THE OXIDATION OF SUCROSE WITH HYPOCHLOROUS ACID

A Thesis

by

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HARDY

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GENERAL INTRODUCTION

Studies in the oxidation of wheat starch with hypochlorous acid by McKillican and Purves suggested that 80 to 90% of the oxidation occurred on carbon no. 6 of the glucose monomer unit in the starch chain, leading to the hypothesis that hypochlorous acid at pH 4- 4.2 was somewhat selective in its attack on the starch molecule.(1)

In the present work, sucrose was chosen as the carbohydrate to be oxidized inasmuch as it is a non-reducing disaccharide having three primary hydroxyl groups available for oxidation. It was hoped that the information gained would be a suitable guide to the study of the oxidation of cellulose by hypochlorous acid at some future date.

HISTORICAL INTRODUCTION

Sucrose has been known as a crystalline sugar derived from sugar cane juice in India as early as 300 A.D., in China from about 400 A.D., and in Egypt from about 640 A.D. Knowledge of sucrose or cane sugar came to Europe gradually through the contact of its peoples with those of the Near East and Africa. By the year 1600 cane sugar was an established sweetening agent in Europe and many mills had been built to produce it. The introduction of sugar cane culture to the Americas has been ascribed to Columbus, who brought it to Santo Domingo on his second voyage.

The necessary restriction of sugar cane culture to tropical or semi-tropical climates stimulated a search for a second source of the substance. During the latter part of the eighteenth century and the early part of the nineteenth, development of the sugar beet as a second source took place. The blockade of the European continent during the Napoleonic wars lent especial urgency to the process.(2)

Sucrose is a white, crystalline solid, melting between 160- 186° C. according to the medium used for purification(3), and has a specific rotation of +66.53° (5) in water. The substance is non-reducing and is best identified by the Raybin test(4), in which an alkaline solution of diazouracil

turns green in the presence of sucrose. Sucrose occurs almost universally throughout the plant kingdom, with its principal sources being sugar cane, sugar beets, and the sap of certain maple trees.

Commercial production of sucrose from cane involves harvesting, cutting into suitable lengths, and passing through roll crushers to force out the juice. The juice is about a 15% sucrose solution in water with about 1% of other carbohydrates present. The juice is treated with slaked lime and then evaporated in vacuum pans, heat control and evaporation rate being such that good crystal growth is obtained. This sugar, the "raw sugar" of commerce, is then refined by washing in mother-liquor from previous refining operations, solution in small amounts of hot water, treatment with lime, and passage of the solution through towers of activated carbon previous to final crystallization. The sucrose thus obtained is the commercial white sugar and is about 99% pure or better.

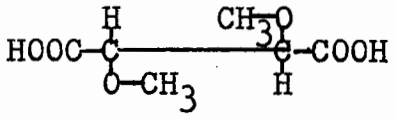
Sucrose from beets is obtained in much the same way except that it must be extracted from the beets with water in a multi-stage counter-current diffusion system. Additional amounts of sucrose may be extracted from the mother-liquors by means of the Steffen process which involves the formation of a difficultly soluble compound, tricalcium saccharate, by treatment of the sucrose solution with an

excess of lime. This compound may be decomposed by treatment with carbon dioxide to yield sucrose and calcium carbonate. Sucrose may be recrystallized from aqueous or aqueous alcohol solution(5).

Sucrose is hydrolyzed by acids and by enzymes to a mixture of equal amounts of D-fructose and D-glucose; a process known as inversion because of the change in specific rotation on hydrolysis from a dextrorotatory to a levorotatory value. The mixture is known as invert sugar.

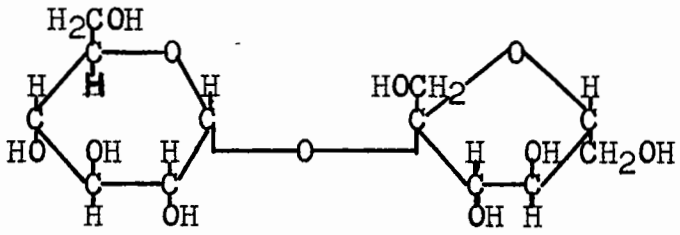
Methylation of sucrose gives an octamethyl sucrose, showing that eight hydroxyl groups are available for esterification and etherification. Octamethyl sucrose does not undergo inversion of rotation on hydrolysis but instead, yields two dextro-rotatory tetramethyl hexoses. One tetramethyl hexose is the well-known 2,3,4,6-tetra-O-methyl-D-glucose, obtained by methylation of D-glucose. The other tetramethyl derivative is of D-fructose. Oxidation of this derivative with nitric acid yields a liquid tri-O-methyl-2-keto-D-gluconic acid, which, in turn, yields a crystalline tri-O-methyl-D-arabonic acid γ-lactone on oxidation with acidic potassium permanganate. This lactone is identical with that obtained by oxidation of tri-O-methyl-L-arabinose except for the sign of rotation. The tri-O-methyl-D-arabonic acid γ-lactone yields (levo) di-O-methyl-d-tartaric acid on further treatment with nitric acid, therefore there must

have been methyl groups at positions 2,3, and 5 and the original tetramethyl fructose derived from sucrose must have had methyl groups on carbons 1,3,4, and 6.

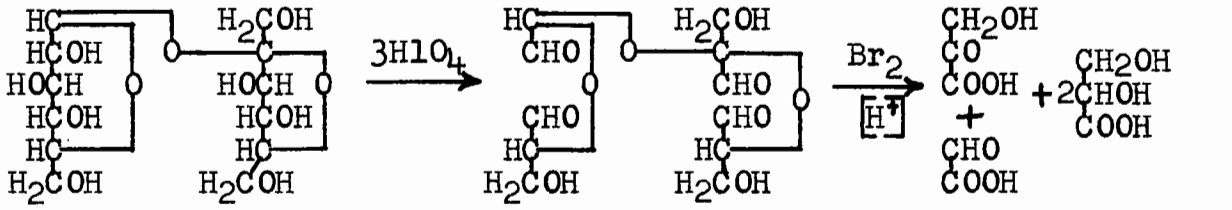


(levo) di-O-methyl-D-tartaric acid

Then, assuming the appropriate ring structure for glucose and fructose; i.e., a pyranose ring for glucose and a furanose ring for fructose, the structure of sucrose must be as follows, with the exact configuration of the anomeric carbon atoms in doubt[6]:



The above structure is confirmed by oxidation with periodic acid, a reagent which cleaves 1,2-glycols. Sucrose consumes 3 moles of periodic acid and liberates 1 mole of formic acid. After oxidation of the tetra-aldehyde with bromine and subsequent hydrolysis, hydroxypyruvic, D-glyceric, and glyoxylic acids are obtained[7].



Sucrose | Oxy-Sucrose Tetraldehyde | Hydroxypyruvic, D-glyceric, and glyoxylic acids

The configuration of the glycosidic linkages of sucrose is alpha for the glucose component and beta for the fructose. Hydrolysis of sucrose by yeast α-glucosidase and not by β-glucosidase from almond emulsion supports the α-glucoside structure. Similarly, hydrolysis of the sugar by yeast invertase, an enzyme hydrolyzing β-fructofuranosides but not the α-fructofuranosides, supplies further evidences for the designated structure[8][9]. Use of the Isorotation rules tended to confirm these inferences, in that only the α,β-configuration gave agreement between the observed and calculated rotational values of sucrose and sucrose octaacetate[10].

In 1954 when Lemieux and Huber[11] were finally able to synthesize sucrose from tri-O-acetyl-D-glucosan $\langle 1,5 \rangle \alpha \langle 1,2 \rangle$ and 1,3,4,6-tetra-O-acetyl-D-fructofuranose by heating the two components together in a 1:1 mole ratio in a sealed tube at 100° for 104 hours, and using column chromatography to separate the sucrose as the octaacetate from its isomers in 5.5% yield. Various attempts had previously been made to synthesize sucrose but without success. It is, however, interesting to note that sucrose had previously been synthesized by biological means. Hassid, Doudoroff, and Baker[12] had found that a cell-free bacterial emulsion (Pseudomonas saccharophila) contains a phosphorylase which in the presence of an inorganic phosphate hydrolyses sucrose to fructose and glucose 1-phosphate, and will regenerate sucrose from these substances. Analagous disaccharides may also be synthesized from glucose 1-phosphate and sorbose or D-xylulose[13].

Oxidation of Sucrose by Various Reagents

Sucrose has been oxidized, or has not been oxidized by a number of reagents.

Williams and Wood[14] found a certain specificity in that ketoses, sucrose, and pentoses are oxidized by iodic

acid in strong sulfuric acid at 100° while aldohexoses and lactose are not. The oxidation of sucrose by iodic acid is not surprising inasmuch as the sucrose would be almost instantaneously hydrolyzed to glucose and fructose under the conditions used and fructose, a ketose, had already been shown to react with the reagent. Jeanes and Isbell[15] found that chlorine dioxide and chlorates in the absence of a catalyst and in mild conditions show no signs of oxidizing aldoses, ketoses, and sucrose. Van Fossen and Pacsu[16] found similar results using alkaline bromates.

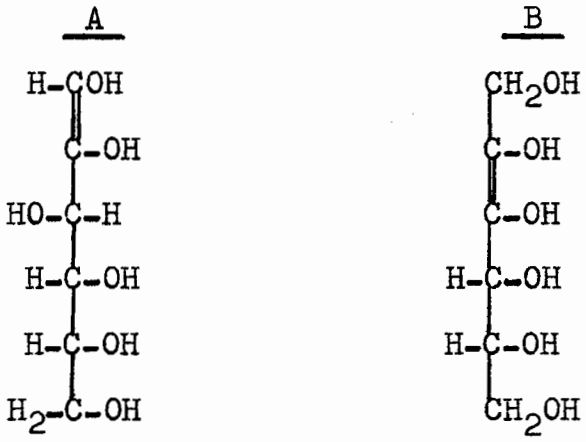
Oxygen of the air very slowly oxidizes sucrose in hot neutral solution during commercial refining of the sugar with the formation of acids and the liberation of carbon dioxide. The increase of acidity causes inversion of the sucrose and decomposition of the resulting hexoses. The presence of lime or an increase of alkalinity speeds up the oxidation[17]. Hot solutions of chromic acid and ceric sulfate oxidize carbohydrate materials to carbon dioxide, formic acid, and formaldehyde; the consumption of oxidant being a fairly precise measure of the carbohydrate present. For instance, when oxidized with ceric sulfate, sucrose behaves as follows:

$$C_{12}H_{22}O_{11}\ \text{(sucrose)} + 13O \longrightarrow 2CO_2\uparrow + 9HCO_2H + HCHO + 2H_2O$$

An accuracy of $\pm$ 0.3% is claimed here[18]. Carbon dioxide

was also the final product when an alkaline solution of sucrose was oxidized by air in the presence of cerous hydroxide, ferrous hydroxide, or sodium sulfite[19][20].

Evans[21], in his work on the action of alkalai on reducing and non-reducing sugars, recorded oxidations of glucose, fructose, galactose, sucrose, and maltose. Results of these reactions led him to the conclusion that the alkali produced a mixture of "ene-diols" of the following types A,B, and others, whose exact composition depended on the concentration of the alkali present.



These active "ene-diols" were the units which were rapidly oxidized and their positions in the molecule governed the stages through which the oxidation proceeded. Periodic acid has been used to oxidize sucrose as has been previously noted[7].

The literature regarding oxidations of sucrose is sparse, probably because of the mixed oxidation products

which would be produced and because of the necessity of guarding against hydrolysis of the sucrose to its component glucose and fructose.

Hypochlorous Acid and Its Effects on Sucrose and Monosaccharides

All of the halogens when passed into or dissolved in water hydrolyze according to the following reaction:

$$X_2 + H_2O \rightleftharpoons HOX + HX$$

The equilibrium constants for this reaction are rather small and so in acid solution the equilibrium lies quite far to the left. Mellor[22] gives the following values: chlorine, $k = 4.5 \times 10^{-4}$; bromine, $k = 2.4 \times 10^{-8}$; and iodine, $k = 3.6 \times 10^{-13}$.

If alkalai is added to the solution, the concentration of the hypo-halite ion increases:

$$X_2 + 2\ MOH \longrightarrow MOX + MX + H_2O$$

Hence the concentration of the halogen, the hypochlorite, and hydrochlorous acid are distinctly dependent on the pH of the solution.

Although the approximate equilibria existing between the hypohalite ion, the undissociated hypohalous acid and free halogen are known for bromine and iodine, the equilibria concerning hypochlorous acid have been much more

thoroughly studied because of their greater technical importance. Green[23] and White[24] summarized the results.

When chlorine was passed into water, hypochlorous acid was formed as noted above. The reaction:

$$Cl_2 + H_2 \rightleftharpoons H^+ + Cl^- + HOCl$$

reached equilibrium in 48 hours at 25° C. The oxidative power of chlorine water depended on the reaction:

$$2\ HOCl \longrightarrow 2\ HCl + O_2\uparrow$$

There might also have been an equilibrium with the anhydride of hypochlorous acid considered to be chlorine monoxide:

$$2\ HOCl \rightleftharpoons Cl_2O + H_2O$$

Hypochlorous acid dissociated as an acid but was found to dissociate also as a base to a small degree:

$$HOCl \rightleftharpoons H^+ + OCl^-$$

$$HOCl \rightleftharpoons Cl^+ + OH^-$$

The spontaneous decomposition of hypochlorous acid gave as final products, chloric and hydrochloric acids, according to the following reactions:

$$HOCl + HCl \rightleftharpoons H_2O + Cl_2$$

$$2\ Cl_2 + HOCl + H_2O \longrightarrow HClO_3 + 4\ HCl$$

In alkaline solution, the chlorine reacted with the base to give the corresponding salt as noted above.

The maximum concentration of undissociated hypochlorous acid was found in the pH range of 3- 6. Ridge and Little[25]

found 99.6% of the available chlorine in this form at pH 4 and 20- 25°. Green gives the dissociation constant of hypochlorous acid as 6.5×10^{-8}; the range given in the literature being 3.2 to 10.5×10^{-8}.

McCarthy[26] used chlorine, hypochlorous acid, and hypochlorite ion in bleaching studies. He found that from pH 0 to 2 the chlorine in chlorine water was mainly in the form of molecular chlorine, although at pH 2, 20% was in the form of hypochlorous acid. From pH 2 to 7.5 hypochlorous acid predominated, at pH 4, 97.5% of the chlorine was in the form of hypochlorous acid and at pH 5, 99.6% was in this form. Above pH 7.5 the hypochlorite ion predominated. The hydrolysis constant for chlorine in water was reported as 4.48×10^{-4}, while the dissociation constant was 3.7×10^{-8}; values similar to those found by other workers.

