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METABOLISM OF RAFFINOSE IN COTTON SEEDS

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Abstract—Raffinose and stachyose, in addition to sucrose, are present in the resting seeds of cotton, and on germination, raffinose and stachyose decreased and then disappeared completely, while sucrose and reducing sugars increased to a considerable extent. No free galactose or any other oligosaccharide containing galactose was detected throughout germination. α-galactosidase activity was fairly high in resting seeds and rose in soaked and germinated seeds, while invertase activity was very low in resting and soaked seeds but increased markedly in germinated seedlings. By infiltrating D-galactose, it was shown that an effective mechanism for galactose utilization existed in the cotyledons of germinated seedling. Galactokinase activity was, however, very low in the germinated seedling. Aerobic conditions were found to be necessary not only for germination but also for disappearance of raffinose and utilization of galactose. Raffinose appeared in the seed during ripening, but was not detected in any other parts of the plant body. Raffinose was also formed in unripe seeds when they were detached from cotton boll and air-dried, suggesting the existence of a system for the formation of raffinose inside the unripe seed. Stachyose was formed from raffinose by transgalactosidation.

INTRODUCTION

IN STUDIES on the carbohydrates of Japanese black pine[1] (*Pinus Thunbergii*), raffinose and stachyose were found by paper chromatography to be the main sugar components in resting seeds. As soon as the seeds began to germinate and the tips of young roots appeared, the concentration of these two oligosaccharides decreased rapidly finally to zero. In young seedlings, no trace of raffinose or stachyose was detected, instead a considerable amount of sucrose was found. With the progress of germination, reducing sugars appeared and increased, but neither free galactose nor any oligosaccharide comprising galactose could be detected. Biochemical investigation of the carbohydrates in needles, branches, trunks, etc.,[2] showed that no raffinose and no stachyose were present in any other part of the pine tree except seeds and needles in winter time.

The results obtained with pine suggested the possibility that raffinose and stachyose might be present as reserve carbohydrates in the seeds of other plants. Investigation of some fatty and starchy seeds[3] showed that these oligosaccharides had a fairly wide distribution in higher plants, although their occurrence was restricted mainly to ripe seeds. In addition, it became clear that the interesting behaviour of raffinose and stachyose during the germination of pine seeds was a general phenomenon with germination of seeds which contained these sugars. In several kinds of seeds,[3] for example, Japanese red pine (*Pinus densiflora*), sunflower, *Robinia pseudacacia*, and sesame,* raffinose disappeared during germination as rapidly as in black pine.

Among fatty seeds, cotton seed is well known as one that contains much raffinose. Experiments on the metabolism of raffinose during the process of germination and ripening of seeds were therefore carried out with cotton seeds in more detail than had been done with some others. Raffinose was found to be metabolized in germination of cotton seed in exactly the same way as in the seeds of pines and other plants, no galactose being detected throughout all stages of germination, although α-galactosidase activity was conspicuous

* The oligosaccharides contained in sesame seeds do not belong to the raffinose series, but to the planteose series.[3]

[1] S. HATTORI and T. SHIROYA, *Arch. Biochem. Biophys.* **34**, 121 (1951).
[2] S. HATTORI, H. MATSUDA, T. SHIROYA and K. NAKAHARA, *Z. Bot.* **43**, 125 (1955).
[3] M. HASEGAWA, T. TAKAYAMA and T. SHIROYA, *Kagaku* **21**, 593 (1951). *Cf. Chem. Abst.* **48**, 5297b (1954).

in both resting and germinated seeds. However, it was found that a system for the utilization of galactose existed in the cotyledons of germinated seedlings, and the galactose liberated from raffinose by the action of α-galactosidase could be metabolized through this system. It was also found that a mechanism for the synthesis of raffinose existed in seeds at the stage of ripening. No raffinose appeared in any other parts of the plant except the seed, and even here it occurred only when the seed was ripe. These results suggest the possibility that raffinose plays an important role in the metabolism of seeds during germination and ripening.

Recently it was found with white pine[4] (*Pinus strobus*) that when $^{14}CO_2$ was fed to the needles the labelled sugars in the phloem 8 hr later were sucrose and raffinose. Raffinose and other oligosaccharides of its series have been found in the sieve tube exudate of trees, and, since in some trees most (white ash) or part (elm, linden) of the sieve tube sugars were in the form of raffinose and stachyose,[5] it is probable that raffinose as well as sucrose takes part in the translocation of photosynthetic products from leaf to root via the stem.

RESULTS

Table 1 shows the sugars in ripe cotton seeds at various stages of germination. In resting seeds, two oligosaccharides beside sucrose were found to exist and isolated by large-scale chromatography and identified as raffinose and stachyose, respectively. Almost no reducing sugars were present in resting seeds. With the progress of germination, the two oligosaccharides decreased and then disappeared. No oligosaccharide other than raffinose and stachyose containing galactose could be detected either in resting or germinated seeds. Sucrose and reducing sugars increased in quantity with the progress of germination. Throughout all stages, no free galactose was found in the aldohexose fraction isolated by large-scale chromatography.

