determine which, of the three possible cytochromes (a_1, *d*, or *o*), is the cyanide-resistant oxidase in *Achromobacter*, Arima and Oka (5, 123) adopted two approaches. The first approach was to examine the effect of cyanide on the level and rate of reduction of the cytochromes of resistant cells after addition of succinate under aerobic conditions (5). The results suggested a cyanide-resistant pathway of electron flow from cytochrome *b* to cytochrome *d*, and that cytochrome a_1 was sensitive to cyanide inhibition. The role of cytochrome *o* could not be determined in these experiments, but as it is the only oxidase found in sensitive cells and its concentration decreases in resistant cells, it is presumably the cyanide-sensitive oxidase.

The second approach used action spectra to detect relief of CO inhibition of oxidase activities in the presence and absence of cyanide (123); intense light of appropriate wavelength can specifically relieve inhibition of respiration via a particular oxidase (25, 26). Oka and Arima (123) used blue and red light for relief of inhibition of cytochromes *o* and a_1 and cytochrome *d*, respectively. For cyanide-sensitive *Achromobacter* (containing only cytochrome *o* as a possible oxidase), both red and blue light relieved inhibition by CO, but in the presence of 0.5 mM cyanide only red light restored activity. The cyanide-resistant oxidase was thus confirmed as cytochrome *d*.

Bacillus cereus. McFeters et al. have isolated a strain of *B. cereus* able to grow in Trypticase soy broth containing 1 mM cyanide and which adaptively develops respiratory resistance to cyanide inhibition (102). Suspensions of stationary-phase cells grown in media with or without cyanide showed biphasic curves of respiratory inhibition by cyanide. The second, sharper inhibition phase required a 10-fold greater cyanide concentration for cells grown in the presence of cyanide. Reduced minus oxidized difference spectra showed that the bacterium normally formed type *b* and *a* (probably $a + a_3$) cytochromes but that when grown in the presence of cyanide the contents of these cytochromes were 50 and 100% greater, respectively, than in cells grown in cyanide-free media. Reduced plus CO minus reduced difference spectra and photodissociation spectra of CO-inhibited bacteria (CO action spectra) confirmed that in cyanide-resistant organisms cytochrome a_3 was the oxidase.

C. violaceum. Niven et al. (122) studied the respiratory system of *C. violaceum* grown under conditions of high and low cyanide evolution; the maximal cyanide levels in the cultures were 1.8 mM and 30 μM, respectively, by the start of the stationary phase.

A particulate fraction derived from stationary-phase, cyanide-evolving cells was unable to oxidize the high-potential electron donor, reduced *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), whereas a comparable particulate fraction from cells grown under conditions of low cyanide evoluation rapidly oxidized reduced TMPD, and the oxidase activity was highly sensitive to inhibition by cyanide or azide. Oxidation of reduced TMPD by other microorganisms is also very cyanide sensitive (70, 78, 82, 192).

The particulate fraction from cyanide-evolving cells of *C. violaceum* oxidized NADH and succinate by oxidases that were very resistant to inhibition by cyanide and azide; the inhibition curves were monophasic (123). The particulate fraction from cells evolving little cyanide also oxidized NADH and succinate, but the cyanide and azide inhibition curves were biphasic. In each case there was an extensive plateau region, at about 50% of the overall oxidase activity, separating the highly inhibitor-sensitive and relatively inhibitor-resistant phases.

It seems likely that the particulate fraction from cyanide-evolving cells synthesizes a linear respiratory system with cyanide- and azide-resistant oxidases, whereas the particulate frac-

tion from cells evolving little cyanide forms an additional respiratory branch with a cyanide-sensitive oxidase; reduced TMPD is oxidized by the latter pathway.

