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THE INTRACELLULAR LOCATION OF PHOSPHORYLASE IN TOBACCO (NICOTIANA TABACUM L.)[1,2]

JOHN H. MADISON, JR.

DEPARTMENT OF LANDSCAPE MANAGEMENT, UNIVERSITY OF CALIFORNIA, DAVIS, CALIFORNIA

Hanes (6, 7) first identified the enzyme phosphorylase in preparations of pea seeds and potatoes. The enzyme catalyzes the reaction glucose-1-phosphate (G-1-P) $\rightleftharpoons$ starch + phosphate, and Hanes predicted that the location of this enzyme would be the plastid of the plant. Yin and Sun (15) presented a histological mehod for identifying phosphorylase activity by the end product starch, and Yin and Sun (17) presented evidence for the location of phosphorylase in the plastids of several species of plants.

Using the above histological method on wheat, bean, and sunflower, Shaw (10) also found phosphorylase activity to be within the chloroplast, or associated with it, and he reviewed the many sources of error which leave the method open to criticism. A principal criticism is that plasmolysis could not be demonstrated in cells exhibiting polysaccharide formation. There is no certainty then that in these injured cells the enzyme is to be found in its "normal" location.

Dyar (5) used the histological method on root tips of pea seedlings, and she found activity in the nucleus and unspecified areas of the cytoplasm.[3] Stocking (11) used the histological method and a method based on the differential centrifugation of a leaf brei and could find no evidence that phosphorylase was within the plastid rather than the cytoplasm. The investigation reported here was made in part to find if the problem of phosphorylase in the plant cell could be resolved by the application of other methods.

Investigations by the author, based on quantitative methods, have shown daily and seasonal variations in the level of activity of phosphorylase, and have shown a considerable reduction in the level of activity when tobacco plants were starved by placing them in the dark. Since the principal test for activity used has been the qualitative formation of starch, previous workers have generally thought it advisable to use materials in which starch was not initially present. The low level of activity in such tissues may yield results which do not reflect conditions in a healthy plant. Hence some of the techniques here reported were designed to obtain data about the intracellular location of phosphorylase in tissues in which starch was present and being actively metabolized.

MATERIALS AND PREPARATIONS

Leaves of tobacco (*Nicotiana Tabacum* L.), stripped of heavy veins, were used as the plant material. G-1-P was obtained from Nutritional Biochemicals Company and its solutions were prepared using the method of Sumner et al (13). A 2% solution was made and diluted with an equal volume of 0.01 M maleic acid buffer pH 6.2 to give a 1% solution. Buffers were prepared using the acid and sodium salt. As sodium may increase permeability this is of concern in interpreting the results.

Some preparations were made by grinding fresh leaf tissue in a mortar with a small amount of phosphate free quartz sand and with 1 ml of solution as indicated per gm of tissue. The preparation was washed down with an additional 2 ml of solution per

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[2] Part of this work has been done in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Cornell University.

[3] Cytoplasm is used in this paper to denote protoplasm other than the nucleus or plastids.

gm of tissue. Other preparations were made by grinding leaf tissue in a Waring blendor with 3 ml per gm of either 12 % sucrose solution in pH 6.2 maleic buffer, or 60 % saturated neutral ammonium sulfate solution. The crude preparations were filtered through a facial tissue supported on cheesecloth.

Methods and Results

In these experiments phosphorylase activity was measured in terms of the free inorganic phosphate released when the plant preparations were incubated with G-1-P under the conditions stated. One ml of preparation was placed in a test tube with 1 ml of 0.01 M maleic acid buffer, pH 6.2; 1 ml of a 1 % soluble starch solution as a primer (6) and 1 ml of 0.02 M NaF as an inhibitor of possible phosphatase activity. These were brought to temperature (35° C) in a water bath and the reaction was started by adding 1 ml of 1 % G-1-P. At the end of the experiment the reaction was stopped by adding 1 ml of 10 % trichloroacetic acid (TCA). In the controls the order of adding the TCA and G-1-P was reversed. The 6 ml of preparation resulting were filtered and 3 ml were tested for phosphate by the use of Sumner's (12) modification of the phospho-molybdate test. The amount of phosphate was determined colorimetrically by reading percent transmission in a Coleman Jr. Model 6A spectrophotometer, at 680 mμ. Either density or micrograms of phosphorus was then determined from tables. Since Beer's Law holds in the range of values obtained, the statistical comparisons were calculated on the basis of the optical density.