White[24] determined the strength of hypochlorous acid solutions by titration with thiosulfate after the acid had reacted with potassium iodide in acid solution, the method giving a measure of the total oxidizing strength of the solution. The addition of a measured amount of hypochlorous acid to an excess of 0.1 N sodium arsenite and back-titration of the excess arsenite with 0.05 N iodine solution in the presence of excess sodium bicarbonate has been found to be a measure of only the hypochlorous acid in solution[27]. Caron and Raquet[28] used hydrazine sulfate to determine

alkaline hypochlorite. The method depended on the reaction:

$$2\ NaOCl + NH_2NH_2 \cdot H_2SO_4 \longrightarrow 2NaCl + H_2SO_4 + N_2\uparrow + H_2O$$

and was not affected by the presence of chlorites or chlorates.

The first use of halogens for the oxidation of simple carbohydrates was that of bromine water by Hlasiwetz[29] to oxidize lactose to lactobionic acid. The reagent thereafter became standard in sugar chemistry for carrying out this type of chemical change. Later, Hlasiwetz and Habemann[30] prepared D-gluconic acid from D-glucose using chlorine water; their yields, however, were low, probably because of over-oxidation. Hönig and Ruzicka[31] also oxidized glucose and galactose with alkaline hypobromite and hypochlorite. They used barium hypobromite with a slight excess of barium hydroxide, or bleaching powder and excess calcium hydroxide. The concentration of hypobromite or hypochlorite was 0.1 N and that of the sugar solution 1%. The products were gluconic and galactonic acids.

In 1924 Craik[32] oxidized sucrose, maltose, and lactose with unbuffered hypochlorous acid at a concentration ratio of 1 available oxygen atom to 1 mole of sugar. No acids were produced by this oxidation. The final optical rotations of the solution were not those calculated for the residual sugars present, suggesting that some addition compounds had been formed which were not isolated. The reactions were followed polarimetrically, by the rate of disappearance of

available oxygen, by any change in the acidity of the solution, or in copper-reducing power, by the action of phenyl hydrazine acetate, and by the isolation and identification of individual products.

Fletcher and Taylor[33] noted that chlorine water attacked D-gluconic acid rapidly and formed reducing matter. No attempt was made to isolate the product.

Reports about the use of chlorine, hypochlorous acid, and hypochlorites on simple carbohydrates were very few, probably because the reagents led to over-oxidation even though the desired reaction was completed. The reagents have been used much more frequently for oxidations of starches and cellulose, but only limited amounts of information became available due to the complexities introduced by the heterogenity of these reactions, whereas those with the simple carbohydrates are homogeneous.

The most important observation in the light of the present work was that the pH of the solution actively controlled the nature of the products. Marsh [34] in 1948 reported that when cellulose was treated with chlorine water the highest carboxyl content was obtained at pH 10 to 11, while pH 4 gave the highest reducing values. At pH 10 the chlorine was, of course, hypochlorite ion and at pH 4, mainly hypochlorous acid. This behavior had also been noted previously by Birtwell, Clibbens, and Ridge[35]. As stated

by Green[23], the action of the halogens on carbohydrates has probably been obscured because of the difficulty in determining the nature of some of the oxidants, the transient character of several oxyhalogen acids, and the frequent difficulty of isolating oxidation products. To this might be added the difficulty of preventing hydrolysis under acid conditions during the oxidation of anything but monosaccharides.

Chlorous Acid Oxidations

In 1941 Jeanes and Isbell[15] reported their investigation of the behavior of chlorous acid and chlorites on carbohydrates. They found that the non-reducing disaccharide, sucrose, and the glycoside, methyl α-D-glucoside, were unaffected except when reaction conditions were sufficiently acid to allow hydrolysis of the glucosidic linkage. Furthermore, salts of aldonic acids such as sodium gluconate and magnesium xylonate were not affected by alkaline chlorites except after prolonged treatment. Fructose was found to be not appreciably affected over a period of several days. Twenty-one days produced a small amount of oxidation. In marked contrast to this behavior, aldoses were found to be readily and rapidly oxidized.

Inasmuch as chlorous acid spontaneously decomposes to give hydrochloric acid, chlorine dioxide and chloric acid, the effects of these three substances on aldoses were also studied. Jeanes and Isbell were able to show that aldoses were not appreciably affected by chlorine dioxide. Chlorates and chloric acid had no effect on aldoses under the mild reaction conditions used for the chlorous acid oxidation. The whole investigation of the quantitative aspects of the reaction was greatly complicated by the decomposition of the chlorous acid. Jeanes and Isbell found that the disappearance of the dark color, presumably chlorous acid, produced on acidification of sodium chlorite with acetic acid during reaction with an aldose signalled the virtual cessation of the reaction. Using glucose, the reaction at pH 2 was essentially complete in 6 minutes, but at pH 4.2 the reaction required several hours.

They were, however, able to determine that the oxidation proceeded according to the following reaction:

$$3\ HClO_2 + RCHO \longrightarrow RCO_2H + HCl + 2\ ClO_2\uparrow + H_2O$$

no matter what the reaction pH was. There was evidence of over-oxidation in that up to 25% in excess of the calculated amount of chlorous acid actually was used. Jeanes and Isbell were unable to elucidate the exact mechanism of the oxidation, but they did find that α-D-glucose was oxidized only slightly faster than β-D-glucose.

Rutherford, Minor, Martin, and Harris[36] in 1942 reported the use of sodium chlorite buffered to pH 2.5 with acetic acid to oxidize the aldehyde groups in a periodate oxycellulose to carboxyl groups. They found that all of the groups had been coverted, in so far as the alkaline hypoiodite estimation for aldehydes was concerned and that they could use this oxidation to determine the amount of aldehyde groups present in an oxycellulose or oxystarch. Launer and co-workers[37] have developed the chlorous acid oxidation as a technique for the direct determination of aldehyde groups. An exact 3:1 oxidation mole ratio could be obtained using 0.005 M sodium chlorite in a pH 3.5 acetate-buffer system for 20 hours at 40°. They were able to determine glucose within ± 1- 2%, and using this sugar to calibrate the method. In 1954, Launer and Tomimatsu[38] restudied this work and changed the reaction temperature to 50° and the buffer to approximately 0.5 M sodium dihydrogen phosphate at pH 2.4. Light adversely affected the oxidation, and they stipulated that the reaction be carried out in the dark or in dull red light. After these changes, they were able to determine glucose accurately in the range 0.6 to 50 γ per ml.

RESULTS AND DISCUSSION

Oxidations With Buffered Hypochlorous Acid

The object of this phase of the research was to determine whether or not a satisfactory oxidation of sucrose could be carried out using conditions suggested by McKillican and Purves[1]; i.e., the use of hypochlorous acid buffered to a pH of 4.0 with calcium acetate. McKillican and Purves had found that using such a system on starch, the greater part of the oxidation occurred on carbon #6 of the glucose molecule, appearing as either aldehyde or carboxyl groups. Sucrose was chosen as the carbohydrate to be oxidized because it contains three primary hydroxyl groups. Furthermore, there was a distinct possibility that the primary hydroxyl group on carbon #1 of the fructose portion of the sucrose molecule would prove to be more easily oxidized than the other two on carbons #6 of glucose and fructose. If so, the oxidation might lead to the formation of a single reaction product.

A total of five oxidations were carried out using the acetate buffer system, the initial molar amounts of hypochlorous acid per mole of sucrose being 1.15, 4.0, 4.0, 4.0, and 2.0, respectively. The reaction was followed by titrating the unused hypochlorous acid at various times

either by the arsenite method or iodimetrically and in some experiments also by the change in optical rotation. In no case did the oxidation seem to conform to the usual form; that is to say, a high initial rate of oxidation following a gradual decreasing to a slow, nearly constant rate until no oxidant remained. On the contrary the reactions seemed to proceed at a constant or a slightly increasing one until all the oxidant had been exhausted. The most important criterion of the rate at which the reactions took place was the initial concentration of the hypochlorous acid; the relative amounts of sucrose to hypochlorous acid did not apparently affect the reaction rate, only the duration of the reaction.

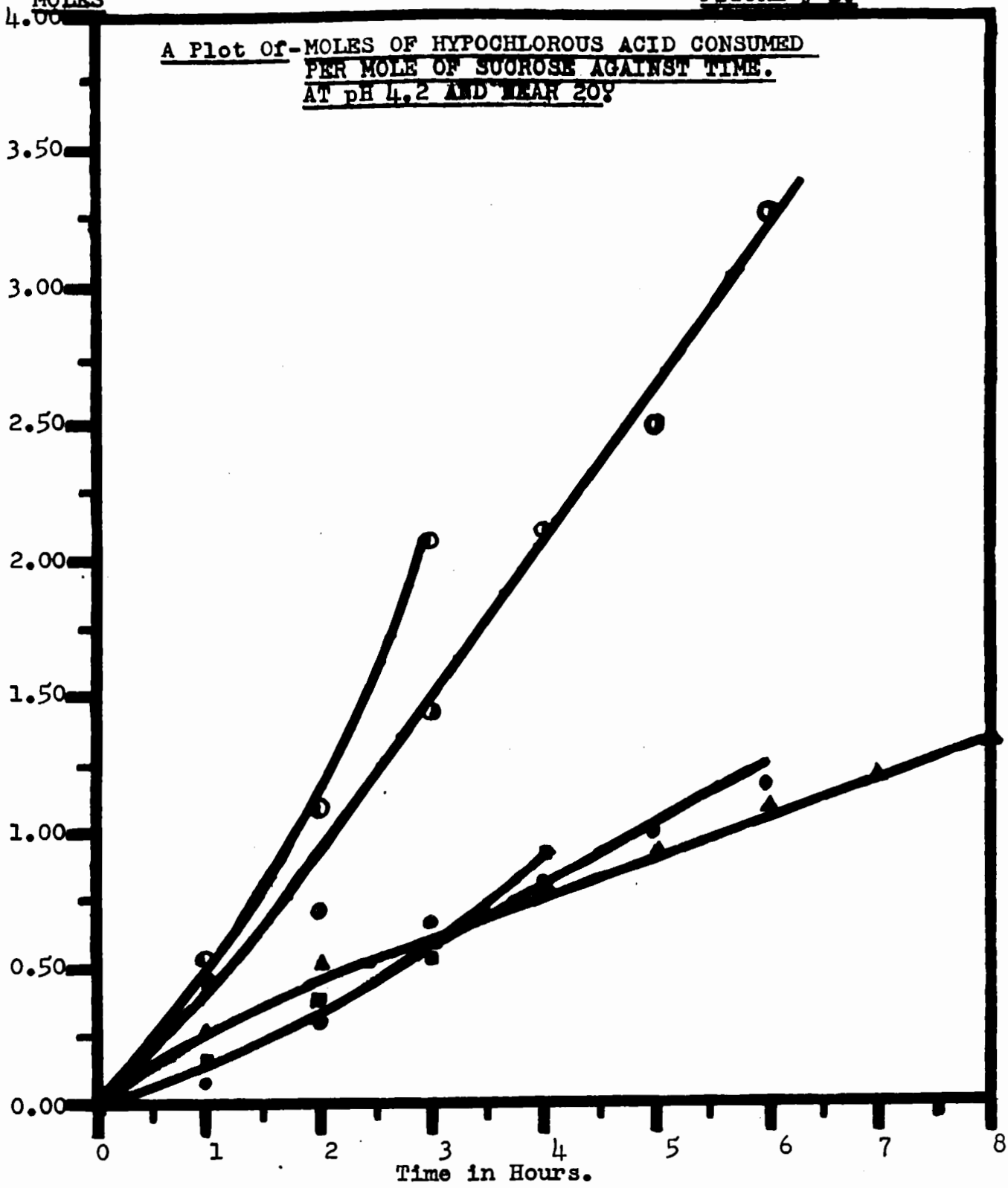
On no occasion at the completion of the reaction was the exact mole ratio of hypochlorous acid to sucrose in the initial solution accounted for. This behavior suggested that the rate of decomposition of hypochlorous acid in the oxidant solution was slightly higher than that obtained in the blank, whose value was used for calculating the moles of hypochlorous acid consumed per mole of sucrose. It was possible to account for this rate difference as a result of local heating effects in the oxidant solution occurring as the reaction proceeded. Hypochlorous acid oxidations seemed to be especially sensitive to temperature increases

when the reaction temperature was approximately that of the room. Figure #1 shows the consumption of hypochlorous acid per molecule of sucrose in the various oxidations plotted against time.

Oxidation #3 was run for the explicit purpose of determining the relationship between the amount of hypochlorous acid present at any given time, as determined by the thiosulfate and the arsenite methods. The latter is known to measure only the hypochlorous acid, whereas the thiosulfate method is a measure of all the oxidizing agents in solution.

A small but distinct difference between the two titrations was found as summarized in the following table:

FIGURE # 1.



A Plot Of- MOLES OF HYPOCHLOROUS ACID CONSUMED PER MOLE OF SUCROSE AGAINST TIME. AT pH 4.2 AND NEAR 20°

■ - Oxidation # 1. (1.15:1 M.R.) ▲- Oxidation # 2. (4:1 M.R.)
● - Oxidation # 3. (4:1 M.R.) ◐- Oxidation # 4. (4:1 M.R.)
◐ - Oxidation # 5. (2:1 M.R.) M.R.* Mole Ratio.

Table I

Comparison of Methods for Determining Oxidant Consumption in Oxidation #3

Time Hr.	Moles HClO per mole sucrose. Thiosulfate Method[a]	Moles HClO per mole sucrose. Arsenite Method[b]	Difference
0	0	0	0
1	0.0614	0.0812	+0.020
2	0.353	0.294	+0.059
3	0.708	0.676	+0.032
4	0.848	0.827	+0.021
5	1.14	1.00	+0.14
6	1.23	1.17	+0.06

[a] Five ml. aliquot of both oxidant and blank mixed with excess acidified potassium iodide and titrated with standard thiosulfate.

