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PREFACE

Because of the availability and low cost, sucrose is attractive as the starting material for the preparation of low cost organic compounds. In view of this possibility, the Sugar Research Foundation, New York, has established several projects to investigate the properties of sucrose. One of the possible modifications is by oxidation. Several attempts to oxidize sucrose have been reported, but none involving the conversion of sucrose to derivatives of D-glucuronic, 2-keto-D-gluconic and 5-keto-L-gulonic acids. These compounds are all naturally occurring and D-glucuronic acid, in particular, is of considerable biological importance.

This thesis reports a method for the preparation of so-called 'sucronic acids' which represent sucrose molecules with one of the three primary hydroxyl groups oxidized to carboxylic acid. The properties of the sucronic acids were investigated briefly.

The author wishes to express his gratitude

to Professor R.U. Lemieux for his invaluable suggestions and his helpful criticisms. The author is also indebted to the Sugar Research Foundation, New York, for a grant in aid of research.

THE PLATINUM-CATALYZED OXIDATION OF SUCROSE BY OXYGEN

PREFACE i

TABLE OF CONTENTS iii

ABSTRACT v

I. INTRODUCTION 1

1. Historical Background Of Sucrose 1
2. The Oxidation Of Sucrose 2
3. The Use Of Noble Metal Catalyst And Oxygen 7
   a. Factors Affecting The Rate Of Oxidation 10
   b. Theory Of Catalysis 11
4. The Nature Of The Oxidation Products 16
   a. Glucuronic Acids 17
   b. Ketoaldonic Acids 24

II. EXPERIMENTAL 34

1. Materials 34
2. Methods 36
   a. Preparative 36
   b. Analytical 41

II. EXPERIMENTAL (cont'd.)

3. Experimental Results 57

a. The Oxidation Of Sucrose By Platinum Catalyst And Oxygen Using Sodium Bicarbonate Buffer 57

b. The Oxidation Of Sucrose Using Platinum Catalyst, Oxygen And Ion Exchange Resin 66

c. The Preparation Of Sucronic Acid Derivatives 80

d. The Oxidation Of Protected Monosaccharides By Oxygen And Platinum Catalyst 84

III. CONCLUSIONS 88

IV. CLAIMS TO ORIGINAL RESEARCH 105

V. BIBLIOGRAPHY 107

ABSTRACT

Sucrose has been oxidized to a monocarboxylic acid in 80% yield while maintaining the glycosidic linkage intact. Analysis of the product of oxidation indicated that the oxidation took place at the three primary hydroxyl groups of the sucrose molecule with equal ease. The three monocarboxylic acids formed, yielded on enzyme hydrolysis, D-glucuronic acid, 2-keto-D-gluconic acid and 5-keto-L-gulonic acid.

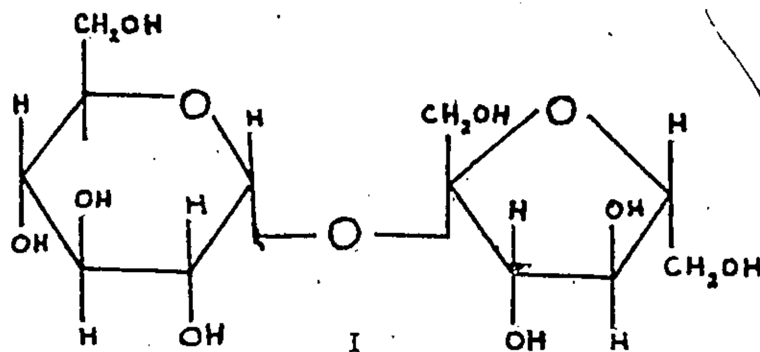
The oxidation was carried out using a platinum catalyst, oxygen and an anion exchange resin to act as an acid acceptor. Reoxidation of the monoacids formed is prevented by this method since the initial acids formed are effectively removed from solution by the ion exchange resin.

The general applicability of the method was demonstrated by oxidizing methyl α-D-glucoside in 86% yield, to the methyl α-D-glucuronide, also 1,2;3,4-di-O-isopropylidene-α-D-galactose was oxidized to the corresponding D-galacturonic acid derivative in 100% yield.

I. INTRODUCTION

1. Historical Background of Sucrose

Sucrose, (1), β-D-fructofuranosyl α-D-glucopyranoside (1)(2)(3)(4), is one of the oldest known pure organic crystalline materials, dating as far back as 400 A.D. in Egypt. This compound is widely distributed through the plant kingdom and the principal sources are the cane and sugar beet, as well as the sap of maple trees.



I

Sucrose is a non - reducing white crystalline compound melting between 160° and 188° (5) depending on the media used for its purification. The compound has a specific rotation of 66.53° in water (6). Sucrose is hydrolyzed both by acids and certain enzymes to a mixture of equal amounts of D-fructose and D-glucose,

a process called inversion by reason of the change in specific rotation from a dextrorotatory value to one which is levorotatory. The mixture of glucose and fructose is known as invert sugar. The rate of hydrolysis of sucrose by hydrochloric acid was studied by several workers (7)(8)(9) and found to be extremely temperature dependent. The time for complete inversion of sucrose using 0.7925 N hydrochloric acid was 42 hours at 18°, 22.8 hours at 22°, and the time required dropped to 5.3 hours at 32°.

Of the two hydrolytic agents commonly employed, the enzyme invertase (10) is superior because of its highly selective action on the sucrose group. The disadvantages are its high cost, the uncertainty that the preparation has retained its activity, and, except under high concentration of enzyme, the long time required for the completion of the hydrolysis.

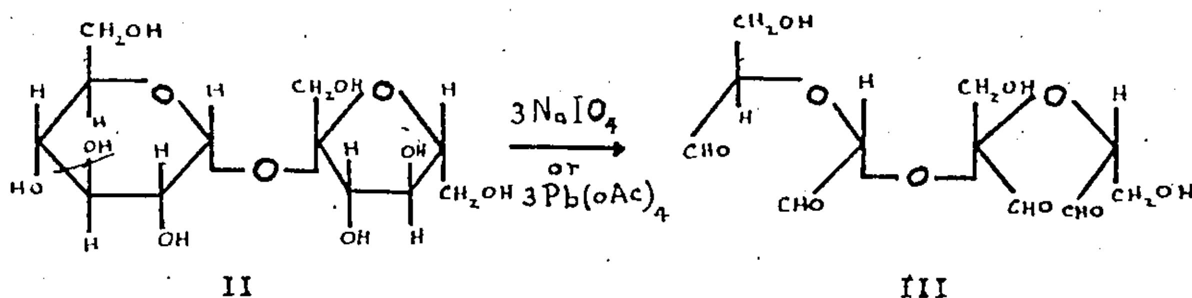
2. The Oxidation Of Sucrose

Since sucrose undergoes hydrolysis in acidic media the substance can only be oxidized in

neutral or basic media. Thus, the reported oxidations of sucrose in acidic media using iodic acid (11) ceric sulfate (12) and nitric acid (13) have no bearing on our problem of oxidizing sucrose while maintaining the glycosidic linkages intact.

Sucrose is inert towards the reagent chlorine dioxide and also towards chlorates in alkaline media (14) when the oxidation is performed in the absence of a suitable catalyst such as vanadium salts. Similarly, Pacsu (15) found that alkaline bromates were inert towards aldoses, ketoses and sucrose.

Sucrose has been reported to have been oxidized while keeping the glycosidic linkages intact by only four reagents; namely, lead tetraacetate, sodium periodate, sodium hypochlorite and nitrogen tetroxide. The oxidations with the glycol cleaving agents, lead tetraacetate (16) and sodium periodate (17) proceed as follows:-



Hydrolysis of the glycosidic linkage of the tetra-aldehyde III followed by oxidation with bromine yielded hydroxy pyruvic acid, D-glyceric and glyoxylic acids (17).

Hardy (18) has studied the oxidation of sucrose with buffered hypochlorite and obtained evidence that sucrose could be oxidized in low yield to one or several acids which contained the glucose moiety intact, however, the acid portions were not identified. The method was not selective and oxidation occurred at the C-1 and C-2 positions of D-glucose and D-fructose respectively since the products of oxidation on acid treatment yielded only small quantities of reducing compounds.

Although the halogen oxidation of sucrose is not in the published literature, several workers have however reported the oxidation of dextrins (19) and starch (20)(21) by this method. When the oxidations are carried out in neutral or alkaline solutions, significant amounts of anhydroglucuronic acid have been detected by conversion to furfural. The preparation of glucuronides by halogen oxidation of D-glucosides was first reported by Bergmann and Wolff (22). Menthyl α-D-glucopyranoside in pyridine solution was

subjected to sodium hypobromite in dilute aqueous alkali and yielded only a small percentage of the glucuronide. However, when the compound was treated under more vigorous conditions, the hydrolyzed reaction mixture yielded only the crystalline benzyl phenyl hydrazone of glyoxylic acid, despite indications of uronic acid formation from the strong blue colour developed by the naphthoresorcinol test. Further evidence that hypobromite oxidations are of no value in the formation of uronides from glycosides comes from Jackson and Hudson (23). These workers were able to obtain only a 12% yield of the brucine salt of methyl α-D-mannuronoside because of oxidative cleavage of the carbon chain of methyl α-D-mannopyranoside. Linberg (24), in oxidizing polysaccharides, obtained evidence that the sodium hypochlorite oxidized the secondary positions to carbonyl groups.

Gisvold (25), in a recent patent, claims to have oxidized sucrose quantitatively with nitrogen tetroxide to a tricarboxylic acid while maintaining the glycosidic linkage intact. The reaction which was complete in 48 hours was carried out in carbon

tetrachloride. Heating the sucrose tricarboxylic acid yields, according to the author, both D-glucuronic acid and 2-keto-D-glucaric acid. The existence of these acids was not confirmed since they were not identified by analytical methods nor by forming any known derivatives.

Yackel, Kenyon and Unruh (26) oxidized cellulose with dry nitrogen tetroxide to form a product which was readily soluble in 2% sodium hydroxide, ammonium hydroxide, sodium carbonate or warm pyridine. The oxidized cellulose was believed to have as high as 25% carboxyl group content with the primary hydroxyl groups being attacked preferentially. The exact nature of the oxycellulose was not determined by the authors, however the glycosidic linkage is known to have undergone some oxidation and the resulting acids were fragments of the long chain polysaccharide.

On the basis of these considerations, it is reasonable to assume that the more labile glycosidic linkage of sucrose would also undergo oxidative cleavage in the nitrogen tetroxide oxidation reported

by Gisvold. Maurer and Drefahl (27) in studying the nitrogen tetroxide oxidation of monosaccharides, concluded that the reagent is not a satisfactory oxidant for carbohydrates. The reactions require anhydrous conditions, prolonged reaction times, and the use of inert solvents such as chloroform and carbon tetrachloride usually lead to nitration products, and subsequently the yields are lowered.

Thus, to date, no successful method of oxidizing sucrose in high yield to a monocarboxylic has been found. The reported methods all yield low molecular weight degradation products which have the original glycosidic linkage of sucrose either hydrolyzed or oxidized to the oxidation products of fructose and glucose.

3. The Use of Noble Metal Catalysts and Oxygen

Despite the fact that it has been known for many years that oxygen in the presence of a noble metal catalyst will oxidize aldehydes and primary alcohols to acids, it has not been until the last few years that it has been applied to the field of

carbohydrates. The first such reported oxidation was done by Busch (28). It was claimed that D-glucose was oxidized quantitatively to D-gluconic acid using a palladium impregnated calcium carbonate catalyst and air with the theoretical amount of base to neutralize the gluconic acid formed. Six years later, in 1947, Heyns and Heinemann (29) used a more active platinized charcoal catalyst for the same conversion.

Using this platinum catalyst, Dalmer and Heyns (30)(31) were able to selectively oxidize the C-1 hydroxyl group of L-sorbose with oxygen, thereby obtaining 2-keto-L-gulonic acid, the precursor of L-ascorbic acid. Under analogous conditions, Trenner (32) was able to convert 2,3-isopropylidene-L-sorbose in aqueous solution to 2-keto-L-gularic acid. The above reactions show clearly the tendency of the system to attack primary hydroxyl positions in preference to the secondary ones. The first reaction is the conversion of one primary hydroxyl group to carboxylic acid, while the second reaction involves the conversion of both primary hydroxyl groups to carboxylic acids.

Mehltretter (33)(34) and coworkers have

prepared glucuronic acid by the platinum catalyzed air oxidation of partially blocked glucose derivatives. 1,2-Isopropylidene-α-D-glucofuranose was oxidized at 50°, using sodium bicarbonate buffer with 13% platinum - charcoal catalyst, to the corresponding glucuronide in 50 to 60% yield. Similarly, Mehltretter, Hockett, Miller and Scattergood (35) oxidized 1,2-cyclohexylidene-α-D-glucofuranose and obtained the sodium salt of 1,2-cyclohexylidene-α-D-glucuronic acid in 60% yield.

The oxidation of both the anomeric forms of methyl D-glucopyranoside, methyl D-galactopyranoside and methyl D-mannopyranoside has been readily achieved by the use of oxygen and platinum catalyst (36)(37). The corresponding glycuronides have been obtained in high yield, however, this method of preparing uronic acids is generally not practical. The chief disadvantage is that the uronic acids survive only in low yield the conditions required for the hydrolysis of the glycosidic linkage. However, the method has been applied commercially for the preparation of D-glucuronic acid (38).

a. Factors Affecting The Rate Of Oxidation

The rate of the oxidation of carbohydrates by oxygen when catalyzed by platinum is largely dependent on the activity of the catalyst. The method used by Trenner (32) for the preparation of an active catalyst has been found by Mehltretter and coworkers (33) to consistently yield an active catalyst. The catalyst is prepared by depositing metallic platinum on Darco G-60 brand activated carbon by reducing sodium chloroplatinate with formaldehyde in an aqueous solution of sodium carbonate. It had previously been found (32) that the reagents must be extremely pure for the preparation of a highly active catalyst. Mehltretter (34) found that he was able to use the same catalyst in five separate oxidations before the catalyst became sufficiently inactive to warrant changing it. More recently, it has been found that Adam's catalyst may also be used in this type of oxidation (38).

The rate of heterogeneous reactions of this type, in which the rate of oxidation is dependent on the aerated solution of carbohydrate coming into contact with the platinum catalyst, is also dependent on manner and rate of stirring or mixing. In small scale

oxidations, appreciable rates of oxidation have been obtained by shaking in creased flasks (33) or by high speed stirring (33). Satisfactory oxidations were obtained with stirrer speeds of 3,500 r.p.m., however, the rate of reaction is significantly increased by even greater agitation (35). Turbo mixers have proven useful for large scale oxidations (36). Instances have been reported (39) where the reaction was sufficiently rapid and exothermic to require external cooling in order to maintain temperatures of 50° to 60°.

The rate of oxidation can also be increased by increasing the ratio of the amount of catalyst to the amount of material to be oxidized. However, the cost of the platinum catalyst is a limiting factor. The rate may be enhanced also by increasing the temperature or pressure at which the oxidation is carried out but this often leads to a significant decrease in the specificity of the oxidation.

b. Theory Of Catalysis

This discussion will be limited to a consideration of the type of heterogeneous catalysis used

in the platinum-catalysed oxidation of alcohols.

