DIURNAL–NOCTURNAL CHANGES IN THE STARCH OF TOBACCO LEAVES

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Summary

The quantitative changes in starch in tobacco leaves have been determined over diurnal–nocturnal periods for rapidly expanding, fully expanded, and yellow leaves. The daily increase in starch lessens as the leaves mature and senesce, and the amount of starch not undergoing diurnal variation in fully expanded leaves is more than in rapidly expanding leaves.

The variation in starch content is not accompanied by changes, within the reproducibility of measurement, in the viscosities and β-amylolysis limits of the whole starches or in the chain lengths of the amylopectins. The isopotential iodine absorptions of the starches are higher before dawn than in the late afternoon and the average granule diameter in rapidly expanding leaves rises towards the end of the daylight period.

No evidence could be found at any time of sampling for significant concentrations of any glucan other than starch and cellulose.

I. Introduction

Leaf starch varies quantitatively over a 24-hr period. Several reports indicate that the amount of diurnal change lessens as the leaves mature (Wanner 1958). Mizuno *et al.* (1961) found that in commercially ripe tobacco leaves (when about two-thirds of the chlorophyll has disappeared) there was very little difference between day and night starch content. These authors also found that in similar leaves the day-time starch had a slightly higher starch-iodine blue colour than the night-time starch. As leaf starch is rapidly accumulated and utilized diurnally an examination of the chemical structure of the starch at different times of the day and night could give an indication of the enzymic processes involved, particularly in the breakdown of the starch (Banks and Greenwood 1959; Greenwood and Thomson 1959, 1962).

Matheson and Wheatley (1962*b*) have found that the estimation of leaf starch from tobacco leaves sampled at 11.00 a.m. indicated that as leaf expansion stops the starch content rapidly increases until senescence is well established. The influence of diurnal change on the starch content at 11.00 a.m. is not known, and the accumulation pattern might not be real but due to a much greater diurnal variation in rapidly growing leaves (giving low values for 11.00 a.m. sampling) than in older leaves.

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II. Materials and Methods

(*a*) *Culture Conditions and Sampling of Plants for Quantitative Starch Changes*

Nicotiana tabacum cv. Hicks was grown in the tropical spring in the field to which 800 lb of 3 : 12 : 12 NPK fertilizer was applied per acre. Ninety plants were selected for uniformity of leaf 17 at each sampling. Three leaf disks were cut, one from the top and one from the bottom of one side of the midrib and one from the middle of the other side of the midrib from each plant at 4.30 a.m. (just before dawn), 11.00 a.m., 5.00 p.m., and 10.30 p.m. The disks were weighed, macerated in ethanol, and exhaustively extracted with ethanol in a Soxhlet extractor. The residue remaining after extraction and the residue after removal of the ethanol were dried and weighed. The starch content was found by subsampling 100 mg of the residue after extraction and estimating the starch by the method of Pucher, Leavenworth, and Vickery (1948). The average size of rapidly expanding leaves was 43 cm in length and 23 cm in maximum width, and when fully expanded 56 cm in length and 30 cm in maximum width.

(*b*) *Culture Conditions and Sampling of Leaves for Isolation of Starch Granules from Samples of* 200 *Plants*

Plants were grown as in Section II(*a*) above. When leaves 17 and 18 were still growing rapidly, 90 plants were chosen for uniformity and at 4.30 a.m., 11.00 a.m., 5.00 p.m., and 10.30 p.m., a half leaf was stripped from leaves 17 and 18 alternatively on consecutive plants. The samples were immediately weighed, macerated five times in 2% sodium chloride and toluene with a trace of mercuric ion, and the granules separated by sedimentation (Porter and Martin 1952).

When leaf 17 was fully expanded, three leaf disks 1½ in. in diameter were cut at each sampling time from 200 plants that had been chosen for uniformity and the granules separated mechanically.

