Development of Cyanide Tolerance in Stemphylium loti

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ABSTRACT

Stemphylium loti, an important pathogen of the cyanogenic plant, birdsfoot trefoil, adapts to cyanide. Adaptation to cyanide could not be effected by inhibiting terminal electron transport of the fungus with azide or an atmosphere of nitrogen. *Stemphylium loti* adapted to cyanide equally well whether glucose, glycerol, or acetate was the carbon source. Germinated spores adapted to cyanide much more quickly than did nongerminated spores. Adaptation to cyanide was not inherited by progeny of adapted spores. 2,4-Dinitrophenol inhibited adaptation as did cycloheximide, which selectively inhibited protein synthesis in this fungus. Protein synthesis was implicated as a prerequisite for adaptation of *S. loti* to cyanide. Phytopathology 61:501-506.

Stemphylium loti, a principal pathogen of birdsfoot trefoil (*Lotus corniculatus* L.), is adaptively tolerant of cyanide (2). This is apparently beneficial to the fungus during pathogenesis of trefoil because trefoil contains the cyanogenic glucosides linamarin and lotaustralin, and because cyanide is released either upon injury to healthy tissue or upon infection (7). Cyanide resistance in *S. loti* is characterized by (i) cyanide-stimulated respiration; and (ii) the disappearance of cyanide from solution (2).

Objectives of this investigation were to determine if adaptation of *S. loti* to cyanide is specifically a function of cyanide and to characterize and elucidate the associated events of adaptation of *S. loti* to cyanide.

MATERIALS AND METHODS.—A culture of *Stemphylium loti* Graham was obtained from J. H. Graham, USDA, Beltsville, Md. Virulence was maintained by passing the fungus periodically through its suscept, birdsfoot trefoil (*Lotus corniculatus* L.). Cultures were maintained on V-8 juice agar (8) under continuous fluorescent light at 21-23 C. Conidia of *S. loti* were harvested in water from 10- to 20-day-old cultures, and filtered through two layers of cheesecloth. The spores then were washed 3 times in distilled water by centrifugation (1 min at ca. 1,500 *g*) and suspended in 0.1 M potassium phosphate buffer at pH 6.5; the concentration of spores in the suspension was about 1 mg dry wt/ml.

Oxygen uptake of spore suspensions was measured either manometrically with a Gilson differential respirometer, Model GRP 20, or polarographically with a Gilson oxygraph, Model KM, equipped with a Clark electrode. All experiments were conducted at 25 C. Measurements with the respirometer were initiated after a 20-min equilibration period. In some experiments, cyanide was added to the reaction mixture in respirometer flasks. For these experiments, a mixture of $Ca(OH)_2$ and $Ca(CN)_2$ was placed in the center well of the flasks to prevent distillation of cyanide from the reaction mixture into the alkali in the center well (9).

Germination of *S. loti* spores was determined using the procedure reported previously (2). Spores did not germinate within 6 hr in the presence of 0.1 M phosphate buffer pH 6.5 and 0.05 M glucose, but did germinate when this medium contained yeast extract (0.5%).

Cyanide and azide determinations.—Cyanide concentration was determined spectrophotometrically, by means of alkaline sodium picrate (2, 7) procedure. Absorbance of the chromogen was measured at 480 nm with a Bausch & Lomb Spectronic 20 colorimeter. The chromogen developed when a solution of 1 ml of test solution and 4 ml picrate solution was heated 5 min in boiling water. Because glucose interfered with this assay, 0.05 M acetate was used as the carbon source in experiments in which cyanide was determined.

The concentration of azide was determined spectrophotometrically by a modification of the method used by Anton et al. (1). Ferric perchlorate (0.036 M in Iron III), 1.9 ml, was diluted with 10 ml distilled water, and the pH was adjusted to 2.2 with 0.1 N perchloric acid; 2 ml of solution containing azide and 5 ml of water then were added in turn. The pH of this solution was readjusted to 2.2, and the solution brought to a final volume of 25 ml. The absorbance of the resulting chromogen was measured at 454 nm; a 5 mM azide solution produced an absorbance of 0.26. Phosphate, but neither glucose nor spores, interfered with the azide determinations; consequently, for determination of azide concentration, spores were suspended in distilled water. The pH of the azide solution was about 6.7.

