Antioxidants protect rat diaphragmatic muscle function under hypoxic conditions

PRIYA MOHANRAJ, A. JOHN MEROLA, VALERIE P. WRIGHT, AND THOMAS L. CLANTON

Departments of Physiology, Medical Biochemistry, and Internal Medicine, Pulmonary and Critical Care Division, The Ohio State University, Columbus, Ohio 43210

Mohanraj, Priya, A. John Merola, Valerie P. Wright, and Thomas L. Clanton. Antioxidants protect rat diaphragmatic muscle function under hypoxic conditions. J. Appl. Physiol. 84(6): 1960-1966, 1998.—In hypoxia, mitochondrial respiration is decreased, thereby leading to a buildup of reducing equivalents that cannot be transferred to O2 at the cytochrome oxidase. This condition, called reductive stress, can paradoxically lead to enhanced formation of reactive O2 species, or a decrease in the ability of the cell to defend against an oxidative stress. We hypothesized that antioxidants would protect tissues under conditions of hypoxia. Rat diaphragm strips were incubated in tissue baths containing one of four antioxidants: N-acetyl-L-cysteine, dimethyl sulfoxide, superoxide dismutase, or Tiron. The strips were directly stimulated in an electrical field. Force-frequency relationships were studied under baseline oxygenation (95% O2-5% CO2), after 30 min of hypoxia (95% N2-5% CO2), and 30 min after reoxygenation. In all tissues, antioxidants markedly attenuated the loss of contractile function during hypoxia (P < 0.01) and also significantly improved recovery on reoxygenation (P < 0.05). We conclude that both intracellular and extracellular antioxidants improve skeletal muscle contractile function in hypoxia and facilitate recovery during reoxygenation in an in vitro system. The strong influence of antioxidants during hypoxic exposure suggests that they can be as effective in protecting cell function in a reducing environment as they have been in oxidizing environments.

DURING RESPIRATORY FAILURE and associated cardiovascular collapse and resuscitation, conditions are appropriate for local tissue hypoxia and ischemia-reperfusion to occur. Ischemia-reperfusion has been shown to be associated with the production of reactive O2 species (ROS) in different organ systems (16, 28, 34). It is generally believed that they are primarily produced during the reperfusion period, when high O2 concentrations promote the formation of superoxide [superoxide anion radical (O2-)] through one electron reduction of O2. However, several studies have demonstrated that hypoxia alone, without subsequent reoxygenation or reperfusion, can result in the production of oxidants in some tissues (26, 27, 38). For example, Park and Kehr (27) have shown that, in the heart, hypoxia results in both oxidant formation and oxidative stress, which are not exacerbated further by subsequent reoxygenation. The mechanism responsible for oxidant production in hypoxia is not well understood and has not previously been demonstrated in skeletal muscle. The influence of hypoxia on oxidant production may be clinically important, because the working diaphragm can be exposed to severe hypoxia during conditions of respiratory failure or cardiopulmonary arrest. Furthermore, in a previous study (5), we have shown increased free-radical generation in the diaphragm during conditions of severe respiratory failure.

Skeletal muscles have been shown to produce ROS both intracellularly and extracellularly at rest (31, 33). Production appears to go up in response to intense, fatiguing exercise (5, 12, 31). Furthermore, during fatigue, force production and endurance are markedly preserved by treatment with a wide variety of antioxidants (31, 33, 36), and treatment with dithiothreitol, a potent reducing agent, improves recovery of muscle function after diaphragmatic fatigue (11). These earlier experiments demonstrate that ROS or other oxidizing agents are generated in skeletal muscle and may contribute to the loss of force development in fatigue.

In this study, we explore the hypothesis that ROS, free radicals, or other oxidizing agents are produced in hypoxic skeletal muscle and that they contribute to the loss of mechanical function associated with severe hypoxia. We reasoned that, if oxidizing species are actively involved in contractile depression, then antioxidant treatment should preserve function, much as it is preserved during conditions of muscle fatigue. Alternatively, if oxidizing species are produced primarily during reoxygenation, then antioxidant treatment would not preserve function during hypoxia but rather during the reoxygenation and/or recovery period.

