Livingston, A. L., Nelson, J. W., Kohler, G. O., *J. Agric. Food Chem.* **16**, 492 (1968).
Marusich, W. L., *Feedstuffs* **37**, 30 (1965).
Marusich, W. L., *Feedstuffs* **39**, 48 (1967).
Marusich, W. L., *Feedstuffs* **41**, 46 (1969).
Mitchell, H. L., Beauchene, R. E., Silker, R. E., *J. Agric. Food Chem.* **2**, 939 (1954).
Thompson, C. R., U.S. Patent 2562970, 1951.
Witt, S. C., Spencer, R. R., Bickoff, E. M., Kohler, G. O., *J. Agric. Food Chem.* **19**, 162 (1971).

Received for review October 29, 1979. Accepted February 11, 1980. Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Transformations of Chemical Constituents during Flue-curing of *Nicotiana tabacum* L. 2. Metabolism of Nitrogenous and Related Constituents

Aumeed N. Amin, R. C. Long,* and W. W. Weeks

Changes in certain nitrogen fractions of tobacco leaf during flue-curing were studied by sampling leaves from two distinct primings in 1976 and 1977 at short time intervals during the yellowing and drying stages of flue-curing. Analytical data were corrected for dry weight loss. Percentage total nitrogen decreased during curing, while nitrate nitrogen showed no significant pattern of change. Total alkaloid concentrations exhibited stalk position and seasonal responses. Proteolysis extended throughout flue-curing, resulting in increased α-amino nitrogen during yellowing, but declined during drying. A net decrease in the concentration of total carbonyls (possible products of amino acid metabolism) occurred in both primings of one season, but not in the other. Miscellaneous nitrogen increased during yellowing and decreased during drying. The results suggest that flue-curing substantially alters the components of the various nitrogenous fractions of the harvested leaf.

Nitrogenous components of tobacco are important not only because of the presence of nicotine, but also for their contribution to flavor and aroma which Leffingwell (1976) reviewed in detail. Changes in some of these chemical constituents of tobacco leaf during flue-curing have been reviewed by Frankenburg (1946) and Johnson (1966, 1975). Early investigations of changes in the constituents during curing indicated the importance of curing in modifying qualitatively and quantitatively the chemistry of the green, mature leaf which, in turn, influences the quality of the cured leaf (Bacon, 1952; Chang, 1961; Sastry, 1960). Weybrew et al. (1966) have suggested that some carbonyls contributing to the aroma and flavor of tobacco may be generated through the decarboxylation and/or deamination of certain amino acids during curing.

By sampling at short time intervals during curing and determining the metabolic activities of leaf tissue and the constituent levels associated with them, one can gain a better understanding of how these factors influence the development of quality. The objective of this study was to investigate the transformations which occur in various nitrogen fractions during curing of tobacco from upper and lower stalk positions.

MATERIALS AND METHODS

As described previously (Amin et al., 1980), *Nicotiana tabacum* L. cv NC 2326 plants were grown and cultured normally in 1976 and 1977. The first and fourth primings (comprising approximately leaves 1–4 and 13–16, respectively) were harvested at maturity and flue-cured in conventional barns. Leaf samples were withdrawn at 6- and 12-h intervals during the yellowing and drying cycles of curing, respectively. After separating the midrib from the lamina, the lamina was quick-frozen, freeze-dried, ground, and stored for analysis.

Samples were analyzed for total alkaloids according to the method of Harvey et al. (1969). Soluble protein nitrogen [extracted according to the method of Gaines (1977)] and total nitrogen were determined colorimetrically as ammoniacal nitrogen/BD acid digest (Technicon Industrial Method No. 312-74A). Nitrate-nitrogen analysis was accomplished according to the method of Collins et al. (1967), while α-amino nitrogen was determined using a ninhydrin-positive procedure. Miscellaneous nitrogen was calculated from the equation:

$$\text{miscellaneous N} = \text{total N} - (\text{protein N} + \alpha\text{-amino N} + \text{nitrate N}) \quad (1)$$