^{14}C-leucine incorporation and uptake.—Spores incubated 6 hr with ^{14}C-leucine were fractionated by a modification of the method of Roberts et al. (10), and the distribution of label in the different cell fractions was determined. The different fractions consisted of (i) the cold, trichloroacetic acid (TCA)-soluble materials; (ii) 75% ethanol (ETOH)-soluble materials; (iii) 75% ETOH-soluble, but ether-insoluble, materials; (iv) 75% ETOH-soluble, ether-soluble materials; (v) hot TCA-soluble materials; and (vi) the residual precipitate.

The incubation medium was 0.08 M phosphate buffer, pH 6.5; 0.05 M glucose; 0.04% Tween 20 (polyoxyethylene sorbitan monolaurate); about 60 mg (dry wt) *S. loti* spores; and 1.2 μc ^{14}C-leucine (specific activity 0.08 μc/μmole), in a total volume of 60 ml. Spores in this medium were shaken gently on a wrist-action shaker at 25 ± 3 C. After 6-hr incubation, spores were collected and washed 3 times with distilled water by centrifugation (5 min at 2300 *g*). Finally, the spores were suspended in 15-20 ml water and the cells broken by shaking them for 1 min (4,000 cycles/min) with 15 ml

of 0.45-0.50 mm diam glass beads in a Bronwill MSK cell homogenizer. The resulting homogenate was filtered through a copper screen (No. 100 mesh) into a 50-ml centrifuge tube, and the precipitate was discarded. The filtrate was centrifuged 10 min at 800 *g* to sediment whole cells, and this precipitate was discarded.

The supernatant was made to 5% in TCA, kept at 5 C for at least 30 min, then centrifuged 10 min at 2,300 *g* at 5 C. The supernatant was removed by aspiration, and constituted the materials soluble in cold TCA. The precipitate from the cold TCA solution was suspended in 6 ml 75% ETOH and maintained at 40-50 C for 30 min. Subsequently, it was centrifuged 10 min at 2,300 *g*. This supernatant contained materials soluble in 75% ETOH. A 2-ml aliquot from the ETOH-soluble fraction was diluted with 2 ml water, then extracted twice with 2 ml ether. The ether washings were discarded; the remaining solution contained materials soluble in ETOH but insoluble in ether. The material that precipitated from 75% ETOH was suspended in 6 ml of a solution containing 3 ml 75% ETOH and 3 ml ether. This suspension was maintained at 40-50 C for 15 min, then centrifuged 10 min at 2,300 *g*. Materials insoluble in ETOH-ether were resuspended in 5% TCA, and heated in a boiling water bath for 30 min. The suspension then was centrifuged 10 min at 2,300 *g*, and the supernatant removed by aspiration. The precipitate from the hot-TCA treatment was washed with acid alcohol (1 drop 6 N HCl in 2 ml 75% ETOH) and then with ether. Washing was accomplished by suspending the pellet in the wash medium, then centrifuging the suspension 10 min at 2,300 *g*. The residual precipitate was suspended in 10 ml water.

^{14}C-leucine uptake and oxygen uptake by spores were determined simultaneously. Oxygen uptake was determined with the respirometer. In one such experiment, respirometer flasks contained 2.6 mg spores (dry wt), 0.05 M glucose, 0.05% Tween 20, 0.08 M phosphate buffer pH 6.5, and 0.03 μc ^{14}C-leucine (specific activity 0.08 μc/μmole) in a total volume of 3.0 ml. In this experiment, half the flasks also contained cyanide (0.46 mM).