Our results demonstrate that hypoxia causes significant muscle dysfunction that is markedly attenuated by antioxidant treatment. This effect appears to be independent of whether the antioxidants work intracellularly or extracellularly. Furthermore, antioxidant treatment improves recovery on reoxygenation.

METHODS

Experiments with Antioxidants

Surgical procedure and muscle strip preparation. The experiments were conducted on diaphragm muscle strips taken from adult male Sprague-Dawley rats (300–500 g). The animals (n = 7) were anesthetized with an injection of pentobarbital sodium (40 mg/kg ip), tracheotomized, and mechanically ventilated. The phrenic nerve was severed, and four diaphragm strips (~0.5 cm) were dissected, with portions of rib and central tendon intact. The strips were divided into four groups. The control tissue was placed in a tissue bath containing ~125 ml of a physiological salt solution (in meq/l: 2.1 NaHCO3, 0.9 NaSO4, 1.2 NaHPO4, 0.9 MgCl2, 2.25 CaCl2, 5.9 KCl, and 109 NaCl with 2.07 g/l glucose). The experimental tissue was placed in a similar bath containing physiological salt solution with N-acetyl-L-cysteine (NAC, 18 mM), a commonly used antioxidant. The baths were maintained at 37°C and were continuously bubbled with 95% O2-5% CO2 during normoxia and 95% N2-5% CO2 during the...
hypoxic phase. Two additional baths containing tissues with and without NAC served as normoxia time controls. Muscle function (twitch characteristics and stress-frequency relationships) was assessed at the end of each phase. To determine the kinetics of gas pressures in the tissue baths, we continuously monitored the PO2 in the baths with a Clark electrode (BMS3 MK2 Blood Micro System, Radiometer America, Cleveland, OH). The PO2 generally dropped rapidly in the tissue baths, reaching <50 Torr within 3 min. A minimum partial pressure, indistinguishable from zero, was reached within 15–20 min into the hypoxic period (Fig. 1).

The strips were mounted vertically, with the central tendon positioned superiorly and attached to a force transducer, which was connected to a micropositioner. d-Tubocurarine (10 μM) was added to eliminate activation of intramuscular nerve branches. The diaphragm strips were positioned between two platinum plates and stimulated by direct electrical field.

To confirm our findings, we repeated the experiments on three other antioxidants, namely, dimethyl sulfoxide (DMSO, 6.4 mM), superoxide dismutase (SOD, 500 U/ml), and 1,2-dihydroxybenzene-3,5-disulfonate (Tiron, 10 mM), chosen because of their different modes of action on diaphragmatic tissues (n = 5 rats in each group). The doses for the four antioxidants were selected based on previous in vitro studies in diaphragm and other tissues that used these antioxidants (10, 24, 31). The surgical procedure was as described above. The experimental protocol was identical to NAC experiments except there were no normoxia time controls. In these experiments, one muscle strip served as control tissue, whereas each of the remaining three strips served as experimental tissue treated with a different antioxidant, with all four tissues being studied simultaneously from the same animal.

Stimulation paradigm. After the strips were mounted in the tissue chamber, the maximum required stimulation current and the optimal length were determined by adjusting the micropositioner between intermittent stimulations. All stimulations were performed at optimal length and at supramaximal current.

Twitch characteristics. Single twitches (0.5-Hz stimulation) were recorded, and the peak-twitch tension, time-to-peak tension, and half time of relaxation were calculated.

Force-frequency relationships. The muscle strips were tetanically stimulated at 20, 30, 40, 50, 60, 80, and 100 Hz with an interval of ~30 s between each tetanic stimulation. Pulses were of 0.2-ms duration, with a train duration of 400 ms.

Data Analysis

At the end of the experiment, the length and wet weight of each muscle strip were obtained. For conversion of force to absolute stress, the cross-sectional area of each diaphragm strip was determined by dividing the muscle mass (weight in g) by the product of muscle length (in cm) and muscle density (assumed to be 1.06 g/cm3) (8). All values are expressed as group means ± SE. Stress-frequency data for NAC experiments were analyzed by using two-way repeated-measures ANOVA with treatment and gas phase as independent variables and frequency as a categorical variable. Twitch data and individual tetanic force measurements for the other antioxidant experiments were also analyzed by using a two-way ANOVA with treatment and gas phase as dependent variables. P < 0.05 was considered to be statistically significant. Post hoc comparisons were made by using SAS JMP (SAS Institute, Cary, NC) contrasts. Unless mentioned otherwise, P values in the text refer to post hoc contrasts.