Many metals have been used as catalysts for both oxidations and dehydrogenations, however, the transition elements of the eighth group of the periodic table are best suited for this purpose. The most probable explanation of the activity of these metals is connected with the fact that they have the ability to adsorb considerable volume of the gases used as reagents. On this basis, the first step in the platinum-catalyzed oxidation of carbohydrates is most probably the adsorption of oxygen at the surface of the platinum metal. According to Langmuir (40), the adsorbed molecules are held to the surface of the catalyst by the same forces of attraction which normally lead to crystal growth, or, in the case of liquids, to the phenomena of surface tension. The adsorbed molecules are also assumed to be oriented at the surface. An interesting piece of evidence to support this idea of orientation is the observation of Palmer and Constable (41) that the activity of copper in the dehydrogenation of primary alcohols was such that the rates of reaction of five primary alcohols were

all equal at a given operating temperature. This then points to an adsorption of primary alcohols with the R-CH_2OH group adjacent to the catalyst and the hydrocarbon chain away from the surface.

It is then possible to assume that a somewhat similar orientation of the carbohydrate material is occurring on the platinum catalyst in the course of these oxidations. However, the effect of physical adsorption alone is insufficient to explain catalysis. The higher concentration of molecular oxygen in close proximity to the carbohydrate material is insufficient to explain the rate of oxidation.

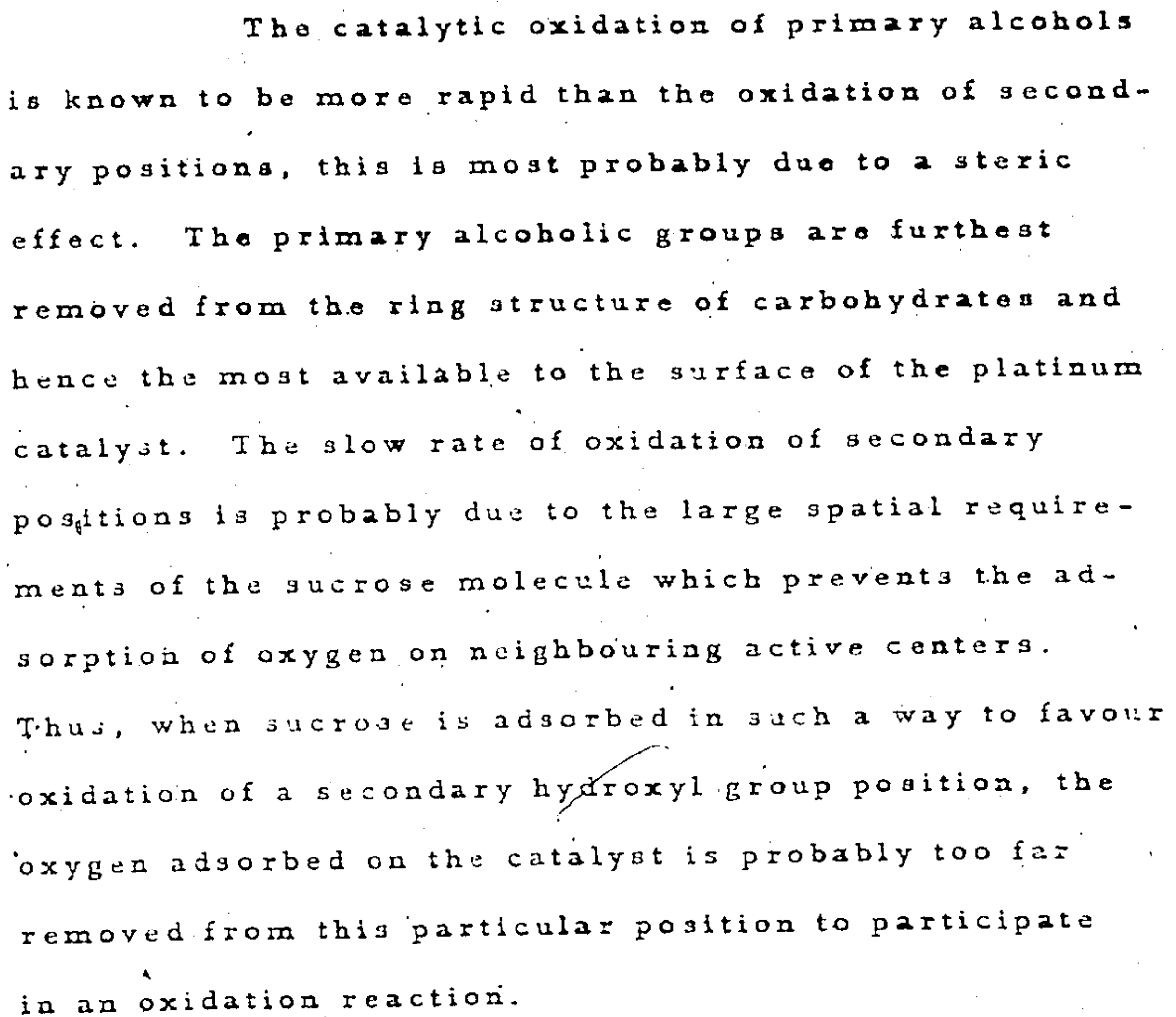
To understand heterogeneous catalysis, one must consider adsorption phenomena. Adsorption studies have shown also that in many cases when some molecules are absorbed on a surface, the molecular bond is broken and is replaced by two bonds with the adsorbent. The admole is said to be converted to two adatoms by the surface reaction and in these cases the reaction is termed chemisorption (40). When the molecular bond is not completely broken but is instead only weakened, the probability of reaction with other

adsorbed species will be quite different from what it would normally be with the molecules in free space (40).

It has been suggested by several workers (42) that chemisorption occurs by electron transfer in which either a polar or covalent bond is formed. The polar bonds are formed by the electrons of the adsorbed material going to fill some of the vacant spaces in the outer electron orbitals and the subsequent bond is polarized by the nature of the two nuclei involved in the new bond. A second scheme involves the loosely bound electrons of the metal catalyst going to form the ordinary covalent bond. The electron in this case is removed only a finite distance from the original crystal lattice orbital, hence the activation energy for this process is not prohibitively high as was thought by Emmett and Teller (43).

Trapnell (44) classified the various metals according to their ability to adsorb gases such as hydrogen and oxygen and found the transition metals, such as platinum, the most active. This he attributed to "holes" in the d-band of these metals. From the above considerations, the role of the platinum catalyst

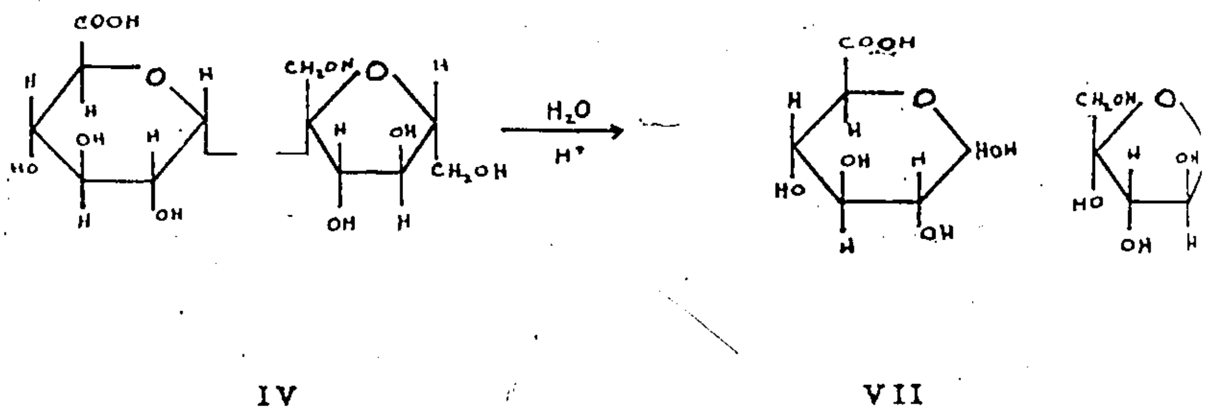
in the oxidation of carbohydrates is to first chemisorb the molecular oxygen, thus weakening or breaking the molecular bond between the oxygen atoms. The alcoholic functional group of the carbohydrate materials present are adsorbed on adjacent sites and an electron transfer mechanism in which the polyol is oxidized by the adsorbed oxygen is set up.

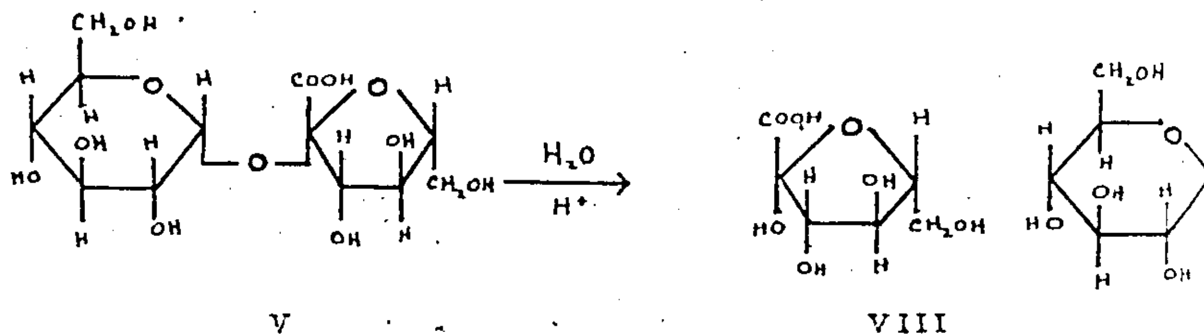


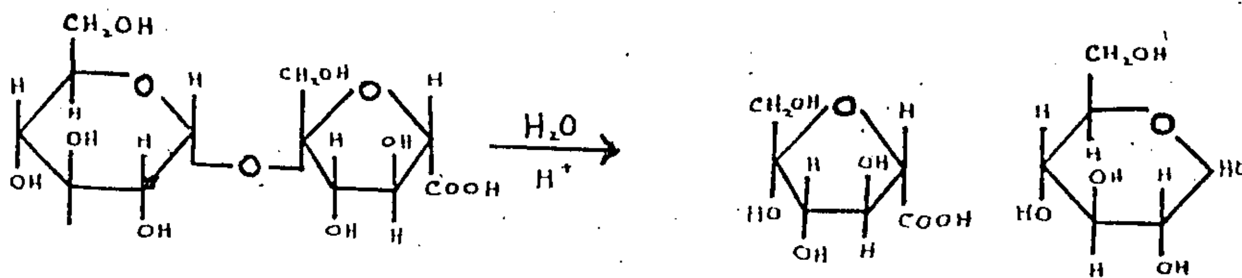
The catalytic oxidation of primary alcohols is known to be more rapid than the oxidation of secondary positions, this is most probably due to a steric effect. The primary alcoholic groups are furthest removed from the ring structure of carbohydrates and hence the most available to the surface of the platinum catalyst. The slow rate of oxidation of secondary positions is probably due to the large spatial requirements of the sucrose molecule which prevents the adsorption of oxygen on neighbouring active centers. Thus, when sucrose is adsorbed in such a way to favour oxidation of a secondary hydroxyl group position, the oxygen adsorbed on the catalyst is probably too far removed from this particular position to participate in an oxidation reaction.

4. The Nature Of The Oxidation Products Of Sucrose

For the reasons we have seen, the oxidation of sucrose by oxygen with platinum as catalyst could be expected to occur most readily at the three primary positions of sucrose to yield the corresponding carboxylic acids IV V VI. In this case, hydrolysis of the monoacids would yield D-glucuronic acid (VII), 2-keto-D-gluconic acid (VIII) and 5-keto-L-gulonic acid (IX).



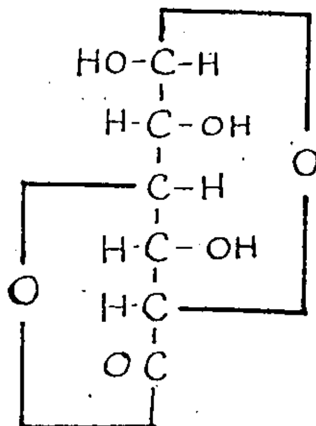




VI IX

a. Glucuronic Acid

D-Glucuronic acid is an unstable molecule and cannot be stored as an oil due to darkening and general decomposition. This is because the uronic acids are capable of causing their own decomposition with the subsequent liberation of carbon dioxide. D-Glucuronic acid (VII) can be lactonized to D-glucuronolactone (X)(45), commonly referred to as "glucurone".



X

The chief source of D-glucuronic acid, until the product became commercially available in 1950, was through the biosynthetic route in which toxic compounds such as camphor or borneol are fed to animals, the urine collected, and the D-glucuronide of the drug isolated. Hydrolysis of the D-glucuronide yielded the free acid (46). D-Glucuronic acid is also present in mucopolysaccharides such as hyaluronic acid, heparin and mucoitin sulfuric acid and in plant gums. In recent years, the free D-glucuronic acid was isolated from the urine of rabbits which had been fed aniline (47).

Uronic acids have been prepared by the reduction of the monolactone of glycaric acids (48), by cyanohydrin synthesis (49) and by oxidation of the primary alcoholic groups of aldoses (50).

No completely accurate and specific analytical method is available for the determination of D-glucuronic acid. At the present time, isolation of the crystalline γ-lactone, or one of the derivatives of the acid, seems to be the only reliable method of demonstrating its presence. Although helpful, the

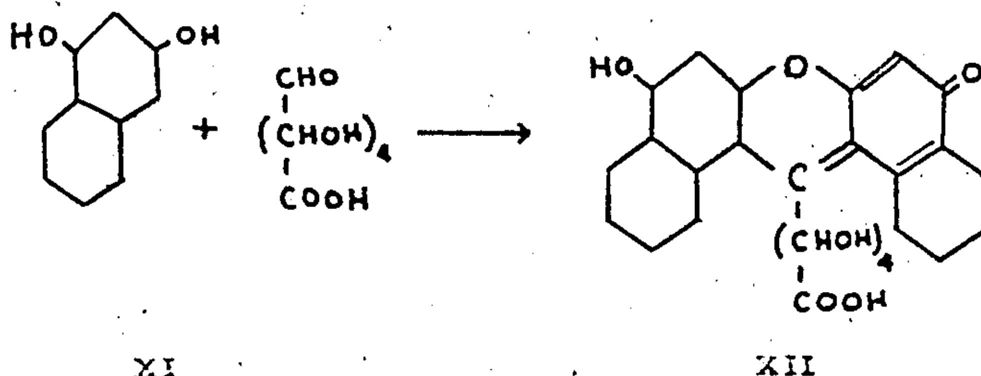
analytical methods now available can lead to erroneous conclusions when used for the analysis of solutions containing several carbohydrate substances.

The most widely used method for the determination of D-glucuronic acid is the colourimetric determination based on the work of Tollens and Rorine (51). It was found that characteristic precipitates and colours are formed when solutions of various sugars are heated with a little naphthoresorcinol (XI) in the presence of an equal volume of concentrated hydrochloric acid. In the case of D-glucuronic acid, a blue deposit was formed which gives a violet solution when dissolved in ether. When identical treatment was given to pentoses and hexoses, the precipitate formed was insoluble in ether.