Oxygen and ^{14}C-leucine uptake were halted by addition of 5 ml 40% TCA to the respirometer flask. The spore suspension then was filtered onto a Gelman Metricel filter with pore size 0.45 μ. The spores were washed with 2 ml of a leucine solution (1 mg/ml), then with water. The filter containing the spores was transferred to a liquid scintillation vial. At 0 time, and at 2, 4, and 6 hr after initiation of the experiment, the radioactivity of spores exposed or not exposed to 0.46 mM cyanide was determined. The values reported are averages of three samples.

Radioactivity determination.—The radioactivity of whole spores or fractions of spores exposed to ^{14}C-leucine was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3002 or Model 3375. The scintillation medium (5) contained toluene, 350 ml; *p*-dioxane, 350 ml; methanol, 210 ml; naphthalene, 73 g; 2,5-diphenyloxazole (PPO), 4.52 g; and 1,4-[bis-2-(5-phenyloxazolyl)]-benzene (POPOP), 0.078 g. For samples containing cell components soluble in different solvents, the activity recovered (count/min) was converted to absolute activity (dpm) by the external standard method of quench correction. No quench corrections were applied to samples containing whole spores, because each sample in a given experiment contained the same wt of spores. The suspension or solution containing radioactivity was placed in a scintillation vial and dried at 70 C before scintillation medium (10 ml) was added.

Adaptation of spores to cyanide.—Some experiments required that *S. loti* spores be adapted to cyanide prior to initiation of the experiment. Adaptation was defined as the ability of spores to take up oxygen after initial inhibition of oxygen uptake by cyanide (2). Oxygen uptake was measured in the respirometer during the adaptation period of about 2 hr in 0.1 mM cyanide. Adaptation to cyanide was effected by subjecting a very dense spore suspension (spores in 0.1 M phosphate buffer, pH 6.5; 0.05 M glucose; and 0.05% Tween 20) to 0.1 mM cyanide. After the spores had adapted, they were removed from the respirometer vessels and rinsed several times in distilled water.

Other experiments required that spores be incubated in azide prior to initiation of the experiment. A very dense spore suspension of *S. loti* in 0.1 M phosphate buffer pH 6.5, 0.05 M glucose, and 0.05% Tween 20 was brought to 1.0 mM azide, and incubated in the respirometer for 4 hr. The spores then were removed from the respirometer vessels and washed. Spores incubated in 0.1 M phosphate buffer pH 6.5, 0.05 M glucose, and 0.05% Tween 20 for the same length of time as spores subjected to azide or cyanide served as controls in these experiments. All experiments were performed at least twice.

RESULTS.—*Specificity of response to cyanide.*—Cyanide complexes with cytochrome oxidase (3), and consequently it is an effective inhibitor of terminal electron transport. Adaptation to cyanide in *S. loti* might be either a response specifically to cyanide ion or a response to cyanide-inhibited electron transport. To test these possibilities, we inhibited electron transport of *S. loti* by exposing spores to an atmosphere of nitrogen or by incubating them in a solution of azide and subsequently determining their response to cyanide.

Spores responded similarly to 0.46 mM cyanide regardless of whether or not they were exposed to anaerobiosis. In one such experiment, spores subjected to anaerobic conditions (an atmosphere of N_2) for 2 hr at 25 C had a respiratory rate of 10 μliters/hr per mg of spores in the absence of cyanide and a rate of 1 μliter/hr per mg of spores in 0.46 mM cyanide. Spores not exposed to anaerobic conditions respired 10 μliters/hr per mg spores in the absence of cyanide, in 0.46 mM cyanide, they took up oxygen at the rate of 2 μliters/hr per mg spores. Respiration of spores subjected alternately to 2 hr of anaerobiosis, 1 hr of aerobiosis, and 2 hr of anerobiosis was inhibited about 70% by 0.46 mM cyanide. These data indicate that anaerobiosis does not cause *S. loti* to adapt to cyanide.