C. violaceum, grown either under conditions of high or low cyanide evolution, formed a complex complement of cytochromes (122). It has not so far been possible to determine which cytochrome is the cyanide-resistant oxidase. Cyanide-evolving cells contained cytochromes a_1, *d*, *o*, and *c* as the CO-binding cytochromes, and hence possible oxidases, whereas cells evolving little cyanide formed only cytochromes *o* and *c* as the CO-binding cytochromes. Cytochromes a_1, *d*, and *o* are known bacterial oxidases (25, 26), but CO-binding, nondenatured *c*-type cytochromes are comparatively rare and it has yet to be clearly established (192) that they are oxidases, although in methylotrophic bacteria a CO-binding cytochrome *c* is involved in methane oxygenase activity (177). The CO-binding cytochrome *c* of *C. violaceum*, unlike the membrane-bound cytochromes a_1, *d*, and *o*, is mainly soluble in cell extracts, although in the intact cell it is probably periplasmic in location (122).

E. coli. Ashcroft and Haddock have shown that *E. coli* strain C-1 can grow aerobically, on a medium containing D-xylose as the carbon source, in the presence of up to 1 mM cyanide with little effect on the growth rate, although the lag period is extended and the growth yield decreases (6). When succinate, which can provide energy only by the respiratory system, is the carbon source, 250 μM cyanide is the maximal cyanide concentration that can be tolerated. On succinate the mean generation time increases from 2.4 h in the absence of inhibitor to about 7 h in the presence of 150 μM cyanide.

The respiratory system was examined in particulate fractions derived from early logarithmic-phase cells of *E. coli* that had been grown on succinate in the presence and absence of 150 μM cyanide (6). Growth in the presence of cyanide had little effect on the ubiquinone and total *b*-type cytochrome contents, but the menaquinone, cytochrome *d*, and cytochrome a_1, levels increased substantially and the cytochrome *o* level decreased somewhat. With growth in the absence of cyanide, cytochromes b_{556} and b_{562} were the only spectrally distinct cytochromes, but in the presence of cyanide cytochrome b_{558} was also observed. NADH oxidase activity was unaffected by growth in the presence of cyanide, but its sensitivity to inhibition by cyanide was dramatically lowered (from 75 to 500 μM cyanide for 50% inhibition).

Thus, the overall effect of inclusion of cyanide in the growth medium is the formation of a respiratory system of similar composition to that found for anaerobic growth of *E. coli* or for oxygen-limited, stationary-phase aerobic cultures (see Haddock and Schairer [61] for references). Pudek and Bragg had previously shown (130, 131) that mid-logarithmic-phase cells of *E. coli* strain NRC-482 formed equal quantities of cytochromes *d* and *o*, whereas in the stationary phase the content of cytochrome *d* increased but there was very little cytochrome *o* present. NADH oxidase of the particulate fraction from stationary-phase cells was much more resistant to cyanide inhibition than particles from logarithmic-phase cells. The increased resistance of stationary-phase cells was partially due to the greater overall cytochrome *d* content and partially due to its increased content relative to the cyanide-sensitive oxidase, cytochrome *o*. Cytochrome a_1 is also formed in stationary-phase cells of *E. coli*, or in logarithmic-phase cells grown in the presence of cyanide, and, as it is also CO binding, it could also be an oxidase. Kinetic evidence, however, suggests that it is not an oxidase in *E. coli* (reference 42, cited in reference 6).

Tetrahymena pyriformis. There have been several reports of the adaptation of growth and respiration of the protozoan. *T. pyriformis*, to cyanide, which have been reviewed by Danforth (40).