Various workers (52)(53)(54) have tried to adopt this test to the quantitative determination of D-glucuronic acid, however, the results were not reproducible. The intensity of the colour formed depends to a marked extent on the manner in which the solution of naphthoresorcinol is prepared and stored. Many workers age their solutions in a bath at

approximately 60° for periods ranging from one hour to 24 hours.

It is believed by Guerrero (55), Jarridge (56) and Meyer and coworkers (57) that the colour intensity is dependent on an oxidation product of naphthoresorcinol rather than the unoxidized product. However, it is difficult to believe that the colour produced by the aldehyde grouping of a uronic acid, as proposed by the aforementioned workers in the formation of xanthene derivatives (XII) should not also be produced by pentoses and hexoses since the residual carbohydrate chain containing the COOH group does not enter into their proposed mechanism.



Several colour reactions can be produced by uronic acids, however in many of these, there is strong

interference by pentoses. Among these, the orcinol test (58) may be used if the solution does not contain pentoses. According to Thomas (59) β-naphthol may be used to distinguish between pentoses and hexuronic acids, the differentiation being based on a coloured ring produced at the interface between concentrated sulfuric acid and β-naphthol and an aqueous solution of pentose or uronic acid. The pentose gives a blue ring whereas the uronic acid gives a crimson red ring.

Dische (60) claims that a deep pink colour is formed when a solution of mannose and glucuronic acid is mixed in concentrated sulfuric acid containing thioglycolic acid. Blanks are necessary since hexoses and pentoses also form coloured products. The colour is determined spectrophotometrically, however, the reproducibility is not better than 10%.

Mann and Tollens (61) found that uronic acids are decarboxylated by the action of boiling 12% hydrochloric acid and this was later made quantitative by Lefevre and Tollens (62) who measured the carbon dioxide evolved. Other sugars also undergo the same reaction under this treatment. This led Norman (63)

and also Whistler, Martin and Harris (64) to measure the rates of evolution of carbon dioxide which allowed them to correct for the evolution of carbon dioxide from non-hexuronic acid material.

The mechanism of the decarboxylation is not well known, however, the equation,

$$C_6H_{10}O \longrightarrow C_5H_4O_2 + CO_2 + 3\ H_2O,$$

is approximately correct. Determination of the furfural produced during the decarboxylation has been suggested a number of times as a method for estimating the amount of uronic acid present. However, the yield of furfural is not quantitative (65) and varies greatly with reaction conditions and also with the percentage content of uronic acid in the material to be analysed(66).

Optical rotation occasionally has been used as an indication of the presence of conjugated D-glucuronic acid in biological fluids. While the free D-glucuronic acid is dextrorotatory, its conjugation products are generally levorotatory (67).

The uronic acids are biologically very important. Of the stereoisomeric hexuronic acids possible, only three have been found in nature. These are

D-glucuronic acid, D-mannuronic acid and D-galacturonic acid. Of these three, D-glucuronic acid is by far the most widely distributed since it takes part in the metabolism of many types of organic compounds.

In the field of biochemistry and pharmacology, interest in D-glucuronic acid has centered around the part it plays in the metabolism of drugs and other foreign organic compounds. Many organisms are capable of producing D-glucuronic acid in response to the administration of such drugs, the production being of appreciable quantities. However, the extent to which D-glucuronic acid takes part in normal metabolism is virtually unknown. Small quantities of this uronic acid are formed in normal urine of both humans and animals (68).

Many theories have been put forth as to the origin of uronic acids in the body, however, recent isotopic labelling procedures have clarified this field. Mosbach and King (69), using D-glucose labelled in all positions with carbon-14, obtained evidence that D-glucuronic acid excreted by guinea pigs as a D-glucuronide of borneol was formed without rupture of the D-glucose

carbon chain. Eisenberg and Gurin (70), investigating the menthyl D-glucuronide isolated from the urine of rabbits which had been fed menthol and D-glucose-1-C^{14}, found that the precursor of D-glucuronic acid is D-glucose, or an equivalent six carbon compound. The site of D-glucuronic acid formation in vivo appears to be the liver where Hemingway and Schmidt (71) were able to isolate the highest concentration.

One of the more important biological functions, involving D-glucuronic acid, is the removal of toxic agents from the liver as D-glucuronides. The literature on the various D-glucuronides is profuse and has been well reviewed by Artz and Osman (72) in 1950 and later by Bray (73) in 1954.

b. Ketoaldonic Acids

The known ketoaldonic acids of the hexose series are of the 2 and 5 keto types. The 2-keto acids (VIII) have been called osonic acids because of their preparation from osones. The 5-keto acids (IX) have been termed keturonic acids, that is, uronic acids derived from ketoses, whereas normal uronic acids are

alduronic acids.

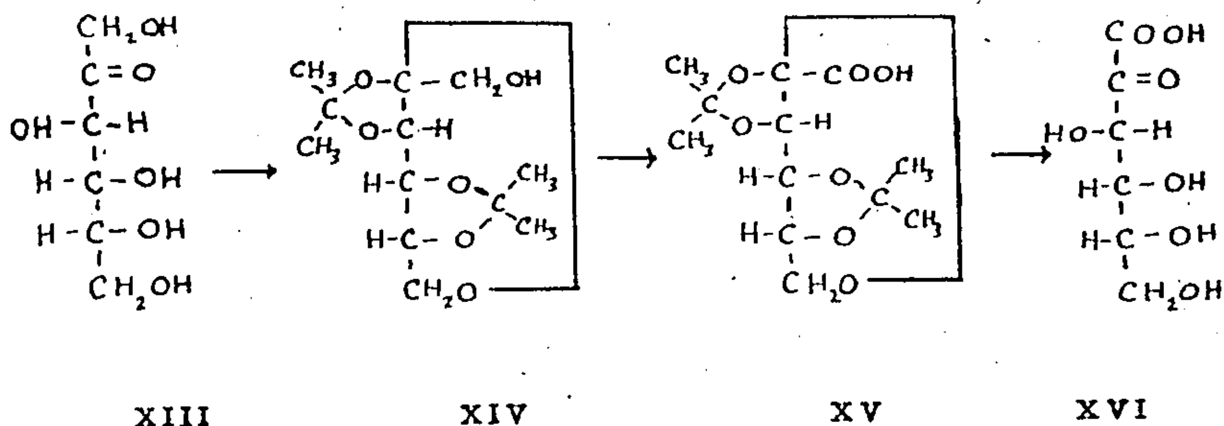
The chemical properties of the keto aldonic acids are very similar to the uronic acids. Both types of acids undergo the same colour reactions with naphthoresorcinol giving the violet colour which is extractable with ether or benzene. The keto aldonic acids also undergo the ready decarboxylation in 12% hydrochloric acid which is characteristic of the hexuronic acids. The 2-ketoaldonic acids on decarboxylation give furfural in 33% yield, whereas the 5-ketoaldonic acids yield approximately 42.5% furfural (74). The evolution of carbon dioxide, as in the case of the uronic acids, may be taken as a quantitative method for estimating both the 2 and 5 keto acids (75). These keto acids are not as stable as D-glucuronic acid as evidenced by the rapid decomposition of 5-keto-D-gluconic acid when it is in the syrupy form. The syrup darkens and froths with the liberation of carbon dioxide (76). 2-Keto-D-gluconic acid and its lactone exist only as syrups, although hygroscopic crystals of the former have been reported (77).

2-Keto-D-gluconic acid has been produced

by many bacterial strains such as Corynebacterium creatinovorans, Penicicillium brevicompactum and Pseudomonas fluorescens. The keto acid can be used by the bacteria Pseudomonas, Xanthomonas, Escherichia, Aerobacter Paracolobactrum, Serratia, Erwinia and Bacillus (78). From these, one sees that the direct oxidation pathway of carbohydrate metabolism is widespread among microorganisms. Hall, Kulka and Walker (79) showed that some of the intermediates that can be isolated from microbial oxidation of 2-keto-D-gluconic acid are D-arabinose, D-ribulose, tartronic acid, and oxalic acid.

2-Keto-D-gluconic acid may be chemically prepared by the direct oxidation of fructose by nitric acid under carefully controlled conditions. In fructose (XIII) the C-1 atom adjacent to the carbonyl group at C-2, is more sensitive to oxidation than the other primary hydroxyl group at C-6 position (80). Preferential oxidation of the secondary alcoholic group adjacent to the carboxyl group in the sugar acids, or aldonic acids can be carried out with chromic acid (81) or with chlorates in the presence of a vanadium catalyst (82).

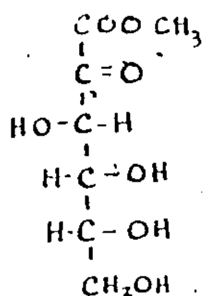
The most effective chemical method of preparing 2-keto-D-gluconic acid XVI is by the oxidation of the 2,3;4,5-di-O-isopropylidene derivative of D-fructose (XVI)(83).



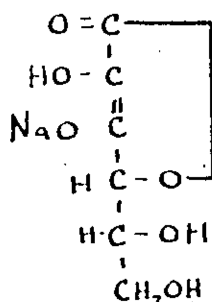
D-fructose (XIII) is condensed with two moles of acetone giving a diisopropylidene derivative with the C-1 hydroxyl group free. The primary alcohol is oxidized by alkaline permanganate and the isopropylidene residues removed by dilute acid hydrolysis.

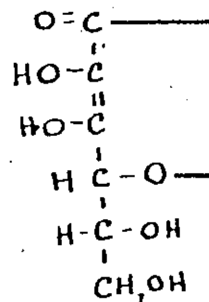
The most characteristic feature of 2-ketoaldonic acids is their ability to undergo simultaneous lactonization and enolization. This double transformation was first performed by Ohle, Erlback and Carls (84) who treated methyl 2-keto-D-gluconate (XVIII)

with sodium methoxide and obtained a product which contained no ester grouping. The product was D-araboascorbic acid (XIX).



NaOMe



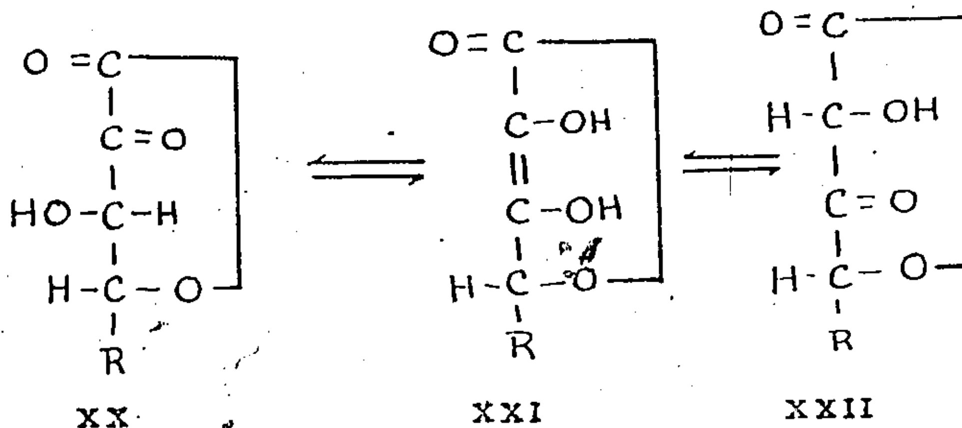


XVIII XIX

The 2-ketohexuronic acids can be transformed into the corresponding ascorbic acid derivatives simply by being heated in aqueous solution. An equilibrium is set up between the keto acid and the corresponding ascorbic acid, but, since this equilibrium does not lie sufficiently on the ascorbic acid side, it has been found preferable to utilise the esters instead of the free acids. An improved method of preparing ascorbic acid analogs consists in heating diisopropylidene 2-keto-D-gluconic acid directly with hydrogen

chloride in solvents such as ethanol (85).

The unique feature of L-ascorbic acid and its analogs lies in the enediolic system and it is this portion of the molecule which is responsible for its reducing properties. It can readily be seen that the enediolic system (XXI) can be written theoretically in the isometric 2-keto (XX) or 3-keto form (XXII).



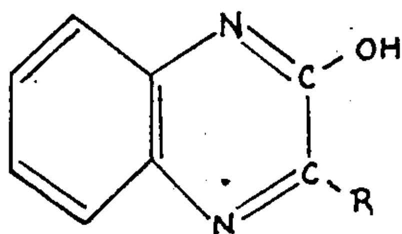
It was found by Demole (86) and Dalmer (87) that the five membered lactone ring containing the enediolic system is essential for antiscorbutic activity and furthermore the lactone ring must have C_4 with the D-configuration. Since the ascorbic acid analog derived from 2-keto-D-gluconic acid fulfils these requirements it is biologically active, having an

antiscorbutic activity 1/20 that of L-ascorbic acid or vitamin C. Recently, D-araboascorbic acid has been used commercially to dilute ascorbic acid preparations in order to prolong the half-life of the vitamin C. The effectiveness of these preparations is due to the D-araboascorbic acid being oxidized somewhat preferentially, hence protecting the ascorbic acid from oxidation.

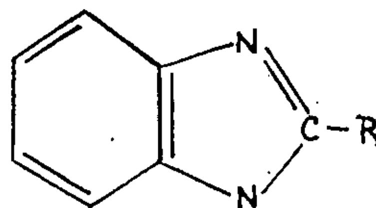
Several methods are available for the estimation of 2-ketoaldonic acids, the best known being the decarboxylation methods, naphthoresorcinol colour determinations and methods such as the Bertrand, the Scales, the Shaffer-Hartman and the Shaffer-Somogye, employing the iodometric determination of cuprous ions in the presence of citrates which form soluble complexes. In the 2-ketoaldonic acids, the reduction value of 2-ketogluconic acid compared to D-glucose is 87% as determined by the Shaffer-Hartmann method (88).

Despite the ready conversion of 2-keto acids to reductone type compounds, their chemical properties differ. The ascorbic acids are strong acids as a result of the presence of the enolic groups rather than of the

lactone ring (89). They reduce Fehling's solution and the double bond is oxidized by acidic iodine solution. The latter reaction is used to differentiate quantitatively between the ascorbic acids and the 2-ketoaldonic acids. Of the methods described in the literature for the estimation of 2-ketoaldonic acids, the most specific is that reported by Lanning and Cohen (90) in which the 2-ketoaldonic acid is condensed with o-phenylenediamine to yield a 2-hydroxyquinoxaline (XXIII). The aldonic acids and uronic acids form benzamidazoles (XXIV).



XXIII



XXIV

The 2-hydroxyquinoxalines have a characteristic ultraviolet adsorption band at 335 $m\mu$ which is independent of the 4-carbon carbohydrate residue.

The 5-ketoaldonic acids resemble uronic acids since both are quantitatively decarboxylated by

12% hydrochloric acid, and also their colour reaction towards naphthoresorcinol reagent (51) are similar. The 5-ketoaldonic acids are generally prepared by bacterial oxidations (91)(88) in yields of better than 90% from D-glucose. These acids have been prepared chemically by two methods, the first, being the action of calcium and strontium hydroxides (92) and the second, by the oxidation of suitably blocked ketoses. Using the latter method, 1,2;3,4-di-O-isopropylidene-D-tagatose was oxidized to 5-ketogalactonic acid (93).