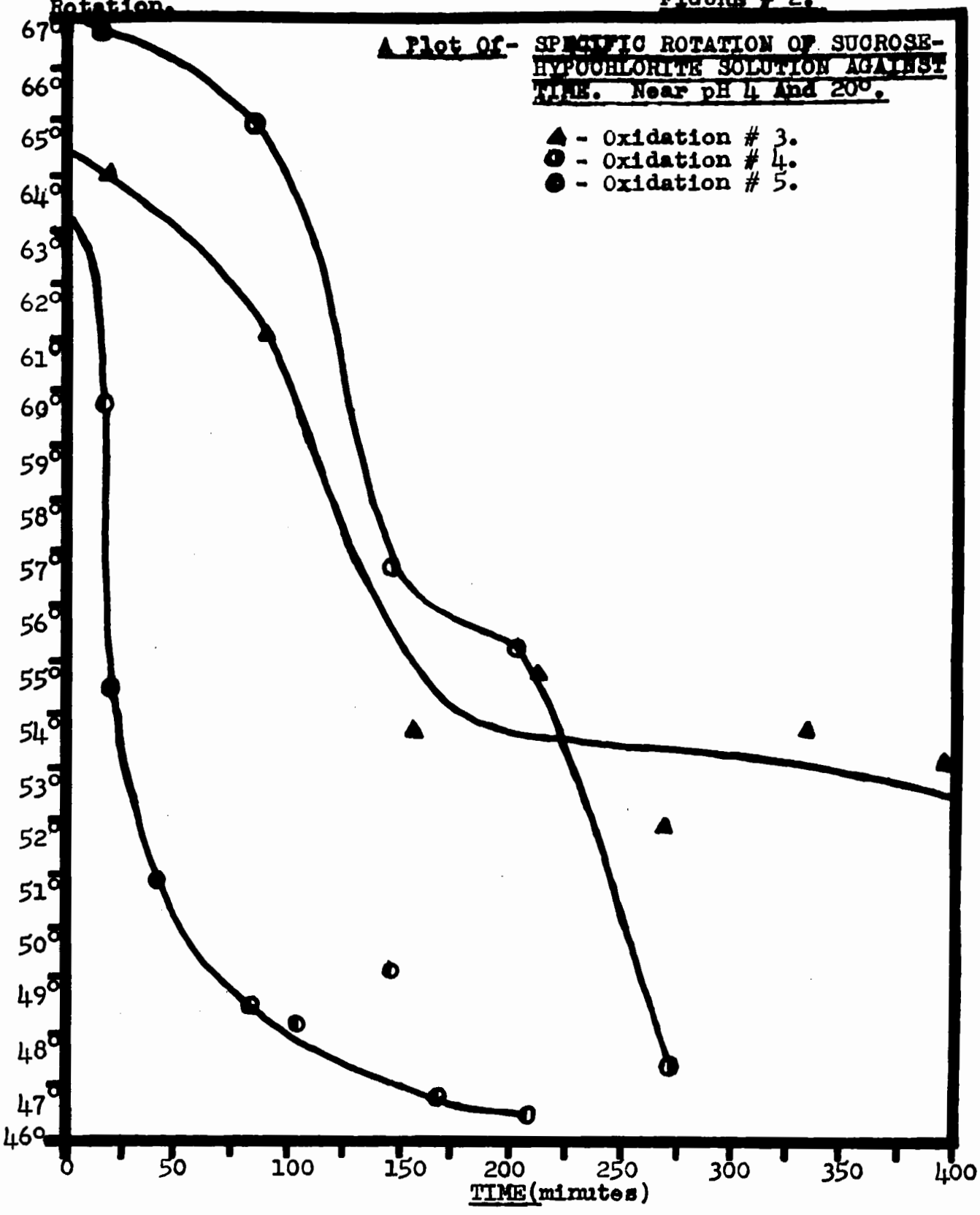
[b] Five ml. aliquot of both oxidant and blank mixed with 25 ml. of standard sodium arsenite and titrated with standard iodine.

In view of the similarity of the results by either method it was decided to substitute the thiosulfate method for the arsenite method for the purpose of following the course of further oxidations. The thiosulfate method also lacks the intrinsic loss of accuracy which was inherent in the arsenite method due to a subtraction step in the calculations.

The course of the last three oxidations was also followed by means of the optical rotation of the solution. The specific rotations during the initial 0.25 hours were found to be roughly those of a similar solution containing only sucrose, $+66.53^{\circ}$ (5). As time progressed, the specific rotation fell off rapidly to a value of $45-53^{\circ}$, after which only a very gradual decrease over a period of time was observed. The only variation in the nature of the rotational decrease was the time at which the maximum fall-off occurred. When the mole ratio of hypochlorous acid to sucrose was set at 4:1, the maximum decrease in specific rotation occurred at a time approximately 2 hours after the beginning of the oxidation. With a mole ratio of only 2:1, however, the maximum decrease occurred within 0.25 hours. Plots showing the behavior of the specific rotation of the solution during the reaction are shown in Figure 2.

All attempts at recovering the alcohol-insoluble calcium salts of the sucrose acids met with failure due to the

FIGURE # 2.


Specific Rotation.
67°
66°
65°
64°
63°
62°
61°
60°
59°
58°
57°
56°
55°
54°
53°
52°
51°
50°
49°
48°
47°
46°
0
50
100
150
200
250
300
350
400
TIME(minutes)
▲ Plot Of- SPECIFIC ROTATION OF SUCROSE-HYPOCHLORITE SOLUTION AGAINST TIME. Near pH 4 And 20°.
▲ - Oxidation # 3.
● - Oxidation # 4.
● - Oxidation # 5.

presence of large amounts of calcium acetate in the reaction mixture.

Oxidations With Unbuffered Hypochlorous Acid

When it became obvious that the calcium acetate buffer seriously interfered with any recovery of the reaction products, a technique was developed to produce a hypochlorous acid solution free of the interfering calcium acetate. The method finally decided upon involved treating a suspension of calcium hypochlorite in water with dilute sulfuric acid; this procedure served to precipitate almost all of the calcium present as calcium sulfate, leaving a solution which was essentially hypochlorous acid. Small amounts of calcium ion still remained in solution inasmuch as calcium sulfate has a distinct solubility in water, amounting to about 0.25 gm. in 100 ml. of water. The same thing could have been accomplished by substituting oxalic acid for the sulfuric acid with an even more efficient removal of the calcium present. The oxalic acid technique was discarded, however, because of the extremely fine crystal size of the precipitated calcium oxalate which rendered filtration impossible or at least extremely slow.

Further oxidations of glucose and sucrose by means of

this unbuffered acid led to yields of sugar acids which were abnormally low (5.3%, nil.) considering the amount of oxidant used, even though the pH was held above 4.0 with calcium acetate solution. The presence of only small amounts of calcium acetate allowed the solution to be concentrated *in vacuo*, and almost all of the alcohol-insoluble calcium salts of sugar acids to be precipitated.

Chromatography of the product from the oxidation of glucose showed that practically none had been converted to gluconic acid. Furthermore, chromatography of the sucrose oxidation solution was found to contain practically no acidic material, only some unreacted sucrose, and large amounts of highly reducing matter. From this result, it was inferred that hypochlorous acid under the conditions used readily oxidized sucrose to aldehyde-containing intermediates but oxidized these intermediates only very slowly to their corresponding carboxyl derivatives.

In order to test this hypothesis, sucrose was oxidized in various mole ratios with hypochlorous acid and careful account of the products was kept. All the reducing sugars in solution were determined by the Somogyi method[39] and acidic materials were determined as that weight of calcium salts precipitated on addition of 4 volumes of ethanol to the test solution (Table II). The amount of reducing material found in the 4:1 mole ratio oxidation was higher

Table II

Oxidations of Sucrose with Unbuffered Hypochlorous Acid[a]

Mole Ratio[b]	% Oxidized Sucrose		% Sucrose Unchanged[e]
	Reduction[c]	Acidic[d]	
1:1	41.8	5.6	52.6
2:1	44.8	7.7	47.5
3:1	43.1	11.3	45.6
4:1	74.2[f]	18.2[f]	7.6[f]
5:1	54.0	21.6	24.4
6:1	50.0	25.3	24.7

[a] Near 20° C. with 0.65-0.88 N hypochlorous acid at pH 4.4 ± 0.4.

[b] Ratio of hypochlorous acid to sucrose added.

[c] Estimated from copper reducing power determined as glucose.

[d] Estimated from the yield of calcium salts insoluble in 80% ethanol.

[e] By difference.

[f] Inadvertent hydrolysis to glucose and fructose suspected.

than that in all other cases; this was probably due to accidental hydrolysis. The results of these determinations supported the view taken, in that the amount of reducing material found stayed relatively constant, between 40 and 55%, calculated as glucose; whereas the amount of acidic material increased steadily from a value of 5.6% for a 1:1 mole ratio of hypochlorous acid to sucrose to a value of 25.3% for a 6:1 ratio.

Thus, in order to recover the sucrose oxidation products readily as their calcium salts, it would be necessary to interpose a second oxidation step which would convert all or nearly all aldehyde groups present to their corresponding carboxyl compounds. The method chosen for this conversion was oxidation with chlorous acid according to Jeanes and Isbell[14], but under very carefully controlled conditions[37][38]. Chlorous acid is usually prepared by treating commercial sodium chlorite (85% pure) with glacial acetic acid until the reaction pH is reached. This method could not be used in the particular case at hand because the presence of sodium ions in the reaction mixture would render unsatisfactory the isolation of the calcium salts of the sucrose acids by precipitation with ethanol, and would contaminate any product with the alcohol-insoluble sodium sulfate. The method first used to prepare chlorous acid was based on the insolubility of sodium sulfate in 70% ethanol solution.

Commercial sodium chlorite was treated with dilute sulfuric acid in water solution, and ethanol up to 70% by volume was added to precipitate all the sodium as sodium sulfate. Calcium base could then be added as desired. The technique was found to be very satisfactory for the production of small amounts of chlorous acid, up to 0.1 moles. Larger amounts could not be produced by scaling up the method, as excessive decomposition of the acid occurred and very large volumes of ethanol required to produce rather small quantities of the acid, introduced an element of waste.

The second method, based on the production and use of calcium chlorite as an aqueous solution, was found to be very satisfactory. All attempts to obtain calcium chlorite from outside sources met with failure, the substance not being commercially available, even on a sample basis. This necessitated production of all calcium chlorite prior to its use as an oxidizing agent.

The technique used either involved the interaction of a saturated chlorine dioxide solution prepared according to Sarkar's method[40] with calcium peroxide made from calcium hydroxide and 30% hydrogen peroxide, or the passage of chlorine dioxide as formed directly into a suspension of technical calcium peroxide in water. In this reaction the peroxide radical acted as a reducing agent[41].

$$CaO_2 + 2\ ClO_2 \longrightarrow Ca(ClO_2) + O_2\uparrow$$

The reaction used by Sarkar[40] to produce chlorine dioxide involves the reduction, by oxalic acid, of chloric acid formed by treatment of dry potassium chlorate with strong sulfuric acid.

$$2\ HClO_3 + H_2C_2O_4 \longrightarrow 2\ ClO_2\uparrow + 2\ H_2O + 2\ CO_2\uparrow$$

The calcium chlorite solution was then added to the solution of oxidized sucrose and treated with more sulfuric acid in order to keep the pH as close to 4.0 as possible. The reaction mixture was allowed to stand overnight so that the conversion of aldehyde groups to carboxyl could proceed as far as possible.

With the addition of the chlorous acid step and the elimination of the calcium acetate buffer, good yields of oxidation products were obtained. Inasmuch as the mole ratio of hypochlorous acid to sucrose was set at 1:1 to reduce over-oxidation and to help in limiting the oxidation to only one position on the sucrose molecule, the maximum yield of oxidation products as calcium salts could not have been more than approximately 100%. The maximum yield of crude product obtained, without reprecipitation, was 47.8%, with an average of 40.0%. This yield, however, fell off to an average of 24.8% after the product had been reprecipitated to eliminate any entrained sucrose and calcium chloride, a part of the product undoubtedly being lost also.

Examination Of Oxidation Products

Oxidation A

The product from oxidation A was found to be essentially non-reducing, nor was much reducing power developed under conditions which would cause inversion of sucrose (a pH lower than 4.0) and either heating to 60- 100° for a few minutes or allowing it to stand overnight. After a 2-3 hour reflux with 0.5 to 1% hydrochloric acid, the product developed a reducing power of 1.075 mgm. of glucose per 2.500 mgm. of calcium salt, or about 40% as glucose. The salts contained 9.78% of calcium determined as calcium sulfate or roughtly one calcium atom per sucrose molecule. These qualities suggested that the salt was that of a sucrose carboxylic acid, much more resistant to hydrolysis than sucrose itself. It was impossible to crystallize either the calcium salt or the free acid produced by double decomposition with oxalic acid.

Attempts at slow precipitation of the calcium salts by the gradual addition of ethanol divided the product into two fractions, one of low solubility in aqueous ethanol with a calcium content approximating one calcium atom per sucrose molecule and a reducing power of only about 15% (calculated as glucose), and a fraction of high solubility in ethanol

with a calcium content about half way between one calcium atom per sucrose molecule and 0.5 and a much higher reducing power (approximately 50% calculated as glucose). Repeating the slow precipitation technique on the original calcium salts three times opened up an even greater gap between the two fractions in the values of various properties determined. This behavior suggested that what had been thought to be a single compound was actually at least two calcium salts, but more probably a series of sucrose carboxyl compounds containing one, two and three carboxyl groups per sucrose molecule.

Oxidations B and C

After further calcium salts were prepared by oxidation of sucrose with hypochlorous acid and with chlorous acid, a 10 gm. sample was subjected to a simple fractionation, collecting each separate precipitate and analyzing it as each successive volume of ethanol was added. The results of this separation continued to suggest a mixture. The calcium content gradually decreased and the optical rotation increased and so confirmed this tendency. It became apparent that the governing factor was the calcium content; i.e., the greater the calcium content or the greater the extent of oxidation, the less the amount of ethanol required to

precipitate the fraction. (See Table III.)

Based on this information, the remaining calcium salts were subjected to a fractionation detailed in Figure 3. The scheme divided the mixture into three main fractions precipitated respectively by one, two and four volumes of ethanol. The per cent calcium present in each was respectively 13.4, 9.05, and 6.58% (see Table IV), which correspond to approximately 1.5 calcium atoms, 1, and 0.5 calcium atoms per sucrose molecule. Apparently one volume of ethanol precipitated calcium salts of sucrose molecules which had three hydroxyl groups converted to carboxyl groups, two volumes precipitated those having two carboxyls, and four volumes those having only one hydroxyl group converted to a carboxyl group.

The yield of the precipitate from four volumes (80%) ethanol was almost three times that of any other fraction.

No crystallization of any of these fractions was obtained even after conversion to the acid form by means of double decomposition with oxalic acid.

Table III

Properties of the Main Fractions of the Calcium Salts from Oxidation A

Fraction	Yield %	Calcium %	$[\alpha]_D^{20}$
A_1	2.5	19.4	insoluble[a]
A_2	2.8	10.6	+ 12.4°
A_3	8.7	8.80	+ 26.5°
A_4	13.3	8.70	+ 28.3°
A_5	9.2	16.8[b]	sl. soluble[a,b]

[a] Rotation was not observed.

[b] High calcium content due to mistaken addition of a small amount of calcium hydroxide.

Table IV

Properties of the Main Sub-Fractions[a]

Sub-Fraction	Yield %	Calcium %	$[\alpha]_D^{20}$	% Reducing Sugar[b] Before	After
B_1	9.7	Mainly	Inorganic	Not determined	
B_2&B_2	13.8	13.4	+ 5.05°	0.00	11.4
B_3	11.5	9.05	+14.00°	0.00	31.5
B_4	34.5	6.58	+28.80°	0.00	46.4
Total	69.5%				

[a] See Figure 3.

[b] Before and after hydrolysis with 1% hydrochloric acid for 3 hr. at reflux temperature.

