Mohan, D. P., Ph.D. Thesis, Purdue University, West Lafayette, Ind., 1975.
Landry, J., Moureaux, T., *Bull. Soc. Chim. Biol.* **52** 1021–1036 (1970).
Nelson, O. E., *Adv. Agron.* **21**, 171–194 (1969).
Singh, R., Axtell, J. D., *Crop Sci.* **13**, 535–539 (1973).
Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.* **30**, 1190–1206 (1958).
Weber, K., Osborne, M., *J. Biol. Chem.* **244**, 4406–4411 (1969).

Received for review January 26, 1977. Accepted August 17, 1977. Journal Paper No. 6588 of the Purdue Agricultural Experiment Station. Supported by the United States Agency for International Development under a contract titled "Inheritance and Improvement of Protein Quality and Content in *Sorghum bicolor* (L.) Moench".

Effects of EDTA and Ascorbic Acid on the Absorption of Iron from an Isolated Rat Intestinal Loop

E. R. Monsen* and J. F. Page

Intestinal loops were isolated in 101 adult male Sprague-Dawley rats. While maintaining a constant level of iron, 12 different iron–chelate solutions were prepared by varying quantities of ascorbic acid and/or EDTA. Observations were made of the rate of iron absorption at 0.25, 0.5, 1, 2, and 4 h from ascorbic acid and iron solutions with a molar ratio of 1:1 and 4:1. Absorption rapidly increased for the first 2 h, after which it plateaued. Subsequently, absorption tests were terminated after 2 h. Iron absorption was significantly higher in the presence of ascorbic acid than in the presence of EDTA. When both chelates were administered, EDTA was capable of negating the enhancing effects of ascorbic acid, even though the molar ratio of ascorbate:EDTA was as high as 4:1.

Iron, as other transitional metal ions, readily forms complexes with various dietary compounds. During the time that the iron complex remains soluble, the iron will not precipitate; this should, theoretically, increase the opportunity for iron to be absorbed. In fact, however, absorption of the sequestered iron may be enhanced or inhibited depending on the nature of the specific iron complex, its reaction with other dietary and luminal factors, and its facility either to enter as an intact complex or to release its iron to the mucosal cell.

Enhanced iron absorption has been seen with certain amino acids, e.g., cysteine and histidine (Kroe et al., 1963; Van Campen and Gross, 1969; Martinez-Torres and Layrisse, 1970; Van Campen, 1973); reducing sugars, e.g., fructose (Sams and Carroll, 1966; Davis and Deller, 1967; Amine and Hegsted, 1975); and the vitamin ascorbic acid (Conrad and Schade, 1968). It has been postulated that these compounds form complexes with iron which keep the iron in solution during transit through the upper part of the small intestine where absorption most rapidly occurs. Of these enhancing factors, ascorbic acid is of particular interest in that it is a compound which is biologically essential for life, occurs naturally in foods and, in addition, may be made synthetically. Ascorbic acid is widely used in the food industry as a food additive; in this capacity ascorbic acid serves as a nutrient supplement and/or an antioxidant. As a reducing agent it is effective in decreasing the browning of fresh fruits and vegetables and in retaining flavor and color. Ascorbic acid is considered to be safe in levels not to surpass the amount necessary to achieve the desired results, and there are no present limitations or restrictions on the quantity which may be used as a food additive (Furia, 1972).

Lowered absorption has been reported from the interaction of certain other dietary compounds which complex with iron. Among these are calcium phosphate salts (Monsen and Cook, 1976), phytates (Cowan et al., 1966), desferrioxamine (Hwang and Brown, 1963; Cook et al., 1972), and ethylenediaminetetraacetate (EDTA) (Cook and Monsen, 1976). Various mechanisms have been proposed as to why these iron-complexing compounds decrease the availability of dietary iron; among the possible suggestions are the formation of macromolecules and/or decreased solubility of the specific iron complex at intestinal pH. EDTA is the most widely used synthetic chelate of polyvalent cations which is currently incorporated into the U.S. diet. As a food additive EDTA decreases oxidative damage to foods by free metals, thus promoting stabilization of color, texture, and flavor. Current regulations allow EDTA to be added to processed potatoes, canned legumes, canned peas and beans, canned shell fish, salad dressings, mayonnaises, sandwich spreads, sauces, and carbonated drinks, beer, and distilled alcohol. Allowable quantities range from 25–800 parts per million (U.S. Food and Drug Administration, 1974).