TABLE 1. SUGARS IN VARIOUS STAGES OF GERMINATION OF RIPE (S′) COTTON SEED AND RESPECTIVE SEEDLINGS[1]

Sugar	Resting (*U*)[2]	Soaked[3] (*G*)[2]	Germinated[4] (*U*)[2] 0 cm	(*G*) 0·1 cm[5]	(*F*) 1 cm	(*F*) 5 cm
Fructose	+	±	+	++	++	++
Glucose	±	±	±	+	++	++
Sucrose	++	+	++	+	+++	+++
Raffinose	+++	++	+++	+++	±	−
Stachyose	+	±	+	+	±	−

(+++: 5–10, ++: 2–5, +: 0·5–2, ±: 0·25–0·5, −: <0·25 mg/g)[6]

[1] The number of seeds was made equal for comparison. [2] *U* = ungerminated; *G* = just germinated; *F* = fully germinated. [3] Soaked for 4 days. [4] Soaked 2 days and on wet filter paper for 2 days. [5] Length of radicle. [6] Corresponding to 1 g of resting seed.

In seeds which had lost viability and could not germinate, not only raffinose and stachyose but also the other sugars were found almost unchanged, as may be seen by comparison with those in resting seeds (Table 1).

In the soaked seeds, in which a fairly high activity of α-galactosidase (α-D-galactoside galactohydrolase) was detected (Table 2), raffinose and stachyose did not disappear (Table 1) and no free galactose was detected. The same occurred in the seeds placed on wet paper under diminished pressure (about 0·1 atm) for 4 days. When these ungerminated

[4] T. SHIROYA, G. KROTKOV and C. D. NELSON, Unpublished data.
[5] M. H. ZIMMERMANN, *Plant physiol.* **32**, 288, 399 (1957), **33**, 213 (1958).

but wet seeds were placed on wet filter paper under aerobic conditions, they began to germinate, the radicle stretching noticeably, and at the same time raffinose and stachyose disappeared rapidly and completely as in normal germination. In this case free galactose could not be detected throughout all stages of germination.

TABLE 2. PATTERN OF GLYCOSIDASE AND TRANSGLYCOSIDASE ACTIVITY IN THE COTTON SEED DURING THE PROCESS OF GERMINATION AND RIPENING

Enzyme	Substrate	Unripe seed (*S'*) Fresh	Unripe seed (*S'*) Air dried	Unripe seed (*S'*) Resting	Ripe seed (*S*) Soaked[1]	Ripe seed (*S*) Germinated[1]
α-glucosidase	Maltose	+	—	±		+
β-glucosidase	Salicin	—	—	—		—
α-galactosidase	Raffinose Melibiose	+	±	+	+	+
β-galactosidase	Lactose	+	+	+		+
β-fructofuranosidase	Sucrose	±	+	±	+	+
α-transglucosidase	Maltose	+	+	±	—	+
α-transgalactosidase	Raffinose Melibiose	±	±	±		+
β-transgalactosidase	Lactose			+		+
Transfructosidase	Sucrose	—	+	—	—	—

+: 50, +: 20–50, ±: 5–20, ±: 2–5, —: <2% of hydrolysis or transfer in 24 hr

Reaction mixture: Substrate (2 *M*) 0·1 ml, enzyme (10%) 0·3 ml, buffer (acetate pH 6·0 or 4·0) 0·1 ml at 30°C.

[1] As for Table 1.

Table 2 gives the pattern of glycosidase and transglycosidase activity at the various stages. The glycosidases which can decompose raffinose are α-galactosidase and β-fructofuranosidase (β-D-fructofuranoside fructohydrolase). α-galactosidase activity is fairly high in resting seeds (Table 2) and increases on soaking and germinating, while β-fructofuranosidase activity is very low in resting and soaked seeds and increases remarkably in germinated seedlings. It could be that raffinose is hydrolysed to galactose and sucrose in early stages of germination. Throughout the process of germination no activity of β-glucosidase (β-D-glucoside glucohydrolase) was detected, while a fairly high activity of β-galactosidase (β-D-galactoside galactohydrolase) and β-transgalactosidase was observed.

The results of infiltrating D-galactose into the intercellular space of the cotyledons of the germinated seedling are shown in Table 3. The spot of D-galactose, which was detected distinctly just after infiltration, disappeared after 2 hr incubation in the dark. Quantitative

TABLE 3. CHANGES IN THE CONCENTRATION OF SUGARS WITH TIME AFTER INFILTRATION OF D-GALACTOSE INTO COTYLEDONS

Sugar	0 hr		2·0 hr		5·5 hr	
	(Ga)	(H_2O)	(Ga)	(H_2O)	(Ga)	(H_2O)
Galactose	1·3	0·0	0·4	0·0	0·0	0·0
Glucose and Fructose	2·8	2·7	2·4	2·3	1·9	2·0
Sucrose	15·5	15·0	14·2	14·1	13·7	13·6
	(mg per 20 cotyledons)					

(Ga): cotyledons infiltrated with D-galactose 5%, 28°C, in dark, (H_2O): infiltrated with water.

estimation of the galactose infiltrated shows that 1·3 mg of galactose infiltrated into 20 cotyledons (corresponding to 10 g) decreased to 0·4 mg after 2·0 hr incubation and zero after 5·5 hr incubation. The incubation was done in the dark and aerobically. Under diminished air pressure (0·1 atm) galactose did not decrease.

When raffinose was infiltrated it decreased and completely disappeared, while galactose did not appear. In other words, there must be an effective mechanism for galactose utilization in cotyledons of germinated seeds.

