STARCH CHANGES IN DEVELOPING AND SENESCING TOBACCO LEAVES

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Summary

The quantitative changes in starch content during the growth and senescence of tobacco leaves have been followed. The starch content was low while the leaves were expanding but rapidly increased after expansion stopped. The maximum was reached when more than half of the original chlorophyll content had gone. The starch content was then reduced rapidly and when the leaf was all yellow there was only a small quantity of starch and this remained when the leaf turned brown.

The viscosities, iodine affinities, β-amylolysis limits, and granule sizes of isolated starch granules and the chain lengths of amylopectins at different ages of leaves have been determined. The viscosities and iodine affinities increased with increasing age of the leaf and the β-amylolysis limits of the whole starch and chain length of the amylopectins were constant throughout the growth of the leaf. The average granule size increased as the leaf matured until maximum starch content was reached and decreased as the starch content decreased. It is suggested that in tobacco leaves another starch accumulation pattern of longer duration than diurnal accumulation exists. The degradation of the starch resembles the breakdown of starch in sprouting potato tubers and not that in germinating barley where amylases are the active enzymes.

The residue after ethanol extraction of leaves at different stages of growth was fractionated into water-soluble material, a perchloric acid extract precipitated by iodine, and a perchloric acid extract not precipitated by iodine but precipitated by ethanol. Glucan was present in significant concentration only in the perchloric acid extract precipitated by iodine and this had the properties of starch. Paper chromatography indicated that non-starchy extracts were composed of galactose, arabinose, xylose, rhamnose, and uronic acid.

I. INTRODUCTION

Starch is a rapidly formed product of photosynthesis (Gibbs and Cynkin 1958) and in grains and tubers where it occurs most extensively it is considered to be a storage material, providing a carbon and energy source at germination. The starch deposition in wheat grains, and presumably other storage organs, is not extensively reversible as shown by radiochemical evidence (McConnell, Mitra, and Perlin 1958). In leaves it is more readily metabolized (Porter 1953; Porter, Martin, and Bird 1959; Tsin-Tze Chan and Bird 1960), showing a diurnal rhythm and acting as an energy reserve for the living plant (Fischer 1958; Wanner 1958). Thus in leaves, starch is transient and if any changes in the structure of starch accompany its metabolism then these could be more readily detected in leaf starch than in the starch of storage organs. Any changes in structure detected could indicate the *in vivo* mode of synthesis and breakdown of starch. In some tobacco

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leaves large amounts of starch are present and during autolysis of excised mature leaves starch undergoes the greatest weight change of any leaf constituent except water, presumably serving as an energy source (Askew *et al.* 1954).

The change in starch content in grains during synthesis follows a sigmoid curve. Barley (Harris and MacWilliam 1957), corn (Wolf *et al.* 1948), wheat (Bice, MacMasters, and Hilbert 1945; Wood 1960), and pea seeds (Turner and Turner 1957) all show this pattern.

Observations on leaves are less frequent and complete than for grains. McCarty (1935) found in a number of perennial grasses that starch in herbage and roots was in low concentration in the early part of the growing season, with higher values later, and with a maximum occurring during the declining phase of growth. Starch concentration increased following the turning point in growth rate of the vegetative shoots. The increase in starch content was up to 8%. Bailey (1958) found that in clover the starch content of leaves collected over a 28-day growth period showed no significant variation. Several estimates of starch content of tobacco leaves have been made (Garner 1951; Askew *et al.* 1954) on samples collected at commercial ripeness or at unidentified age (Spoehr and Milner 1939) and these have indicated that starch percentage of dry weight is about 30.

The isopotential iodine absorptions of wheat starch (Bice, MacMasters, and Hilbert 1945; Wood 1960), corn starch (Wolf *et al.* 1948; Maywald, Christensen, and Schoch 1955; Erlander 1960), and barley starch (Harris and MacWilliam 1957, 1958) were found to increase with age of grain. In wheat and corn starches the intrinsic viscosities also increased with age.

