4a. Even after 200 days, ca. 50% of the added radioactivity could be extracted, and unchanged 4a still accounted for most of the ^{14}C. We observed little or no pendimethalin as a degradation product. This constrasted with our earlier observations of nitrosoatrazine where denitrosation to atrazine was always a major degradation pathway (Kearney et al., 1977).

A portion of the 4a-treated (100 ppb) soil in which soybeans were grown was also extracted after 4 months, and 58% of the ^{14}C was recovered. As was the case in the biometer flask experiments, unchanged 4a seemed to account for the majority of the ^{14}C as judged by TLC and autoradiography.

In addition to the experiments just described, a considerable number of additional $^{14}CO_2$ evolution experiments haave been conducted with NDPA-^{14}C, and the results, except when influenced slightly by temperature, soil type, etc., have been essentially identical with those described. Some observations from some of the additional experiments are: exposure of biometer flasks to light did not influence the rate of $^{14}CO_2$ production from either 1a or 4a [most incubations were performed in the dark because of the known photolability of nitrosamines (Chow, 1973)]. Addition of 1 ppm trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) to the soil had no effect on the rate of $^{14}CO_2$ production from NDPA-^{14}C. The rate of $^{14}CO_2$ production did not vary with NDPA-^{14}C concentration over the range of 5 ppb to 10 ppm.

In summary, nitrosamines are degraded to CO_2 in nonsterile, but not in sterile soils. The low-molecular-weight nitrosodialkylamines seems to have half-lives of about 3 weeks, and under these experimental conditions, significant losses of nitrosamines can result from volatilization during the first few days. The *N*-nitroso derivative of pendimethalin is more stable in soil and appears to be able to persist at least several months.

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Total Carbonyls and Phenols in Experimental Burley and Bright Tobacco

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Soluble carbonyls and phenols were determined in several experimental tobacco materials that were produced by practices that altered concentrations of these health- and quality-related components. An improved spectrophotometric method was used for measuring total carbonyls as quinoidal anions of their 2,4-dinitrophenylhydrazones. Phenolic estimations were based on the extent of hydrogen bonding of phenols to insoluble polyvinylpyrrolidone. Carbonyl and phenolic levels decreased in bright tobacco that was grown at higher-than-normal plant population density or reconstituted into sheets after flue curing. Carbonyl concentrations reduced in air-cured burley leaves that were harvested from successively higher leaf positions on the stalk, whereas phenolics increased with ascending stalk position. Phenolics were reduced in tobacco that was treated for removal of soluble protein.

Cured tobacco contains many compounds with carbonyl and phenolic functional groups that contribute to the organoleptic and biological properties of the leaf and smoke. Phenolic compounds are considered important to leaf quality and usability (Tso, 1969). Volatile and semivolatile carbonyl-containing compounds influence the flavor and aroma of tobacco smoke (Weybrew and Stevens, 1962; Demole and Berthet, 1972; Kimland et al., 1972; Demole and Demole, 1975; Demole and Enggist, 1975; Davis et al., 1976; Dickerson et al., 1976; Lloyd et al., 1976). Health-related effects in the respiratory systems of mammals have also been attributed to specific carbonyl compounds in smoke (Kensler and Battista, 1963; Fenner and Braven, 1968; Schoental and Gibbard, 1972; Sabine et al., 1973; Sprince et al., 1975).

Recent statistical evidence based on correlations of the chemical composition of experimental cigarettes and the biological activity of their derived smoke suggested that high levels of soluble phenols in leaf are undesirable [DHEW Publication No. (NIH) 76-1111, 1976; DHEW

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Publication No. (NIH) 77-1280, 1977; USDA Technical Bulletin No. 1551, 1977]. Carbonyl levels in the unburned cigarettes, however, were not investigated in this regard. In this paper we report the effects of different curing and production practices upon total carbonyls and total phenols in several experimental tobacco samples. A modified method for determining total carbonyls in tobacco is described based on the spectrophotometric determination of total carbonyls (as 2,4-dinitrophenylhydrazones).

