LOCALIZATION OF PHOSPHORYLASE AND OF STARCH FORMATION IN SEEDS

H. C. YIN AND C. N. SUN

(WITH THREE FIGURES)

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Introduction

The discovery of the enzyme phosphorylase provides an important clue toward the solution of the problem of starch transformation in plants (**5**). The enzyme has been purified and its properties and reactions *in vitro* are fairly well understood (**7**) (**11**). Only its physiological role in the living plant remains obscure. One approach to this problem is to investigate the distribution of the enzyme in different tissues and cells to correlate it with the site of normal starch synthesis. For this purpose a histochemical method has been devised and described (**13**). With this method a number of observations have been made on various plant parts. The present report embodies the results of the work on germinating seeds.

Briefly the method for the detection of phosphorylase (**13**) consists of incubating free-hand sections of plant parts in a buffered medium of glucose-l-phosphate (prepared according to the method of HANES (**6**) and of subsequent staining with iodine in potassium iodide. The amount of starch formed is taken as a measure of the phosphorylase activity. Incubation is conducted at 25° C and lasts for a half to one hour, or longer in case the enzymic activity is particularly weak.

Soybean was chosen as the main experimental material because of the absence of detectable starch, which interferes with the test, in the dormant seeds and during the first days of germination. Castor bean which is also practically starch-free was also tried. For comparison, broad bean was used, but in this case the embryo was removed from the cotyledons and starved for several days to rid it of stored starch.

Distribution of Phosphorylase

The results on the distribution of phosphorylase activity are presented diagrammatically in figure 1.

In the soybean (fig. 1, A and B) the most intense reaction occurs in the root cap, less in the root tip and the lateral buds and still less in the hypocotyl and the stem tip. Very little activity is found in the cotyledons and is confined to the surface cells of the middle portion. There are wide differences of reaction intensity in the individual cells. Some cells are practically filled with starch so that the whole cell stains dark with iodine, some cells are partially filled, and still others contain only a few isolated starch grains (fig. 3, A). These differences are represented in the diagrams (fig. 1) by

the size of the dots. It is seen that cells of the first type are mostly found in the root tissues and only that of the last type are present in the cotyledons.