Oxygen uptake of spores exposed to azide was mea-

sured in the respirometer. Azide initially inhibited oxygen uptake nearly completely (Fig. 1); but within 3 hr, the fungus subsequently developed the capacity to take up oxygen.

The respiratory response to cyanide of spores which had apparently developed the capacity to respire in the presence of azide was determined. Oxygen uptake was measured with the respirometer. Spores which had previously been incubated in buffer alone or in buffer containing 1.0 mM azide were tested for their respiratory response to 0.1 mM cyanide.

In the absence of cyanide, spores previously incubated in azide took up 12 μliters oxygen/hr per mg, and spores previously incubated in buffer alone took up 8 μliters oxygen/hr per mg. In the presence of 0.1 mM cyanide, spores previously incubated in azide took up 22 μliters oxygen/hr per mg, whereas spores not previously exposed to azide took up only 2 μliters oxygen/hr per mg. These rates of oxygen uptake were determined in the first 30 min following the equilibration period. Thus oxygen uptake of spores which had been incubated previously in 1.0 mM azide was stimulated by cyanide. Conversely, the oxygen uptake of spores adapted to cyanide was not inhibited by azide. Spores, either adapted or not adapted to cyanide, were subjected to 1.0 mM azide, and the respiratory response to azide was measured for 30 min after equilibration of the mixture. In one such experiment, adapted spores respired 11 μliters/hr per mg in the absence of azide, and 13 μliters/hr per mg in azide. Nonadapted spores respired 9 μliters/hr per mg in the absence of azide and 2 μliters/hr per mg in azide.

Spores previously incubated in azide took up oxygen in the presence of cyanide, but germination of these spores was no better in cyanide than that of spores not previously incubated in azide. Germination of spores previously exposed or not exposed to azide was inhibited 95-100% by 2.2 mM cyanide. Previous incubation in azide also did not enable the spores to remove cyanide from solution (Fig. 2). Conversely, adaptation to cyanide did not improve spore germination in the presence of azide, because 0.5 mM azide completely inhibited germination of either adapted or nonadapted spores. Also, spores adapted to cyanide did not remove azide from solution. Oxygen uptake of spores preincubated in azide was only slightly inhibited in 5 mM azide (oxygen uptake was 80-100% the rate of similar spores not in azide), but the azide concentration remained at 5 mM during a 1-hr incubation period.

Characterization of adaptation of spores to cyanide.—The possibility that different substrates might affect the adaptation process was investigated by supplying glucose, glycerol, or acetate (all at 0.05 M) as carbon sources. Although glucose supported the most rapid rate of oxygen uptake, adaptation with each carbon source occurred within 2 hr when the spores were exposed to 0.1 mM cyanide.

We also tested the possibility that adaptation of *S. loti* to cyanide might be heritable. Spores were harvested, then exposed to 0.1 mM cyanide. Oxygen uptake was measured in the respirometer and after adaptation, these spores were removed and placed on V-8 juice agar. All of the procedures were accomplished aseptically. After 10 days of growth and concomitant sporulation, spores were harvested, and their respiratory response to 0.46 mM cyanide was determined. In the first hr of exposure to 0.46 mM cyanide, oxygen uptake was inhibited 75-90% regardless of whether the spores tested were the progeny of spores that had been exposed or not exposed to cyanide. Thus adaptation to cyanide was not inherited.

