Toxicity of Nalidixic Acid on Candida albicans, Saccharomyces cerevisiae, and Kluyveromyces lactis

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The antibacterial drug nalidixic acid (Nal) can suppress the growth of Candida albicans at levels of the drug normally found in urine. Growth suppression increases as drug levels are increased, and Nal also causes a similar proportional inhibition of the synthesis of all cellular macromolecules. However, growth temperature (25 versus 37 C) and the divalent cations Mg2+ and Mn2+ can increase C. albicans resistance to Nal. Also, nitrogen depletion of Candida shows that Nal-treated and untreated cells exhibit no difference in leucine uptake during readaptation to nitrogen. In Nal-treated, nitrogen-starved cells, ribonucleic acid and deoxyribonucleic acid (DNA) biosynthesis is less affected than in unstarved Nal-treated cells, but of the two nucleic acids DNA synthesis is the most affected. Nal-resistant strains of C. albicans exhibit a slight toxicity for macromolecular synthesis. Nal treatment of a synchronized population of Saccharomyces cerevisiae results in an increase in the culture mean doubling time of, at most, 20%, but Nal causes the loss of synchronous cell division. With a synchronized population of Kluyveromyces lactis, Nal causes an increase in the mean doubling time of upwards of 300%, with synchrony of cell division being maintained. It is known that S. cerevisiae asynchronously synthesizes mitochondrial DNA during the cell cycle, whereas with K. lactis it is synchronous. Thus, with C. albicans Nal toxicity is dependent both on the dose and the physiological state of the cell. Furthermore, Nal inhibits growth of yeast with synchronous mitochondrial DNA synthesis more adversely than yeast with asynchronous mitochondrial DNA synthesis.

Nalidixic acid (1-ethyl-1,4-dihydro-1-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; Nal), a known inhibitor of deoxyribonucleic acid (DNA) synthesis in bacteria, is used in the treatment of urinary tract infections with certain gram-negative infections. With bacteria Nal has a specific but reversible effect, both in vivo (4, 12) and in vitro (1). DNA replication studies in vitro suggest that a previously unknown protein component may be involved with Nal inhibition of DNA biosynthesis in Escherichia coli (1).

With some eukaryotic organisms Nal causes perturbations in cell organelles possessing circular prokaryotic-like DNA. In the presence of Nal, Euglena gracilis, for example, loses its chloroplasts to produce a bleached race of permanently heterotrophic progeny (7). With the yeast Saccharomyces cerevisiae, Nal has been found to cause no preferential inhibition of mitochondrial DNA synthesis (20) or to effect the rates of total cellular DNA (25), but it does inhibit the rate of increase in mitochondrial respiration during stationary adaptation to oxidative growth conditions (18). Other studies (13) found Nal to increase petite formation in this same organism at a low but significant level, a phenomenon known to involve mitochondrial DNA (21, 27). With Kluyveromyces lactis, exposure to Nal causes mitochondrial DNA synthesis to be selectively inhibited to nondetectable levels (19). Prior studies on S. cerevisiae have established that mitochondrial DNA replication is asynchronous (28), with the initiation of replication occurring throughout the cell cycle, whereas in K. lactis it is synchronous (23). Experiments on these two organisms in this investigation suggest that the severity of Nal toxicity on yeast resides in the nature of cellular mitochondrial DNA replication.

The metabolism of Nal appears identical in man and other animals and in various fungi (14). In man, after a single 1-g dose of Nal 95% of the drug or its antibacterial degradation products eventually are found in the urine (11). Further, these metabolic derivatives have the same activity as Nal against bacteria (8).

At the usual dosages of Nal, the urinary
levels reached within 1 h, 150 to 300 \( \mu g \) (15) is sufficient, we have found, to markedly reduce the cellular proliferation of the eukaryotic opportunistic pathogen Candida albicans. Thus, in a concomitant candiduria Nal would exert an antymycotic effect, even though it is not a specific antymycotic agent. This investigation deals primarily with C. albicans and focuses on (i) the consequences Nal has on cellular macromolecular synthesis and (ii) cell physiological properties that are able to alter the influence of Nal on C. albicans.

