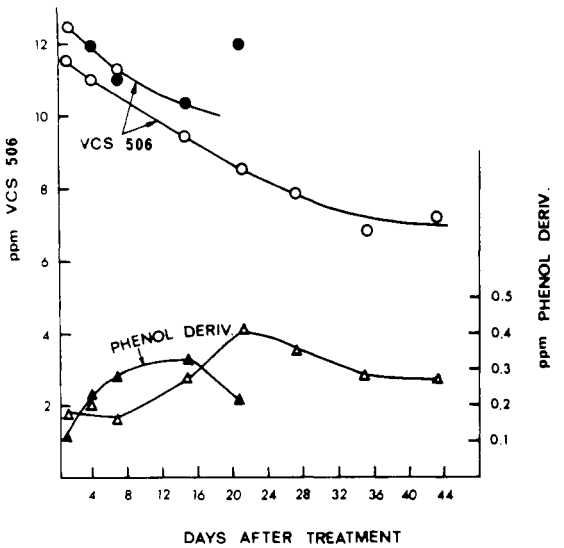

Figure 3. Residues of Velsicol VCS-506 and its phenol derivative in grapes: (●,▲) variety Muscat (black); (○,△) variety Dabuki (white).

During the same period of time the amount of the phenol derivative in the covered fruit was 0.08–0.09 ppm whereas in the uncovered fruit it was 0.35–0.37 ppm. This suggests the possible translocation of this derivative from leaves to fruit.

Tomato fruits and grapes were dipped in tap water for 24 hr employing mild agitation; it was found that the amount of the insecticide on the fruit was the same before and after the treatment.

Residues in Grapes. Bunches of grapes (two varieties) 2 weeks before ripening were dipped in the insecticide emulsion, and at selected time intervals samples were taken for analysis. At the time of treatment the fruits had reached their final size and therefore the effect of growth on the residue level during the experiment was eliminated. The results of this experiment are presented in Figure 3. The degradation rate in both varieties was very slow. Only 20% of the insecticide disappeared during the first 2 weeks after treatment. On the 44th day after application, 60% of the amount applied was still present in the white (Dabouki) variety.

In grapes there was a gradual increase in the amount of the phenol derivative in both varieties during the first 2–3 weeks, with a moderate decrease later on. These results were similar to those obtained for tomato fruits and leaves.

Analysis by the Tlc Enzyme Inhibition Technique. Tomato extracts from this and other experiments and various fractions obtained from column chromatography were subjected to the tlc enzyme inhibition test, in a search for additional cholinesterase inhibitor breakdown products (A. Ben-Aziz and N. Aharonson, unpublished results).

The parent compound and two of its metabolites were separated on silica gel G and analyzed by the enzymic inhibition test (Mendoza *et al.*, 1968), using either 5-bromoindoxyl acetate or 1-naphthyl acetate as the substrate. In this experiment, the presence of only the parent compound and its O-analog in the tomato plants was confirmed, and the residue level was similar to that obtained by chemical analysis.

In the plant the organophosphorus insecticide Velsicol VCS-506 was found very stable, a phenomenon which would suggest a relatively long biological lasting effect correlated with improved insecticidal performance. Field trials, however (P. M. Vermès, personal communication), gave disappointing results against larvae of *Spodoptera littoralis* when compared to the efficacy of other organophosphorus insecticides with comparable stomach and contact toxicity and which were found less persistent in the plant.

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COMMUNICATIONS

Effect of Flue Curing on the Amylose/Amylopectin Ratio of Tobacco Starch

Methods are evaluated for isolating starch and fractionating amylose and amylopectin in tobacco. Flue curing did not alter the amylose/amylopectin ratio, which was found to be approximately 30%/70% before and after curing, but did decrease the solubility of amylose from 23% before curing to 5% after curing. Amylose was retrograded during the flue curing process either because of high temperature and dehydration, or enzymatic activity. The data suggest that the amylases responsible for conversion of starch to reducing sugars during the flue-curing process do not alter the amylose/amylopectin ratio but only the total starch content.

The amylose/amylopectin ratio of tobacco starch has not been well documented although this ratio has been established for many other plant species including potato, corn, wheat, rice, banana, tapioca, sago and lily bulbs (Bates *et al.*, 1943), peas (McCready *et al.*, 1950), apples (Carter and Neubert, 1954), and sugar cane (Cashen and Friloux, 1966). This lack of information for tobacco starch is regrettable because tobacco leaf starch is subjected to drastic conditions during the flue-curing process, when it is reduced by enzymatic activity from amounts as high as 30% (Bacon *et al.*, 1952) to as low as 1% in 4–5 days.