EXPERIMENTAL SECTION

Growth and Processing of Tobacco Samples. Three burley tobacco (*Nicotiana tabacum* L.) cultivars and one breeding line were selected based on their wide range in total alkaloid content and satisfactory growth characteristics. The entries were Burley 21, Ky 14, Ky 12 and a low-alkaloid breeding line, LA Burley 21; mean alkaloid contents determined in leaves of these entries were 4.18, 3.71, 3.66, and 0.87%, respectively. Plants were grown in a randomized block design with four replications in 1976 at the Kentucky Agricultural Experiment Station Farm at Lexington, Kentucky. Normal cultural and air-curing practices for burley tobacco were followed except that the plants were hand suckered. After curing, the leaves were grouped for analyses in such a manner that they represented leaves from eight stalk positions. Position 1 represented the lowest group of leaves and position 8, the uppermost leaves. All leaf midribs were removed and the leaf lamina from each position of each plant were ground to 40/60 mesh. Samples for chemical analyses were prepared by compositing the ground leaf lamina of the same stalk position from all four replications.

In a separate study, bright tobacco (*N. tabacum* L. cultivar Speight G-28) plants were grown with normal plant spacing (14 800 plants/ha) or close spacing (111 000 plants/ha, under normal cultural practices at the Oxford Tobacco Research Station, Oxford, NC, in 1975. Leaves of normally spaced plants were primed and flue-cured by conventional means. Close-spaced plants were harvested and cured by either of two methods: (1) a corn cutter chopped the whole plants (including stalk and leaves) into 5–8-cm pieces, and the pieces were flue-cured in a modular wire container in a manner similar to that used in bulk curing (Johnson et al., 1960), and (2) the whole plants (including stalks and leaves) were homogenized and cured by the homogenized leaf curing (HLC) process for bright tobacco (DeJong et al., 1975; Tso et al., 1975). The cured tobacco materials were reconstituted into sheets by either a paper process (P. J. Schweitzer Division Laboratory, Lee, MA), or a slurry process (American Machine and Foundry, Inc., Richmond, VA). Radioelements (^{210}Pb, ^{210}Po, and ^{226}Ra) and soluble proteins were removed during the paper process.

Method for Total Carbonyls. The spectrophotometric assay for total carbonyls as 2,4-dinitrophenylhydrazones developed for tobacco samples (Andersen et al., 1977) was used, except that thiourea was added to the reaction mixtures. This modification prevented oxidative decomposition of certain carbonyl compounds likely to be present (such as dehydroascorbic acid) and avoided discoloration of the 2,4-dinitrophenylhydrazine solution by any oxidation products that might form (Olliver, 1967).

To show that thiourea did not interfere with the color development, the absorbance maxima and molar extinction coefficients at 480 nm for alkaline 2,4-dinitrophenylhydrazones were obtained for the following carbonyl compounds in the presence and in the absence of 3.3 mM thiourea: *n*-heptaldehyde, methyl ethyl ketone, acetone, acetophenone, furfural, *p*-coumaraldehyde, β-ionone, *p*-acetylsinapaldehyde, syringaldehyde, vanillin, cinnamaldehyde, coniferaldehyde. All compounds had a single maximum in the visible wavelength range except acetone and methyl ethyl ketone. The extinction coefficients were measured at 480 nm because this wavelength was near the mean of the absorbance maxima and corresponded to the mean of the absorbance maxima determined for these compounds when thiourea was absent. The mean of the coefficients in the presence of thiourea was $2.68 \times 10^{-4} \pm 0.93$ standard deviation for the derivatives, excluding those for *n*-heptaldehyde, methyl ethyl ketone, and acetone; the latter were not included because *n*-heptaldehyde yielded little colored derivative and methyl ethyl ketone and acetone produced two maxima. This compared to a mean value of $2.70 \times 10^{-4} \pm 0.90$ for coefficients obtained with no thiourea.