(A preliminary report of this work was presented at the 1975 Annual Meeting of the American Society for Microbiology, New York, N.Y. A part of this work was taken from a thesis submitted to Wichita State University by A.R.B. in partial fulfillment of the M.S. degree in biology.)

**MATERIALS AND METHODS**

Organisms and growth conditions. C. albicans 526 and the C. albicans Nal-resistant spontaneous mutants were obtained from Alvin Sarachek of this department. S. cerevisiae was a departmental stock culture and K. lactis ATCC 12425 was purchased.

Cells were cultivated in a Camasino Acids medium (Cas) similar to that of Busebee and Sarachek (2). Cas contains 1.5% dextrose, 0.03% vitamin-free Casamino Acids (Difco), 0.2% \( \text{K}_2\text{HPO}_4 \), 0.7 mM \( \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \), 1.01 mM \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 0.02 mg of adenosine, 0.02 mg of uracil, 0.02 mg of biotin, and the following levels of vitamins and minerals, in milligrams per liter: inositol, 10; thiamine hydrochloride, 1; calcium pantothenate, 1; para-aminobenzoic acid, 1; pyridoxine hydrochloride, 1; nicotinic acid, 1; FeSO\(_4\) \( \cdot \) \( 7\)\( \text{H}_2\text{O} \), 3; \( \text{ZnSO}_4 \), 0.5; \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \), 0.5; \( \text{MnSO}_4 \cdot 0.4 \); \( (\text{NH}_4)_2\text{MoO}_4 \cdot 4\text{H}_2\text{O} \), 0.15; and \( \text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O} \), 0.9. After autoclaving, 100 ml of sterile 0.1 N KOH was added to each 900 ml of basemedium. Cas agar was prepared with 1.5% agar (Difco). C. albicans was routinely cultivated at 37 C, whereas the other yeasts were grown at 30 C. Overnight, aerated growth flasks were inoculated with sufficient cells to produce between 10\(^6\) to 4 \( \times \) 10\(^6\) organisms/ml (absorbancy at 425 nm = 0.1 to 0.4) after incubation for 14 to 16 h at 37 C or 20 to 22 h at 30 C.

Reagents. All organic and inorganic chemicals were the highest purity available. Labeled compounds and Liquifluor scintillation fluid concentrate were purchased from New England Nuclear Corp. Nal was obtained from Schwarz/Mann.

Radioisotope labeling techniques. (i) Protein. Cell crops were grown overnight in Cas supplemented with 20 \( \mu g \) of \( L \)-leucine (Leu) per ml. The cells were washed and resuspended to a concentration of 2 \( \times \) 10\(^4\) to 3 \( \times \) 10\(^4\) cells/ml and adapted for 0.5 h in Cas lacking the supplemented amino acids. After the adaptation period, the cells were diluted 10-fold into Cas containing 1.1 \( \times \) 10\(^{-4}\) mcg of \( ^{15} \text{C} \)Leu (specific activity, 53.5 mcCi/mmol) and enough \( ^{35} \text{S} \)Leu to bring the final Leu concentration to 5.0 mcg/ml. Experimental samples were processed for hot trichloroacetic acid-precipitable radioactivity on Whatman 3MM filter paper squares as previously described (24). After drying, the filter papers were placed in 15 ml of scintillation fluid and counted. To determine the effects of Nal on the intracellular pools of amino acids, C. albicans (2 \( \times \) 10\(^4\) to \( 3 \times \) 10\(^4\) cells/ml) was prelabeled with 2.2 \( \times \) 10\(^{-3}\) mcCl of \( ^{14} \text{C} \)Leu for 30 min. Chasing was accomplished with 0.5 mcg of \( ^{14} \text{C} \)Leu. The total soluble and acid-precipitable radioactivity in cells was obtained by filtering the experimental sample through a 25-mm (diameter) membrane filter (0.45-\( \mu \)m pore size; Millipore Corp.). A duplicate sample was processed for acid-precipitable radioactivity to determine the soluble \( ^{14} \text{C} \)Leu pool size by difference.