McCashland and Pace showed that very low concentrations of cyanide (e.g., 10^{-9} M) stimulate growth and respiration of *T. pyriformis* strain W but that higher concentrations inhibit both of these processes (98). McCashland (95, 96) grew strain W in media containing gradually increasing concentrations of cyanide (from 10^{-12} to 10^{-4} M). Cells adapted to 10^{-4} M cyanide have 82% of the volume of normal, nonadapted cells, and their nucleus-cytoplasmic volume ratio is greater. Respiration of adapted cells is more resistant to inhibition by 1 mM cyanide than normal cells, and adapted cells lose their resistance to cyanide when regrown on cyanide-free media. Glucose is used more rapidly by adapted than normal cells, but this does not reflect a switch to a fermentative type of metabolism, because the lactate output is similar in normal and adapted cells (about 50% of the glucose is converted to lactate) (99). The gross metabolism of fats is unchanged, but adapted cells utilize amino acids more rapidly during growth than do normal cells.

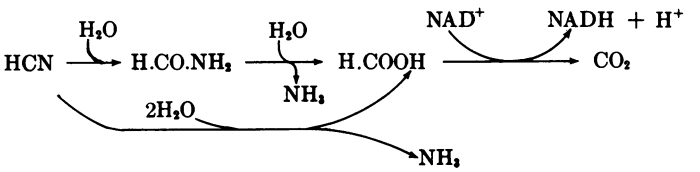
The sensitivity of seven strains of *T. pyriformis* to cyanide has been examined (97). Millimolar cyanide inhibits growth of all the strains by 80% or more; strains E and W are particularly sensitive. Respiration of strains E and W is 75 to 80% inhibited by 1 mM cyanide,

whereas respiration of the other strains is only 0 to 10% inhibited by 1 mM cyanide and 8 to 50% inhibited by 5 mM cyanide.

Cyanide Detoxication

Many microorganisms are resistant, or can induce resistance, to cyanide by degrading it to nontoxic products. Thus, cyanide-acclimatized sewage systems (72, 94, 121, 127) and specific microorganisms (157, 190, 194) have been reported to convert cyanide to carbon dioxide and ammonia, nitrite, or nitrate. In addition, it is possible that cyanide may be detoxified by conversion to β-cyanoalanine or other products reported to be intermediates of cyanide assimilation (page 661). However, such pathways of detoxication would be wasteful of the cells' resources (e.g., loss of serine or cysteine in the case of β-cyanoalanine formation). It is possible that these pathways act to recycle intermediates, with the ultimate conversion of cyanide to ammonia and carbon dioxide rather than its assimilation, although this would seem to be a needlessly complex method of detoxication.

The simplest method of detoxifying cyanide is to convert it to formate and then to carbon dioxide (by formate dehydrogenase). Conversion to formate could be direct (by a nitrilase) or via formamide (by cyanide hydratase and formamidase):



In support of this possible pathway, spores of *S. loti* have been shown to induce a cyanide hydratase, forming formamide as the detoxication product; the enzyme has been partially purified (53). In addition, *Fusarium solani* catalyzes release of ammonia from cyanide (the carbon product is unknown), and the enzyme responsible has been purified (153).

In 1933, Lang reported the discovery of rhodanese [EC 2.8.1.1, thiosulfate:cyanide sulfurtransferase) from a wide variety of animal tissues and *E. coli* (83). The distribution, properties, and biological function of rhodanese have been reviewed recently (142, 191), and it will therefore only be briefly discussed here.

Lang showed (83) that rhodanese catalyzes the formation of thiocyanate and sulfite from thiosulfate and cyanide:

$$S_2O_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN^-$$

Rhodanese has since been shown to catalyze a wide range of reactions involving transformations of sulfur-containing molecules. A sulphane-containing anion donates sulfur to rhodanese, forming a rhodanese-sulfur complex, which then interacts with a thiophilic acceptor anion to regenerate free rhodanese:

$$RSO_xS^- + \text{rhodanese} \rightleftharpoons RSO_x^- + \text{rhodanese} - S$$

$$\text{rhodanese} - S + X^- \rightleftharpoons \text{rhodanese} + SX^-.$$

The sulfur donors include thiosulfate, thiosulfonate, persulfide, polysulfide, ethanesulfonate and thiotaurine, and the sulfur acceptors include cyanide, sulfite, organic sulfinates, thiols, dithiols, dithionite, and lipoate. The cyanide-to-thiocyanate reaction is atypical among the rhodanese-catalyzed reactions in that it is irreversible.