Table I. Volumes of Reactants Prior to Addition of Pyridine and KOH in Carbonyl Assay

	reagent, mL			
flask content	thiourea in methanol sol.	methanol	2,4-DPH sol.	HCl sol.
sample A	8.0		1.0	1.0
blank A	8.0		1.0	1.0
sample color background B	8.0	1.0		1.0
blank B	8.0	1.0		1.0

The details of the procedure for total carbonyls in tobacco (without the present modifications) were given by Andersen et al. (1977). An abbreviated description of the modified method follows: A 0.25-g sample (cured or freeze-dried) that was moisture equilibrated was extracted with 25 mL of thiourea in methanol solution (0.8 g of thiourea in 1 L of carbonyl-free methanol) in a stoppered flask with gentle shaking for 30 min. The contents of the flask was filtered. One aliquot (A) of the filtered extract (from 1 to 5 mL) was pipetted into a flask for color development with 2,4-dinitrophenylhydrazine. A second aliquot (B) of the same volume was pipetted into another flask for the preparation of a sample color background solution containing all color development reagents except 2,4-dinitrophenylhydrazine solution. Two reagent blanks were prepared, one for the sample (A) and one for the sample color background (B). Thiourea in methanol solution, carbonyl-free methanol, 2,4-dinitrophenylhydrazine (2,4-DPH) solution (0.1 g of recrystallized 2,4-DPH in 200 mL of carbonyl-free methanol) and HCl solution (1 mL of 10% concentrated HCl in carbonyl-free methanol) were added to obtain the 10-mL volume of reactants (see Table I); the amount of thiourea solution added to obtain 8 mL depended on the volumes of sample or blank already added. The flasks were maintained at 60 °C for exactly 15 min. After cooling, 10 mL of pyridine solution 80% (v/v) was added to each of the flask contents (see Table I). Next, 5 mL 33% KOH (in carbonyl-free methanol) was added to each flask. The absorbancies of the solutions at 480 nm were read between 2.5 and 4 h after color development. The sample A was read vs. the blank A, and the sample color background B was read vs. the blank B (see Table I). The absorbancy of the solution containing B was subtracted from the absorbancy of the solution containing A to yield the net absorbancy caused by carbonyl-containing compounds. The quantity of total carbonyl-containing compounds equivalent to acetophenone in a sample was determined by calculations and reference to a calibration curve prepared from aceto-

Table II. Total Carbonyls and Phenols in Leaves of Burley Cultivars Varying in Stalk Position

cultivar	ascending leaf position on stalk during growth							
	1	2	3	4	5	6	7	8
	Total Carbonyls,[a,b] mg/g dry wt							
Burley 21	2.58 e	2.29 cd	2.40 de	2.05 bc	2.00 b	2.07 bc	1.83 b	1.17 a
low-nicotine Burley 21	1.97 cd	2.06 d	2.06 d	1.76 bc	1.68 b	1.51 ab	1.39 a	1.37 a
Ky 12	1.88 ab	2.01 b	1.95 ab	1.81 ab	1.81 ab	1.69 a	1.72 a	1.72 a
Ky 14	2.96 d	2.66 c	2.36 b	2.60 bc	2.03 a	1.81 a	2.04 a	1.84 a
	Total Phenols,[c] mg/g dry wt							
Burley 21	5.0 a	7.1 a	5.1 a	21.9 d	19.4 cd	15.6 b	17.2 bc	15.0 b
low-nicotine Burley 21	9.1 a	11.2 ab	14.3 cd	13.0 bc	14.3 cd	16.1 de	17.2 de	18.7 d
Ky 12	24.2 b	18.9 a	17.8 a	23.7 b	24.0 b	24.7 b	25.4 b	25.2 b
Ky 14	13.3 bc	15.0 cd	15.2 cd	20.1 e	17.1 d	15.8 cd	10.0 a	11.2 ab

[a] Mean values in each horizontal row not followed by same letter are significantly different at the 0.05 level or probability. [b] Expressed as acetophenone equivalent; LSD for total carbonyls among all Tables II-III entries is 0.28 mg/g at $P < 0.05$ determined from triplicate laboratory analyses. [c] Expressed as chlorogenic acid equivalent; LSD for total phenols among all Tables II and III entries is 2.9 mg/g at $P < 0.05$ determined from triplicate laboratory analyses.