Total carbonyls were determined by a modification of the procedure of Henick et al. (1954). Analytical grade CH_2Cl_2 was distilled with 2,4-dinitrophenylhydrazine (1.0 g/L) to eliminate carbonyls. Carbonyl-free ethanol was prepared by distilling 100% ethanol with 2,4-dinitrophenylhydrazine (0.5 g/L). One gram of ground tobacco was extracted with 100 mL of carbonyl-free ethanol for 24 h in the dark. The extract was filtered and made to 100-mL volume with carbonyl-free ethanol. Five milliliters was diluted to 100 mL. Five milliliters of diluted extract was pipetted into a 25-mL volumetric flask, and 3 mL of 4.3% trichloroacetic acid in carbonyl-free CH_2Cl_2 and 5 mL of 0.15% 2,4-dinitrophenylhydrazine in carbonyl-free ethanol were added. The flask was heated in a water bath at 40 °C for 30 min and cooled to room temperature. Color was developed by adding 10 mL of 4% KOH (prepared fresh in carbonyl-free ethanol). The flask was adjusted to volume with carbonyl-free CH_2Cl_2 and mixed. The absorption at 435 nm was determined spectrophotometrically after exactly 10 min against a blank prepared in the

Department of Crop Science, North Carolina State University, Raleigh, North Carolina 27650.

0021-8561/80/1428-0656$01.00/0 © 1980 American Chemical Society

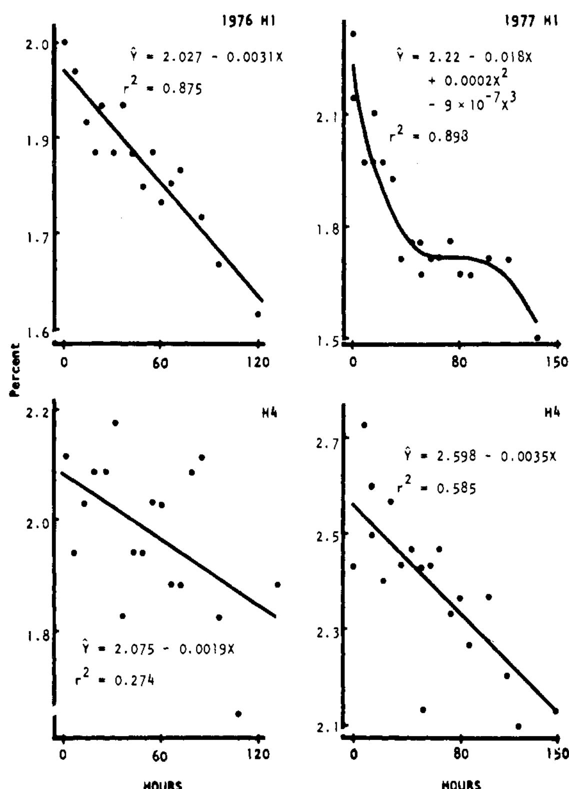

Figure 1. Changes in total nitrogen during flue-curing of first and fourth primings in 1976 and 1977.

same manner. Corrections were made for background sample color. Isobutyraldehyde was used as the analytical standard.

The increase in calcium concentration [due to respiratory loss of dry weight; Amin et al. (1980)] was used to correct all analytical data. Regression equations were employed to express quantitative changes in leaf components at the $P \leq 0.05$ level.

RESULTS AND DISCUSSION

Although the exact patterns of change differed somewhat among the two primings and growing seasons, total nitrogen decreased during flue-curing (Figure 1). The relative loss of total nitrogen during curing was greatest in the first priming. Bacon et al. (1952), Chang (1961), and Hara et al. (1971) have reported similar changes. However, only Bacon et al. (1952) corrected their data for dry weight loss, whereas Sastry (1956) and Tomita (1968a), who reported that total nitrogen concentration did not change during curing, did not. In contrast, Yamada and Hatano (1967) found that during stalk curing of tobacco, nitrogen was translocated from leaves into the stalk during the early and middle stages of curing. The results of the present study do not eliminate the possibility that some mobile nitrogen compounds, especially α-amino nitrogen and nitrate, may have been translocated between the laminae and midrib. This possibility is supported by the fact that total nitrogen in the midrib was observed to increase during curing (data not shown). However, it is possible that part of the nitrogen lost from the lamina was due to the release of ammonia from the lamina tissue.