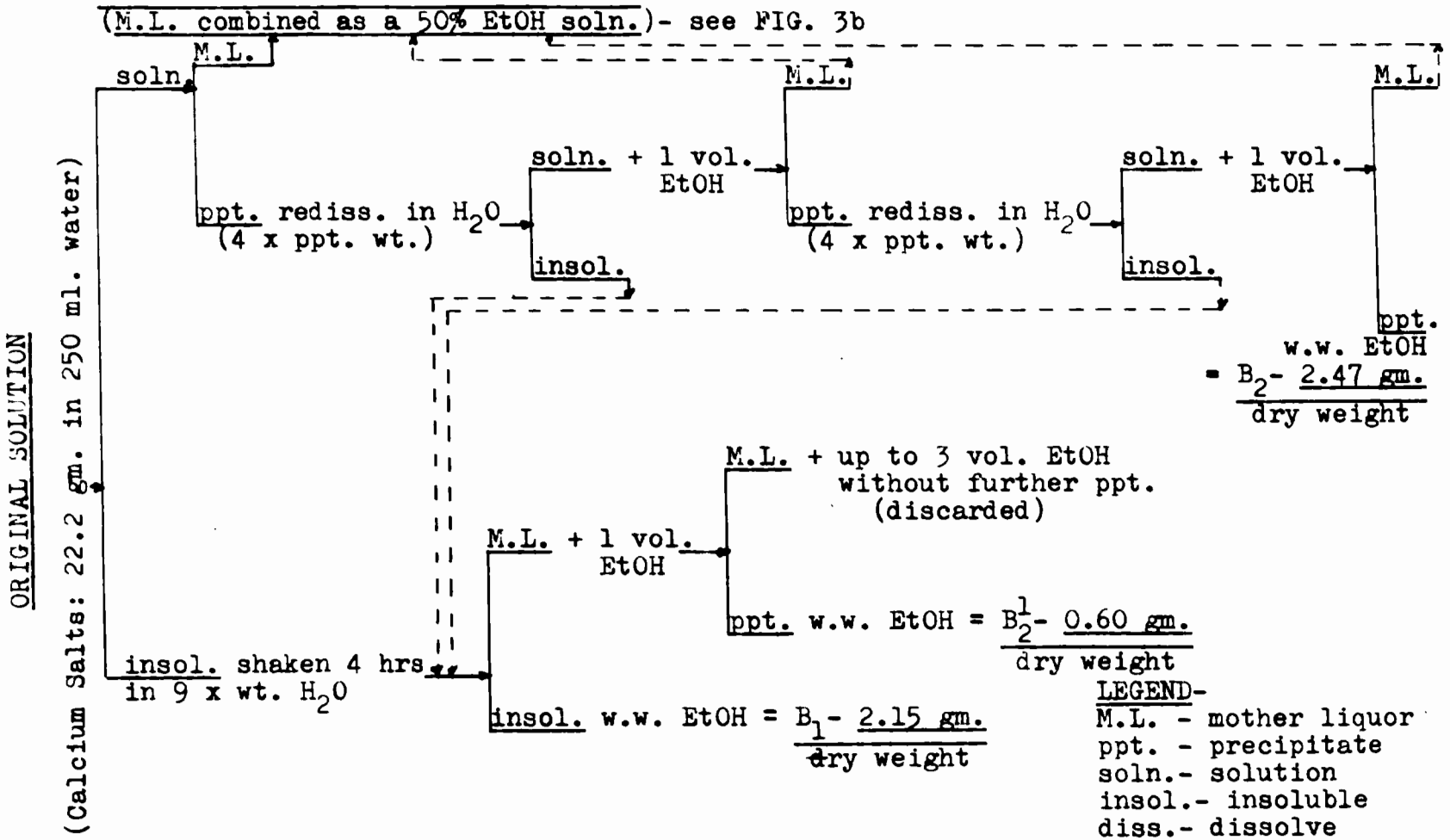
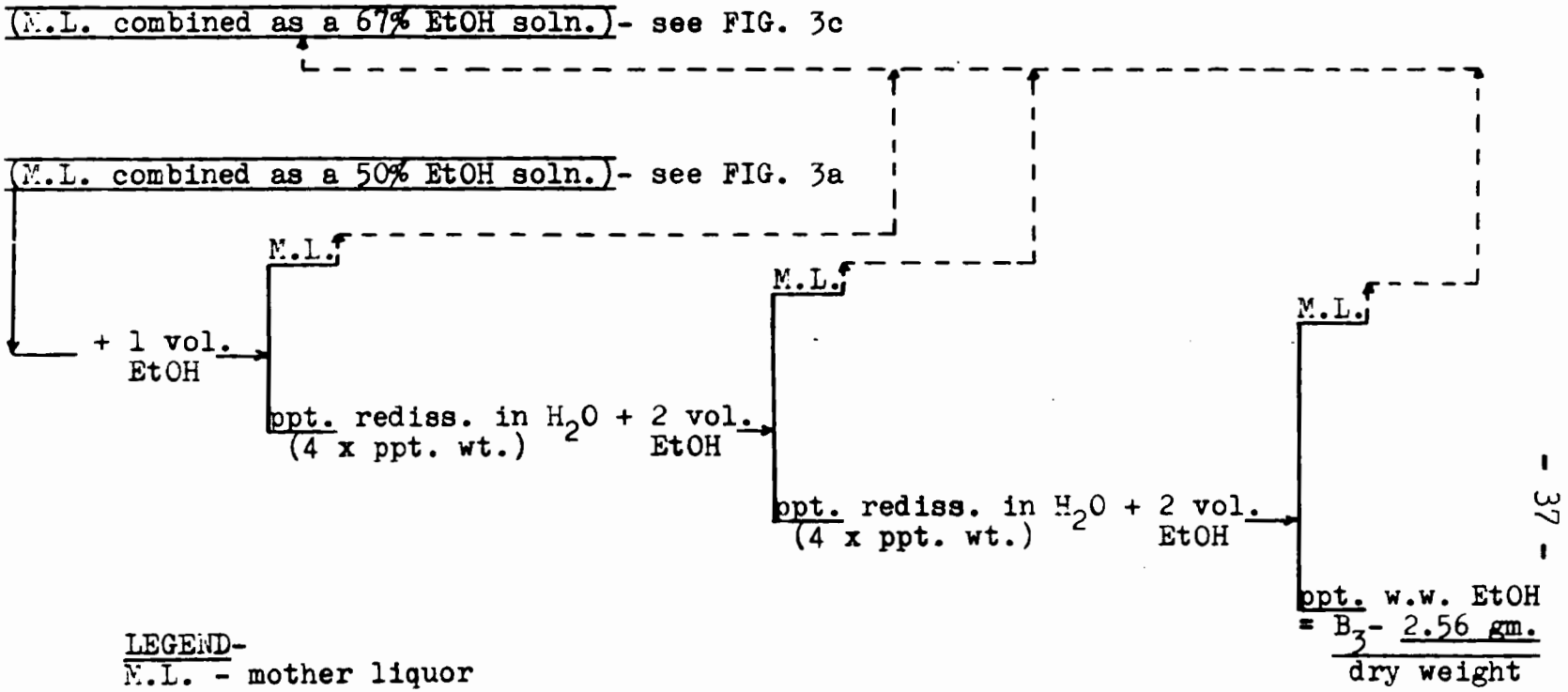
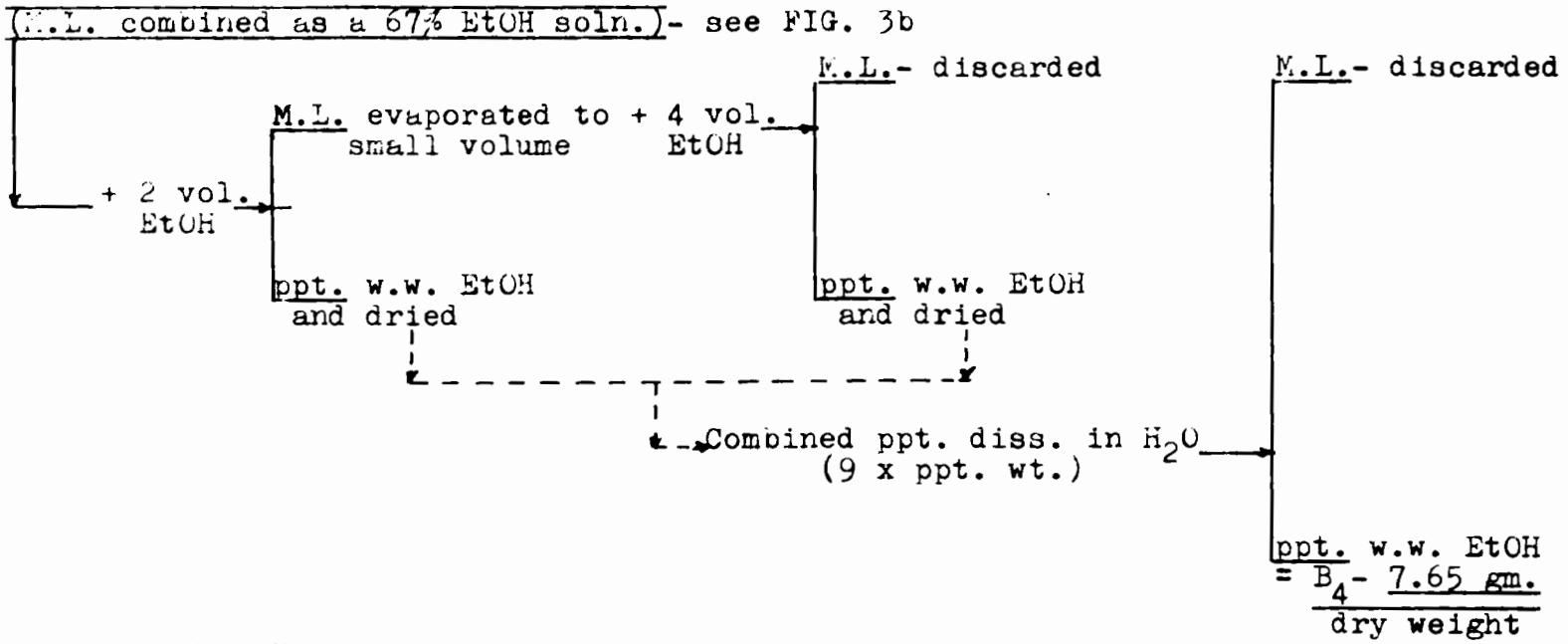


FIG. 3a- Fractionation of Combined Calcium Salts B & C from Aqueous Ethanol (Fractions B_1, B_2 & $B_2^{\frac{1}{2}}$)



LEGEND-
M.L. - mother liquor
ppt. - precipitate
soln.- solution
insol.- insoluble
diss.- dissolve
rediss.- redissolve
w.w.- washed with
vol.- volume
wt.- weight

FIG. 3b- Fractionation of Combined Calcium Salts B & C from Aqueous Ethanol (Fraction B_3)



LEGEND-
M.L. - mother liquor
ppt. - precipitate
soln.- solution
insol.- insoluble
diss.- dissolve
rediss.- redissolve
w.w.- washed with
vol.- volume
wt.- weight

FIG. 3c- Fractionation of Combined Calcium Salts B & C from Aqueous Ethanol (Fraction B_4)

Separation And Examination Of The "Four Volume" Fraction (B_4)

The four volume fraction, after conversion to the acid, was chosen for further examination because it was in greatest yield and would lead to the simplest products on hydrolysis and chromatography.

The acid form of this fraction, as produced, was found to contain only a negligible amount of ash and about 2.4% of moisture. The acids gave neutralization equivalents of 378 and 365; an acid formed by converting one hydroxyl group of sucrose to a carboxyl group would give a neutralization equivalent of 356. The acids, however, gave neutralization equivalents of 187 and 187 using a back-titration technique. The acid mixture had also developed a reducing power of 16.7% expressed as glucose, as contrasted with 0% for the calcium salts.

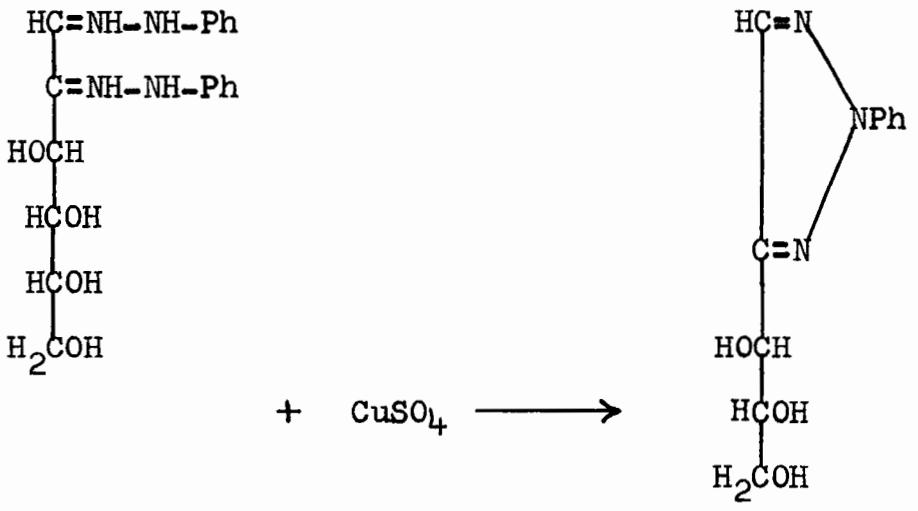
Two possibilities were presented by this data. The first was that some hydrolysis took place during the isolation of the acidic form (though the procedure was carried out as rapidly as possible at a low temperature) and that reducing power was developed as a result of this hydrolysis. The decrease in the neutralization equivalent suggested that two carboxyl groups were actually present in the sucrose molecule and that one of them was in a lactone form not directly titratable by sodium hydroxide. This possibility

seemed, however, to be ruled out because the calcium content of the original salt corresponded to only one carboxyl group per sucrose molecule. The amount of hydrolysis noted was not sufficient to decrease the neutralization equivalent to a value as low as was found.

A second interpretation was that the reducing power resulted not from hydrolysis and formation of aldehyde groups, but rather from a reducing group which did not react with the copper reagent in the presence of calcium. The increased sodium hydroxide consumption on back-titration would be the reaction of this reducing group with sodium hydroxide, possibly through enol formation. A grouping which might possibly be responsible for such behavior would be an α-keto acid or a potential α-keto acid, which might be produced if the hydroxyl on carbon 1 of the fructose part of sucrose was oxidized to a carboxyl group.

The investigation ended with a more detailed study of the "four volume" fraction B_4 in its acid form. An aqueous solution was hydrolyzed either by boiling with the acidic resin Amberlite IR-120, or more conveniently, by autohydrolysis since its natural pH was near 2. This hydrolysis produced nearly equal amounts of neutral reducing sugars and of sugar acids, and the latter were separated either by use of ion-exchange columns or by relative insolubility of the barium salts in aqueous ethanol. The alternative methods seemed to be about equal in efficiency.