TABLE 4. GALACTOKINASE ACTIVITY IN COTTON SEED EXTRACTS

Time (hr)	Galactose remaining (μg/reaction mixture)	Galactose phosphorylated (μg/reaction mixture)
0·0	90	0
0·5	88	2
1·5	85	5
2·0	83	7

Reaction mixture: Galactose (10 m*M*) 0·05 ml, ATP (40 m*M*) 0·05 ml, cell-free extract 0·2 ml, buffer (maleate pH 6·5) 0·1 ml, $MgCl_2$ (0·1 *M*) 0·1 ml, final volume 0·5 ml at 30°C.

These phenomena were observed only with intact tissue. When galactose was added to a homogenate, no decrease was observed. Raffinose was hydrolysed to sucrose and galactose when mixed with the homogenate, and the galactose thus produced was not further metabolized. Destruction of the tissue thus appears to inactivate the galactose utilization mechanism.

Another enzyme that may be involved in the metabolism of galactose is galactokinase (ATP: D-galactose 1-phosphotransferase). Table 4 shows the weak activity of this enzyme in a cell-free extract of germinated seedlings. An extract of the acetone powder of germinated seedlings showed an even weaker activity than the homogenate. Galactose phosphate could not be detected in the sugar phosphate fraction in various stages of germination.

The patterns of sugars in various organs of cotton plant at the stage of ripening are shown in Fig. 1. Except for the seeds the comparison was made on the basis of fresh weight, while with unripe and ripe seeds the same number of seeds (20 seeds corresponding to 10 g of ripe seeds) were compared. In the leaf, no raffinose appears. The same is true with stem, peduncle, calyx, pericarp, juice in lint in cotton boll, and unripe seed (*S'*). Raffinose appears only in seeds, and only when the seed is quite ripe. No galactose or oligosaccharides comprising galactose were detected in seeds or any other part of plant body during the process of ripening.

To answer the question whether the raffinose in ripe seeds was transferred from any other part of the plant body during the process of ripening, or formed in the seed from some substance thus transported, the unripe seeds (*S'*) were detached from the cotton boll and air-dried, to see whether raffinose was detectable. As shown in Table 5, raffinose was formed in seeds detached from the plant after drying. This fact demonstrates that a mechanism for formation of raffinose does exist in the unripe seeds.

The effect of speed of drying and of juice in cotton boll on the formation of raffinose is shown in Fig. 2. When the seed with lint, which contains sap, was dried slowly, the formation of raffinose was greater than in the seed without lint and dried more rapidly.

Since raffinose could be found in the seed, experiments to detect an enzyme taking part in the synthesis of raffinose and oligosaccharides of the same series were performed with unripe seeds. Fig. 3 shows that a trisaccharide corresponding to raffinose in R_f value and in its reactions to various reagents was formed from melibiose and sucrose by the action of α-transgalactosidase. Using melibiose as D-galactose donor and melibiose or D-fructose as D-galactose acceptor, manninotriose and galactosyl fructose were formed, respectively.

TABLE 5. CHANGES IN SUGARS IN COTTON SEED DURING THE PROCESS OF RIPENING

Sugar	Unripe	Unripe air dried	Ripe ungerminated
Fructose	+	−	−
Glucose		−	−
Sucrose	± to +	+	++
Raffinose	−	+ to ++	+++
Stachyose	−	±	+

(+++: 5–10, ++: 2–5, +: 0·5–2, ±: 0·25–0·5, −: <0·25 mg/g*)

* Corresponding to 1 g of ripe seed. Equal number of seeds is compared.

When raffinose was incubated with α-galactosidase, the D-galactosyl residue was transferred to raffinose and stachyose appeared in reaction mixture (Table 6), but the ratio of transgalactosidation to galactosidation is very small. From 50 μM of initial raffinose, 0·06 μM stachyose was formed, while 1 μM of galactose was liberated after 2 hr incubation (Table 6).

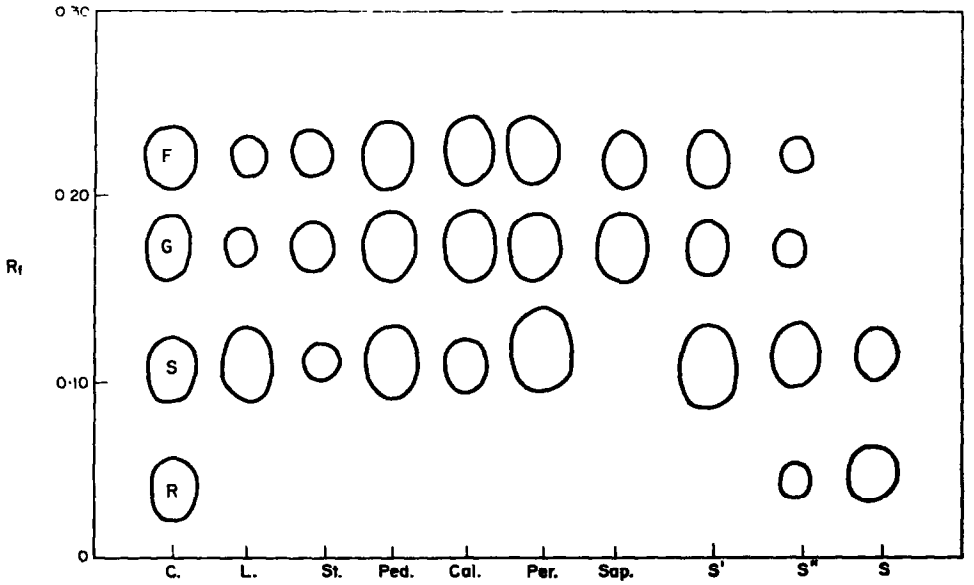

FIG. 1. SUGARS IN VARIOUS ORGANS OF COTTON PLANT AT THE STAGE OF RIPENING.
C, control: *L*, leaf; *St*, stem; *Ped*, peduncle; *Cal*, calyx; *Per*, pericarp; *Sap*, juice in lint of cotton boll; *S'*, unripe seed at the beginning of ripening; *S''*, unripe seed at the last stage of ripening; *S*, ripe seed; *F*, fructose; *G*, glucose; *S*, sucrose; *R*, raffinose. Butanol–acetic acid–water (4 : 1 : 1) was used as solvent.