Similar distribution of phosphorylase activity is shown by the embryo

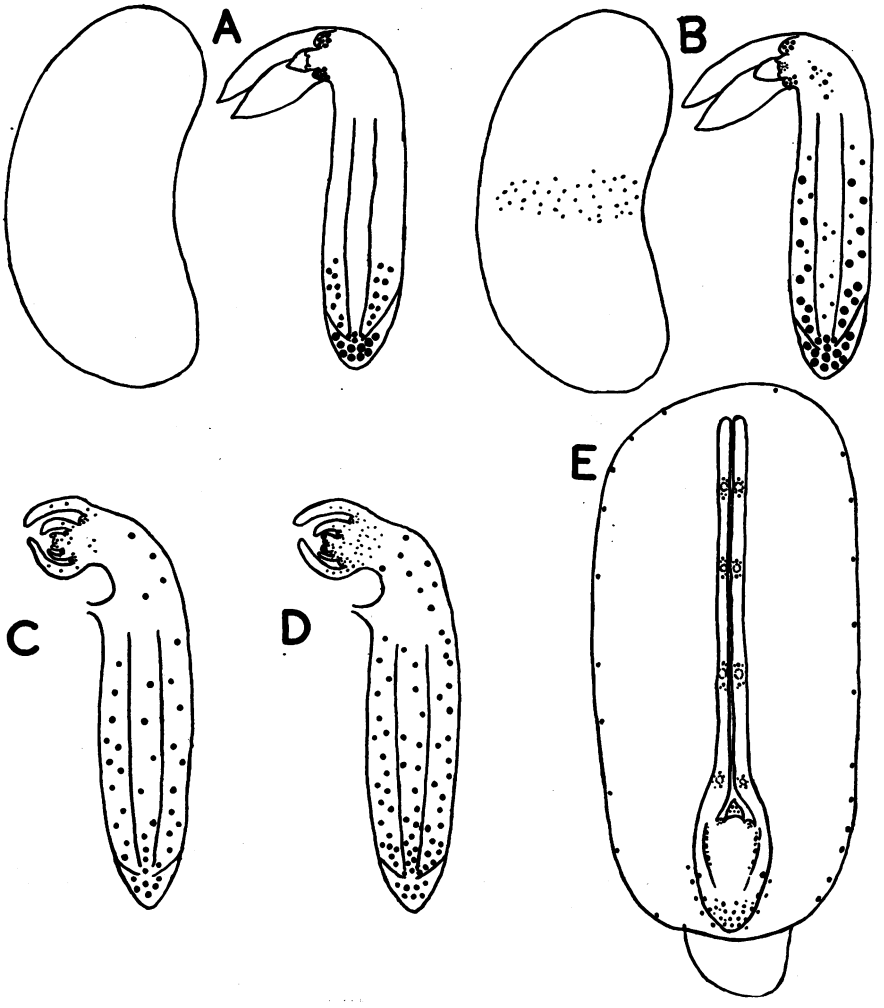

FIG. 1. Distribution of phosphorylase activity in seeds. A and B. cotyledon and embryo of soybean one and two hours after incubation respectively. C and D. embryo of broad bean one and two hours after incubation. E. tissues of castor bean, 12 hours after incubation. Size of dots indicates relative intensity of reaction of the cells, as explained in the text.

of the broad bean (fig. 1, C and D). More intense reaction is located in the root and the hypocotyl than in the young leaves and stem meristems. Unlike the soybean, the individual cells are more uniform in their activity. No experiments have been made with the cotyledons, because they contain much stored starch which renders the test inapplicable.

Very weak activity is found in the tissues of the castor bean. Starch is detectable only after prolonged incubation (3–12 hours). It is mainly confined to the root and stem meristems and near the vascular bundles in the cotyledons. Faint reaction is also shown by isolated cells in the peripheral portions of the endosperm (fig. 1, E).

Localization of Starch Normally Present in the Seeds

In order to ascertain the part played by phosphorylase in the normal physiological process of starch formation in the seeds, the distribution of the enzyme activity is compared with that of starch accumulation in the seeds before and after germination (**10**). Seeds were soaked in water for 12 hours and allowed to germinate at 25° C. Free-hand sections were made at daily intervals, fixed and stained with iodine in potassium iodide. The results are given in figure 2.

In general no starch can be detected in the dormant soybean; it is formed only after germination (fig. 2, A and B). The synthesis of starch appears to be most active in the root cap, less in the root tissues, the hypocotyl and the cotyledons, still less in the stem meristems and very slow in the young leaves. The phenomenon is especially clear in seeds germinated at lower temperatures. The appearance of starch in the cotyledons is earlier by one to two days than in the stem tip. Quantitatively the starch formed in normal development is always less than that formed in sections incubated in glucose-l-phosphate. In no case, are cells found to be filled with starch (fig. 3, B). The starch grains are compound; each leucoplast contains several loci.

A few observations were also made on the broad bean. Both the embryo and the cotyledons contain starch even before germination. Figure 2, D shows that most of the starch is present in the cotyledons and the hypocotyl, less in the stem and root tissues and none in the young leaves. The starch grains are in general very large and few in number. Frequently a single starch grain almost fills up a cell (fig. 3, F).

In the castor bean, starch is found only after germination is far advanced. It is concentrated in the root and the tip portions of the endosperm (fig. 2,E).

From the above results it may be concluded that the site of normal starch formation coincides, in general, with that of phosphorylase activity, indicating that the enzyme plays an important role in the starch synthesis during germination. There are, however, minor discrepancies. For example, in the soybean starch is formed earlier in the cotyledons than in the stem tissues although the latter is more active in phosphorylase reaction, and in the broad bean less starch is found in the root cap and root tip and none in the young leaves as the activity of phosphorylase would demand (compare figs. 1 and 2). Furthermore, in the individual cells, less starch is always present in normal developing seeds than that in sections supplied with glucose-l-phosphate (fig. 3, A and B) and a smaller number of starch grains are visible (fig. 3, E and F). These differences suggest that there are factors other than phosphorylase involved in normal starch formation. The avail-

ability of substrate and the efficiency of the phosphorylating mechanism, for instance, may at times, be limiting (2) (7).