Standard methods were used to separate the reducing sugars on a large cellulose column, using n-butanol saturated with water as the moving phase[48]. A total of 415 eluates, each weighing 10 gm., were collected, and paper partition chromatography revealed little but fructose in eluates 121 to 164. Eluates 165 to 180 contained glucose but no fructose, and no other sugars were found. Although neither sugar crystallized when isolated, both were satisfactorily identified chromatographically, as crystalline glucose phenyl osazone, and as the crystalline glucosotriazole, formed by oxidizing the osazone with copper sulfate.[49]



Glucose phenyl osazone
decomp. 200°

Glucosotriazole
m.p. 195°

Since sucrose had been definitely shown to be absent from the original, nearly non-reducing fraction B_4, the glucose and fructose found after hydrolysis must have originated from sucrose carboxylic acids. It followed that

at least one component of the mixed acids retained the glucose half of sucrose unoxidized, while at least one other component was oxidized in the glucose but not in the fructose portion. Although the re-oxidation with chlorite showed that hypochlorite oxidized the primary alcohol groups in sucrose to a much greater extent than secondary alcohol functions, the present result suggested that no preference was displayed between the three different primary groups present.

The sugar acids were also separated on a cellulose column, a customary eluant, n-butanol-formic acid-water (50:1:5)[50] being used. The first acidic substance, recovered from eluates 64 to 121, was non-reducing, and had a neutralization equivalent in the range of a tetrose carboxylic acid. Another acidic fraction from eluates 129 to 195 was shown by paper chromatography to consist of two different acids of which the less mobile resembled glucuronic acid in its behavior. It was unfortunate that these acids failed to crystallize or to form crystalline derivatives, and that their further study had to be deferred.

EXPERIMENTAL

All analyses were the mean of concordant duplicate or triplicate determinations.

Materials

The sucrose was usually commercial white cane sugar which is extremely pure. During the preparatory phases of this work, C. P. Sucrose as supplied by the Brickman Chemical Co., Montreal, was used because of the ease of handling the larger crystals.

The calcium hypochlorite used initially was Fisher's Technical Grade Bleaching Powder; C. P. grade calcium hypochlorite was preferred, when it was available, as it gave a higher strength of hypochlorous acid, weight by weight, than the technical product and also because it contained less insolubles and dirt.

All other reagents were C. P. Reagent Grade as furnished by the Brickman Chemical Co. of Montreal. The ethanol used was "absolute" ethanol as supplied in drums. All other organic liquids were used as supplied if their grade and state allowed it, otherwise they were given a simple redistillation.

Analytical Methods

Optical Rotation

All optical rotations were observed in a one-decimeter tube using sodium D light at 20° C. Solutions for such observations were prepared by dissolving a weighed quantity of solid, approximately 0.1 gm., in water and diluting to a specific volume, usually 10 ml. If all the material did not dissolve, the suspension was thoroughly shaken and a 2 ml. aliquot withdrawn. The 2 ml. aliquot was centrifuged in a weighed centrifuge tube and the clear supernatant liquid carefully removed. The precipitate was washed with a few drops of absolute ethanol (in which it was not soluble), dried, and weighed. This weight of insolubles was used as a correction factor to give the exact weight of substance in solution.

Neutralization Equivalents

Neutralization equivalents were determined by titration of a solution of acid in water by N/100 sodium hydroxide. The equivalence point was determined by measuring pH changes in solution, using a standard pH meter with glass and calomel electrodes, on the addition of successive volume increments of the base. This technique accounted for all of the free acid in solution. Any lactone or enol which would not be

directly titratable by base was determined by back-titration. An excess of base was added to the acid solution, mixed, allowed to stand for several hours, and then back-titrated with acid. The amount of base consumed over that used for the direct titration of the acid represented the amount of lactone or enol present with the acid.

In a specific determination, sufficient acid was dissolved in water and diluted to 50 ml. such that a 10 ml. aliquot contained approximately 5×10^{-2} milli-equivalents. Exactly N/100 sodium hydroxide was prepared by diluting 100 ml. of N/10 sodium hydroxide to 1 liter with water which had been thoroughly boiled and then cooled. The N/10 sodium hydroxide was prepared from a carbonate-free concentrate sold by the Fisher Scientific Co. which on dilution to 1 liter with boiled water gives exactly N/10 sodium hydroxide.

Approximately N/10 hydrochloric acid was prepared by diluting 11 ml. of concentrated C. P. hydrochloric acid to 1 liter. This acid was calibrated by titration against 25 ml. of N/10 sodium hydroxide to a phenolphthalein end-point. The approximately N/100 hydrochloric acid was prepared by diluting 100 ml. of this N/10 acid to 1 liter, using boiled water.

A 10 ml. aliquot of the unknown acid solution was pipetted into a beaker and titrated with N/100 sodium hydroxide increment-wise using a 10 ml. micro-burette, recording the

change in pH of the solution as the titration progressed.

At the same time, another 10 ml. aliquot was pipetted into a glass-stoppered 125 ml. Erlenmeyer flask together with 10 ml. of the N/100 sodium hydroxide. The mixture was swirled to insure proper mixing and then set aside for two to four hours. After sufficient time had elapsed, the solution was transferred with washings of boiled water to a beaker and the residual sodium hydroxide was titrated as before with N/100 hydrochloric acid using a micro-burette.

The neutralization equivalent by direct titration was given by:

$$\text{N.E.} = \frac{\text{weight of acid in 10 ml. aliquot}}{\text{volume of NaOH used} \times \text{normality of NaOH}}$$

The neutralization equivalent by back titration was calculated as follows:

$$\text{N.E.} = \frac{\text{weight of acid in 10 ml. aliquot}}{10 - \frac{\text{volume of HCl used} \times \text{normality of HCl}}{\text{normality of NaOH used}}}$$

Determination Of Calcium

Calcium was determined by simple ignition and by ignition in the presence of dilute sulfuric acid.

In a typical determination, 10 to 25 mg. of the dry sample was weighed into a platinum micro-boat and the boat and its contents placed in a micro-muffle furnace.

The furnace was made from heavy pyrex tubing bent in a right angle, with wire gauze wrapped around those areas of the tubing which come in direct contact with the flame. The pyrex tubing was clamped in a horizontal position with the bend extending downward. A Bunsen burner equipped with a wing top heated the vertical portion of the tubing to provide a gentle convection current of air through the apparatus and another Bunsen flame was placed directly below the boat in the tube to provide the heat for ignition.

The contents of the boat were heated gently for one-quarter to one-half hour, during which time the greater part of the carbonaceous material was burned. After the initial ashing was completed, the sample in the boat was ignited at about 700° to constant weight. The boat was then cooled in a desiccator over anhydrous calcium chloride and weighed. The ash was assumed to be calcium carbonate and was calculated as such.

Per cent calcium in sample = 0.400 x weight of ash.

Since the amount of conversion of calcium carbonate to calcium oxide becomes appreciable near 700°, it became necessary to convert the calcium carbonate-oxide mixture to calcium sulphate. After the ignition to constant weight was accomplished, 2 or 3 drops of distilled water was added to the cool ash and allowed to stand for a few minutes,

followed by 4 or 5 drops of 1:5 (v/v) sulfuric acid in distilled water. The boat was then heated gently in the micro-muffle furnace until fumes of sulfur trioxide were evolved, allowed to cool, and a further 2 or 3 drops of 1:5 sulfuric acid were added. After more gentle heating until all excess sulfuric acid had been expelled, the boat and its contents were then ignited to constant weight at about 700°. The percentage of calcium present was calculated on the basis of anhydrous calcium sulfate.

Per cent calcium = 0.294 x weight of ash.

Determination Of Reducing Sugar Present

The reducing sugar present in the various calcium salts and acids before and after hydrolysis was determined using the Somogyi copper reducing method(39). One liter of the alkaline copper solution used had the following formula: in one liter, 38 gm. of trisodium phosphate, 26.8 gm. of disodium hydrogen phosphate, 25 gm. of Rochelle salts, 7.5 gm. of cupric sulfate pentahydrate, 5 gm. of potassium iodide and 250 ml. of 0.1 N potassium iodate. The solution has the capacity to determine quantitatively 0.5 to 2.5 milligrams of glucose.

Exactly 5 ml. of an aqueous sugar solution containing an amount of reducing sugars between the set limits, plus exactly 5 ml. of the copper reagent were heated together in

25 x 200 mm. pyrex test tubes, loosely stoppered by inserting a smaller test tube, for ten minutes in a boiling water bath. After the heating time was over, the test tubes were removed immediately and cooled to approximately room temperature in cold water. The contents of the tubes were then transferred to 250 ml. glass-stoppered flasks with washing. Two ml. of an aqueous solution, 2.5% in potassium iodide and 2.5% in potassium oxalate, and 5 ml. of 5.2% sulfuric acid were added. The contents of the flask were swirled to insure mixing and allowed to stand for a few minutes. The liberated iodine was titrated with approximately 0.005 N sodium thiosulfate to a starch end-point. Blanks of the copper solution and water were always done inasmuch as the normality of the sodium thiosulfate will always vary slightly. The weight of the reducing sugars present was determined as glucose by means of a graph made by plotting known weights of glucose against ml. of thiosulfate required to titrate the copper solution reduced by these weights.

Paper Chromatography

Paper chromatograms were used continually throughout this research as an aid to identification of the oxidation products and to check for the presence or absence of certain components such as unreacted sucrose. The chromatograms were developed on strips of Whatman #1 or #4 filter paper

23 inches in overall length and 4.5 inches wide with the lower edge serrated to a depth of 0.5 inch in order to improve drainage off the sheet. The starting line for the samples placed on the chromatograms was 5 inches below the top of the paper strips. The solutions were "spotted" on the paper at intervals varying from 0.5 inch to 0.75 inch along the starting line, using pipets made from capillary tubing. The amount of solution deposited on any one spot varied according to the amount of material in solution. For example, with a concentration of about 5 mgm. of sugar per ml. 0.5 to 1 drop was sufficient for a well-defined spot after development and spraying. The spots were dried by means of a current of warm air.

The chromatograms were developed by the descending method in a cylindrical glass tank and were supported by hanging them vertically over glass rods supported on a stainless steel stand containing a glass dish to hold the developer. The short ends of the chromatograms were placed in the dish and weighted down with short pieces of 0.25 inch glass rod. The tank was sealed by a plate of glass over the top, ground to fit the tank top and coated with stop-cock grease.

Various solvent systems were used to develop the chromatograms; n-butanol: acetic acid: water, 2:1:1[42] and 4:1:5 (top layer)[43]; n-butanol: pyridine: water, 3:1:1[42]; ethyl acetate: acetic acid: water, 3:1:3[42]; ethyl acetate:

pyridine: water, 5:2:5[42]; and n-butanol saturated with water[44]. Varying equilibration and developing times were used; i.e., n-butanol: acetic acid: water, 2:1:1, gave excellent chromatograms in 10 to 13 hours, whereas others required as much as 24 hours.

Three spotting reagents were used exclusively as sprays after drying the chromatograms. Aniline phosphate dissolved in water-saturated n-butanol[45]; equal volumes of 0.1 N silver nitrate and 5 N ammonium hydroxide[46]; and a mixture of 4 volumes of 1% sodium metaperiodate in water and 1 volume of 1% potassium permanganate in 2% sodium carbonate solution[47]. The aniline phosphate spray was used for dissacharides and uronic acids; the ammoniacal silver nitrate spray was used for reducing sugars; and the periodate-permanganate spray to detect carbohydrates and their acids in general. Attempts were made to use acid-base indicators as sprays to determine acidic spots but unusual difficulty was experienced due to the basic or acidic nature of the solvent used.

Sodium Arsenite Titration for Hypochlorous Acid

Five ml. of the hypochlorous acid solution was pipetted into a mixture of 25 ml. of N/10 sodium arsenite solution and 5 gm. of sodium bicarbonate dissolved in 25 ml. of water. Two ml. of 1% starch suspension was added and the solution

was back-titrated with N/20 iodine solution. The normality of the hypochlorous acid was

$$\frac{(25.00 - ab)N_{As_2O_3}}{5.00}$$

where a = the number of ml. of N/20 iodine solution used

b = the ratio of the normality of the iodine solution to that of the arsenite solution

Sodium Thiosulfate Titration for Hypochlorous Acid

Five ml. of the hypochlorous acid solution was pipetted into an excess of potassium iodide (approximately 3 gm.) dissolved in 5 ml. of 2N sulfuric acid and 25 ml. of distilled water. The solution was allowed to stand for a few minutes, to allow the liberation of iodine to come to an end, and 2 ml. of 1% starch solution was added. The liberated iodine was titrated with N/10 sodium thiosulfate solution.

$$\text{Normality of hypochlorous acid} = \frac{C \times N_{Na_2S_2O_3}}{5.00}$$

where C = volume of N/10 sodium thiosulfate required.

Hypochlorous Acid Buffered With Calcium Acetate

The oxidizing solution was prepared by suspending approximately 64- 80 gm. of technical calcium hypochlorite in

750- 1000 ml. of cold distilled water. Glacial acetic acid was added slowly, with stirring, until the calcium hypochlorite was dissolved; approximately 100 ml. of the acid was required to reach a pH of 4.2. Numerous tests showed that this pH was maintained within 0.4 unit throughout an oxidation. A small residue of silica and other impurities was always obtained and this residue was removed by filtration through a mat of glass wool. The clear solution was brown and smelled strongly of chlorine. The solution was divided in half to provide both the oxidizing reagent and the blank solution. Analyses for the hypochlorous acid were performed, using either the arsenite or the thiosulfate method, or both, as described previously.

The quantity of sucrose for oxidation was calculated on the basis that 2 moles or 4 equivalents of hypochlorous acid were required to convert a primary hydroxyl group to a carboxyl group. The sucrose was added slowly, with shaking, to the hypochlorous acid solution contained in a stoppered flask rendered light-proof by wrapping in aluminum foil. The blank was treated in exactly the same manner except that no sucrose was added. Samples were withdrawn at stated intervals from each flask and were analyzed for hypochlorous acid.

Details of Oxidations

Tables to record the experimental details of the first five oxidations. (See Figures 1 and 2.)

Table V

Oxidation of Sucrose with 1.15 Moles of Buffered Hypochlorous Acid[a]

Hours	Ml. I_2 used in oxidation[b]	Ml. I_2 used in blank[b]	Mole Ratio HClO used:sucrose
0	5.85	5.85	0.000
1	8.86	6.19	0.174
2	12.59	7.03	0.378
3	16.80	7.87	0.542
4[c]	23.87	8.76	0.913
20	23.97	13.06	1.120

[a] Sucrose, 20.7 gm., was added to 350 ml. of 0.492 N Hypochlorous acid near 20° and buffered with calcium acetate to pH 4.2.

[b] Five ml. aliquots mixed with 25 ml. of 0.098 N sodium arsenite and titrated with 0.102 N iodine.

[c] Oxidation was essentially complete at 4 hours.