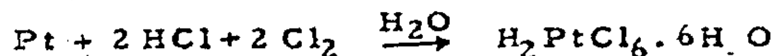
In the current carbohydrate literature, two methods have been proposed for the determination of 5-ketoaldonic acids in the presence of other reducing sugars. The first of these methods is a modified Benedict's reagent proposed by Militzer (94). In this method, the rate of reaction of 5-keto-D-gluconic with Benedict's reagent at 25° is visually observed and the amount present is determined by comparison with standard solutions. However, the method is semi micro and is not very reproducible. A third method claiming high specificity and accurate results, as well as small quantities, is that of Perlman (95). The

method depends on the spectrophotometric measurement of the reduction of Nelson's (95) arsenomolybdate reagent at 50°. Interference however is encountered when 2-ketoacids are present since Perlman found that, under the conditions of the analysis, 2-keto-D-gluconic acid had 16% of the activity of 5-keto-D-gluconic acid and fructose and sucrose 8% and 3% respectively.

II. EXPERIMENTAL

1. Materials

The catalyst was prepared according to the method of Mehltretter and coworkers (33), in which 6.4 g. of platinum metal were placed in 90 ml. of aqua regia and heated over steam for eight hours. The solution was



$$Pt + 2\,HCl + 2\,Cl_2 \xrightarrow{H_2O} H_2PtCl_6 \cdot 6H_2O$$

evaporated to dryness under reduced pressure yielding a viscous liquid which crystallyzed on cooling to room temperature. The yield of chloroplatinic acid was 20 g.

Fifty g. of Darco G-60 brand of activated charcoal were digested overnight in 1:1 hydrochloric acid, collected by filtration and washed free of acid with distilled water. After drying the carbon for 20 hours at 110°, 49 g. were added to a solution of 20 g. of chloroplatinic acid in 430 ml. of water. The mixture was carefully neutralized with sodium bicarbonate while being stirred vigorously. The mixture was then heated to 80° in a water bath and 32 ml. of 38% formaldehyde

were added over a 45 minute interval, while, simultaneously, solid sodium bicarbonate was added to neutralize the formic acid liberated and to maintain slightly alkaline conditions (pH8). After all the formaldehyde was introduced, the mixture was held at 80° with constant stirring for 2.5 hours. The mixture was cooled to room temperature and the solids gathered by filtration. The cake was washed with 60 ml. of hot concentrated potassium chloride solution followed by similar washings of decreasing concentration of potassium chloride solutions until the cake was washed with distilled water. These washings were continued until a negative chloride ion test was obtained using silver nitrate. The catalyst was then dried for 12 hours in a 110° oven. This afforded a catalyst containing 13% platinum by weight.

N,N'-di-p-Tolylcarbodiimide. - The N,N'-di-p-tolylcarbodiimide was prepared by the method outlined by Sheehan and Hlavka (97) in which p-toluidine is condensed with carbon disulphide to form a thiourea which is then treated with mercuric oxide to give the carbodiimide in 70% yield.

Methyl α-D-Glucopyranoside. - The compound was prepared according to the Fischer method as described by Helferich and Schafer (98). A 49% yield of methyl α-D-glucopyranoside melting at 164.5° - 165.5°, $[\alpha]_D^{23}$ = 158° (c, 8 in water), was obtained.

1,2;3,4-di-O-Isopropylidene-D-Galactopyranose. - The compound was prepared according to the method of Sell and Link (99) in which two moles of acetone are condensed with one mole of galactose using sulphuric acid and copper sulphate catalysts. The syrupy di-O-isopropylidene-D-galactose was distilled at 119° to 125° under vacuum in 41% yield.

2. Methods

a. Preparative Methods

The Oxidation Of Sucrose By Oxygen Using Platinum Catalyst And Bicarbonate Buffer. -

These oxidations were carried out in an H-shaped reaction vessel in which two 30 ml. bulbs were attached to the lower portion of the H. One side of the vessel was employed as the oxidation chamber, while the second was used as a trap for carbon dioxide in the

system. The vessel was immersed in a wax bath of constant temperature and the whole was shaken by a wrist action shaker. An atmosphere of oxygen was maintained by using a mercury-filled gas burette of 100 ml. capacity. In the course of the oxidation, a slight positive pressure of 2 cm. of mercury was maintained by adjustment of the levelling bulb.

In a typical run, sucrose was oxidized as follows. Two mM. of sucrose were dissolved in ten to 15 ml. of distilled water and six mM. of sodium bicarbonate and 80 mg. of platinum catalyst were added. Twelve ml. of 0.5 N sodium hydroxide were introduced into the adjacent chamber of the H-shaped vessel and the vessel was immersed in a wax bath kept at 80°. The reaction flask was then thoroughly agitated by means of the wrist action shaker. Once the pressure of oxygen inside the system had equilibrated due to temperature changes and the solubility of oxygen in the aqueous solution, a positive pressure of pure oxygen of 2 cm. mercury was maintained. When the extent of oxidation was increased by prolonging the reaction time, additional sodium bicarbonate was added at suitable intervals.

The uptake of oxygen by the system was measured by adjusting the mercury level until atmospheric pressure was reached and the change in volume in the gas burette recorded. The alkali in the trap was titrated with standard hydrochloric acid (0.1 N) to a phenolphthalein end point and then further titrated to bromphenol blue end point in order to obtain the number of milliequivalents of carbon dioxide liberated during the oxidation.

Oxidations Using Platinum Catalyst, Oxygen And Anion Exchange Resin. -

These oxidations were carried out using a Parr Pressure Reaction Apparatus. Approximately 40 g. of sucrose were dissolved in 150 ml. of distilled water and the solution was slurried with 70 to 80 g. of anion exchange resin (Amberlite I R-45) which is a weak tertiary ammonium resin. The anion resin was previously regenerated in a column by eluting with sodium hydroxide until a negative chloride ion test was obtained using alcoholic silver nitrate. The platinum catalyst, 0.5 - 1.0 g., was added to the slurry and the reaction vessel was then attached to the apparatus and the gas chamber filled with oxygen to a

pressure of 50 p.s.i. The solution was heated electrically at the base to maintain the temperature in the reaction flask between 70° and 75°. The rate of uptake of oxygen in the system was followed by a pressure gauge attached to the gas chamber. The reaction vessel was shaken by means of an eccentric rod causing a push-pull motion.

The reaction was stopped after a pressure drop of 5 p.s.i. which generally required reaction times of from two to five days depending on the quantity of catalyst used. The slurry was allowed to cool and then quantitatively poured into a 20 mm. diameter chromatographic column (length approximately 60 cm) and the column worked to remove all air bubbles. The column was eluted with distilled water until the effluent gave a negative anthrone test (100) for sucrose. Once the column was freed of sucrose, the resin was eluted with ammonium hydroxide and the rate of elution held constant at 3 ml. per minute. To completely remove the acids from the resin requires from two to three liters of the 3 N base before a negative anthrone test (100) is obtained. The basic eluate was evaporated to a thick oil under vacuum at 40°. The yield of

acids was dependent on the extent of oxidation.

Column Adsorption Chromatography. -

The method employed was reported by Whistler and Durso (101) in which the adsorbent used consisted of equal parts of Darco G-60 and Celite (John Mansville #535). The two components were intimately mixed when dry, and then sufficient water was added to form a loose slurry which could easily be poured into chromatographic columns. The slurry was poured into a column 230 mm. x 34 mm. diameter to give a column 170 mm. long after packing. Ethanol, 5%, in water was used to develop the columns.

Column Partition Chromatography. -

Column partition chromatograms were prepared by the method of Lemieux, Bishop and Pelletier (102) in which acid-washed Celite (John Mansville #535) acts as the media supporting the static phase. The columns used in the preparative work were 41 mm. in diameter. The flow rate was from 2.5 to 3 ml. per minute and the effluent was generally collected in 20 ml. fractions. The bands were located by one of two methods. The first method was to calculate the approximate location of the bands by the method outlined

by Lemieux, Bishop and Pelletier (102). When it is impractical to remove all compounds by the above mentioned elution technique, the extrusion method is used. The column is blown free of developing solvent by using pressure and removed from the glass tube by applying air pressure from rubber-bulb to the bottom of the tube. The compounds are located on the extruded Celite columns by spraying with 2% potassium permanganate in 2 N alkali through a mask to locate the zones. The bands are cut out and washed with water, or organic solvent, and filtered. The percentage recovery of compounds is generally 90% of the amount loaded on the column.

b. Analytical Methods

Paper Chromatography. -

The paper used in this investigation was Whatman #1 chromatographic paper which was cut 8" wide by 22" long. The descending type paper chromatography was used throughout this investigation. The spots were applied using capillary tubes delivering about 0.002 - 0.02 ml. of 4% solution. The

chromatograms are placed in a chromatographic cabinet previously saturated with the aqueous phase of the two phase system of organic solvent saturated with water. The developing solvent front was allowed to move approximately 19 inches before the paper was removed from the cabinet. In some cases where more complete separations were desired, the bottom of the paper was serrated to prevent channeling, and the solvent front allowed to drip from the edge of the paper.

The following solvent systems were used:-

1) n-Butanol:water, - prepared by saturating n-butanol with distilled water.
2) n-Butanol:ethanol:water, - prepared by mixing 5 volumes of n-butanol with 1 volume of ethanol and 4 volumes of distilled water.
3) Ethanol:acetic acid:water (103), - prepared by mixing 10 volumes of ethanol with 3 volumes of glacial acetic acid and 2 volumes of distilled water.
4) Ethanol:phenol:water (104), - prepared by mixing 5 volumes of ethanol with 2 volumes of phenol and 5 volumes of distilled water.

Spray Reagents For Paper Chromatography. -

Aniline Phosphate. - The spray reagent used was a modification of the reagent described by Bryson and Mitchell (105), in which 2 volumes of 2 N aqueous phosphoric acid and 1 volume of 2 N aniline in ethanol are mixed just before use and the resulting salt precipitate is dissolved by adding 3 volumes of glacial acetic acid. The sprayed and dried chromatogram was heated for ten minutes at 105°. All reducing pentoses and hexoses and many of their derivatives can be detected as brown spots. Non-reducing disaccharides which are easily hydrolyzed by the acid conditions also give the characteristic brown spots.

Aniline Hydrogen Phthalate. - This spray reagent (102) is prepared by dissolving 930 mg. of aniline and 1.6 g. of phthalic acid in 100 ml. of water saturated n-butanol. The dry paper chromatogram is sprayed with the reagent and then heated at 105° for ten minutes. The spray develops reducing sugars and compounds capable of liberating reducing sugars under the conditions of the test. The spots are not quite as intense as those developed by the aniline phosphate reagent.

Periodate-Permanganate. - The 1,2-glycol-cleaving spray (107) is prepared by mixing before use 1 volume of 1% potassium permanganate containing 2% sodium carbonate and 4 volumes of 2% aqueous sodium periodate. The dry paper is sprayed heavily with the solution and placed in a damp atmosphere to prevent drying during the time required for the reagent to cleave the glycols. The latter appear as yellow spots on a purple background. Washing off the excess reagent results in a white background with the spots remaining as permanent brown stains.

Urea Oxalate. - The dry paper chromatogram is sprayed heavily with a 2% aqueous solution of urea oxalate. A dark green spot indicates the presence of ketosugars when the paper is heated at 105° for ten minutes. Sucrose gives a positive test due to the liberation of D-fructose under the conditions of the test. D-Glucose gives a faint brown colour which is detectable only in high concentrations.

Hydroxamic Acid Spray For Esters. - The reagent (108) is prepared by mixing before use equal volumes of N methanolic hydroxylamine hydrochloride and 1.1 N methanolic potassium hydroxide. The dry paper

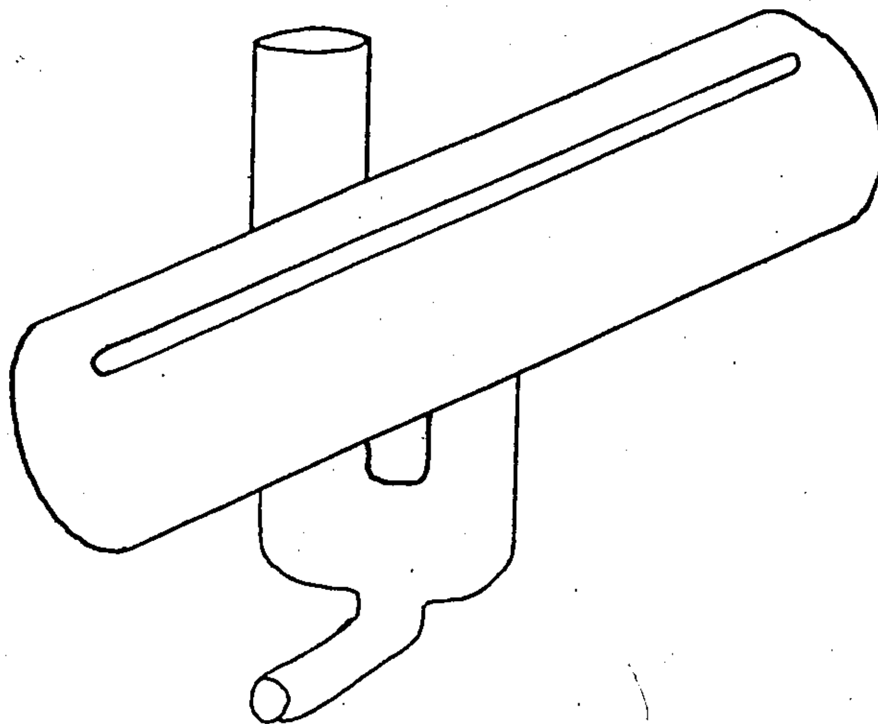
chromatogram is heavily sprayed with the reagent and left at room temperature for ten minutes. The paper is next sprayed with 1 to 2% ferric chloride solution containing 1% hydrochloric acid. A mauve colour appears in those areas containing esters or lactones.

Napththoresorcinol Spray Reagent. - The spray reagent (109) gives a green colour with non-reducing sugars, a grey colour with reducing sugars and with ketoses and sucrose a red colour. Pentoses are developed as blue spots while further treatment with steam at 70° develops uronic acids as a dark blue colouration. For these colour reactions the reagent is prep ared by making and mixing equal parts of 0.2% alcoholic naphthoresorcinol solution and 2% aqueous trichloroacetic acid. The paper is sprayed heavily and heated to 100° for ten minutes.

Paper Electrophresis. - Paper electrophoretic work was carried out using a Labline Electrograph capable of delivering 500 D.C. volts and 50 m.amps. A cabinet capable of housing the complete apparatus was constructed 15" x 13" x 33". The cabinet has two shelves to support the U-shaped electrolyte reservoirs.

The reservoirs are U-tubes of pyrex glass

which support at one end a horizontal piece of glass tubing (5 cm. diam. x 26 cm. long) which is sealed at both ends and has a slot 0.75 cm. wide and 21 cm. long. The other end of the U-tube is left open to accept the platinum electrodes which are dipped into the electrolyte at this position. The electrodes are held in place by passing the leads through a slotted rubber stopper.