(ii) Nucleic acids. Ribonucleic acid (RNA) was labeled by growing C. albicans overnight in Cas. After washing, 2 \( \times \) 10\(^4\) to 3 \( \times \) 10\(^4\) cells/ml were exposed to 1.7 \( \times \) 10\(^{-4}\) mcCl of \( ^{32} \text{P} \)adenine (Aden) with a specific activity of 57 mcCi/mmol and enough \( ^{32} \text{P} \)Aden to bring the final Aden concentration to 5 \( \mu \)g. The volume of cells processed in this fashion was always at least 15% larger than needed for the experiment. Radioactivity in RNA was obtained by the difference between the total acid-precipitable radioactivity minus the counts in DNA determined by alkali hydrolysis as previously described (24). To determine the effects of Nal on DNA synthesis, cells were prelabeled for 1 h with the same concentration of \( ^{14} \text{C} \)Aden as above, and then they were shifted into identical medium with or without the drug. No combination of \( ^{14} \text{C} \)Aden supplementation followed by various starvation periods in the absence of Aden allowed for immediate uptake of label into DNA. There is always a 40- to 50-min lag before counts appear in DNA. Consequently, for DNA measurements on C. albicans that were physiologically starved of a nitrogen source (see below), DNA measurements were performed on 10\(^3\) cells by the Dische diphenylamine procedure with calf thymus DNA as a standard.

Physiological manipulations. To investigate the influence of temperature on Nal toxicity in C. albicans, the minimum inhibitory concentration of Nal was determined at 25 and 37 C. At each test temperature growth was followed turbidimetrically after an equivalent number of cell generations. Divalent cation effects on Nal toxicity were determined in Cas medium with the absence of all cations except the ion under test. For nitrogen starvation effects, cells were first grown in Cas, washed three times in sterile 0.9% saline, and then resuspended to an absorbancy at 425 nm = 0.3 Cas medium prepared with the absence of all organic nitrogen. Such treatment for 10 to 12 h produced a cell population that was greater than 99.9% viable and comprised of nonbud- ding cells of uniform size.

Synchronization. The synchronization of S. cerevisiae and \( K. \) lactis was accomplished by the procedure of Williamson and Scopec (29) using Cas medium and nitrogen-depleted Cas medium, with growth occurring at 30 C. After sonication for 20 to 30 s in a Bronwill Biosonik III to break up cell
clumps, experimental samples were counted in a hemocytometer to determine the percentage of budding cells and cell numbers.

RESULTS

Toxicity. Table 1 lists the results of minimum inhibitory concentration determinations for Nal on C. albicans as a function of the time of incubation. As the incubation period increases from 16 to 72 h, the minimum inhibitory concentration increases. However, at Nal levels above 0.3 mg/ml the toxicity is more severe, as indicated by a smaller increase in cell growth. The effects of Nal on C. albicans is defined as a toxicity because: (i) growth continues in the presence of Nal as the incubation period increases (Table 1), (ii) at 0.5 mg/ml the drug does not completely suppress cellular biosynthetic activity (see below), and (iii) growth is not prevented even at 0.4 mg of Nal per ml (see below).

Cellular growth. The toxicity of Nal for exponential cells of C. albicans was found to be immediate and more severe as the levels of the chemotherapeutic agent were raised. As the concentration of Nal increases in growth flasks, there is a progressive increase in the mean doubling time (MDT). Nal at 0.1 mg/ml produced approximately a 25% increase in the MDT above the 55-min time seen in the controls. At 0.25 mg/ml the MDT increased to 140%, and 0.4 mg of Nal per ml caused a 380% increase in the MDT. Also, as reported for prokaryotic cells, subbacteriocidal doses of Nal on bacteria are reversible (5), and with C. albicans removal of Nal (0.4 mg/ml) by washing results in an MDT identical to control cells after a lag period of almost one generation time.

### Table 1. Minimum inhibitory concentrations of Nal on C. albicans as a function of time

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<th>Nal (µg/ml)</th>
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* Growth was visually scored on a +1 to +4 scale.