Table III. Total Carbonyls and Phenols in Bright Tobaccos Varying in Culture and Postharvest Treatment

growth and processing variables	total carbonyls,[a,b] mg/g dry wt	total phenols,[a,c] mg/g dry wt
normal spacing during field growth		
conventional flue-cured leaf	3.09 g	45.6 h
paper reconstituted sheet of flue-cured tobacco		
leaf	1.83 d	30.5 g
"deproteinized" leaf	2.39 ef	23.7 de
"radioelement-free" and "deproteinized" leaf	1.44 bc	21.8 cd
leaf and stalk (2:1 ratio)	2.14 e	29.8 g
slurry reconstituted sheet of flue-cured tobacco		
leaf	2.58 f	24.8 e
leaf and stalk (2:1 ratio)	1.59 cd	26.3 ef
close spacing during field growth		
paper reconstituted sheet of modular-cured tobacco		
leaf and stalk	1.38 bc	14.8 ab
"radioelement-free" and "deproteinized" leaf and stalk	1.38 bc	12.8 a
paper reconstituted sheet of homogenized-cured tobacco		
leaf and stalk	1.16 ab	29.2 fg
"radioelement-free" and "deproteinized" leaf and stalk	1.03 a	20.2 c
slurry reconstituted sheet of modular-cured tobacco		
leaf and stalk	1.32 bc	15.8 b
slurry reconstituted sheet of homogenized-cured tobacco		
leaf and stalk	1.24 ab	25.2 e

[a] Mean values in each vertical column not followed by same letter are significantly different at the 0.05 level of probability. [b,c] See Table II for description of corresponding footnotes.

phenone standards (0–0.25 μL/25 mL). Details of the calculations were given earlier (Andersen et al., 1977). The mean carbonyl content of a sample in a given subgroup was compared with that for other samples in the subgroup by means of the Student's t test.

Estimation of Total Phenols. A spectrophotometric method was used that separated phenols extracted with aqueous methanol by their hydrogen bonding to polyvinylpyrrolidone at pH 3.5. The amount of phenolpolyvinylpyrrolidone insoluble complex was determined by measuring the difference in phosphomolybdate–tungstate reduction in the solutions at 725 nm before and after addition of polyvinylpyrrolidone (Andersen and Todd, 1968). The mean phenol content of a sample was compared with that for other similar samples as in the case of carbonyl contents.

Chemicals. The aldehydes, ketones, reagents, and solvents were redistilled or recrystallized. Acetophenone and furfural were obtained from the J. T. Baker Chemical Co., Pillipsburg, NJ; acetone and thiourea from Fisher, Pittsburg, PA; cinnamaldehyde, 2,4-dinitrophenylhydrazine, β-ionone, syringaldehyde, and vanillin from Aldrich, Milwaukee, WI; methanol from Mallinckrodt, St. Louis, MO, and pyridine from Burdick and Jackson, Muskegon, MI. Coniferaldehyde, p-coumaraldehyde, sinapaldehyde, and p-acetylsinapaldehyde were synthesized by the reduction of their acetylated acid chlorides with lithium tri-*tert*-butoxyaluminohydride (Brown and McFarlin, 1956; Pearl and Darling, 1957).

RESULTS AND DISCUSSION

Carbonyls in Experimental Tobacco Materials. The results of the determinations of total carbonyls and phenols in 45 burley and bright tobacco samples are summarized in Tables II–III. There was a mean carbonyl content of 1.90 mg/g dry weight (acetophenone equivalent) which was approximately one-tenth of the mean level of phenols, i.e., 18.7 mg/g dry weight (chlorogenic acid equivalent). The range of total carbonyls among the samples was 1.03–3.09 mg/g. Conventionally flue-cured bright tobacco (Table III) had the highest amount of total carbonyls (3.09 mg/g) compared with the lower amounts in conventionally air-cured burley (1.97 mg/g when averaged over tobacco variety and leaf position on the stalk; see Table II). Levels of carbonyls among the reconstituted bright tobacco samples ranged from 1.03 to 2.58 mg/g; these levels were lower than the 3.09 mg/g concentration of carbonyls found for the conventional flue-cured bright leaf sample. In the air-cured samples, degradation and volatilization of carbonyl compounds probably occurred because analogous