Analysis showed that no significant breakdown of G-1-P occurred in 4 hours at room temperature after the addition of TCA. After the addition of H_2SO_4 in the phosphomolybdate test, however, acid hydrolysis of G-1-P began at once and so all readings were made between 1.5 to 2 minutes after adding the sulfuric acid.

The time of incubation was from 1/2 hour with preparations of high activity up to 1 hour when the tobacco leaves showed low phosphorylase activity. As comparisons were made within the experiments, such variation between experiments was considered allowable.

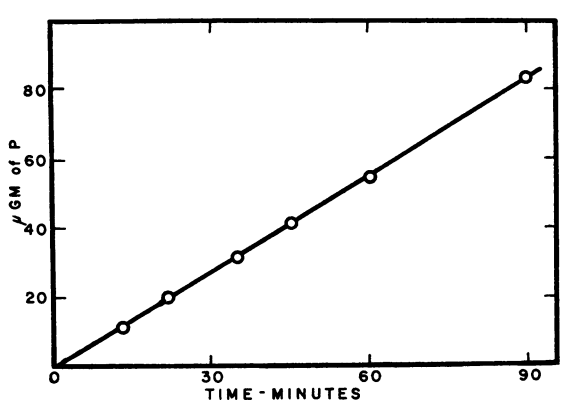


Fig. 1. Hydrolysis of G-1-P by phosphorylase. About 1190 μgm of P were supplied as G-1-P. The hydrolysis is a straight line reaction for the range of values used (up to 100 μgm hydrolyzed).

Table I

Relative Phosphorylase Activity of Residue (R) and Supernatant (S) of Centrifuged Tobacco Leaf Brei

Ml of residue		Activity */ml			
		Residue			
Initial	Resuspended	Resuspended	Initial	Supernatant, initial	Ratio of activity, (R/S)
2	12	26.3	157	37	4/1
1	10	10	120	17	7/1
0.5	10	7.4	148	5	30/1

* As μgm of P hydrolyzed from G-1-P in 30 min at 35° C.

One ml of 1 % G-1-P contains 1.19 mg of inorganic phosphorus. The greatest amount hydrolyzed in any single experiment was less than 100 μgm. The reaction was found to be a straight line for these values (fig 1).

Other experiments showed no deterioration in the phosphorylase activity of the preparation during the first 4 hours at room temperature. For this reason low temperatures were not used in making the preparations; however, the experiments were executed with promptness.

The first group of experiments was run with the following hypothesis as a basis. The enzyme phosphorylase is water soluble. When ground preparations are centrifuged the phosphorylase will be found in the supernatant in any case, since a large percentage of the plastids are ruptured in grinding. If the phosphorylase is normally located in the cytoplasm, then that portion of the centrifuge tube containing the residue should have less phosphorylase per unit volume, as phosphorylase would be displaced by the solid residue. On the other hand, if the phosphorylase is normally located within the plastid, then the residue should have the higher concentration of phosphorylase, its value being proportional to the number of uninjured chloroplasts.

Preparations were made by grinding leaf tissue in a mortar with pH 6.2, 0.01 M maleic buffer and fine sand, as described, and the filtered brei was centrifuged. The results in all cases showed a higher concentration of phosphorylase activity in the green pellet of the residue. The greatest total amount of activity was invariably in the supernatant, but the highest concentration was in the residue. Examples of data are given from three such experiments in table I.

