On the Occurrence of Raffinose in the Seeds of *Nelumbo nucifera* Gaertn.

BY

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In regard to the chemical constituents of lotus seeds, no special investigation has been undertaken until recently. In 1918, C. H. CHOW[1], under the guidance of Prof. K. OSHIMA, studied the constituents of this seed in our laboratory. His investigation gave the following results:—

1. The lotus seed is composed of three parts, that is, integuments, plumule and cotyledons. These parts were separated and weighed, 3.74% being integuments, 3.03% plumule and 93.23% cotyledons. Average weight of 100 seeds was about 87.35 grams.

2. The finely ground substance of cotyledon gave the following composition.

	Air-dry substance. %	Water-free substance. %
Water	7.75	—
Protein	18.71	20.28
Fat	2.05	2.22
Crude fiber	2.70	2.93
Nitrogen-free extract	64.90	70.35
Ash	3.89	4.22
Total nitrogen	2.99	3.24
Protein nitrogen	2.93	3.18
Non-protein nitrogen	0.06	0.06
Reducing sugar	0.12	0.14
Non-reducing sugar	5.53	5.99
Dextrin	4.18	4.53
Starch	44.13	47.84
Galactan	3.16	3.43
Pentosan	2.11	2.29

1) Unpublished.

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3. The nature of the carbohydrates was also studied but no satisfactory results could be obtained. Especially, the nature of the rather large amount of non-reducing sugar remained unsolved.

Consequently, the author has investigated the non-reducing sugar in lotus seeds and found that much of it is raffinose.

Raffinose was first isolated by JOHNSTON[1] from *Eucalyptus manna*, and it was named melitose by BERTHELOT[2]. It was obtained from beet sugar in the refining process by LOISEAU[3], who gave to it the name raffinose. B. TOLLENS[4] and v. LIPPMANN[5] found raffinose in the juice of sugar beet (*Beta vulgaris* L.) at almost the same time. C. SCHEIBLER[6] found a method to obtain raffinose from the non-crystallizable beet-sugar molasses by using a strontium salt. LIPPMANN[6], HERZFELD[7], and SCHÖNE and TOLLENS[8] proved that the raffinose contained in the molasses came from the sugar beet and not from the process of sugar manufacture. Subsequently it was found in sugar-beet—normally in small amount (about 0.002%), but occasionally in large quantities. The occurrence of a trisaccharide in cotton seed (*Gossypium herbaceum* L.) was proved by RITTHAUSEN and WEGER[9], and BÖHM[10]; the former two authors named it melitose, and the latter gossypose. The identity of melitose, gossypose and raffinose was proved by RISCHBIETH and TOLLENS[11], C. SCHEIBLER[12], and v. LIPPMANN[13], the last name being generally used at the present time. O'SULLIVAN[14], C. RICHARDSON and C. A. CRAMPTON[15], and E.

1) Jour. Pr. Chem., **1** (1843), 29, p. 485.

2) Ann. Chem. Phys., **3** (1856), 46, p. 66; Compt. rend., **103** (1886), p. 533.

3) Compt. rend., **82** (1876), p. 1058; Zeitschr. d. Ver. D. Zuckerind., **35** (1885), p. 1008; Ber. D. Chem. Ges., **9** (1876), p. 732.

4) Ber. D. Chem. Ges., **18** (1885), p. 26; Zeitschr. d. Ver. D. Zuckerind **35** (1885), p. 31; ibid., **36** (1886), p. 212.

5) Ber. D. Chem. Ges., **18** (1885), p. 3087, Ref. 188; Zeitschr. d. Ver. D. Zuckerind., **35** (1885) p. 257.

6) Ber. D. Chem. Ges., **18** (1885), p. 1409.

7) Zeitschr. d. Ver. D. Zuckerind., **42** (1892), p. 150.

8) Ibid., **50** (1900), p. 978.

9) Jour. Prak. Chem., (2), **29** (1884), p. 351; **30** (1884), p. 32.

10) Ibid., (2), **30** (1884), p. 37.

11) Ann. Chem., **232** (1886), p. 169 *ff.*

12) Ber. D. Chem. Ges., **18** (1885), p. 1409; ibid., **19** (1886), p. 2868.

13) loc. cit.

14) Centralb. f. Agric. Chem. (1886), p. 287; Chem. News. **52** (1885), p. 293.

15) Ber. D. Chem. Ges., **19** (1886), p. 1180.

