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THE RESPIRATION OF THE PEA PLANT. OXIDATION OF HEXOSE PHOSPHATE AND PENTOSE PHOSPHATE BY CELL-FREE EXTRACTS OF PEA LEAVES[1, 2, 3]

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Evidence for the presence of the Embden-Meyerhof-Parnas pathway in higher plants has accumulated rapidly in the last few years (23). The major outstanding difference reported between the glycolytic pathway in the green tissues of higher plants and in yeast, animal tissue and microorganisms is the presence in green tissues of a glyceraldehyde phosphate dehydrogenase linked not only to diphosphopyridine nucleotide but also to triphosphopyridine nucleotide (1, 13).

Preliminary evidence has also been gathering that an alternate pathway of glucose-6-phosphate metabolism characterized in yeast (6, 9, 10), animal tissue (11, 15, 16) and microorganisms (8, 12), and termed the "hexosemonophosphate shunt" may also be operating in leaves (3, 13), roots (13) and other tissues (5, 7) in higher plants.

The present investigation was undertaken to establish whether the enzymes of the "hexosemonophosphate shunt" are present in pea leaves and, if so, (1) whether the pathway is the same as in tissue of other organisms and (2) whether it is connected with the glycolytic pathway.

MATERIALS AND METHODS

Fructose-1,6-diphosphate (FDP), fructose-6-phosphate (F-6-P), and adenosine triphosphate (ATP) were purchased from the Schwarz Laboratories. Glucose-6-phosphate (G-6-P) and 6-phosphogluconate (6-PG) were prepared by the methods of Seegmiller and Horecker (21). Ribose-5-phosphate (R-5-P) was kindly supplied by Dr. R. DeMoss. Ribonic acid-5-phosphate prepared from ribose-5-phosphate by the hypoiodite oxidation procedure of Moore and Link (19) was kindly supplied by Dr. L. M. Paege. Coenzyme I (diphosphopyridine nucleotide, DPN) was obtained from the Schwarz Laboratories. Analysis showed that it contained 61% DPN. Coenzyme II (triphosphopyridine nucleotide, TPN) was prepared by the method of LePage and Mueller (17) and found to contain 31% Co II and to be free of Co I. TPN (80%) purchased from the Sigma Chemical Co. was also used. The barium salts of the phosphate esters were converted into potassium salts by dissolving in 1 *N* HCl, adding a slight excess of 10% K_2SO_4 and removing the resulting $BaSO_4$ by centrifugation. The supernatant solution was neutralized with 1 *N* KOH and diluted to make the final substrate concentration either 0.1 M or 0.05 M.

PREPARATION OF PEA LEAF EXTRACT: *Pisum sativum* variety Alaska (Rogers Brothers Seed Co., Idaho Falls, Idaho) was used throughout this investigation. The seeds were soaked overnight in tap water, then transplanted to tanks containing gravel in the greenhouse. Nutrient solution (14) was poured over the gravel once during the period of growth.

It is in order to note that cell-free preparations of leaves obtained from plants grown in the winter (November through March) were unsatisfactory for manometry, although solar radiation was supplemented with artificial light, total radiation being 300–800 fc.

Leaves of 11–15 day plants were used. A typical batch of plants had 4–5 sets of leaves, weighed about 2 gm, and disregarding the cotyledons, the weight was approximately evenly divided among the leaves, stems and roots. In general, about 70 gm of leaves, separated from the stems, were ground with an equal weight of acid-washed, cold, sharp sand and 20 ml of ice-cold 0.5 M $KHCO_3$ in a cold mortar. This operation and subsequent ones in the preparation of the cell-free extracts were carried out at 0–4°C. The extract was strained through 4 thicknesses of cheesecloth and centrifuged at 18,000 × g for 20 minutes. On occasion the supernatant material was passed through glass wool to remove fat particles. The solution had a dark green color, its volume was 50–75 ml and its pH varied from 6.8 to 7.5. The pH was brought to 7.2 with either ice-cold 1 *N* KOH or 2 *N* acetic acid. This preparation was used immediately or placed in the deep freeze until the next day. The frozen material when thawed the next morning formed a heavy precipitate which was removed by centrifugation at 18,000 × g for 20 minutes. The removal of this precipitate did not affect the activity of the extract. In some of the experiments, the extract was dialyzed 6

[1] Received March 16, 1953.
[2] A preliminary report was given at the meetings of the American Institute of Biological Science, Ithaca, N. Y., September, 1952.
[3] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

hours against 10 liters of 0.02 *N* $NaHCO_3$ or 0.02 *N* $NaHCO_3$ containing 47 mM cysteine · HCl. The pH of the dialyzing mixture was 7.5.