In the first of the experiments which are described, ground and filtered leaf material was centrifuged lightly (250 × g for 3 min) and the residue which was principally starch was discarded. The supernatant was centrifuged for 20 minutes at 800 × g and 2 ml of

chloroplastic residue was obtained. The 2 ml of residue was brought to 12 ml by adding 10 ml of buffer and was then re-ground in a Potter-Elvehjem type homogenizer. One ml of the first supernatant hydrolyzed 37 μgm of phosphorus in 30 minutes. One ml of the second supernatant (derived from 1/6 ml of chloroplastic residue) hydrolyzed 26.3 μgm of phosphorus in the same time. On a ml for ml basis then the ratio of the activity of the supernatant and of the chloroplastic residues was 37/157 or about 1/4.

In a second example the ratio of activities was about 1/7. These two examples represent the range of differences obtained in experiments of this sort. It was found that regrinding the residue was not necessary and that shaking the residue in buffer to resuspend it gave the same values as regrinding. An effort was made to improve these first figures by using gentler techniques. Since the sodium ion affects permeability, no buffer was used. A 10 % sucrose solution was deionized by passing over amberlite resins. The resulting neutral solution was chilled in an ice bath. Cell contents were released by gently tearing tobacco leaves under the surface of the solution. One-half ml of green residue was obtained by centrifugation, and this was re-suspended in 10 ml of buffer. Total activity was still greater in the supernatant. Two and one half times the activity was contained in the 40 ml of supernatant as in the 0.5 ml of residue. In this instance the ratio was widened and the concentration of phosphorylase activity was, however, thirty times as great in the residue.

These results suggesting a concentration of phosphorylase in the chloroplast are at variance with the conclusions of Stocking (11). At this point in his work the author had the opportunity of comparing his data with that of Dr. Stocking and of discussing it with him. A comparison showed that the principal discrepancy between the works lay in Stocking's use of the plastid fraction of leaves which had been dark-starved to remove starch. In preliminary experiments the author had found that dark starvation reduced phosphorylase activity to low levels, and had so designed his experiments as to use leaf material gorged with stored starch. This difference in material was examined as a possible source of the differences of result. Typical and comparable leaves were taken at 10:00 A.M. from a tobacco plant which had its starch depleted by seven days of dark starvation and one growing in the greenhouse in the summer sun. Leaf (1.5 gm) tissue was ground in 5 ml of buffer and washed with 10 ml, filtered and centrifuged. The 0.2-ml pellet was suspended in 4 ml and activity was determined over a 55-minute period at 35° C. The results of a typical experiment are given in table II. These results indicate that at least some phosphorylase activity is primarily in the chloroplast in a plant containing starch and actively synthesizing it, but that it appears in the cytoplasm of a plant which does not contain starch. In addition it may be noted that the total activity is much greater in the plant kept under normal conditions.

Table II

Relative Phosphorylase Activity of Supernatant (S) and Residue (R) of Centrifuged Tobacco Leaf Brei from Full Sun and Dark Starved Leaves

Treatment	Corrected activity*/ml R	Activity*/ml S	Ratio, (R/S)
Sun	290	23	13/1
Dark, starved	nil	12	0

* As μgm of P hydrolyzed from G-1-P in 55 min at 35° C.

Table III

Ratios of Phosphorylase Activities* in Plastid Containing Residues (R) and Supernatants (S) Derived from Centrifuged Tobacco Leaves Ground by Various Methods

Treatment	Ratio, (R/S)
Cut into 12 % deionized sucrose with razor blade	37/1
Bruised with pestle in 12 % deionized sucrose	9/1
Ground in mortar with sand and 12 % sucrose in maleic buffer	6/1
Blendorized 5 sec in 12 % deionized sucrose	8/1
Blendorized 45 sec in maleic buffer	5/1

* Activities based on μgm of P hydrolyzed from G-1-P/ml of preparation.

Dark starved vs full sun plants were also compared using more and less drastic methods of "grinding." The activity of dark starved leaves was in the supernatant. In the leaves from the full sun plants, the phosphorus hydrolyzed per unit volume of pellet was always greater than that per unit of supernatant. Data from full sun leaves is presented in table III. The method of "grinding" is given, with the ratio of the concentration of phosphorylase in the pellet to that in the supernatant.