changes during the air curing of tobacco were reported for dry matter, CO_2, NH_3, H_2O, and carbohydrates (Tso, 1969), soluble leaf proteins (Palmer, 1963), chlorophyll and carotenoids (Jeffrey and Griffith, 1947), and chlorogenic acid and rutin (Andersen et al., 1969). Andersen et al. (1977) reported that total carbonyls in the lamina and midveins of Ky 14 burley tobacco were 30–60% higher on date of harvest than after either conventional air curing or an experimental bulk curing process. Carbonyl amounts may have also been affected by genetic factors in a manner similar to that of other chemical components such as alkaloids (Legg et al., 1971; Chaplin, 1975) and phenolic compounds (Sheen et al., 1973).

Burley 21, low-nicotine Burley 21, and Ky 14 cultivars had significant, but generally nonlinear, decreases of total carbonyls with ascending leaf positions on the stalk (Table II). However, carbonyls in Ky 12 showed much less variation with leaf position. Total carbonyls averaged over all stalk positions in each variety of the burley tobacco samples ranged from 1.72 mg/g in low nicotine Burley 21 to 2.29 mg/g in Ky 14. There were significant differences between either the Ky 14 or Burley 21 varieties and the low-nicotine Burley 21 or Ky 12 samples.

The relationships of carbonyl levels to growth, curing, and processing parameters were difficult to determine in the bright tobaccos because of multiple variables (Table III). Nevertheless, some generalizations can be made. Samples of conventionally flue-cured bright tobacco that were reconstituted by either the paper or slurry process contained lower levels of carbonyls than the nonreconstituted flue-cured sample. These losses were increased at some stage of the process that removed ^{210}Pb, ^{210}Po, and ^{226}Ra. Removal of soluble protein, per se, did not seem to affect levels of carbonyls. Combined effects of close spacing and either modular or homogenized curing caused lower levels of total carbonyls than combined effects of conventional spacing and flue curing in the reconstituted samples.

Recent investigations suggested that chemical constituents containing aldehyde and ketone functional groups contribute much of the flavor and aroma of burley tobacco and smoke. These carbonyl compounds were grouped in "families" of biochemically related compounds, including terpenoids, isoprenoids, and solanone derivatives from higher terpenes (Kimland et al., 1972; Demole and Berthet, 1972; Demole and Enggist, 1975; Davis et al., 1976); 3-substituted pyridines related to nicotine (Demole and Berthet, 1972; Demole and Demole, 1975); and low boiling compounds up to about six carbon atoms (Weybrew and Stephens, 1962). Although a correlation might be expected between levels of carbonyl-containing compounds and the intensity of tobacco aroma, there are studies in tobacco (Dickerson et al., 1976; Lloyd et al., 1976) and other plant products (Lorenz and Maga, 1972) suggesting that this kind of relationship is not entirely consistent.

The present investigation will furnish carbonyl leaf data for future evaluations of the biological activity of derived smoke. The concept that phenylpropanoid-derived chemicals related to lignin may be of some importance to health-related effects in the human respiratory tract is based on epidemiological evidence (Clifford and Beecher, 1964; UICC Monograph, 1967; Acheson et al., 1972; Brinton et al., 1977).

Phenols in Experimental Tobacco Materials. The range of total phenols among the samples was 5.0–45.6 mg/g dry weight (Tables II–III). The conventionally flue-cured bright tobacco had the highest amount of total phenols (45.6 mg/g) compared with the lower amounts in conventionally air-cured burley (16.3 mg/g when averaged over tobacco variety and leaf position on the stalk). Reduced levels of phenols were present in the reconstituted bright tobacco samples (12.8–30.5 mg/g). In addition to air curing effects that promote losses (Andersen et al., 1969), variations in total phenols in the present samples are caused by cultural and genetic variables. The effects of nitrogen fertilization and genetic factors on the phenolic content of tobacco leaf has been reported (Sheen et al., 1973).

