Isolation and Characterization of Floc-Causing Substances in Granulated Cane Sugar.

Huan-wen Herbert Hsu

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SUBSTANCES IN GRANULATED CANE SUGAR

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in

The Department of Food Science

by

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ABSTRACT

A floc-causing substance was isolated from granulated cane sugar samples and characterized.

The substance was isolated by precipitation with ethanol in a 50% sugar solution, extraction of the precipitate with NaOH, and separation of the active principle in the alkalized extract with column chromatography. Dowex 50W-x2, Amberlite IR-45, and Sephadex G-150 were used in the column chromatography.

Characterization of the floc-causing substance was conducted by paper chromatography, infrared analysis, and various chemical tests. These analyses indicated that the major floc-causing substance in granulated sugar was an amylose-related compound which could complex with a number of other compounds to enhance the floc formation. Results further showed that protein or peptides were among the complexing materials.

Observed characteristics of the floc formation were: faster floc formation at refrigerator temperatures than at room or incubator (30°C) temperatures; more rapid floc formation at the two extremes of the pH range (1.5 and 11.5); filtrability of the floc-causing substance. The addition of EDTA to the sugar solution did not prevent floc
formation, thereby suggesting that inorganic divalent minerals are not involved. The floc-causing substance was soluble in perchloric acid, dimethylsulfoxide and sodium hydroxide.

The results obtained in this research should contribute to the development of techniques for determining the presence and removal of floc-causing substances from granulated cane sugar. This application is particularly important to the soft-drink industry.
INTRODUCTION

The spoilage of bottled, carbonated beverages usually is attributed to contamination by organisms or to impurities in the added water. Good sanitation and adequate water treatment can solve these problems. It has been found, however, that some is caused by impurities in

the sugar used (22).

When the sugar used for manufacturing soft drinks contains certain kinds of impurities, a specific type of precipitate is formed. This precipitate is referred to as "floc" (25). It is loosely aggregated, like a floating cloud or cotton. It can rise to the top, suspend throughout the solution or settle to the bottom. To a prospective soft drink consumer, the impression obtained may be that of a toxic bacterial slime. Nevertheless, a differentiation can be made between the toxic bacterial slime and floc by shaking the bottle. The aggregation will disperse into the solution for the latter; whereas the aggregation will not disperse for the former (14).

The literature concerning the floc problem is limited and mostly deals with beet sugar floc. Eis et al. (9) found that saponin and its derivatives were chiefly responsible for the refined beet sugar floc. If saponins were present in an acidified syrup, they would precipitate
first and then other surface-active materials present would complex onto the floc. The problem of beet sugar flocculation has been solved because of their work. However, the problem of cane sugar flocculation still exists.

Stansburg and Hoffpauir (22) found that the floc of refined granulated cane sugar contained mostly starch, lipids (wax), protein, ash constituents, and decolorizing carbon from the refining operation. They suggested that decolorizing carbon may act as a coagulating center with other substances binding to the decolorizing carbon. Cohen et al. (5) reported that acid floc-positive sugar contained 250 times more protein than the acid floc-negative sugar and suggested that a direct relationship may exist between the protein concentration and the time of floc appearance. Liuzzo and Hsu (12) also found that floc-positive cane sugar has a relatively higher protein or peptide content than that of floc-negative sugar.

The substance which is responsible for the floc formation has never been isolated although numerous theories have been advanced. This research was conducted to isolate and to characterize the floc-forming substances in granulated cane sugar.
LITERATURE REVIEW

During the 1940's the manufacturers of carbonated drinks became increasingly critical of the quality of the sugars they used in making their beverages. Members of the soft-drink and sugar manufacturing industries had already met several times before 1949, and in these meetings the dissatisfaction of the bottlers was conveyed to the sugar manufacturers (14).

Eis et al. (9) found that saponins were "triggering" materials of beet sugar floc. If a saponin was present in an acidified sugar solution, the saponin first precipitated and then other surface-active materials present added onto the floc.

Isolation of Beet Sugar Floc-Forming Substances

The method used by Eis et al. (9) for the separation of the beet sugar floc substances was described as follows:

The solution containing the floc was filtered through F-fritted glass and the precipitate was washed with dilute hydrochloric acid. The soluble portion of precipitate was recovered from the filter by dissolving with dilute sodium hydroxide. The sodium hydroxide solution was filtered through the same type of filter to remove insoluble matter. The floc was precipitated by adjusting to
pH 2 with hydrochloric acid, heating to the boiling point, filtering hot and recovering in a dilute sodium hydroxide solution. When sufficient sodium hydroxide solution of the floc was available to produce a visible precipitate on the filter, the floc was precipitated with hydrochloric acid, separated and washed on the filter, and allowed to dry until it broke from the glass. Drying was completed under vacuum.

Characteristics and Identification of Beet Sugar Floc-Forming Substances

Separated floc caused foaming in solutions and was soluble in dilute sodium hydroxide, glacial acid, methanol, and ethyl alcohol. It was insoluble or very slightly soluble in dilute acids, and not freely soluble in ether and chloroform. It produced effervescence and flocculation when sufficient neutral solution of the floc was added to carbonated beverages, had hemolytic activity, and was principally organic matter. This substance has been identified as saponin or its derivative (9).

Shiga et al. (21) found that the beet root saponin content was between 0.012% (central part) and 0.057% (peel). Crude saponin extracted was about 0.34-0.65% of the peel. Walker and Owens (25) pointed out that in addition to sugar beet saponin and its derivatives, the floc contained fat and various adsorbed colloidal impurities from the refined sugar. Because of its colloidal nature, it tended
to act as a scavenging agent as it slowly formed aggregates picking up impurities present in the original sugar or in the water used to prepare the syrup.

Three methods have been introduced by Eis et al. (9) for the determination of saponin: the hemolysis test; the Tollens naphthoresorcinol test; and a direct gravimetric method. Walker (24), on the other hand, published the details of a test procedure based on the reaction of antimony pentachloride with saponin to produce a reddish complex. Shiga et al. (21) preferred to use 2,4-diaminophenol as a reagent for the floc test.

**Methods of Removing Saponin from Beet Sugar**

Several methods for removing saponin from beets have been suggested by Eis et al. (9): removal from the beets before processing; decrease of the amount extracted in diffusion; increase of normal elimination in liming and carbonation; decrease of the amount separated with the white sugar; and removal by new process steps. Shiga et al. (21) showed that anion exchange resins could remove floc-forming materials completely from commercial beet sugar.

Walker (24) found no saponins in refined cane sugar. Therefore, the methods of refining and floc testing in beet sugar cannot be applied to the refined cane sugar. The floc substances of refined cane sugar have been discussed in several articles. However, none of the authors had
ever separated the real "triggering" substances of the cane sugar floc.

**Isolation of Cane Sugar Floc**

Stansbury and Hoffpauir (22) prepared a 10 gallon bath of 54% sugar syrup, using sterile glassware, equipment, and distilled water. Fifty-six pounds of granulated cane sugar were dissolved in 21 liters of distilled water in a 12 gallon borosilicate glass solution bottle, and then acidified to pH 1.5 to 1.6 with 155 g of 85% phosphoric acid dissolved in 500 ml of water. The syrup was then filtered with suction through a large coarse-porosity fritted-glass funnel to remove gross foreign materials. The syrup was allowed to stand until floc coagulated and settled (about 30 days). The syrup above the floc was removed by siphoning. The syrup which remained with the floc was diluted to about 25 to 27 Brix with aqueous phosphoric acid (pH 1.6). The floc was washed with \( \text{H}_3\text{PO}_4 \) (pH 1.5) and separated by high speed centrifugation.

**Characterization and Composition of the Cane Sugar Floc**

Stansbury and Hoffpauir (22) pointed out that floc contained some tan or brownish material, black specks (presumably carbon particles), and other particles. Micro ashing indicated that the floc was about one-third inorganic material containing appreciable quantities of silica.
Major combustible constituents appeared to be free carbon,
starch, and lipids. The material gave a slight Biuret test
for protein, contained only trace amounts of phosphorus,
and gave a negative test for pectin.

Cohen et al. (5) observed that all refined sugars
that floc with acid also floc with alcohol. However, all
refined sugars that flocced with alcohol did not necessarily
floc with acid. Alcohol floc was predominantly polysac­
charide in nature, but acid floc had been shown to contain
protein, inorganic material and some polysaccharides (es­
pecially starch or starch-like materials). Although it
appeared that a protein-ion interaction might be the main
factor responsible for the flocculation, no correlation had
been found between acid floc and the concentration of
either inorganic matter or polysaccharides. Acid floc
could develop at any pH level between 1.5 and 6.5 in acid
floc-positive sugar solutions. The lower the pH of the
solution, the sooner the floc appeared. The appearance of
acid floc was delayed, to some extent, after the solution
was filtered through a 0.45 μ membrane filter. They fur­
ther observed that the most significant difference between
the acid floc-positive sugar and acid floc-negative sugar
was the fact that the former contained 250 times more pro­
tein than the latter.

Liuzzo and Hsu (12) also found that the amino acid
concentration of floc hydrolyzate was directly related to
the floc intensity. Acid floc formed between pH 1.5 and 11.5 with faster formation at the extremes of the pH range.

Methods of Granulated Sugar Floc Test (1)

Test no. 1:
1. Prepare a 50-55° Brix syrup,
2. Acidify to pH 1.5 with $\text{H}_3\text{PO}_4$,
3. Observe after 6-10 days.

This test is satisfactory with both beet and cane sugar.

Test no. 2:
1. Use 400 g sugar,
2. Add distilled water to a volume of one gallon,
3. Add 0.5 ml concentrated HCl (pH approx. 2.70),
4. Heat to 100°C for one hour,
5. Observe after 24 hr, over light beam.

This test is only satisfactory with beet sugar.

Test no. 3:
1. Acidify 50-55° Brix syrup with $\text{H}_3\text{PO}_4$ (minimum amount suggested, 240 ml) to obtain a pH of 1.5,
2. Add ethyl alcohol (95%) to obtain 40% by volume,
3. Mix and incubate at 32°C for 18-24 hr,
4. Observe for the presence of floc with light beam.

This test is satisfactory only for cane sugar.