Significant patterns of change in nitrate nitrogen concentration were not observed during curing of the tobaccos (Figure 2). There appears to be no adequate explanation for the widespread fluctuations observed during curing. However, Gous et al. (1970) reported that nitrate-nitrogen concentration of freeze-dried tobacco was lower than that of heat-dried samples from the same source. They suggested that nitrate was codistilled with the water vapor in the freeze-drying process. Yet, freeze-drying has been found to have no effect on other chemical constituents of the leaf (Gous et al., 1970; Johnson, 1970).

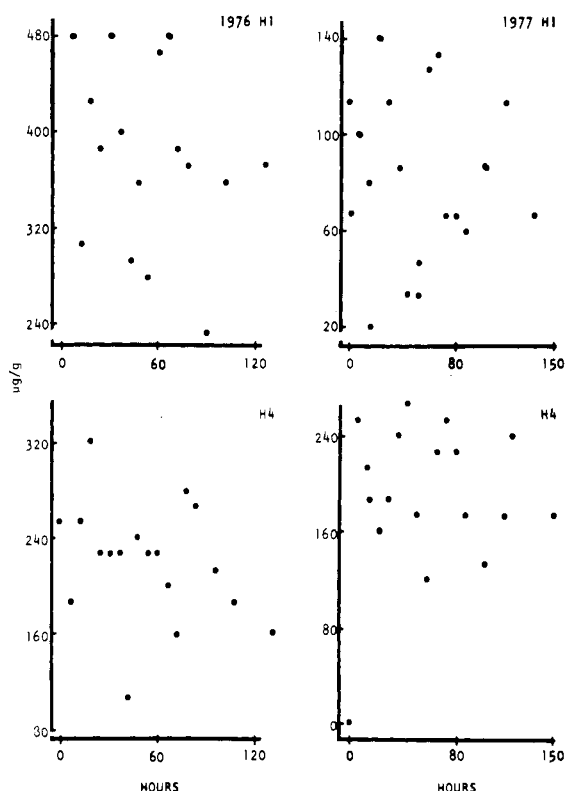

Figure 2. Changes in nitrate-nitrogen during flue-curing of first and fourth primings in 1976 and 1977.

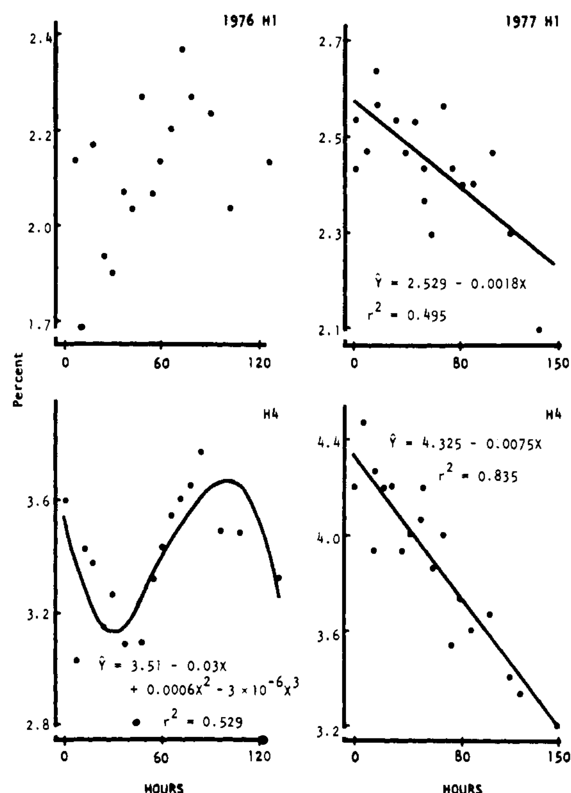

Figure 3. Changes in total alkaloids during flue-curing of first and fourth primings in 1976 and 1977.