The starch from malted barley has been compared with the original grain starch (Aspinall, Hirst, and McArthur 1955; Greenwood and Thomson 1959) and as malting is a degradative process the depletion of starch in tobacco leaves could resemble it. During the germination of barley grains the starch content of the kernel drops from 64 to 58% dry weight, the apparent amylose content calculated from iodine affinities rises from 22 to 26%, and the degree of polymerization of the amylose fraction is reduced slightly with a rise in the β-amylolysis limit. The average chain length of the amylopectin fraction is reduced from the original value of 26 glucose units to 18 glucose units as shown by methylation analysis, β-amylolysis, and periodate oxidation. These results are consistent with a limited β-amylolysis of the amylopectin (reducing the chain length) and a very limited α-amylolysis of the amylose in the original starch.

In potato tubers Halsall *et al.* (1948) have shown that there is no significant variation in the iodine absorption and chain length of the amylopectin from two varieties of potatoes over a 2-month growing period (cf. Stepanenko and Afanasyeva 1957). Banks and Greenwood (1959) compared the starches of the potato tuber before and after shoot development and found they had identical iodine affinities, viscosities, and β-amylolysis limits and the amylopectins had the same chain lengths as measured by periodate oxidation.

Radwan and Stocking (1957) isolated starch from medium-aged leaves of 1-month-old sunflower plants and found the granules to be smaller than those from

storage organs and comparable in size to potato leaf starch. The iodine absorption of the starch was low (2·5 g/100 g) and gave a calculated amylose content of 14%. The β-amylolysis limit of the sunflower leaf starch was lower than potato tuber starch and this reduced value was found to be due to the amylose fraction. These authors also found the iodine absorption of tobacco leaf starch from plants of unspecified age to be 3·4 g/100 g.

II. Materials and Methods

(a) *Culture Conditions and Sampling of Glasshouse Plants for Following Quantitative Changes in Starch Content*

The variety of tobacco plant used was a fixed line from hybridization of *Nicotiana tabacum* and *N. debneyi* and which is known as Clayton Hybrid with the low disease index. The plants were grown in well-washed vermiculite in 22-in. diameter pots using the nutrient regime of Lovett (1959). Eight plants were selected from 24 for uniformity and leaf 17, counting up from the cotyledons, was marked. The plants were grown in a glasshouse in the tropical winter. At each sampling, three leaf disks, 1·6 cm in diameter, two from one side of the midrib at the tip and base of the leaf, and one from the middle of the other side of the midrib, were cut. Sampling was always carried out at 11 a.m. The physiological age was estimated during expansion from the size of the leaf, recorded as the multiple of length and breadth at the widest point, and during senescence by the chlorophyll concentration per unit area of leaf. The three leaf disks were immediately macerated in ethanol and allowed to stand in the dark for half an hour, made up to 10 ml with ethanol, centrifuged, and the light absorption of the supernatant solution measured at 665 mμ in a 1 cm cell to estimate the chlorophyll concentration. The solution was washed back into the original tube and evaporated in a vacuum desiccator for dry weight measurements. The starch content was then determined by the method of Pucher, Leavenworth, and Vickery (1948), i.e. by extraction with perchloric acid, precipitation as the starch–iodine complex, hydrolysis with dilute acid, and estimation of the glucose produced by the Nelson (1944) colorimetric modification of the Somogyi method.

(b) *Culture Conditions and Sampling of Field-grown Plants for Following Quantitative Changes in Starch Content*

Nicotiana tabacum cv. Hicks plants were grown in the tropical spring in the field to which 500 lb of 2 : 16 : 8 NPK fertilizer was applied per acre. Ten plants were sampled at random each week from a plot of 288 plants. Leaf 17 was picked at 11 a.m., the midrib discarded, and the lamina weighed and macerated in ethanol. The macerate was extracted with ethanol and the residue and extract dry weights found by evaporation of the solvent under diminished pressure at room temperature. The starch content was found by subsampling 100 mg and estimating starch by the method of Pucher, Leavenworth, and Vickery (1948). The results were calculated as percentage starch of total dry weight.