(*c*) *Culture Conditions and Sampling for Isolation of Starch Granules from Bulked Samples of Seedling Leaves*

The variety used was a fixed line produced by hybridization of *Nicotiana tabacum* and *N. debneyi* and which is known as the Clayton hybrid with the low disease index. The plants were grown in commercial seedbeds in sterilized soil under normal commercial conditions. Sampling was carried out at 4.30 a.m., 11.00 a.m., 5.00 p.m., and 10.30 p.m. The leaves were still rapidly expanding or just fully expanded. Plants were 5 in. high and the biggest two or three leaves were selected and the midribs discarded. At each sampling 200 g (fresh weight) of leaves was collected. The samples were macerated in sodium chloride solution and toluene containing a trace of mercuric ion (Porter and Martin 1952).

(*d*) *Culture Conditions and Sampling of Leaves for Isolation of Starch by Perchloric Acid Extraction of Individual Plants*

N. tabacum cv. Hicks was grown in the tropical spring in the field to which 500 lb of 2 : 16 : 8 NPK fertilizer was applied per acre. When leaves 15, 16, 17,

and 18 were still expanding, four plants were chosen for uniformity and at 4.30 a.m., 11.00 a.m., 5.00 p.m., and 10.30 p.m., three disks were taken from each of these leaves. These were weighed and, after maceration in ethanol, dried in a desiccator at room temperature for estimation of dry weight of the whole leaves. The midribs were discarded and the water-soluble material and perchloric acid extract isolated as previously described (Matheson and Wheatley 1962*a*). The perchloric acid extract was separated into starch precipitated as the iodine complex and the polysaccharide not precipitated by iodine but precipitated by ethanol. When leaves 15, 16, 17, and 18 were fully expanded the procedure was repeated.

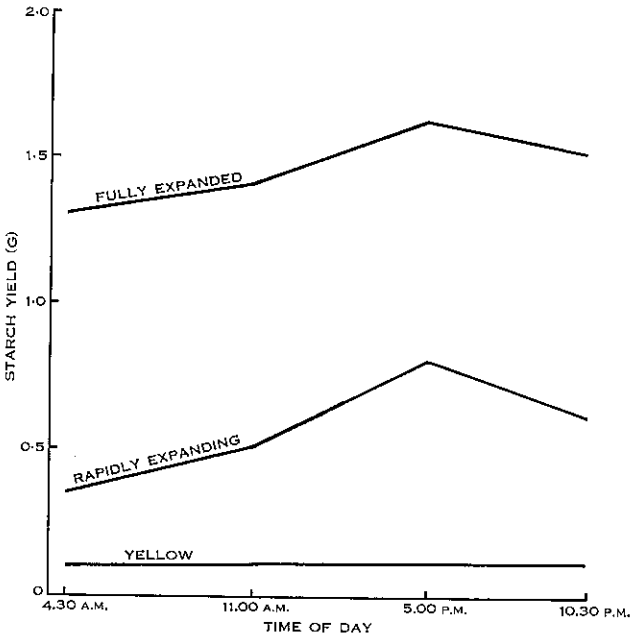

Fig. 1.—Weight of starch in 270 leaf disks, each of 2·3 cm diameter, from 90 plants.

(*e*) *Characterization of Isolated Starch Granules*

The methods described in Matheson and Wheatley (1962*a*, 1962*b*) were used to estimate glucan content of starch samples, glucan content of the fraction soluble in water and of the perchloric acid extract not precipitated by iodine, limiting viscosity number, isopotential iodine absorption, extent of conversion to maltose by β-amylase, the apparent chain length from the formic acid produced on periodate oxidation, and measurement of granule size.

III. Results

The quantitative changes in weight of starch in rapidly expanding, fully expanded, and yellow leaves are shown in Figure 1.

In fully expanded leaves, if the starch contents are calculated as a percentage of dry weight, there is not a rise but a fall during the day, due to a larger increase

in other photosynthetic products. Starch weight increase is proportionately much less than total dry weight increase. This probably accounts for statements in the literature that some species do not undergo a diurnal increase in starch (Wanner, 1958).