SCHULZE and FRANKFURT[1] found raffinose in the seeds of wheat and rye; and then H. HERISSEY and C. LEFEBVRE[2] found it in fresh leaves and young branches of *Taxus baccata*; E. BOURQUELOT and M. BRIDEL[3] in two legumes, *Erythrina fusca* LOUR. and *Entada scandens* BTH.; K. MIYAKE[4] in the tubers of Arrowhead (*Sagittaria sagittifolia* L. f. *sinensis* MAKINO); and H. E. ANNETT[5] recently found raffinose in jute seeds (*Corchorus capsularis* L.), in sugar-beet molasses, in cotton seeds, in barley grain, in wheat sprout and in *Eucalyptus manna*.

In addition to the sources of raffinose above mentioned, I have found in lotus seeds a new source of the same sugar.

EXPERIMENTAL PART.

I. Preparation of the Syrup.

One kilogram of the air dry, finely pulverized material of the cotyledon of lotus seeds (the red-flowering variety) was mixed with 3 liters of 90% alcohol. After allowing to stand for a day, occasionally stirring, the alcohol solution was found to show a neutral reaction to litmus paper. It was next heated in a boiling water bath for 3 hours, using a reflux-condenser, and then filtered by suction. To the residue, 2 liters of 90% alcohol were added and it was heated for 3 hours according to the above process and filtered. With the residue the same treatment was repeated twice to make the extraction complete. The extracts were combined and evaporated at low temperature under reduced pressure to about 300 c.c. After allowing to stand for a few days, a brownish precipitate was deposited in a small quantity on the bottom of the flask. The yellow colored clear solution was decanted and concentrated at a low temperature to a syrup—Syrup (I).

II. Qualitative Reactions of the Syrup.

The above prepared syrup (I) was neutral, sweet and of a transparent yellow color, and showed the following qualitative reactions :—

1) Zeitschr. f. Physiol. Chem., **20** (1895), p. 511.
2) Jour. Pharm. Chem., **6** (1907), 26, p. 56.
3) Compt. rend., **149** (1909), p. 361.
4) Jour. Biol. Chem., **15** (1913), p. 221; Trans. Sapporo Nat. Hist. Soc., **5** (1913), p. 23.
5) Biochem. Jour., **11** (1917), p. 1.

1) It gave MOLISCH's reaction with α-naphthol and sulphuric acid distinctly.

2) It reduced FEHLING's solution weakly; but after inversion with hydrochloric acid, the reducing power was very strong.

3) It rotated the plane of polarization strongly toward the right, and even after inversion, toward the right also.

4) It gave no characteristic absorption-spectrum of pentose with phloroglucin and hydrochloric acid.

5) It gave BRAUN's reaction by heating with picric acid and a small quantity of caustic soda solution.

6) It gave no PINOFF's reaction for ketose with ammonium molybdate and acetic acid; but after inversion, the reaction was distinct.

7) It gave SELIWANOFF's reaction for ketose with resorcin and hydrochloric acid very distinctly.

8) It produced mucic acid upon oxidation with nitric acid of 1.15 sp. gr. and it melted at 213°C.

9) From the filtrate separated from the mucic acid crystals, saccharic acid was obtained as acid potassium saccharate.

10) It produced no characteristic mannosephenylhydrazone with phenylhydrazin, either before or after inversion.

From the above qualitative reactions, we may conclude that the syrup (I) contains a little reducing sugar and a large amount of non-reducing sugar, which may yield glucose, fructose or galactose.

III. Phenylosazone Tests.

1) According to FISCHER's[1] method, 3 grams of the syrup (I), 2 grams of phenylhydrazin hydrochloride, 3 grams of sodium acetate and 20 c.c. of water were mixed in a large test tube and heated in a boiling water bath. Yellow crystals of osazone began to form in 10 minutes. The heating was continued for an hour and a half. When cooled, the crystals were examined under a microscope and found to be all uniform and quite identical with those of glucosephenylosazone made from pure glucose. No other crystals of osazone than those of glucosephenylosazone were formed. The crystals were filtered off and washed with a little water. By repeating the recrystallization from 60% alcohol, the crystals of osazone obtained were dried over concentrated sulphuric acid in a vacuum.

1) Ber. D. Chem. Ges., **17** (1884), 1, p. 579.

The determination of nitrogen in the osazone gave the following result:—

0.0528 g. substance gave 0.00816 g. N.