Table VI

Oxidation of Sucrose with 4.0 Moles of Buffered Hypochlorous Acid[a]

Hours	Ml. I_2 used in oxidation[b]	Ml. I_2 used in blank[b]	Mole Ratio HClO used:sucrose
0	8.35	8.35	0.000
1	17.71	11.46	0.258
2	19.66	14.37	0.512
3	22.13	15.14	0.585
4	26.51	16.79	0.801
5	29.12	18.13	0.932
6	32.41	19.30	1.08
7	35.20	19.43	1.19
8	38.06	22.02	1.32
20	24.82[c]	16.00[c]	2.46

[a] Sucrose, 7.35 gm., was added to 350 ml. of 0.492 N hypochlorous acid near 20° and buffered with calcium acetate to pH 4.2.

[b] Five ml. aliquot mixed with 25 ml. of 0.115 N sodium arsenite and titrated with 0.0507 N iodine.

[c] Titrated with 0.102 N iodine.

Table VII

Oxidation of Sucrose with 4.0 Moles of Buffered Hypochlorous Acid[a]

Followed By Arsenite Method

Hours	Ml. I_2 used in oxidation[b]	Ml. I_2 used in blank[b]	Mole Ratio HClO used:Sucrose
0	9.16	9.16	0.000
1	11.40	10.79	0.0812
2	14.22	11.63	0.294
3	17.48	11.78	0.676
4	19.67	12.77	0.827
5	21.67	13.73	1.00
6	23.86	13.98	1.17

Followed Iodimetrically

Hours	Ml. thiosulfate used in oxidation[c]	Ml. thiosulfate used in blank[c]	Mole Ratio;HClO used: sucrose
0	19.04	19.04	0.000
1	17.97	18.26	0.0614
2	16.17	17.84	0.353
3	14.36	17.70	0.708
4	13.24	17.25	0.848
5	11.94	16.84	1.14
6	10.75	16.49	1.23

Followed Polarimetrically[d]

Minutes	Observed Rotation	Specific Rotation
18	+1.05°	+64.0°
90	+1.00°	+61.0°
155	+0.88	+53.7°
212	+0.90°	+54.8°
268	+0.85°	+51.8°
333	+0.88°	+53.7°
393	+0.87°	+53.1°
23 Hours	+0.65°	+39.6°

a Sucrose, 16.4 gm., added to 1 liter of 0.382 N hypochlorous acid near 20° and buffered to pH 4.2 with calcium acetate.

b Five ml. aliquot mixed with 25 ml. of 0.0974 N sodium arsenite and titrated with 0.0571 N iodine.

c Five ml. aliquot mixed with excess acidified potassium iodide and titrated with 0.102 N sodium thiosulfate.

d Rotations observed at 20° C. and calculated on basis of 16.4 gm. of sucrose in 1 l.

Table VIII

Oxidation of Sucrose with 4.0 Moles of Buffered Hypochlorous Acid[a]

Followed Iodimetrically

Hours	Ml. thiosulfate used in oxidation[b]	Ml. thiosulfate used in blank[b]	Mole Ratio;HClO used: sucrose
0	28.08	28.08	0.000
1	23.11	26.37	0.463
2	20.47	25.40	0.701
3	14.66	24.89	1.45
4	9.51	24.27	2.10
5	5.90	23.37	2.49
6	0.00	22.88	3.25

Followed Polarimetrically[c]

Minutes	Observed Rotation	Specific Rotation
13	+1.62°	+66.7°
82	+1.58°	+65.0°
143	+1.38°	+56.7°
201	+1.34°	+55.2°
274	+1.15°	+47.3°

[a] Sucrose, 24.3 gm., added to 1 l. of 0.567 N hypochlorous acid near 20° and buffered to pH 4.2 with calcium acetate.

[b] Five ml. aliquot mixed with excess acidified potassium iodide and titrated with 0.101 N sodium thiosulfate.

[c] Rotations observed at 20° C. and calculated on the basis of 24.3 gm. of sucrose in 1 l.

Table IX

Oxidation of Sucrose with 2.0 Moles of Buffered Hypochlorous Acid[a]

Followed Iodimetrically

Hours	Ml. thiosulfate used in oxidation[b]	Ml. thiosulfate used in blank[b]	Mole Ratio HClO used: sucrose
0	30.11	30.11	0.000
1	21.66	28.42	0.537
2	13.94	27.87	1.11
3	trace	26.21	2.07

Followed Polarimetrically[c]

Minutes	Observed Rotation	Specific Rotation
17	+2.59°	+59.7°
19	+2.36°	+54.4°
42	+2.20°	+50.7°
81	+2.10°	+48.4°
103	+2.09°	+48.2°
145	+2.13°	+49.1°
171	+2.03°	+46.8°
210	+2.01°	+46.4°

[a] Sucrose, 43.4 gm., added to 1 l. of 0.607 N hypochlorous acid near 20° and buffered to pH 4.2 with calcium acetate. It was assumed that 0.100 equivalents would decompose in the oxidant solution during the reaction period and this was allowed for.

[b] Five ml. aliquot mixed with acidified potassium iodide in excess and titrated with 0.101 N sodium thiosulfate.

[c] Rotations observed at 20° and calculated on the basis of 43.4 gm. of sucrose in 1 l.

When all of the hypochlorous acid had been consumed, or at some stated time, the solution was neutralized to a pH of 7.0 - 7.4 with solid calcium hydroxide and 10 ml. of acetone was added to decompose any residual hypochlorous acid to chloroform. When the mixture no longer liberated iodine from acidified potassium iodide, signaling complete decomposition of the oxidant, the solution was cooled in the refrigerator for two to four hours and 2 to 4 volumes of ethanol was added. After standing overnight, only traces of material precipitated from solution. Any attempts at concentration caused a voluminous precipitate of calcium acetate to form, either before or after addition of the diluting ethanol, thoroughly masking any other material which might have precipitated with it. For this reason, a technique was developed which permitted the oxidations to be carried out in the absence of an acetate-buffer system.

Unbuffered Hypochlorous Acid

For the purpose of comparing the various acids used to prepare hypochlorous acid, 10 gm. of C. P. calcium hypochlorite was suspended in 50 ml. of ice-cold distilled water, and the solids in each suspension were assumed to contain approximately 20% of calcium hydroxide, 40% of calcium chloride, and 40% of calcium hypochlorite.

One sample was acidified with cold glacial acetic acid to a pH of 4.2 to produce an acetate-buffered solution for comparative purposes, all insoluble material being removed on a sintered glass crucible.

A second sample was treated with ice-cold 8.2% until a pH of 4.2 was reached. The finely divided precipitate was separated only with the greatest difficulty and had to be removed finally by centrifugation.

A third sample was treated with ice-cold 5.2% sulfuric acid until a pH of 4.2 had been reached. The precipitate of calcium sulfate was easily removed by filtration inasmuch as the particle size was quite large.

A final sample was acidified to a pH of 4.2 with ice-cold 11.7% citric acid. The resulting calcium precipitate was readily filterable and was so removed.

Table X

Precipitation of Calcium from Calcium Hypochlorite Solution[a]

Acid used	Acid % of Theory	Calcium salt ppt'd.[a] gm.	State	HClO Solution[b] Normality[c]	pH
Acetic	142	0.08	Sandy (impurities)	0 hr. 0.257	4.2
				6 hr. 0.224	4.4
				21 hr. 0.186	4.4
Oxalic	86.2	---[d]	Fine Unfilterable	0 hr. 0.160	4.5
				6 hr. 0.147	4.1
				21 hr. 0.122	4.0
Sulfuric	72.2	9.09	Coarse Filterable	0 hr. 0.217	4.3
				6 hr. 0.193	3.8
				21 hr. 0.154	4.2
Citric	71.6	5.32	Coarse Filterable	0 hr. Neg.[e]	4.0
				6 hr. 0.00	3.3

[a] Ten grams of C. P. calcium hypochlorite in 50 ml. of water.

[b] After filtration, the solutions were all made up to 200 ml. for purposes of comparison.

[c] Five ml. aliquot mixed with an excess of acidified potassium iodide and titrated with 0.115 N sodium thiosulfate.

[d] The precipitate was unfilterable and could not be quantitatively recovered.

[e] Citric acid was incorrectly assumed to be able to precipitate the calcium from calcium hypochlorite without reducing the hypochlorous acid produced.

Exploratory Oxidation Of Glucose

Ten grams of C. P. calcium hypochlorite was suspended in 50 cc. of ice-cold distilled water and 76 ml. of ice-cold 5.2% sulfuric acid (v/v) was added with stirring so that the pH was 4.0. The precipitated calcium sulfate and other insolubles were removed on a sintered glass crucible and the filtrate was made up from 96 ml. to 100 ml. for convenience. The solution was then analyzed for hypochlorous acid by the thiosulfate method. On the basis of a 1:1 mole ratio of hypochlorous acid to glucose, and 90 ml. of 0.386 N hypochlorous acid, 3.47 grams of anhydrous sugar was added near 0°. The pH of the solution was continually controlled by the addition, in small increments, of a total of 11.4 ml. of a 10% solution of calcium acetate. The reduction of the hypochlorous acid was complete in three hours as judged by starch-potassium iodide paper, and the final pH was 4.2. The solution was then neutralized by the addition of small portions of solid calcium hydroxide to a pH of 7.4, was filtered to remove a slight amount of calcium sulfate, and was diluted with 2 volumes of ethanol. After thorough cooling, the precipitate was centrifuged and collected. Yield was 0.24 grams of a buff-colored precipitate or 5.28% as calcium gluconate. It was apparent that this oxidation had produced only minor amounts of sugar acids.

Exploratory Oxidations Of Sucrose

(a) Ten grams of calcium hypochlorite was suspended in 50 ml. of ice-cold distilled water and 68 ml. of ice-cold 5.2% sulfuric acid (v/v) was stirred into the solution; the resulting suspension had a pH of 4.6. The precipitated material was then removed on a sintered glass funnel and the clear filtrate made up to 100 ml. and analyzed for hypochlorous acid by the thiosulfate method. On the basis of a 3:1 mole ratio and 90 ml. of 0.395 N hypochlorous acid, 2.11 gm. of sucrose was added. The pH of the reaction mixture was kept between 4.2 and 4.6 by the addition of small increments of a 10% calcium acetate solution, a total of 5.64 ml. being used. After an elapsed time of eight hours, there were only slight traces of hypochlorous acid left, as judged by the reaction with starch-potassium iodide paper. Five ml. of acetone was added and the solution was neutralized to a pH of 7.2 by the addition of small amounts of solid calcium hydroxide. The solution was stirred at this pH until it was negative to starch-potassium iodide paper and was then worked up. The addition of 2 volumes of ethanol, after removal of any precipitate by filtration, yielded only a highly opalescent solution with a negligible amount of precipitate. The solution, however, was found to be highly reducing to an alkaline copper reagent and when

it was chromatographed using 5:2:5; ethyl acetate; pyridine;water[42], large spots of highly reducing matter were noticed.

(b) In order to determine the relationship between the amount of hypochlorous acid used and the extent and nature of the sucrose oxidation, a number of oxidations were carried out using various mole ratios of hypochlorous acid to sucrose.

In each oxidation, 50 ml. of an ice-cold suspension of hypochlorite containing approximately 10 grams of calcium hypochlorite was decalcified with sulfuric acid as just described. After removing the precipitated calcium sulfate and other insolubles, the clear solution was made up to 100 ml. and was analyzed for hypochlorous acid by the thiosulfate method. Sucrose calculated on the basis of the following mole ratios of hypochlorous acid, 4:1, 3:1, 2:1 and 1:1 was then stirred into the various oxidant solutions. The pH of the solutions was held as close to 4.2 as was possible by the addition of small amounts of solid calcium hydroxide from time to time. The reaction was usually complete at a time 2.25 to 4.0 hours after the addition of the sucrose. The pH of the solution was then raised to about 7.5 by the addition of small amounts of calcium hydroxide. A few ml. of acetone was added at this point and the solution was stirred for about 0.5 hour to insure the total

decomposition of all hypochlorous acid. The solutions were then evaporated to between 20 and 45 ml. and filtered free of all insoluble substances prior to the analytical determinations.

Oxidations at mole ratios of hypochlorous acid to sucrose of 5:1 and 6:1 were also performed. There was no real change of method except that the amount of the calcium hypochlorite suspension was increased by 50% in order to make the weight of sucrose convenient, and appropriate increases in the other reagents were made.

The results of these oxidations were computed in the following manner and were summarized in Table II.

The per cent of sucrose present in solution as reducing material was calculated on the basis of the amount of reducing matter, determined as glucose, in solution. It was assumed that the sucrose reducing matter contained only one aldehyde group per sucrose molecule and that the determination of glucose was also exact for the sucrose aldehyde compound even though this compound has a molecular weight of almost twice that of glucose. Thus the per cent of sucrose present as reducing material in solution is given by:

$$\frac{A. \times \text{Vol. of Evap'd.Soln.} \times \text{Mol. Wt. of sucrose aldehyde} \times 100;}{\text{Wt. of Sucrose in Soln.}}$$

where A = Mgm. of glucose per ml. of Evap'd.Soln.

The molecular weight of the sucrose aldehyde compound, assuming one aldehyde group per molecule, is 340.

The per cent of sucrose in solution as acids is calculated on the basis of that weight of material which is precipitated from the evaporated solution on addition of 4 volumes. This substance is assumed to be composed of one atom of calcium and two molecules of sucrose acid, each of which contains one carboxyl group. Thus the per cent of sucrose present in solution as acids is calculated in the following manner:

$$\frac{\dfrac{B. \times \text{Vol. of Evap'd.Soln.} \times \text{Mol. Wt. of Sucrose Acid}}{\text{Mol. Wt. of Calcium Salt of Sucrose Acid}}}{\text{Wt. of Sucrose in the Evap'd.Soln.}} \times 100;$$

where B = gm. of calcium salts precipitated from 2 Ml. of evaporated solution on addition of 4 volumes of ethanol.

The results of these estimations have been presented in Tables XI and XII on pages 77 and 78.

Table XI

Oxidations of Sucrose with Unbuffered Hypochlorous Acid

Mole Ratio[a]	HClO[b] N	Sucrose Grams	Duration[c] Hours	Variation in pH	Concentrated to ml.
1:1	0.648	9.95	2.25	4.0-4.8	43.4
2:1	0.659	5.06	3.0	4.1-4.8	26.5
3:1	0.651	3.39	3.5	4.2-4.8	32.2
4:1	0.663	2.55	2.5	4.0-4.8	22.0
5:1	0.875[d]	4.22	3.25	4.0-4.8	37.0
6:1	0.868[d]	3.46	4.0	4.0-4.8	47.0

[a] The ratio of hypochlorous acid to sucrose added.

[b] Volume of oxidant solution after filtration is 90 ml. unless otherwise stated.

[c] To exhaustion, or near exhaustion, of oxidant.

[d] Volume 140 ml.