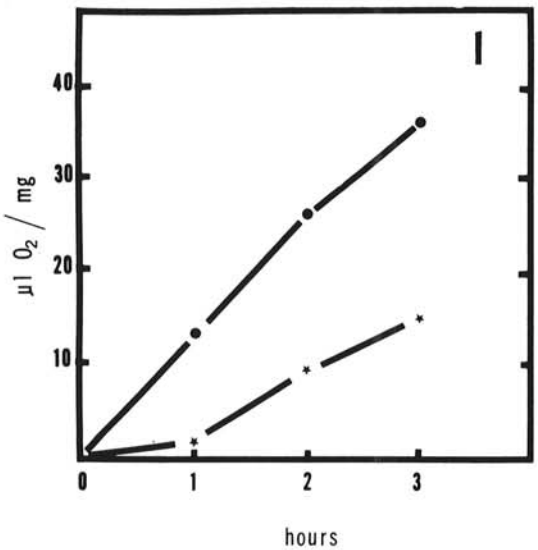
Because the spore is a relatively inactive stage of the fungus, we tested the possibility that adaptation to cyanide might occur more rapidly with germinated than with nongerminated spores. The results of a typical experiment (Fig. 3) demonstrated that germinated spores adapt markedly more rapidly to cyanide than do nongerminated spores. No inhibition of germinated spores by 0.46 mM cyanide was detectable with the respirometer. However, when the respiratory response of spores to cyanide was measured within 1-2 min with the oxygen electrode, both germinated and nongerminated spores initially were inhibited (to 30-50% of control) in 0.1 mM cyanide. Thus, germinated spores initially were inhibited by cyanide, but they adapted much more rapidly than did nongerminated spores.

We tested further the possibility that adaptation of *S. loti* required active synthesis by determining the effect of 2,4-dinitrophenol (DNP) on adaptation. In a typical experiment, spores were exposed to 0.46 mM cyanide, and the oxygen uptake was measured in the respirometer. In one set of flasks, however, DNP was tipped into the spore suspension 30 min after the initiation of measurements. Oxygen uptake of spores was inhibited by cyanide from the time of DNP addition. The final concentration of DNP was 0.1 mM. DNP inhibited adaptation of *S. loti* to cyanide (Fig. 4).

The possibility that protein synthesis might be involved in adaptation was investigated in a series of experiments. In one experiment, oxygen uptake and ^{14}C-leucine uptake were determined simultaneously. Initially, ^{14}C-leucine uptake and oxygen uptake were both inhibited by cyanide, but after about 2 hr oxygen uptake and ^{14}C-leucine, uptake occurred at uninhibited rates (Fig. 5).

Cycloheximide (CHI) is a potent inhibitor of protein synthesis (6) in other organisms. We used this antibiotic to help us elucidate the role of protein synthesis in adaptation of *S. loti* to cyanide. But, since the mode of action of CHI on *S. loti* had not previously been demonstrated, we first determined the effect of CHI (10 μg/ml) on incorporation of ^{14}C-leucine into the various fractions of *S. loti* cells.