Long suggested that rhodanese, found in mammalian tissues, acts to detoxify cyanide (83). The later discoveries that rhodanese is located in the mitochondria of mammalian cells (165) and that cyanide-inhibited cytochrome oxidase is reactivated by addition of rhodanese and thiosulfate (166) would appear to confirm this hypothesis. However, more recently it has been realized that rhodanese has an important role in the metabolism of various sulfur-containing compounds (cf. 142, 191).

Rhodanese has been discovered in a wide range of bacteria. The frequent observations of rhodanese in *Thiobacillus* species (18, 31, 32, 90, 100, 145, 173, 175), in photosynthetic bacteria (159, 198), and in *Desulfphomaculatum* (11) suggest that, in these organisms at least, it has a role in sulfur metabolism rather than one as a cyanide detoxifying agent. Rhodanese has also been found in several non-photosynthetic heterotrophic microorganisms (7, 8, 83, 149, 180, 181). Some reports suggest that *E. coli* forms rhodanese, whereas others indicate that it does not (83, 149, 180): Westley has pointed out that nonspecific reactions can be mistaken for rhodanese activity (191).

In *B. subtilis* and *B. stearothermophilus*, rhodanese is constitutive (8, 181). Rhodanese activity in photosynthetic bacteria does not correlate with the ability to metabolize thiosulfate or the pathway of thiosulfate metabolism (198). On the other hand, in *Thiobacillus denitrificans* rhodanese can be induced threefold by

growth in media containing 40 or 400 μM cyanide (18) and growth of *Rhodopseudomonas palustris* in cyanide-containing media results in a doubling of rhodanese activity (198).

In summary, it seems likely, but has yet to be clearly established, that rhodanese has a role in cyanide detoxication by some microorganisms in addition to its function in the metabolism of sulfur-containing compounds.

Mercaptopyruvate sulfurtransferase (EC 2.8.1.2, 3-mercaptopyruvate:cyanide sulfurtransferase) catalyzes the formation of pyruvate and thiocyanate from mercaptopyruvate and cyanide (142, 191):

$$HS.CH_2.CO.COOH + CN^{\ominus} \rightarrow CH_3.CO.COOH + SCN^-$$

It is specific for mercaptopyruvate, but sulfite may replace cyanide, and the enzyme can release colloidal sulfur from mercaptopyruvate alone. In animals it functions as a transsulfurase (142). It has also been found in *E. coli* (80), where its function is not yet clear, but presumably it has a role in mercaptopyruvate metabolism rather than in detoxication of cyanide.

CONCLUDING REMARKS

In experiments designed to simulate the conditions of the prebiotic earth, significant concentrations of hydrogen cyanide have been routinely found (108, 109). Moreover, under similar conditions cyanide is readily converted to amino acids, adenine, and other biologically important compounds (124, 125).

It therefore seems probable that among the most primitive organisms (which must have been anaerobes since the early earth did not have an oxygen-containing atmosphere) were some that could metabolize cyanide, perhaps in conjunction with other carbon and nitrogen sources. At present there is some, though far from adequate, understanding of the pathways of cyanide assimilation by aerobic microorganisms, but virtually nothing is known about cyanide assimilation by anaerobic microorganisms. Obviously, it is important that more understanding be gained about cyanide assimilation by microorganisms, both from an evolutionary standpoint and from the light it can shed on the metabolism of one-carbon compounds.