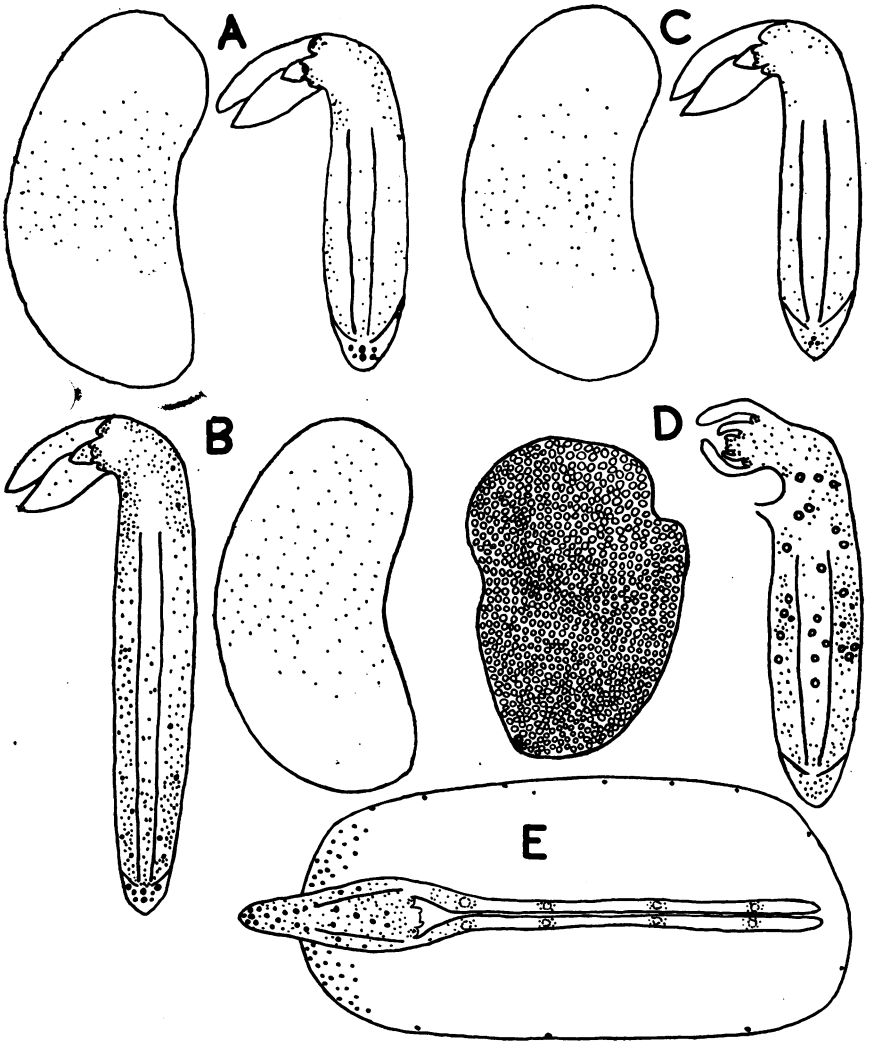

Fig. 2. Starch normally present in seeds. A and B. Cotyledon and embryo of soybean, one and two days after germination. C. Same, after soaking and boiling, or after incubating 24 hours in glucose. D. Cotyledon and embryo of broad bean after soaking for 12 hours in water. E. Castor bean, seven days after germination. Size of dots indicates relative amount of starch present, as in figure 1: circles represent groups of cells with single big starch grains as shown in figure 3, F.