(*c*) *Culture Conditions and Sampling of Plants for Large-scale Isolation of Starch Granules*

Nicotiana tabacum cv. Hicks plants were grown in the tropical spring in the field to which 800 lb of 3 : 12 : 12 NPK fertilizer was applied per acre. When the plants were about 1·5–2 ft high, leaves 15–18 were marked on 40 plants chosen for uniformity of size and appearance. Each week, leaves 15–18 on two plants were picked at 11 a.m. and, after separation of the lamina from the midribs and weighing, were macerated in toluene and dilute aqueous mercuric chloride solution. The samples consisted of four leaves from each plant except in the first and last weeks when four leaves from two plants were combined to provide sufficient starch. The starch grains were purified by repeatedly shaking with toluene and sodium chloride solution and allowing the starch grains to settle. Protein and mechanical impurities separated at the interface (Porter and Martin 1952). The granules were washed successively with hot methanol, acetone, and ether and dried. The last three samples were of four leaves from leaf position 17 collected from four separate plants and chosen for their closeness to the description given in Table 2. They represent three stages in leaf senescence.

(*d*) *Culture Conditions and Sampling of Plants for Separation of Glucan Fractions*

Nicotiana tabacum cv. Hicks plants were grown during the tropical spring in the field to which 500 lb of 2 : 14 : 8 NPK fertilizer mixture was applied per acre. Leaves 15–18 were numbered. The plants were sampled at 11 a.m. The four leaves from each plant were picked, and three disks were cut from each leaf. The disks were combined, weighed, macerated in ethanol, the solvent evaporated, and the residue dried to constant weight at room temperature in a vacuum desiccator to estimate the dry weight of the whole lamina. The midribs were discarded and the lamina weighed, macerated in ethanol, and extracted for 48 hr with hot ethanol. The residue was stirred with cold water for 30 min, centrifuged, and the supernatant evaporated to dryness under diminished pressure. This residue was extracted with warm water, centrifuged, and the supernatant freeze-dried to give the water-soluble fraction. The residue after the original cold water extraction was boiled with water for 15 min, cooled, and an equal volume of 60% perchloric acid added slowly with vigorous stirring. The temperature of the mixture was kept below 10°C in an ice–salt-bath. After 30 min the mixture was centrifuged and the residue extracted again with cold 30% perchloric acid for 30 min and once more with water. Iodine (3%) in potassium iodide solution (3%) was added to the combined extracts, followed by sodium chloride (15 g/100 ml.). The starch–iodide precipitate was centrifuged and washed twice with 0·25M ethanolic sodium chloride, dissolved in 0·25M sodium hydroxide, centrifuged to remove insoluble material, and this residue was washed once with alkali. The solution was de-ionized by electrodialysis in a "Perspex" tank containing "Permaplex" C–10 and A–10 resin-coated membranes. The temperature was kept below 30°C by cooling. The product was isolated by freeze-drying.

(*e*) *Estimation of Glucan Content of Starch Samples*

The method of Pirt and Whelan (1951), modified as described by Matheson and Wheatley (1962), was used for these estimations.

(f) Estimation of Glucan Contents of Fractions Soluble in Water and of Perchloric Acid Extracts not Precipitated by Iodine

Approximately 100 mg of material was hydrolysed for 5 hr with 5 ml of 1N sulphuric acid. The solution was neutralized with barium carbonate and the filtrate and washings made up to 10 ml. The glucose content was estimated after paper chromatographic separation of the sugars (solvents n-butanol–pyridine–water–benzene, 5 : 3 : 3 : 1 v/v) and development of the chromatogram with *p*-anisidine hydrochloride and colorimetric comparison with standards run on the same chromatogram. The procedure of Pridham (1956) was followed for these estimations. The presence of other sugars in these fractions was determined on the same chromatograms.

(g) Estimation of Limiting Viscosity Number, Isopotential Iodine Absorption of Starch, and of Starch Conversion to Maltose by β-Amylase

The methods used in these estimations have been described by Matheson and Wheatley (1962).

(h) Estimation of Apparent Chain Length from Formic Acid Produced on Periodate Oxidation

A method similar to that of Anderson, Greenwood, and Hirst (1955) was followed. Starch (*c.* 150 mg) was suspended in 0·56M potassium chloride (30 ml) and 0·2M sodium metaperiodate (10 ml) solutions. The flask was shaken in the dark at 20 ±0·5°C and samples (10 ml) withdrawn by pipette at 168, 192, and 216 hr. Ethylene glycol (1 ml) was added to the sample and after shaking in the dark for 10 min nitrogen gas, free of CO_2, was bubbled for 10 min. The solution was titrated potentiometrically under nitrogen against 0·01N sodium hydroxide solution using a Cambridge pH-meter. The amount of alkali used at pH 6·25 was determined graphically. The formic acid liberated was plotted against time and the chain length calculated from the amount of formic acid produced at 200 hr.

