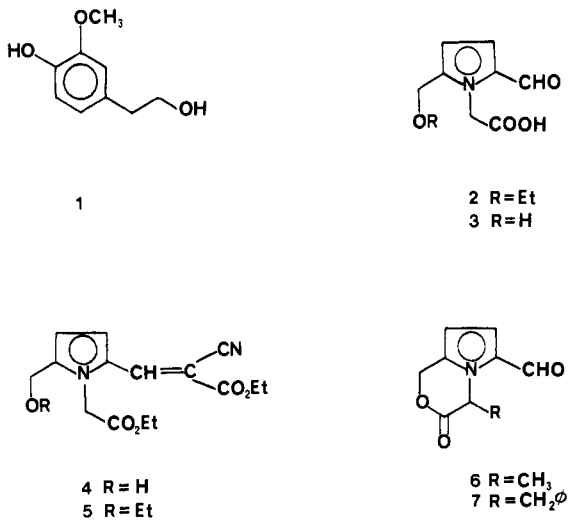

Figure 1

Identifications were based on NMR, IR, and UV spectral data and were confirmed by comparison with authentic samples and, additionally, by coinjection of these on HPLC. Of these compounds, (4-hydroxy-3-methoxyphenyl)-2-ethanol (1) and 2-formyl-5-(ethoxymethyl)-pyrrole-1-acetic acid (2) are new to tobacco. The phenol 1 has been found previously in tobacco smoke condensate (Hecht et al., 1981). The pyrrole derivative 2 is an addition to the growing number of compounds originating from the Maillard reaction of sugar and amino acids which have been found in processed foods. That this is indeed the origin of 2 seems certain as the corresponding alcohol 3 has been found in model studies of glucose–glycine reactions (Kato et al., 1977). Two related pyrrole lactones, presumably derived from glucose–alanine 6 and phenylalanine 7 reactions, have been reported in tobacco (Lloyd et al., 1976). The aroma of lactones of this type has been postulated as being of significance for the flavor of browning systems (Shigematsu et al., 1971).

The purified pyrrole acid 2 has negligible aroma; however, it does on pyrolysis produce sweet caramelic notes. The lactone derivative of 2 which we synthesized by the method of Olsson et al. has, in contrast to the related lactones isolated by Shigematsu et al. (1971), no detectable aroma. On pyrolysis it produces a weak savory/caramelic odor reminiscent of cooked food. Both 1 and 2 effected marginal improvements in the overall smoking quality of a low-tar filtered cigarette.

ACKNOWLEDGMENT

We thank J. Kemp and J. I. Stirling of Gallaher Ltd., Belfast, for help in the larger scale extraction and initial fractionation of tobacco and F. G. Riddell for ^{1}H NMR spectra.

Registry No. 1, 2380-78-1; 2, 84499-92-3; 4, 70789-16-1; 5, 84499-93-4.

LITERATURE CITED

Anderson, R. C.; Gunn, D. M. *Chem. Abstr.* **1977**, *86*, 168145.
Anderson, R. C.; Gunn, D. M. British Patent 1 508 617, 1978.
Hecht, S. S.; Carmella, S.; Mori, H.; Hoffman, D. *JNCI, J. Natl. Cancer Inst.* **1981**, *66*, 163–169.
Kato, H.; Sonobe, H.; Fujimaki, M. *Agric. Biol. Chem.* **1977**, *41* (4), 711–712.
Lloyd, R. A.; Muller, C. W.; Roberts, D. L.; Giles, J. A.; Dickerson, J. P.; Nelson, N. H.; Rix, C. E.; Dyers, P. H. *Tob. Sci.* **1976**, *20*, 43–51.
Olsson, K.; Pernemalm, P.; Theander, O. *Acta Chem. Scand., Ser. B* **1978**, *B32*, 249–256.
Shigematsu, H.; Kurata, T.; Kato, H.; Fujimaki, M. *Agric. Biol. Chem.* **1971**, *35*, 2097–2105.
Stedman, R. L. *Chem. Rev.* **1968**, *68* (2), 153–207.

Robert C. Anderson
Alan G. Kelly*
James S. Roberts

Collaborative Research Unit on Plant Derived Flavours
Department of Chemistry
University of Stirling
Stirling FK9 4LA, Scotland

Received for review April 26, 1982. Revised manuscript received September 28, 1982. Accepted November 29, 1982.

Application of Carbon-13 Nuclear Magnetic Resonance to the Germination of Soybean Seeds in Vivo

The application of carbon-13 nuclear magnetic resonance to studies of intact seeds has provided a technique for direct observation and determination of chemical constituents in living matter. An extension of this technique to the behavior of soybean seeds in the presence of water allows the determination of certain chemical changes that occur during the process of germination. Observations correspond to those obtained by multistep isolation and characterization procedures, raising the possibility of registering variations in sugar and oil concentration on the same sample in a continuous fashion.