On occasion, extracts were prepared in the usual manner with 150 mg of sodium versenate (pH 7.4) added to protect the sulfhydryl-containing enzymes. Extracts prepared in this manner were inactive for manometry, presumably due to the removal of co-factors.

Oxygen uptake was determined manometrically at 37° with air as the gas phase. The main compartment of each Warburg vessel usually contained 1.0 ml of dialyzed enzyme solution, 0.1 ml of 0.2 M $MgCl_2$, 0.05 ml riboflavin (1 mg/ml), and 0.05 ml of 0.25 M ATP, or undialyzed enzyme solution, 1.0 ml, and water, 0.45 ml. The side arm contained 0.05 ml TPN solution (1 mg/ml) and 0.2 ml of 0.05 M substrate. The center well contained 0.2 ml 20% KOH as CO_2 absorbent or in experiments with cyanide a suitable KOH-KCN mixture. After the endogenous level had subsided (usually 60–90 minutes), the reaction was initiated by tipping in the TPN and substrate. For each point on the curves, duplicates were run.

The rate of reduction of TPN was followed at 340 mμ in 1 cm cells in a Beckman spectrophotometer, model DU, at 23–25°. The cell contained 1.5 ml veronal buffer, pH 8.0, 0.05 ml enzyme solution, 0.05 ml TPN (1 mg/ml) and 0.1 ml of 0.05 M substrate. Water was added to make a total volume of 3 ml. Blanks omitting TPN were used.

Results

Figure 1 indicates that the cell-free preparation contains enzymes capable of oxidizing fructose-1,6-diphosphate, fructose-6-phosphate, glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate. The following materials are not oxidized: glucose, gluconic acid, ribose, ribonic acid-5-phosphate, hydroxypyruvic acid and formic acid. When formic acid was used as substrate DPN was also added; however, it had no effect.

It is also apparent from figure 1 that the extract

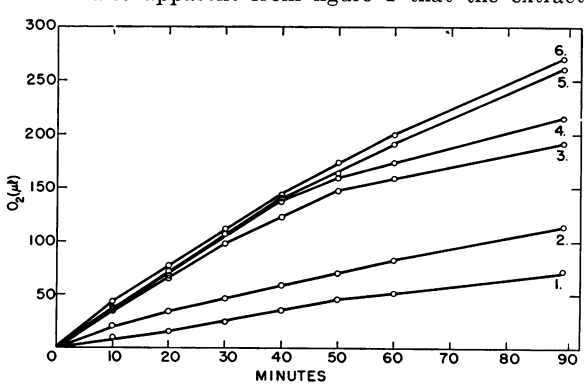

Fig. 1. Oxidation of hexose and pentose phosphate by pea leaf extract. Extract 1.0 ml; 0.5 mg TPN and 0.2 ml 0.05 M substrate. Total volume 1.7 ml; 1. endogenous; 2. 6-phosphogluconate; 3. glucose-6-phosphate; 4. fructose-6-phosphate; 5. ribose-5-phosphate; 6. fructose-1,6-diphosphate.

Table I

Effect of Boiled Pea Leaf Extract on the Oxidation of Glucose-6-phosphate by Pea Leaf Extract

Reactants *	O_2 uptake (μL/110 min)
Undialyzed P.L.E.,† G-6-P, TPN	232
Dialyzed P.L.E., TPN	67
Dialyzed P.L.E., G-6-P	42
Dialyzed P.L.E., TPN, G-6-P + 0.2 ml B.P.L.E.‡	33
Dialyzed P.L.E., TPN, G-6-P + 0.5 ml B.P.L.E.	58
Dialyzed P.L.E., TPN, G-6-P + 1.0 ml B.P.L.E.	118

* 1.0 ml pea leaf extract dialyzed against $NaHCO_3$, 0.5 mg TPN, 0.2 ml 0.05 M glucose-6-phosphate. Total volume 2.5 ml.