RESULTS

Twitch Characteristics

Hypoxia caused significant reductions in peak tension (P = 0.002), half time of relaxation (P = 0.001), and time-to-peak tension (P = 0.004), as shown in Fig. 2. In each case, these changes did not return to baseline during the reoxygenation phase. Although tension is expressed as %baseline, similar conclusions can be drawn from absolute stress measurements (not shown). During hypoxia, NAC significantly ameliorated the fall in peak-twitch tension (P < 0.05) and lengthened the half time of relaxation (P < 0.01) but had no significant effect on time-to-peak tension. On reoxygenation, treatment with NAC did not significantly improve recovery of these variables.

Stress-Frequency Relationships

Treatment with NAC resulted in significantly lower baseline stress measurements at low frequencies (20 and 30 Hz; P < 0.05). However, the baseline stress measurements at high frequencies (40–100 Hz) were not affected by NAC treatment (Fig. 3A). This phenomenon has also been observed in previous studies on the diaphragm with NAC (23). In the hypoxic phase, there was a significant fall (ANOVA, P < 0.001) in muscle stress (compared with baseline) at all frequencies of stimulation. This was markedly attenuated (overall ANOVA, P < 0.001) in tissues treated with NAC (Fig. 3B). Similarly, during reoxygenation, stress remained decreased at all frequencies of stimulation compared with baseline (overall ANOVA, P < 0.001). Stress during recovery was significantly improved (overall ANOVA, P < 0.01) in tissues treated with NAC at all frequencies of stimulation (Fig. 3C).

The effects of other antioxidants (DMSO, SOD, and Tiron) on muscle function were similar to that of NAC (Table 1). Peak-twitch stress and the stress-frequency relationship during hypoxia and on reoxygenation in muscles treated with these antioxidants were similar
Fig. 2. Twitch characteristics of muscle in normoxia (Initial), in hypoxia, and on reoxygenation (Recovery) in control (open bars) and N-acetyl-L-cysteine-treated (hatched bars) tissues. A: peak-twitch tension. B: half time of relaxation. C: time-to-peak tension or contraction time. Data are means ± SE; n = 7 rats.

Fig. 3. Stress-frequency relationships of control and N-acetyl-L-cysteine (NAC)-treated tissues during 3 phases of stimulation: baseline/initial normoxic phase (A); hypoxic phase (B); and reoxygenation/recovery phase (C). Data are means ± SE; n = 7 rats. *Individual post hoc contrasts. ‡Effect from initial with use of repeated-measures ANOVA. §Effect of treatment with NAC compared with control tissue.
Table 1. Effect of antioxidants on muscle function

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Control</th>
<th>Tiron (10 mM)</th>
<th>DMSO (6.4 mM)</th>
<th>SOD (500 U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 Hz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>17.0±2.3</td>
<td>12.9±1.9†</td>
<td>16.9±1.0</td>
<td>19.4±1.7</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>2.6±0.5</td>
<td>3.9±1.1</td>
<td>4.5±0.6</td>
<td>4.9±1.0</td>
</tr>
<tr>
<td>Recovery</td>
<td>6.8±1.7</td>
<td>8.3±0.1</td>
<td>10.6±0.1†</td>
<td>12.5±1.3†</td>
</tr>
<tr>
<td>50 Hz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>23.5±2.6</td>
<td>21.4±2.5</td>
<td>23.3±1.5</td>
<td>25.9±2.3</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>3.9±0.8</td>
<td>8.2±2.2*</td>
<td>8.0±0.8*</td>
<td>7.9±1.6*</td>
</tr>
<tr>
<td>Recovery</td>
<td>11.4±2.7</td>
<td>16.7±0.0†</td>
<td>17.4±0.5†</td>
<td>18.7±1.2†</td>
</tr>
<tr>
<td>100 Hz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>27.7±2.0</td>
<td>26.5±2.0</td>
<td>27.1±2.2</td>
<td>29.3±2.2</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>6.8±1.9</td>
<td>14.0±1.9†</td>
<td>12.7±2.1†</td>
<td>12.9±2.1†</td>
</tr>
<tr>
<td>Recovery</td>
<td>17.3±1.5</td>
<td>22.9±1.5†</td>
<td>22.9±1.5†</td>
<td>23.7±1.5†</td>
</tr>
</tbody>
</table>