(i) Measurement of Granule Size

Starch samples were stained with 0·2% iodine in 2·0% potassium iodide solution and photographed at 72 diameters. The negatives were enlarged onto a screen until 10 cm represented 40 μ in true size (using a stage micrometer). The sizes were measured to the nearest millimetre, i.e. 0·4 μ in actual size. The measurement was always made in one direction to overcome effects due to the non-spherical shape of the granules. A large final magnification was necessary due to the very small size of the leaf starch granules. Curves were constructed from smoothed histograms in which the average number of granules in 0·4-μ classes were plotted. A total of between 450 and 500 diameters were measured from six fields of each sample.

III. Results

(a) Quantitative Changes in Starch Content during Growth

(i) *Starch Changes with Age of Clayton Hybrid in the Glasshouse.*—Figures 1 and 2 show the change in starch content of leaf 17 of the variety Clayton Hybrid. The changes in leaf size and chlorophyll content are plotted on the same time scale.

This time scale was constructed by calculating the average time of growth of leaf 17 of the eight plants from when the multiple of the length and breadth was

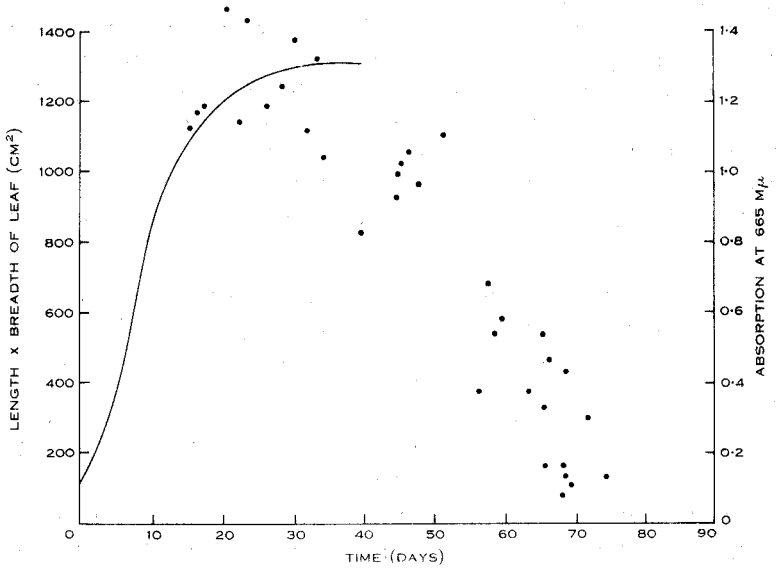

Fig. 1.—Growth curve (continuous line) and changes in chlorophyll content (●) of tobacco leaves. Absorption of a 10-ml ethanolic extract of 6·1 cm² of leaf measured.

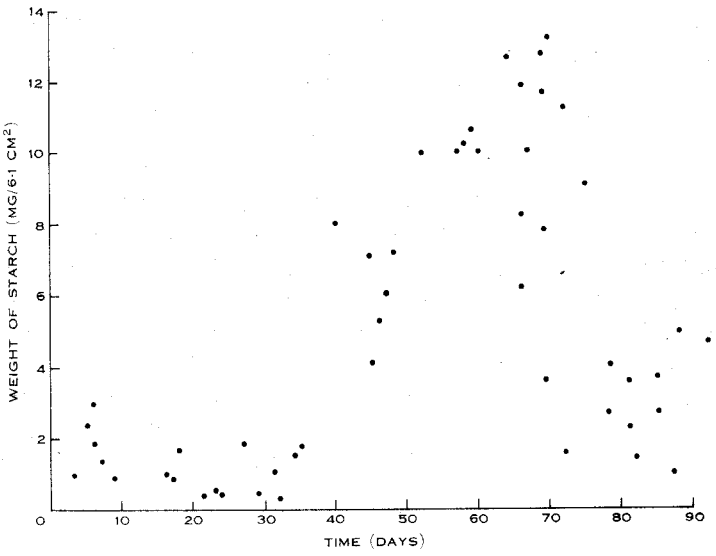

Fig. 2.—Changes in starch content of tobacco leaves during growth.