Macromolecular effects. Nal toxicity on macromolecular synthesis in C. albicans was also related to the concentration of the chemotherapeutic agent present. Figure 1 shows that inhibition of [14C]Leu incorporation into protein increased with increasing drug levels. Even at limits approaching saturation of Nal in the medium, C. albicans still synthesizes protein, albeit at a much reduced rate. The influence of Nal on protein synthesis is not due to the inability of the labeled precursor to enter the cell but on some event subsequent to transport. When cells are pulsed with [14C]Leu (Fig. 2) and then chased so as to follow the disappearance of acid-soluble radioactivity in the presence or absence of Nal (0.25 mg/ml), the intracellular acid-soluble pools are still depleted in the presence of the drug, but at a rate reduced from that seen in the controls.

With respect to cellular RNA synthesis by C. albicans in the presence of Nal, the results are similar to those seen for protein synthesis. Figure 3 shows that RNA synthesis is depressed in comparison to control cultures, and the decrease is related to the concentration of the drug. The inhibition of DNA synthesis also increases with the amount of Nal used (Fig. 4).

Nal-resistant mutants. The spontaneous mutants M3 and M5 were tested for alterations in their macromolecular synthesis in the presence and absence of the drug. Figure 5 shows that M3 and M5 have 36 and 30% decreases, respectively, in the value of [14C]Leu uptake in
per ml, whereas the parental strain exhibited a 60% decrease. Thus, although M3 and M5 grow in the presence of Nal, the drug does cause a slight toxicity, which can be detected by these measurements; however, the toxicity that is evident is less severe than that present in the wild-type strain.

Physiological alterations of toxicity. Several physiological alterations of C. albicans can suppress or reduce drug toxicity. In Fig. 6, C. albicans is seen to be more susceptible to Nal

the presence of 0.4 mg of Nal per ml, whereas a 72% decrease was found with the wild-type culture. Similar results were obtained for M3 and M5 when RNA and DNA synthesis were followed; namely, in the presence of Nal macro-molecular synthesis is depressed, but the depression is less than that seen in the wild-type strain. For example, with respect to DNA synthesis M3 showed a 17% reduction and M5 a 40% reduction after exposure to 0.25 mg of Nal per ml. Labeling conditions are as described in Materials and Methods.

Fig. 2. Effect of Nal on the loss of acid-soluble intracellular [4C]Leu pools in C. albicans. Cells (2.0 ml) for each test system were labeled and assayed as described in Materials and Methods.

Fig. 3. Effect of increasing Nal concentrations on [4C]Ade incorporation into RNA of C. albicans. Labeling conditions are as described in Materials and Methods.

Fig. 4. Effect of increasing Nal concentrations on [4C]Ade incorporation into the DNA of C. albicans. Conditions are as described in Fig. 3.

Fig. 5. Effect of Nal on protein synthesis in wild-type C. albicans 526 and the Nal-resistant mutants M3 and M5. Open symbols represent controls; closed symbols represent cells treated with 0.4 mg of Nal per ml. Labeling conditions are as described in Fig. 1.
when grown at 37 C than when grown at 25 C. Even though the incubation periods were such as to allow an equivalent number of cell generations at both 37 and 25 C, more growth occurred at the lower temperature. A comparison of growth between the 37 C minimum inhibitory concentration value of 0.25 mg/ml and the growth at 25 C reveals a fivefold increase in growth at 25 C.

Cas medium was prepared with the absence of its divalent cations to assess their role in Nal toxicity. Cas normally is 9.9 x 10^{-2} mM for Mg^{2+} and 3.3 x 10^{-2} mM for Mn^{2+}. When cells were grown in Nal-containing medium supplemented with one or the other of these cations from concentrations of 0 to 20 x 10^{-2} mM, the results in Fig. 7 were obtained. The maximum protection afforded by either of these cations occurs between 12 x 10^{-2} to 15 x 10^{-2} mM, and at these levels the Nal toxicity is significantly alleviated. The protection by these cations is of a magnitude that allows upward of 70% of the cell growth to occur in the presence of Nal plus the cation, as compared with drug-free controls at the same cation concentration. In this system Ca^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} were without effect in preventing toxicity. An interesting result was noted with Mn^{2+}. As the concentration of Mn^{2+} increases in drug-free control tubes, cellular growth is repressed, maximum growth inhibition occurring at 4 x 10^{-2} to 6 x 10^{-2} mM. In spite of the Mn^{2+} inhibition of normal growth when also present with Nal, this cation allowed more growth to occur than would occur in its absence.