There are conflicting reports on diurnal changes in the

amylose/amylopectin ratio of tobacco starch. One report showed that early matured green tobacco leaves varied diurnally from 32%/68% amylose/amylopectin in the morning to 22%/78% in the afternoon (Kakie and Sugisaki, 1970), while another (Mizuno *et al.*, 1960) reported that the amylose content of matured green tobacco leaf starch increased in the daytime and decreased at night. These differences may be attributed to the different ages of the leaves, since more diurnal starch variations occur in immature than in fully expanded mature leaves (Matheson and Wheatley, 1963).

Subsequently, Kakie and Sugisaki (1971) reported that the ratio varied with maturity from 32%/68% to 20%/80% amylose/amylopectin, as the amylose content decreased with increasing time after topping. Earlier Matheson and Wheatley (1962) elicited a different response as they reported the iodine affinity of isolated granules increased with age, but they used starch extracted from untopped plants.

Since amylose and amylopectin are affected differently by enzymes (White *et al.*, 1964), a study was conducted to determine changes in the amylose/amylopectin ratio of tobacco starch before and after flue curing. The amount of reducing sugars, a major constituent of tobacco chemical quality, is a direct function of the amount of starch hydrolyzed during the flue-curing process.

MATERIALS AND METHODS

Flue-cured tobacco *Nicotiana tabacum* L. cv. Hicks Broadleaf was grown on a Klej loamy fine sand soil at the Tobacco Research Farm, Coastal Plain Experiment Station, Tifton, Ga. Cultural practices were in accordance with those recommended as desirable for the production of flue-cured tobacco. Samples were selected from four random areas of a quarter-acre block. Each area represented a replication of this study.

Mature leaves were harvested as they ripened and four harvests were required to strip the stalk. Leaves from each replicate were divided into two lots. The first lot was dried in a microwave oven to immediately stop enzymatic activity. Two leaves were microwaved at a time for three 1-min intervals as described by Stephenson *et al.* (1971). The second lot was flue cured in the conventional manner. Samples were composited over stalk positions. After midribs were removed, the dried laminae were ground in a Wiley mill to pass a 2-mm screen.

The starch content was determined by the AOAC starch method (1970) with modifications developed by Gaines (1972). Reducing sugars were determined by the method of Harvey *et al.* (1969).

Methods of Isolating Starch. Several methods of isolating starch were evaluated in order to select the method that had the least retrograding effect on amylose. These methods included the procedure by Pucher *et al.* (1948), later adopted by the AOAC as the official starch method (Hoffpauir, 1956), a modification of this method (Sensabaugh and Rush, 1972), a starch isolation method by Johnston (1956), and starch precipitation by ethanol (Gaines and Meudt, 1968).

The starch isolation method selected for this study was the Gaines and Meudt method (1968) which involved homogenizing 5 g of ground tobacco tissue in 100 ml of water, boiling the homogenate for 30 min, centrifuging, and decanting the centrifugate. The residue was washed in water, reboiled, and centrifuged again. This was repeated three to four times. The combined centrifugates were filtered through fine muslin and heated to boiling and 95% ethanol was slowly added to precipitate the starch. The extracted starch was washed twice in methanol, twice in ether, and vacuum dried at 100°.

Starch isolated by the method of Pucher *et al.* (1948) when fractionated gave results very similar to those reported in Table II, but this procedure employs periods of long standings which tend to have a more retrograding effect on amylose than the isolation procedure described above.

The modified AOAC preparation of starch reported by Sensabaugh and Rush (1972) was inappropriate for this study because the perchloric acid solubilized starch when filtered through Celite deposits a layer of gelatinous amylopectin above the Celite. This never passes through the filter, and consequently upsets the amylose/amylopectin ratio of the precipitated starch.

To determine if gelatinizing starch in boiling water increased the concentration of degraded amylose, the mild extraction process of Johnston was tried as it employs only cold ammonium oxalate extraction. Tissue was ground in cold 1% ammonium oxalate and filtered through muslin and the filtrate centrifuged. Impurities were removed by suspending starch in water, centrifuging, and scraping the top of the surface to remove any observable heterogeneous material. This isolation method is the least specific of those tested and yielded the highest amount of insoluble amylose. Impurities remaining after washing may have been attributed to the high degree of insoluble amylose, yet we conclude that boiling did not influence the amount of insoluble amylose.