Test no. 4—modified Coca-Cola floc test (22):
1. 63 g of sugar is dissolved in 44 ml of sterile
distilled water,
2. Add 0.4 g of 85% phosphoric acid in 10 ml of distilled water (final pH should be 1.5 to 1.6),
3. Heat the syrup in boiling water bath for 15 to 20 min,
4. Remove from the bath, cap the bottle, and allow to stand to check floc development,
5. Examine periodically with a strong light beam.
This test will detect the floc 1-2 days sooner than the original Coca Cola test.

Test no. 5—Beverage Test (15):
1. Sterilize a 7 oz soda bottle in an autoclave under 15 psi pressure and 120°C steam for 15 min,
2. Cool bottle and add 21 g of dry sugar and 25 ml of distilled, membrane (0.45 μ) filtered water (if a liquid sugar is being tested, add 21 g of dry sugar equivalents and accordingly decrease the amount of water added),
3. After the sugar is dissolved, heat to 85°C in a water bath and maintain for three minutes,
4. Cool to room temperature and add 2 ml of a citrate buffer consisting of 0.076 g of sodium citrate and 0.25 g of hydrous citric acid, mix well (the citrate buffer solution should have been previously filtered through a 0.45 μ membrane filter),
5. Cool mixture to about 40°C and add carbonated water to a designated level,
6. The bottle is quickly capped with a capping device (the caps must be previously sterilized by inserting in boiling water for 5 min),

7. Observation is made daily for 14 days with a microscope light illuminator and gentle swirling of the bottle which will aid in observing any formation of a white flocculent precipitate. Sometimes a light fluffy floc forms which tends to remain suspended above the bottom of the bottle. This type is the result of biological activity and should be removed for microscopic examination of microbological cells, pseudomycelium, mycelia, ascospores and oidiospores.

The results of floc tests were recorded as follows:

0 - no definite general floc.
1 plus - barely visible, very fine, general floc.
2 plus - very definite but fine pin-point type floc.
3 plus - larger (1/64") but pin-point (not loose) floc
4 plus - large, loose, snowflake-type floc with clearing of solution.
MATERIALS AND METHODS

Sugar Samples

The sugar samples used in the research included floc-positive and floc-negative granulated cane sugars and sugar syrups. The floc-negative sugars were either bought from supermarkets or donated by cane sugar refiners: they were coded A, B, C, D, E, F, G, H, I, J, K. All of the floc-positive sugars donated by Coca Cola Company were coded Aust.R, Aust.F, Jap., and X. The floc-positive and floc-negative sugar syrups were donated by the Refined Syrups and Sugars Inc., and were coded SI for floc positive syrup and SP for floc-negative syrup.

Reagents Used in the Research

The preparation of several major reagents used in the research are described below:

Iodine solution (27):

Two grams of iodine were dissolved in 20 ml of a solution containing 20 g of potassium iodide and diluted to 1 liter.

Folin Phenol reagents (2, 13, 19):

Reagent A - 2% Na₂CO₃ in 0.1 N NaOH

Reagent B - 0.5% CuSO₄·5H₂O in 1% potassium tartrate.
Reagent C - Mixed 50 ml of reagent A with 1 ml of reagent B.

Reagent D - Folin Phenol reagent, diluted to final acidity of 1 N.

Phenol-sulfuric acid (4, 26):

Phenol solution - 5 g of reagent grade phenol was dissolved in 95 g distilled water.

Sulfuric acid - reagent grade concentrated sulfuric acid.

Silicate measurements (10, 18):

Oxalic acid - 10 g oxalic acid (analytical reagent) was dissolved in distilled water and made up to 100 ml.

Ammonium molybdate - 10 g ammonium molybdate (AR) was dissolved and diluted to 100 ml using molar ammonium hydroxide solution. (Molar aqueous ammonia may be prepared by diluting 55 ml of concentrated AR ammonia (sp. gr. 0.880 at 20°C) to 1 liter with distilled water.)

Hydrochloric acid solution - 10 ml concentrated hydrochloric acid was diluted to 100 ml with distilled water to produce a 10% v/v solution.

Reducing solution - 0.15 g 1 amino-2 naphthol-4 sulphonic acid, 0.7 g anhydrous sodium sulfite and 9 g sodium metabisulfite were dissolved together in distilled water and diluted to 100 ml.

Standard silica solution - 1.01 g Na$_2$SiO$_3$·9H$_2$O which
(according to calculations) contained 0.1 g silicon was dissolved in distilled water and diluted to 100 ml. Silicon concentration in the solution was 0.1%. One milliliter was pipetted from the solution and diluted to 100 ml; the concentration of the silicon in the solution would be 0.001% or 10 μg/ml. From the solution 1, 3, 5, and 8 ml were pipetted, respectively, to a 10 ml volumetric flask, and diluted to the mark; the concentrations of the silicon in the solutions were 1, 3, 5, and 8 μg per ml.

**Ion Exchange Resins and Sephadex Gels**

Dowex 50W-x2 (200-400 mesh) was used to fractionate the NaOH extract of sugar impurities precipitated by alcohol from floc-positive sugar solution. The resin was mixed with water and packed into a column. The packed column was ready to be used after it was washed with 200 ml 2 N NaOH, and 200 ml 2 N HCl for a column 2 cm x 27 cm, then washed with distilled water to remove excess Cl\(^-\) (8).

Amberlite IR-45 (20-50 mesh), an anion exchanger, was used to remove the anions from the fractions that contained the floc-causing substance (FCS).

Dowex 1-x8 (-Cl) (50-100 mesh) column (1 cm x 7 cm) was washed with 1 N acetic acid (100 ml) to change it from the Cl\(^-\) form to the acetate form. The column was then washed with distilled water to remove excess acetic acid. The acetate form of Dowex 1-x8 was used to separate any
uronie acid from the hydrolysate of the FCS (4).

Amberite IR-120 (H+) (16-50 mesh), a cation exchanger, was used to remove the barium ion from the FCS hydrolysate (4). Both IR-45 and IR-120 (H+) were washed thoroughly with water before use.

Sephadex G-150 (40-120 μ) was also used to isolate the FCS. For packing the column, dry sephadex beads were stirred in a beaker with distilled water. Until the particles were completely swollen (about one day), the supernatant fluid was decanted to remove the finest particles. The remaining suspension was transferred from a funnel into a vertically mounted column which was packed as usual. The top of the bed was covered with a piece of filter paper to protect it from disturbance (16, 28).

**Floc Testing Methods**

The floc testing methods used in the research included the alcohol floc test, the acid floc test, and the soft drink beverage test.

The methods of the alcohol floc test and the acid floc test were described in the literature review. The soft drink beverage test was done by adding 30 ml commercial Coca Cola into 30 ml of the questionable 50% sugar solution. The pH was not adjusted, and the mixed solution was set in a refrigerator at 3°C for the formation of the floc. The solution was examined for floc after 5-10 days.
The Relationship of Floc-Formation Time to Temperature, Kind of Acid Added and pH of the Solution

Temperature:
One hundred milliliter floc-positive sugar solution (50% concentration) was adjusted to pH 1.5 with 85% H₃PO₄ and was then divided equally into two portions. One portion was placed in a 30°C incubator, the other in a refrigerator at 3°C. The solutions were examined daily for floc development.

Kind of acid added:
A floc-positive sugar solution (50% concentration) was divided into 4 portions, each containing 40 ml of sugar solution. The pH was adjusted to 1.5, respectively, with 2 N H₂SO₄, 6 N HCl, saturated trichloroacetic acid, and saturated oxalic acid, and placed in a 30°C incubator. The floc-formation times were compared to each other.

pH:
A floc-positive sugar solution (50%) was divided into 12 portions, each containing 70 ml. Their pH's were adjusted to pH 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, and 11.5, respectively, with H₃PO₄ and NaOH. A non pH adjusted portion was used as a control. These solutions were placed in a refrigerator at 3°C. The floc-formation times were correlated with pH.
Special Treatments of Sugar Solution
Related to Floc-Formation Time

Heat related to floc-formation time:

A 100 ml floc-positive sugar solution was divided into two equal portions. One portion was boiled for two minutes, the other was not. The solutions were adjusted to pH 1.5 with 85% $\text{H}_3\text{PO}_4$. The solutions were incubated at 30°C; floc formation times were noted.

Filtration related to floc-formation time:

A 500 ml floc-positive sugar solution (50%) was filtered through a fine asbestos filter plate in a Seitz filter with a vacuum pump. The filtrate was adjusted to pH 1.5 and was placed in a refrigerator at 3°C. Formation of floc and floc-formation times were recorded.

Effect of EDTA on floc formation:

Four hundred grams of sugar was dissolved in 400 ml of a 0.1% EDTA solution. The solution was divided into 12 portions, each containing 55 ml. Solutions were adjusted to pH values of 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5 with $\text{H}_3\text{PO}_4$ and NaOH. These solutions were placed in a refrigerator at 3°C together with a solution whose pH was not adjusted. The floc-formation times were recorded.
Isolation of the Floc-Causing Substance

Separation of impurities from sugar solutions:

The precipitation of sugar impurities which are responsible for the formation of acid floc is most desirable in the isolation procedure. It is possible to precipitate many other impurities which are not related to the floc formation by the addition of more ethanol. Therefore, the percentage of ethyl alcohol added to the acidified sugar solution to precipitate the impurities must be carefully selected. In this research, it was found that a sugar:water:95% ethanol ratio of 1:1:1 (w/v/v) gave the best results. If a sugar syrup was used the volume of ethanol added should be equal to the weight of a 50% sugar syrup multiplied by 0.5. For example, if the weight of a 50% syrup was 200 g, then 100 ml of 95% ethanol would be needed.

The isolation of the floc-causing substance from acidified sugar solution without the addition of ethanol is difficult. The floc is formed by an amylose retrogradation type of aggregation. This aggregation can be redispersed into the solution and cannot be completely sedimented by centrifugation. Therefore, the use of ethanol to precipitate the floc-causing substance is advantageous.

Two thousand grams of floc-positive raw sugar was dissolved in 2000 ml of distilled water. The solution was
adjusted to pH 1.5 with 85% H₃PO₄, to which was added 2000 ml ethanol (95%) and mixed well. The mixture was placed at room temperature (22°C) for about one week for the precipitation of the impurities. The supernatant fluid of the solution was removed by siphoning and the remaining solution was centrifuged at 10,000 rpm for 20 min to collect the precipitate. The precipitate was then washed twice with 40% ethanol employing centrifugation to remove the alcohol.