When C. albicans was physiologically starved of nitrogen and, at the time of a shift back to nitrogen, was simultaneously exposed to Nal, the results on protein macromolecular synthesis were those depicted in Fig. 8A. The 0.3-mg/ml level of Nal used normally generates a decrease in protein synthesis of at least 50% in nonstarved populations, and, as is evident in this figure, such toxicity is absent in starved populations. With respect to RNA synthesis (Fig. 8B), 0.25 mg of Nal per ml produced a 70% decrease in nonstarved cells but only a 37% depression in starved cells.

In starved control cells DNA synthesis does not begin for 100 min after the shift back to nitrogen (Fig. 9), and when such cells are exposed to 0.25 mg of Nal per ml the drug produces a 46% decrease in the biosynthesis of this nucleic acid, whereas non-nitrogen-starved cells show a 56% decrease. With C. albicans physiological starvation reduces Nal toxicity, but of the macromolecules measured DNA synthesis is the most adversely altered.

Synchronous growth. Synchronously dividing yeast populations show uniform cycles of cell division (16), enzyme production (10), and DNA synthesis (26). The influence of Nal on synchronous growth of S. cerevisiae and K. lactis was investigated. Figure 10 shows that exposing synchronized populations of S. cerevisiae to 0.25 mg of Nal per ml caused a 20% depression in the MDT. Also, this figure reveals that in control populations synchronous

![Figure 6](http://aac.asm.org/) Influence of temperature on Nal growth inhibition of C. albicans. Cells were grown in 2.5-ml sample volumes with or without Nal for an equivalent number of cell generations at each temperature. Medium was as described in Materials and Methods.

![Figure 7](http://aac.asm.org/) Divalent cation reversal of Nal growth inhibition of C. albicans. Cells (2.0 ml) were grown as described in Materials and Methods.
an overall sense is unlike that reported by Michaeis and co-workers for S. cerevisiae (20) but approaches that seen in E. coli (30). With E. coli at high levels of Nal (0.02 mg/ml) all macromo-

![Graph for DNA synthesis](image)

**FIG. 8.** Protein and RNA synthesis in nitrogen-depleted C. albicans exposed to Nal. (A) [14C]Leu incorporation in starved Nal-treated and untreated cells. (B) [14C]Ade incorporation into RNA in starved and unstarved C. albicans. Nitrogen depletion of C. albicans was as described in Materials and Methods. Protein and RNA determination was as described in Fig. 1 and 3, respectively.

![Graph for protein and RNA synthesis](image)

![Graph for DNA synthesis](image)

![Graph for chemical determination of DNA synthesis](image)

![Graph for effect of Nal on bud formation and cell numbers](image)

**DISCUSSION**

The toxicity of Nal on DNA, RNA, and protein metabolism and growth of C. albicans in cell divisions occur when cell numbers and bud formation are monitored, but in the presence of Nal S. cerevisiae lost this synchrony with the first cycle of cell division and remained asynchronous throughout the sampling period. Unlike S. cerevisiae, when synchronized populations of K. lactis are exposed to Nal, synchronous cell division and bud formation occur in the presence or absence of the drug (data not shown); however, in the presence of Nal the MDT was, depending on the experiment, between 2.7 to 3.0 times longer than in the controls. Thus, Nal has a diverse effect on these two yeasts: in the one case it causes a very large increase in the MDT without effecting cell synchrony, and in the other case it effects synchrony with only a small increase in the MDT.
molecular synthesis is permanently depressed, whereas lower levels only prevent the synthesis of DNA. Saccharomyces, on the other hand, exhibits a biphasic toxicity to Nal (0.5 mg/ml) when protein, DNA, or RNA syntheses are followed. Immediately after exposure there is a severe, but transient, period in which DNA, RNA, and protein are not synthesized. This is followed by a recovery phase wherein rates of synthesis of these molecules are nearly identical to control cells. C. albicans displays a permanent Nal toxicity for each macromolecular species tested, and this toxicity is dependent on the amount of Nal in the medium (Fig. 1, 3, and 4). There is no recovery phase nor is there any loss in cell viability (data not shown). The MDT remains lengthened in contrast to S. cerevisiae, which after recovery expresses an MDT equivalent to the drug-free controls. These observations point up differences in these organisms that are reflected by their responses to this drug. The specific nature of these differences is unknown. It should be noted, however, that even in E. coli diverse responses occur to Nal; some strains exhibit DNA degradation in the presence of Nal (5), whereas other strains show very little or no degradation (12, 30).