100 cm² to when the leaf was completely yellow. The average time was 74 days and the range 67–83 days. In Figure 1, the growth curve has been constructed from

average length times breadth measurements of the eight leaves and the solid circles are the absorption at 665 mμ in a 1 cm cell of a 10-ml ethanolic extract of 6·1 cm^2 of leaf. This is a measure of comparative chlorophyll concentrations. In Figure 2, the starch content in milligrams per 6·1 cm^2 of leaf has been plotted for the eight leaves against the average time scale. If the starch values are plotted as percentage dry weight, the pattern is similar, with the percentages varying between 1 and 8% in the young leaf, rising to 30–40% at the maximum, and falling again to 5–10% on yellowing.

TABLE 1

STARCH CHANGES IN TOBACCO LEAVES (CV. HICKS) IN FIELD PLOTS

Week of Growth of Whole Plant	Description of Leaves Sampled	Starch Content (% dry wt.)	Standard Deviation
15	Not fully expanded	3·3	0·53
16	Not fully expanded	4·5	0·21
17	Not fully expanded, six plants flowering	3·4	1·11
18	Fully expanded, all plants flowering	5·0	1·72
19	Five leaves with one-half of chlorophyll gone, five leaves green	12·1	1·17
20	Four leaves with one-half of chlorophyll gone, one green, five yellow to brown	5·1	1·59
21	Eight leaves brown, two leaves yellow	2·3	0·59

(ii) *Starch Changes in* Nicotiana tabacum *cv. Hicks Grown in Field Plots.*—Table 1 shows the starch percentage changes in field-grown *N. tabacum*. The first column describes the week of growth from seed emergence. In week 15, the first week of sampling, the length times breadth measurement of leaf 17 was at least 100 cm^2. The second column describes the condition of the leaves. Starch content for weeks 15, 16, 17, and 18 were not significantly different, but in week 19 it was significantly higher than at weeks 18 and 20. Starch content for week 20 did not differ significantly from that for week 21.

(*b*) *Structural Changes in Starch Granules during Growth*

Table 2 describes some physical and chemical properties of these granules. Excluding week 22, the correlation coefficient between time of sampling and viscosity is 0·918 ($P<0·001$) and between time of sampling and iodine affinity 0·662 ($P<0·01$).

(i) *β-Amylolysis Limits.*—The β-amylolysis limits of the whole starch (Table 2) remained constant within the accuracy of measurement (±2%) throughout the

TABLE 2

PROPERTIES OF STARCH GRANULES FROM TOBACCO LEAVES (CV. HICKS)

n.d., not determined

Week of Growth	Sample No.	Limiting Viscosity Number (g/100 ml)	Isopotential Iodine Absorption* (g/100 g starch)	β-Amylolysis (% conversion to maltose)	Formic Acid Produced on Periodate Oxidation (ml of 1N acid per 0·01 mole of starch)	Apparent Amylopectin Chain Length (glucose units):	
						Assuming an Amylose Content of 20%	Calculated from Apparent Amylose Content
15	15A	1·49	3·64	61	n.d.	n.d.	n.d.
	15B	1·50	4·17	61	0·41	19	19
16	16A	1·56	4·65	62	0·39	21	19
	16B	1·60	4·50	60	n.d.	n.d.	n.d.
17	17A	1·67	4·80	61	n.d.	n.d.	n.d.
	17B	1·62	4·34	62	0·40	20	19
18	18A	1·59	4·05	60	0·38	21	21
	18B	1·88	5·89	58	n.d.	n.d.	n.d.
19	19A	1·77	4·45	56	0·40	20	19
	19B	1·86	5·20	58	0·39	21	19
20	20A	1·95	5·34	58	0·39	21	18
	20B	1·90	5·26	59	0·40	20	18
21	21A	1·88	5·55	59	0·42	19	17
	21B	2·04	4·75	59	0·41	19	18
22	22A	1·76	5·60	55	n.d.	n.d.	n.d.
	22B	n.d.	n.d.	52	n.d.	n.d.	n.d.
	A†	1·95	4·76	57	0·39	21	19
	B†	2.32	4·97	57	0·41	19	18
	C†	1·68	5·38	57	0·38	21	19

* Accuracy of determination ±0·06.