TABLE 1

DIURNAL CHANGES IN THE PROPERTIES OF STARCH GRANULES FROM LEAVES OF N. TABACUM CV. HICKS

Sample Type	Time of Sampling	Viscosity (g/100 ml)	Isopotential Iodine Absorption (g/100 g starch)	Apparent Chain Length of Amylopectin Assuming 20% Amylose Content (glucose units)	Average Granule Size and (in brackets) Maximum Granule Size (μ)
Seedling leaves at 6 weeks	4.30 a.m.	1·67	4·00	20	2·5 (5·6)
	11.00 a.m.	1·66	3·72	20	2·4 (5·6)
	5.00 p.m.	1·63	3·33	22	2·5 (5·2)
	10.30 p.m.	1·67	3·85	20	2·4 (6·0)
Rapidly expanding leaves	4.30 a.m.	1·42	3·00	22	1·7 (4·0)
	11.00 a.m.	1·45	2·88	19	1·8 (4·0)
	5.00 p.m.	1·41	1·62	20	2·2 (5·6)
	10.30 p.m.	1·38	2·14	19	2·2 (4·8)
Fully expanded leaves	4.30 a.m.	1·70	4·90	20	2·4 (6·8)
	11.00 a.m.	1·70	4·42	21	2·4 (5·6)
	5.00 p.m.	1·66	3·74	20	2·5 (5·6)
	10.30 p.m.	1·66	3·71	22	2·5 (6·4)

Diurnal changes in the properties of starch granules in seedling leaves, rapidly expanding leaves, and fully expanded leaves are shown in Table 1. Bulked samples from 200 plants were taken to avoid plant sample variation.

TABLE 2

GLUCAN FRACTIONS OBTAINED BY PERCHLORIC ACID EXTRACTION OF RAPIDLY EXPANDING LEAVES (NOS. 15–18) OF N. TABACUM CV. HICKS

Time of Sampling	Water Soluble Glucan (% dry wt.)	Perchloric Extract Precipitated by Iodine (% dry wt.)	Glucan Extracted by Perchloric Acid and not Precipitated by I–KI (% dry wt.)
4.30 a.m.	0·02	2·0	0·08
11.00 a.m.	0·03	7·9	0·07
5.00 p.m.	0·03	5·8	0·08
10.30 p.m.	0·04	5·0	0·11

The viscosities and chain lengths of the amylopectins over diurnal–nocturnal periods for a particular age of leaf lie within the reproducibility of measurement

($\pm 0 \cdot 04$ g/100 ml and ± 1 glucose unit) but the iodine affinities differ significantly, being at the lowest values at the end of the day. The standard deviation of measurement of iodine affinity was 0·1 g/100 g starch.

From Table 2, the only significant quantity of glucan extractable by perchloric acid from rapidly expanding leaves is that precipitated by iodine. The residue after perchloric acid extraction was indistinguishable from cellulose with some hemicellulose. The perchloric acid extracts precipitated by iodine have the properties of starch (Table 3).

TABLE 3

DIURNAL CHANGES IN THE PROPERTIES OF PERCHLORIC ACID EXTRACTS PRECIPITATED BY IODINE FROM RAPIDLY EXPANDING LEAVES (NOS. 15–18) OF N. TABACUM CV. HICKS

Time of Sampling	Isopotential Iodine Absorption (g/100 g starch)	β-Amylolysis Limits (% conversion to maltose)	Time of Sampling	Isopotential Iodine Absorption (g/100 g starch)	β-Amylolysis Limits (% conversion to maltose)
4.30 a.m.	4·7	63	5.00 p.m.	3·9	60
11.00 a.m.	3·8	59	10.30 p.m.	3·6	63

The average granule size was determined within the limits of reproducibility of measurement for a sample number of 500. These limits were $\pm 0 \cdot 05\ \mu$. The starch from rapidly expanding leaves shows a higher average granule size in samples from the late afternoon and early night.

IV. DISCUSSION

The daily amount of increase in starch content in rapidly expanding leaves, or in fully expanded leaves (Fig. 1), is much less than the increase in starch content during the development of leaves from the rapidly expanding to the fully expanded phase of growth. Thus the increase in starch content with age (Matheson and Wheatley 1962*b*) is a real accumulation and not due to the time of sampling in the day.