Neither enzymatic epimerization of D-galactose to D-glucose mediated by UDPG, nor raffinose formation from UDPGal and sucrose, has been demonstrated with germinated and unripe seeds, respectively.

TABLE 6. α-GALACTOSIDASE AND α-TRANSGALACTOSIDASE ACTIVITY IN COTTON SEED EXTRACTS

Time (hr)	Galactose liberated (μg/ml)	Stachyose formed (μg/ml)
0·0	0	0
0·5	40	10
1·0	120	25
2·0	180	40

Reaction mixture: Raffinose (25%, 0·5 *M*) 0·1 ml, buffer (0·2 *M* *Tris*-maleate pH 6·5) 0·1 ml, cell-free extract 0·8 ml at 30°C.

DISCUSSION

The trisaccharide raffinose and the tetrasaccharide stachyose, which are contained in resting seeds, disappear rapidly and completely in an early stage of germination. Neither galactose nor melibiose nor manninotriose was detected throughout all stages of germination. As the activity of α-galactosidase in resting and soaked seeds is much higher than that of invertase, it appears that raffinose and stachyose are hydrolysed by α-galactosidase into sucrose and galactose, and the latter is metabolized through the galactose utilization system present in germinated seedlings. This phenomenon is fairly common in the germination of seeds which contain raffinose and stachyose.[1,3] Planteose, an isomer of raffinose,

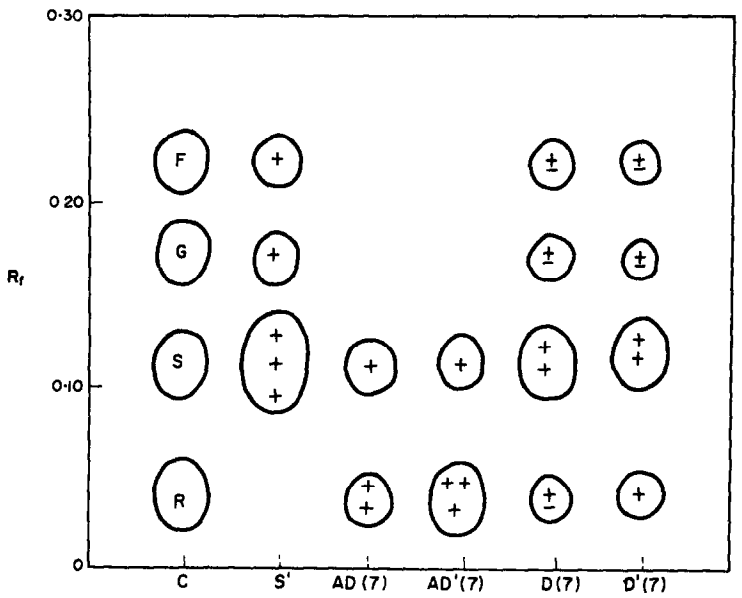

FIG. 2. SUGARS IN DETACHED UNRIPE SEEDS BEFORE AND AFTER VARIOUS KINDS OF DRYING FOR 7 DAYS. *S'*, unripe seed; *AD*(7), air-dried (slowly) without lint; *AD'*(7), air-dried with lint; *D*(7), dried *in vacuo* (rapidly) without lint; *D'*(7), dried *in vacuo* with lint. +++, 5–10; ++, 2–5; +, 0·5–2; ±, 0·25–0·5 mg/g. Butanol–acetic acid–water (4 : 1 : 1) was used as solvent.

which is also found in some seeds,[3,6-8] shows similar behaviour in germination of sesame seed.[8]

No galactose could be detected in any stage of germination, although raffinose and stachyose are metabolized and the content of fructose, glucose and sucrose was shown to rise during germination. These facts suggest that the galactose utilization system is very effective and the rate of utilization is as high as that of galactose liberation by α-galactosidase.

It is of interest that the galactose utilization system was demonstrated in the cotyledons of germinated seeds by infiltration. When raffinose was infiltrated it was also metabolized in cotyledons, no galactose being detected. The same phenomenon was demonstrated with cotyledon slices of germinated kidney bean.[9]

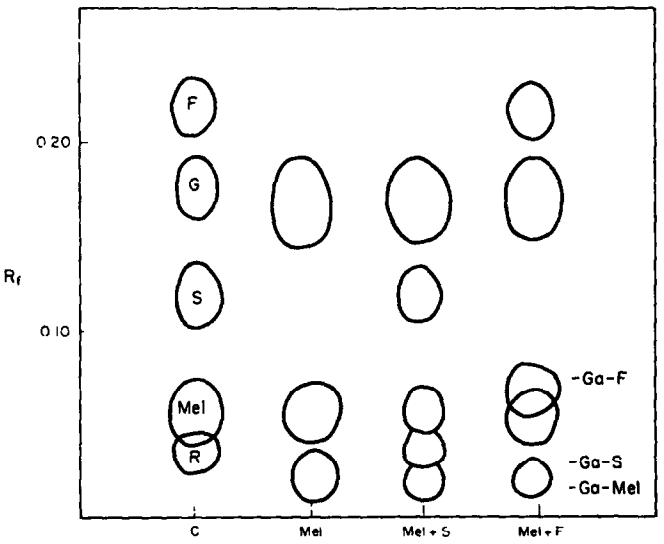

FIG. 3. OLIGOSACCHARIDES FORMED BY α-TRANSGALACTOSIDASE.