† P.L.E.—pea leaf extract.

‡ B.P.L.E.—undialyzed pea leaf extract heated 2 minutes at 100°C.

contains the entire complement of enzymes necessary for the transfer of electrons from the substrate to oxygen. After dialysis, the pea leaf extract loses its capacity to oxidize hexose and pentose phosphate, however, the oxidizing activity of the dialyzed preparation is restored by the addition of pea leaf extract heated at 100°C for 2 minutes (table I). These heat-stable cofactors are also present in boiled yeast juice. Of the various likely cofactors tested, a mixture of TPN, ATP and riboflavin or riboflavin phosphate increases the oxygen uptake when any of the hexose or pentose phosphates is employed as substrate (tables II, III, IV).

Table II

Effect of Cofactors on the Oxidation of Fructose-1,6-diphosphate by Dialyzed Pea Leaf Extract

Addition	O_2 uptake (μL/120 min)
FDP, riboflavin, ATP, TPN	370
Riboflavin, ATP, TPN	112
FDP, ATP, TPN	201
FDP, riboflavin, TPN	199
FDP, riboflavin, ATP	117

See methods section for protocol. Extract dialyzed against $NaHCO_3$.

With some extracts the rate of oxidation of FDP

Table III

Effect of Adenosine Triphosphate on the Oxidation of Fructose-1,6-diphosphate by Pea Leaf Extract

Micromoles of ATP	O_2 uptake (μL/50 min)
0	50
5	90
10	117
15	117
20	132
30	125

See methods section for protocol. Extract dialyzed against $NaHCO_3$.

TABLE IV

EFFECT OF RIBOFLAVIN AND RIBOFLAVIN-5-PHOSPHATE ON THE OXIDATION OF FRUCTOSE-1,6-DIPHOSPHATE BY PEA LEAF EXTRACT

MICROGRAMS		O_2 UPTAKE (μL/360 MIN)
RIBOFLAVIN-5-P	RIBOFLAVIN	
..	...	259
1.3	...	256
2.6	...	274
6.5	...	315
..	1.0	271
..	2.0	304
..	5.0	389
..	10.0	400
..	20.0	410

See methods section for protocol. Extract dialyzed against $NaHCO_3$.

is higher than with other substrates. Also, when FDP oxidation is carried out with preparations in which the triosephosphate dehydrogenase was presumably inactivated by addition of 0.01 M iodoacetamide (fig. 2) or by dialysis against 0.02 M $NaHCO_3$, the rate of oxygen uptake was decreased to the level obtained with G-6-P. This indicates the FDP is being metabolized via two pathways.