The patterns of change for total alkaloids were somewhat different between the two seasons (Figure 3). Al-

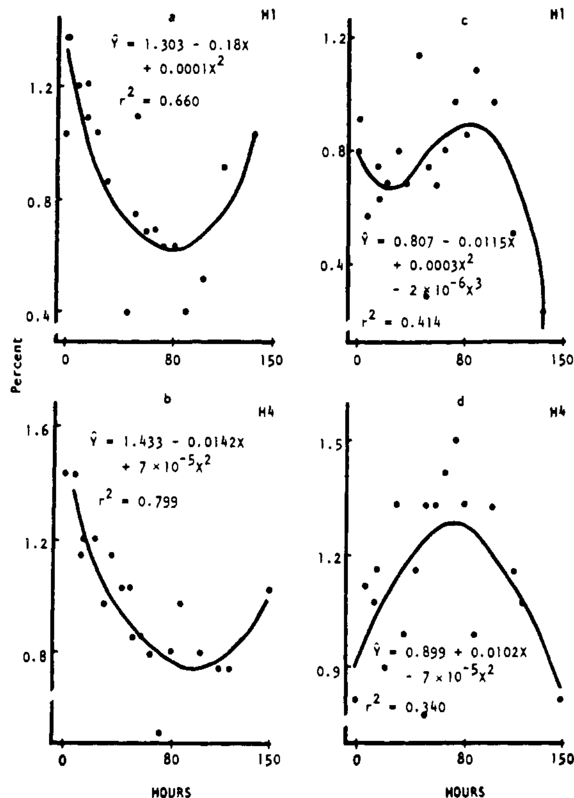

Figure 4. Changes in protein nitrogen (a, b) and miscellaneous nitrogen (c, d) during flue-curing of first and fourth primings in 1977.

though the changes in the first harvest in 1976 exhibited no significant pattern, the changes during curing of the fourth priming followed a cubic function, i.e., an initial decrease in total alkaloids before an increase during yellowing. The increase continued until about 90–100 h when a decline began. In contrast, total alkaloids declined in a linear manner during the curing of both primings in 1977. The alkaloid concentrations of the cured tobaccos in 1977 were also very much lower than in the harvested leaf as opposed to no change or a slight decrease in 1976. These contrasts emphasize the differences in either curing conditions or the "curability" of the tobaccos between the 2 years.

The data for the fourth priming in 1976 are, however, consistent with the report of Hara et al. (1973) regarding increases in alkaloids during the yellowing phase of curing. Tomita (1968a) reported, on the other hand, that nicotine concentration did not increase during curing. Yet, Bacon et al. (1952) observed small losses of nicotine during curing. The decrease generally noted during yellowing in this study undoubtedly arose from translocation from the laminae to the midrib, as was suggested for total nitrogen. Thus, the early hours of curing take on added significance as regards the mobilization of metabolites. The decreases during drying are more difficult to explain, but may be due to metabolic and thermal alteration. Although acidity of the lamina decreased during drying (Amin et al., 1980), it seems unlikely that significant quantities of nicotine were volatilized or codistilled with the water vapor during the freeze-drying.

From the data presented in Figure 4a and 4b, it is obvious that proteolysis, which began in the field with the onset of senescence, was continued during curing of the 1977 tobaccos. That excision, thermal treatment, and limited dehydration accelerate the rate of decomposition (Bacon, 1952; Sastry, 1956; Kawashima et al., 1967b; Hara et al., 1973) is borne out by the correlation between protein level and moisture loss during curing in the fourth priming ($r^2 = 0.826$; $P \leq 0.01$). It has been suggested that a large proportion of the protein hydrolyzed during curing is accounted for by the soluble fraction 1 protein (Burton et al., 1957; Kawashima et al., 1967a,b). The slight increase in protein concentration during the drying phase is difficult to explain. However, it is known that soluble low-molecular-weight proteins increase at the expense of insoluble, high-molecular-weight proteins (Hara et al., 1971; Kawashima et al., 1967b) and may partially account for the results observed in this study.