This study focuses on the individual effect of two iron-complexing compounds—EDTA and ascorbic acid—and the effect of their competitive interaction on the absorption of iron from an isolated intestinal loop. Such a model allows the blood supply to remain intact while measuring the intestinal absorption of specified iron solutions in designated sections of the intestinal tract (Wheby et al., 1964; Van Campen and Mitchell, 1965). Influences of gastric juice, gastric activity, bile secretions, pancreatic juice, and undefined food residues are removed and the contents of the intestinal lumen may be under greater control.

METHODS

Adult male Sprague-Dawley rats with a mean weight of 227 g (range 190–282 g) were utilized for the study. Prior to the experiment the animals were housed in stainless steel cages with wire mesh bottoms, and water and Purina Rat Chow were given ad libitum. Surgery was preceded

Division of Human Nutrition, Dietetics and Foods, University of Washington, Seattle, Washington 98195.

0021-8561/78/1426-0223$01.00/0

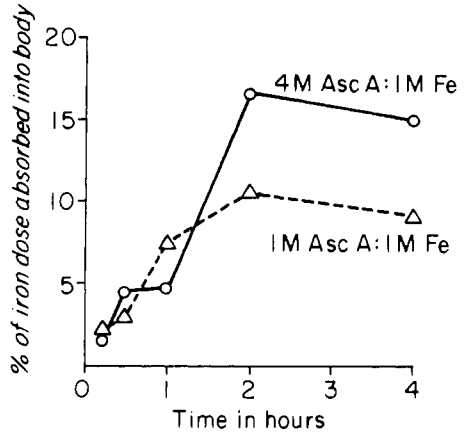

Figure 1. Percent of iron absorbed from an ascorbic acid:iron solution of either 1 M:1 M or 4 M:1 M ratio in an isolated rat intestinal loop. The ascorbic acid:iron solutions were in the intestinal loop for 15 min, 30 min, 1, 2, or 4 h.

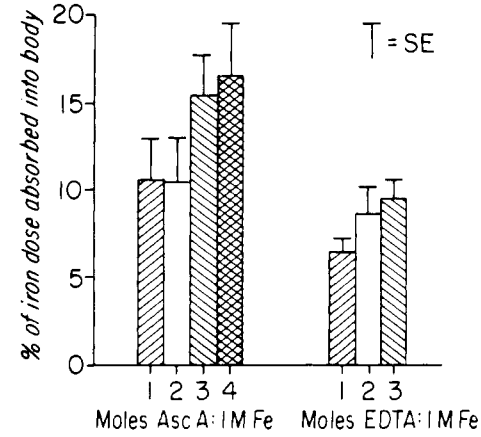

Figure 2. Percent of iron absorbed from either ascorbic acid:iron or EDTA:iron solutions in an isolated rat intestinal loop. All solutions contained the same quantity of iron and were left in the intestinal loop for 2 h.

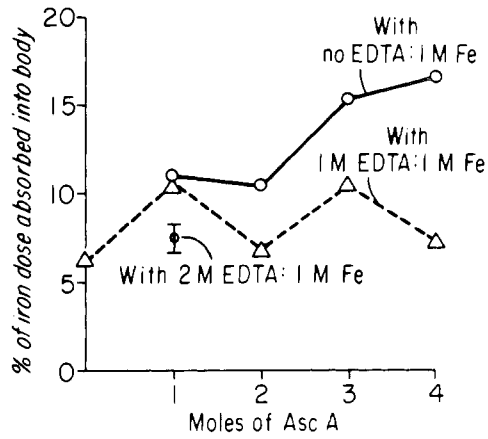

Figure 3. Percent of iron absorbed from combined solutions of ascorbic acid, EDTA, and iron in an isolated rat intestinal loop. All solutions contained the same quantity of iron but differed in ascorbic acid and EDTA concentrations. The solutions were left in the intestinal loop for 2 h.

by a 24-h fast. The 101 animals were divided into 20 experimental groups. The animals were anesthetized with ether and a 3-cm intestinal segment at the proximal end of the jejunum was isolated. The loop was purged with 3 mL of saline via syringe and the iron–chelate dose was subsequently administered. The incision was closed, and the animals, after a predetermined time, were again placed under ether anesthesia, the abdominal cavity reopened, and the gut loop excised. The gut loop was placed in a petri dish and γ emissions were counted by a human whole-body counter. Subsequently, the body (the animal minus the gut loop) was counted for γ emissions. The total γ counts of the intact loop and body equalled the total activity injected. Means, standard errors, standard deviations, and Student's t-test were calculated (Downie and Heath, 1970).