Extraction of the floc-causing substance from the alcohol precipitate:

The extraction of the floc-causing substance from the precipitated impurities (Fig. 1) was the first step in the isolation of the substance by column chromatography.

Several solvents which are usually used for starch extraction were used for the floc-causing substance extraction. These solvents included 50% perchloric acid (at 0°C), dimethylsulfoxide, 1 N NaOH (29), 0.5% ammonium oxalate, and water.

The washed alcohol precipitate (1 g wet wt), discussed above, was mixed with each of the solvents (10 ml) and stirred for 10 min. The supernatant fluid of the mixture was collected by centrifugation at 10,000 rpm for 10 min, and was shown to contain the floc-causing substance by testing with a floc-negative sugar solution (see floc testing method).
Figure 1. A Comparison of Extraction Methods for the Floc-Causing Substance
Fractionation of the alkaline extract of the sugar impurities:

The alcohol precipitate from 2000 g raw sugar was washed twice with 40% ethanol, and centrifuged to collect the precipitate. The precipitate was then divided into two portions: one portion was not treated whereas the second portion was extracted with 80-85% methanol (or acetone) in a soxhlet extractor for 16 hr to remove the waxes, phenolic compounds, etc. (5). These two alcoholic precipitates (treated and untreated) were kept separately in 50 ml centrifuge tubes, mixed with 17 ml of a 1 N NaOH solution, and stirred vigorously for 5 min. The supernatant fluids, which contained the floc-causing substance, were recovered by centrifugation. The fractionation of both supernatant fluids followed the same procedure as described below (Fig. 2 and 3). The results were compared to each other to determine the effect of the methanol extraction in the isolation of the FCS.

The Dowex 50W-x2 (200-400 mesh) column (2 x 27 cm) was washed with 200 ml each of 2 N NaOH and 2 N HCl and 500 ml of distilled water. The fluid over the column bed was removed, the last part being drained until the meniscus just reached the top of the bed. The fraction collecting tubes were set under the column in order to collect the effluent and the fractionation started when 15 ml of the supernatant was added to the top of the column. After the
50% floc-positive sugar solution (Sample Aust. R.)

- Adjust to pH 1.5 with H₃PO₄ + 95% ethanol

- Supernatant (discarded) | Remaining solution

- Supernatant (discarded) | (centrifuged) precipitate

  (washed twice with 40% ethanol and centrifuged to collect the precipitate)

  (extracted with 1 N NaOH)

- Supernatant (discarded)

  Fractionated by Dowex 50W-x2

  - 1 ml was used from each fraction for the floc test

<table>
<thead>
<tr>
<th>Floc-positive fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions 16-22 (F:18)</td>
</tr>
<tr>
<td>(dialyzed against flowing tap water)</td>
</tr>
<tr>
<td>(undialyzed substances were preserved for future characterization)</td>
</tr>
</tbody>
</table>

  Fraction 3

  | Refractionated by Amberlite IR-45 (OH) |
  | Floc test for each fraction |

<table>
<thead>
<tr>
<th>Floc-positive fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions near 18 (pinpoint floc)</td>
</tr>
<tr>
<td>(the floc-causing substance was precipitated with 2 volumes of 95% ethanol)</td>
</tr>
<tr>
<td>Centrifuged</td>
</tr>
</tbody>
</table>

  | Supernatant precipitate |
  | (extracted with 80-85% methanol in soxhlet for 16 hr) |

  | Supernatant (discarded) |
  | Residue (redispersed in water) |

  Floc test

Figure 2. Scheme Number 1 for the Isolation of the Floc-Causing Substance
50% floc-positive sugar solution (Sample Aust. R.)

<table>
<thead>
<tr>
<th>Adjust to pH 1.5 with $\text{H}_3\text{PO}_4$ + 95% ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>supernatant (discarded)</td>
</tr>
<tr>
<td>remaining solution</td>
</tr>
<tr>
<td>supernatant (discarded)</td>
</tr>
<tr>
<td>precipitate (washed twice with 40% ethanol and centrifuged to collect the precipitate)</td>
</tr>
<tr>
<td>remaining solution</td>
</tr>
<tr>
<td>supernatant (discarded)</td>
</tr>
<tr>
<td>precipitate (washed twice with 40% ethanol and centrifuged to collect the precipitate)</td>
</tr>
<tr>
<td>methanol or acetone soluble (5)</td>
</tr>
<tr>
<td>(phenolic glucoside, lignin degraded compounds, waxes, etc.)</td>
</tr>
<tr>
<td>residue</td>
</tr>
<tr>
<td>(extracted with 1 N NaOH)</td>
</tr>
<tr>
<td>supernatant (fractionated by Dowex 50W-x2)</td>
</tr>
<tr>
<td>precipitate (discarded)</td>
</tr>
<tr>
<td>fraction test for each fraction</td>
</tr>
<tr>
<td>fraction 3 (floc-positive fraction)</td>
</tr>
<tr>
<td>fractionated by Amberlite IR-45 (OH)</td>
</tr>
<tr>
<td>floc test for each fraction</td>
</tr>
<tr>
<td>(floc-positive fractions)</td>
</tr>
<tr>
<td>fraction near 18 (pinpoint floc)</td>
</tr>
<tr>
<td>fraction 3 (2F3) (preserved for characterization)</td>
</tr>
</tbody>
</table>

Figure 3. Scheme Number 2 for the Isolation of the Floc-Causing Substance
solution was drained into the resin bed, the column wall
was washed with 15 ml distilled water, and the liquid was
again drained into the bed, and another 15 ml of water was
added. The process was then followed by a gradient elution
with 60 ml of 0.3 and 0.7 N NaOH and completed with 100 ml
1 N NaOH. The flow rate was 1 ml/min; 12 ml was collected
for each fraction (8).

Characterization of fractions:

    Floc test - Acid floc-negative sugar was used for the
test. One milliliter of solution was pipetted from each
fraction to a test tube to which was added 15 ml of a 50%
acid floc-negative sugar solution. The solution in each
tube was adjusted to pH 1.5-2.0 with H$_3$PO$_4$ and was kept in
a refrigerator at 3°C for 10 days. The conclusion was that
the floc-causing substance was present in the fractions in
which floc was evident.

    Iodine test - Four milliliters of solution from the
fractions were pipetted to a test tube. One milliliter of
50% perchloric acid (at 0°C) was added to each tube, fol­
lowed by two drops of iodine solution and mixed well. The
color was measured after 15 min at 460 nm.

    Folin phenol reagent test - One milliliter of solu­
tion from the fractions was pipetted to test tubes. Five
milliliters of reagent C was added to each tube and shaken
well. After setting for 10 min, 1 ml of reagent D was
added, mixed well and allowed to stand for 30 min. The absorption was read at 500 nm (2, 13, 19).

**Phenol-sulfuric acid test** - Five-tenths milliliter of solution was pipetted from each fraction and was diluted to 1 ml with water, to which was added 1 ml phenol solution and mixed well. Concentrated sulfuric acid, 5 ml, was added from a fast-delivery pipet (10-20 sec) onto the liquid surface. The tubes were allowed to stand 10 min, then they were shaken and placed in an incubator at 30°C for 10-20 min. The absorbances of the yellow orange solutions were read at 490 nm (4, 26).

Two volumes of alcohol and phosphoric acid were added separately to portions of the fractions to determine which ones contained the main FCS.

**Removal of anions from the fractions containing the floc-causing substance:**

Fraction 3 was found to contain the floc-causing substance. However, since this fraction was eluted first from the cation exchanger (Dowex 50W-x2), the possibility existed that some anion impurities might be present (the pH of this fraction was near pH 2). In order to obtain a relatively purified floc-causing substance, refractionation of this fraction by an anion exchanger was necessary.

To perform the fractionation, the solution in fraction 3 (6 ml) was pipetted into an Amberlite IR-45 column (1 x 24 cm). After the solution entered the resin bed,
elution was performed with 35 ml distilled water and then with 60 ml 1 N acetic acid. The flow rate was 1 ml/3 min; 4 ml was collected for each fraction.

Isolation of the floc-causing substance by Sephadex G-150:

Fraction 3 which was eluted from Dowex 50W-x2 was fractionated with Sephadex G-150 (1 x 42 cm, Sephadex G-25, about 3 cm, was placed on the bottom of the column). Four milliliters of the solution in fraction 3 was pipetted onto the gel column. After the solution had entered the gel bed, 4 ml of water was used to wash the column wall and the liquid was allowed to enter the bed. The column was then eluted with distilled water. The flow rate was 1 ml/5 min; 4 ml fractions were collected (16, 28).

Deionization of fractions containing the floc-causing substance:

The fractions near number 18 (fractionated by Dowex 50W-x2) were found to contain the floc-causing substance. Since the portion of the floc-causing substance which was eluted from the Dowex 50W-x2 column was mixed with NaOH and other cations, deionization of this portion was done by dialysis. The fractions which contained the floc-causing substance were combined and the solution was added to a dialyzing tube and dialyzed against flowing tap water for 48 hours. The undialyzable substances were evaporated at room temperature (22°C) and saved for characterization.
Characterization of Isolated Floc-Causing Substance

IR spectrum of FCS:

The isolated FCS (2F·3 Aust.R., Fig. 3), which was precipitated from the fractions containing the substance by adding two volumes of ethyl alcohol and collected by centrifugation, was kept in a watch glass and dried in a desiccator for four days at room temperature (22°C). The dried material was mixed with 200 times its weight of KBr and ground in the dental Wig-L-Bug for 10 min. The ground mixture was pressed in a die for 15 min by using 20,000 psi pressure to form a pellet. The pellet was used to run an IR spectrum on a Perkin-Elmer Infrared Instrument Model 137 (23).