Values are means ± SE in N/cm². Effects of antioxidants on muscle stress at 30, 50, and 100 Hz during baseline (initial/normoxia), hypoxia, and on reoxygenation (recovery) phases are shown. Tiron, 1,2-dihydroxybenzene-3,5-disulfonate; SOD, superoxide dismutase.

*P < 0.05, †P < 0.05, ‡P < 0.01, compared with control tissue.

to those in NAC-treated tissues. Similar to NAC, Tiron significantly lowered baseline stress at low frequencies (30 Hz, Table 1). Also similar to NAC, these antioxidants protected muscle function during hypoxia as well as on reoxygenation. Although the responses did not reach statistical significance at 30 Hz during hypoxia, they were significant at higher frequencies of stimulation (e.g., 50 and 100 Hz, Table 1). Furthermore, antioxidant-treated tissues also showed significantly increased recovery at nearly all frequencies of stimulation.

**Discussion**

To the best of our knowledge, this is the first study demonstrating the effect of antioxidants on muscle function in hypoxic skeletal muscle. Our results show that there is significant impairment of skeletal muscle function during hypoxia, characterized by a reduction in force at all frequencies, as well as a decrease of both twitch contraction and relaxation time. The changes in tetanic force can be ameliorated by coincubation with antioxidants. Furthermore, antioxidants improve recovery of hypoxic muscles on reoxygenation. These findings suggest that oxidants, oxidizing agents, or free radicals are produced in skeletal muscle during hypoxia and contribute to decreased force production.

**Potentially Sources of ROS During Hypoxia**

There are a number of potential sources of ROS in hypoxia. One source may be mitochondria (9, 21, 22, 26). It has been suggested that, even under normal aerobic conditions, ∼2% of the total mitochondrial O₂ consumption is shuttled toward the formation of O₂⁻. This is believed to be generated at complex I and complex III of the electron transport chain (9). During hypoxia, less O₂ is available to be reduced to H₂O at cytochrome oxidase, resulting in an accumulation of reducing equivalents within the mitochondrial respiratory sequence, a condition known as reductive stress (21). This can result in the formation of ROS by the auto-oxidation of one or more mitochondrial complexes such as the ubiquinone-ubiquinol redox couple (9, 21). Khan and O'Brien (22), in their studies on isolated hepatocytes subjected to similar conditions of hypoxia, showed evidence of reductive stress by demonstrating an increase in the cellular NADH/NAD⁺ ratio. They concluded that hypoxia induced hepatocyte injury in their model from sustained reductive stress and O₂ activation.

It has been shown that, even at a tissue Pₒ₂ of 1 Torr (normal, 35 Torr), there is a significant production of free radicals despite the low Michaelis constant (Kₘ) of cytochrome oxidase complex in the mitochondria (30). Our tissues were subjected to hypoxic levels comparable to other studies in hypoxia (22, 26, 28). In our model, there was an exponential washout of O₂ over time. It took ∼20 min for O₂ to fall to <6 Torr (Fig. 1). It is conceivable that this initial period of relative hypoxia could result in the formation of O₂⁻ free radicals by direct single-electron reduction of the available molecular O₂ to form O₂⁻ (9). These ROS, on reaction with cell membranes and polyunsaturated fatty acids, could propagate into a self-perpetuating chain reaction, resulting in a significant accumulation of free radicals (6).