Application of carbon-13 NMR (^{13}C NMR) to the study of biological systems has shown that this powerful technique permits the direct observation and determination of chemical constituents in living matter. Recent examples involving plant cells include intact seeds (Shoolery, 1973; Schaefer and Stejskal, 1974, 1975; Schaefer et al., 1975; Kainosho, 1976; Rutar et al., 1977; Albornoz and Leon, 1980) as well as other plant tissue (Kainosho and Konishi, 1976; Kainosho and Ajisaka, 1978).

The evolution of a biological process should thus be amenable to ^{13}C NMR analysis. Indeed, it was recently shown (Chen et al., 1979) that after 2 days the spectrum

0021-8561/83/1431-0459$01.50/0

of excised endosperm of wild oat platelets indicates the presence of new absorptions which result from the hydrolysis of reserve carbohydrate. To what extent the vital process itself could be followed in vivo by using routine ^{13}C NMR techniques is now under investigation. This paper reports our work on germinating soybean seeds and points out some of its possibilities and limitations.

MATERIALS AND METHODS

Analysis of Germinating Seeds. The soybean [*Glycine max* (L), Merr. Paraná] seeds are carefully placed in 10-mm NMR tubes and allowed to germinate at approximately 30 °C in the presence of light and water. At regular intervals, enough deuterated water to obtain a lock signal is added to the sample and the spectrum registered. After each analysis the water is removed and the NMR tube is placed back in a greenhouse. Germination is followed until the confined environment interferes too strongly with plant development (approximately 13 days).

^{13}C NMR Spectra. Spectra were run on a Varian CFT-20 spectrometer operating in the Fourier transform mode, with deuterated water as an internal lock. Operating parameters were chosen to optimize sensitivity for the seed spectra (Shoolery, 1973), which resulted in a choice of 0.2-s acquisition time, no pulse delay, and a pulse width of 15 μs (90°). All spectra are completely hydrogen decoupled.

Chemical Analysis. A sample of seeds for each interval was previously sterilized with alcohol (70%) followed by a 0.5% sodium hypochloride solution and planted in plastic boxes in the presence of light and a humidity-saturated atmosphere. After each interval, the root portion was removed at the point of emergence and seeds were dried at 65 °C until a difference of less that 0.02 g was observed for two successive weighings. Total oil was determined by extraction and weighing and total sugars by the phenol/sulfuric acid method (Dubois et al., 1956), using a 6:1:3 mixture of sucrose, raffinose, and stachyose as a standard.

RESULTS AND DISCUSSION

The first spectrum (Figure 1A) was run before the seed was allowed to absorb any water and reveals signals that are due to triglycerides, the only fluid substances present in an observable amount. Peaks corresponding to the esters of oleic and linoleic acid can be readily identified (Leal et al., 1981).

After about 2.5 h of contact with water, several new absorptions appear in the region between 60 and 105 ppm (Figure 1B). These were previously observed (Shoolery, 1973), being ascribed to the increased molecular freedom of the carbon atoms in the carbohydrate content of the seed as it absorbs deuterated water and swells. We have verified that they correspond exactly to the chemical shifts of an aqueous solution of sucrose, raffinose, and stachyose, the soluble sugars in soybeans (Hsu et al., 1973). Most of the signals are of reasonable intensity and overlap, while the low-intensity signals at 67.1 and 99.0 ppm that are due to rafinose and stachyose are difficult to sort out from background noise. Sucrose is the main component, as also shown by chemical methods.

Spectra were registered regularly during the 13-day period (Figure 1). These can be used to determine relative quantities of substances present in the seed by comparing the signal-to-noise ratios for the following peaks and components: oil peaks a–f at 130.0, 128.3, 34.1, 29.6, 27.5, and 25.5 ppm, respectively, and oligosaccharides, peaks g–l, at 92.6, 81.9, 77.1, 74.6, 73.0, and 71.6 ppm, respectively (Figure 1). It must be noted that, under the conditions in which the NMR spectra were registered, no absorptions due to solids should appear. Thus sugar concentration