The change in starch content during the day and night at one particular leaf age is not accompanied by any measurable changes in viscosities or chain lengths of the amylopectins (Table 1) within the limits of accuracy of measurement. Starch isolated as granules from leaves 14–18 on individual plants, sampled at four times of the day when leaves in these positions were rapidly expanding and fully expanded, showed no change in β-amylolysis limits within the accuracy of measurement. The isopotential iodine absorptions at various ages varies significantly, being highest before dawn and lowest in the late afternoon. The amount of change is greater in rapidly expanding leaves than in fully expanded leaves. This observation of low iodine affinity during the day and high affinities during the night is the opposite of that reported by Mizuno *et al.* (1961). However, they

isolated starch by extraction with hot calcium chloride solution, which may result in a preferential extraction of amylose (Banks and Greenwood 1959). A change in iodine absorption could be due to a change in the amylose content relative to amylopectin or to a change in the structure of the amylose fraction. An increase in the degree of polymerization of amylose would increase the iodine absorption, whereas branching would decrease this value. Only fractionation of the starch and determination of the iodine affinities could resolve the actual cause of the iodine absorption differences. In any case, these results suggest that the metabolisms of tobacco leaf starch amylose and amylopectin proceed at different rates. Another possible source of variation in iodine affinity is contamination by variable amounts of fatty acid in the granules. Further extraction of granules with hot methanol did not change the iodine affinity values, indicating that this is not the cause of variation. By using the method of isolation reported above, it cannot be established if this change is taking place uniformly over all granules, or if some granules are relatively inert and others show a large change in iodine absorption.

In rapidly expanding leaves the ratio of starch content at maximum and minimum weights is approximately 2·5 (Fig. 1). The ratio of volumes of average granule sizes is also approximately 2·5 (Table 2), suggesting that most of the starch weight increase is by accretion on to existing granules and not by synthesis of new small granules. However, the method of isolation of the granules (by sedimentation) could lead to a selective loss of the smaller granules so that the samples may not be representative of the size distribution in the original leaves. The increase in average granule size, detectable in starch from rapidly expanding leaves, is probably due to the large variation in maximum and minimum starch content in young leaves. A similar increase in granule size is not apparent in the starch from fully expanded leaves, but it should be noted that even if all the new starch was incorporated into existing granules there could only be an increase in average granule size of the same order as the accuracy of measurement.

Greenwood and Thomson (1959) have shown that, during starch depletion in germinating barley, utilization of the starch is accompanied by a fall in the chain length of the amylopectin and a rise in the iodine absorption of the whole starch and a slight reduction in granule size. These authors have suggested that β-amylase action with limited α-amylolysis would explain these findings. On the other hand, in sprouting potato tubers all the properties of the starch are unchanged apart from a slight decrease in average granule size (Banks and Greenwood 1959). The utilization of starch in tobacco leaves at night differs from the depletion of starch in both sprouting potatoes and germinating barley, in that it is accompanied by no changes in the chain lengths of the amylopectins and viscosities of the whole starches, but the iodine affinity rises and the average granule size is reduced.

From samples taken at all times of the day and night the main glucan fraction which can be extracted by perchloric acid is precipitated by iodine. This fraction shows iodine absorption values and β-amylolysis limits typical of perchloric acid-extracted starch (Matheson and Wheatley 1962*a*). This fraction also gives an indication of variable iodine absorption. If any dextrin is involved in synthesis or degradation of the starch granules then it is in very low concentration. This

does not exclude the possibility of the participation of dextrin in the metabolism of starch. Unless the leaves can efficiently utilize maltodextrins it indicates that amylases are not the degradative enzymes. From our present knowledge of starch enzymology (Whelan 1961), this would suggest that diurnal utilization of starch is due to phosphorylase or a reversal of uridine or adenosine diphosphate glucose starch synthetase (Rongine de Fekete, Leloir, and Cardini 1960).

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