Other possible sources of ROS include activation of certain enzymatic pathways during hypoxia. Xanthine dehydrogenase is known to be present in a reasonably high concentration in rat diaphragm (1). Its exact location within skeletal muscles is not clear, but it seems to be predominantly located within endothelial cells in the vasculature (1); the latter is abundant in diaphragm. During hypoxia, an insufficient supply of ATP can result in the malfunction of ATP-dependent Ca²⁺ pumps of the cell. The excess Ca²⁺ stimulates the transformation of xanthine dehydrogenase to xanthine oxidase by the action of Ca²⁺-activated proteases. Xanthine oxidase catalyzes the breakdown of hypoxanthine (formed as a catabolic product of adenine nucleotides in increased amounts during hypoxia) into xanthine and uric acid, releasing O₂⁻ (15). ROS may also be formed by cytosolic enzymes such as cytochrome oxidase. Under hypoxic conditions, there is an increased Ca²⁺-dependent activation of the enzyme phospholipase A₂, which releases arachidonic acid from phospholipids. Cyclooxygenase reacts with this accumulated fatty acid, simultaneously generating singlet O₂ and hydroxyl radical (-OH) (7). Finally, hypoxia can also cause increased nitric oxide radical (-NO) production. Recent studies on endothelial cells showed that ·NO production may be stimulated by elevated levels of cytosolic Ca²⁺ (20) or by increased activity of constitutive NO synthase (39) occurring during hypoxia. ·NO is known to be toxic to the cell by its direct interaction with iron-sulfur proteins, inhibition of ribonucleotide reductase and other enzyme systems, nitrolylation of thiols, as well as inhibition of protein synthesis (4). It can also exert indirect toxic effects via peroxynitrite formed by its combination with O₂⁻. Peroxynitrite is a potent oxidant with "OH-like activity" and has been shown to oxidize thiols, sulfides, lipids, and deoxyribose (4). In this
study, antioxidants may have functioned in part by preventing peroxynitrite formation during hypoxia, i.e., by scavenging superoxide or perhaps by direct antioxidant activity on peroxynitrite itself.

Muscle Function During Hypoxia

During hypoxia, there was a decline in peak-twitch tension and tetanic tension, and an acceleration of both twitch contraction time and half time of relaxation. The muscle function did not recover completely on subsequent 30-min reoxygenation. The decline in muscle tension during hypoxia may have been due to several reasons. 1) ROS-mediated injury to some of the muscle fibers may have occurred. This may also explain the incomplete recovery of muscle function on subsequent reoxygenation. 2) It has been shown in cardiac muscle that during hypoxia there may be an increase in intracellular Ca\(^{2+}\) levels due to inhibition of membrane ATPase and the Na\(^+/Ca^{2+}\) exchanger (13). The excess Ca\(^{2+}\) can activate proteases, which destroy the sarcomlemma and cytoskeleton as well as cause breakdown of membrane phospholipids by the action of Ca\(^{2+}\)-activated phospholipases (19). Therefore, function may be reduced by Ca\(^{2+}\)-induced cell injury. 3) Several changes in the intracellular milieu occur under conditions of hypoxia or fatigue, including a rise in inorganic phosphate, decline in pH, fall in phosphocreatine levels, and changes in the concentrations of adenine nucleotides. These changes can directly depress the contractile apparatus, causing a decline in muscle function (14).

The cause of acceleration of twitch kinetics during hypoxia is unclear. Green et al. (17) showed an increased activity of sarcoplasmic reticulum (SR) ATPase in both slow- and fast-twitch skeletal muscle during ischemia. This could result in increased reuptake of Ca\(^{2+}\) by the SR ATPase, resulting in faster relaxation. Alternatively, during hypoxia there may be a reduction in the contribution from slow-twitch oxidative fibers, which depend more on \(O_2\) for energy metabolism. In contrast, fast-twitch glycolytic fibers would be expected to be more preserved in hypoxic muscle, as glycolytic pathways could continue to provide energy. This could result in a predominant fast-twitch fiber influence on force. Interestingly, slow-twitch oxidative fibers are rich in mitochondria, which may, as mentioned, represent a source of ROS in hypoxia.

Effect of Specific Antioxidants on Muscle Function

Our results indicate that both small (intracellular) and large (extracellular) antioxidants have equal effects on ameliorating the decline in muscle tension during hypoxia and enhancing recovery of tissue function on reoxygenation. The mechanism of action may or may not be the same for each of these antioxidants.

