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Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds[1] I. An Analytical Study

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Abstract. **Time-sequence analyses of carbohydrate breakdown in germinating rice seeds shows that a rapid breakdown of starch reserve in endosperm starts after about 4 days of germination. Although the major soluble carbohydrate in the dry seed is sucrose, a marked increase in the production of glucose and maltooligosaccharides accompanies the breakdown of starch. Maltotriose was found to constitute the greatest portion of the oligosaccharides throughout the germination stage. α-Amylase activities were found to parallel the pattern of starch breakdown. Assays for phosphorylase activity showed that this enzyme may account for much smaller amounts of starch breakdown per grain, as compared to the amounts hydrolyzed by α-amylase. There was a transient decline in the content of sucrose in the initial 4 days of seed germination, followed by the gradual increase in later germination stages. During the entire germination stage, sucrose synthetase activity was not detected in the endosperm, although appreciable enzyme activity was present in the growing shoot tissues as well as in the frozen rice seeds harvested at the mid-milky stage. We propose the predominant formation of glucose from starch reserves in the endosperm by the action of α-amylase and accompanying hydrolytic enzyme(s) and that this sugar is eventually mobilized to the growing tissues, shoots or roots.**

Recent biochemical studies have elucidated the mechanism of carbohydrate metabolism in fatty seeds with particular emphasis on the fat-carbohydrate conversion and its control (3). There have been many classical works on the breakdown of reserve polysaccharides in starch-bearing cereal seeds (10). It is generally accepted that the amylolytic breakdown of starch may account for the major metabolism of carbohydrate in the endosperm tissues of these plants (1, 20, 21). A rapid increase in the α-amylase activities in the endosperm of germinating barley seeds was observed many years ago, and current biochemical studies have demonstrated the mechanism of the gibberellic acid-induced synthesis of α-amylase in the aleurone layers of cereal grains, resulting in the hydrolysis of the starch reserve in the endosperm tissues (7, 16, 17, 23–25). Germinating rice seeds are suitable material for investigating the enzymic mechanism of carbohydrate breakdown in starch-bearing tissues. This study is concerned with the mechanism of starch breakdown in rice endosperm, using both chemical and enzymic analyses. The significance of the data is discussed in terms of the physiology of germinating rice seeds.

Materials and Methods

Rice Seed Germination. Seeds of the rice plant, *Oryzae sativa* L. var. Fujiminori, were soaked in a disinfectant solution (0.1 % Takeda-Mer, Takeda Pharmaseutical Co. Ltd) for 1 hr at room temperature. After thoroughly rinsing the seeds in running water, they were sowed on a wet filter paper placed in a deep Petri dish (d = 20 cm). The seeds were then germinated in a temperature-controlled chamber (30°) in the dark. In order to avoid fungus infection during germination, all seeds in one dish were used for a single analysis. For the gibberellic acid (GA) treatment, rice seeds were soaked in GA (1 μg/l) solution (Kyowa Hakko Co. Ltd.), and germinated as described above, except filter paper in a Petri dish was wet with GA of a concn. of 1 μg/l.

Analysis of Carbohydrate. Growing embryonic tissues (scutellum, roots, and shoots) were carefully removed with razor from rice seeds. Then seed coat-bearing endosperm tissues were analyzed for carbohydrates at different stages of germination in the following method. A portion of the tissues was

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heated at 90° for 30 min and then dried at 60° for 4 hr (2). The dried grains were finely ground and a subsample was hydrolyzed with 0.7 N HCl (100°, 60 min). After neutralization of the hydrolysate, the total sugar content (starch plus total soluble sugars) was assayed by the Somogyi-Nelson method (15). Separately, another portion of the tissues was ground with 5 vol of 0.6 N $HClO_4$:ethanol (1:1, v/v) in a mortar (2–5°). The whole homogenate was strained through 4 layers of cheesecloth. The extraction was repeated twice and centrifuged to collect supernatant fraction. The extract was neutralized with 5 N KOH and after removing the precipitable material by centrifugation, the supernatant was concentrated *in vacuo* at 30 to 35°. After desalting with IR-120 and IR-4B resins the clear supernatant was applied to a column of Dowex-1 (borate-form, 0.8 × 5.5 cm) for the analysis of individual sugar components (12). An aliquot was simultaneously used for determining the content of total soluble sugars by the phenol-H_2SO_4 method (9). Both sucrose and fructose were assayed by the resorcinol method (18). Starch content was calculated by subtracting the content of total soluble sugars from the total sugar content determined by the Somogyi's method.