The second group of experiments was based on the following hypothesis. If the phosphorylase can be kept in situ by precipitation and if differential centrifugation can produce a series of pellets having different proportions of various cell fractions, then an analysis of chlorophyll content (chloroplasts), DNA (nucleus) or total protein (cytoplasm) can be compared to an analysis of phosphorylase activity and a correlation sought.

The final procedure which was developed did not include the analysis for DNA as the amount present in leaf tissues is low and insufficient for the methods of analysis that were available. The leaf tissue was taken either from dark starved plants or those growing in the greenhouse and was ground in the blendor with either 12 % sucrose or neutral 60 % saturated $(NH_4)_2SO_4$. The filtered brei was placed in centrifuge tubes and the centrifuge was run at varying speeds for varying lengths of time to obtain various

degrees of sedimentation. Ten such tubes were centrifuged for each experiment. The tubes contained 12 ml initially and after centrifugation the green pellet was resuspended in 4 ml of its own supernatant. Some of the supernatants were included as preparations. One ml of preparation was used to test phosphorylase activity by the method described, the incubation period being 1 hour at 35° C. To a second 1-ml aliquot was added 10 ml of alcohol. The tubes were brought up to the boiling point of the alcohol mixture in a hot water bath to facilitate coagulation of the protein and extraction of the chlorophyll. The coagulum was centrifuged down and the optical density of the supernatant was obtained spectrophotometrically at 664 mμ to compare the relative amounts of chlorophyll. When the residue was from one of the groups where the leaves had been ground in ammonium sulfate solution, the pellet was given an additional washing in 3 to 4 ml of 5 % TCA solution to remove excess salts. After centrifugation the pellet was treated the same as the alcohol precipitated pellet from the groups where the leaves were ground in sucrose. The pellets were suspended in 10 ml of 3.3 *N* NaOH solution and heated at 80° C for 2 to 3 hours to dissolve the pellet. The volume of the tubes was brought back to 10 ml if evaporation had occurred and 0.4 ml of 20 % copper sulfate pentahydrate solution was added to each tube and the tube shaken. The tubes were centrifuged and the optical density of the supernatant was obtained at 530 mμ to give relative amounts of protein. For each group of ten tubes the coefficient of correlation was calculated between hydrolyzed P and chlorophyll content, between hydrolyzed P and protein, and between protein and chlorophyll content. The results in table IV present the one poorest correlation from each group. From 2 to 5 groups were run for each condition. Further repetitions were not made because of the great consistency of the results, and because these results contributed little to the problem under consideration.

In the preparations made with ammonium sulfate, all three factors, protein, chlorophyll content, and phosphorylase activity were highly correlated. This indicates that the three are distributed homogeneously in the same particles of the solid phase so that an aliquot samples all three uniformly.

In the preparations made with sucrose the high correlation between chlorophyll content and protein indicates again that in an aliquot the solid matter contains a homogeneous mixture of these two. The relatively poor correlations between phosphorylase and the other two factors indicates its distribution largely in the soluble fraction of these preparations. The positive correlation of P to C in sun grown plants and the negative correlation in dark starved plants was consistent and may be interpreted as bearing out the data from the other methods; i.e., the negative value of r for dark starved plants may again be explained by the displacement of the solution, having uniform phosphorylase content, by the solid fraction containing the chlorophyll, water insoluble protein and no phosphorylase. The value of r would be positive for sun plants due to some of the phosphorylase being associated with the solid (plastid) fraction.

Inasmuch as material in solution is evenly distributed, correlation should arise out of the differences in the solid fraction. This was checked in one experiment by centrifuging an extra aliquot from each sample and determining protein separately for the supernatant and the residue. The data are given in table V. They were obtained from a run using full sun plants ground in sucrose. The data effectively support the deduction. The negative correlations arise

TABLE V

COEFFICIENT OF CORRELATION BETWEEN PHOSPHORYLASE AND PROTEIN AND BETWEEN CHLOROPHYLL AND PROTEIN IN 1 ML OF A TOTAL TOBACCO LEAF BREI AND IN THE RESIDUE AND SUPERNATANT FROM 1 ML OF THE CENTRIFUGED BREI