Glucosephenylosazone		N.
$C_{18}H_{22}N_4O_4$	Calculated	15.64%
	Found	15.45%

The melting point of the osazone was determined and found to be 204°C.

The crystal form, the quantity of nitrogen and the melting point indicate that the osazone in question is glucosephenylosazone.

2) Three grams of the syrup (I) were dissolved in 15 c.c. of water and inverted with 1 c.c. of hydrochloric acid of 1.188 sp. gr. in a water bath at 69°C. for 5 minutes, according to HERZFELD's[1] modification of CLERGET's method. After inversion, the solution was neutralized with sodium carbonate, and 3 grams of phenylhydrazin hydrochloride, 4.5 grams of sodium acetate, and 15 c.c. of water were added to it and the mixture was heated in a boiling water bath for an hour and a half as in the above experiment. Yellow crystals of osazone were formed abundantly. The osazone soluble in hot water was relatively large in amount. When cooled, the crystals were filtered off and washed with a little water. The crystals of osazone here obtained were boiled with water and filtered immediately. After cooling, a large amount of osazone crystallized in the filtrate and the crystal form was similar to that of melibiosephenylosazone made from melibiose. The crystal form of the osazone insoluble in hot water coincides with that of glucosephenylosazone.

a. Osazone insoluble in hot water.

The osazone insoluble in hot water was washed sufficiently with hot water. By repeating the recrystallization from 60% alcohol, the crystals of osazone obtained were dried for a long while over concentrated sulphuric acid in a vacuum.

The determination of nitrogen in the osazone gave the following result:—

0.2526 g. substance gave 0.0394 g. N.

Glucosephenylosazone		N.
$C_{18}H_{22}N_4O_4$	Calculated	15.64%
	Found	15.60%

1) Zeitschr. d. Ver. D. Zuckerind., **38** (1888), p. 699.

The melting point of the osazone was determined and found to be 204°C.

Therefore I am sure that the osazone insoluble in hot water thus isolated is glucosephenylosazone.

b. Osazone soluble in hot water.

After recrystallization from hot water several times, pure osazone was obtained, which was dried for a long time over concentrated sulphuric acid in a vacuum.

The determination of nitrogen in the osazone gave the following result :—

0.2128 g. substance gave 0.02266 g. N.

Melibiosephenylosazone		N.
$C_{24}H_{32}N_4O_9$	Calculated	10.77%
	Found	10.65%

The melting point of the osazone was determined and found to be 176°–178°C.

The crystal form, the quantity of nitrogen and the melting point indicate that the osazone in hand soluble in hot water is melibiosephenylosazone.

3) Three grams of the syrup (I) were dissolved in 30 c.c. of water and inverted with 2 c.c. of strong hydrochloric acid in a boiling water bath for an hour. After inversion, the solution was neutralized with sodium carbonate, and heated with phenylhydrazin hydrochloride and sodium acetate as in the above experiment. When cooled, the crystals of osazone produced were filtered and washed with water. Under a microscope, the crystals of osazone were determined and found to consist of two kinds of osazone, glucosazone and galactosazone. Therefore the osazone obtained was separated into two parts by its solubility in hot 20% alcohol.

a. Osazone insoluble in hot 20% alcohol.

After recrystallization from 60% alcohol several times, pure osazone was obtained, which was dried for a long time over concentrated sulphuric acid in a vacuum.

The determination of nitrogen in the osazone gave the following result :—

0.2266 g. substance gave 0.0353 g. N.

Glucosephenylosazone		N.
$C_{18}H_{22}N_4O_4$	Calculated	15.64%
	Found	15.58%

The melting point of the osazone was determined and found to be 204°C.

Consequently the osazone in question is glucosephenylosazone.

b. Osazone soluble in hot 20% alcohol.

By repeating the recrystallization from 20% alcohol, the crystal form of the osazone was found to be all uniform and quite identical with those of galactosephenylosazone. After drying, the determination of nitrogen in the osazone gave the following result :—

0.3105 g. substance gave 0.04856 g. N.

Galactosephenylosazone		N.
$C_{18}H_{22}N_4O_4$	Calculated	15.64%
	Found	15.64%

The melting point of the osazone was determined and found to be 191°C.

The crystal form, the quantity of nitrogen and the melting point indicate that the osazone in hand is surely galactosephenylosazone.