The iron–chelate solutions were made as follows: ferrous sulfate labeled with ferric-59 chloride was diluted to the appropriate volume with saline. Solutions of either ascorbic acid or EDTA were then added to the iron solution. In cases where both chelates were added to one solution, the chelates were thoroughly mixed together before addition of the iron and saline mixture. Final solutions were adjusted to approximately pH 6 with dilute NaOH and HCl. Solutions were made daily. Of the 12 different iron–chelate solutions utilized, each contained 0.045 mg of iron/0.20 mL, which was the quantity injected into each rat. Solutions with ascorbic acid to iron molar ratios of 1:1, 2:1, 3:1, and 4:1 (0.14, 0.28, 0.42, and 0.56 mg of ascorbic acid, respectively) and EDTA to iron molar ratios of 1:1, 2:1, and 3:1 (0.318, 0.636, and 0.954 mg of EDTA per 0.20 mL, respectively) were prepared. Of the combined chelate solutions, the ascorbic acid to EDTA to iron molar ratios were 1:1:1, 2:1:1, 3:1:1, 4:1:1, and 1:2:1. The ascorbic acid:iron solutions of 1:1 and 4:1 were administered for 0.25, 0.5, 1, 2, and 4 h periods, and the remaining solutions were administered for 2-h intervals only.

RESULTS

The quantity of iron absorbed into the body from ascorbic acid:iron solutions of a 1:1 and 4:1 molar ratio were observed at 0.25, 0.5, 1, 2, and 4 h. A steady rise in absorption was seen with a peak at 2 h ($p < 0.001$). No significant changes were seen between the 2- and 4-h period (Figure 1). Within the first 15 min, 2.1 ± 0.7% (mean ± SE) of the iron in the 1:1 ascorbic acid:iron solution was absorbed which approximated the quantity absorbed from the 4:1 molar ascorbic acid:iron solution (1.7 ± 0.1, mean ± SE). At 2 h, however, significant differences were observed between the two solutions of different ascorbic acid concentration; a fivefold increase in the amount of absorbed iron was seen in the lower ascorbic:iron solution while a 9.8-fold increase was seen in the 4:1 ascorbic:iron ratio after 2 h ($p < 0.05$). The 2-h peak of absorption for the 1:1 ascorbic:iron was 10.6 ± 2.3% (mean ± SE) and for the 4:1 ascorbic:iron was 16.6 ± 2.9% (mean ± SE). The time period of 2 h was selected for the subsequent studies of the effects of the chelates on iron absorption.

The effects of maintaining the same quantity of iron but increasing the molar ratio of either ascorbic acid or EDTA in the solution injected into the intestinal loop is shown in Figure 2. Overall, the quantity of iron absorbed in the presence of ascorbic acid was higher than the quantity of iron absorbed when EDTA was the chelate ($p < 0.01$). However, increasing the ratio of either chelate led to an increase in the quantity of iron absorbed. When the molar ratio of EDTA:iron was raised from 1:1 to 3:1, absorption was increased from 6.4 ± 0.8% to 9.6 ± 1.0% ($p < 0.05$).

The effects on iron absorption of the competitive interaction of EDTA and ascorbic acid is shown in Figure 3. EDTA in the absence of ascorbic acid resulted in a low rate of iron absorption; with the addition of an equimolar quantity of ascorbic acid (molar ratio of ascorbic acid: EDTA:iron of 1:1:1), absorption was increased over 70% ($p < 0.05$). However, the addition of another molar

quantity of EDTA (ascorbic acid:EDTA:iron of 1:2:1) negated the enhancing effect of the ascorbic acid. In subsequent studies ascorbic acid concentration was increased fourfold, leading to a rise in iron absorption which was totally curtailed by the addition of EDTA; 16.6 ± 2.9% of the iron from the 4:1 molar ascorbic acid:iron was absorbed while only 7.3 ± 1.4% was absorbed when 1 molar ratio EDTA:Fe was added ($p < 0.05$).