Table V indicates that the leaf extract also oxidizes

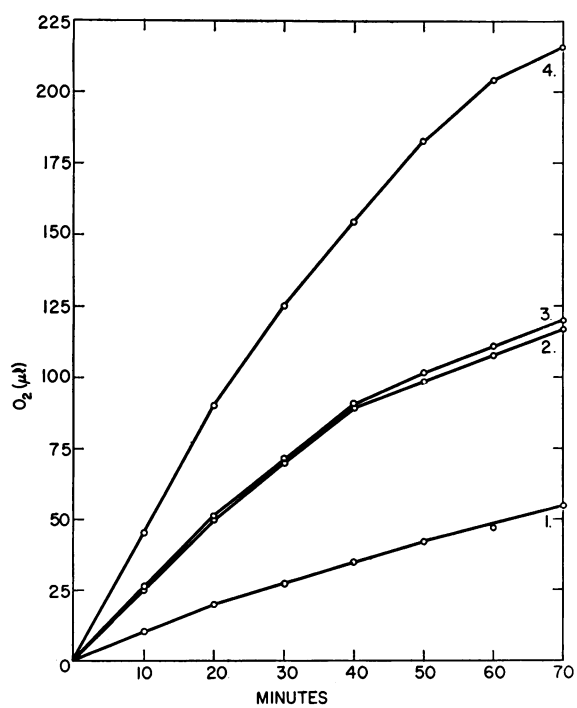

FIG. 2. Effect of iodoacetamide on fructose-1,6-diphosphate oxidation. Pea leaf extract 1.0 ml; 0.5 mg TPN and 0.2 ml 0.05 M substrate. Total volume 1.7 ml; 1. endogenous; 2. glucose-6-phosphate; 3. fructose-1,6-diphosphate and iodoacetamide—final concentration 0.01 M; 4. fructose-1,6-diphosphate.

TABLE V

DPN-LINKED TRIOSEPHOSPHATE DEHYDROGENASE IN PEA LEAF EXTRACT

COMPONENTS OF SYSTEM	O_2 UPTAKE (μL/90 MIN)
Complete system *	180
- arsenate	60
- ATP	30
- FDP	30
- riboflavin	140
- NaF	190
+ iodoacetamide 0.0025 M (final conc.)	140
+ iodoacetamide 0.01 M (final conc.)	100

* Complete system. Extract dialyzed against $NaHCO_3$-cysteine, 1.0 ml; sodium arsenate 36 μM; sodium fluoride 60 μM; FDP 10 μM; ATP 12.5 μM; riboflavin 0.13 μM; DPN 0.17 μM; cysteine 8 μM. Total volume 2.5 ml.

FDP via a DPN-triosephosphate dehydrogenase. Cofactor requirements (DPN and arsenate) are the same as for the DPN-triosephosphate dehydrogenase isolated from tissues of other organisms. On the other hand, the rate of oxidation of FDP is increased by only a small amount on the addition of arsenate (fig. 3) when TPN is used in place of DPN.

Coenzyme specificity was determined by following the reduction of DPN and TPN spectrophotometrically at 340 mμ. The results (fig. 4) show a reduction of TPN by FDP, F-6-P, G-6-P, 6-PG, and R-5-P indicating the presence of dehydrogenases. In the presence of arsenate, FDP and R-5-P reduce DPN. None of the dehydrogenases is active with DPN alone. As indicated in figure 4, a lag of 30–45 seconds in TPN reduction occurs when R-5-P is substrate.

EFFECT OF ENZYME INHIBITORS: Cyanide (0.01 M) produces a 30% increase in glucose-6-phosphate dehydrogenase activity and a slight increase in the 6-phosphogluconic acid dehydrogenase. The rate of O_2

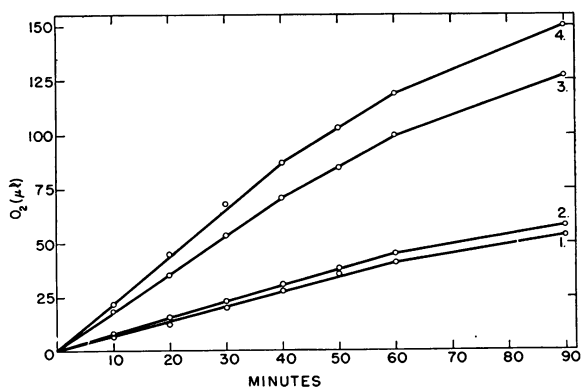

FIG. 3. Effect of arsenate on the oxidation of fructose-1,6-diphosphate by pea leaf extract in the presence of TPN. Extract dialyzed against $NaHCO_3$-cysteine 1.0 ml; 0.5 mg TPN; riboflavin 0.13 μM; ATP 12.5 μM. Total volume 2.0 ml; 1. endogenous; 2. endogenous, sodium arsenate 36 μM; 3. fructose-1,6-diphosphate 10 μM; 4. fructose-1,6-diphosphate 10 μM, sodium arsenate 36 μM.