Enzyme Assays. Preparation of Crude Enzyme. Ten g of rice seeds were ground with 20 ml of 0.01 M tris buffer (pH 7.5) in a chilled mortar and the whole homogenate was squeezed through 2 layers of cheesecloth. The supernatant fraction obtained by the centrifugation (10,000 rpm, 15 min, 0°) is referred to as the crude extract which was used for most enzyme assays. Usually 13 ml of clear supernatant were obtained. In order to convert the enzyme activity to unit enzyme activity per grain, the number of grains was estimated from a calibration curve, grain numbers *vs.* fresh weight, at each germination stage. From this curve, for example, 1 ml extract was found to be equivalent to 24 grains at 16 days.

α-Amylase. A portion of the crude extract was diluted 100 fold with 0.01 M tris buffer (pH 7.5) and assayed for α-amylase activity following the method of Shuster and Gifford (19). One ml of soluble starch solution (67 μg dissolved in 100 ml 0.06 M KH_2PO_4) and 0.5 ml H_2O were preincubated at 25° for 10 min, after which time was added 0.5 ml of the enzyme solution to start the reaction. At various times (0, 5, 10, 15, and 20 min), 1.0 ml of KI-I_2 reagent was added. The mixture was diluted by adding 5.0 ml of H_2O and the decrease in optical density at 620 mμ was measured. Boiled enzyme preparation served as a control. As a quantitative measure of relative enzyme activity, unit enzyme activities (mg starch broken down/grain) were calculated from the analytical data of 5 min incubation at each germination stage.

For comparison, the action of bacterial α-amylase on soluble starch was determined. Two-tenth of a ml of 1 % soluble starch was hydrolyzed with 0.1 ml of 0.01 % crystalline bacterial α-amylase (Nagase Sangyo Co. Ltd.) dissolved in 0.01 M acetate buffer (pH 6.9) at 25°. Aliquots (20 μl) of the reaction mixture were withdrawn at 10, 30, and 60 min and the products were analyzed by paper chromatography using the solvent system as described below.

Phosphorylase. The activity of this enzyme was determined according to the method of Whelan (26), using the reaction system for amylose synthesis. The reaction mixture contained: citrate buffer (pH 6.0), 750 μmoles; glucose-1-P, 100 μmoles; 0.2 ml of 5 % soluble starch; and 1.0 ml of crude enzyme extract. The liberated P_i in the 30 min incubation was determined by the method of Nakamura (14). In this case also, the relative enzyme activity (μg starch broken down/grain) was computed from the analytical data.

Sucrose Synthetase. Crude enzyme extracts were prepared as above from germinating rice endosperm, and also from growing rice shoots at the appropriate stage or from frozen rice seeds at the mid-milky stage (harvested in 1967). The assay mixture contained (in μmoles); tris buffer (pH 7.5) 50; fructose or fructose-6-P, 2; UDP-glucose, 1; $MgCl_2$, 1.25; Na-molybdate, 10; and 0.1 ml of enzyme preparation in a total volume of 0.5 ml. The mixture was incubated at 35° for 30 min, and 0.2 ml aliquots were withdrawn for the determination of the sucrose formed by the resorcinol method (18).

Paper Chromatographic Analysis of Sugar Components. Ten g of rice seed endosperm devoid of shoots and roots were ground with 20 ml of 95 % ethanol and boiled in a water bath for 20 min. The extracts were clarified by centrifugation. Ten μl aliquots of the supernatant were applied to paper chromatographs which were developed in a descending manner for 40 hr, with 1-butanol:pyridine:H_2O (6:4:3, v/v). The sugars were located by the reagent used by Trevelyan *et al.* (22).

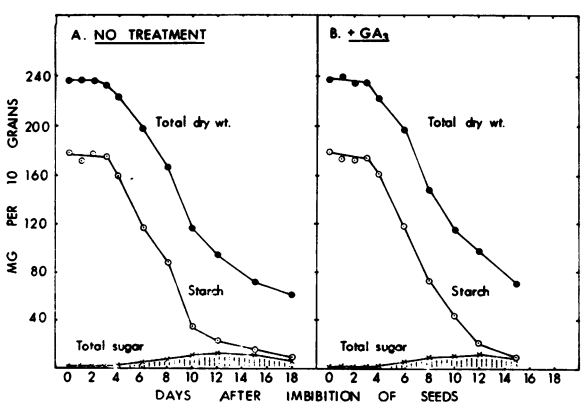

FIG. 1. Changes of total dry weight, starch, and total soluble sugar in germinating rice seed endosperm. A) Without gibberellic acid treatment. B) With gibberellic acid (1 μg/l) treatment. Details of analytical methods are described in text. All values are plotted as mg per 10 grains.