DISCUSSION

Absorption of iron from the isolated intestinal loop occurred rapidly within the first 2 h. Similarly, Wheby and Crosby (1963) observed that 60–80% of the iron placed into a ligated intestinal loop was absorbed within the first 2 h with a gradual increase over the next 24 h. During the initial 2-h period, however, it cannot be assumed that the iron remained complexed with the ascorbic acid. It is reasonable that there may have been some disassociation, degradation, and/or absorption of the complexing agent.

As chelating agents, ascorbic acid appears to be far more effective than EDTA in forming an iron complex which increases the net absorption of iron. That ascorbic acid enhances iron absorption in man has long been recognized. Ascorbic acid appears effective in increasing absorption of non-heme iron from foods (Cook et al., 1972; Cook and Monsen, 1977; Sayers et al., 1973), as well as iron supplements (Sayers et al., 1974a,b; Bjorn-Rasmussen, 1974). One reason for this effect may be that ascorbic acid serves as a reducing agent thus converting ferric iron to the more available ferrous form. The exact mechanism by which ascorbic acid enhances the absorption of iron is not completely understood. Conrad and co-workers (1968) have suggested that ascorbic acid forms a complex with iron at a low pH which remains intact and soluble over a wide range of pH.

Ferric EDTA has been seen to be an effective source of iron for both animals (Davis et al., 1968) and plants (Stewart and Leonard, 1952). In the experiments reported here, EDTA was less effective than ascorbic acid in increasing the absorption of iron from the isolated intestinal loop; however, EDTA appeared to maintain iron in a soluble complex and thus minimizes the precipitation of iron within the ligated loop. The effect of EDTA appears to be dose related. In man it has been shown that as the molar quantities of EDTA are increased with respect to iron from 2:1 to 20:1, the amount of iron absorbed significantly decreases; at molar ratios of EDTA:iron of 1:1 or lower, significant inhibition was not seen (Cook and Monsen, 1976).

Although iron in the presence of equimolar levels of EDTA is not absorbed as readily as iron in the presence of equimolar levels of ascorbate, it seemed possible that if ascorbate were present along with EDTA that the iron would be more readily assimilated than when EDTA was the sole chelate. This was investigated by injecting solutions of iron with EDTA plus ascorbate into the isolated intestinal loop. When EDTA and ascorbic acid were competing for the iron at equimolar amounts to the iron, absorption was similar to the 1:1 ascorbic acid:iron. However, doubling the molar concentration of EDTA with respect to the ascorbate and iron inhibited iron absorption. Further, the increased absorption seen when ascorbic acid was increased fourfold in molar concentration to iron was blocked by the addition of a small quantity of EDTA.

In the ligated loop, the presence of food residues and gastric and biliary secretions are eliminated. Within a physiological setting, however, these factors would be present as well as other metallic ions such as calcium, nickel, zinc, and copper which might be chelated by heavy metal ligands such as EDTA, thus allowing the iron to be complexed by other chelates or be precipitated from solution. Such possibilities are feasible in a normal diet, particularly with its much higher content of calcium, the molar concentration of calcium:iron approximating 69:1 for a typical American dietary (Cook et al., 1972; Monsen and Cook, 1976). It has been estimated that daily ingestion of 50 mg of EDTA may be routine in the American diet (Cook and Monsen, 1976). At an iron intake of 10 mg, this would be a molar ratio of EDTA:iron of 0.7:1.0. Little interference with iron absorption would be anticipated at this level. However, for certain segments of the population, the level of EDTA ingestion may be several times higher.

Intake of ascorbic acid is equally variable. Current recommended dietary allowances show molar ratios of ascorbic acid:iron of 0.8:1.0 for the female during menstrual years and the adolescent male and 1.4:1.0 for the adult male ("Recommended Dietary Allowances", 1974). Yet the level of ingested ascorbic acid appears to be far greater in proportion to recommended intake than is the level of ingested iron. Massive quantities of ascorbic acid are currently being ingested by some individuals as a voluntary ascorbic acid supplement. These high quantities of ascorbic acid may be helpful in absorption of iron which is consumed at the same meal as indicated by a steady rise in non-heme iron absorption exhibited on a logarithmic scale with increasing quantities of ascorbic acid up to 1000 mg. (Cook and Monsen, 1977). Concern has been raised as to the effects of these megadoses of ascorbic acid on the absorption and metabolism of other vitamins and minerals. The interaction of dietary components needs further study.