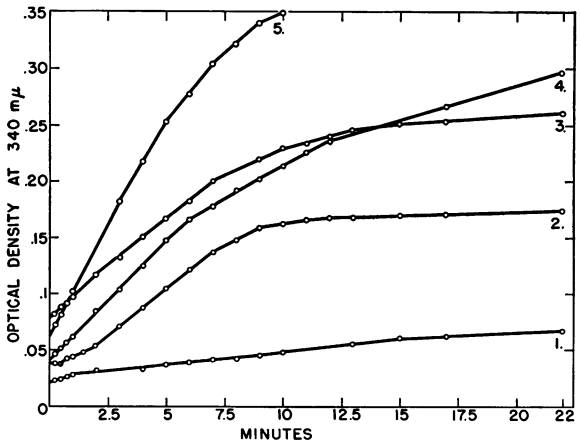

FIG. 4. Reduction of TPN by hexose and pentose phosphate. For details, see methods section. 1. 6-phosphogluconate; 2. ribose-5-phosphate; 3. fructose-1,6-diphosphate and 0.01 M iodoacetamide; 4. fructose-6-phosphate; 5. glucose-6-phosphate.

uptake is either not affected or slightly increased with any of the hexose phosphates (fig. 5) as substrates. These facts seem to indicate cyanohydrin formation and not inhibition of catalase as a possible explanation of increased O_2 uptake. Inhibition of catalase would result in a twofold increase in O_2 uptake.

Azide and Fluoride: Azide (0.01 M) and fluoride (0.01 M) do not affect the activity of any of the dehydrogenases or the O_2 uptake. The pH of the mixtures was 6.8 to 7.0 when azide was used.

Iodoacetamide: Iodoacetamide (0.01 M) did not inhibit glucose-6-phosphate dehydrogenase or 6-phosphogluconic acid dehydrogenase. It did inhibit (see fig. 2) the O_2 uptake when FDP was employed as substrate by approximately 50%. The poison did not inhibit the O_2 uptake when G-6-P or 6-PG were oxidized.

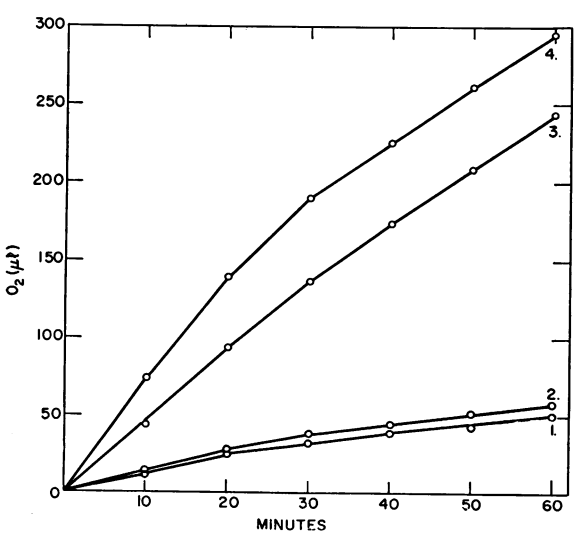

FIG. 5. Effect of cyanide on the oxidation of fructose-1,6-diphosphate. Pea leaf extract 1.0 ml; 0.5 mg TPN. Total volume 1.7 ml; 1. endogenous; 2. endogenous, cyanide—final concentration 0.01 M; 3. 0.2 ml 0.05 M fructose-1,6-diphosphate; 4. 0.2 ml 0.05 M fructose-1,6-diphosphate, cyanide—final concentration 0.01 M.

Indoleacetic Acid: High concentrations (0.01 M) did not affect the O_2 uptake when FDP, F-6-P, G-6-P, 6-PG or R-5-P was employed as substrate.

DISCUSSION

The present work establishes the existence in the pea leaf of a pathway which has been designated as "the hexosemonophosphate shunt." As in animal tissue and yeast, all of the dehydrogenases involved are TPN-specific and are not inhibited by fluoride and iodoacetamide.