Considering from the above experiment, the reducing sugar in lotus seed is small in amount and consists of a sugar (perhaps glucose) which produces glucosephenylosazone only. On the other hand, a large amount of non-reducing sugar is present, from which after complete inversion, glucosephenylosazone and galactosephenylosazone are produced, but, after incomplete inversion, glucosephenylosazone and melibiosephenylosazone. By summarizing these results and the results of the above qualitative reactions, we may conclude that the syrup (I) here examined contains a sugar higher than a disaccharide.

IV. Isolation of Raffinose.

According to SCHULZE's method[1], a portion of the syrup (I) (about 30 grams) was dissolved in hot 90% alcohol and then heated to boiling with the addition of a hot saturated solution of strontium hydrate, using over 3 parts of strontium hydrate for 1 part of the syrup used. After boiling for 30 minutes, the precipitate produced was filtered

1) Landw. Vers.-Stat., **34** (1887), p. 403; abs. in Ber. D. Chem. Ges., **21** (1888), Ref., p. 299.

hot and then, after suspending in water, decomposed with carbon dioxide. The solution, filtered from strontium carbonate, was evaporated at a low temperature to a small volume, purified with 90% alcohol several times, and then concentrated to the state of syrup (II).

To the syrup (II), absolute alcohol was added and well stirred and then decanted. The residue, insoluble in absolute alcohol, was extracted with pure methyl alcohol and decanted. By repeating the same operation with pure methyl alcohol, the residue was completely dissolved. The extracts with methyl alcohol were combined and evaporated to about 10 grams—syrup (II)'.

After allowing to stand for about 7 months in a cold place, white needle crystals were abundantly formed in the syrup (II)'. To the syrup (II)', a little 95% alcohol was added, well mixed, and dried on a clay plate. On the following day, 3 grams of white crystals were obtained.

The crystal above obtained gave the following qualitative reactions :—

1) It was perfectly white and somewhat sweet.

2) It left no ash on ignition.

3) It gave MOLISCH's reaction very distinctly.

4) It showed no reducing power, but after inversion, it reduced FEHLING's solution.

5) It rotated the plane of polarization toward the right strongly, and even after inversion toward the right also.

6) It produced mucic acid upon oxidation with nitric acid of 1.15 sp. gr.

7) From the filtrate of mucic acid crystals, saccharic acid was separated and detected as acid potassium saccharate by the usual method.

8) After inversion, it gave PINOFF's and SELIWANOFF's reaction for ketose.

9) It gave no absorption band of pentose by the phloroglucin method.

Judging from the above results, the crystals above obtained were those of non-reducing sugar which is composed of glucose, fructose and galactose.

The sugar obtained was dried for 2 weeks over concentrated sulphuric acid in a vacuum.

0.1772 gram of the dried sugar was dissolved in water, a few drops of alumina cream added, and then made up to 10 c.c. and polarized

in a 100 m.m.-tube in a SCHMIDT and HAENSCH half-shadow polariscope. No birotation was observed. The rotation was 5.4 on the scale toward the right.

The specific rotatory power of the sugar is

$$[\alpha]_D = +\frac{5.4 \times 0.346 \times 10}{0.1772 \times 1} = +105.44^\circ \text{ (at } 20^\circ\text{C.)}$$

The sugar was once more recrystallized from pure methyl alcohol, and dried.

0.1344 gram of the sugar was polarized with the same treatment as in the previous experiment. A dextro-rotation of 4.1 on the scale was observed.

The specific rotatory power of the sugar is

$$[\alpha]_D = +\frac{4.1 \times 0.346 \times 10}{0.1344 \times 1} = +105.56^\circ \text{ (at } 20^\circ\text{C.)}$$

Next, the rotation of the sugar after inversion was determined as follows:—

0.1052 gram of the sugar was inverted at 69°C. for 5 minutes, according to HERZFELD's[1] inversion method. After inversion, it was neutralized with sodium carbonate, a few drops of alumina cream added, and then made up to 10 c.c. and polarized in a 100 m.m.-tube. After 24 hours, the rotation was 1.65 on the scale toward the right.

The specific rotatory power is

$$[\alpha]_D = +\frac{1.65 \times 0.346 \times 10}{0.1052 \times 1} = +54.24^\circ \text{ (at } 20^\circ\text{C.)}$$

After inversion of the sugar according to HERZFELD's method, the osazone test was applied. The osazone soluble in hot water melted at 176°C., and the insoluble one at 204°C. Considering the melting points and the crystal forms, the former coincides with the osazone of melibiose, and the latter with that of glucose. And then, after complete inversion with hydrochloric acid, it was tested with phenyl-hydrazin and acetic acid. The osazone soluble in dilute alcohol melted at 190°C., and the insoluble one at 204°C. From the melting points and the crystal forms, the former is galactosephenylosazone and the latter glucosephenylosazone.