Iron tends to form complexes and, indeed, may have to be held in a soluble complex in order to be absorbed. It would appear from the data that ascorbic acid is the complexing agent of choice. Equimolar quantities of ascorbic acid ingested along with iron may improve iron absorption, unless excess EDTA is present.

LITERATURE CITED

Amine, E. K., Hegsted, D. M., *J. Agric. Food Chem.* **23**, 204 (1975).
Bjorn-Rasmussen, E., *Nutr. Metab.* **16**, 94 (1974).
Conrad, M. E., Schade, S. G., *Gastroenterology* **55**, 35 (1968).
Cook, J. D., Layrisse, M., Martinez-Torres, C., Walker, R., Monsen, E., Finch, C. A., *J. Clin. Invest.* **51**, 805 (1972).
Cook, J. D., Monsen, E. R., *Am. J. Clin. Nutr.* **29**, 614 (1976).
Cook, J. D., Monsen, E. R., *Am. J. Clin. Nutr.* **30**, 235 (1977).
Cowan, J. W., Esfaham, M., Salij, J. P., Azzam, S. A., *J. Nutr.* **90**, 423 (1966).
Davis, P. N., Norris, L. C., Kratzer, F. H., *J. Nutr.* **94**, 407 (1968).
Davis, P. S., Deller, D. J., *Aust. Ann. Med.* **16**, 70 (1967).
Downie, N. M., Heath, R. W., "Basic Statistical Methods", Harper and Row, New York, N.Y., 1970, pp 167–185.
Furia, T. E., Ed., "Handbook of Food Additives", 2nd ed, CRC Press, Cleveland, Ohio, 1972.
Hwang, Y., Brown, E. B., *Lancet* **1**, 135 (1963).
Kroe, D. T., Kinney, T. D., Kaufman, N., Klavins, J. V., *Blood* **21**, 546 (1963).
Martinez-Torres, C., Layrisse, M., *Blood* **35**, 669 (1970).
Monsen, E. R., Cook, J. D., *Am. J. Clin. Nutr.* **29**, 1142 (1976).
"Recommended Dietary Allowances", 8th ed, Food and Nutrition Board, National Academy of Sciences–National Research Council Publ. 2216, Washington, D.C., 1974.
Sams, W. M., Carroll, N. V., *Nature (London)* **212**, 404 (1966).
Sayers, M. H., Lynch, S. R., Charlton, R. W., Bothwell, T. H., *Brit. J. Nutr.* **31**, 367 (1974a).
Sayers, M. H., Lynch, S. R., Charlton, R. W., Bothwell, T. H., Walker, R. B., Mayet, F., *Brit. J. Haematol.* **28**, 483 (1974b).
Sayers, M. H., Lynch, S. R., Jacobs, P., Charlton, R. W., Bothwell, T. H., Walker, R. B., Mayet, F., *Brit. J. Haematol.* **24**, 209 (1973).
Stewart, I., Leonard, C. D., *Science* **116**, 564 (1952).

U.S. Food and Drug Administration, Code of Federal Regulations (21), Office of the Federal Register, Washington, D.C., 1974.
Van Campen, D. R., *J. Nutr.* **103**, 139 (1973).
Van Campen, D., Gross, E., *J. Nutr.* **99**, 68 (1969).
Van Campen, D., Mitchell, E. A., *J. Nutr.* **86**, 120 (1965).
Wheby, M. S., Crosby, W. H., *Blood* **22**, 416 (1963).
Wheby, M. S., Jones, L. G., Crosby, W. H., *J. Clin. Invest.* **43**, 1433 (1964).

Received for review February 18, 1977. Accepted August 2, 1977. Supported in part by the Department of Agriculture Grant 12-14-100-9918(61).