Various physiological states of the cell can significantly alter the response of microbes to an antimicrobial agent (22). With C. albicans physiological changes, including growth at temperatures lower than 37 C (Fig. 6), nutritionally induced stationary growth conditions (Fig. 8), and the presence of specific cations (Fig. 7) can protect the cell or somewhat alleviate drug toxicity. The unusual growth inhibition noted for Mn\(^{2+}\) on C. albicans, which when also present with Nal allowed the cation to still protect the cell, suggests a possible mechanism for this physiological protection. Cations may form an extracellular complex with Nal, which would then obviate toxicity by preventing the entry of Nal into the cell or otherwise neutralizing the effects of Nal.

The urinary excretion of the cation, Mg\(^{2+}\), in humans approaches 3.4 nM based on an average daily volume of 1.21 (17). This excretion level of Mg\(^{2+}\) is higher than the amount we found necessary to confer maximum cation protection to C. albicans against Nal's in vitro toxic effects. The ability of Mg\(^{2+}\) to neutralize or overcome drug toxicity is, therefore, of some practical concern but does not obviate Nal's potential benefit in a candiduria. All experiments in this report incorporated levels of Mg\(^{2+}\) that provide significant protection, and even at these Mg\(^{2+}\) levels Nal at concentrations found excreted in urine markedly depresses cellular proliferation and macromolecular biosynthesis. Therefore, in a patient treated with Nal to suppress a gram-negative bacteriuria, the drug could significantly influence the balance of the disease if Candida was a co-infecting agent.

The synchronized cell populations of S. cerevisiae and K. lactis respond to Nal treatment in a manner that suggests that some intracellular event controlling cell division is affected. Figure 10 shows that S. cerevisiae loses the ability to undergo synchronous cell proliferation with Nal treatment. From studies mentioned earlier, Nal is known to perturb eukaryotic organelles with circular prokaryotic-like DNA. The regulatory event responsible for the loss of cell synchrony in S. cerevisiae is, we suggest, some feature of the mitochondrial DNA replication cycle.

The loss of synchrony in S. cerevisiae probably reflects Nal's interaction with the mitochondrial function responsible for coordinating mitochondrial and nuclear DNA synthesis. That a signal exists between these two molecules is suggested by work that established a constant ratio of mitochondrial to nuclear DNA under a variety of growth conditions (9). The nature of the control mechanism, however, is unknown at present. Although the ratio of the DNA species is constant in S. cerevisiae, it is also known that mitochondrial DNA replication occurs throughout the cell cycle (28), indicating an asynchrony in its intracellular regulation. With K. lactis, replication of mitochondrial DNA occurs within a prescribed brief portion of the cell cycle immediately after nuclear DNA replication (23). Our results with synchronized populations of S. cerevisiae show that Nal causes asynchronous cell divisions in cells that have an asynchrony in mitochondrial DNA replication. In K. lactis, however, Nal does not cause asynchronous cell division but only a substantial increase in the rate of growth.

If the lack of cytokinesis, and hence the loss of synchrony, in S. cerevisiae and the large increase in the MDT observed in Candida and Klyuyveromyces is related to the influence of Nal on a mitochondrial signal needed for optimal growth, then one site of Nal action in yeast may involve the association between mitochondrial DNA and this organelle's membrane structure. Another antimycotic agent, ionophore A28187, may act on yeast in an analogous fashion. This agent inhibits growth of a variety of yeasts by its action on the integrity of the mitochondrial membrane (3) or, alternatively, by inhibiting the breakdown of microtubules (6). Interestingly, Mg\(^{2+}\) reverses this ionophore's growth inhibition (6), and Mg\(^{2+}\) has been shown to be essential for maintenance of the mitochondrial membrane (3).
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LITERATURE CITED