Table I. *Effect of Gibberellic Acid Treatment on Shoot Growth of Germinating Rice Seeds*

Rice seeds were treated with GA (1 μg/1) as explained in text and germinated at 30°. Values are average length of 10 determinations.

Time after seed imbibition	Length of shoot	
	No GA	+ GA
Days	*mm*	*mm*
4	7	11
8	17	65
12	112	151
15	123	180

Results

The unit dry weight of seed (g per 10 grains) does not change during the initial 4 days of germination (fig 1). However, the dry weights start to decline abruptly soon after the 4 days. At much later stages (15–18 days) the dry weight is about one-fifth of that at the initial stage. It can be seen that the decrease in starch closely parallels the decrease in seed dry weight. The onset of breakdown of reserve polysaccharide appears to occur after about 4 days of seed imbibition. Most of the reserve polysaccharide is broken down and translocated in the later stages, with the seed coat accounting for the major part of the total dry weight. The content of the soluble carbohydrates comprises a small proportion in the initial stages, up to about the 4 days. From then on, there is a gradual increase in soluble carbohydrate accounting for the major portions of the total carbohydrate in the seed endosperm. GA-treated rice seeds showed essentially the same patterns of both dry weight decrease as well as the starch breakdown during germination. However, results presented in table I clearly show the enhancement of shoot length due to the GA-treatment, in accord with the observation of other workers (5, 6).

The changes in individual sugar constituents in rice endosperm during germination are presented in figure 2. The major soluble sugar component of the dry seed is sucrose, and a very small amount of glucose is detectable. As germination proceeds, there is an accumulation of glucose and fructose; during later stages, the accumulation of glucose becomes more marked. Maltose-series oligosaccharides, emerging just after sucrose, also showed a gradual increase from 6 to 12 days of germination.

The changes in the amounts of the individual sugar components, sucrose, glucose, fructose, and maltooligosaccharides (mg/10 grains) during germination, are presented in figure 3. The content of the total soluble carbohydrate determined by the phenol-H_2SO_4 method is also given in the figure. The following points emerge. A) A rapid increase in the quantity of glucose starts from third to fourth day, reaching maximum at about the twelfth day. B) Maltooligosaccharides show a similar pattern to that of glucose, but levels of the former are much lower than those of glucose. C) There is a discrepancy between the content of total sugar and the sum of the individual sugar components (marked by the shadowed areas on the graphs). This difference represents the formation of maltooligosaccharides of large molecular weight. Their formation starts after about 4 days of seed imbibition. D) The content of sucrose, the major soluble sugar component in the dry seed, declines to about half of the initial level at about the 4 days, gradually increases afterwards and levels off at later stages. E) GA-treatment of rice