Reaction mixture: substrate (2 *M*) 0·1 ml, acceptor (2 *M*) or water 0·1 ml, enzyme (10% of acetone powder) 0·3 ml, buffer (acetate, pH 6·0) 0·1 ml, incubated at 30°C. for 48 hr. *C*, control, *Mel*, melibiose; *S*, sucrose; *F*, fructose; *Ga–F*, galactosyl–fructose (planteobiose); *Ga–S*, galactosyl–sucrose (raffinose); *Ga-Mel*, galactosyl–melibiose (manninotriose). Butanol–acetic acid–water (4 : 1 : 1) was used as solvent.

Galactose occurs widely in plants as a constituent not only of reserve substances but also of cell-wall substances, and its utilization in connection with cell-wall formation has been demonstrated with several plants. Discs of carrot and radish have been reported to absorb galactose as rapidly as glucose.[10] Galactose promoted excellent growth of pollen tubes *in vitro*,[11] suggesting that galactose might be converted to cell-wall substance by pollen grain. Some evidence as to the properties of a conversion product of galactose has been obtained with *Avena* celeoptiles.[12,13] When labelled D-^{14}C-galactose was fed to *Avena* coleoptiles, galactose was respired to $^{14}CO_2$ by coleoptile tissue only to a very small extent,

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while it is metabolized in considerable quantity to form insoluble products. This suggests that the major metabolic pathway of exogenous galactose is not respiration but transformation into cell-wall materials, perhaps hemicelluloses, galactans or pectic substances.

Recently galactolipids of homologous series were found in alfalfa leaves[14] as major components of subcellular organelles which perform biochemical functions. Since it was shown with rat brain tissue[15] that D-galactose-1-^{14}C was readily incorporated into galactolipids, it is possible that D-galactose is incorporated not only into cell-wall substance but also into lipid fractions of subcellular organelles. Therefore, further investigations on galactose utilization in germinated seedlings should be made with reference to the formation of cell-wall substances and lipids, which are indispensable for growth of cell during germination process. Although it is still possible that galactose serves as a respiratory substrate at an early stage of germination, its participation as respiratory substrate is slight, as compared quantitatively with that of other reserve substances, for example, fats.

The first step of the metabolic pathway of D-galactose utilization in cotton seed has not yet been elucidated. Utilization of galactose by tissues of higher plants appears to occur by a mechanism similar to that observed in animal tissues and galactose-adapted yeast.[16-18] In wheat plants D-galactose would be oxidized to D-galacturonic acid by way of its UDP derivative and would thus directly contribute to cell-wall formation.[19] UDPGal is the primary D-galactose donor to a lipid acceptor endogenous to the microsomal fraction of rat brain tissue.[15] Although galactokinase activity in the cell-free extract of germinated seed of cotton was not high enough to explain the rapid utilization of galactose introduced into cotyledons, it is still probable that galactose in cotton seedlings is utilized through its UDP derivative formed after phosphorylation.

When the seeds are placed on wet paper under reduced pressure, the raffinose and stachyose are unchanged, although α-galactosidase activity is detected. On introducing air the seeds begin to germinate and at the same time raffinose and stachyose disappear. These facts suggest that oxygen is necessary not only for germination but also for disappearance of these sugars. With some hydrolytic enzymes, an inhibitory effect of anaerobic incubation has been reported. As aerobic conditions are required for galactose utilization, raffinose is doubtless readily hydrolysed under the same conditions.

In contrast to the results with cotton seeds and some other fatty seeds, galactose, melibiose and manninotriose are detectable in *Stachys*,[20] *Verbascum*,[21] *Ajuga*,[22] which contained raffinose and its higher homologues in almost all parts of the plant body. Melibiose and manninotriose might be regarded as intermediates in the utilization of raffinose and stachyose in these plants.[22] It is noteworthy however, that the patterns of sugars in seeds and seedlings of these plants are exactly the same as those of cotton, pine, and other fatty seeds.

Although the mechanism of formation of raffinose and stachyose has not yet been completely established, participation of transgalactosidase in the formation of stachyose is most probable. If raffinose is formed from some galactose donor and sucrose, stachyose

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[22] S. HATTORI and Y. FUJITA, unpublished data.

would be formed from raffinose by transgalactosidase. It was demonstrated that raffinose was formed from melibiose and sucrose by transgalactosidase. Melibiose, however, has never been found in seeds or any other parts of the plant body during ripening, so that the origin of the galactosyl residue remains obscure. Discontinuity between the amount of raffinose and its higher homologs in *Verbascum* suggests that mechanism of formation of the former might be different from that of the latter.[21] Formation of raffinose mediated by UDPGal and sucrose has not yet been demonstrated clearly with cotton.

MATERIALS

Experimental

The variety of cotton seed (*Gossypium herbaceum* Oliver) used was "Moppo" No. 380, which was grown in the Experimental Farm of the University of Tokyo. Resting seeds (ripe seeds S) were collected in October from well-formed fruits. The percentage of germination was 75 per cent at 25°C. Lint on the seed coats was removed by a short treatment with conc. H_2SO_4.