† *A*, leaf contains half its original chlorophyll; *B*, leaf contains one-third to one-eighth of its original chlorophyll; *C*, leaf has lost all of its original chlorophyll (see Fig. 4).

growth of the leaves. Samples 22A and 22B from dead leaves show lower values, but these samples, as isolated, were a yellow-brown colour, and chemical contamination which could not be avoided in these samples would probably reduce the accuracy of measurement. The purpose in isolating these granules was to show that, although in low yield, starch granules were present in dead leaf.

(ii) *Chain Length of Amylopectin.*—The calculation of the chain length of the amylopectin from formic acid production on periodate oxidation has been done in two ways. Firstly a constant amylose content of 20% for the whole starch was

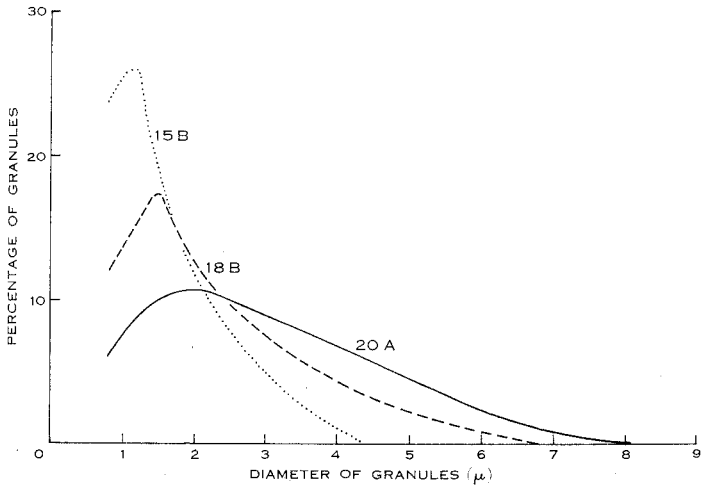

Fig. 3.—Distributions of size of starch granules from tobacco leaves during the 15th (15*B*), 18th (18*B*), and 20th (20*A*) week of growth.

assumed, and secondly the apparent amylose content, based on a constant iodine absorption of 19·2 g/100 g by all amylose samples, was assumed to be the true amylose content. The chain length was measured to an accuracy of ±1 glucose unit.

(iii) *Granule Size.*—In Figure 3 the frequency distributions of diameters of starch granules from rapidly expanding and fully expanded leaves have been plotted. The change in granule size during development is shown. The curve for sample 15B (see Table 2) is very similar in pattern to those obtained for samples 15A, 16A, 16B, 17A, and 17B. The curve for sample 18B is very similar in pattern to those obtained for samples 18A and 19B whilst the curve for sample 20A closely resembles those obtained for samples 19A, 20B, and 21B.

Figure 4 shows the frequency distributions for starch granule diameter in senescing leaf. Curve *A* is from leaf containing half its original chlorophyll and resembles those obtained for samples 19A, 20A, 20B, and 21B. Curve *B* is from leaf containing one-third to one-eighth of its original chlorophyll and resembles that obtained for sample 21A; and curve *C* is from leaf which has lost all of its chlorophyll and resembles those obtained for samples 22A and 22B.

Increase in mean granule size during growth was accompanied by a spread of size range, and the decrease in mean granule size during senescence by a decrease in the size range.

(c) *Glucan Contents of Tobacco Leaf Fractions*

The glucan contents of the various fractions of tobacco leaves isolated by extraction with cold water and with cold perchloric acid are shown in Table 3.