Data in Figure 4c and 4d present the changes in miscellaneous nitrogen during curing in 1977. This fraction consists of alkaloids, nucleic acids, and other miscellaneous nitrogen compounds. In both harvests, miscellaneous nitrogen increased during yellowing and decreased during the drying period. As exhibited with the other nitrogen fractions, except α-amino nitrogen, miscellaneous N concentrations were generally higher in the upper leaves than in the lower leaves.

Proteolysis resulted in a sharp increase in soluble nitrogen in the form of α-amino nitrogen (Figure 5). Amino nitrogen concentration increased to the end of the yellowing period and then declined at a rapid rate. These results and the decrease in protein during yellowing are strikingly similar to those observed during the yellowing of burley during air-curing (Hamilton and Lowe, 1978). In tobacco leaf, the amino acids known to make major con-

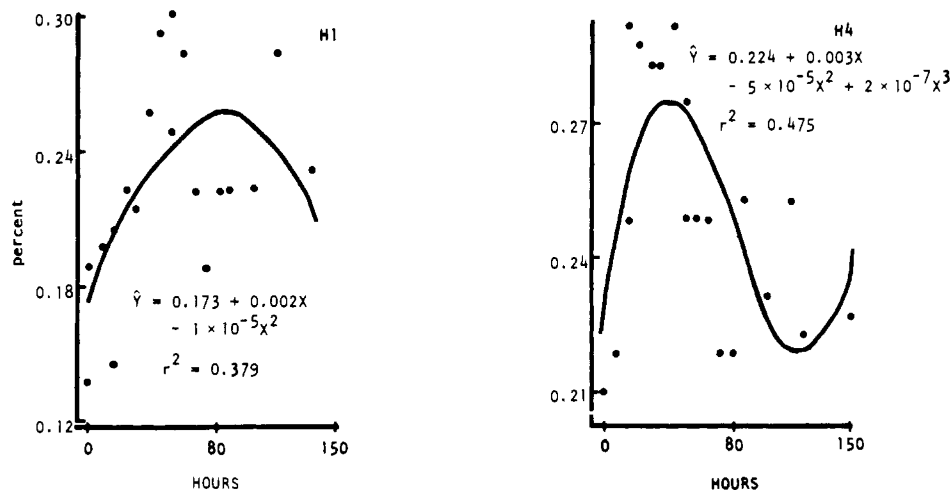

Figure 5. Changes in α-amino nitrogen during flue-curing of first and fourth primings in 1977.

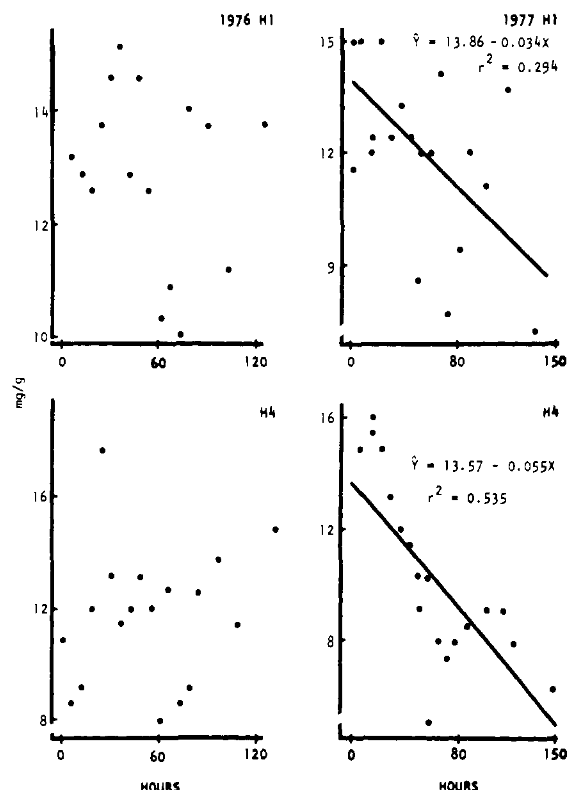

Figure 6. Changes in total carbonyls during flue-curing of first and fourth primings in 1976 and 1977.