The contact between the power supply and the platinum electrodes was made through a glass tube which has platinum leads fused into the glass at one end, one extremity of the platinum wire extending into the mercury filled glass rod, the other end was woven into a 2 cm. square platinum sheet. The contact between the apparatus and the power supply was made by inserting the leads into the mercury filled glass rod. The bottom of each U-tube was fitted with a draining and levelling tube, these are connected by rubber tubing and a three way stop cock. The U-tubes placed at such a distance apart as to accommodate a shelf 25 x 51 cm. The shelf, which is made of 3/4" plywood, is covered by a piece of 1/4" plate glass, next by a 1/2" piece of foam rubber of the same dimension as the shelf. The rubber is in turn covered with a thin transparent sheet of polyethylene plastic of the same width, but of such a length that it extends to the horizontal slots of the electrolyte reservoirs.

The paper (Whatman #1) is 10" x 24". The paper to be treated is placed on top of the plastic and a second sheet of the same dimensions is used to cover the paper. Both ends of the paper are dipped to a

depth of 1/4" into the reservoirs. A second piece of 1/4" plate glass of the same dimensions is placed over the plastic and the whole is compressed with 50 lbs. of lead weights.

The paper was sprayed heavily with a suitable electrolyte and the paper dried. The compounds to be separated were either spotted or streaked as an 8% aqueous solution along a line 8" from one extremity of the paper. After the application had dried, the paper was sprayed a second time with the same electrolyte and immediately placed between the polyethylene sheets. The power supply is set at a potential capable of giving eight to ten milliamperes of current, this normally required from 350 to 450 D.C. volts. The time required for a singly charged ion to migrate 4" was approximately 9 to 12 hours.

The elctrolytes used in the paper electrophoresis work were 0.5 N in the reservoirs and the papers were sprayed with 0.1 N solutions of the same electrolyte. The electrolytes used were:

1) 0.5 N solution of 50% monosodium phosphate and 50% disodium phosphate.

2) A solution consisting of equal volumes of 0.5 N solutions of borax and boric acid.

3) A 1/40 N sodium bicarbonate solution.

The paper electropherograms were developed with the same spray reagents that were used for paper chromatographic work.

The Determination Of Uronic Acids By The Evolution Of Carbon Dioxide. -

When a uronic acid is heated with hydrochloric acid, carbon dioxide is evolved due to the decarboxylation of the acid. Whistler, Martin and Harris (64) have adapted the following procedure to the determination of uronic acid groups. Nitrogen which is used as a carrier gas for the evolved carbon dioxide, enters the system through an empty safety bottle. It next passes through an alkaline solution of pyrogallol, then to two absorption towers filled with soda lime, then into a second safety bottle provided with a mercury manometer and from there into the reaction flask by way of a side arm whose outlet is 10 to 15 mm. above the surface of the liquid in the flask. The flask is a three necked 125 ml. round bottomed flask. From the reaction vessel the gas passes through a 40 cm. reflux

condenser into a bubbling tower containing approximately 60 ml. of concentrated sulfuric acid. The latter serves to remove interfering decomposition products which are carried over from the reaction flask. The gas next passes through a U-tube filled with anhydrous copper sulfate to remove chlorine or hydrogen sulfide, then through a second U-tube which contains phosphorous pentoxide and finally through the carbon dioxide absorption tube which contains Ascarite. The Ascarite tube is protected by a soda lime tube which is followed by a flowmeter to estimate the rate of flow of nitrogen through the apparatus. The Ascarite tube is connected to the rest of the train by dry ground glass joints to facilitate the rapid transfer of absorption tubes. The remainder of the connections in the train are made with Tygon tubing.

The reaction flask is immersed in a constant temperature wax bath, the temperature being held constant at 138°. The flask is placed in the bath so that the oil level is three to four mm. lower than the liquid level inside the flask. This precaution is taken to prevent baking of small bits of sample which may be splashed against the sides of the flask. Nitrogen at

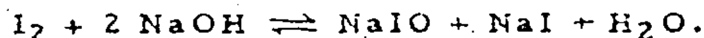
about a rate of ten liters per hour is passed through the apparatus until the Ascarite tube shows no further gain in weight.

A sample calculated to give approximately 30 to 40 mg. of carbon dioxide is dissolved in 70 ml. of 12% hydrochloric acid. The solution is then quantitatively transferred to the reaction flask and the nitrogen flow started. After one hour the weighed Ascarite tube is replaced by a second such tube and the difference in weight recorded.

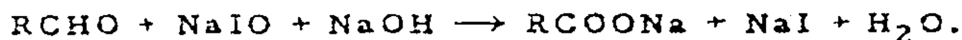
This procedure is repeated until the Ascarite tubes show no further increase in weight.

Iodometric Titration Of Aldoses. -

In the presence of iodine in alkaline solution, aldose sugars are oxidized quantitatively to their respective aldonic acids. The postulated reactions for this oxidation are



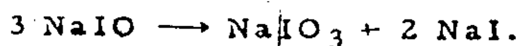
$$I_2 + 2\,NaOH \rightleftharpoons NaIO + NaI + H_2O.$$



$$RCHO + NaIO + NaOH \longrightarrow RCOONa + NaI + H_2O.$$

The difficulties which must be overcome in standardizing the analytical procedure can be summarized briefly. If all of the iodine and alkali are added simultaneously, much of the iodine is transformed to

iodate according to the following equation, and hence, lost to the oxidation reaction.



$$3\ NaIO \longrightarrow NaIO_3 + 2\ NaI.$$

If, on the other hand, an excess of alkali is added first, keto sugars, such as fructose, can undergo the Lobry de Bruyn and Alberda van Ekenstein rearrangement to glucose and mannose which are rapidly oxidized. If a large excess of iodine is added, then overoxidation can occur with primary hydroxyl groups being slowly oxidized to carboxylic acids. Kline and Acree (110) then devised the following procedure which avoids excess iodine or alkali.

A weighed sample or aliquot containing approximately 180 mg. of aldohexose is transferred to an Erlenmeyer flask and is exactly neutralized to a pale pink phenophthalein end point. Five ml. of 0.1 N iodine solution is added from a burette and then dropwise 7.5 ml. of 0.1 N sodium hydroxide. The process is repeated until 22 ml. of iodine and 35 ml. of alkali solution have been added; the operation should require five to six minutes. After a two minute interval, to allow for completion of reaction, the solution is acidified with 0.1N hydrochloric acid and the liberated iodine

is titrated with 0.1 N thiosulphate to a starch indicator end point. Two or three drops of phenolphthalein are added and the excess acid is titrated with 0.1 N sodium hydroxide. The amount of aldohexose present can be calculated from the difference in titer between the iodine and thiosulphate solutions. The aldohexose content can also be calculated from the alkali titer less the titer of hydrochloric acid added. When the reaction is confined to the oxidation of aldehyde groups alone, the ratio of alkali to iodine is 3:2.

The Colourimetric Determination Of 2-Keto Acids. -

This analytical procedure by Lanning and Cohen (90) consists of a freshly prepared 2 to 5% aqueous solution of ortho-phenylenediamine dihydrochloride, or a solution containing 15 mg. of the free diamine per ml. of 0.25 N hydrochloric acid. To two ml. of a neutral solution containing 10 to 100 γ of 2-ketohexonic acid is added one ml. of reagent. The ten ml. reaction tube is heated in a boiling water bath for 30 minutes and cooled to room temperature. The optical density is measured at 330 and 360 m.μ. The ratio of the optical density at 330 to that at 360 m.μ. should be 1.51$\pm$0.07 for reaction mixtures containing a

2-ketohexonic acid. The condensation of neutral sugars can be avoided by using no more than two equivalents of acid per mole of diamine. Lanning and Cohen found that the optical density and concentration relationship was linear over the range of 10γ to 100γ of 2-ketohexonic acid with a precision of 1.6% in triplicate determination.

The Colourimetric Determination Of 5-Keto Acids. -

The procedure has been outlined by Militzer (94). A modified Benedict's solution is prepared as follows: copper sulfate pentahydrate (18 g.), anhydrous sodium carbonate (15 g.), potassium citrate (200 g.), potassium thiocyanate (125 g.), potassium ferrocyanide (5 ml. of a 5% solution) are dissolved in water and brought up to one liter volume. The solution is left to stand at room temperature for two days and filtered free of the precipitate.

A standard solution of 5-ketohexonic acid is made by weighing a known amount of calcium 5-ketohexonate and precipitating the calcium by the addition of oxalic acid. The calcium oxalate is filtered and the filtrate is made to volume. The ketohexonic acid solution should contain a minimum of 50 mg. of

acid per ml. of solution.

The colourimetric estimation is done in comparison tubes which are painted white on the bottom up to a height of two inches. All the solutions are brought to 25° in a constant temperature bath before use and are held at that temperature throughout the determination. Five ml. of the solution are placed in the comparison tube and five ml. of the standard or unknown solution is pipetted in and shaken to provide uniform agitation. The time necessary for the complete disappearance of the blue colour is determined with a stop watch in which the starting time is taken as the first moment during which pipetting of the solution into the reagent begins. The time for pipetting should be 15 to 20 seconds. The calculations are based on the time required for the disappearance of the blue colour in the case of a standard solution. D-Fructose in higher concentration than 50 mg. per ml. causes a 10% error by hastening the time of reduction while 2-ketoaldonic acids do not interfere.

The Gravimetric Estimation Of Ketoaldonic Acids. -

The 2-ketohexonic acids were determined by their estimation as oxalic acid. Approximately

0.8 mM. of the 2-ketoaldonic acid was dissolved in 20 ml. of solution and 11 mM. of sodium periodate added. The flask was stoppered with a ground glass stopper and the solution was shaken until all the solids dissolved. The solution was left for 24 hours at room temperature. The excess periodate was destroyed by the addition of ethylene glycol (approximately 7.8 mM.). After ten minutes, to ensure complete oxidation of the ethylene glycol, a calculated amount of calcium chloride was added to precipitate the insoluble calcium oxalate.

After three to four hours at room temperature, the calcium oxalate was filtered through a dried and weighed Gooch crucible. During the course of the oxidation, iodine was liberated and, on standing for three to four hours, crystallized. The iodine was quantitatively washed from the Gooch with chloroform. The crucible was dried to a constant weight at 105°.

The oxalic acid content in the crucible was checked by placing the crucible in a wide mouthed Erlenmeyer and adding 50 ml. of water and 20 ml. of concentrated sulfuric acid. The solution was heated

at 95° for five minutes. While the solution was still above 60°, it was titrated with a standard solution of potassium permanganate. The number of mM. of 2-ketohexonic acid in the sample is equivalent to the mM. of oxalic acid found in the precipitate.

Samples of D-fructose and 5-keto-D-gluconic acid were treated with sodium periodate and it was found that under the conditions of the method no interfering oxidation products were obtained. Oxidations of 2-keto-D-gluconic acid gave 97.3% of the theoretical oxalic acid.

3. Experimental Results

a. The Oxidation Of Sucrose By Platinum Catalyst And Oxygen Using Sodium Bicarbonate Buffer.

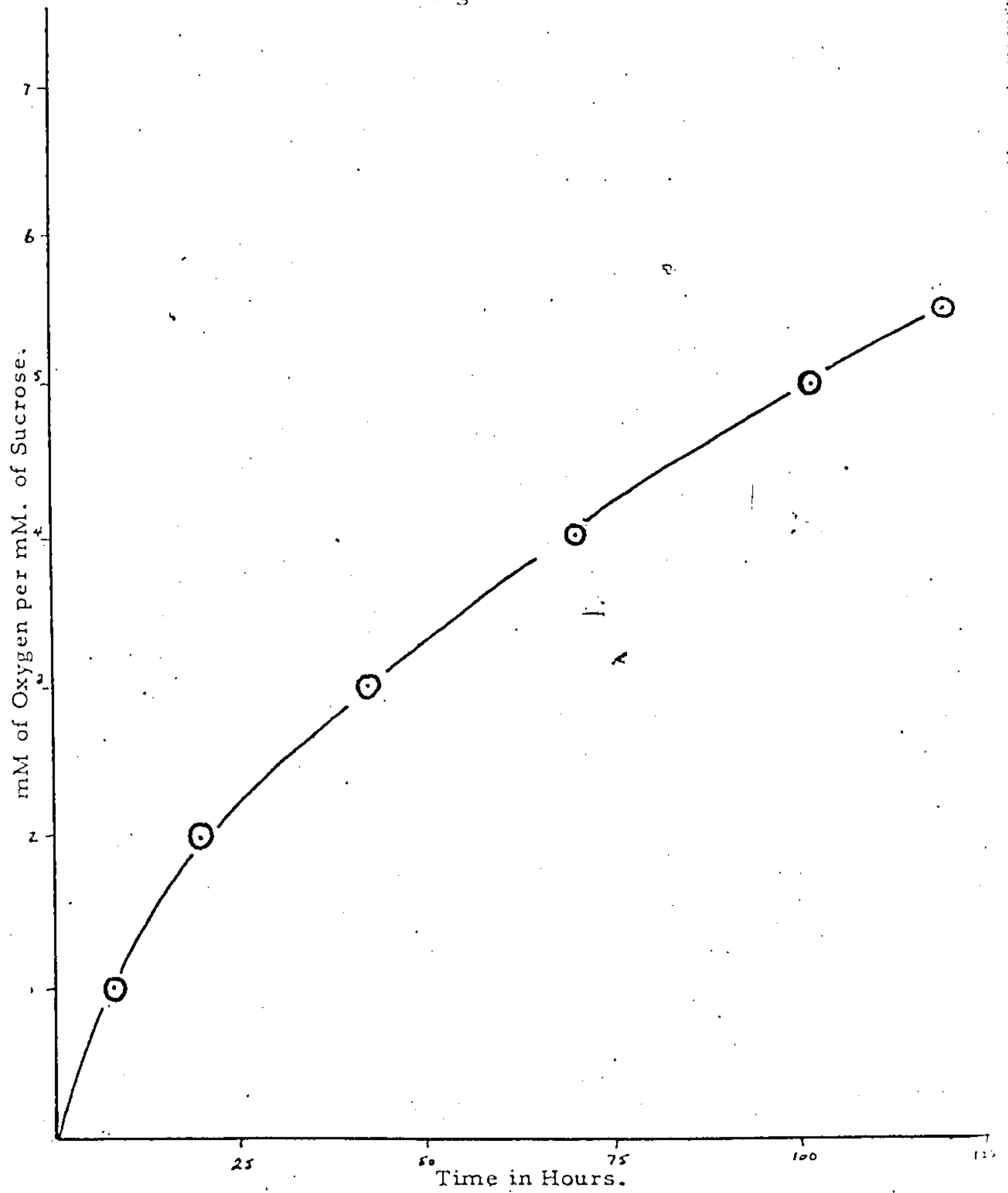
The initial studies on the oxidation of sucrose were carried out using 1.7 mM. of sucrose, 5.1 mM. of sodium bicarbonate (3 molar excess) and 40 mg. of platinum catalyst. The temperature was held constant between 78° and 80°. The oxidation was carried out in an atmosphere of pure oxygen with 5 cm

of mercury pressure. The uptake of oxygen is plotted against time in figure 1. The oxidation was continued until 7 mM. of oxygen per mM. of sucrose were consumed. Additional sodium bicarbonate was added in 5.1 mM. quantities as required in order to maintain neutral, or slightly alkaline, conditions. Titration of the alkali in the trap indicated that one carboxyl group was formed for every molecule of oxygen reduced.