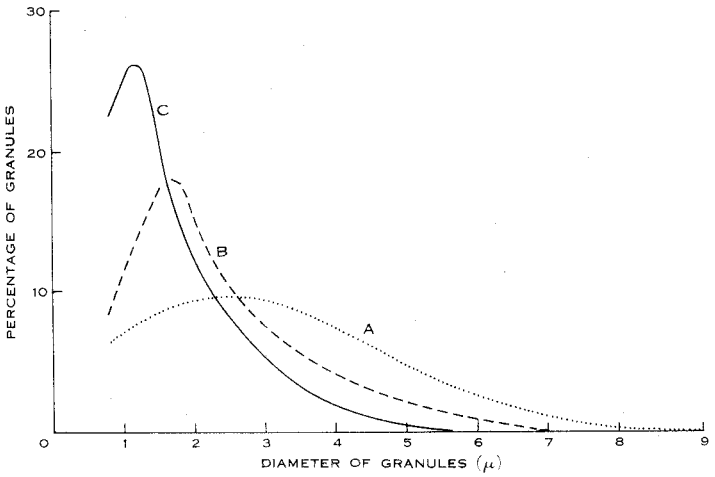

Fig. 4.—Distributions of size of starch granules from tobacco leaves during senescence. *A*, leaf containing half its original chlorophyll; *B*, leaf containing from one-third to one-eighth its original chlorophyll; *C*, yellow leaf (no chlorophyll).

The perchloric acid extract was further fractionated by precipitation firstly with iodine and then with 3 volumes of ethanol prior to glucan determination. The iodine affinities and extent of β-amylolysis of the perchloric acid extracts which were precipitated by iodine are given in Table 4.

IV. Discussion

In storage organs starch accumulates, and this is in contrast to leaves where utilization in the dark is considered to maintain net starch synthesis at a low level. In Clayton Hybrid plants, the starch content is low while the leaf is still expanding. During the expansion phase, leaves grow considerably overnight, so that any excess of photosynthetic products over the requirements of daytime growth and translocation is probably used during the night in growth, thus maintaining starch at a low level. Once leaf expansion stops starch content rises rapidly and continues to rise after chlorophyll breakdown is well established. The starch content quickly drops during the final stages of chlorophyll disappearance and when the leaf is all yellow, most of the starch has been removed. At least part of the residual starch remains when the leaf turns brown. *N. tabacum* cv. Hicks also shows a similar pattern of low

starch content during leaf expansion with increasing content until the later stages of chlorophyll breakdown and a rapid decrease as the leaf turns yellow. There were differences in the maximum starch content but these were probably caused by different nutrient applications or seasonal differences.

During the growth of the eight leaves of Clayton Hybrid, starch contributed an average of 66% of the dry weight per unit increase in area from the time of maximum rate of leaf expansion to the time of maximum dry weight per unit area. This increase in starch content occurred mainly after leaf expansion stopped.

TABLE 3

GLUCAN FRACTIONS EXTRACTED FROM TOBACCO LEAVES (CV. HICKS) BY WATER AND PERCHLORIC ACID

Stage of Growth	Water-soluble Glucan	Perchloric Acid Extract Precipitated	Glucan Extracted by Perchloric Acid but not

CORRIGENDUM

Table 3, column 2: *For* 9·010 *read* 0·010

Yellow	0·018	3·1	0·040

Loss of starch was responsible for much of the weight loss that accompanied yellowing of the leaves. In some leaves the starch loss was greater than the weight loss indicating that more low molecular weight carbohydrate was formed than was utilized by respiration or translocation.

The viscosities and iodine affinities of isolated starch granules increase with increasing age of the leaves up to the time of maximum starch content. This increase in viscosity and iodine affinity with age is similar to the properties of starches in accumulating organs, e.g. cereal grains. This evidence, and the increase in starch content as the leaf ages suggests that, as well as acting as a storage organ on a diurnal basis, the leaves of tobacco act as temporary storage organs during a longer time period—from when leaf expansion stops until the leaf turns yellow.

During the expansion phase of leaf growth, starch granules are very small and of uniform size. As starch accumulates, the maximum granule size gradually increases, and the increase in weight of starch is mainly due to an increase in the numbers of medium-sized and large granules. In young leaves of 3–8% starch content, granules 1·2 μ in diameter (the most frequent class) constitute about 25% of the total

granules. In older leaves when the total starch content is 25–30%, they constitute 8%. The chain lengths of the amylopectin fractions (calculated from formic acid production on periodate oxidation) and the β-amylolysis limits of the whole starches were unchanged during starch build-up.

One possible mode of synthesis of starch granules is via a soluble glucan. Gibbs (1951) found in sunflower leaves a dextrin fraction, soluble in 10% ethanol, that rapidly incorporated $^{14}CO_2$.