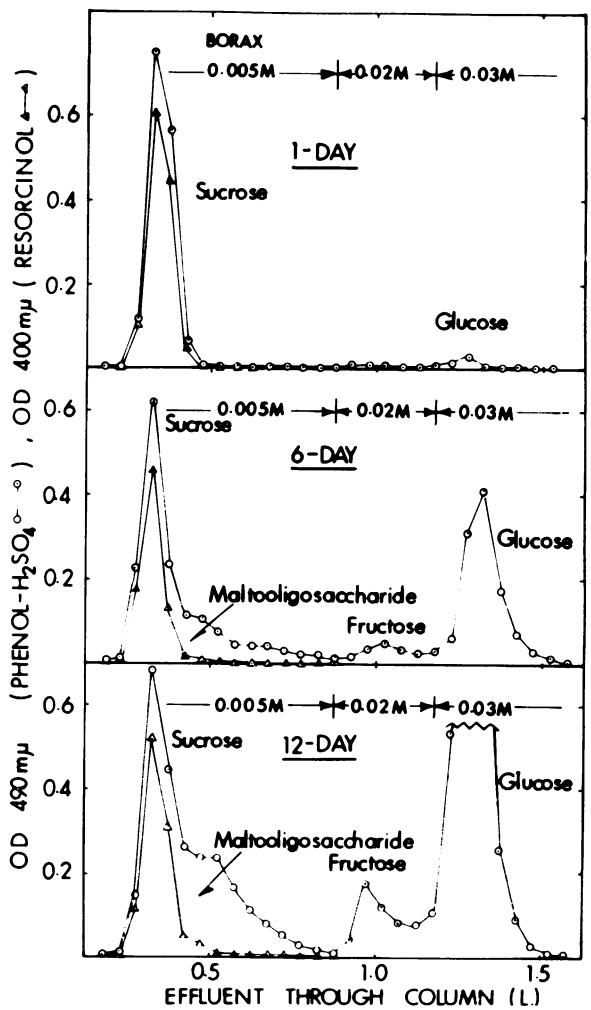

FIG. 2. Dowex-1 ion exchange column chromatography of soluble sugar fraction isolated from germinating rice seed endosperm. Each of 2 g fresh weight (1 day) and 1 g fresh weight (6 and 12 days) of seed coat-bearing rice endosperm was used for extracting soluble sugar fraction following the method described in text. For the colorimetric analyses of sugars, each of 1 ml and 2 ml effluent was used for phenol-H_2SO_4 method and resorcinol method respectively.

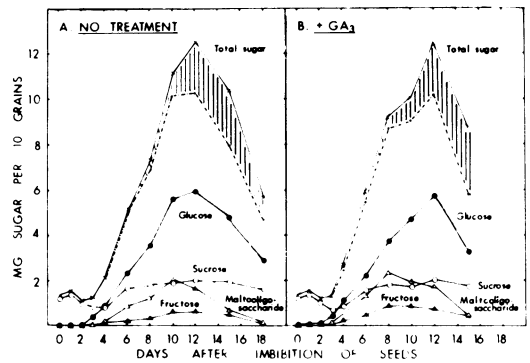

FIG. 3. Changes of individual sugar components in germinating rice seed endosperm. A) Without gibberellic acid treatment, B) With gibberellic acid treatment. Details of analytical methods are described in text. All values are plotted as mg sugar per 10 grains. Shadowed area is difference between the content of total sugar (acid hydrolyzate) and the sum of the individual sugar components determined by ion-exchange column.

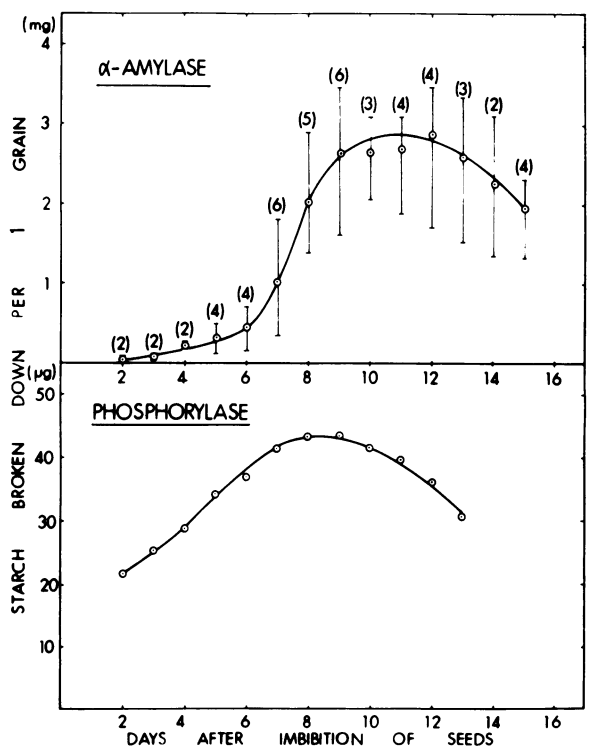

FIG. 4. α-Amylase and phosphorylase activities of extracts of germinating rice seed endosperm. Assay methods of enzyme activities are described in text. In each case, the enzyme activities are expressed as (α-amylase) mg starch broken down per 5 min per grain and (phosphorylase) μg starch broken down per 30 min per grain. In upper figure of the α-amylase activity curve, the points are the average values obtained in the stated numbers of experiments, and the vertical bars show the extent of the variation encountered

seeds does not affect the pattern of carbohydrate breakdown (fig 3b).