Experiments with Glucose and other Sugars

In order to obtain an insight into the phosphorylating mechanism of the seeds, several experiments were made with sections of soybean incubated in glucose or other sugars (sucrose, galactose, levulose, mannose, etc.) in

phosphate buffers with or without the addition of magnesium sulfate and adenosine-triphosphate. The results are exemplified by figure 2, C. It may be seen that only very little starch can be detected even after prolonged

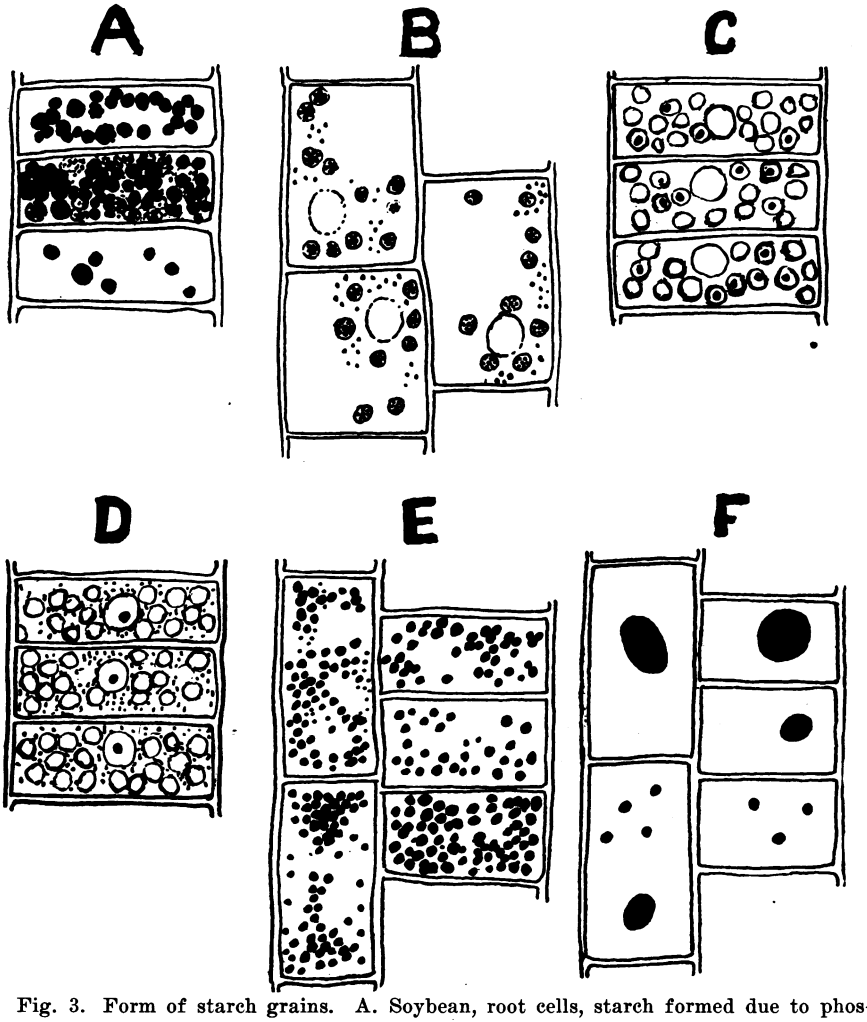

Fig. 3. Form of starch grains. A. Soybean, root cells, starch formed due to phosphorylase activity after incubation in glucose-1-phosphates. B. Same, starch formed after germination. C. Same, starch developed after soaking and boiling. D. Same, leucoplasts (circles) and mitochondria (small dots). E. Broad bean root cells, starch formed due to phosphorylase activity. F. Same, starch normally present before starving.

incubation (24 hours). The appearance of starch is comparatively uniform in both the cotyledon and the embryo. These results show that under the conditions of these experiments, the sections of tissue are unable to utilize or phosphorylate glucose with the formation of starch (**2**). Indeed, it is doubtful that even the little starch observed comes from the added sugar at all.