FRACTION	COEFFICIENT OF CORRELATION, r	
	PHOSPHORYLASE/PROTEIN	CHLOROPHYLL/PROTEIN
Protein of uncentrifuged brei	0.905	0.963
Protein of residue	0.895	0.943
Protein of supernatant	−0.598	−0.680

TABLE IV

COEFFICIENT OF CORRELATION BETWEEN PHOSPHORYLASE ACTIVITY AND CHLOROPHYLL CONTENT, PHOSPHORYLASE ACTIVITY AND PROTEIN CONTENT, AND BETWEEN CHLOROPHYLL AND PROTEIN CONTENTS IN TOBACCO LEAVES GROUND IN VARIOUS MEDIA FROM PLANTS IN FULL SUN OR DARK STARVED

TREATMENT	GRINDING MEDIUM	COEFFICIENT OF CORRELATION, r *		
		PHOSPHORYLASE/CHLOROPHYLL	PHOSPHORYLASE/PROTEIN	CHLOROPHYLL/PROTEIN
Full sun	12 % Sucrose	0.339	0.337	0.910
Full sun	60 % $(NH_4)_2SO_4$	0.964	0.967	0.883
Dark starved	12 % Sucrose	−0.389	−0.155	0.948
Dark starved	60 % $(NH_4)_2SO_4$	0.915	0.989	0.983

* At 1 % level of significance, r = 0.765.

because as there is more residue, there is less supernatant in one ml.

Discussion

The experimental evidence presented here indicates that both the plastid and the cytoplasm may be normal sites of the enzyme phosphorylase. Although the evidence for phosphorylase in the plastid is based on normal plants and that for phosphorylase in the cytoplasm on dark starved plants, the data do not preclude the possibility that phosphorylase may occur at both sites under either condition.

The data showing phosphorylase distribution may be subject to the following interpretation. In centrifuging, a starch pellet is thrown down beneath the plastid material. Even though this is not used, the remaining material is heavily contaminated with free starch. The association of an enzyme with its substrate is well known and Baum and Gilbert (2) have used this relation to purify phosphorylase by adsorbing it on amylose. The concentration of phosphorylase in the pellet may, therefore, be due to adsorbtion on the starch.

When the phosphorylase content of the plastid fraction is increased by the use of gentle techniques, the increase may result because more starch is retained in the green pellet within the unruptured plastids.

The attraction between starch and phosphorylase provides then an adequate explanation of the data presented here. It fails however to explain why—if phosphorylase is of general distribution—starch is found almost exclusively in the plastids.

Stocking (11) has set forth the 4 requirements for amylose formation.

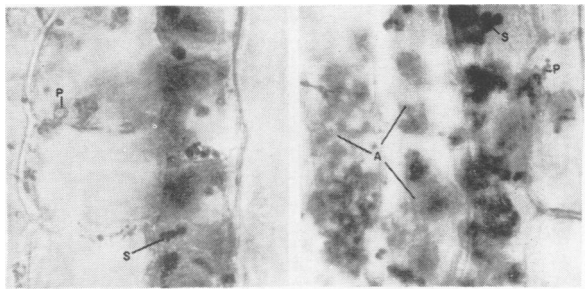
1. pH about 6
2. Active phosphorylase
3. α-D-glucose-1-phosphate as substrate
4. Starter—an α,1-4 glucoside chain to prime the reaction.

1) The range of pH over which phosphorylase is active is wide enough so that a difference of pH between the plastid and cytoplasm would not account for activity at only one site.

2) An inhibition system can be postulated. Known elements are available, as β-amylose inhibits phosphorylase (9) and is itself inhibited by sucrose (4). Stocking has considered inhibition however and shows that it is an unlikely mechanism.

3) The plastid may well be the principal site of starch formation because it is the exclusive site of the formation of the substrate G-1-P. In Calvin's (3) experiments he found G-1-P to be labeled with C-14 after 5 seconds of photosynthesis in $C^{14}O_2$ medium. G-1-P is not reported as participating in reactions in higher plants other than in its equilibrium reactions with G-6-P, and with amylose and phosphate.