The patterns observed in (A), (B), and (C) above may be closely linked to the formation of α-amylase in the germinating rice seeds. We herefore examined the patterns of α-amylase and phosphorylase activities during germination (fig 4). We find from repeated analyses that the activity of α-amylase is very low at the initial stages, up to about 4 days, and then increases abruptly after this lag-period. Maximal enzyme activity was seen at about 10 days and did not decline appreciably even at the very late stages. Phosphorylase activity,

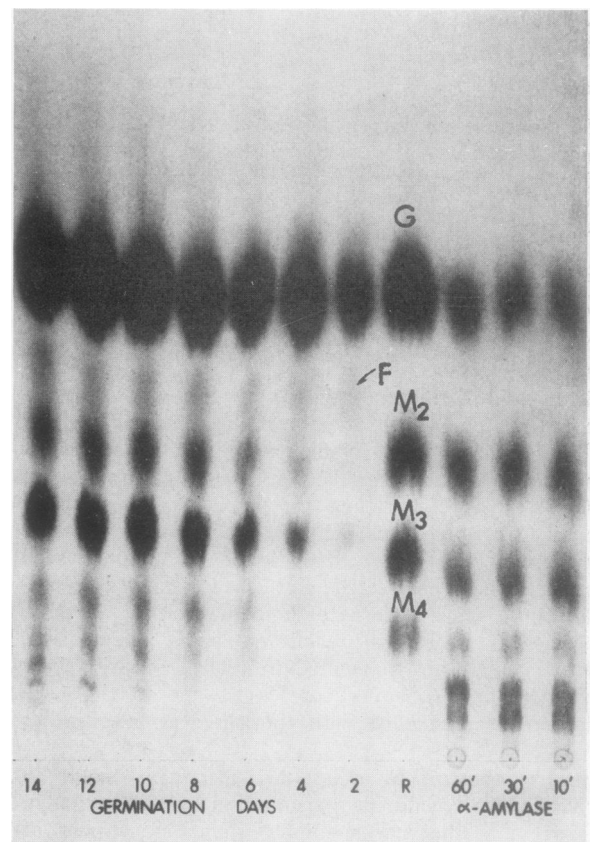

FIG. 5. Paper chromatography analysis of reducing sugar components in germinating rice seed endosperm. Details of experimental procedures are described in text. Germination days refer to the days after seed imbibition. Reference (R) samples are: G, glucose; M_2, maltose, M_3, maltotriose, and M_4, maltotetraose.

expressed as μg starch broken down per 1 grain, also shows a peak at about 8 to 10 days. Although the phosphorylase was assayed in the synthetic direction, it seems evident that the quantity of starch broken down in the endosperm tissues by α-amylase is much greater than that by phosphorylase, indicating the more important role of α-amylase in the

Table 11. *Sucrose Synthetase Activities in Germinating Rice Seeds*

Experimental details are described in text. Reaction mixture contains (in μmoles); tris buffer (pH 7.5), 50; either fructose or fructose-6-P, 2; UDP-glucose, 1; $MgCl_2$, 1.25; Na-molybdate, 10; and 0.1 ml of enzyme preparation in a total volume of 0.5 ml.

Time after seed imbibition	Source of enzyme	System	Sucrose formed
			μmoles per 30 min per ml enzyme
2	Endosperm	UDP-glucose + fructose	0
		UDP-glucose + fructose 6-P	0
4	Endosperm	UDP-glucose + fructose	0
		UDP-glucose + fructose 6-P	0
6	Endosperm	UDP-glucose + fructose	0
		UDP-glucose + fructose 6-P	0
	Shoot	UDP-glucose + fructose	0.14
		UDP-glucose + fructose 6-P	0.14
8	Endosperm	UDP-glucose + fructose	0
		UDP-glucose + fructose 6-P	0
	Shoot	UDP-glucose + fructose	0.28
		UDP-glucose + fructose 6-P	0.33
9	Endosperm	UDP-glucose + fructose	trace
		UDP-glucose + fructose 6-P	trace
	Shoot	UDP-glucose + fructose	0.64
		UDP-glucose + fructose 6-P	0.39
10	Endosperm	UDP-glucose + fructose	0
		UDP-glucose + fructose 6-P	0
	Shoot	UDP-glucose + fructose	0.32
		UDP-glucose + fructose 6-P	0.31
14	Endosperm	UDP-glucose + fructose	0
		UDP-glucose + fructose 6-P	0
...	Frozen grains at mid-milky stage	UDP-glucose + fructose	0.20
		UDP-glucose + fructose 6-P	0.38

breakdown of starch reserve in the germinating rice seeds. It is to be noted in this connection that sugar phosphates were hardly detectable in the soluble sugar fractions separable by a Dowex-I ion exchange column chromatography.