To germinate the seedlings, the resting seeds were soaked in water at room temperature for 1–2 days and then placed on wet filter paper in the darkness at 25°C. The seedlings used had radicles up to a length of 5 cm.

For soaked seeds, the resting seeds were soaked in water 2 cm deep for 3–5 days, the water being changed every 12 hr. Every seed absorbed water and swelled, the tip of the radicle just appearing (1–2 mm long) from the split seed coat. When these soaked seeds were transferred on to wet filter paper, they began to germinate immediately with the same percentage as in normal germination. Seeds placed on wet filter paper in a vacuum bottle under diminished pressure did not germinate, but on introducing air, they began to germinate normally.

The unripe seeds (*S'*) were harvested at the beginning of ripening. The colour of the seed coat was white and the content was very juicy. Fat content was very low and no raffinose was detected. Unripe seeds (*S''*) were at the last stage of ripening, when the cotton boll was about to open. The seed coat was already brown and the seeds getting dry. The fat content was increasing and raffinose was detectable, although in small amount.

Extraction of the material

The seeds were freed from their coats, crushed in a mortar, and the fatty substances extracted 3–4 times with cold ether. The residue was extracted three times with boiling 80% ethanol, the ethanol was distilled off *in vacuo*, and the residual aqueous solution treated with lead acetate solution. The lead was removed from the filtrate by hydrogen sulphide, the filtrate neutralized and concentrated *in vacuo* and made up to a known volume with water, any precipitate being removed by filtration through Filter Cel. When the amount of material was small, the whole extract was transferred to a small evaporating dish, dried over calcium chloride in a vacuum desiccator, re-dissolved in water and assayed by paper chromatography. Final volume of the extract was usually 1 ml for 20 seeds and seedlings, which corresponds to 10 g of resting seed.

Paper chromatography

Toyo Filter Paper Nos. 2 and 50 and Whatman Filter Paper No. 1 were used throughout Whatman No. 54 was also used for detecting sugar phosphates.

For the separation of sugars, butanol–acetic acid–water (4 : 1 : 1)[23] and 80% aqueous phenol were used as solvents. A mixture of butanol–pyridine–water (5 : 3 : 1)[24] was also used to separate the aldohexose fraction, isolated from paper chromatograms, into glucose and galactose. For sugar phosphates, isopropanol–trichloroacetic acid–ammonia–water (75 ml : 5 g : 0·3 ml : 25 ml)[25] and methanol–88% formic acid–water (16 : 3 : 1; v/v)[26] were used as acidic solvent and methanol–ammonia–water (6 : 1 : 3)[26] as basic solvent.

In most cases the ascending method was applied, at room temperature, the descending method[27] being used only when a longer distance of solvent run was required for the clear separation of tri- and tetrasaccharides, the R_f values of which were comparatively small. Controls of pure sugars were run each time beside the sample to be analysed.

As authentic sugars, Merck's or Schering–Kahlbaum's preparations were used. The stachyose sample had been isolated from the tubers of *Stachys affinis* Franchet and supplied by Dr. S. Murakami. For the preparation of melibiose,[28] raffinose was dissolved in 1·5% HCl and heated for 30 min at 70°C. The reaction mixture was concentrated *in vacuo* and mixed with ether and ethanol; the melibiose separated out, while fructose remained in solution. This procedure was repeated and the melibiose filtered off, washed with ethanol and dried. The preparation gave a single spot on a paper chromatogram.

Detection of spots

Spots on paper chromatograms were detected by means of the following reagents. 1. Sugar, general: Bacon–Edelman's reagent[29] (benzidine 0·5 g, glacial acetic acid 10 ml, 40% (w/v) trichloroacetic acid 10 ml, and ethanol 80 ml). Almost all sugars are detected with this reagent except non-reducing oligosaccharides which will not be hydrolysed readily. 2. Reducing sugars: Horrocks' reagent[30] (benzidine 0·5 g, glacial acetic acid 20 ml, and ethanol 80 ml). Although these two reagents are not so sensitive as ammoniacal silver nitrate, the spots detected with this reagent appear well defined and do not grow dim on paper as those with the latter reagent. 3. Ketoses and oligosaccharides comprising ketose as a component, i.e. sucrose, raffinose and stachyose. (*a*) Seliwanoff's reagent.[31] Ten ml of solution *A* (H_2SO_4 conc. 100 ml, and 95% ethanol 375 ml) and 0·4 ml of the solution *B* (resorcinol 2·5 g, in 95% ethanol 50 ml) were mixed just before spraying. On heating to 80°, red spots appeared on a pale pink background. (*b*) Urea–HCl reagent[32] (Urea 5 g, 2 *N* HCl 20 ml, and 95% ethanol 100 ml). Bluish-green spots become visible on the light grey background when heated to 100–105° for 10–20 min. Seliwanoff's reagent is more sensitive than urea–HCl, but yields not so stable spots. 4. Phosphoric esters of sugars. Hanes–Isherwood reagent[33] (60% (w/v) perchloric acid 5 ml, 4% (w/v) ammonium molybdate 25 ml, *N* HCl 10 ml and H_2O 60 ml) was sprayed at the rate of 1–2 ml per 100 cm². Inorganic phosphate appeared as a yellow spot. The paper was then dried at 85° for 1–2 min, at which time glucose-1-phosphate was revealed by a yellow to blue colour. The paper was then exposed to a 30 W u.v. lamp at a distance of about 2 cm for 10–20 min. All of the organic phosphate