Hydrolyzation of the FCS for amino acid analysis:

A 2 ml solution of the isolated floc-causing substance (0.04%) (both 2F·3 and 1F·18, Fig. 2 and 3) was hydrolyzed separately with 5 ml 0.5 and 6 N HCl at 100°C for 6 and 20 hr. The hydrolysate was dried and was redissolved with 0.2 ml of 0.1 N HCl and was spotted on a Whatman no. 1 chromatography paper (9 x 22-1/2 inch). The chromatogram was resolved by one-dimensional, descending migration with a solvent containing n-butanol:glacial acetic acid:water in a 4:1:1 ratio by volume. After resolution, the paper was dried at room temperature (22°C),
sprayed with ninhydrin (0.2%) and heated at 100°C for 15 min for detection of amino acids. A standard consisting of known amino acids was chromatographed simultaneously.

Hydrolyzation of FCS for simple sugar analysis:

**Hydrolysis with enzymes (20, 27)** - Three drops of 0.1% α-amylase (B. subtilis α-amylase, partially purified, 2500 SKB units/g N.B.C.), pectinase (fungal pectinase, partially purified, N.B.C.), and amyloglucosidase (Novo Enzyme Corporation) solution were each added to 1 ml solutions of the isolated FCS (about 0.04%) (2F.3 Aust. R., Fig. 3) at pH 7 (for pectinase pH was adjusted to about 3.5). They were kept in test tubes and rotated on a test tube rotator for 48 hr at 30°C. The tubes containing the hydrolysates and enzymes were heated in a boiling water bath for 20 min to inactivate the enzymes. The hydrolysates were spotted on the Whatman no. 1 chromatographic paper and cellulose TLC plates. The solvent for the chromatographic development was the same as described in the paper chromatographic analysis of amino acids. The detecting reagent was a mixture of 4% diphenylamine:4% aniline:85% phosphoric acid in a ratio of 5:5:1.

**Hydrolysis with sulfuric acid (4)** - A 5 ml aliquot of isolated FCS (about 0.04%) (2F.3 and 1F.18, Fig. 2 and 3) was hydrolyzed with 5 ml of 2 N sulfuric acid at 100°C for
6 and 20 hr (20 hr hydrolysis was used only for the determination of uronic acid). The hydrolysate was diluted with 10 ml water, neutralized with Ba(OH)$_2$ and BaCO$_3$, and filtered through a Whatman no. 42 filter paper. The filtrate was then passed through an Amberlite IR-120 (H$^+$) column (1 cm x 5 cm) to remove the Ba$^{++}$ ion from the hydrolysate (flow rate 3 ml/10 min). The effluent was collected and passed through a Dowex 1-x8 (50-100 mesh in acetate form) column (1 cm x 7 cm) to separate uronic acid (flow rate 3 ml/10 min). The effluent was evaporated in a watch glass at room temperature and was used for chromatographic analysis of simple sugars.

The carboxyl group containing sugars, if present, would stay in the Dowex 1-x8 (acetate form) resin bed after the hydrolysate passed through. The column was washed with distilled water (about 100 ml) until the effluent was negative to the anthrone test. The column was then eluted with 20 ml 3 N acetic acid. The effluent was evaporated to dryness at room temperature (22°C) in a watch glass (4). The dried material was redissolved in 0.5 ml of pyridine and the entire solution was spotted in one spot on Whatman no. 1 chromatographic paper (or a cellulose TLC plate) for uronic acid analysis. A pectic acid (about 1 mg) was hydrolyzed and analyzed following the same procedure as a reference.
Quantitative analysis of silicate (18):

One milliliter of FCS (0.04%)(2F.3 Aust. R.) and 1 ml of distilled water were pipetted into separate 10 ml plastic tubes. Two drops of 10% NaOH was added to each tube and the mixed solutions were heated in a boiling water bath for 15 min. Two-tenths milliliters of cold ammonium molybdate was added followed by the gradual addition of 10% hydrochloric acid until a yellow color was produced. After standing for 10 min, 1 ml of oxalic acid was added, the solutions thoroughly mixed and then treated with 0.2 ml of reducing solution. The solutions were allowed to stand for 1 hr before measuring the optical density at 650 nm. The tube containing 1 ml distilled water was used as a blank. The soluble silica content of the sample was obtained by referring the optical density to a standard curve.

The standard curve of silica was prepared as follows: One milliliter of silicate solution containing 1, 3, 5, 8, and 10 µg of silicon was pipetted into separate test tubes. To each of the tubes 0.2 ml ammonium molybdate was added followed by 10 drops of 10% HCl to form the yellow color. After standing for 10 min, first 1 ml of oxalic acid was added to each tube, followed by 0.2 ml of reducing solution. The solutions were allowed to stand for 1 hr before measuring the absorbance at 650 nm. The blank consisted of distilled water and the other reagents.
Tests to determine the floc possibilities of amylose and amylopectin:

Commercial grade amylose and amylopectin (commercial grade, A. E. Staley Mfg. Co., Decatur, Illinois) were used for the experiment. Since amylose was relatively difficult to dissolve in cold water, it was dissolved in 1 N NaOH. The NaOH was removed by Amberlite IR-120 (H⁺). The amylopectin was dissolved in distilled water (both of them about 0.15%). Separate solutions of amylose and amylopectin were pipetted (1 ml) into a floc-negative sugar solution (15 ml); the solutions were mixed well and adjusted to pH 1.5-2 for the floc test (at 3°C). The type of floc produced was compared to that of isolated FCS.

Floc formation time affected by formaldehyde:

A floc-positive sugar solution (50%) was divided into ten portions, each containing 60 ml of sugar solution. Reagent grade formaldehyde (37%) was added to the five portions in 1, 5, 10, 20, and 30 ml series, respectively. The sugar solutions in the other five portions were added to 1, 5, 10, 20, and 30 ml distilled water as a control. The solutions were adjusted to pH 1.5-2 and tested for floc formation at 3°C.
The effect of partial methylation on the floc formation of isolated FCS (7):

Twenty milliliters of isolated FCS (2F.3 Aust. R., 0.04%) was added to 20 ml of 40% NaOH solution. The alkaline solution was then divided into two equal portions. One portion was treated with dimethylsulfate to methylate the FCS; the other was untreated to serve as a control for comparing the floc formation with the methylated portion.

The methylation procedure was performed as follows: The 20 ml of FCS in NaOH solution was stirred with a magnetic stirrer, at the same time, 1 ml dimethylsulfate was added during a period of 1 hr. After further stirring for 4 hr the solution and control portions were neutralized with 6 N sulfuric acid. Three milliliters of each was pipetted into 15 ml of floc-negative sugar solution for the floc test (at 3°C).

The effect of enzymatic hydrolysis of isolated FCS on floc formation characteristics:

The hydrolyzation process was the same as that discussed in "the hydrolyzation of FCS for simple sugar analysis" portion. The only difference was that after hydrolyzation for 48 hr the enzymes in the solutions were not inactivated by heat. The hydrolysates of the FCS (including enzymes) were added to a floc-negative sugar solution for the floc test (at 3°C).
RESULTS AND DISCUSSION

The Characteristics of Cane Sugar Floc

Correlation between Acid Floc Test, Alcohol Floc Test and Soft Drink Beverage Test

Since the composition of impurities in refined sugar is different from sugar to sugar, the flocculation characteristics are also different. For example, some acidified sugar solutions can form floc at 30°C; however, some cannot but they can floc at 3°C; floc formation from some floc-positive sugar solutions can be accelerated by heat but in some the heat causes no acceleration.

Since there is limited knowledge of the identity of the floc-causing substance (FCS), many floc testing methods are not dependable. For instance, G. J. Marov (Chairman of Nutritive Sweetener Committee, Society of Soft Drink Technologist, 1970) pointed out that of the several tests which have been proposed, none give consistently reliable results, i.e., a sugar that gives negative results by any of the floc tests frequently flocculates in the beverages and, conversely, sugar that would be expected to cause floc (as predicted by the floc tests) sometimes does not. The results in Table 1 show that only sample B was an alcohol floc-negative sugar. Samples B, K, P, SI, and SP were
acid floc-positive sugars (according to acid floc test at 30°C). However, according to soft drink beverage test, the real beverage floccers were sugar samples X, Jap, Aust.F., and SI.

Actually most granulated sugars floc to some degree when using the alcohol or acid floc tests. Therefore, the experience gained through repeated tests aids in developing reliable floc-testing methods. It is recommended that the alcohol floc test samples be read after 18-24 hr (1). The results of alcohol floc tests shown in Table 1 demonstrate that most floc-negative sugars could only form pinpoint flocs which gradually precipitated to the bottom. However, in the floc-positive sugars, the pinpoint floc quickly grew larger to form a loose, cloud-type floc, which gradually precipitated to the bottom. Most floc-negative sugars also formed pinpoint floc in the acid floc test. However, like the pinpoint floc of floc-negative sugars in the alcohol floc test, they did not clump together and grow larger but precipitated to the bottom or stuck to the bottom wall. Nevertheless, the pinpoint floc of the floc-positive sugars usually grew very fast, by clumping with other pinpoint floc, to form a large, loose, cloud-type floc (Fig. 4).