After 6-hr incubation of spores in ^{14}C-leucine in the presence or absence of CHI, spores were washed and fractionated, and the radioactivity present in each fraction was determined. Table 1 gives the results of one such study. In the absence of CHI, 74% of the recovered radioactive materials were soluble in cold, trichloroacetic acid (TCA). Most of the radioactivity in this fraction is probably free ^{14}C-leucine or small mol wt metabolites of leucine. The protein fraction (re-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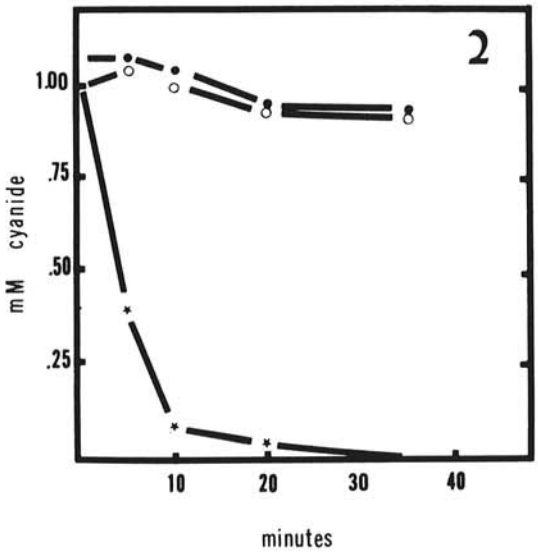
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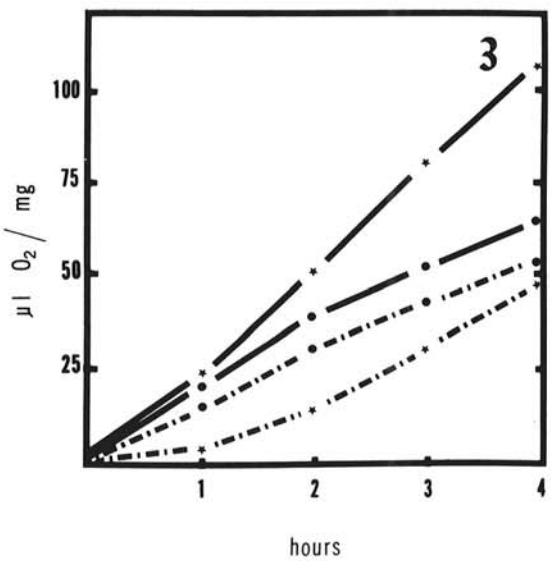
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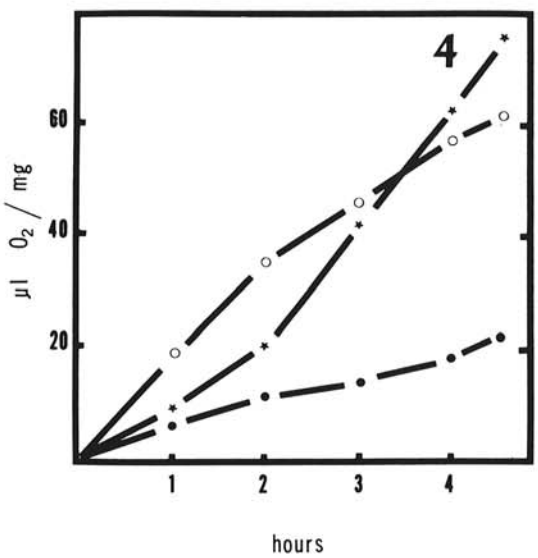
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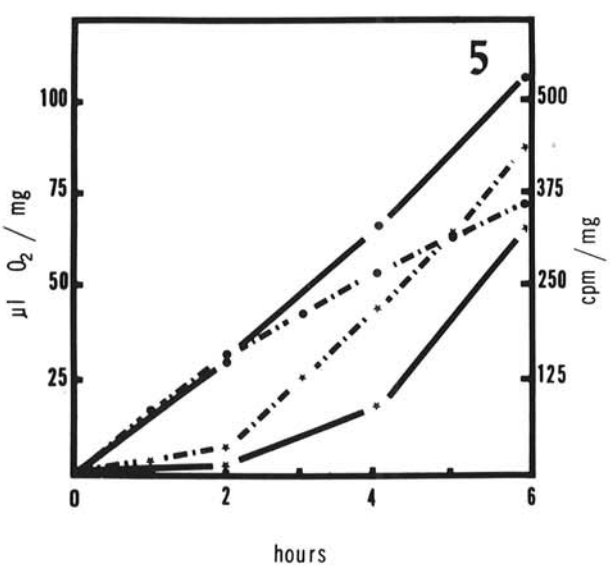
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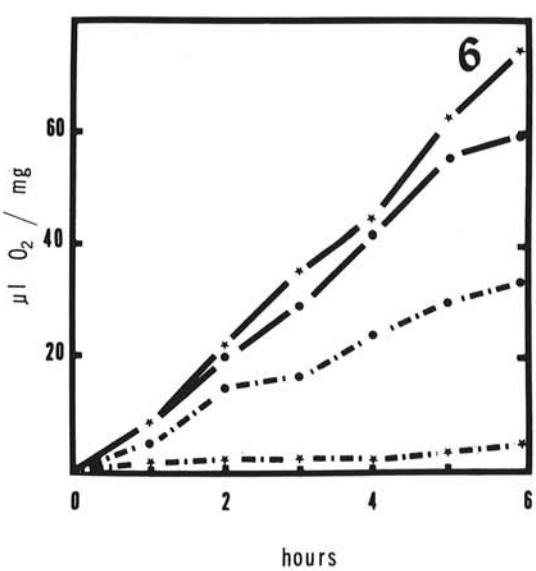
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Fig. 1-6. **1)** Effect of 0.32 mM azide on oxygen uptake of *Stemphylium loti* spores. Oxygen uptake was measured with a respirometer, and each vessel contained 0.6 mg/ml spores, 0.08 M phosphate buffer pH 6.5, and 0.05 M glucose, with (★——★) or without (●——●) azide, in a total volume of 3 ml. **2)** Effect of *S. loti* spores on cyanide solutions. Cyanide concentrations in spore suspensions were determined at time 0 and at 5, 10, 20, and 35 min after addition of cyanide. Spore suspensions consisted of *S. loti* adapted to cyanide (★——★), or *S. loti* previously incubated 4 hr in 1.0 mM azide (○——○). Control (●——●) consisted of incubation medium without spores. Incubation medium consisted of 0.05 M acetate pH 6.5, 0.08 M phosphate pH 6.5, spores 0.85 mg/ml, and cyanide in a total volume of 10 ml. Cyanide (1.0 mM) did not inhibit the oxygen uptake of spores adapted to cyanide or incubated 4 hr in 1.0 mM azide. **3)** Effect of 0.46 mM cyanide on oxygen uptake