Paper chromatography, using the solvent system butanol:ethanol:water, of the oxidation products showed the formation of a compound, or compounds, with an R_f of zero. When the oxidation was extended beyond 3 mM. of oxygen per mM. of sucrose, paper chromatography still indicated unreacted sucrose was present when the paper chromatogram was sprayed with the spray reagent permanganate periodate.

Sucrose was then oxidized on a preparative scale using a Parr pressure reaction apparatus. Ten grams (0.03 moles) of sucrose and 20.16 g. (0.12 moles) of sodium bicarbonate with 0.6 g. of platinized charcoal catalyst were shaken with 100 ml. of distilled water at 70° for 40 hours under fifty pounds

Figure 1.



pressure of oxygen. The solution was filtered free of catalyst and evaporated to 1/3 volume at 40°. Paper chromatography, using butanol:ethanol:water and subsequent spraying with permanganate-periodate, once again showed the presence of unreacted sucrose. On cooling the reaction solution to 2°, a white crystalline optically inactive salt crystallized. The salt was filtered and passed over Dowex 50 x 12 ion exchange resin to remove sodium ions and a pale blue eluate resulted. On cooling the solution after concentrating it to 20 ml., a crystalline acid separated with a melting point of 189°. A neutralization equivalent, mixed melting point and the dimethyl ester (m.p. 44-45°) identified the acid as oxalic acid; 0.05 moles of oxalic acid were isolated from the oxidation of 0.06 moles of sucrose. Paper chromatography of the acid solution using butanol:water and spraying with the naphthoresorcinol spray reagent showed spots at R_f 0.088 (red), and at R_f 0.202, 0.368 and 0.60, blue spots indicating uronic or keto uronic acids were developed. A second paper chromatogram sprayed with the permanganate-periodate reagent showed a minimum of seven compounds with R_f values 0.021, 0.119, 0.25,

0.378, 0.54, 0.665 and 0.705.

The Separation Of Products From Starting Materials.

A sucrose oxidation using 0.5 g. of sucrose (1.4 mM.) with 0.365 g. of sodium bicarbonate (4.35 mM.) and 40 mg. of platinum catalyst was run until the uptake of oxygen corresponded to two mM. of oxygen per mM. of sucrose. The product was filtered free of catalyst and loaded on a 40 g. column of 50% Darco G-60 and 50% Celite. The column was eluted at two to three ml. per minute, using 5% aqueous ethanol under 24 mm. pressure of mercury. The eluate was collected in ten ml. fractions, the total volume being 680 ml. of eluant. The sodium bicarbonate was detected by a sodium flame test and by the evolution of carbon dioxide upon the addition of one drop of acid in the eluate. The carbohydrate acids were detected by the sodium flame test and by optical rotation. The unreacted sucrose was detected by optical rotation measurements and by the anthrone test (100). The results are seen in table I. Paper chromatography, using butanol:water, of the fractions collected indicated a complete separation

between the carbohydrate acids and the unreacted sucrose. The sucrose portion, when evaporated to dryness, crystallized and gave $[\alpha]_D^{23} = 60^\circ$ (c, 5 in water).

Table I

The Separation Of Oxidation Products From Starting Material

Volume in ml.	Sodium Flame Test	Anthrone Test	Compound
0 - 90	negative	negative	
90 - 110	positive	negative	sodium bicarbonate
110 - 120	negative	negative	
120 - 220	positive	positive	reducing acids
220 - 245	negative	negative	
245 - 550	negative	positive	sucrose

The weight of sucrose recovered was (0.20 g) 40% of the starting material. The acids, 0.30 g., were then passed through ten g. of cation exchange resin Dowex 50 x12. The acid solution was paper chromatographed using the three solvent systems #1, #3 and #4.

In each case, a minimum of four major components could be detected. Using the system butanol:water, the R_f of the compounds were 0.014, 0.062, 0.97, 0.25. The compounds were detected using the spray reagent permanganate - periodate.

A portion of the acid solution containing 597 mg. of the amorphous acids was thoroughly dried and decolourized with charcoal and titrated with standard alkali. The neutralization equivalent was 288 g. The salts were passed through Dowex 50 x12 and freed of sodium. The acid solution was concentrated to a total volume of five ml. and placed on a 20 g. Celite column and eluted with butanol:water at a rate of one ml. per minute. The eluate was collected in two ml. fractions, the total volume of eluate was 1000 ml. The acid in the various tubes was titrated with standard hydrochloric acid to a brom thymol blue end point. The eluate contained 22% of the acids in the original sample. The column was then extruded, using air pressure, and sprayed through a mask with 2% potassium permanganate in 2 N sodium hydroxide. Three bands were observed on

the 30 cm. column. They were found between three to seven cms., 9.8 to 12.2 cms. and 14.2 to 17 cms. from the top of the column. The bands were cut out and sufficient distilled water was added to form a loose slurry and the Celite was filtered on a Buchner funnel. The filtrate and washings were combined and concentrated to a syrup to remove the butanol. Each fraction was taken up in five ml. of water and paper chromatographed with butanol:water mixture and sprayed with both permanganate - periodate and aniline hydrogen phthalate reagents. The R_f values were 0.00, 0.07, 0.10.

Titration of the three fractions with standard alkali gave 0.259 m.e. in the slowest, 0.043 m.e. in the next band and 0.043 m.e. in the last. The total recovery of acid corresponded to 40% of the acid placed on the column. Fraction one, both as such and when dissolved in 1:1 hydrochloric acid, was chromatographed on paper using butanol:water. The papers were sprayed with aniline phosphate and permanganate-periodate. In the acid treated solution, a reducing compound with an R_f identical to D-glucose was observed. A second paper sprayed with urea oxalate

failed to develop any spot corresponding to D-fructose. The specific rotation for the fraction was $[\alpha]_D^{24} = +32.4^\circ$ (c, 0.73 in water) and, when hydrolyzed with hydrochloric acid, $[\alpha]_D^{24} = +25.5^\circ$(c, 0.69 in water). The yield of acid isolated in this fraction is 32% of the acids recovered from the Celite column. The hydrolysis mixture was concentrated to two ml. of solution, and the complete mixture placed on a 25 g. Celite column and eluted with 300 ml. of butanol:water. The eluate was collected in 18 ml. fractions. The column was extruded and sprayed through a mask with 2% potassium permanganate in 2 N alkali. Only one band was found and it was cut out, washed with distilled water and filtered free of Celite. The weight of compound isolated was less than 1 mg. of a dark syrup. Tubes 10 and 11 contained a reducing compound which, when concentrated to a syrup and left in the cold for several days, crystallized giving an R_G value of 1.0 when paper chromatographed with butanol: water. The rotation of the compound was $[\alpha]_D^{24} = 51.7^\circ$ (c, 1.08 in water). The weight of compound isolated was 8.5 mg. A mixed melting point with authentic

D-glucose caused no depression.

b. The Oxidation Of Sucrose Using Platinum Catalyst, Oxygen and Ion Exchange Resin. -

Blank oxidations containing catalyst, resin and water were run for four days at 78° and no detectable uptake of oxygen could be observed. Sucrose, 5 g., and 14.5 g. of Dowex 1 x10 (200 - 400 mesh) anion exchange resin, was oxidized at 78° using 400 mg. of platinum catalyst and an oxygen pressure of 5 cms. of mercury. The oxidation was carried out for 120 hours, during which time the average uptake of oxygen was 2.7 ml. per hour. The total uptake was 333 ml. of oxygen or slightly less than equimolar quantities based on sucrose. The reaction flask was cooled and filtered free of catalyst and resin. The resin was washed with 18 ml. of distilled water and the filtrate and washings were concentrated to a syrup on a water bath. Paper chromatography using butanol: ethanol:water gave a single spot for sucrose when the paper was sprayed with urea oxalate spray reagent. The sucrose crystallized and 3.08 g. were obtained $[\alpha]_D^{24} = +62°$ (c, 2.3 in water).

The ion exchange resin and catalyst were

slurried with water and eluted with 3 N ammonium hydroxide. After 170 ml. of eluate, the column was freed of carbohydrate material. The salts were located by using the anthrone test for reducing sugars. The weight of salts collected was 0.98 g. and their $[\alpha]^{25}_{D} = +31.4^{o}$(c,1 in water). The ammonium salts were slurried with the cation exchange resin Dowex 50 x12 and the rotation of the solution gradually became less dextrorotatory until the equilibrium rotation of $[\alpha]^{24}_{D} = +12.6^{o}$ (c, 0.92 in water) was reached. The hydrolysed acids were chromatographed on paper using butanol:water and the presence of both D-glucose and D-fructose was determined by R_f values and spraying with the spray reagents aniline phosphate and urea oxalate.

The neutral compounds were removed from the solution by passing the mixture over an anion exchange resin Dowex 1 x10 and eluting the column with distilled water until the eluate gave a negative anthrone test. Paper chromatography showed two compounds which were D-glucose and D-fructose. The acids were removed from the resin by eluting the column with 1 N hydrochloric acid. This step caused

considerable darkening of the product. The yield of acids was 0.7 g. of the 1.98 g. from the original ion exchange column. Attempts to remove the hydrochloric acid from the carbohydrate acids by freeze-drying were unsuccessful and extensive decomposition occurred.

Difficulties were experienced with the anion exchange resin Dowex 1 x10 when similar oxidations were tried using the Parr pressure reaction apparatus. Oxidations of sucrose under 50 pounds per square inch pressure of oxygen at 78° gave a variety of acid products with R_f values in butanol:water ranging from 0.0 to 0.85. Elution of the resin with 3 N and 6 N ammonium hydroxide did not completely remove the acids from the ion exchange column. The resin could only be freed of acid products by eluting with 2 N sodium hydroxide.

A second resin Amberlite IR-45 (coarse mesh), which is a weak tertiary ammonium hydroxide type resin, was used instead of the stronger Dowex 1 x10 which is a quarternary ammonium hydroxide exchange resin. In the oxidation, 0.1 moles of sucrose,

0.2 moles of Amberlite IR-45 (74 dry g.) and 200 mg. of catalyst were taken up in 225 ml. of distilled water and the whole shaken at 70° for 72 hours under an oxygen pressure of 50 pounds per square inch. The oxidation was stopped, the solution was cooled and slurried into a chromatographic column and washed with two to three liters of distilled water. The water elution was discontinued when a negative anthrone test was obtained (100). The resin was then eluted with 3 N ammonium hydroxide until a negative anthrone test was obtained. Approximately 2.5 to 3 liters of ammonium hydroxide were required to completely remove the acids from the resin. Further elution of the resin with 2 N sodium hydroxide did not remove any material from the column. Evaporation of the ammonium hydroxide solution yielded approximately two g. of the acid salts as an amorphous solid. The clear colourless glass, when dried overnight in a pistol at 36°, had a specific rotation $[\alpha]_D^{23} = +38.4°$ (c, 12.0 in water). The glass when treated with hydrochloric acid undergoes hydrolysis yielding a glass with $[\alpha]_D^{24} = +12.6°$ (c, 1 in water). Paper chromatography using butanol: ethanol:water showed that both D-glucose and D-fructose

were present in the hydrolyzate. Continuous paper chromatography of the ammonium salts for 11 days with butanol:ethanol:water failed to separate the acids.

Separation of the sucrose acids was then attempted by paper electrophoresis. The electrolyte was a monosodium - disodium phosphate buffer of pH.6.8. The salts were applied as one application of 8% solution. Optimum conditions for achieving the separation of the compounds D-gluconic acid and D-glucuronic acid were 250 volts for nine hours at room temperature. Under these conditions, however, it was impossible to achieve a satisfactory separation between D-glucuronic acid and 5-keto-D-gluconic acid. The buffer system consisting of 50% borax and 50% boric acid was investigated, varying the voltages from 250 volts to 500 volts and the time from 9 to 16 hours. The latter buffer system increased the mobility of D-glucose and D-fructose, however, the relative rates of migration of carbohydrate acids was not affected.

The Neutral Equivalents Of The Sucrose Acids. -

A five g. sample of the ammonium salts was dissolved in ten ml. of water and decolourized with

Norite-A charcoal. The colourless solution was freeze-dried overnight and a sample, weighing 379.4 mg., was dissolved in 50 ml. of water and quantitatively transferred into a flask containing 125 ml. of 50% sodium hydroxide. The ammonia was distilled by the Kjeldahl method into a trap containing standard hydrochloric acid. The calculated milliequivalents of ammonia were 1.015, and 1.064 milliequivalents were found. The method was standardized against crystalline ammonium D-gluconate and 1% accuracy was obtained in triplicate determination.

The Inversion Of Sucrose Acids By Enzymes and Acid. -

The enzyme invertase, a β-D-fructofuranosidase was used for the inversion of the sucrose acids and the activity of the commercial preparation obtained from Pfanstiehl Laboratories was determined according to the method of the Association of Official Agricultural Chemists (111). Ten g. of sucrose were transferred to a flask calibrated at 100 and 110 ml. and 75 ml. of water were added to dissolve the sugar. Two drops of glacial acetic acid were added and the whole was diluted to 100 ml. of solution. The invertase

solution was prepared by diluting one ml. of enzyme preparation to 200 ml. To the 100 ml. sugar solution was added 10 ml. of the dilute enzyme solution and the solution was thoroughly and rapidly mixed. At the termination of exactly 60 minutes, a portion of the solution was made distinctly alkaline to litmus paper with sodium carbonate and the solution was polarized at 20°. When the enzyme preparation is sufficiently active, the alkaline solution polatizes +31° specific, without applying correction for the dilution of the sugar solution. In this work, the activity of the enzyme preparation was somewhat low as the 60 minute reading was +47.7°.

A standard solution of ammonium sucronates, free from sucrose, containing 12 g. per 100 ml. was prepared. The rotation was $[\alpha]^{20}_{D} = +38.6°$ (c, 12 in water). A 9.53% solution of ammonium sucronate was prepared by adding to [illegible] ml. of 12% solution 1 ml. of diluted enzyme preparation and 0.5 ml. of glacial acetic acid in order to give a solution with a pH of 5.8. This is the pH range in which invertase has the optimum activity (112). The rate of hydrolysis

was followed polarimetrically and the results given in table II.

Table II

The Hydrolysis Of Sucronic Acid By Invertase Enzyme

Time in Hours	Observed
0	7.70
5	6.01
10	4.85
15	4.10
20	3.60
25	3.35
30	3.20
35	3.02
40	3.01
60	3.01

The contribution of the enzyme invertase to the optical rotation can be ignored as blank solutions containing invertase only had no measurable optical activity in the concentrations used for hydrolysis. The final rotation was $[\alpha]_D^{25} = +15.4^\circ$ (c, 9.5 in water). Once the

equilibrium rotation was reached, the hydrolyzate was placed on paper and the chromatogram developed with butanol:water. Both D-glucose and D-fructose were detected by spraying with aniline phosphate and urea oxalate spray reagents. A third paper chromatogram was sprayed lightly with distilled water and held in hydrogen chloride fumes. The chromatogram was then sprayed with naphthoresorcinol reagent and dried at 105°, then treated with steam for five minutes. The low R_f salts of the acids appeared as blue spots on a pink background.