The results of paper chromatographic analyses of the reducing sugar components shown in figure 5 further support the possibility that α-amylase is the enzyme mainly responsible for the changing patterns of carbohydrates in seeds after 4 days of germination. The chromatogram of oligosaccharides and glucose from seeds after 4 days germination mimics, essentially, the pattern obtained by the action of bacterial α-amylase on soluble starch. However, it will be noted that throughout the entire germination period the content of maltotriose was found to be markedly higher compared to maltose.

It will be recalled that after an initial decrease in sucrose content during germination, there was a gradual increase in the content of this sugar after an initial decrease in sucrose content during germination (*cf.* fig 3). One may, therefore, postulate that there is a rapid utilization of sucrose in endosperm which is the major respirable carbohydrate in the embryo in the initial few days of seed germination. Later on, resynthesis of sucrose may occur to keep pace with its great demand, as a transport sugar for the growing tissues (shoots and roots) through the scutellum. However, throughout the entire period of seed germination, we have been unable to detect activity for sucrose (sucrose-P)-synthetase in extracts of rice seed endosperm (table II). Further purification of the enzyme extract by $(NH_4)_2SO_4$ precipitation did not yield any detectable activity. The loss of enzyme activity during the preparative step cannot be totally excluded, although a similarly prepared crude extract from ripening rice seeds, harvested in 1967 and stored in a deep freeze, still exhibited marked enzyme activity. Moreover, the crude extract from growing shoots of rice seeds showed appreciable sucrose-synthetase activity. The young shoots were reported to be the best source of the enzyme by Cardini *et al.* (8).

Discussion

Questions have been raised about the *in vivo* role of amylases in germinating plants (1). The present data obtained from both analytical and enzymic studies point to the predominant contribution of α-amylase in the carbohydrate metabolism in the endosperm of rice seeds. Our basic findings are essentially in accord with the recent concept on the inducible formation of α-amylase in cereal grains at the onset of germination (23–25, 27). Although the exogeneous addition of GA did not cause any noticeable change in the pattern of carbohydrate breakdown, it is highly probable that the synthesis of α-amylase in the aleurone layers of rice seeds induced by the hormone is analogous to other cereal seeds. The lack of response may not be too surprising since the *in vivo* supply of the hormone in rice seeds may have been sufficient to induce the synthesis of α-amylase.

The phosphorolytic breakdown of reserve starch has often been thought to be an important mechanism of carbohydrate breakdown in germinating seeds (1, 20, 21). This notion has been derived basically from an analogy to the carbohydrate metabolism in animal tissues (11). Furthermore, the mechanism of starch synthesis mediated by ADP-glucose (UDP-glucose)-starch transglucosylase may support this presumption (1, 13). Although the results of *in vitro* enzyme analysis may not be directly applicable to the carbohydrate metabolism occurring *in vivo*, our analytical data on α-amylase and phosphorylase activities appear to relegate the utilization of starch reserves *via* the latter pathway to a minor role.

Although the mode of action of α-amylase varies from one enzyme source to another, it is generally found that a large quantity of maltose and limit dextrins of low molecular weight plus a small amount of glucose and maltotriose are formed by the action of the enzyme (4). Paper chromatography of the products of enzyme digestion showed that crystalline bacterial α-amylase produces a relatively large amount of glucose from soluble starch. On the other hand, the time-course analyses of sugars in germinating endosperm showed a marked accumulation of glucose as well as maltotriose. The content of the latter was found to be much greater than maltose. Certainly there is no reason to assume that only α-amylase is responsible for the starch hydrolysis (17). It is known in this connection that the addition of GA to the barley endosperm resulted in an enhancement of the maltase activity (7).

It is generally agreed that sucrose is the major form of sugar translocated in plants. The results of sugar analyses in ripening rice seeds [*cf.* figs 1 and 2 of (2)] well support this assumption. However, the data reported in the present paper on germinating seeds contradict this view. The failure to detect sucrose-synthetase activities in the rice endosperm throughout the germination stage and the predominant accumulation of glucose might indicate a unique metabolic pattern in the endosperm. Naturally, one cannot exclude (A) the loss of sucrose synthetase activity during the process of isolating crude extract from rice endosperm, (B) the presence of a highly active invertase in the tissue, and (C) an unknown route of sucrose synthesis, not involving UDP-glucose. Another possible interpretation of our analytical data would be that glucose is the major form of sugar translocated from endosperm to other growing organs. The morphological unit of septum existing between endosperm and embryonic tissues (shoots or roots) is scutellum, and it is conceivable that glucose from the endosperm is converted into sucrose in that organ and that transport to the root and shoot is in the form of sucrose.

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