tributions to the increase in total α-amino nitrogen are proline, hydroxyproline, phenylalanine, alanine, asparagine, glutamine, α-aminobutyric acid, and ammonia (Yoshida, 1961). Weybrew et al. (1966) reported an increase in 20 ninhydrin-positive components and a decrease in 7 during flue-curing. The decrease in α-amino nitrogen during the drying cycle has been reported by Tomita (1968a,b). There may be several explanations for the decrease. For example, it may be due to deamination and condensation reactions between amino acids or amides and monosaccharides and/or phenols as suggested by Tomita (1968a) and Weybrew et al. (1966). It has also been suggested (Forsyth, 1964; Lovett and May, 1958; Sastry, 1956) that amino acids may be utilized in respiration when sugars in the tissue become depleted. Although the reducing sugar concentrations of these samples were approximately 4% near the end of curing, total soluble sugar concentrations were quite high (Amin et al., 1980). Consequently, it seems unlikely that appreciable amounts of amino acids were respired during drying. The remobilization of α-amino nitrogen into the midrib also seems unlikely although vaporization of ammonia during drying at high temperature may account for substantial α-amino nitrogen loss.

Total carbonyls (as isobutyraldehyde equivalents; Figure 6) were determined to examine the relationship between amino acid degradation (as viewed through α-amino nitrogen loss) and total carbonyls during curing. No distinctive patterns of change were observed in either priming in 1976, but total carbonyls decreased rapidly in both primings in 1977. These data do not preclude the possibility of the continuous generation of carbonyls during curing from decarboxylation and deamination of specific amino acids or from terpenoids and 3-substituted pyridines [as suggested by Kimland et al. (1972) and Demole and Berthet (1972)]. But they strongly suggest the rate of loss of carbonyls, through volatilization (due to the low boiling points of such compounds), reduction reactions, and conversion of the carbonyls into alcohols, esters, and acids, far exceeds the rate of formation of such compounds during the thermodehydration process of flue-curing. The positive correlation observed between changes in α-amino nitrogen and total carbonyls in the fourth priming in 1977 lends support to that concept ($r^2 = 0.465$; $P \leq 0.05$).

Weybrew and Stephens (1962) reported several observations that may explain, in part, the much higher total carbonyl values reported here (approximately 12 mg/g of dried, ground tobacco) than those of Weybrew and Stephens (1962; approximately 15 mg/100 g of shredded tobacco) and Anderson et al. (1979; approximately 2 mg/g of ground, moisture equilibrated tobacco). First, their analyses were restricted to the cumulative total of seven specific, low-boiling carbonyls. Second, they observed nearly a threefold increase in carbonyls from ground samples as opposed to shredded ones. And lastly, they noted that reordering (equilibrating to about 2% moisture) reduced by more than 50% the liberation (during steam distillation) of certain carbonyls in relation to dried samples containing 12–14% moisture. Further, the 24-h solvent extraction used in the present study was probably more efficient in extracting carbonyls than the 30-min solvent extraction procedure used by Anderson et al. (1979).

ACKNOWLEDGMENT

Appreciation is expressed to R. E. Williamson for the α-amino nitrogen analyses.