Five ml. of a 12% aqueous ammonium sucronate solution was treated with hydrochloric acid until the solution was pH 2.3. The solution was left at room temperature and the equilibrium rotation was $[\alpha]^{23}_{D}$ = +12.6° (c, 10.5 in water). The complete hydrolysis of the glycosidic linkage by the enzyme was checked by adding to the equilibrated solution of enzyme and hydrolyzed sucronic acid, sufficient o.1 N hydrochloric acid to bring the solution to pH 2.0. No observable changes in rotation occurred during 18 hours at room temperature.

To a solution of five ml. of 12% ammonium sucronate was added 0.5 g. of α-amylase enzyme and the solution was left for five days at room temperature. No observable change in rotation could be detected. Paper chromatograms of the α-amylase treated solution were developed with butanol:water and sprayed with permanganate - periodate reagent. No detectable quantities of D-glucose or D-fructose were found.

A solution containing 2.04 g. of anhydrous ammonium sucronate was inverted with invertase and once the equilibrium rotation was reached the solution was passed over a 20 g. column of Amberlite IR-45 anion exchange resin. The column was eluted with distilled water until the pH had dropped from 9 to that of distilled water, and, also, when a negative anthrone test for reducing sugars was obtained (100). The eluate was concentrated in vacuum to a glass. The solid was dried over phosphorous pentoxide for two days and then weighed. From 2.04 g. of sucrose acids, 1.066 g. of neutral sugars were obtained. The syrup was paper chromatographed using butanol:water

for two days and the chromatogram sprayed with aniline phosphate and permanganate - periodate reagent. Two spots corresponding to D-glucose and D-fructose were obtained.

Analysis Of Neutral Components. -

The neutral syrup, which was obtained from the enzymatic hydrolysis of sucronic acid, polarized $[\alpha]^{22}_{D} = +1.9^{o}$ (c, 1.04 in water). The composition of the mixture of D-glucose ($[\alpha]^{20}_{D} = +52.7^{o}$) and D-fructose ($[\alpha]^{20}_{D} = -92.4^{o}$) could be calculated since the specific rotations of the pure components are known. On this basis, the syrup contained 35% D-fructose and 65% D-glucose.

The syrup was next analyzed for D-glucose by the iodometric titration method. The method was standardized against known D-glucose, as well as mixtures of D-glucose and D-fructose, until the method was accurate to within two per cent. The number of milliequivalents of D-glucose found in a 480.1 mg. sample of the syrup was 3.21, which corresponds to 288.9 mg. of D-glucose. The content of D-glucose was therefore 67% of the total sample.

Analysis Of The Acid Moieties Of Sucronic Acid.

The total uronic acid and ketoaldonic acid content per sucronic acid molecule was determined by the decarboxylation method which was first standardized against D-glucuronolactone, methyl 2-keto-D-gluconate and calcium 5-keto-D-gluconate. The yields of carbon dioxide were 98.5% and 95.0% for the glucuronolactone, 101.6%, 100.8% for the methyl 2-keto-D-gluconic acid, and 80.0%, 82.0% for the calcium 5-keto-D-gluconate.

The low analyses of the calcium 5-keto-D-gluconate were checked by determining the calcium conte by precipitation as calcium oxalate. From this analysis it was found that the calcium content was 114% based on the theoretical amount from pure calcium 5-keto-D-gluconate.

The ammonium sucronate was prepared for the analysis by decolourizing with Darco G-60 and evaporating the solution in vacuo over steam until the glass foamed. The glass was then powdered and dried in vacuo at room temperature for 14 hours. The yields of carbon dioxide in duplicate determinations were 80.0% and 79.8% of theory for pure

ammonium sucronate.

Two grams of anhydrous ammonium sucronate were dissolved in a minimum of hot 18% aqueous ethanol and the solution left to cool slowly to room temperature. The solution was then kept at 2° for two days. The solution deposited an amorphous solid which was separated from the mother liquor. The aqueous ethanol solution was concentrated to a glass in vacuo and the glass dried at 60° in a vacuum pistol for 12 hours. A 319.4 mg. sample liberated 9.3 mg. of carbon dioxide, while the theorétical value, assuming the compound to be ammonium sucronate, was 37.6 mg.

The amorphous solid which was precipitated from the aqueous ethanol solution was dried in vacuo at 60° for 12 hours and a 299.8 mg. sample was decarboxylated. The compound yielded 34.44 mg. or 97.4% of the theoretical weight of carbon dioxide for ammonium sucronate.

A solution containing 1.3 g. of the ammonium sucronate was hydrolyzed with invertase enzyme and the salts freed of neutral sugars by passing

the hydrolyzate over a 15 g. column of anion exchange resin Amberlite IR-45. The acids were removed from the ion exchange column by eluting the resin with 80% acetic acid. Four liters of effluant were required to free the column of reducing acids. The eluate was concentrated to a thick syrup to remove the acetic acid, and the syrup was taken up in 25 ml. of distilled water and freeze-dried for 20 hours. Decarboxylation of a sample of the white powdery acids gave an 89.5% yield of carbon dioxide based on the assumption that the material was only hexuronic acid.

Ammonium sucronate was analyzed for its 2-ketoacid content by the periodate oxidation procedure. A solution containing 1 mM (373.8 mg.) of ammonium sucronate was dissolved in 25 ml. of distilled water and 3 ml. of diluted enzyme preparation added. The solution was left at 25° until the equilibrium rotation was reached. Twenty ml. of the solution were pipetted into a ground glass bottle and 2.5 gm. of sodium periodate were added. The weight of calcium salts precipitated by the additon of calcium chloride was 7.4 mg. Contamination of the calcium

oxalate by calcium iodate was corrected for by titrating the precipitate with acidic permanganate. The quantity of oxalic acid, 3.95 mg., formed in the oxydation indicated that the substance possessed 38.5% 2-keto-D-gluconic acid.

A second analysis for 2-keto-D-gluconic acid was attempted using the method of Lanning and Cohen (90). Standard samples of pure 2-keto-D-gluconic acid, D-glucuronic acid and calcium 5-keto-D-gluconate were prepared, as well as standard mixtures of the above compounds. When the method was applied to the mixtures, it was found that D-glucuronic acid interfered with the peak for the 2-ketoaldonic acids at 360 m.μ. However, the method did indicate the presence of 2-ketoaldonic acids in the ammonium sucronate, since the characteristic peak was registered at 360 m.μ.

c. The Preparation Of Sucronic Acid Derivatives. - Sodium Borohydride Reductions. -

Ammonium sucronate, 3.5 g., was inverted with invertase at pH 5.6. When the equilibrium was reached, the solution (50 ml.) was treated with excess

sodium borohydride and tested with anthrone reagent (100) for completeness of reaction. The reduction was continued for 76 hours at room temperature. The solution still gave a positive anthrone test indicating the presence of reducing compounds. Further reduction was carried on for 24 hours, however, the product remained partially reducing. Paper chromatography of the product using butanol:water and subsequent spraying with permanganate - periodate did not indicate compounds with an R_f which would be separable from the remaining uronic type acids.

Isolation Of D-Glucuronolactone From An Hydrolyzate Of Ammonium Surcronate. -

Three hundred mg. of freeze-dried ammonium sucronate was dissolved in 18 ml. of acetic acid and 12 ml. of distilled water. The solution was left at 45° until the equilibrium rotation of the solution was reached. After six days, the solution was passed over a 40 g. column of anion exchange resin Amberlite IR-45. The neutral hydrolyzate products were removed from the column with distilled water. Paper chromatography using butanol:water gave two spots corresponding to D-glucose and D-fructose when the paper was

sprayed with aniline phosphate. The acid components were removed from the column with 2.1 liters of 3.1 N acetic acid. The solution was concentrated at 30° in vacuo to a total volume of 40 ml; the solution was then frozen and the solvent removed by freeze - drying overnight.

The acids (130 mg.) were dissolved in 3 ml. of distilled water and heated to 60°. Three ml. of hot dioxane were added and the solution was concentrated to a glass. An additional 6 ml. of dioxane were added and distilled. This was repeated four times and the resulting syrup was taken up in the minimum amount of hot 80% ethanol. The solution was decolourized with charcoal and filtered. The solution was seeded with authentic D-glucuronolactone and, after three days at 5°, the compound partially crystallized. The solution was filtered and washed with 3 ml. of ice cold 90% ethanol and the product dried in a pistol at 60° for two hours. The compound melted at 160° - 164.5°, while authentic D-glucuronolactone melted at 164° - 165°. A mixed melting point caused no depression of the melting point. The weight of

crystalline D-glucuronolactone obtained was 18 mg. or 13.8% of the total weight of acid.

The crystalline product obtained was paper chromatographed using acetic acid:ethanol:water and compared to authentic D-glucuronolactone. Both compounds gave a positive hydroxamic acid test and had identical R_f values.

The lactone was further characterized by forming the diethyl dithioacetal derivative according to the method of Wolfrom and Onodera (113). The lactone, 18 mg., was suspended in 2 ml. of ice cold concentrated hydrochloric acid and 3 ml. of ethanethiol added. The suspension was shaken continuously for 45 minutes at 0°. The reaction mixture was poured in 30 ml. of ice water and extracted three times with 20 ml. of chilled ethyl acetate. The ethyl acetate extractions were combined and dried over anhydrous sodium sulfate. The solution was concentrated at 30° in vacuum and 21.1 mg. of a yellow syrup were obtained. This represents a 72% yield. The diethyl dithioacetal was then thoroughly dried over phosphorous pentoxide for 13 hours and then dissolved in 40 ml. of

anhydrous pyridine with 10 ml. of acetic anhydride. The solution was left at 2° overnight. The reaction solution was concentrated to a thick glass at 70° in vacuum and 20 ml. of chloroform added to the syrup. The syrup was then decolourized by passing it over a 10 g. column of Magnasol containing 25% Celite. The column was washed with an additional 15 ml. of chloroform. The chloroform solution was taken down to dryness and the syrup dissolved in a minimum of hot ethanol. The compound crystallized in 62% overall yield, and was identical to authentic 2,4,5-tri-O-acetyl D-glucuronolactone diethyl dithioacetal, melting point 110.5°, $[\alpha]_D^{23} = +56.3°$ (c, 1.07 in ethanol).

d. The Oxidation Of Protected Monosaccharides By Oxygen and Platinum Catalyst. -

The Oxidation Of Methyl α-D-Glucopyranoside. -

Ten g. of methyl α-D-glucopyranoside were dissolved in 50 ml. of distilled water containing 25 gms. of Amberlite IR-45 anion exchange resin. One g. of platinized - charcoal catalyst was added to the slurry and the whole was shaken at 68° to 70° under 50 pounds pressure of oxygen for 72 hours.

oxidation, the methyl α-D-glucopyranoside was washed from the resin with distilled water until a negative anthrone test was obtained. The acid, a methyl α-D-glucuronide, was removed from the resin with 6 N ammonium hydroxide until a negative anthrone test was obtained. The weight of ammonium salt was 1.6 g. Paper chromatography with butanol: water showed complete separation between the reactant and the product.

The syrup was analyzed for purity by the evolution of carbon dioxide as previously described. A solution of 12% hydrochloric acid containing 92.453 mg. of the salt, liberated 15.4 mg. of carbon dioxide, which corresponded to 86.6% of theoretical.

The product, 1.2 g., was dissolved in 100 ml. of 2 N hydrochloric acid, heated at 65° for two hours and left at room temperature overnight. The syrup was evaporated to dryness at 30° and 50 ml. of dioxane were added and removed by distillation. After four such additions, the syrup was freed of solvent and pumped to dryness under a vacuum for six hours. The material was then dissolved in 10 ml. of hot ethanol

and decolourized with Darco G-60. Paper chromatography using butanol:water indicated the presence of D-glucuronolactone in high concentration when the paper was sprayed with analine phosphate reagent.

A 103.1 mg. sample of the syrupy material was converted to crystalline 2,4,5-tri-O-acetyl D-glucuronolactone diethyl dithioacetal by the method of Wolfrom (113). After filtering and drying the crystalline material, the weight of product obtained was 86 mg., which corresponded to a 12% yield calculated from the ammonium salt of the methyl α-D-glucuronide. The compound melted at 109.5^{o} after one recrystallization from hot ethanol. Mixed melting point between the authentic 2,4,5-tri-O-acetyl D-glucuronolactone diethyl dithioacetal and the compound gave no depression. The rotation of both was $[\alpha]_{D}^{23} = +56.5^{o}$ (c, 2.54 in ethanol).

The Oxidation Of 1,2;3,4-Di-O-isopropylidene-D-Galactose.

A solution containing 25.5 g. of 1,2;3,4-di-O-isopropylidene-D-galactose in 175 ml. of 5% dioxane in water was slurried with 74 g. of Amberlite

IR-45. Two grams of catalyst were added to the suspension and the whole was oxidized at 70° for 96 hours. The resin mixture was eluted with distilled water until a negative anthrone test for reducing sugars was obtained (100). The 1,2;3,4 -di-O-isopropylidene D-galacturonic acid was removed from the resin as the ammonium salt by elution with 6 N ammonium hydroxide until a negative anthrone test was obtained. The ammonium 1,2;3,4 -di-O-isopropylidene-D-galacturonate crystallized from the hot ammonia solution when the ammonium hydroxide was removed by evaporation at 40° in vacuum. The weight of compound isolated was 2.6 g. Carbon dioxide analysis in 12% hydrochloric acid gave 99.1% of the theoretical quantity of carbon dioxide. The compound melts at 244° with sublimation at 205°, which is in agreement with the melting point reported in the literature (114).

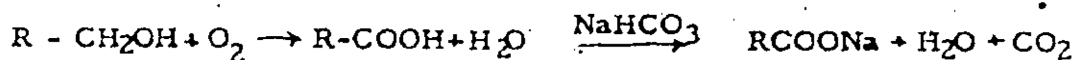
III. CONCLUSIONS

The problem of oxidizing sucrose and obtaining an acid with the glycosidic linkage intact involves finding reaction conditions which are sufficiently mild that the labile linkage between D-glucose and D-fructose may survive the treatment. Any oxidation conditions which require acid media are automatically ruled out. Of the methods in the literature which are applicable, the method chosen seemed to be the most promising since it fulfilled the necessary conditions of moderate temperatures and non acidic media.

The initial work using the method of Mehltretter (33) was investigated and found to be unsatisfactory for the following reasons. When sucrose was oxidized by oxygen with a platinum catalyst, the products of the initial oxidation were present in the reaction mixture and appeared to be oxidized more rapidly than the sucrose. Paper chromatography

showed that reoxidation was occurring since a minimum of eight compounds were detected while 40% of the original sucrose remained.