Selenium and Arsenic Levels in Soybeans from Different Production Regions of the United States

R. D. Wauchope

Whole soybeans [*Glycine max* (L.) Merr.] from Arkansas, Florida, Indiana, Iowa, Maryland, Mississippi, and North Carolina have been analyzed for total arsenic and selenium. Arsenic levels were 0.1 ppm (fresh weight) or less for all samples and were independent of selenium levels. Selenium levels in soybeans reflected levels in the soil in the region where the soybeans were grown, varying from 0.07 ppm or less in low-selenium areas (Maryland, Indiana, Florida, and North Carolina) to 0.16 ppm (Arkansas), 0.28 ppm (Iowa), and 0.90 ppm (Mississippi). The selenium was associated with the oil-free fraction of the soybean. Selenium levels in the soybeans from Stoneville, in the Mississippi "Delta" floodplain, were high in comparison with levels in soybeans from other areas considered to have adequate soil selenium levels, suggesting that soils in this area may unusually rich in available selenium. These results show that soybeans may be a significant source of selenium and possibly other trace nutrients in human and animal diets.

Ferretti and Levander (1976) recently reported selenium (Se) levels in soybean [*Glycine max* (L.) Merr.] food products and found quite variable levels in the range 0.02–0.7 ppm, with good correlation between Se levels and soy protein content. Since these products are being increasingly used as meat substitutes, there is concern that soy products with the lower ranges of Se will not adequately provide as much Se as the replaced meat. Ferretti and Levander listed three possible sources of variation in the soy product selenium levels: (1) differences in selenium content of the soybeans at harvest; (2) losses of Se in processing; and (3) addition of other ingredients to the soy products that may or may not contain substantial amounts of selenium. This report is concerned with source (1).

The Se content of forage and grain crops varies widely and is a function of the content of Se in the soil where they are grown, as well as other soil factors such as pH and texture. Wheat (*Triticum aestivum* L.) is the most studied example (Kubota et al., 1967). Few data are available on Se levels in fresh soybeans, however. Ferretti and Levander reported 0.07 ppm fresh weight; Olson (1969) reported a single value of 1.5 ppm. Klayman and Gunther (1973) list values of 0.07 ppm for soybeans from the eastern United States, 0.54 for Nebraska soybeans (an area of highly seleniferous soils), and 0.1 ppm for midwestern soybeans, indicating considerable variation of levels with production area. At the time of appearance of the article by Ferretti and Levander (1976) the authors had been applying a new flameless atomic absorption technique to the measurement of arsenical herbicide residues in soybean grain grown at Stoneville (Wauchope and McWhorter, 1977). Since the technique is equally useful for selenium, we tried it on the same soybean digests used for arsenic analysis and were surprised at the high levels found (in excess of 1 ppm for some samples). To verify these results we have analyzed a variety of Stoneville-grown soybeans, along with soybean samples from six other states for comparison. Although most of the important production areas of the U.S. (Dovring, 1974) are represented by these samples, the number of samples is too small for the results to be taken as definitive for each area. We also include results of arsenic (As) analyses, since they were simple to obtain from the same digested samples and are of interest both from a nutritional and toxicological standpoint.

MATERIALS AND METHODS

Soybeans were obtained through the ARS Soybean Production Research Unit at Stoneville, Miss. Fresh soybeans were ground in a Wiley mill and stored in a freezer until used. Two or more 4-g samples of the beans were digested with 80 mL of nitric–perchloric acids (3:1) using Bethge condensers to contain $HClO_4 \cdot 2H_2O$ fumes (Griffen et al., 1974, 1975). We found that slow digestion was necessary and used round-bottom flasks and heating mantles, instead of hot plates and the Erlenmeyer flasks provided with the Bethge apparatus by the manufacturer. Rapid digestion with high-wattage hot plates caused an average loss of 30% of the Se content.

At the end of digestion the approximately 20 mL of 70% perchloric acid remaining in the apparatus was diluted to 50 mL with distilled water. Samples (1 mL) of the diluted digest were analyzed by sodium borohydride reduction of the Se or As to hydrogen selenide or arsine, followed by flameless atomic absorption assay (Wauchope, 1976). Optimization of carrier gas flow rates and the introduction of a very small flow (a few milliliter/min) of air into the carrier gas gave a working range of 5–200 ppb Se or As in the digest with this method, with a detection limit (3 × blank variance) of 2 ppb, equivalent to 0.03 ppm in the soybeans.

Southern Weed Science Laboratory, U.S. Department of Agriculture, Agriculture Research Service, in cooperation with the Mississippi Agricultural and Forestry Experiment Station, Stoneville, Mississippi 38776.