LITERATURE CITED

Amin, A. N., Long, R. C., Weeks, W. W., *Tob. Sci.*, in press (1980).
Andersen, R. A., Tso, T. C., Chaplin, J. F., *J. Agric. Food Chem.* **27**, 891 (1979).
Bacon, C. W., Wenger, R., Bullock, J. F., *Ind. Eng. Chem.* **44**, 292 (1952).
Burton, M. P., Moseley, J. M., Like, C., Koening, D. F., *J. Agric. Food Chem.* **5**, 301 (1957).
Chang, A. Y., M. S. Thesis, North Carolina State University, Raleigh, NC, 1961.
Collins, P. F., Williams, J. F., Gerritsen, B. H., *Tob. Sci.* **11**, 75 (1967).
Demole, E., Berthet, D., *Helv. Chim. Acta* **55**, 1866 (1972).
Forsyth, W. G. C., *Annu. Rev. Plant Physiol.* **15**, 443 (1964).
Frankenburg, W. G., *Adv. Enzymol. Relat. Subj. Biochem.* **6**, 309 (1946).
Gaines, T. P., *J. Assoc. Off. Anal. Chem.* **60**, 590 (1977).
Gous, P. J., Kroontje, W., Terrill, T. R., *Tob. Sci.* **14**, 63 (1970).
Hamilton, J. L., Lowe, R. H., *Tob. Sci.* **22**, 189 (1978).
Hara, J., Fukuzumi, T., Sakuma, H., Yamada, S., Takamoto, T., *Okayama Jpn. Tob. Exp. Sta. Bull.* **30**, 69 (1971).
Hara, J., Fukuzumi, T., Sakuma, H., Yamada, S., Takamoto, T., Samejima, I., *Okayama Jpn. Tob. Exp. Sta. Bull.* **32**, 63 (1973).
Harvey, W. R., Stahr, H. M., Smith, W. C., *Tob. Sci*, **13**, 13 (1969).
Henick, A. S., Benca, M. F., Mitchell, J. H., *J. Am. Oil Chem. Soc.* **31**, 88 (1954).
Johnson, W. H., *Proc. Int. Tob. Sci. Congr., 4th*, 293 (1966).
Johnson, W. H., *Proc. Int. Tob. Sci. Congr., 5th*, 142 (1970).
Johnson, W. H., *Tob. Int.* **177**(11), 12 (1975).
Kawashima, N., Fukushima, H., Tamaki, E., *Phytochemistry* **6**, 339 (1967a).
Kawashima, N., Imai, A., Tamaki, E., *Plant Cell Physiol.* **8**, 595 (1967b).
Kimland, B., Aasen, A. J., Enzell, C. R., *Acta Chem. Scand.* **26**, 1281 (1972).
Leffingwell, J. C., *Recent Adv. Tob. Sci.* **2**, 1 (1976).
Lovett, W. J., May, L. H., *Aust. J. Sci.* **29**, 237 (1958).
Sastry, A. S., *Proc. Indian Acad. Sci., Sec. B* **44**, 148 (1956).
Sastry, A. S., *Proc. Indian Acad. Sci., Sec. B* **51**, 219 (1960).
Tomita, H., *Morioka Jpn. Tob. Exp. Sta. Bull.* **3**, 158 (1968a).
Tomita, H., *Morioka Jpn. Tob. Exp. Sta. Bull.* **3**, 199 (1968b).
Weybrew, J. A., Stephens, R. L., *Tob. Sci.* **6**, 53 (1962).
Weybrew, J. A., Woltz, W. G., Johnson, W. H., *Proc. Int. Tob. Sci. Congr., 4th*, 766 (1966).

Yamada, S., *Hatano Jpn. Tob. Exp. Sta. Bull*, **60**, 140 (1967).
Yoshida, D., *Plant Cell Physiol.* 1, 209 (1961).

Received for review September 4, 1979. Accepted December 26, 1979. Paper no. 6110 of the North Carolina Agricultural Research Service, Raleigh, NC. Part of a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree. Mention of a trade name does not imply an endorsement of that product by the North Carolina Agricultural Research Service to the exclusion of others which may be suitable for the intended purpose.

Multielement Absorption by Crops Grown on Soils Amended with Municipal Sludge Ashes

A. Keith Furr, Thomas F. Parkinson, Carl A. Bache, Walter H. Gutenmann, Irene S. Pakkala, and Donald J. Lisk*

Beans, cabbage, carrots, millet, onions, potatoes, and tomatoes were grown in pots containing soils amended 5% by weight with municipal sludge ashes from Indianapolis, Indiana, or Kalamazoo, Michigan. Forty-two elements were determined in the edible plant tissues and growth media by neutron activation, furnace atomic absorption, anodic stripping voltammetry, and other methods. Boron, molybdenum, and selenium increased most consistently in the crops as a result of the sludge ash amendments. The plants did not exhibit symptoms of phytotoxicity.