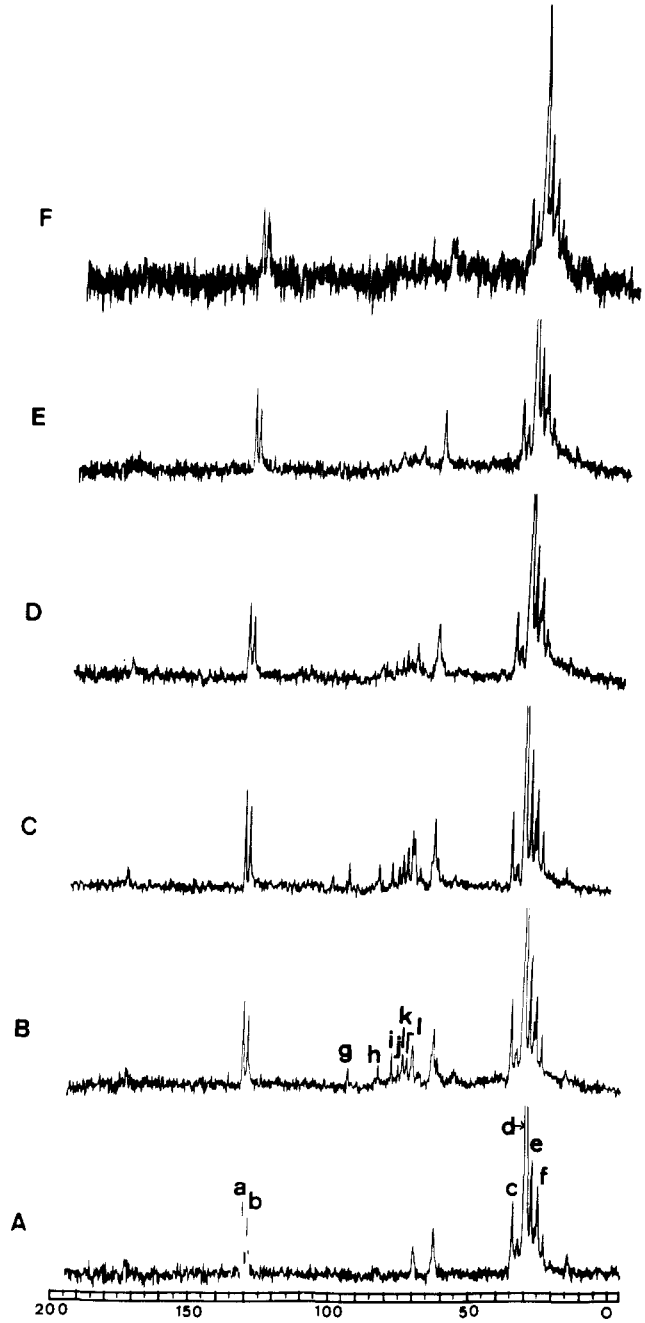

Figure 1. Carbon-13 NMR spectra at various stages of germination. (A) Before contact with water. (B) After 2.5 h, (C) 12 h, (D) 120 h, (E) 192 h, and (F) 312 h. The scale is in ppm downfield from Me_4Si.

remains the same up to 12 h, the corresponding peaks being due solely to the fraction that is dissolved in water. Results are plotted against time in Figure 2. Points below 12 h were not included since they reflect the rate at which sugars dissolve and not their participation in the processes under investigation.

These curves reflect the same trends as the ones obtained by following the relative compositions by classical chemical analysis (Figure 3) and can be rationalized in the following way. On coming into contact with water, the oligosaccharides begin to dissolve. They are thus ready for use in the initial germination metabolism, revealing that the approximately 10% moisture normally found in seeds does not suffice for solution of free sugars except for a small amount used for latent metabolism.

The oligosaccharides must constitute the sole initial energy reserve for rapid processes since the oil is only

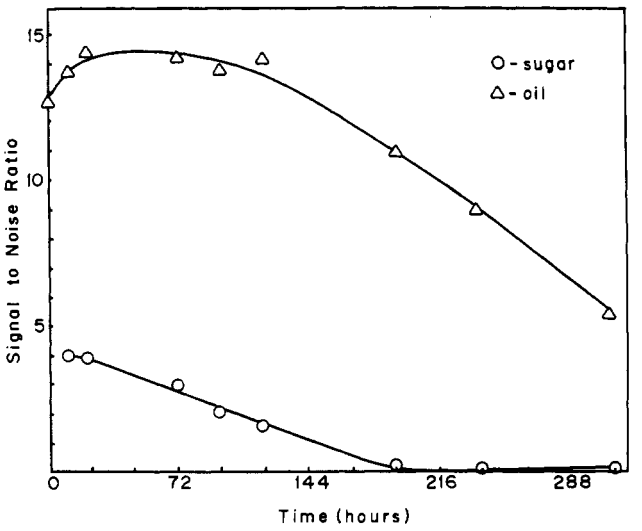

Figure 2. Relative intensities of oil and sugar peaks obtained from signal-to-noise ratios.