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[26] R. S. BANDDURSKI and B. A. AXELROD, *J. Biol. Chem.* **193**, 405 (1951).
[27] S. M. PARTRIDGE, *Nature* **164**, 443 (1949).
[28] B. TOLLENS, *Kurzes Lehrbuch der Kohlenhydrate*, p. 435. Leipzig, Verlag von Johann Ambrosius Barth (1914).
[29] J. BACON and J. EDELMAN, *Biochem. J.* **48**, 114 (1951).
[30] R. H. HORROCKS, *Nature* **164**, 444 (1949).
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[32] R. DEDONDER, *Bull. Soc. Chim. Biol.* **34**, 144, 157, 171 (1952).
[33] C. S. HANES and F. A. ISHERWOOD, *Nature* **164**, 1107 (1949).

compounds appeared as blue spots, while inorganic phosphates gave a yellow-green colour.[26]

Isolation of sugars: large-scale chromatography

The extract was put on a filter paper 30–60 cm wide, along a line 5 cm from the end of the sheet, which was rolled in cylindrical form and put in the developing solvent. After drying, three strips were cut off from both sides and middle part of the chromatogram and the spots on the strip detected by the above reagents. The zones of sugars were cut off, hung at the end of a reflux condenser, and extracted three times with flowing hot ethanol. The combined extract was concentrated *in vacuo* and the residue dissolved in water, to be checked by paper chromatography, whether the substance isolated was a single substance or not.

Hydrolysis of raffinose and stachyose

The substance isolated by large-scale chromatography was dissolved in 1 ml of 2% HCl and heated under reflux on a boiling water bath for 1 hr. On cooling, the hydrolysate was evaporated to dryness over calcium chloride or solid NaOH in a vacuum desiccator, dissolved in 1–2 ml of water and the process repeated until HCl was completely removed. (Even a very small amount of HCl left in the sample makes clear separation of the sugars difficult.) The residue was made up to volume in water for paper chromatography.

To hydrolyse solely the sucrose-type linkage present in the oligosaccharides, the dried substance was dissolved in 1·5% hydrochloric acid and heated in a water-bath at 70° for 30 min. Under these conditions, no galactoside linkage was hydrolysed.

All subsequent procedures were exactly the same as those for complete hydrolysis.

Enzymatic hydrolysis

The isolated raffinose and stachyose were treated with a 2% solution of α-galactosidase in acetate buffer (pH 4·0). The preparation of α-galactosidase should be free from invertase and sugars. The mixture was kept covered with toluene at 30°. After 12 and 24 hr, respectively, a sample of the reaction mixture was chromatographed on paper. The final concentration of raffinose and stachyose in the reaction mixture should be high enough to be detected distinctly with Seliwanoff's reagent or urea–HCl reagent. An α-galactosidase, preparation obtained from the seeds of Japanese black pine was used since it is free from other glycosidases.[1]

Identification of stachyose and raffinose

The isolated tetrasaccharide or trisaccharide was hydrolysed completely and partially with acid, enzymatically with α-galactosidase, and the products of hydrolysis were assayed by paper chromatography. In every case, authentic stachyose and raffinose were treated by the same procedure and compared with the samples. By partial hydrolysis, reducing tri- and disaccharides (Seliwanoff reaction negative) were obtained from the tetra- and trisaccharide, respectively. These oligosaccharides derived from the sample were isolated, hydrolysed completely and chromatographed to identify the sugars, and the results obtained with the sample were compared with those of authentic sugars.

Quantitative estimation of sugars by paper chromatography

For quantitative estimation, extracts were prepared from 20 seeds and seedlings (corresponding to 10 g of ripe seeds) as described previously and the final volume was made

up to 1 ml. Using a graduated glass capillary, equal amounts of these extracts and control sugar solutions were put on filter paper side by side and chromatographed, the length of spots of the sample being compared with those of control sugars.[34]

After the approximate concentration of the sugars had been ascertained, a large-scale chromatogram was prepared from the final extract in a quantity enough to determine the sugar concentration by Sumner's method.[35] The sugars were extracted from the paper twice with 2–3 ml of 80% ethanol. The extract was transferred to a test tube, evaporated, and then dried completely in a vacuum desiccator. For the estimation of the non-reducing sugars, sucrose, raffinose and stachyose, the dried substance was dissolved in 1 ml of 1·5% HCl and heated for 30 min at 70° to hydrolyse sucrose-type linkage. After neutralizing with 0·3 ml of 10% NaOH, the sample was made up to 2 ml with water, and the sugar colorimetrically determined by the method of Sumner. The amount of original oligosaccharide was calculated from the amount of reducing sugars liberated. As blank, an equal area of filter paper was cut off from a blank part of the chromatogram and treated by the same procedure.

Detection of glycosidase and transglycosidase activity by paper chromatography

The acetone powders of seeds and seedlings were mixed with 3 times their volume of water, covered with toluene and kept at 25° for 20 hr. After centrifuging, 3 volumes of alcohol were added to the supernatant at 0–5°. The resulting precipitate was centrifuged off and dried in a vacuum desiccator. A 10% solution of this preparation was centrifuged to obtain a clear supernatant, which was used as enzyme solution for glycosidase and transglycosidase.