Total phenols in Burley 21, low-nicotine Burley 21, and Burley Ky 14 cultivars varied with different leaf positions on the stalk (Table II). There was a general trend of increased levels of phenols from the lowest leaves to either middle leaf positions, as in Burley 21 and Ky 14 or to the top leaf position in low-nicotine Burley 21. Our results regarding the effect of eight stalk positions on the concentration of phenolic compounds in leaf are in general agreement with those reported by Sheen et al. (1973) for three stalk positions. These variations were also similar to those previously reported for bright tobaccos (USDA Technical Bulletin No. 1551, 1977).

Mean values of total phenols averaged over stalk positions in each variety of the burley tobacco samples ranged from 13.3 mg/g in Burley 21 to 23.0 mg/g in Ky 12. Based on these values, there was a significant difference between Ky 12 and each of the other varieties, but no differences among Burley 21, low-nicotine Burley 21 and Ky 14. Sheen et al. (1977) determined total polyphenols as sums of chlorogenic acid isomers and rutin (determined individually) in leaves of three burley cultivars. They reported a total polyphenol concentration range of 4.1 to 5.7 mg/g, and these levels were at least 50% lower than the total phenol levels that we found in burley leaves. The differences among these results are probably caused by the differences in methodology. Tannins and phenolic phenylpropanoids as well as chlorogenic acid isomers and rutin contribute to the measurement of total phenols by the Folin-PVP method that we used.

The conventionally flue-cured bright tobacco that was reconstituted by either the paper or slurry process contained lower levels of phenols than the nonreconstituted flue-cured sample. These losses of phenols associated with reconstitution occurred along with losses of carbonyl compounds. The removal of soluble protein associated with the paper reconstitution increased the losses of phenols. In paper and slurry reconstituted samples, combined effects of close spacing and modular curing caused lower total phenol levels than those in a sample produced with conventional spacing and flue curing. In close-spaced paper or slurry reconstituted bright tobacco samples, homogenized leaf cured entries contained higher levels of total phenols than comparable modular cured samples.

Total soluble phenolic compounds in unburned cigarette tobaccos correlated positively with biological activity of resultant condensates on mouse skin in long-term tests with low-dose condensate levels [DHEW Publication No. (NIH) 76-1111, 1976; DHEW Publication No. (NIH) 77-1280, 1977]. An understanding of genetic, growth, and processing parameters that alter total phenolics in tobacco might be useful for the production of less hazardous cigarettes.

About 50 individual phenolic compounds, including phenolic aldehydes and other compounds with multifunctional groups, have been identified and quantitated in tobacco smoke (Stedman, 1968; Ishiguro et al., 1976). Phenol, cresol isomers, 2,4-xylenol, catechol, and pyrogallol

were biologically active phenolic promotor compounds (Boutwell and Bosch, 1959) or cocarcinogens (Van Duuren et al., 1973) that were identified in the weak acid fraction of some cigarette smoke condensates (Rayburn et al., 1953; Commins and Lindsey, 1956; Boch et al., 1971; Swain et al., 1973; Ishiguro et al., 1976). It is generally believed that leaf phenols including polyphenols, tannins, and lignin serve as precursors for many of the phenols in the smoke of cigarettes (Zane and Wender, 1963; Clark, 1968). Only a few leaf phenols, however, have been found to transfer directly to smoke without chemical change (Dieterman et al., 1959; Zane and Wender, 1963). Based on pyrolysis of ^{14}C-labeled glucose, it was estimated that 40% off the total phenol of smoke was contributed by the carbohydrates of the leaf (Bell et al., 1966). The complexity of the composition of the weak acid fraction is becoming more apparent as evidenced by the phenols recently identified in smoke, including 4-vinylcatechol (Leach et al., 1969), acetovanillone, 4-vinylphenol, *O*-methoxy-*p*-vinylphenol, *O*-acetyl-*p*-cresol, 5-isopropenyl-2-methylanisole (Demole and Berthet, 1972), pyrogallol, catechol, and coniferyl alcohol (Ishiguro et al., 1976). Catechol and pyrogallol have shown significant oncogenic activity in biological assay systems (Van Duuren et al., 1973; Andersen and Linney, 1977).

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