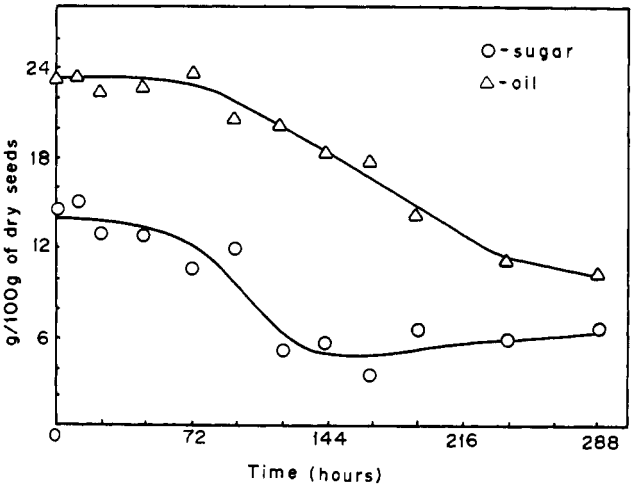

Figure 3. Relative change in oil and sugar proportions during the various stages of germination. Values are given in g/100 g of dry seeds.

consumed after the sugars reach a certain concentration (at approximately 72 h). This suggests the presence of an autofeedback mechanism where the enzymatic system used in degradation of the oil is activated when the sugars reach a low concentration.

Application of the ^{13}C NMR technique to this type of study has inherent limitations in the semiquantitative nature of the technique itself and in the difficulty presented by attempting to make a seed germinate in an NMR tube, where it is compressed and periodically soaked in deuterated water. On the other hand it is both fast and clean, allowing variables to be measured simultaneously and in a continuous fashion on the same specimen. Although results agree qualitatively with those obtained through the more lengthy multistep purification and characterization procedure, there appear to minor discrepancies. These, along with the adaptation of the technique to monitoring a wider range of variables, are presently under investigation.

LITERATURE CITED

Albornoz, F.; Leon, V. *Acta Cient. Venez.* **1980**, *31*, 20.
Chen, S.; Elofson, R. M.; Mactaggart, J. M. *J. Agric. Food Chem.* **1979**, *27*, 435.
Dubois, M.; Gilles, K. A.; Hamilton, J.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350.
Hsu, S. H.; Hadley, H. H.; Hymowitz, T. *Crop. Sci.* **1973**, *13*, 407.
Kainosho, M. *Tetrahedron Lett.* **1976**, 4279.
Kainosho, M.; Ajisaka, K. *Tetrahedron Lett.* **1978**, 1563.
Kainosho, M.; Konishi, H. *Tetrahedron Lett.* **1976**, 4757.
Leal, K. Z.; Costa, V. E. U.; Seidl, P. R.; Campos, M. P. A.; Colnago, L. A. *Cienc. Cult. (Sao Paulo).* **1981**, *33*, 1475.
Rutar, V.; Burgar, M.; Blinc, R.; Ehrenberg, L. *J. Magn. Reson.* **1977**, *27*, 83.
Schaefer, J.; Stejskal, E. O. *J. Am. Oil Chem. Soc.* **1974**, *51*, 210.
Schaefer, J.; Stejskal, E. O. *J. Am. Oil Chem. Soc.* **1975**, *52*, 366.
Schaefer, J.; Stejskal, E. O.; Beard, C. F. *Plant Physiol.* **1975**, *55*, 1048.
Shoolery, J. N. *Varian Appl. Notes.* **1973**, 73-3.

Luiz A. Colnago
Peter R. Seidl*

Instituto Militar de Engenharia
Praça Gen Tiburcio No. 80—Praia Vermelha
22.290—Rio de Janeiro, Brasil

Received for review August 2, 1982. Accepted December 1, 1982. This work was reported at the Second Chemical Congress of the North American Continent, Las Vegas, NV, Aug 1980, AGFD 48. Financial support was provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Determination of Dithiocarbamate Fungicides in Vegetable Foodstuffs by High-Performance Liquid Chromatography

A high-performance liquid chromatographic (HPLC) method for analyzing residues of thiram and salts of *N,N'*-alkylenebis(dithiocarbamic acids) and *N,N*-dimethyldithiocarbamic acid in vegetable foodstuffs is presented. Iron, zinc, and manganese salts of dithiocarbamic acids were transformed into readily water-soluble sodium salts in an alkaline solution of EDTA and L-cysteine. The dithiocarbamate anions were extracted into an organic solvent as ion pairs of tetrabutylammonium and S-alkylated with methyl iodide in one process at room temperature. The methyl esters formed were analyzed by HPLC with UV detection at 272 nm. The average recoveries of zineb, ziram, and thiram added as talc mixtures to six different food crops at the 0.5 mg/kg level were within the ranges 58.7–70.0, 69.4–84.8, and 61.5–78.2%, respectively. The limits of detection are below 0.02, 0.01, and 0.01 mg/kg, respectively.

A new method for high-performance liquid chromatographic (HPLC) determination of dithiocarbamates was described earlier by Gustafsson and Thompson (1981). In that method the sample was treated with an alkaline EDTA solution in order to transform iron, zinc, and manganese salts of dithiocarbamic acids into their readily

0021-8561/83/1431-0461$01.50/0