Arnon, Whatley and Allen (1) have recently presented evidence for a hypothesis that the chloroplast is a complete synthesizing unit, using the energy of sunlight to manufacture its end products without intermediate cytoplasmic steps. The formation of G-1-P and hence starch in the plastid would be consistent with such a hypothesis.



Figs. 2 and 3. Longitudinal sections of promeristem of tobacco shoot apex showing results of Yin's histological test for phosphorylase. P—Plastid not forming polysaccharide. S—Plastid which has formed starch. A—Amylose network. Fig. 2 (*left*) Incubated with G-1-P. A blue-brown staining polysaccharide has formed within the plastids of the endodermis or starch sheath. Fig. 3 (*right*). Incubated with G-1-P and soluble starch solution. An additional lavender-blue staining network of polysaccharide has formed in the cytoplasm generally.

This explanation still fails to answer one question. If amylose production is limited by the location of G-1-P rather than the location of phosphorylase why does not amylose appear generally when G-1-P is supplied in Yin's test? It often does. In an early report Yin (16) found polysaccharide formation in loci in the cytoplasm. He concluded in a later paper (17) that phosphorylase was exclusively in the plastid.

Stocking (11) reports cytoplasmic starch in leaf sections prepared by using Yin's histological method.

Shaw (10) reported "the most common result was the formation of irregular masses of polysaccharide in the cytoplasmic matrix around the chloroplast." Shaw considers this due to diffusion of polysaccharide out of the plastids.

Paech and Krech (8) find small kernels forming outside of the plastids in their immediate vicinity. They consider that the phosphorylase has leaked out of the plastids.

Dyar (5) reports starch formation in unspecified areas of the cytoplasm and in the nucleus.

The histological test has then yielded results showing the formation of polysaccharide in the cytoplasm supplied with G-1-P. The interpretations of the results have varied. Figures 2 and 3 show some results of the use of the histological test in this laboratory. Londitudinal free-hand sections of the same tobacco stem apex are shown after incubation for 4 hours in G-1-P. Soluble starch solution was added to the G-1-P medium of the one section (fig 3). Figure 2 shows the results of incubation in G-1-P alone. A brownish blue staining (IKI) polysaccharide has formed in the plastids of the endodermis or starch sheath. In figure 3 with soluble starch primer present

the same formation of polysaccharide in the plastids of the endodermis has taken place, and in addition a cytoplasmic polysaccharide has formed. A loose network of bright lavender-blue staining polysaccharide occurs in the cytoplasm of the cells generally.

4) This brings us to a consideration of the requirement for primer. Starch may form only in the plastid in vivo because this is the sole site of primer. This seems unlikely however. Whelan and Bailey (14) have found that α,1-4, tri glucoside units are sufficient to prime the reaction. Polysaccharide of such chain length is readily diffusible and occurs as a product of α-amylose hydrolysis along with diffusible chains of 6 glucose units in length.

Of the four possibilities the one which best fits the experimental evidence is that, that the plastid is the exclusive side of the formation of G-1-P substrate. In metabolically active cells the phosphorylase may become concentrated in the plastids because of the adsorbtion (or other chemical binding) of the enzyme on the starch surface and because of the protective effect of substrate in reducing catabolic destruction of the enzyme. When G-1-P is supplied to the whole cell, polysaccharide may or may not form in the cytoplasm in the period of observation. If either primer or enzyme are in low concentrations in the cytoplasm the rate of synthesis may be so low that stainable polysaccharide is not formed within the duration of the experiment.

Shaw (10) found that he obtained his results only during the summer period. This laboratory has found that seasonal factors affect phosphorylase activity. The work described here was done on vegetative plants, for it appears that once the flower is induced, even prolonged dark starvation will not remove all the starch from leaves. As we are better able to understand phosphorylase it should provide a tool for investigating other plant reactions.

Summary

Evidence is offered that phosphorylase may occur in both the plastid and in the cytoplasm surrounding it. The data indicate a concentration of phosphorylase with the plastids of leaves actively synthesizing starch and a concentration in the cytoplasm of leaves dark starved to remove starch.

The hypothesis is offered that starch is normally found in the plastids because that is the site of formation of the G-1-P substrate.

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