Inhibition of O_2 uptake in the presence of iodoacetamide is apparently due to a poisoning of the DPN- or TPN-linked glyceraldehyde phosphate dehydrogenase. Inhibition (50%) of the rate of fructose-1,6-diphosphate oxidation by iodoacetamide demonstrates that in these preparations about half of the ester is being oxidized via the "hexosemonophosphate shunt" pathway and the remainder via the Embden-Meyerhof-Parnas pathway. James and Beevers (17) had reported a partial inhibition of the oxidation of fructose-1,6-diphosphate by cell-free extracts of the Arum spadix in the presence of iodoacetamide. Stumpf (24) found no inhibition of fructose-1,6-diphosphate oxidation by iodoacetamide; however, the preparations were made by use of the Waring blender which may have inactivated the triosephosphate dehydrogenase.

The data indicate (table V) that arsenate is needed as a cofactor in fructose-1,6-diphosphate oxidation when DPN is used as electron acceptor. This requirement is similar to that of the DPN-linked triosephosphate dehydrogenase isolated from pea seeds (22). However, arsenate has little effect on fructose-1,6-diphosphate oxidation when TPN is added to the extract although the inhibition of O_2 uptake caused by iodoacetamide indicates the presence of triosephosphate dehydrogenase. The reduction of TPN with fructose-1,6-diphosphate as substrate and the absence of an arsenate effect on the reduction has been reported (3, 5). This observation has been confirmed in this laboratory. However, these same extracts require arsenate as cofactor when glyceraldehyde-3-phosphate is employed as substrate in place of fructose-1,6-diphosphate.[4] Thus, the possibility exists that fructose-1,6-diphosphate can be converted to triosephosphate and be metabolized by an alternative pathway which does not include the arsenate-requiring triosephosphate dehydrogenase.

Ribose-5-phosphate which arises from the decarboxylation of 6-phosphogluconate (5, 15) is readily oxidized by the pea leaf extract; however, the same extract is not able to oxidize ribonic acid-5-phosphate. This behavior is similar to that found in rat and kidney extracts (11). After a short lag, ribose-5-phosphate reduces TPN (fig. 4). This lag is presumably due to a slow conversion of ribose-5-phosphate to

[4] Gibbs. Unpublished data.

ribulose-5-phosphate (15, 20). Present evidence indicates that the ketopentose sugar incubated with spinach leaf preparations yields sedoheptulose phosphate and triosephosphate (4). The latter can reduce TPN via the TPN linked triosephosphate dehydrogenase of the Embden-Meyerhof glycolysis pathway.

Lack of inhibition by cyanide when hexose phosphate is substrate together with the requirement of microgram quantities of riboflavin or riboflavin-5-phosphate and milligram quantities of adenosine triphosphate suggests a flavin-type respiratory system with a phosphorylated riboflavin as cofactor. A similar observation was reported for the Arum spadix (17). Flavin-adeninedinucleotide with adenosine triphosphate has also been reported to stimulate respiration in pea leaf extracts (24).

Summary

1. Cell-free extracts of 11–15-day pea leaves oxidize fructose-1,6-diphosphate, fructose-6-phosphate, glucose-6-phosphate, 6-phosphogluconate, ribose-5-phosphate in the presence of triphosphopyridine nucleotide. Glucose, gluconic acid, ribose, ribonic acid-5-phosphate and formic acid did not accelerate the O_2 uptake of the leaf extract.

2. The oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate indicates the presence in leaf tissue of a " hexosemonophosphate shunt " which closely resembles that found in animal tissue and yeast. The oxidations are TPN-specific and not inhibited by iodoacetamide or fluoride.

3. In some extracts, the O_2 uptake with fructose-1,6-diphosphate is approximately double that with glucose-6-phosphate. On addition of 0.01 M iodoacetamide to such extracts or to dialyzed extracts, the rate of O_2 uptake becomes equal for the two substrates.

4. The failure of the pea leaf extract to oxidize ribonic acid-5-phosphate while oxidizing ribose-5-phosphate indicates that ribose-5-phosphate can be metabolized by conversion to compounds of the Embden-Meyerhof glycosis pathway.

5. Dialyzed leaf extracts lose the ability to oxidize hexose or pentose phosphate. This ability can be restored with microgram quantities of riboflavin or riboflavin-5-phosphate in addition to milligram quantities of adenosine triphosphate. Added boiled pea juice or boiled yeast extract produce the same effect.