The floc formation procedure seemed to be related to the temperature (Table 2). For example, when an acid floc test was performed at 30°C the pinpoint floc of sample Aust.F. did not grow larger, but at 3°C the floc grew to a
Table 1

The Results of the Acid Floc, the Alcohol Floc, and the Soft Drink Beverage Tests

<table>
<thead>
<tr>
<th>Samples&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Alcohol floc (30°C)</th>
<th>Acid floc (30°C)</th>
<th>Soft drink beverage (3°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>days type&lt;sup&gt;2&lt;/sup&gt;</td>
<td>days type&lt;sup&gt;2&lt;/sup&gt;</td>
<td>days type&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>1 ++</td>
<td>18 ++</td>
<td>30 0</td>
</tr>
<tr>
<td>B</td>
<td>1 0</td>
<td>- 0</td>
<td>30 0</td>
</tr>
<tr>
<td>C</td>
<td>1 +</td>
<td>24 +</td>
<td>30 0</td>
</tr>
<tr>
<td>D</td>
<td>1 +++</td>
<td>11 +++</td>
<td>30 0</td>
</tr>
<tr>
<td>E</td>
<td>1 +</td>
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</tr>
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<td>F</td>
<td>1 ++</td>
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<td>30 0</td>
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<td>1 +</td>
<td>6 +</td>
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<td>I</td>
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<td>J</td>
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<tr>
<td>K</td>
<td>1 +</td>
<td>- 0</td>
<td>30 0</td>
</tr>
<tr>
<td>P</td>
<td>1 +</td>
<td>- 0</td>
<td>- -</td>
</tr>
<tr>
<td>X</td>
<td>1 +++</td>
<td>11 +++</td>
<td>6 +++</td>
</tr>
<tr>
<td>Jap</td>
<td>1 +++</td>
<td>9 +++</td>
<td>6 +++</td>
</tr>
<tr>
<td>Aust.F.</td>
<td>1 +++</td>
<td>15 +++</td>
<td>6 +++</td>
</tr>
<tr>
<td>SI</td>
<td>1 +++</td>
<td>30 0</td>
<td>12 +++</td>
</tr>
<tr>
<td>SP</td>
<td>1 ++</td>
<td>30 0</td>
<td>30 0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Origin of sugars: A, B, C, D, E, F, G, H, I, J, and K were floc-negative refined sugars which were either bought from supermarkets or donated by cane sugar refiners. P, a chemically pure sugar. X, donated by Coca-Cola Company and known as beverage floccer. Original source of the sugar was the American Sugar Company. Jap, donated by Coca-Cola Company; original source of the sugar was the Nippon Beet Sugar Mfg. Co. Ltd., Shimonoseki Refinery. Aust.F., donated by Coca-Cola Company; original source of the sugar was the Amstar Corporation (the raw sugar of this refined sugar was coded Aust.R., which was also donated by Coca-Cola Company). SI, donated by CPC International Inc.; it is an industrial invert. SP, donated by CPC International Inc.; it is a prime invert.

<sup>2</sup>Code of floccing: 0 = no definite general floc; + = barely visible very fine, general floc; ++ = very definite but fine pinpoint type floc; +++ = larger (1/64") but pinpoint; ++++ = large, loose, cloud-type floc with clearing of solution (7).
Figure 4. The type of acid floc from a floc-positive sugar.
loose, cloud-type floc. Also, the acid floc test seemed unsuitable for sugar-syrup (invert sugar) floc tests. Invert sugar syrup SI would not form acid floc in 30 days at 30°C (it may form acid floc at 3°C after 30 days), but it was considered a beverage floccer because it formed floc when mixed with Coca-Cola beverage (Table 1).

Therefore, the acid and alcohol floc tests can only be used as references for the sugar floc tests. For a more reliable test, the sugar in question should be put into a beverage to confirm its floccing capability.

**Temperature Effect on Acid Floc Formation Time**

Table 2 shows that the acid-floc formation time was directly related to the temperature. This interesting phenomenon can be explained by the fact that at lower temperatures hydrogen bonds between FCS were more readily formed to abet floc formation. This phenomenon is very similar to the retrogradation of amylose molecules, which is enhanced by low temperature (27).

**Effect of Different Acids on Acid Floc Formation Time**

Table 3 shows that floc of a sugar solution can be formed by the addition of different acids; however, phosphoric acid appeared to hasten the process.
### Table 2

**Acid Floc Formation Time Related to Temperature¹**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Acid floc test at 3°C (days)</th>
<th>Acid floc test at 30°C (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aust.F.²</td>
<td>7</td>
<td>15¹</td>
</tr>
<tr>
<td>X</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Jap</td>
<td>5</td>
<td>9</td>
</tr>
</tbody>
</table>

¹ Solution pH = 1.5.
² Aust.F. formed pinpoint floc at 30°C.

### Table 3

**Acid Floc Formation Time in Relation to the Kind of Acid Added¹**

<table>
<thead>
<tr>
<th>Samples</th>
<th>HCl</th>
<th>H₂SO₄</th>
<th>TCA</th>
<th>Oxalic acid</th>
<th>H₃PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aust.F.²</td>
<td>22</td>
<td>22</td>
<td>31</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Jap</td>
<td>22</td>
<td>21</td>
<td>21</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>SI³</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>X</td>
<td>12</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

¹ Sugar solution pH 1.5 at 30°C
² Aust.F. at 30°C forms pinpoint floc.
³ SI, negative in 30 days.
Effect of Solution pH's to Acid Floc Formation Time

Table 4 shows that all three beverage floccers formed floc rapidly at pH 1.5, 2.5, 10.5, and 11.5. It is obvious that the two extremes in the pH range resulted in the most rapid floc formation. A possible explanation is the presence of amphoterically charged impurities in the sugar solutions and their ability to form insoluble particles at the two extreme pH ranges. These particles probably aggregate with minerals and FCS to form the floc.

Viscosity could affect the speed of precipitation, but Browne and Zerban (3) suggested that the effect of variations in pH on the viscosity of sugar solutions is negligible, except above pH 11 where the viscosity increases.

Relation of Heating to Acid Floc Formation Time

Table 5 shows that samples X and Aust.F. accelerated the floc formation after the solutions were boiled for 2 min, but sample "Jap" decelerated in floc formation. Therefore, in the acid floc test, boiling of a sugar solution may not necessarily shorten the floc formation time. It is possible that some of the compounds in the "Jap" sugar broke down during boiling.
<table>
<thead>
<tr>
<th>pH of sugar solution</th>
<th>X (days)</th>
<th>Aust.F. (days)</th>
<th>Jap (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>9</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>2.5</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>3.5</td>
<td>15</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>4.5</td>
<td>19</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>5.5</td>
<td>24</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>6.5</td>
<td>20</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>7.5</td>
<td>24</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>8.5</td>
<td>24</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>9.5</td>
<td>15</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>10.5</td>
<td>9</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>11.5</td>
<td>9</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>12.0</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>pH not adjusted</td>
<td>-</td>
<td>13</td>
<td>24</td>
</tr>
</tbody>
</table>
Table 5
Effect of Heating on Time of Acid Floc Formation

<table>
<thead>
<tr>
<th>Samples</th>
<th>Boiling, 2 min</th>
<th>Non-heated</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>8 (days)</td>
<td>11 (days)</td>
</tr>
<tr>
<td>Jap</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Aust.F.¹</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>SI</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Aust.F. produced pinpoint floc.
**Filtration Related to Floc Formation Time**

These results showed that floc-positive sugar solutions still formed floc after filtration through a millipore filter or fine asbestos filter plate even though the acid floc formation time was prolonged. Some of them flocculated even after 40 days at 5°C. Filtration probably can remove portions of the FCS, some insoluble organic materials, and a certain amount of minerals. But filtration did not remove all of the FCS and did not prevent a floc-positive sugar solution from flocculating again.

**Addition of EDTA Related to Floc Formation**

The results shown in Table 6 demonstrated that EDTA did not prevent floc formation. From the results it is suggested that floc formation is not primarily due to the complexing of organic compounds with divalent minerals. Actually, the FCS can complex with other organic compounds or with themselves. To prove this assumption, an isolated FCS was added to solutions containing chemically pure glucose, maltose, and NaCl and the pH was adjusted to 1.5 with H₃PO₄. The results showed that the FCS per se could form floc in all of the solutions.
Table 6
Effect of EDTA on Floc Formation

<table>
<thead>
<tr>
<th>pH</th>
<th>Floc formation time (Aust.F. at 3°C) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>EDTA precipitated</td>
</tr>
<tr>
<td>2.5</td>
<td>EDTA precipitated</td>
</tr>
<tr>
<td>3.5</td>
<td>10</td>
</tr>
<tr>
<td>4.5</td>
<td>11</td>
</tr>
<tr>
<td>5.5</td>
<td>11</td>
</tr>
<tr>
<td>6.5</td>
<td>14</td>
</tr>
<tr>
<td>7.5</td>
<td>11</td>
</tr>
<tr>
<td>8.5</td>
<td>11</td>
</tr>
<tr>
<td>9.5</td>
<td>10</td>
</tr>
<tr>
<td>10.5</td>
<td>9</td>
</tr>
<tr>
<td>11.5</td>
<td>6</td>
</tr>
<tr>
<td>pH not adjusted</td>
<td>EDTA precipitated</td>
</tr>
</tbody>
</table>
Isolation of the Floc-Causing Substance

Extraction of Floc-Causing Substance from Alcohol Precipitated Floc-Positive Sugar Solutions

The five different solvents used to extract the alcohol precipitated sugar impurity (Fig. 1) were: distilled water, ammonium oxalate (0.5%), perchloric acid (50%), dimethylsulfoxide, and NaOH (1 N). The results in Table 7 show that the FCS could be extracted by any of the five solvents from the alcohol precipitate of Aust.F. and Aust.R. However, the FCS could not be extracted by H$_2$O or ammonium oxalate from the alcohol precipitate of the Jap sugar. Before extraction the alcohol precipitate should be kept in a moist state; otherwise the FCS becomes more insoluble in water and relatively difficult to extract, especially when extracted with water or ammonium oxalate. Perchloric acid (50%) seemed to have the strongest extraction power, but the extract had additional non-floc materials. Dimethylsulfoxide and 1 N NaOH extractions were more complete.

When using 1 N NaOH to extract the alcohol precipitate no heat should be added and the extraction should be performed in a plastic tube to prevent extraction of silicate from glassware.
Table 7
Extraction of the Alcohol Precipitates of Floc-Positive Sugars

<table>
<thead>
<tr>
<th>Samples</th>
<th>Water</th>
<th>Ammonium oxalate (0.5%)</th>
<th>Perchloric acid (50%)</th>
<th>Dimethylsulfoxide</th>
<th>1 N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aust.F.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aust.R.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Jap</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1 Test conducted at 3°C.

2 A "+" indicates that the extract could form the cloud-type floc when added to a solution of floc-negative sugar.
Although we have five different kinds of floc-positive sugars, only Aust.R. is a floc-positive raw sugar. Aust.F. is a floc-positive refined sugar which was refined from Aust.R. The alkaline extracts of alcohol precipitates of both Aust.R. and Aust.F. were fractionated with Dowex 50W-x2, respectively. The results show that the FCS from Aust.R. and Aust.F. have the same kind of floc. Most of the isolation and characterization research was done with Aust.R. raw sugar because it contained more FCS than the refined sugar samples examined.