From radiochemical evidence Porter, Martin, and Bird (1959) have suggested that in tobacco leaves a starch-like polysaccharide, which does not undergo the same metabolism as starch, is synthesized.

TABLE 4

PROPERTIES OF PERCHLORIC ACID EXTRACTS PRECIPITATED BY IODINE FROM TOBACCO LEAVES (CV. HICKS)

Stage of Growth	Isopotential Iodine Absorption (g/100 g starch)	β-Amylolysis (% conversion to maltose)
Rapidly expanding	4·1	57
Fully expanded, green	4·7	57
Approx. two-thirds of chlorophyll lost	4·1	61
Yellow	4·2	59

A search has been made for these products in tobacco leaves (cv. Hicks). Glucan fractions were obtained from weekly samples of these leaves and small amounts of water-soluble glucan were found to be present (Table 3). These amounts were considered to be insignificant, as traces of oligosaccharide left from ethanol extraction could produce this amount of glucan. A low concentration of a metabolite does not exclude its importance as a synthetic intermediate but, in tobacco leaves, no significant amount of dextrin fraction is present during the synthesis of starch. Paper chromatographic examination indicated that the water-soluble fraction was composed of galactose, arabinose, xylose, rhamnose, and uronic acid.

The glucan fractions soluble in perchloric acid were always completely precipitated as the iodine complex. The quantities of glucan precipitated by ethanol after iodine treatment were insignificant. Paper chromatography of the hydrolysates of the ethanol precipitates, indicated that they were composed of galactose, xylose, arabinose, rhamnose, and uronic acid. The solubility, difficulty of hydrolysis, and sugars produced on acid hydrolysis indicated that the residues after perchloric acid extraction were hemicellulose and cellulose. The carbohydrate content of

the perchloric acid extracts precipitated by iodine was more than 99% glucan. The iodine affinities and β-amylolysis limits of these iodine precipitates (Table 4) are typical of starch. Matheson and Wheatley (1962) have shown that these properties for granules approximate those of a perchloric extract prepared from granules. Thus no evidence could be found for the presence in tobacco leaves of significant amounts of a glucan that was not starch, apart from structural cellulose.

On the breakdown of starch on senescence, the average size of granule drops until, in yellow and dead leaves, only the granule of smaller size is left. It is not possible from the size distributions to distinguish between two types of depletion, the selective removal of larger granules and the steady reduction in size of all granules. From microphotographs of granules of samples of leaves in the process of losing starch (sample 21A, one-third to one-eighth chlorophyll) and of the yellow leaves which have lost most of their starch (samples 22A and 22B), the granules are as regular in shape as during accumulation, indicating that erosion of the grains is a peeling process or that once attack is initiated a granule rapidly disappears, as soluble material.

Two different chemical patterns resulting from the initial *in vivo* enzymic breakdown of starch have been distinguished between sprouting potato tubers and germinating barley (see Section I). The large variation in replicate values obtained for iodine affinities and viscosities of starches from synthesizing leaves and leaves in which starch is being depleted does not allow any conclusion about which pattern depletion of tobacco leaf starch resembles.

The chain lengths of the amylopectin fractions calculated from periodate oxidations, indicate that these are constant within the accuracy of measurement (± 1 glucose unit) and this suggests the mechanism of breakdown in normal senescence is similar to that of the starch in potato tubers on sprouting. The β-amylolysis limits are also unchanged. Paper chromatographic examination of ethanol extracts of senescing leaves indicated no significant amounts of maltose and the water-soluble extracts of leaves during starch depletion (Table 3) showed no production of soluble maltodextrins. In tobacco leaves the absence of maltose or maltodextrins during breakdown would indicate that amylases are not the effective enzymes, unless senescing leaves have an efficient method of translocation or utilization of maltose. As recent evidence suggests that phosphorylase is not an enzyme for starch synthesis (Ewart, Siminovitch, and Briggs 1954; Rongine de Fekete, Leloir, and Cardini 1960), it may be the initial attacking enzyme. A mechanism involving phosphorylase would be applicable, in theory, to tobacco leaf starch as tobacco leaf chloroplasts contain this enzyme (Madison 1956).

V. Acknowledgments

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