About 5 million metric tons of sewage sludge is produced annually in the United States presently (Cahill, 1976). It is presently disposed by ocean dumping, discarding in landfills, incineration, and to a minor extent as a soil amendment on lawns, ornamentals, forests, and agricultural land. Municipal sludge may contain elevated concentrations of Cd, Ni, Zn, Cu, Cr, and other elements resulting largely from industrial wastes. Much research is therefore underway to determine the extent of absorption of toxic elements by edible crops (Furr et al., 1976a) and this subject has been reviewed by Cahill (1976) and Page (1974).

When municipal sewage sludge is disposed of by incineration, the remaining ash may constitute from 30 to 60% of the original sludge burned on a dry weight basis (Furr et al., 1976b). This residue therefore still comprises a sizable disposal problem. Investigations of the possible use of municipal sludge ash as a soil fertilizer amendment have not been published. Since many nutrient and toxic elements might expectedly remain in sludge ash, the present study was conducted to learn the possible extent of absorption of a range of such elements by a variety of crops grown in the greenhouse in potted soils amended with sludge ash.

EXPERIMENTAL PROCEDURES

Sludge ashes from the cities of Indianapolis, Indiana, and Kalamazoo, Michigan, were used. Table I lists data pertaining to the sources, treatment, and handling of these sludges during the production of ash. The Indianapolis and Kalamazoo ashes had fertilizer equivalents, respectively, of 0.1–11.9–1.5% and 0.06–8.7–5.7% $N-P_2O_5-K_2O$. The ashes were air-dried, pulverized in a hammermill through a 3-mm screen, and mixed by tumbling.

The soils used were a Darien gravelly silt loam (fine-loamy, mixed, mesic aeric ochraqualfs) and a Teel silt loam (coarse-silty, mixed, mesic fluvaquentic entrochrepts). Table II gives data concerning the ashes and soils used to prepare the potting mixtures. The soils, sampled near Ithaca, New York, were air-dried, sifted through a 2-mm screen, and mixed by quartering. The ash was mixed with the respective soil (Table II) using a cement mixer. The rate of ash addition was equivalent to 111.2 metric tons/h (50 tons/acre).

The crops used were "Long Tendergreen" bush bean (*Phaseolus vulgaris*), "Golden Acre" cabbage (*Brassica oleracea* var. *capitata*), "Scarlet Nantes" carrot (*Daucus carota* var. *sativa*), Japanese millet (*Echinochloa crusgalli* var. *frumentacea*), "1620 Pedro" onion (*Allium cepa*), "Katahdin" potato (*Solanum tuberosum*), and "New Yorker" tomato (*Lycopersicon esculentum*). All of the crops were grown in 7.6-L plastic pots except potatoes which were grown in 11.4-L pots. The weights of growth media contained in the 7.6- and 11.4-L pots were, respectively, 6 and 10 kg for Indianapolis-Darien ash–soil mixture and 8 and 13 kg for Kalamazoo-Teel mixture. Equal weights of the particular soil alone was used for growth of the control crops. The number of plants grown in each pot were: bean, 3; cabbage, 1; carrot, 10; millet, 5; onion, 10; potato, 1; and tomato, 1. All treatments were replicated four times. All plants were fertilized weekly with 1000 mL (1500 mL for the 11.4-L pots) of a solution containing reagent grade KH_2PO_4 (0.001 M) and KNO_3 (0.005 M) (Hoagland and Arnon, 1950). All plants were watered daily, care being taken to avoid splashing soil on the aerial portions of the plants.

At maturity the crops were harvested. Only the edible portions were collected for analysis. Prior to analysis all crop portions were rinsed with distilled water to remove adhering dust. Carrots, onions, and potatoes were brushed, rinsed, and peeled. The respective, replicated, edible plant portions were combined and subdivided by homogenizing

Office of Occupational Health and Safety (A.K.F) and Nuclear Reactor Laboratory (T.F.P.), Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, and the Pesticide Residue Laboratory (C.A.B., W.H.G., I.S.P., D.J.L.), Department of Food Science, New York State College of Agriculture and Life Sciences, Cornell University, Ithaca, New York 14853.

0021-8561/80/1428-0660$01.00/0 © 1980 American Chemical Society