Cyanide inhibits not only cytochrome oxidases but also a wide range of other enzymes (although usually at somewhat higher concentrations; 41). Hence, cyanide would be expected to inhibit growth of anaerobes as well as aerobes, unless they contain detoxication mechanisms. Because of the presence of cyanide in the primordial soup, it seems probable that the development of primitive anaerobic organisms was paralleled by evolution of mechanisms of detoxication of cyanide; these could have either been precursors of the pathways of cyanide assimilation found in contemporary microorganisms or the latter could have developed independently. Schievelbein et al. (147) have pointed out that cyanide now occurs relatively seldom in nature (although the reader of this article will be aware that it occurs less seldom than might otherwise be imagined), yet rhodanese activity is found in a wide range of microorganisms, plants, and animals. They have proposed that the original function of rhodanese was detoxication of cyanide rather than sulfur metabolism and, partially at least, that it is a biochemical relic.

Although the function of nitrogenases is to fix dinitrogen, they are also able to carry out a range of other reductive processes, including conversion of cyanide to ammonia and methane (67). If nitrogenase emerged at the time of evolution of primitive anaerobes, it would seem unlikely that its original function was to fix nitrogen: there would have been a lack of selective pressure in the ammonia-rich primitive universe. Silver and Postgate have pointed out (155) that the nitrogenase system might have initially evolved for reduction of some other substrate and only recently adapted to fix nitrogen; detoxication of cyanide is one such possible early function. On the other hand, nitrogenase might have evolved more recently and the ability to reduce cyanide, etc., is coincidental to its development (129).

At the time the atmosphere of the earth changed from anaerobic to aerobic and cytochrome oxidases evolved, cyanide was presumably still prevalent in the environment and primitive types of oxidase would have had to be relatively cyanide tolerant. Therefore, an understanding of the mechanisms of cyanide-resistant respiration could give some insights into the evolution of early aerobes, in addition to aiding our comprehension of the biochemical mechanism of oxidase function. It is not yet clear whether any particular one of the known cytochrome oxidases is specifically cyanide resistant, and hence possibly a direct descendant of a primitive oxidase, although cytochrome *d* could be a candidate since it is (i) the cyanide-resistant oxidase of *Achromobacter* (5, 123), (ii) the least cyanide-sensitive oxidase of *E. coli* (6, 130, 131) and *Azotobacter vinelandii* (1, 78), and (iii) induced in *C. violaceum* under cyanide-evolving growth conditions (122). It remains to be seen whether these *d*-type cytochromes are unusually cyanide insensitive or whether *d*-

type cytochromes in general are more cyanide resistant than other oxidases. Unlike cytochrome *d*, which is a bacterial oxidase, the cyanide-resistant alternate oxidases (which are not cytochromes) have so far been found exclusively in eukaryotes (70) and are, therefore, unlikely to be descendents of early oxidases.

Although it is self-evident why some microorganisms assimilate or grow on cyanide, it is not so immediately clear why the same or other microorganisms (and plants) produce cyanide. After all, production of so toxic a chemical will result in self-inhibition unless cyanide-resistant oxidases and other enzymes are concomitantly produced.

Cyanide excretion could be used to destroy or inhibit growth of neighboring organisms, thereby (i) increasing the competitiveness of the excreting organism, (ii) preventing possible predatory activity of the affected organisms, or (iii) enabling parasitism of other cells. In the snow mold, copperspot, and fairy ring diseases (page 653), a varient of (iii) occurs, whereby the parasitic fungi excrete an enzyme (β-glucosidase) that causes destructive release of cyanide from the host plant cyanogens, possibly with coordinate release of cyanide from the pathogens themselves. Traces of cyanide could also be produced by many bacteria and fungi as a convenient form of nitrogen and/or carbon (in the same way that heterotrophic microorganisms produce and utilize CO_2). Only under certain unusual conditions, such as growth in media containing an excess of the precursor (glycine), would relatively large and inhibitory concentrations of cyanide be formed. Finally, cyanide could be produced as a secondary metabolite, as proposed by Castric (27), acting solely to remove an excess of a substance (presumably glycine) that would otherwise be toxic to the cell if present in too high a concentration.

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