NAC, a well-known antioxidant in ischemia-reperfusion models, is a low-molecular-weight intracellular antioxidant with a nucleophilic sulfhydryl group that can interact with and detoxify reactive electrophiles and free radicals directly via conjugation or reduction reactions. NAC has been shown to scavenge ·OH and hypochlorous acid; its antioxidant effect on other ROS is unlikely. Its indirect effects involve raising the intracellular concentration of cysteine, thereby supporting glutathione (an endogenous antioxidant) biosynthesis (2, 35). In addition, NAC has been shown to increase the activity of other endogenous intracellular antioxidant enzymes such as SOD (25). Recent in vivo and in vitro studies on skeletal muscle (10, 23, 36) have shown that NAC inhibits skeletal muscle fatigue in both human and animal models. The exact mechanism by which NAC influences the contractile process in muscle is unknown. Khawli and Reid (23) proposed that improved contractility and inhibition of fatigue seen with NAC treatment could be due to its effect on \(O_2\). During oxidative stress, the sulfhydryl groups on the Ca\(^{2+}\) release channels and SR ATPase can be oxidized, thereby causing loss of Ca\(^{2+}\) homeostasis in the contractile apparatus. This may be prevented or reversed by treatment with a free radical scavenger and thiol donor such as NAC (23).

Previous studies (31, 33, 37) on the diaphragm have shown both DMSO and SOD to be effective antioxidants under conditions of diaphragmatic fatigue. DMSO is a commonly used, highly permeable, nonenzymatic, sulfur-containing antioxidant and is primarily a ·OH scavenger, although it can also scavenge other ROS (3). Its mechanism of action on the contractile process is poorly understood (32). SOD, on the other hand, primarily catalyzes the dismutation of \(O_2^-\) to hydrogen peroxide and \(O_2\). It is a high-molecular-weight metalloenzyme, and so it may be restricted to an extracellular distribution (31). Radak et al. (29) demonstrated that administration of SM-SOD [poly-(stylene-co-maleic acid) butyl ester-linked derivative of SOD] to rats resulted in reduced oxidative damage to skeletal muscles during exhaustive exercise. They showed a significant reduction in plasma and skeletal muscle thiobarbituric acid-reactive substances and reduced plasma xanthine oxidase activity after exercise in the treated group. In our model, it is likely that SOD prevented a decline in muscle function during hypoxia by a combination of the above effects.

Tiron is a less well-known antioxidant, and to our knowledge its effects on skeletal muscle have so far not been studied. It is a low-molecular-weight phenolic compound and has been shown to scavenge ·OH, \(O_2^-\), and hydrogen peroxide (18, 24). It has been used for in vivo animal studies as a nontoxic metal chelator (24). In addition, it can be used as a spin trap. Combined with free radicals, it is easily oxidized to its semiquinone radical, which can be detected by electron paramagnetic resonance imaging (24). We have also shown that Tiron inhibits diaphragmatic fatigue similar to other antioxidants (unpublished data). In this study, the improvement in muscle function on coincubation with Tiron is perhaps due to its free-radical scavenging and metal-chelating properties.
In conclusion, hypoxia results in skeletal muscle dysfunction, which can be partially prevented by treatment with antioxidants. We speculate that hypoxia results in the production of reactive \( \text{O}_2 \) or other oxidizing species through a mechanism initiated by reductive stress. These findings suggest a new role for antioxidants in providing protection during conditions of reductive stress, much as they have been shown to provide protection in oxidative stress. This may apply to the proven effectiveness of antioxidant treatment in many other tissue beds and clinical conditions in which problems with \( \text{O}_2 \) delivery and utilization are present. For example, cell dysfunctions caused by ischemia, sepsis, or intense metabolic activity seen in fatiguing skeletal muscle have all been shown to be partially prevented by treatment with antioxidants and are likely to represent conditions of a high-reducing environment within the cells. Therefore, the traditional view that antioxidants protect tissue from reactive \( \text{O}_2 \) produced during reperfusion or reoxygenation must be expanded to include the concept of protection during the preceding period of hypoxia or ischemia.

This research was supported by National Heart, Lung, and Blood Institute Grant HL-53333.

Address for reprint requests: T. L. Clanton, The Ohio State Univ., Pulmonary and Critical Care Medicine, N325 Means Hall, 1654 Upham Dr., Columbus, OH 43210 (E-mail: Clanton.1@osu.edu).

Received 7 August 1997; accepted in final form 17 February 1998.

REFERENCES