In order to be able to better study the reaction, the system was modified to include an alkali trap in order to remove carbon dioxide evolved from the following reaction:-



$$R-CH_2OH + O_2 \rightarrow R-COOH + H_2O \xrightarrow{NaHCO_3} RCOONa + H_2O + CO_2$$

Previously, only small pressure changes could be observed because one mole of oxygen consumed liberated an equal amount of carbon dioxide. By removing the carbon dioxide, volume changes occurred, and the uptake of oxygen could be followed. Figure I shows a continuous curve for the ratio of sucrose and oxygen consumed up to 7 mM of oxygen per mM of sucrose. From the nature of the curve, there is no decrease in the rate of oxygen uptake, hence, the first products of the oxidation are susceptible to oxidation. If the oxidation were as preferential for primary hydroxyl groups over secondary ones, as Mehltretter suggests, then the rate of oxidation should have shown some decrease after an oxygen uptake of 3 mM of

oxygen per mM of sucrose. The isolation of oxalic acid as one of the products of oxidation means that oxidation occurred at a secondary position with the subsequent cleavage of the carbon - carbon bond.

The use of 50% Darco G-60 and 50% Celite as adsorption columns has proven useful in isolating the products of oxidation from the reactants, sucrose and sodium bicarbonate. This enabled the isolation of the mixed products of oxidation and also permitted the isolation of unreacted sucrose in sufficiently pure state to establish directly the extent of oxidation. In oxidations where two and three mM of oxygen were consumed per mM of sucrose, the percentage of unreacted sucrose was 40% and 33% respectively. From these values, the sucrose which did undergo oxidation was oxidized fo the extent of three to four mM of oxygen per mM of sucrose. These data show that the initial products were susceptible to further oxidation.

From the above, it was apparent that it would be impossible to oxidize sucrose under these conditions and obtain a sucrose carboxylic acid in appreciable yields. However, chromatographic work

showed that the system did not attack the glycosidic linkage completely since acids were isolated which, on hydrolysis, liberated reducing sugars.

Oxidations Using Platinum Catalyst, Oxygen And Ion Exchange Resin.

The problem of reoxidation was solved by introducing weakly basic tertiary ammonium anion exchange resin instead of the usual bicarbonate buffer. Using the bicarbonate buffer, the products are present in the reaction solution as the soluble sodium salts and hence they are available for further oxidation. However, by substituting the ion exchange resin for the bicarbonate buffer, the acids formed are neutralized by the basic ion exchange resin and in this manner the carbohydrate acid is held to the resin. Since the oxidation reaction proceeds by heterogeneous catalysis, the insoluble salt can no longer come into contact with the catalyst because a three phase system is formed where two of the phases cannot come into proper contact. Hence, in this manner, it is possible to protect the initial oxidation products from being oxidized a second time.

The weakly basic resin also serves as a

means of maintaining neutrality since the acids formed are neutralized, thus preventing a drop in pH and consequently the hydrolysis of the glycoside bond is prevented. The resin itself is not oxidized by the reaction mixture since blank determinations using resin and catalyst alone did not consume any oxygen. The resin must also be weakly basic since the ion exchange resin cannot be eluted with acid since this would hydrolyze the glycosidic linkage of the sucronic acid. The resin must be eluted with base and, if sodium or potassium hydroxide were used, a separation problem involving the acid salts and the base would be involved. Ammonium hydroxide has the advantage of being volatile and hence may be easily removed from the non-volatile products of the oxidation. Since both the resin and the base used for elution are weak bases, the anions are readily removed from the resin.

A third advantage of using the ion exchange resin is the ease with which the oxidation products are freed of any unreacted sucrose. Whereas the sodium bicarbonate method required adsorption columns and tedious elutions, this method simply

involved washing the resin with distilled water until a negative test for sucrose was obtained (100).

The method using the ion exchange resin is not costly in time or material since the removal of the acid products by elution of the column with ammonium hydroxide also regenerated the resin into the hydroxide form. The resin could then be slurried with a fresh sucrose solution and a second oxidation started using the same catalyst. Sucrose solutions have been oxidized in this manner using the same resin and catalyst five times and no appreciable loss of activity could be detected.

The oxidation of sucrose at one of the three primary hydroxyl groups of sucrose can give rise to three sucronic acids, each of which has the same molecular formula $C_{12}H_{20}O_{12}$. The three acids may be regarded as positional isomers with the carboxyl group at either the 6 position of the D-glucose residue or the 1 and 6 positions of the D-fructose portion of the sucrose molecule.

Carbohydrate acids are difficult to chromatograph and special solvent systems have been

developed for their separation by paper chromatography. These systems, usually containing acids such as formic acid, acetic acid or phenol, are designed to keep the carbohydrate acid as a single spot, since in neutral media there is always an equilibrium between the acid and the lactone. Paper chromatography of sucronic acid using these methods is not feasible since the medium is sufficiently acidic to cause continuous hydrolysis of the glycosidic link and, hence, cause streaking. Paper chromatography of the free acids was also found impossible because the acid is sufficiently strong to cause its own hydrolysis. Based on these considerations, the sucronic acids were paper chromatographed as the ammonium salts, however, a solvent system capable of separating the positional isomers was not found and the acids simply developed as a single spot on paper. Similar negative results were obtained using paper electrophoresis. The acids migrated as a single diffuse spot using the buffering systems previously mentioned.

Hydrolysis of these three acids gave D-glucose and D-fructose as the neutral hexoses, and D-glucuronic acid, 2-keto-D-gluconic acid and

5-keto-L-gulonic acid as the acid moieties. These acids have as a common feature their ability to decarboxylate quantitatively in 12% hydrochloric acid. Decarboxylation of these acids, as isolated from the anion exchange resin, yielded approximately 80% of the calculated amount of carbon dioxide. From this, it is possible to say that the oxidation of sucrose must proceed in at least 80% yield on the three primary positions of sucrose. Purification of the sucronic acids by a single precipitation from 18% water in ethanol yields a product with 97.4% of the calculated carbon dioxide on decarboxylation with 12% hydrochloric acid. The ammonium sucronates yielded 95.3% of the ammonia calculated on the basis of $C_{12}H_{23}O_{12}N$ when treated with sodium hydroxide.

Analysis Of Oxidation Products. -

Paper chromatography, after the hydrolysis of the ammonium sucronates, gave strong evidence that the glycosidic link was still present in the sucronic acids since both D-glucose and D-fructose were identified; however, further evidence was obtained by treating the sucronic acids with the enzyme invertase. Enzymes are specific in their action and invertase

is known to hydrolyze only β-D-fructofuranosides. Since sucronic acid salts were inverted by the enzyme, the glycosidic linkage of sucrose was not affected or broken by the oxidation reaction. Originally, it was hoped that oxidation at the C-1 position of D-fructose would lead to an acid whose steric requirements about the glycosidic link would be sufficiently altered to obstruct the stereospecific enzyme from functioning. This would have been a convenient method of differentiating between the three ammonium sucronates. However, the enzyme completely hydrolyzed all three sucronates.

The neutral hexoses from the enzyme hydrolizate were quantitatively separated from the acid components by ion exchange methods and the weight ratio of hexose to hexuronic acid was within 4% of the theoretical 48% hexoses and 52% of hexuronic acids expected in sucronic acid.

From the ratio of D-glucose to D-fructose in the neutral hydrolizate it was possible to determine the percentage of oxidation which occurred on the D-glucose and D-fructose moieties of sucrose.

By optical rotation measurements the percentage of D-glucose and D-fructose in the hydrolizate was 65% D-glucose and 35% D-fructose. These calculations were based on the known equilibrium rotations of D-glucose and D-fructose and the fact that paper chromatography failed to show the presence of any other components. The above value was checked by oxidizing the aldose quantitatively in the presence of the ketose and measuring the quantity of iodine consumed in the oxidation. From this experiment the mixture analyzed 67% D-glucose and by difference 33% D-fructose. From these measurements it was concluded that the D-fructose moiety of sucrose is oxidized twice as readily as the D-glucose portion. This is to be expected since the D-fructofuranose structure contains twice as many primary hydroxyl groups as the D-glucopyranose one. This established the extent of oxidation occurring at the D-glucose C-6 position as approximately 33% of the total, however, the ratio of oxidation between the one and six positions of D-fructose remained unknown.

The three acid salts liberated on the hydrolysis of ammonium sucronate by the enzyme

invertase must be D-glucuronic acid (VII), 2-keto-D-gluconic acid (VIII) and 5-keto-L-gulonic acid (IX).

Of the three acids, only the 2-keto acid liberates oxalic acid on treatment with sodium metaperiodate. This glycol cleaving agent forms the oxalic acid by cleaving the carbon - carbon bond between C-2 and C-3 of the 2-keto-D-gluconic acid. The oxalic acid liberated in the oxidation was determined by precipitation as the calcium salt and the oxalic acid content of the precipitate determined by oxidation to carbon dioxide with acidic permanganate. The yield of oxalic acid indicated that 38% of the oxidation occurred at the C-1 position of the D-fructose moiety. Therefore, the relative rates of oxidation of 6, 1' and 6' positions appear to be 34:38:28. These values correspond to practically a statistical distribution of oxidation between the three available positions.

A confirmation of the value obtained by periodate oxidation for the C-2 position was sought by using the colourimetric determination of the 2-hyroxyquinoxaline formed by the condensation of the 2-ketoaldonic acids with ortho-phenylenediamine.

The adsorption spectra of the condensation products of sucronic acids with ortho-phenylenediamine showed characteristic peak at 360 m.μ.; however, the method could not be made quantitative due to interference by the uronic acid present in the mixture. The interference of D-glucuronic acid was proven by determinations done on known mixtures of D-glucuronic acid and 2-keto-D-gluconic acid. The method did however confirm the presence of 2-ketoacids in the mixture.

The estimation of the 5-keto-L-gulonic acid by the modified Benedict's reagent could not be carried out since it was not possible to reproduce the results described by using standard solutions of 5-keto-D-gluconic acid.

The Preparation Of Sucronic Acid Derivatives.

The uronic acids and ketohexonic acids are known as an unstable classification of compounds which are often sufficiently strong acids to cause their own decomposition in concentrated solutions or as amorphous solids. The aldonic acids, on the other hand, are stable under such conditions and are therefore easier to identify.

Uronic acids can be converted to aldonic acids by reduction of the aldehyde or hemiacetal group to a primary alcohol. The reagent of choice to reduce the aldehyde group is sodium borohydride (115)(116)(117). The ammonium salts of the acids obtained on the hydrolysis of ammonium sucronate were treated with excess sodium borohydride and the reaction tested for completion using the anthrone reagent (100). The solution still contained reducing compounds after reaction times of up to one week. Since uronic acids (116) and 5-keto aldonic acids (115) can be reduced quantitatively, the remaining acid in the mixture, the 2-keto-D-gluconic acid must be the reducing compound which was not reduced completely.

A possible explanation for this is the ability of the 2-ketoaldonic acids to form enediols under either acidic or basic conditions (84). Since sodium borohydride reductions are done under alkaline conditions, it is quite probable that an equilibrium between the 2-keto form and the enediolic system was set up. Reductones are known not to be attacked by lithium aluminum hydride (118).

Acids from Sucronic Acid. -

Indirect evidence obtained from the analysis of the neutral sugars derived from the hydrolysis of sucronic acid indicated that 34% of the oxidation formed a β-D-fructofuranosyl-α-D-glucuronide (IV), however, no satisfactory method of analysis for uronic acids could be found which was free from interference by ketohexonic acids. The sucronic acids were hydrolyzed and the acids were isolated. D-Glucuronic acid was obtained as D-glucuronolactone in 13.8% yield. Since the sucronic acid is monocarboxylic, D-glucuronolactone could only come from the hydrolysis of the β-D-fructofuranosyl-α-D-glucuronide. The lactone, when isolated from the complex mixture of acids, had a low melting point. Recrystallization was slow and the recovery of the acid was poor. During this work the first good derivative of D-glucuronic was reported by Wolfrom (113). Using the latter derivative, it was possible to isolate a 62% yield of 2,4,5-tri-O-acetyl-D-glucuronolactone diethyl dithioacetal based on D-glucuronolactone. This further characterized the D-glucuronic acid.

Adducts Of Sucronic Acid With Diimides.

In order to try and form crystalline derivatives of sucronic acids, their reaction with N, N'-dicyclohexylcarbodiimide and N, N'-di-p-tolylcarbodiimide was investigated. These reagents, which were thoroughly reviewed by Khorana in 1953 (120), provide an easy route to the acid amides which are neutral compounds and hence might be more easily separated by chromatographic methods. In the course of their reactions, it was found that practically quantitative yields of adducts of sucronic acid and N,N'-di-p-tolylcarbodiimide could be achieved using dimethyl sulfoxide as the reaction media. These adducts were hydrolyzed by both acid and alkali. Acid treatment cleaved the glycosidic linkage, while alkaline conditions cleaved the amide linkage found in the adduct. The formation of these adducts did not however prove useful in characterizing the sucronic acids, since they did not form crystalline derivatives.

The Oxidation Of Various Carbohydrates By Oxygen Using Platinum Catalyst And Ion Exchange Resin.

In order to show the wide applicability of

the method developed, both methyl α-D-glucopyranoside and 1,2;3,4-O-diisopropylidene-D-galactose were oxidized by oxygen and platinum catalyst using the resin Amberlite IR-45 as the acid acceptor. Methyl α-D-glucoside, which had only the aldehyde grouping protected, was oxidized in 86.6% yield as determined by carbon dioxide measurements on the methyl α-D-glucuronide. The compound was then characterized as 2,4,5-tri-O-acetyl D-glucuronolactone diethyl dithioacetal.

The usefulness of the method was further demonstrated by the oxidation of 1,2;3,4-di-O-isopropylidene D-galactose. Ten per cent dioxane was added to increase the solubility of the compound in the oxidation mixture. Elution of the product with ammonium hydroxide yielded ammonium di-O-isopropylidene D-galacturonate which crystallized immediately on concentrating the effluent.

In conclusion, it is possible to state that a new method for the oxidation of glycosides to the corresponding uronic acid in yields from 80 to 100% has been developed. This method undoubtedly will

prove to have wide applicability. The procedure allowed the oxidation of sucrose to the three possible monocarboxylic acids for the first time.

IV. CLAIMS TO ORIGINAL RESEARCH

1. The addition of an anion exchange resin to oxygen oxidations of glycosides when catalyzed by platinum was shown to protect the primary oxidation products from further oxidation.

2. The monocarboxylic acids which result from the oxidation of each of the three primary alcohol groups in sucrose were prepared for the first time.

3. The three primary hydroxyl groups in sucrose were found to oxidize at approximately equal rates.

4. A novel method for the determination of 2-keto-aldonic acids in the presence of D-glucose, D-fructose, D-glucuronic acid and 5-keto-L-gulonic acid was developed using a periodate oxidation to form oxalic acid.

5. The hydrolysis of the glycosidic linkage of the ammonium salts of the three sucronic acids were found to be catalyzed by invertase enzyme.

6. Methyl α-D-glucopyranoside was oxidized in 86% yield to the corresponding methyl α-D-glucuronide.

7. 1,2;3,4-Di-O-isopropylidene-D-galactose was oxidized in 100% yield to the corresponding 1,2;3,4-di-O-isopropylidene-D-galacturonide.

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