The results shown in Figure 5 (Aust.R. alcohol precipitate, one week old) indicate that only fractions 3 and 4 formed the specific, loose, cloud-type floc. Fractions 8, 9, 10, 11, and 12 formed only pinpoint floc. This pinpoint floc never aggregated and finally adhered to the test tube wall or precipitated to the bottom. However, the distribution of floc-positive fractions could be affected by the condition of the sugar solution alcoholic precipitate. If the alcohol precipitate was kept for a longer time (over 1 month) in the sugar solution, or after centrifugation and then separated from the sugar solution and kept in a dry condition for several days (about one week), the floc-positive fractions would be distributed to fractions 3, 4, 16, 17, 18, 19, 20, 21, and 22 (Fig. 6). The cloud-type
Figure 5. The Dowex 50W-x2 fractionation of an alkaline extract of a one-week-old Aust.R. sugar alcohol precipitate.
Figure 6. Dowex 50W-x2 fractionation of FCS from a two-month-old alcohol precipitate of Aust.R. sugar.
floc in fractions 16 to 22 (code 1F.18, Fig. 2) were lighter and usually were suspended in the sugar solution. The FCS in fractions 3 and 4 were water soluble, but fractions 16 to 22 were water insoluble.

The iodine test, Folin phenol reagent test, and phenol sulfuric acid test of the fractions (fractionated from one-week-old alcohol precipitates of Aust.R.) are shown in Figures 7, 8, and 9. The iodine test (Fig. 7) shows two peaks with the stronger response in fraction 3. The deep blue color observed is an amyllose iodine complex response. It is believed that some starch probably complexed with another compound and carried a positive charge; this portion of starch was eluted near fraction 9 (second peak) which also produced a blue color. But the blue color of the iodine complex precipitated to the bottom of the test tubes.

The Folin phenol test results (Fig. 8) show that there were two peaks at fractions 6 and 16. However, these two peaks were not related to floc formation, since no floc was found with these two fractions in the floc test.

Figure 9 shows the results of the phenol-sulfuric acid test for general carbohydrates. This test also developed two peaks, one in fraction 3 and the other in fraction 5. This test was used to detect the location of the FCS when it was degraded or complexed with other compounds to such an extent that it did not form color when iodine
Figure 7. Iodine tests of the Dowex 50W-x2 fractions which were fractionated from a one-week-old alcohol precipitate of the Aust.R. sugar.
Figure 8. Folin phenol reagent tests of the Dowex 50W-x2 fractions which were fractionated from a one-week-old precipitate of the Aust.R. sugar.
Figure 9. Phenol-sulfuric acid tests of the Dowex 50W-x2 fractions which were fractionated from a one-week-old precipitate of the Aust.R. sugar.
was added. The results showed that the first peak, fraction 3, was the fraction that contained the main FCS.

The results of the Dowex 50W-x2 fractionation of the floc-positive sugar, sample Jap, are shown in Figures 10, 11, 12, and 13. Figure 10 shows the distribution of floc-positive fractions in the fractionation of a 2-month-old alcohol precipitate of sample Jap sugar. The floc-positive fractions were in fractions 3, 4, 16, 17, 18, 19, 20, 21, 22, 23, and 24. The cloud-type floc found in fractions 16 to 24 (coded IF.18, Fig. 2) seemed lighter than the floc in fractions 3 and 4 and were also water insoluble. Since the FCS could be dissolved in a NaOH solution, the fractions were eluted with NaOH solution. The quantity of the FCS fluctuated, and could be affected by the amount of time the substance remained in the NaOH solution. For example, if the column was shut off overnight before collecting fraction 19, the fraction obtained more FCS when elution was started again the next morning. The eluting mechanism in this portion, in addition to ion exchange, probably also was affected by solubility. Although the peak distribution of the FCS was not consistent in this portion, fractions 16, 17, and 18 usually contained more FCS.

Another interesting phenomenon was that the FCS in fractions 16 to 24 could form floc per se by merely adjusting the pH to neutral or below neutral with HCl, H$_2$SO$_4$, or H$_3$PO$_4$. This phenomenon was probably due to the fact that
Fl o c diameter (cm)

NaOH solution started to come out

Figure 10. The Dowex 50W-x2 fractionation results of a two-month-old alcoholic precipitate of sugar (sample Jap) solution.
Figure 11. Results of the iodine test on the Dowex 50W-x2 fractions of a two-month-old alcoholic precipitate of a Japanese sugar solution.
Figure 12. Results of the Folin phenol reagent test on the Dowex 50W-x2 fractions of a two-month-old alcoholic precipitate of a Japanese sugar solution.
Figure 13. Results of the phenol-sulfuric acid test for general carbohydrates in the Dowex 50W-x2 fractions of a two-month-old alcoholic precipitate of a Japanese sugar solution.
the FCS can form floc not only in the sugar solution but also in a salt solution.

The iodine test of the fractions (Fig. 11) shows only one peak in fraction 3. No iodine reaction was detected in fractions 16 to 24.

Figure 12 shows the results of the Folin phenol reagent test. Two peaks were found in the test, one at fraction 6, the other at fraction 16. The results were similar to that of Figure 8, which showed no strong correlation with the floc formation fractions.

Figure 13 shows the results of the phenol-sulfuric acid test for general carbohydrates. The first peak, fraction 3, was related to floc formation. However, the second and most intense peak, fraction 5, was not correlated with floc formation. The FCS in fractions 16 to 24 was undetectable by the reagent. (The solutions in these fractions were pooled and dialyzed against tap water. The undialyzable substances were preserved for future characterization.)

Refractionation of the Solution in Fraction 3 which Contained the FCS

Since fraction 3 was eluted first from the Dowex 50W-x2 column, it was believed that some negatively charged compounds (such as silicic acid or phosphoric acid) might be present. These compounds were separated from the FCS by fractionation with Amberlite IR-45 (OH⁻) immediately after the effluent from Dowex 50W-x2 column was collected
in fraction 3. Figure 14 shows the floc test results of the fractions. They indicate that the heaviest floc was found in fraction 3 (coded IF.3, Fig. 2) (iodine test: violet), and some pinpoint floc was found in fractions 16, 17, and 18 (iodine test: blue). It was concluded that the main FCS was still present in the fractions near 3, which indicated little or no charge.

Originally, the iodine test of fraction 3 in Figure 7 was blue. But after it was fractionated with Amberlite IR-45 (OH⁻), the iodine test showed that fractions near 3 were violet and fractions near 17 were blue. Several possibilities for the development of the violet color are the presence of amylopectin, degraded amylose, retrogradated amylose, or amylose complexed with a trace amount of protein. Fraction 3 (from Amberlite) showed specific characteristics of amylose because it had a retrogradation effect and could also be precipitated by 30% ethanol (17). The presence of all three amylose derivatives were possible, because all of them when compared with the original amylose molecule, had a relatively shorter linear structure to complex with iodine and, consequently, to form the violet instead of the blue color.

The FCS in fractions near 3 (Fig. 14) was precipitated with 2 volumes of 95% ethanol, and centrifuged to remove the ethanol. The precipitate was extracted with 80-85% methanol in a soxhlet extractor for 16 hours. The
Figure 14. Results of the elution of the Dowex 50W-x2 column fraction 3 from Amberlite IR-45 (OH)^-. 
methanol soluble portion was found to be insoluble in water. However, only the methanol insoluble portion formed the specific floc when tested in a floc-negative sugar solution. The methanol soluble portion only formed pinpoint particles which were suspended in the solution. It is believed that since these two portions were originally complexed together the methanol soluble portion could possibly contribute a hydrophobic effect to the FCS. The methanol insoluble portion was dissolved in water (about 0.04%) and tested with iodine. The iodine test color was still violet. Since this portion does have a retrogradation effect, it was concluded to be an amylose degraded product.

**Fractionation of Alkalized Extract of Methanol Extracted Alcohol Precipitate (Fig. 3)**

A two-month-old alcohol precipitate of Aust.R. sugar solution was extracted with methanol for 16 hours before fractionation with Dowex 50W-x2. The methanol soluble portion was found to develop only pinpoint floc particles when tested for floc possibilities in floc-negative sugar solutions. The main FCS was still in the methanol insoluble portion, since the 1 N NaOH extract of that portion could form the specific floc. The fractionation results of the alkalized extract show (Fig. 15) that the FCS was present only in fractions 3 and 4 (coded 2F.3, Fig. 3). These results suggest that the FCS in the fractions near 18 (Fig. 6)
Figure 15. Floccing results of the fractions from the alkalized extract of the methanol extracted alcohol precipitate.
was complexed with a substance which rendered the whole complexed group insoluble in water, to carry a positive charge, and to be eluted in fractions 16-22 together with the NaOH solution. However, it can now be seen that this complex can be broken by extraction with methanol.

Figure 16 shows results of the iodine test for the fractions shown in Figure 15 and reveals two positive peaks at fractions 3 and 7. However, only fractions 3 and 4 contained the FCS (Fig. 15).

Figure 17 shows that there were two peaks for the Folin phenol test determined on the fractions shown in Figure 15: one in fraction 8 and the other in fraction 16. Neither of them showed an ability to form floc.

Figure 18 shows that there was only one peak, fraction 3, using the phenol sulfuric acid test. This peak correlated with the fraction which contained the FCS in Figure 15.

The above evidences lead to the suggestion that the FCS is primarily a starch-like compound, since the FCS produced a strong color reaction with iodine and phenol-sulfuric acid reagent, and a very weak reaction with the Folin phenol reagent. The FCS could not be precipitated by trichloroacetic acid, consequently, the presence of a protein was ruled out.
Figure 16. Iodine test results of fractions from the alkalized extract of the alcoholic precipitate of the Aust.R. sugar which was previously extracted with methanol.
Figure 17. Folin phenol test results of the fractions from the alkalized extract of the alcoholic precipitate of the Aust.R. sugar which was previously extracted with methanol.
Figure 18. Phenol-sulfuric acid test results of fractions from the alkalized extract of the alcoholic precipitate of the Aust.R. sugar which was previously extracted with methanol.
Fractionation by Sephadex G-150

The solution in fraction 3 after elution from Dowex 50W-x2 (Fig. 5) was fractionated with Sephadex G-150 (1 cm x 42 cm). The collected solution in each fraction was tested to ascertain if floc formation was possible in a floc-negative sugar solution. The results in Figure 19 show that only fraction 5 formed floc within ten days. The solution in this fraction could also complex with iodine to form a violet color (Fig. 20). This strengthens the theory that the FCS is mainly a degraded starch. Figure 21 shows a large concentration of carbohydrates in fraction 5. These results also strengthen the theory that the FCS is carbohydrate in nature.