Table XII

Examination of Products from Oxidations of Sucrose

Mole Ratio[a]	Solids in Solution[b]		Reduction[b]	Ppt'd Ca Salts[c]	
	%	% Ash in Solids	Mgm./ml.	%	% Ash in Salts
1:1	33.1	4.17	47.5	3.90	16.7
2:1	25.9	5.93	42.5	5.67	20.9
3:1	17.8	6.30	22.5	6.67	37.5
4:1	29.7	12.4	45.0	7.64	29.8
5:1	25.8	17.4	31.3	9.70	24.9
6:1	17.1	11.0	18.8	10.2	25.9

[a] Ratio of hypochlorous acid to sucrose added.

[b] Volumes given in right hand column of preceding table.

[c] Precipitated from solution by adding ethanol up to 80%.

Final Technique For Oxidations With Hypochlorous Acid

A suspension of 20 gm. of calcium hypochlorite per 100 ml. of distilled water was uniformly used. The concentration of the sulfuric acid was, however, increased from 5.2% (v/v) to 10% in order to obtain a more concentrated solution; this increase amounted to approximately 50%. In consequence, care had to be exercised lest the decomposition of the hypochlorous acid became too rapid by reason of a considerable increase in the temperature of the solution during the oxidation. All temperature increases were kept to a minimum by the use of a water bath held at between 15° and 20°. The lower temperature tended to prolong the total reaction time to about seven hours. The ratio of hypochlorous acid to sucrose of 1:1 was settled upon as being the most productive of simple oxidation products. The solution after the hypochlorous acid oxidation was complete was then subjected to oxidation by chlorous acid to convert all aldehyde groups to carboxyls.

The Preparation Of Chlorous Acid Solutions

In a typical preparation, the requisite amount of chlorous acid was calculated on the basis of the reaction[14]:

$$3HClO_2 + RCHO \longrightarrow RCO_2H + HCl + 2\,ClO_2\uparrow + H_2O$$

in terms of technical sodium chlorite which was about 85% pure, plus a suitable excess of about 30%. The weighed amount of sodium chlorite was then dissolved in an appropriate amount of ice-cold distilled water contained in a 150 ml. centrifuge bottle covered with aluminum foil to render it light-proof. The requisite amount of ice-cold 5.2% sulfuric acid to precipitate all sodium ions was then added with stirring, the solution was made up to 75% ethanol at 0° and was kept for several hours in the refrigerator in order to complete the precipitation of the sodium sulfate. This technique caused the precipitation of approximately 99% of all sodium sulfate originally in the solution. After being centrifuged, the chlorous acid solution was then ready for use in the oxidation of aldehyde groups to carboxyl groups. As already mentioned this method was satisfactory, for quantities up to 0.1 moles.

Preparation Of Calcium Chlorite Solution

Chlorine dioxide was prepared by Sarkar's method[40]. Twenty-five grams of dry potassium chlorate and 20 gm. of oxalic acid dihydrate were mixed together in a 250 ml. standard-taper, round-bottom flask painted black to exclude light. Eighty milliliters of ice-cold 30% (v/v) sulfuric acid was added and the flask was swirled to mix all components. The flask was then attached to an apparatus which

consisted of a spray trap, a gas washer, and a bubbler dipping into 250 ml. of ice-cold distilled water. The gas washer column was filled with a saturated solution of sodium chlorite, which converted any chlorine gas evolved back to chlorine dioxide. The generating flask was placed in a water bath with an initial temperature of about 15° and was gently warmed over a period of 2 hours to 60°. Chlorine dioxide began to be evolved at about 30° and dissolved readily in the water to form a dark red-brown solution smelling strongly of the gas. At the completion of the reaction, the actual volume of the chlorine dioxide solution was measured and the concentration determined by an iodimetric titration.

On the basis of the number of moles of chlorine dioxide in solution and according to the reaction:

$$CaO_2 + 2\ ClO_2 \longrightarrow Ca(ClO_2)_2 + O_2\uparrow$$

sufficient calcium peroxide plus a 30% excess was prepared by suspending the requisite amount of calcium hydroxide in about 100 ml. of cold distilled water and adding a calculated quantity of cold 30% hydrogen peroxide. The calcium peroxide immediately precipitated as a buff-colored, amorphous substance, but this suspension reacted readily with the chlorine dioxide solution when the two were stirred together. The calcium peroxide suspension had to be cooled while the chlorine dioxide solution was being added, as a considerable

amount of heat was evolved. Solutions of calcium chlorite were accumulated until sufficient was obtained for the particular oxidation, allowing for a 30% excess, and the combined solutions were then evaporated to approximately 200 ml. in vacuo. All insolubles (calcium peroxide and calcium hydroxide) were removed on a filter and the filtrate was used for the oxidation in question.

This preparation of calcium chlorite was later modified to the extent that technical calcium peroxide, of roughly 60% purity, was substituted for the prepared material. The chlorine dioxide was then passed directly into the well-stirred cooled suspension of calcium peroxide in distilled water and the apparatus was enlarged so that double the original quantities of potassium chlorate, oxalic acid, and sulfuric acid could be used.

Oxidations With Chlorous Acid Or Calcium Chlorite

The calculated amount of chlorous acid or calcium chlorite based on an estimated conversion of 45% of the sucrose to an aldehyde form by hypochlorous acid and on a 30% excess of the required 3 moles of chlorous acid per mole of sucrose aldehyde, was stirred into the solution of sucrose, whether neutral or acid, that had been previously

oxidized with hypochlorous acid. The pH of the solution was then held as close to 4.0 for 2 hours as was possible, by the addition of small amounts of 5.2% sulfuric acid.

After being allowed to stand over night, the pH had risen to between 5.5 and 6.0. Solid calcium sulfite or saturated calcium bisulfite solution was added to destroy all excess chlorous acid. The solution was then aerated until the odor of chlorine dioxide had disappeared, and no reaction with starch-potassium iodide paper was evident. This aeration required from 4 to 8 hours for completion.

After the aeration, the solution was neutralized to a pH of about 7.5 by the addition of small amounts of solid calcium hydroxide, and was then worked up in the manner to be described later.

Final Oxidations Of Sucrose With Hypochlorous Acid

A series of three preparative oxidations was run with the object of accumulating sufficient product for further study. In these oxidations, the mole ratio of hypochlorous acid to sucrose was 1:1 and the probable conversion to reducing sucrose was estimated to be about 45% in order to calculate the quantities of calcium chlorite required to complete the oxidation of the sucrose to the acid form. The results are shown in the following table:

Table XIII

Re-oxidation of Hypochlorous Acid Oxy-Sucrose With Chlorous Acid

	Oxidation A	Oxidation B	Oxidation C
Hypochlorous Acid[a]			
Normality	0.912	1.03	0.920
Volume, ml.	300	325	350
Sucrose, gm.	49.6	56.5	55.1
Reaction Time, hr.	3	6	7
Range of pH	4.0-4.5	4.0-4.5	4.0-4.5
Calcium Chlorite			
gm.	60.8	53.5	53.5
millimoles	348	306	306
Calcium Salts			
yield, gm.	16.5	27.1	23.7
yield, % of sucrose	29.2	47.8	43.0
Reppt'd yield, % of sucrose	15.6[b,c]	31.5[b,c]	27.0[c,d]

[a] At room temperature.

[b] Reprecipitated once by adding 9 volumes of ethanol to the aqueous solution.

[c] Chromatographically free of sucrose.

[d] Reprecipitated twice by adding 9 volumes of ethanol to the aqueous solution.

Examination Of The Calcium Salts

Oxidation A

A sample of the calcium salts, 0.5047 gm., and containing 9.78% calcium, was dissolved in distilled water and diluted to 10 ml. A small amount of material continually precipitated during the period of optical measurement. This material was removed on the centrifuge and weighed; and the specific rotation was corrected. This rotation was $[\alpha]_D^{20}$ +24.0° initially, + 21.9° after 18 hr. at room temperature and pH2-3, and + 25.6° after heating for 10 min. at 50-60° and 18 hr. at room temperature and the same pH. Parallel determinations of copper reducing power made on 2.49, 2.44, and 2.44 mgm. samples by the Somogyi[39] method, corresponded to 0.038, 0.125, and 0.25 mgm., respectively, as glucose. A slow hydrolysis, going from 1.5 through 5 to 10% as glucose, was presumably occurring. A sample of the calcium salt, 1.00 gm., was dissolved in 30 ml. of 1% hydrochloric acid; the solution was heated at 100° under reflux for 3 hours and then diluted to 100 ml. After this more hydrolytic drastic process, the specific rotation was - 6.0° and remained constant for 18 hours. Neutralization with calcium carbonate then caused the rotation to rise to +1.00°. Reducing material determined by the Somogyi method[39] had increased to 1.075 mgm. as glucose in 2.500 mgm. of solids, or to 43%.

Attempts to crystallize both the calcium salt and the

acid obtained by treatment of the calcium salt with a calculated amount of oxalic acid ended in failure. The calcium salts were then separated into two fractions using slow precipitation. One gram was dissolved in approximately 10 ml. of distilled water, and ethanol was added very slowly from a burette through an adapter whose tip was below the surface of the water solution. A very slow stream of nitrogen was also passed through the adapter to prevent excessive incrustation of the tip. In this way about 25- 35 ml. of ethanol could be added over a period of 18 hours and the precipitate formed readily about the submerged adapter tip. The supernatant liquor was decanted and the precipitate was redissolved in water and reprecipitated. The less soluble portion was reprecipitated once and the supernatant liquors evaporated, the more soluble material being reprecipitated from the concentrate with six volumes of alcohol. The less soluble substance contained 10.4% of calcium and 15.6% of reducing material, expressed as glucose, on hydrolysis with 0.5% hydrochloric acid. The specific rotation of the unhydrolysed substance was $+11.7^{\circ}$. The more soluble substance contained 8.82% of calcium and 46.8% of reducing substance as glucose after hydrolysis, the specific rotation of the unhydrolysed material being $+21.5^{\circ}$. The use of methanol or isopropanol instead of ethanol did not improve the quality of the precipitate.

On the basis of the above results, more of the original material was fractionated using a similar technique. A 2 gram sample was dissolved in 18 ml. of distilled water and allowed to stand so that any insolubles present could settle out. A total of 63.2 ml. of ethanol was then added very slowly over a period of 24 hours. The suspension was filtered, the precipitate redissolved in water to give 26.3 ml. of solution, and 28.5 ml. of ethanol was added over a period of 12- 15 hours. The suspension was again filtered, the precipitate dissolved again in water to give 20 ml. of solution and 33.75 ml. of ethanol added over a period of 12- 15 hours. The precipitated component was then recovered on a filter, washed thoroughly 3 times with ethanol and dried. The yield was 1.05 gm. The combined mother-liquors from the three precipitations were evaporated to dryness in vacuo and dried further with ethanol. Yield was 0.85 gm. Analyses for per cent calcium, optical rotation,and per cent reducing material as glucose were carried out as before. The less soluble fraction had a specific rotation of $+11.2^{o}$, contained, 11.4% of calcium, and gave 11.4% of reducing material as glucose on hydrolysis. The fraction more soluble in ethanol had a specific rotation of $+27.9^{o}$, gave 7.98% of calcium, and gave 46.8% of reducing material as glucose on hydrolysis.

Insufficient material remained at this point for further operations, and so two additional oxidations were carried out to accumulate more material for a more thorough fractionation.

Oxidations B And C

The calcium salts from oxidations B and C were combined to give a total of 32 grams of refined precipitate.

For a preliminary fractionation, 10 grams of the combined calcium salts was dissolved in 90 ml., of distilled water to the greatest extent possible, and the solution was allowed to stand for two hours. Any insolubles formed were removed by filtration; these totalled 0.25 grams (2.5%) and were designated Fraction A_1.

Ethanol, 123 ml., was added slowly overnight and the suspension allowed to stand for a second day. The precipitated material was removed by filtration and washed 3 times with ethanol. During the filtration approximately 34% of the unfiltered precipitate and mother-liquor was lost by spillage, and this loss was taken into account in calculating the percentage recovery. The precipitate recovered weighed 1.70 grams and represented 27.9% recovery. This precipitate was designated Fraction A_2. A further 81.4 ml. of ethanol was added slowly to the roughly 50% ethanol-water solution remaining and the suspension allowed to stand overnight. The precipitate after filtration and washing 3 times with ethanol amounted to 0.53 grams and represented 8.7% recovery. This precipitate was designated Fraction A_3. A further 81.4 ml. of ethanol was added to the now approximately 67% ethanol water solution and the suspension allowed to stand overnight.

The precipitate recovered after filtration and a three-fold washing with ethanol weighed 0.810 grams and amounted to a 13.3% recovery. This precipitate was designated Fraction A_4. Another 81.4 ml. of ethanol and a small amount of solid calcium hydroxide were added to the approximately 75% ethanol water solution and allowed to stand overnight. The precipitate recovered on filtration and three-fold washing with ethanol weighed 0.56 grams and amounted to a 9.2% recovery. This precipitate was designated Fraction A_5. Further additions of ethanol up to a volume ratio of 8:1 ethanol to water yielded only negligible amounts of material.

The various fractions were analyzed for per cent calcium, and specific rotation was also determined when the fraction was sufficiently soluble in water. These results are given in Table III.

In the main experiment 22.2 grams of calcium salts were fractionated from 250 ml. of water with the results already discussed (Figure 3 and Table IV).

Fractions B_2, B_3, and B_4 (Figure 3 and Table IV) were converted to acid forms by grinding together a known weight of the fractions and calculated amounts of oxalic acid dihydrate until fine uniform powders were obtained. The mixture was then wetted with 15 ml. of distilled water and the slurry stirred continuously for 10 minutes to insure conversion to the acid form. A small amount of Super-Cel was

added and the suspension filtered through a sintered glass filter funnel. The precipitate was washed 3 times with a few ml. of water and finally sucked dry. The filtrates from all the fractions were allowed to evaporate down to moist, glassy solids in a desiccator over anhydrous calcium chloride under vacuum. No crystallization was noted.

All fractions, before and after conversion to the acid form, on chromatography both in n-butanol: pyridine: water (2:1:1)[42], and in n-butanol: acetic acid: water (3:1:1)[42], were found to contain no sucrose but, however, a multiplicity of acidic and neutral sugars. Inasmuch as Fraction B_4 had a calcium content rather close to that for one half a calcium atom per molecule of sucrose and was in the highest yield, all further experimental work was carried out on this fraction in its free acid form.

The honey-colored glassy syrup, Fraction B_4, in the free acid state, contained 2.44% moisture, as determined by drying for 48 hours over phosphorous pentoxide at 0.5 mm. pressure, and 0.49% of ash, an amount considered negligible. The neutralization equivalent was 378, 365, by titration with N/100 sodium hydroxide; on addition of excess alkali and back-titration, the neutralization equivalents decreased to 187 and 187. The per cent reducing sugar determined without hydrolysis was 16.7% as glucose as compared with 0% for the unhydrolyzed calcium salts prior to conversion to the free

acid form. A dilute solution of the acids in distilled water was strongly acid, about pH 2.