Regardless of the starch isolation method employed, insoluble amylose was always higher on cured leaf than on uncured leaf and in approximately the same relation as shown in Table II.

Methods of Fractionating Starch. Several methods of fractionating the isolated tobacco starch were tried including the aqueous leaching method of McCready and Hassid (1943), the thymol amylose precipitation method of Haworth *et al.* (1946), and the *n*-amyl alcohol amylose precipitation method of Schoch (1945). Schoch's fractionating method was selected for its simplicity, accuracy, and degree of correlation between duplicated samples.

The weight of the dried starch powder was recorded; then the starch was transferred to a 250-ml erlenmeyer flask and water was added to make a 1% suspension. Starch was then gelatinized on a steam bath and autoclaved for 3 hr at 20 lb pressure, 127°. The insoluble residue present after autoclaving was removed by centrifugation, washed twice in methanol and then twice in ether, and vacuum dried. This insoluble fraction was weighed after drying and was found to be degraded amylose. The supernatant liquid was heated to near boiling and a 10% volume of *n*-amyl alcohol was added to the hot sol and amylose was fractionated from amylopectin in the manner described by Schoch (1945). After shaking for 48 hr the precipitated amylose was separated by centrifugation and washed twice in methanol and then twice in ether, vacuum dried, and weighed as soluble amylose. While the amylopectin fraction can be retrieved from the centrifugate by flocculation with excess methanol, in this study the amylopectin fraction was determined by subtracting the sum of the degraded and soluble amylose from the starch weight.

For the method of McCready and Hassid (1943), it was difficult to separate the aqueous leached amylose from amylopectin by centrifugation as some of the gelatinous amylopectin invariably decanted off with the liquid amylose fraction. The thymol amylose precipitation method of Haworth *et al.* (1946) was about as equally efficient in separating amylose from amylopectin for our purposes as the method of Schoch, but the autoclaving step employed by Schoch ensured that as much amylose as possible went into solution.

RESULTS AND DISCUSSION

A high degree of enzymatic activity occurred during the flue-curing process as the starch content averaged 12.1% before curing and decreased to 1.5% after curing while

Table I. Effect of Flue Curing on the Starch and Reducing Sugar Contents of Tobacco

Sample[a]	% starch	% reducing sugars
Before curing	12.1	4.8
After curing	1.5	10.4

[a] Means of four replications.

Table II. Effect of Flue Curing on the Composition of Tobacco Starch

	Relative proportion (%) of starch as			
Sample[a]	Insoluble amylose	Soluble amylose	Total amylose	Total amylopectin (by difference)
Before curing	7	23	30	70
After curing	24	5	29	71

[a] Means of four replications.

there was a concomitant increase in reducing sugars from 4.8% before curing to 10.4% after curing (Table I). These values typify starch conversion during flue curing of tobacco, although it is not uncommon to find the starch content of green mature tobacco to be two- or threefold higher and the reducing sugar content in the 20% range (Gaines, 1971).

Flue curing did not alter the actual amylose/amylopectin ratio (Table II). Tobacco starch averaged 30%/70% amylose/amylopectin before curing and 29%/71% after curing. Flue curing did affect the solubility of amylose as soluble amylose decreased from 23% before curing to 5% after curing. Insoluble amylose changed in the opposite manner (Table II). Amylose was retrograded during the flue-curing process either because of high temperature (32–82°) and loss of moisture or because enzymes hydrolyzed starch to sugars.

The ease with which amylose retrogrades into an insoluble state is well known and it was determined that the insoluble residue remaining after autoclaving the isolated starch was retrograded amylose in the following manner. First, the fractionated amylose had similar properties to this insoluble residue which was very sparingly soluble in hot water. By contrast, fractionated amylopectin was totally soluble in hot water. Second, after stirring the insoluble residue with 5% aqueous KOH for 48 hr, some of the insoluble residue went into solution, and after centrifugation, one drop of iodine–potassium iodide solution turned the supernatant blue. Amylopectin turns a reddish-violet color with the iodine reagent.

Our study has shown that the flue-curing process had a retrograding effect on amylose. While the amylose/amylopectin ratio remained the same, the residual starch was different in character to green leaf starch present before curing. If the amylases are capable of hydrolyzing retrograded amylose (and because of its solubility properties this appears unlikely) an increase in reducing sugar content might be expected, and a different amylose/amylopectin ratio would result in the residual starch. It may be speculated that processed starchy foods may be higher in retrograded amylose than soluble amylose. Consequently, the nutritive value of these products may be reduced.

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