of germinated (solid lines) and nongerminated (dashed lines) *S. loti* spores. Oxygen uptake of spores in the presence (★——★) and absence (●——●) of cyanide was measured with the respirometer. Germination was effected by incubating the spores 4 hr in buffered medium containing 0.05 M glucose and 0.5% yeast extract. Nongerminated spores were incubated in buffered medium containing 0.05 M glucose. **4)** Effect of 0.1 mM 2,4-dinitrophenol (DNP) on adaptation of *S. loti* to cyanide. Adaptation was defined as regained ability of spores to respire in cyanide. Results are presented for spores incubated in the absence of cyanide and DNP (○——○), spores in 0.46 mM cyanide (★——★), and spores in 0.46 mM cyanide and 0.1 mM DNP (●——●). DNP or water was added to the spores 30 min after measurements were initiated. The medium contained 0.8 mg/ml spores, 0.08 M phosphate pH 6.5, and 0.05 M glucose, with or without cyanide and DNP. **5)** Effect of cyanide on uptake of oxygen and ^{14}C-leucine by *S. loti* spores. Oxygen uptake (dashed lines) was measured with the respirometer. Radioactivity (solid lines) of washed spores was determined in a liquid scintillation spectrometer. Results are presented for spores incubated in the presence (★——★) or absence (●——●) of cyanide. Medium consisted of 0.08 M phosphate pH 6.5, 0.05 M glucose, 0.05% Tween 20, and 0.01 μc/ml ^{14}C-leucine (specific activity 0.08 μc/μmole), with or without 0.1 mM cyanide, in a total volume of 3 ml. **6)** Effect of cycloheximide (CHI) on adaptation of nongerminated *S. loti* spores to cyanide. Results are presented for spores incubated in the presence (dashed lines) or absence (solid lines) of 10 μg/ml CHI, and for spores in the presence (★——★) or absence (●——●) of 0.1 mM cyanide. Oxygen uptake was measured with a respirometer. The reaction medium consisted of 1 mg/ml spores, 0.08 M phosphate pH 6.5, 0.05 M glucose, 0.05% Tween 20, 0.27 mM leucine, and cyanide or CHI in a total volume of 3 ml.