These acids were chromatographed on a column after hydrolysis as detailed below.

Separation Of Neutral From Acidic Carbohydrate Substances

Separation Using Ion Exchange Resins

The mixed disaccharide acids obtained from the calcium salt B_4 were hydrolysed by means of Amberlite IR-120. A solution of 1.65 gm. of the acids in 15 ml. of water with 1 gram of Amberlite IR-120 was heated under reflux for 2.5 hours over a boiling water bath, and for 0.5 hours by direct heating over a flame, a treatment which had been previously shown to give as complete a hydrolysis as was possible without excessive decomposition. The solution was filtered free of the resin, and a small amount of Darco decolorizing carbon was added to remove the color. The solution was then diluted to approximately 100 ml. with water and basic Amberlite IR-4B was added until a pH of 6.9 was realized. The resin and solution were then transferred to two columns containing at the bottom 2" layers of fresh Amberlite IR-4B, and the columns were washed with distilled water until the washings gave a negative Molisch test for carbohydrates. The washings were

evaporated *in vacuo* to a syrup weighing 1.1 gm. and containing all the neutral sugars obtained on hydrolysis of the disaccharide acids. This syrup was saved for further examination (see below).

The resin still in the columns was then treated with N sulfuric acid until the effluent in each case was distinctly acid- about 500 ml. The columns were then washed with more distilled water until the pH of the effluent was that of the distilled water. This treatment removed all the acidic substances previously absorbed on the weakly basic resin. The combined sulfuric acid and washings (2.5 liters) were evaporated to roughly 800 ml. This concentrate was neutralized to a pH of 6 with barium carbonate and to 7.0 with saturated barium hydroxide solution. The neutralized solution was allowed to stand overnight to improve the crystal size and filterability of the barium sulfate. After the latter was removed on a filter, the solution was evaporated to about 35 ml. at which point any residual barium sulfate was removed.

The filtered solution was then passed through Amberlite IR-120 to remove the soluble barium ions still present and the Amberlite resin washed with water until the effluent had reached the pH of the wash water. This solution of sugar acids was evaporated to a syrup weighing 1.0 gm. which was also saved for examination (see below).

The above technique was essentially that of Hough, Jones, and Wadman[48].

Separation By Means Of Barium Salts

A sample of the mixed disaccharide acids, 1.30 gm., was dissolved in 10 ml. of water. Inasmuch as the solution was found to have a pH of approximately 2, it was decided to omit any further acid for purposes of hydrolysis. The solution was heated with reflux over a boiling water bath for 2.5 hours, and then over a direct flame for 0.5 hours.

After cooling, an excess of barium carbonate was stirred in and the suspension allowed to stand overnight. The suspension was then evaporated *in vacuo* almost to dryness; ethanol was added and evaporated under vacuum several times to remove most of the water present. The precipitate was then worked free from the flask and quantitatively transferred to a coarse sintered-glass filter funnel, in which the solids were extracted with a total of 300 ml. of hot 90% ethanol used in small volumes. After changing receivers, the solids were then extracted with 150 ml. of warm distilled water until the residue was white. This residue, consisting principally of barium carbonate, was discarded.

The ethanol solution was evaporated to approximately 10 ml., and was then used in conjunction with the neutral sugar fractions obtained from the chromatographic column (see below).

The aqueous solution of the barium salts was evaporated to dryness *in vacuo* and dried further by solvent exchange and vacuum evaporation. Yield, 0.70 gm. of slightly impure

material. It was impossible to obtain a crystalline derivative from these salts.

Column Chromatography

A powdered cellulose column was used exclusively and was prepared by tamping down small portions of dry cellulose powder, passed through a 60 mesh screen, into a tube 1.75 inches inside diameter and 20 inches tall, until a column height of 14.5 inches was obtained. The base of the column consisted of a pad of spun glass wool, fitting down into the constriction at the base of the tube and covered by several discs of Whatman #1 filter paper cut to fit the tube as exactly as possible. This base was found to support the column adequately. Another disc of the same filter paper was placed on top of the cellulose to protect the upper end of the column. n-Butanol, saturated at room temperature with distilled water, was added at the top through a constant-head apparatus with a liquid level about 2.5 inches above the surface of the cellulose. The column was washed with the water-saturated n-butanol until the eluate was water-white and approximately 500 ml. more was collected thereafter[48]. The liquid level was then allowed to fall to the level of the cellulose in the column, and 1.1 grams of the neutral sugar

syrup dissolved in water was distributed dropwise over the entire upper end of the cellulose column. The flask containing the syrup was then washed out with a few drops of water and this, too, was added to the column head. A fresh disc of filter paper was put in place and the liquid level was then brought up to its previous position.

The column was then clamped to the upright stand of a Shandon Fraction Collector and the constant-head reservoir clamped in place above it. The first 100 ml. of eluate was discarded and collection begun. Inasmuch as the fraction collector was designed to collect specific weights of eluate, the apparatus was set to collect fractions weighing 10 grams, which required approximately 20 minutes per fraction. A total of 415 fractions was collected over a period of 5 days and nights, with every eighth fraction being chromatographed on Whatman #4 filter paper using n-butanol: pyridine: water, 2:1:1[42], as a developer for about 12 hours and the permanganate-periodate mixture as a spray reagent.[47]

Fructose, the first hexose, came off the column at the 121st fraction and continued until fraction #164; concentration to a very small volume and chromatography showed a trace of glucose present. Fractions #165- 180 were a mixture of glucose and fructose and #181- 220 showed only glucose on chromatography. No further sugars were obtained from the column, though it showed an intensely blue fluorescence band,

and the fractions collected were contaminated with the same fluorescent material. Neither sugar, though relatively pure, was crystallized on evaporation of the n-butanol and water, nor could they be crystallized from ethanol and methanol, respectively, after being washed free of residual n-butanol with petroleum ether. Attempts to remove the fluorescent material present by treating it with a small amount of Amberlite IR-120 were only partially successful.

Fructose was identified by its position on a chromatogram with respect to the spot given by authentic fructose and by its behavior to the ammoniacal silver nitrate spray reagent[46]. This identification was confirmed by the fact that even at room temperature it gave good yields of glucosazone which on conversion to a glucosatriazole[49] gave an undepressed mixed melting point of 194 -195° C. with authentic glucosatriazole. The very ready formation of glucosazone is characteristic of fructose as opposed to glucose.

Glucose was identified by its spot position with respect to the spot for authentic glucose and by the behavior of the spot on spraying with ammoniacal silver nitrate solution[46]. The identification was confirmed by an excellent yield of glucosazone only after treating for about 15 minutes in a boiling water bath. This glucosazone on conversion to glucosatriazole[49] gave an undepressed mixed melting point, 194- 195°, with authentic glucosatriazole.

After the 415th fraction of water-saturated n-butanol had been collected, the developer was changed to n-butanol: formic acid: water, 50:1:5, as recommended by Whistler and Durso[50] for acids on a cellulose column. The eluate was not again collected until the pH was approximately 4, or that of the solvent added. At this point, a further 147 fractions were collected and chromatographed as before but no further material was present.

After the 147th fraction, the liquid level was again allowed to fall to that of the top of the cellulose column and the old filter paper disc was removed. One gram of a yellow-brown syrup containing the acidic components in water was added dropwise directly to the whole upper surface of the cellulose column and a new protective filter paper disc put in place. The constant-head reservoir was replaced and the developer level brought to its former position. The first 100 ml. of solvent was discarded and the collection of fractions began anew.

The first acidic substance appeared in the eluate at Fraction 64 and continued to Fraction 121. A clear-cut separation was then obtained between it and the second acidic substance, as determined by paper-chromatography developed by n-butanol: pyridine: distilled water, 3:1:1[42]. The second acidic substance appeared from fractions 129 through 195. Fraction collection was continued until a total of 389 had

been taken. No further material was obtained up to this point when the separation was discontinued.

The first acidic substance (A_1) on chromatography using n-butanol: acetic acid: distilled water, 4:1:5[43], and a development time of 14 hours gave a very fast moving spot (R_f 0.34) as compared to glucose (R_f 0.18). The substance was non-reducing and as a sufficient amount of material was on hand (approximately 40 mg.) to give an approximate neutralization equivalent by titration with sodium hydroxide of 182 and 199. Equivalents given by addition of excess sodium hydroxide solution and back-titration with hydrochloric acid were 146 and 143. This result suggested that A_1 was a four-carbon acid fragment of a hexose chain. Calcd., neutralization equivalent for $C_4H_8O_5$, 136.

The second acidic fraction (A_2) resolved itself into two substances (A_2^1 and A_2^2) when chromatographed in n-butanol: acetic acid: distilled water, 4:1:5[43]. Both constituents gave pink spots using aniline phosphate spray and their solution in water gave positive benzidine and Fehling's solution tests. Constituent A_2^1 had an R_f value of 0.11- 0.12 which was approximately that given for glucuronic acid[43]. Constituent A_2^2 had an R_f value of 0.19- 0.20. No crystalline derivative could, however, be formed from the mixture.

Preparation Of Sugar Osazones And Osatriazoles

The two neutral reducing sugar fractions from the column were each made up to 10 ml. with ethanol and 2.5 ml. of these solutions were taken for each precipitation. Fraction #1 contained fructose with a trace of glucose, and #2 contained glucose. A blank mixture of 0.4 gm. of glucose and 0.2 gm. fructose was dissolved in 10 ml. of ethanol and 25% of this solution was used for comparison.

The sugar solutions were added to a solution of 4 ml. of distilled water containing 1 ml. of phenyl hydrazine (free of tar), 1 ml. of glacial acetic acid, and 0.4 gm. of sodium acetate tri-hydrate. The tubes were allowed to stand overnight at room temperature. Within the first 4 hours, the blank solution gave a yellow precipitate which was removed and washed with a few ml. of ethanol. This precipitate gave an undepressed mixed decomposition point, 195- 197° with an authentic sample of glucosazone.

After standing overnight, fraction #1 gave an osazone, and more osazone formed in the blank. Fraction #2 gave no osazone. The two osazones were separated, washed as before, and both gave the same undepressed mixed decomposition point with authentic glucosazone. At this point, the tubes were heated in a boiling water bath for 15 minutes and allowed to cool. After heating, both fractions #1 and #2, as well as

the blank gave osazones which gave the same undepressed mixed decomposition point with authentic glucosazone.

Since mixed decomposition points, as opposed to mixed melting points, are unreliable criteria of identity, the above procedure was repeated in order to obtain approximately 50 mg. of the osazone in each case for conversion to the osatriazole.

Using a modification of the method of Hann and Hudson[49], about 50 mgm. of the phenyl osazone was transferred to an 8 inch test tube fitted with a reflux condenser, and 4-5 ml. of distilled water, 3 to 4 drops of 2 N sulfuric acid, 170 mgm. of cupric sulfate pentahydrate, and 3 ml. of isopropyl alcohol were added. The mixture was gently boiled under reflux for one hour. After heating, the yellow-green solution was evaporated to approximately 1.5 ml. on a steam bath, using an air stream. The tube and its contents was placed in the refrigerator for 3 to 4 hours. After the thorough chilling, the yellow-brown precipitate recovered, was boiled with 5- 6 ml. of distilled water and 20 mgm. of Darco, and was filtered again (hot). After the recrystallization had completed itself in the refrigerator overnight, the fine needle-like crystals were separated and washed with a few drops of water. The conversion to the osatriazole was carried out on authentic glucosazone, on the cold precipitate from fraction #1 and the hot precipitate from fractions #1 and #2. Authentic gluco-

satriazole had a melting point of 194- 195° and was undepressed by the osatriazoles from fraction #1 and hot precipitates from fractions #1 and #2.

SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

1. Sucrose was oxidized in various mole ratios using hypochlorous acid buffered to pH 4.0- 4.2 with calcium acetate. A total of five oxidations were carried out and the following qualitative information developed as to the behavior of the oxidation.

The reaction occurred at a constant or slightly increasing rate.

The rate of reaction seemed to be dependent on the initial concentration of the buffered hypochlorous acid.

The duration of the reaction seemed to be dependent on the amount of sucrose added to the hypochlorous acid.

At no time was the observed mole ratio of hypochlorous acid to sucrose the same as that originally used. This discrepancy was attributed to a more rapid decomposition of hypochlorous acid in the sucrose solution than in the blank. No oxidation products could be recovered as ethanol-insoluble calcium salts, because of difficulties caused by the large amount of calcium acetate present.

2. Two oxidations were run with glucose and sucrose using unbuffered hypochlorous acid prepared from calcium hypochlorite and dilute sulfuric acid. The reaction pH was held between 4.0- 4.2 with a little 10% calcium acetate. Paper chromatography revealed that most of the glucose was

unoxidized and that the major part of the sucrose was converted to reducing material.

3. A series of six oxidations was carried out using varying ratios of unbuffered hypochlorous acid to sucrose and controlling the pH by additions of solid calcium hydroxide. Both reducing and acidic material were estimated in the oxidation products. The results indicated that the amount of reducing material as glucose stayed relatively constant between 40 and 55%, whereas the amount of acidic material increased steadily from a value of 5.6% for a 1:1 mole ratio of hypochlorous acid to a value of 25.3% for a 6:1 ratio.

4. A second oxidation step involving chlorous acid was introduced, to convert aldehyde groups as opposed to carbonyl to carboxyl groups[14]. The re-oxidation raised the yield of products isolated as calcium salts from 5.6% to a maximum of 47.8% with an average of 40%, even when a mole ratio of hypochlorous acid to sucrose of 1:1 was used. Most of the reducing products formed by the hypochlorous acid therefore consisted of aldehyde groups formed from one or the other of the three primary hydroxyl groups in sucrose, if hydrolysis to glucose was discounted. The yields of calcium salts fell off to a maximum of 31.5% and an average value of 24.8% after reprecipitation to free the salts of coprecipitated sucrose and calcium chloride.

5. Attemps to crystallize the calcium salts of the oxidation products led to the discovery that several were probably present. When the mixed salts were subjected to a fractionation, it was found that the fractions corresponded approximately to 1 carboxyl group, 2 carboxyl groups, and 3 carboxyl groups per sucrose molecule.

6. Hydrolysis of the fraction containing roughly 1 carboxyl group per sucrose molecule yielded a neutral sugar fraction consisting only of glucose and fructose, thereby indicating a total unspecificity of attack on the various primary hydroxyl groups of the sucrose molecule. In the acidic fraction, three separate acids were found, two of which seemed to be uronic acids and the third perhaps a tetrose acid fragment. No positive identification of the products was possible though the R_f value of one of the uronic acids suggested that it was glucuronic acid. The presence of uronic acids suggested oxidation of the hydroxyl group of carbons #6 of either D-glucose or D-fructose or both. The tetrose acid fragment suggested in the oxidation of a secondary hydroxyl to a carboxyl group followed by hydrolysis.

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