One tenth ml of 2 *M* substrate solution, 0·3 ml enzyme solution, and 0·1 ml acetate buffer were mixed in a small test tube, covered with toluene, and incubated at 30° for 12–24 hrs. The pH of the buffer solution was adjusted to 4·0 for detection of glycosidase, and pH 6·0 for transglycosidase. After 12, 18, and 24 hr of incubation, the reaction mixture was assayed by paper chromatography directly and the resulting monosaccharides and oligosaccharides identified. No dilution or concentration of the reaction mixture was necessary.

The activity of α-glucosidase (α-D-glucoside glucohydrolase) was tested with maltose, that of β-glucosidase with salicin, that of α-galactosidase with melibiose and raffinose, that of β-galactosidase with lactose, and that of invertase with sucrose. The activity of α-transglucosidase was tested with maltose, that of α-transgalactosidase with raffinose and melibiose, that of β-transgalactosidase with lactose, and that of transfructosidase with sucrose.

Cell-free extract for assay of galactokinase and α-transgalactosidase

Germinated seedlings were homogenized in a mortar, squeezed through cheese cloth and centrifuged at 10,000×g. The supernatant was dialysed against distilled water till the dialysate became free of sugars. All processes were carried out between 0–5°.

Estimation of galactokinase

The method of Trucco *et al.*[36] was applied to the following modified reaction mixture: 0·05 ml 10 m*M* D-galactose solution, 0·05 ml 40 m*M* ATP solution, 0·2 ml cell-free extract,

[34] R. B. FISCHER, D. S. PARSONS and G. A. MORRISON, *Nature* **161**, 764 (1948).
[35] J. B. SUMNER, *J. Biol. Chem.* **47**, 5 (1921), **65**, 393 (1925).
[36] R. E. TRUCCO, R. CAPUTTO, L. F. LELOIR and N. MITTELMAN, *Arch. Biochem.* **18**, 137 (1948).

0·1 ml maleate buffer (pH 6·5), 0·1 ml 0·1 *M* $MgCl_2$ solution, 0·5 ml total volume. After phosphate was precipitated off by the addition of 0·2 ml 5% $ZnSO_4$ solution and 0·2 ml 0·3 *N* $Ba(OH)_2$ solution, the galactose which remained in the reaction mixture was determined by Somogyi and Nelson's method.[37,38]

Estimation of α-galactosidase and α-transgalactosidase

One tenth ml 0·5 *M* raffinose solution, 0·1 ml 0·2 *M* trismaleate buffer (pH 6·5) and 0·8 ml cell-free extract were mixed and incubated at 30°. After incubation, reaction mixture was concentrated and applied to large-scale chromatography to isolate the galactose liberated and stachyose formed. Sugars isolated after hydrolysis were determined by Somogyi and Nelson's method.

Infiltration of D-*galactose into cotyledons*

Thirty cotyledons (corresponding to 15 seedlings) were submerged in 5% D-galactose solution in a suction bottle, which was then evacuated to remove gas from the intercellular spaces of the tissue. On introducing air, the galactose solution in which the cotyledons were immersed was introduced into the tissue. This procedure was repeated twice to remove the gas completely from the intercellular spaces. Intimate contact between the cells of cotyledon tissue and galactose solution was thus achieved. After rinsing completely with distilled water, the cotyledons were transferred to a moist chamber and incubated at 28° in the dark. As control, distilled water in place of galactose solution was infiltrated into the same numbers of cotyledons and incubated in parallel with sample.

Each 10 cotyledons (corresponding to 5 seedlings) of both sample and control were killed in boiling 80% ethanol after incubation for 0, 2, and 5·5 hrs. The procedure of extraction was as above. The extract of 10 cotyledons each was made up to final volume of 0·1 ml and paper chromatographed. For estimation of sugars, the extract was made up to 2·5 ml and 1 ml was used to estimate reducing sugars by Sumner's method before and after hydrolysis. The amount of galactose infiltrated was calculated from the difference between reducing sugars of sample and control, the amount of sucrose from the difference in reducing sugars before and after hydrolysis.

Separation of sugar phosphate fraction

The material (10–50 g) was extracted with 5 ml of cold 10% trichloroacetic acid in the first extraction, and with 5 ml of 5% solution in the second extraction. The combined extract, after the addition of barium acetate (at pH 8·2), was chilled in a refrigerator for half an hour and the precipitate ("Barium insoluble fraction") centrifuged. The supernatant ("Barium soluble fraction") was treated with 4 times its volume of 95% ethanol and chilled for 1 hr. The precipitate ("Barium-soluble, alcohol-insoluble fraction") was removed by centrifuging, washed with ethanol and dried. It is important to keep the pH at 8·2 throughout the precipitation. This was done by adding 1/300 volume of 1% phenolphthalein and KOH till the solution turned slight pink. The dried material was suspended in a small amount of water and treated with Amberlite 1R-120 (H+).[39] One ml of wet resin was used for 1 mg sugar phosphates. The resin was removed by vacuum filtration and the barium-free

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[38] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).
[39] T. OMURA and S. FUKUSHI, *J. Agr. Chem. Soc. Japan* **27**, 516 (1953).

filtrate was concentrated and made to 0·1 *M* in orthophosphate in order to be assayed by paper chromatography.

Drying of unripe seeds

Unripe seeds (*S'*) with and without lint were dried in air at room temperature. The lint held the juice containing fructose and glucose (concentration was about 5%). Another lot of seeds was also dried over calcium chloride in a vacuum desiccator. Dried unripe seeds, after removing the lint and washing the sugars left on the surface, were crushed in a mortar and extracted as above and analysed by paper chromatography.

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