Characterization of Isolated Floc-Causing Substance

Characterization of Isolated FCS by Hydrolyzation and Paper Chromatography

The solution coded 1F.18 (Fig. 2) was dialyzed for two days against tap water. The undialyzable FCS was hydrolyzed with sulfuric acid and HCl (see Material and Methods). The paper chromatographic results (Fig. 22 and 23) show that the main simple sugar in the hydrolysate was glucose as was also observed in the hydrolysates of amylose and amylopectin.

The paper chromatographic results of amino acids of
Figure 19. The results of floc formation of the fractions from the Sephadex G-150 column.
Figure 20. The iodine test results of fractions from the Sephadex G-150 column.
Figure 21. The phenol-sulfuric acid test results of the fractions from the Sephadex G-150 column.
the hydrolysates of the FCS (in 1F.18) are shown in Figure 24. They indicate that several amino acids were present, thereby suggesting that the starch-like FCS was complexed with peptides or proteins.

The isolated FCS in 2F.3 (Fig. 3) was also hydrolyzed with both sulfuric acid and HCl. Figure 25 shows that the main sugar in the hydrolysate was glucose as was evident in Figures 22 and 23. The chromatogram of the Jap and Aust.R. fractions were the same as the amylose and amylopectin hydrolysates. The FCS in 2F.3 was also hydrolyzed with α-amylase, pectinase, and amylglucosidase. The results (Fig. 26) also show that the FCS was a starch-like compound. No galacturonic acid was found in the FCS hydrolysate hydrolyzed with pectinase.

Figure 27 shows results of a cellulose coated thin layer chromatographic plate spotted with hydrolysates of Jap and Aust.R. fractions from Dowex and Amberlite column isolations. The test was specific for uronic acid. The figure indicates that no uronic acid was present. This shows the absence of pectic substances in the FCS. Results in Figure 26 also support this theory.

The possibility of proteins or peptides complexed with the FCS in 2F.3 was also investigated. Paper chromatography for amino acids of hydrolysates were also compared with that of the hydrolysates of amylose and amylopectin. The results (Fig. 28) show that the FCS in 2F.3 contained
Figure 22. Carbohydrate paper chromatographic results of hydrolyzed undialyzable FCS from fraction IF.18. Code: (1) glucose; (2) a hydrolysate of amylose; (3) a hydrolysate of IF.18 (Jap); (4) a hydrolysate of IF.18 (Aust. R.); and (5) a hydrolysate of amylopectin.
Figure 23. Carbohydrate paper chromatographic results of hydrolyzed FCS from fraction 2F.3 and 1F.18. Code: (1) non-hydrolyzed 2F.3 (Aust.R.) - no spot was found; (2) a hydrolysate of 2F.3 (Aust.R.); (3) a hydrolysate of amylose, and (4) a hydrolysate of 1F.18 (Jap).
Figure 24. Paper chromatographic results of amino acids in Aust.R. and Jap hydrolysates of fraction 1F.18 (Fig. 2). Code: (1) standard amino acids; (2) Jap; (3) Aust.R.; (4) standard amino acids.
Figure 25. Paper chromatographic results of sugars in hydrolyzed fractions from the Amberlite IR-45 column (coded 2F.3, Fig. 3).
Code: (1) glucose; (2) pectic acid hydrolysate - no neutral sugar was found; (3) a hydrolysate of amylopectin; (4) a hydrolysate of 2F.3 (Jap); (5) a hydrolysate of 2F.3 (Aust.R.); (6) a hydrolysate of amylose, and (7) galactose.
Figure 26. Paper chromatographic results of sugars in enzyme hydrolyzed fractions of Aust. R. from the Amberlite IR-45 column (coded 2F.3, Fig. 3). Code: (1) galacturonic acid; (2) amylose hydrolyzed with α-amylase; (3) 2F.3 (Aust. R.) hydrolyzed with α-amylase; (4) amylose hydrolyzed with pectinase; (5) 2F.3 (Aust. R.) hydrolyzed with pectinase; (6) amylose hydrolyzed with amylglucosidase; (7) 2F.3 hydrolyzed with amylglucosidase; and (8) glucose.
Figure 27. Cellulose TLC for uronic acid determination of hydrolyzed Jap and Aust.R. fractions. Code: (1) glucose; (2) hydrolysate of 1F.18 (Jap); (3) hydrolysate of pectic acid; (4) hydrolysate of 2F.3 (Aust.R.); and (5) galacturonic acid.
Figure 28. An amino acid chromatogram of the FCS;s in Aust.R. and Jap fractions 2F.3 (Fig. 3)1
Code: (1) standard amino acids;
(2) hydrolysate of 2F.3 (Aust.R.) (about 1 mg);
(3) hydrolysate of amylose (about 3 mg);
(4) hydrolysate of amylopectin (above 3 mg);
(5) hydrolysate of 2F.3 (Jap) (about 1 mg);
and (6) standard amino acids.
amino acids; it was thereby concluded that the FCS was complexed with a protein or peptide. It was interesting to note that the chromatogram of the hydrolysates of the commercial grade of amylose and amylopectin also show the same 9 amino acids as those of the FCS hydrolysates. This indicates that trace amounts of protein or peptides are naturally complexed with starch (17). This led to the assumption that small amounts of protein or its degraded products are in chemical combination with some of the starch molecules (17). The results further emphasized that starch is the main FCS.

Two milliliters of Aust.R. fraction 2F.3 (Fig. 3) were hydrolyzed with 0.5 N and 6 N HCl for 5 and 20 hours. The hydrolysates of each were spotted on Whatman no. 1 paper and analyzed for amino acids by paper chromatography (Fig. 29). The results show that hydrolysis with 0.5 N HCl had smaller amino acid spots than those hydrolyzed with 6 N HCl. However, those which had been hydrolyzed for 20 hours showed no greater intensity of the amino acid spots over those which had been hydrolyzed for 5 hours.

The spots near the line of origin were extremely intense. The intensity seemed to be unchanged by the concentration of the HCl or by the time of hydrolysis. These spots were probably some nitrogen carrying compounds (like peptides) which were difficult to hydrolyze.

The quantity of the protein-like material which
Figure 29. The effect of acid concentration and time of hydrolysis on the yield of amino acids from Amberlite IR-45 fractionation of Aust.R. (2F.3, Fig. 3).

Code: (1) glucosamine; (2) standard amino acids; (3) 2F.3 hydrolyzed with 6 N HCl for 20 hours; (4) 2F.3 hydrolyzed with 6 N HCl for 5 hours; (5) 2F.3 hydrolyzed with 0.5 N HCl for 20 hours; (6) 2F.3 hydrolyzed with 0.5 N HCl for 5 hours, and (7) standard amino acids.
complexes with the FCS might not correlate with floc formation capabilities as is evidenced in Figure 30. Here is shown the paper chromatogram of hydrolysates of solutions recovered from the two peaks of the Sephadex G-150 fractionation (Fig. 20). The hydrolysate of the peak (fraction 5), which was floc-positive, seemed to have less amino acids than the second peak (fraction 9). Equivalent concentrations of each were chromatographed.

Silica Contained in the FCS (2F.3 Aust.R.)

In flocculation research silica or silicate usually is an important subject which should be discussed especially since these molecules can polymerize or complex with organic materials to form floc (10). The silica contained in the FCS (2F.3 Aust.R.) was determined by the method described in "Analytical Methods Used in Sugar Refining" (18).

The results of the determination show that very little silica was present in the FCS (2F.3 Aust.R.). If any was present, the percentage (in silicon percentage) was below 0.5 ppm. The standard curve in ppm of silicon is shown in Figure 31.

IR Spectrum of Isolated FCS (2F.3 Aust.R.)

Initially in this study, an attempt was made to differentiate between floc-positive and floc-negative sugars by a study of the IR spectra of the sugar alcoholic flocs.
Figure 30. Amino acid content of the hydrolysates of solutions in the two peaks (fractions 5 and 9 in Fig. 20) of the Sephadex G-150 fractionation of 1F.3 (Aust.R.).
Code: (1) standard amino acids; (2) hydrolyzed fraction 5; (3) hydrolyzed fraction 9; and (4) standard amino acids.
Figure 31. Standard curve of silicon concentration.
But the results were unsatisfactory, because the peaks from the polysaccharides overlapped the peaks from the non-polysaccharides, and consequently, the spectra were similar (Fig. 32).

The IR spectrum of the isolated FCS (2F.3 Aust.R.) was compared with that of amylose (Fig. 33) and was found to be very similar. The most striking differences were found in the region above 9 microns. The significance of using IR spectrum to differentiate floc-positive sugar from floc-negative sugar must be further evaluated. However, from the isolated FCS spectrum it was believed that a great portion of the FCS was a polysaccharide, probably starch.

The principal absorption bands discerned in the spectra and the vibration groups probably associated with them are listed in Table 8 (23).