sidual precipitate and ethanol-soluble, ether-insoluble substances) contained 24% of the recovered radioactivity. In the presence of CHI (10 μg/ml), however, the protein fraction contained only 2% of the recovered radioactivity, and the materials soluble in cold TCA accounted for 97% of the radioactivity. It appears from these results that CHI inhibits more than 90% of the protein synthesis in *S. loti*.

Spore suspensions which contained CHI (10 μg/ml) were subjected to 0.1 mM cyanide, and the spores did not adapt to cyanide (Fig. 6); however, 10 μg/ml CHI inhibited respiration by about 50%, a result which was unexpected and remains unexplained.

TABLE 1. Effect of cycloheximide (CHI) at 10 μg/ml on incorporation of ^{14}C-leucine into *Stemphylium loti* spores[a]

Fraction	+CHI		−CHI	
	Absolute activity	%[b]	Absolute activity	%[b]
Cold TCA[c]-soluble	89,000	97	124,000	73.8
Ethanol & ether-soluble	506	0.5	1,850	1.2
Hot TCA[c]-soluble	132	0.2	1,460	0.9
Residual precipitate & ethanol-soluble, ether-insoluble	2,030	2.2	40,680	24.4
Total recovered activity	92,068		167,990	

[a] Spores (60 mg) were incubated for 6 hr in 60 ml of medium which consisted of 0.08 M phosphate buffer pH 6.5, 0.05 M glucose, 0.04% Tween 20, and 1.2 μc ^{14}C-leucine (specific activity 0.08 μc/μmole), with or without 0.6 mg cycloheximide. The spores were washed with nonradioactive leucine (1 g/ml), then fractionated as described in the text.

[b] Per cent of total activity recovered.

[c] TCA = trichloracetic acid.

DISCUSSION.—Two lines of evidence indicate that adaptation of *S. loti* to cyanide is a response specifically to the cyanide ion and not a response to inhibition of electron transport. Firstly, exposure of *S. loti* to azide, an ion which inhibits electron transport, elicited only part of the response that is elicited by exposure of the fungus to cyanide. Spores exposed to azide developed an azide-insensitive respiration which was also cyanide-insensitive. But these spores did not transform either azide or cyanide, nor did they germinate better in azide or cyanide than spores not previously exposed to azide. On the other hand, *S. loti* exposed to cyanide not only developed an ability to respire in cyanide, but also transformed or detoxified cyanide. Probably the ability to transform cyanide enabled the conidia to germinate when cyanide was added to the incubation medium. Secondly, incubation of *S. loti* in anaerobic conditions for short periods did not effect any of the capabilities associated with adaptation to cyanide.

Adaptation to cyanide apparently is a function of processes associated with growth of the fungus. Spores already germinated and actively growing adapted to cyanide much more rapidly than did nongerminated spores. At this stage, the fungus has the capacity for rapid synthesis of protein, which apparently is a prerequisite for adaptation. When protein synthesis was inhibited, cyanide adaptation was inhibited, regardless of whether protein synthesis was inhibited selectively by cycloheximide or inhibited as a result of decreased energy supply caused by DNP. Presumably, each of glucose, acetate, and glycerol supported adequate protein synthesis since adaptation occurred with each source.

We could not determine whether or not DNA-dependent RNA synthesis also was a prerequisite for adaptation in *S. loti*. Actinomycin D, an inhibitor of

DNA-dependent RNA synthesis (6), in concentrations as high as 100 μg/ml, had no effect on adaptation of spores to cyanide. Actinomycin C, a similar inhibitor (4), at 30 μg/ml had variable effects on the ability of *S. loti* to adapt to cyanide, and did not inhibit either protein or nucleic acid synthesis in our tests.

Resistance of trefoil to infection probably depends on several factors which must be overcome by the pathogen during pathogenesis. If release of HCN is a factor in the resistance of trefoil, then *S. loti* apparently has evolved a mechanism that specifically overcomes this resistance factor. Presumably, HCN is not released until hyphae penetrate tissue and effect injury to the cells. The pathogen, at this stage in its development, is actively synthesizing protein and, therefore, has the capacity to adapt rapidly to cyanide. Since its rate of adaptation was the same for different carbon sources, those available to the fungus in the "infection" drop or as a consequence of its invasion of the tissues should support rapid adaptation.

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