6. Leaf extracts oxidize fructose-1,6-diphosphate in the presence of DPN provided arsenate is present. When TPN is substituted for DPN, arsenate stimulates the O_2 uptake only slightly.

7. Cyanide (0.01 M) does not inhibit the oxidation of hexose phosphate and may accelerate the early stages of oxidation. It does inhibit ribose-5-phosphate oxidation.

8. Azide (0.01 M) or fluoride (0.01 M) does not affect the O_2 uptake of these substrates.

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EFFECTS OF SHADE AND PARTIAL DEFOLIATION ON CARBOHYDRATE LEVELS AND THE GROWTH, FRUITING AND FIBER PROPERTIES OF COTTON PLANTS[1]

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IN COOPERATION WITH

DEPARTMENT OF PLANT PHYSIOLOGY AND PATHOLOGY, TEXAS AGRICULTURAL EXPERIMENT STATION, COLLEGE STATION, TEXAS

Carbohydrate levels in cotton plants during the fruiting period were reduced: 1. by growing the plants under muslin shades and 2. by cutting away half of each new leaf. The effects of these treatments were studied and are discussed in terms of 1. light in relation to growth of the cotton plant, 2. partial defoliation and growth, 3. relative fruitfulness (bolls per 100 gm of fresh stems and leaves) and 4. changes in boll and fiber properties.

The foregoing experiments were conducted along with others (partial defruiting and drought) that increased carbohydrate levels. Both of these treatments (4) increased fiber strength. Partial defruiting increased slightly and drought decreased: seed cotton per boll, fiber length, weight per inch and percent of mature fibers; but between five varieties of cotton it was found that those operating at the lowest carbohydrate levels had the strongest fibers, i.e., strength was evidently correlated with enzymatic and other growth factors that furthered carbohydrate utilization. As a further background for the results of this paper, it has been found that boll shedding is not commonly related to the carbohydrate or nitrogen levels in the plant (5).

The shade experiment (light intensity reduced to 32% of full exposure by muslin sheeting) was repeated at a high, intermediate and low level of water supply and was started at about the time of first flowers. References were made to the findings of others on the effects of light intensities in a previous paper (6). In the partial defoliation (half-leaf) experiment, one-half of each of the previous leaves and of each subsequent new leaf was removed weekly starting also at the time of first flowers. The work was conducted at the U. S. Cotton Field Station, Shafter, California, with the Acala p18 variety of cotton. Consideration is given in the discussion to evidence bearing on the auxin relations involved in the fruiting of the cotton plant.

[1] Received March 26, 1953.

METHODS

The two experiments, shade and half-leaf, were each laid out in four blocks with the control and treated plots alternating in irrigation borders 320 feet long; different irrigation borders were used for the two tests. Twelve rows of cotton with the plants spaced 12 inches apart were used for the shade experiment and four rows of similarly spaced plants for the half-leaf (partial defoliation) experiment. The four shades were 25 feet long; plants in the outside rows under the shades as well as the three end plants under and outside of the shades were not used for the measurements. The three levels of water supply—heavy, medium and light—were supplied by irrigating: a, both sides of three rows weekly, b, only one side of three rows weekly and, c, one side of each of three rows every two weeks. One-half of each control plot was to the east and one-half to the west of each of the shades. The analyses of variance for *yields* assume randomization which would have been difficult or impractical in the instance of the irrigation treatments.

Twelve plants were sampled at three times during the flowering period for carbohydrate analyses in both experiments. At each collection half of the plants were harvested in the late morning and half in the early afternoon; the order of collection was reversed between morning and afternoon and between successive days. In the half-leaf experiment the sampling dates were July 14, 15, 29, and 30 and August 11 and 12, tissues from three plants in each treatment being collected each time and the twelve then combined as single samples. In the shade experiment six plants were sampled in the late morning and six in the early afternoon as above but during the single days: July 16 and 31 and August 13. The leaf samples (48 leaves per sample) were from the middle third of the main stalk. The middle third of the main stalk was used for the stem samples. The dated bolls were from the plants sampled.

Measurements of height and number of nodes and