Raffinose contains 5 molecules of water of crystallization and the

1) loc. cit.

specific rotation of raffinose hydrate ($C_{18}H_{32}O_{16}+5H_2O$) is $+104°$ — $105.7°$. According to C. SCHEIBLER and H. MITTELMEIER[1], when heated with dilute hydrochloric acid for a short time, raffinose is hydrolyzed to d-fructose and melibiose; and after inversion by HERZFELD's method, the specific rotation of raffinose hydrate decreases from $+104°$ — $105.7°$ to $+53°$ — $54°$.

Consequently, the sugar in hand is raffinose.

V. Raffinose Content in the Syrup.

The content of raffinose in the syrup is approximately determined by the application of CREYDT's[2] method.

1) Direct polarization. (=the sum of the sucrose and raffinose polarizations $=P$)

A grams of the syrup were dissolved in water and made up to B c.c., and polarized in 200 m.m.-tube at 20°C. The rotation of C on the scale was observed. Hence the direct polarization (P) was calculated as follows:—

$$P=\frac{C\times B\times 26}{A\times 100}$$

2) Invert polarization. (=the sum of sucrose and raffinose invert polarizations $=P'$)

A' grams of the syrup were dissolved in a little water, inverted by HERZFELD's[3] method, and then made up with water to B' c.c. It was polarized in 200 m.m.-tube at 20°C. The rotation of C' on the scale was observed. Hence the invert polarization (P') was calculated as follows:—

$$P'=\frac{C'\times B'\times 26}{A'\times 100}$$

Supposing that the non-reducing sugar in the syrup consists only of raffinose and sucrose, they are calculated respectively by the following formulae.

$$\text{Raffinose content in the syrup}=R\%=\frac{0.3266\times P+P'}{1.554}$$

1) Ber. D. Chem. Ges., **22** (1889), p. 1678.
2) Zeitschr. d. Ver. D. Zuckerind., **37** (1887), p. 153. ibid., **40** (1890), p. 194.
3) loc. cit.

and

$$\text{Sucrose content in the syrup} = S\% = \frac{0.5124 \times P - P'}{0.839}.$$

The syrup (I)[1], free of reducing sugar, gave a direct polarization of $+87.5^\circ$ (P) and an invert polarization of $+44.4^\circ$ (P').

The percentages of raffinose and sucrose are calculated by the formulae above mentioned as follows:—

$$R\% = \frac{0.3266 \times 87.5 + 44.4}{1.554} = 46.96\%$$

and

$$S\% = \frac{0.5124 \times 87.5 - 44.4}{0.839} = 0.51\%.$$

It is thus seen that, if non-reducing sugar in the syrup (I) consists only of sucrose and raffinose, a larger portion of the sugar present is raffinose and the sucrose content is very small.

Next, a large portion of raffinose in the syrup (I) was precipitated in alcohol solution by using strontium hydroxide and filtered hot. From the filtrate, alcohol was removed by evaporation, and then it was diluted with water. The solution, filtered off from strontium carbonate precipitated with carbon dioxide, was evaporated to a small volume, and then purified with 95% alcohol several times. The syrup obtained was small in amount and is indicated as syrup (III).

The syrup (III)[2], free of reducing sugar, gave a direct polarization of $+33.3^\circ$ (P) and an invert polarization of $+11.4^\circ$ (P').

Consequently, $R\%$ and $S\%$ in the syrup (III) are calculated as follows:—

$$R\% = \frac{0.3266 \times 33.3 + 11.4}{1.554} = 14.33\%$$

and

$$S\% = \frac{0.5124 \times 33.3 - 11.4}{0.839} = 6.67\%.$$

Thus, in the case of the syrup (III), $R\%$ is greater than $S\%$ also.

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1) The syrup (I) contained a small amount of reducing sugar, which was determined by the volumetric method of Bertrand and calculated to be glucose. The figures of P and P′ were obtained by subtracting the rotation given by glucose present, from the total rotation.

2) The reducing sugar in syrup (III) was treated the same as that of syrup (I).

Summary.

From the results of the above investigation, we may conclude that the most important sugar in lotus seeds is raffinose, and also that the presence of a little glucose and sucrose is highly probable.

Hokkaido Imperial University,
June 30th, 1920.