Some varieties of soybean after soaking and sectioning do show fine particles stainable with iodine (fig. 3, C). Due to their small size, their color cannot be clearly defined under the ordinary microscope. It is questionable that these particles are truly starch. It is also possible that they are the starch grains originally present in the dormant seeds. Even in the varieties that show no detectable starch when dormant, similar particles can often be observed after the seeds are boiled for five minutes or treated with bleaching powder, mineral acids, polyvalent salts, some organic solvents, etc. It is likely, therefore, that small amounts of starch do exist in dormant seeds but because of its colloidal status, it is not readily detected by ordinary methods. Experiments on soybean milk (**8**) have shown that both the emulsifying power and the isoelectric properties of the soybean colloids do change with germination, boiling, and chemical treatments. Contradictory results concerning the presence of starch in dormant soybeans (**9**) also encourage a similar explanation.

For these reasons, the very fine particles of starch found in soybean sections incubated in glucose or sucrose and that in the just germinated seed may be the starch originally present but masked in the dormant seed.

Cytological Observations

To study the localization of phosphorylase activity within the individual cells, thin free-hand sections were made and incubated for short intervals in glucose-l-phosphate (fig. 3, A). The observations were compared with paraffin sections of the same tissue doubly stained to bring out the cellular constituents (fig. 3, D). The results show that the enzyme is present exclusively in the leucoplasts. No reaction can be found in the nucleus or in the mitochondria (**1**) (**3**). Each plastid contains several loci of activity. After long incubation, these loci fuse to form a compound starch grain. In the normal germinating seeds only a part of the leucoplasts develop into starch grains due perhaps to lack of substrate (fig. 3, B).

Conclusions and Discussion

From the present investigation the conclusion may be drawn that phosphorylase plays a prominent role in the physiological starch synthesis in germinating seeds. Evidence comes from two sources. Firstly, the distribution of the enzymatic activity coincides in general with that of normal starch formation. Secondly, the reaction is exclusively confined to the plastids where starch is normally accumulated.

The localization of phosphorylase activity and of starch formation in the root and stem tips indicates that the enzyme is connected with active growth and that starch is a temporary food reserve of the active developing cells. It is readily formed when the raw material is available and quickly consumed during starvation. Even oily seeds contain phosphorylase and accumulate starch in their growing regions. The presence of the enzyme in the root cap shows that this organ is not simply a protective structure but is an active

center of metabolic changes. The concentration of other enzymes, e.g., phosphatase (**4**) in this region also supports this contention. In the castor bean, phosphorylase is found near the vascular tissues indicating that the enzyme has a probable function in translocation (**12**). Unfortunately the present method is not applicable to plant parts that contain stored starch so that the role of the enzyme in the storage organs cannot be similarly investigated.

Unlike the other enzymes so far studied cytologically (**1**), phosphorylase is shown to be exclusively confined to the plastids. In the germinating seed it is found in the leucoplasts, which may turn green in light. In the leaves (**14**) it is localized in the chloroplasts of both the guard cells and the mesophyll. These results supply a conclusive mechanism by which starch is formed in the plastids.

The minor descrepancies between the histological distribution of phosphorylase reaction and that of starch formation indicate that there are other factors which may be limiting in some tissues. It may be the availability of substrate or the activity of the phosphorylating mechanisms (**2**) or other conditions of the tissues (**7**). The failure to obtain starch formation in sections incubated in glucose with or without adenosine-triphosphate may mean that the former is not the normal raw material or the latter does not permeate the tissue. The evidence is, however, not conclusive and optimal experimental conditions may not have been obtained.

Summary

The distribution of phosphorylase activity and of starch formation in the soybean and other seeds was studied histochemically by the staining techniques. Phosphorylase reaction is most intense in the root cap, less in the root tip and the lateral buds, still less in the hypocotyl and the stem tip and very little in the cotyledons. Upon germination, starch is found first in the young leaves. The general coincidence of the sites of phosphorylase activity with those of starch formation indicates that the enzyme is responsible for the synthesis of starch during germination. Minor discrepancies are perhaps due to the availability of the substrate, the efficiency of the phosphorylating mechanisms or other conditions in the tissues. Cytological observations show that phosphorylase is exclusively localized in the plastids. These observations further confirm the contention that phosphorylase is the normal physiological mechanism of starch formation.

BOTANY DEPARTMENT,
NATIONAL PEKING UNIVERSITY,
PEKING, CHINA.

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