Effect of Enzymatic Hydrolyzation of Isolated FCS on Floc Formation Characteristics

The floc test of the enzymatically hydrolyzed FCS showed that only those hydrolyzed with amyloglucosidase could not form floc in the floc-negative sugar solution after 60 days (also negative in Coca-Cola beverage). Those hydrolyzed with pectinase or α-amylase formed floc in 3 days. This discovery was very interesting and important because it suggested that the FCS was a starch, and trace amounts of enzymatic protein would not cause the sugar solution to form floc.
Figure 32. IR spectra of alcohol floc of cane sugar solution. Code: (1) sample J, floc-negative sugar; (2) sample Aust.R., floc-positive sugar; (3) sample D, floc-negative sugar; (4) sample Jap, floc-positive sugar; (5) sample SI, floc-positive invert syrup.
Figure 33. IR spectra of commercial grade amylose and isolated FCS from Aust.R.
### Table 8
Absorption Bands and Related Vibration Groups Useful in Floc Investigation (23)

<table>
<thead>
<tr>
<th>Approximate position of absorption band maximum (µ)</th>
<th>Vibration group probably causing observed absorption band</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8-3.2</td>
<td>Free OH and bonded OH stretching</td>
</tr>
<tr>
<td>3.0-3.5</td>
<td>NH stretching</td>
</tr>
<tr>
<td>5.6-6.1</td>
<td>CO (carbony stretching)</td>
</tr>
<tr>
<td>6.2-6.3</td>
<td>COO(^-) stretching</td>
</tr>
<tr>
<td>7.0</td>
<td>CH(_2) symmetrical bending</td>
</tr>
<tr>
<td>7.3-7.6</td>
<td>CH bending</td>
</tr>
<tr>
<td>7.5</td>
<td>OH bending</td>
</tr>
<tr>
<td>8.6</td>
<td>CO stretching and/or COH bending (ring frequency)</td>
</tr>
<tr>
<td>8.1-9.5</td>
<td>PO (inorganic) bending and stretching</td>
</tr>
<tr>
<td>9.1-11.0</td>
<td>SiO(_3)(^-) (inorganic) bending and stretching</td>
</tr>
<tr>
<td>9.4</td>
<td>OH bending</td>
</tr>
<tr>
<td>9.8</td>
<td>COH stretching</td>
</tr>
<tr>
<td>10.0</td>
<td>CO, CC, CH(_2) stretching</td>
</tr>
<tr>
<td>11.1</td>
<td>CH bending</td>
</tr>
<tr>
<td>15.4</td>
<td>OH out-of-plane bending</td>
</tr>
</tbody>
</table>
Amyloglucosidase is capable of hydrolyzing the glucosidic bonds in both the linear and branched fractions of starch to glucose (Fig. 26); however, α-amylase can only hydrolyze starch to maltose and dextrin (Fig. 26). Actually, the degraded products from amulose, the dextrins, still have the retrogradation effect. Since amyloglucosidase can hydrolyze starch to glucose, it can completely destroy the floc formation capability of the isolated FCS (2F.3 Aust.R.).

The possibility of using amyloglucosidase to hydrolyze the FCS in a granulated sugar solution has been investigated, but the efforts were unsuccessful. The reason was probably that in the sugar solution many impurities were complexed with FCS which prevented the enzyme from completely hydrolyzing the FCS.

Tests to Determine the Floc Possibilities of Amylose and Amylopectin

According to the evidences discussed above, the isolated FCS is a starch-like compound. The starch granule is comprised of molecules of amylose and amylopectin. In order to know which one is responsible for forming the specific floc in sugar solutions, the aqueous solutions of commercial grade amylose and amylopectin were tested in a flocc-negative sugar solution. The results showed that only amylose could form the specific cloud-type floc; amylopectin did not form any kind of floc. Actually amylopectin was dissolved in the sugar solution (17, 27, 29), whereas
amylose is not truly soluble in water. Upon neutralization of an amylose alkalized solution, it was found that, sooner or later, turbidity developed and the amylose ultimately precipitated. This is the phenomenon of retrogradation which is so well known in starch literature. There seems little doubt that the retrogradation of starch is primarily due to the amylose component. Amylopectin solutions in water are relatively stable (17, 27, 29).

The term "retrogradation" has been employed in the starch field for over 60 years to describe the process whereby starch in the dissolved or hydrated state reverts to a water-insoluble form (17, 27, 29).

Retrogradation merits much further investigation. At present, it seems clear that it is an intermolecular process, the rate of which depends strongly on: (a) concentration of amylose; (b) concentration of amylopectin—which decreases the rate; (c) temperature—retrogradation rate increases with decreasing temperature; (d) nonstarch component in the medium; and (e) molecular size. Amylose preparations of very high molecular weight retrograde more slowly than preparations of moderate molecular weight when compared at similar weight concentrations (27). Polyvalent cations were found to have a significant accelerating effect on retrogradation (27).

Retrograded starch is insoluble in water and resistant to enzymic hydrolysis; it does not form the blue
iodine complex (27).

Some dextrins still show evidence of retrogradation, and this is attributed to undestroyed linear material (27).

Prevention of Floc Formation by Adding Formaldehyde

Formaldehyde has a pronounced stabilizing effect on concentrated aqueous solutions of amylose by preventing retrogradation (11, 17, 29).

It is common knowledge that formaldehyde is a protein denaturing agent. If protein is the main floc-causing substance in floc-positive sugar solutions, the floc formation time should be shortened by the addition of formaldehyde. However, if amylose is the main floc-causing substance, the floc formation time should be prolonged (or even prevented) by the addition of formaldehyde to the floc-positive sugar solution. The experimental results are shown in Table 9.

Table 9 shows that when the sugar solution was diluted with water the floc formation time was shortened, but when formaldehyde was added, the floc formation time was prolonged. When 20 or 30 ml formaldehyde was added to a 60 ml Aust.F. sugar solution, no floc was formed in 60 days. Therefore, it is suggested that the amylose-like compound (or degraded amylose) is the main floc-causing substance, and is in the center of the floc aggregation. The flocculation of a sugar solution is probably the phenomenon of the retrogradation of an amylose-like compound.
Table 9

Floc Formation Time Related to the Volume of Formaldehyde (37%) Added to Sugar Solutions

<table>
<thead>
<tr>
<th>Formaldehyde added (ml)</th>
<th>Jap sugar 60 ml at 50% conc. (days)</th>
<th>Aust.F. sugar 60 ml at 50% conc. (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>28</td>
</tr>
<tr>
<td>20</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>30²</td>
<td>-</td>
</tr>
</tbody>
</table>

Control Sugar Solution

<table>
<thead>
<tr>
<th>Distilled Water (ml)</th>
<th>Jap sugar 60 ml at 50% conc. (days)</th>
<th>Aust.F. sugar 60 ml at 50% conc. (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

1Solution pH = 1.5-2.0, Temperature = 3°C

2Only several pinpoint flocs. If 50 g of Jap was dissolved in 50 ml of formaldehyde and 20 ml of water, no floc was formed in 60 days.
Delaying the Floc Formation Time of Isolated FCS

The solubility characteristics of starch may be enhanced, and retrogradation prevented by partial methylation (17). If the isolated FCS is an amylose-like compound, floc formation can be delayed or prevented by partial methylation of the isolated compound.

Three milliliters of partially methylated FCS (Aust.R. 2F.3) and 3 ml of 20% NaOH treated FCS (Aust.R. 2F.3) were each added to a 15 ml floc-negative sugar solution, and adjusted to pH 1.5 for floc testing. The results show that the sugar solution containing partially methylated FCS formed floc after 14 days, but the non-methylated one formed heavy floc in 3 days (Table 10).

The delaying of the floc formation time was due to the methylation. The methyl groups on the partially methylated amylose molecules retarded the formation of hydrogen bonds between the linear molecules of amylose. This is further evidence that the FCS is an amylose-like compound.

Isolated FCS Ability to Form Floc in a Non-Sucrose Solution

Since "retrogradation" of amylose is a process whereby amylose in the dissolved or hydrated state reverts to a water-insoluble form (27), it can be concluded that amylose per se can form floc in glucose, maltose, NaCl
Table 10

Comparison of the Floc Formation Time between Methylated FCS and Non-Methylated FCS

<table>
<thead>
<tr>
<th>Non-Methylated FCS</th>
<th>Methylated (partially) FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>14 days</td>
</tr>
</tbody>
</table>

\[^1\text{FCS, 2F.3, isolated from Aust.R.}\]
solutions, etc. If isolated FCS is an amylose-like compound, it should form floc under the above controlled solutions. The results show that the isolated FCS could form the specific cloud-type floc in glucose, maltose, and NaCl solutions. These results give additional evidence that the FCS is an amylose-like material.

The results which were obtained in this research project show that the floc-causing substance in granulated cane sugar is an amylose-related compound which can complex with a number of other substances to enhance floc formation. This work should contribute to the development of techniques for determining the presence and removal of floc-causing substances from granulated cane sugar.
SUMMARY

A floc-causing substance was isolated from granulated cane sugar samples and characterized.

The substance was isolated by precipitation with ethanol in a 50% sugar solution, extraction of the precipitate with NaOH, and separation of the active principle in the alkalized extract with column chromatography. Dowex 50W-x2, Amberlite IR-45, and Sephadex G-150 were used in the column chromatography.

Characterization of the floc-causing substance was conducted by paper chromatography, infrared analysis, and various chemical tests. These analyses indicated that the major floc-causing substance in granulated sugar was an amylose-related compound which could complex with a number of other compounds to enhance the floe formation. Results further showed that protein or peptides were among the complexing materials.

Observed characteristics of the floe formation were: faster floe formation at refrigerator temperatures than at room or incubator (30°C) temperatures; more rapid floe formation at the two extremes of the pH range (1.5 and 11.5); filtrability of the floc-causing substance. The addition of EDTA to the sugar solution did not prevent
floc formation, thereby suggesting that inorganic divalent minerals are not involved. The floc-causing substance was soluble in perchloric acid, dimethylsulfoxide and sodium hydroxide.

The results obtained in this research should contribute to the development of techniques for determining the presence and removal of floc-causing substances from granulated cane sugar. This application is particularly important to the soft-drink industry.
BIBLIOGRAPHY


VITA

Huan-Wen Hsu was born in Fukien province, the Republic of China, on January 20, 1937. Before the Communists took control of mainland China he and his parents left his home town for Taiwan. He attended Taichung First Middle School, in Taichung, Taiwan, and was graduated in July, 1958.

The following September he entered the Fishery Technology Department of Taiwan Provincial Maritime College, Keelung, Taiwan. He was graduated from Maritime College in July, 1962. After graduation he worked as a technician in charge of canned food processing in the Subsistence Factory of the Chinese Army for four years, and then as an assistant professor in his alma mater for another three years. During this time, he completed requirements for the B.S. degree, which was granted to him in 1967.

In September, 1970, he enrolled in the Graduate School of Louisiana State University. The next spring he was assigned a research assistantship in the Department of Food Science. He has been inducted into Phi Kappa Phi, Gamma Sigma Delta and Phi Tau Sigma honorary fraternities. At present he is a candidate for the Doctor of Philosophy degree in Food Science at Louisiana State University.
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Major Field: Food Science

Title of Thesis: Isolation and Characterization of Floc-Causing Substances in Granulated Cane Sugar

Approved:

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