THE OXIDATION OF STARCH WITH ALKALINE HYPOCHLORITE

A Thesis

by

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GENERAL INTRODUCTION

The oxidation of starch and cellulose with alkaline hypochlorite is of considerable industrial interest. Starches oxidized with this reagent have wide application in the sizing of textile fibers and paper. Alkaline hypochlorite is also widely used in the bleaching of cellulose pulp.

In this study, starch rather than cellulose was chosen, as in cellulose the factor of inaccessibility might have introduced unnecessary complications. The first object of this work was the isolation of the oxidized starch and by-products in highest yields possible, so that most of the oxidant could be accounted for.

The second object was the investigation of the reaction mechanism, a task which involved the isolation and identification of the individual degradation products. About 80% of the oxidizing power of the hypochlorite consumed was represented by carbon dioxide and carboxylic acid groups. Methods of hydrolysis plus paper and rather large-scale column chromatography were developed, which made it possible

to separate and identify the other oxidation products as D-, Dl and meso-tartaric acid, D-glucuronic acid, D-erythronolactone and glyoxylic acid. A new method of preparing the latter substance was incidentally discovered.

HISTORICAL INTRODUCTION

Oxidation of Starch and Simple Sugars with Alkaline Hypohalite

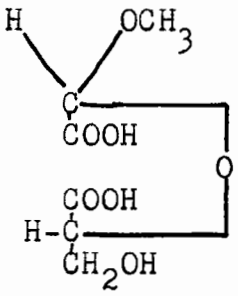
Sodium hypoiodite has been used for a very long time to oxidize aldoses stochoimetrically to aldonic acids (1) according to the equation:

$$RCHO + I_2 + 3\ NaOH \longrightarrow RCOONa + 2\ NaI + 2H_2O$$

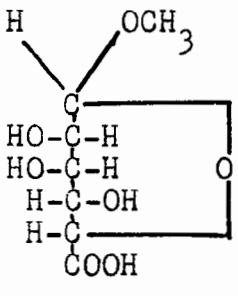
Goebel (2) prepared D-gluconic and D-maltobionic acids by the treatment of the corresponding aldoses with barium hypoiodite. Hoenig and Rusiczka (3) oxidized both D-glucose and D-galactose to the corresponding aldonic acids with alkaline barium hypobromite in 83% and 87% yield, using ultraviolet light as a catalyst. These authors noted that the formation of aldonic acids was favoured by low concentrations of alkali and sugar. When barium hypobromite was replaced by calcium hypochlorite, the reaction was slower and the yields were reduced to 61 and 58%. Hoenig and Tempus (4) studied the prolonged action of barium hypobromite on D-glucose.

These authors thought that in the course of this reaction first D-gluconic, then 2-keto-D-gluconic and finally D-arabonic acids were formed. Reichstein and Neracher (5) disputed these results and claimed that the main product was 5-keto-D-gluconic acid and not the 2-keto acid, since the former keto-acid could be further oxidized to D-xylo-trihydroxy-glutaric acid.

Smolenski (6) oxidized methyl-α-D-glucopyranoside with bromine using sodium carbonate as a buffer, and isolated the brucine salt of methyl-D-glucuronide in 30% yield. Jackson and Hudson (7) oxidized methyl-α-D-mannopyranoside with 8 equivalents of bromine in barium hydroxide solution and isolated D-methoxy-D-hydroxy-methyl-diglycolic acid (I) and the methyl-α-glycoside of D-mannuronic acid (II) in 25% and 12% yields as their strontium and brucine salts. The



I



II

formation of these compounds showed that alkaline hypobromite sometimes oxidized secondary alcohol groups in such a manner

as to break the carbon chain and to produce carboxyl groups. Simultaneously oxidation at the primary hydroxyl group of the glycoside took place.

The older work on the hypohalite oxidation of starch in alkaline solution consisted for the most part of studies on the nature of the oxidized polymer. Those studies included the determination of viscosity and reducing power, of retrogradation as well as the adsorption of dyes. The results were not of major structural significance as in only a very few instances were degradation products isolated and identified. Since all this work prior to 1952 was very well described by Ellington (8,9) and by Miss McKillican (10,11) the following account is restricted to articles of immediate relevance to the present research.

Hoenig and Ruziczka (12) oxidized thin-boiling starch with barium hypobromite illuminated by an ultraviolet quartz mercury lamp at room temperature and in the presence of ca. 0.02 N barium hydroxide. The hypobromite used was equivalent to 0.33, 0.5 and 1.0 atoms of oxygen per anhydroglucose unit; the corresponding amount of barium hydroxide being 0.44, 0.66 and 1.32 equivalents. Barium ions were removed by precipitation with the calculated quantity of sulphuric acid and the filtrate was then neutralized with calcium oxide. Addition of 96% alcohol

to the solutions obtained from oxidations at the two lower concentrations produced precipitates in 71% and 70% yield. After six reprecipitations from aqueous alcohol the pure calcium salt of maltobionic acid was isolated and was identified by analyses and by conversion to the corresponding brucine salt. It appeared that the crude product was extensively contaminated by calcium bromide and dextrins and thus the true yield of calcium maltobionate must have been considerably lower than 70%.

The solution oxidized with hypobromite equivalent to one atom of oxygen per anhydroglucose unit gave a very low yield of a crystalline product when tested with phenylhydrazine. The nitrogen content of this material was similar to that of gluconic acid phenylhydrazide but the authors did not confirm its identity. There was therefore no conclusive evidence that gluconic acid was formed by oxidation of starch with hypobromite in alkaline solution. Calcium maltobionate was again isolated as the second reaction product.

Farley and Hixon (13) oxidized starch with 4 equivalents of bromine at neutral pH. On hydrolysis of the oxidized starch they obtained a small yield of glucuronic acid, which they identified as the cinchonine salt. Methylation and methanolysis of the oxidized starch, followed by conversion to the barium salts, yielded several fractions whose barium

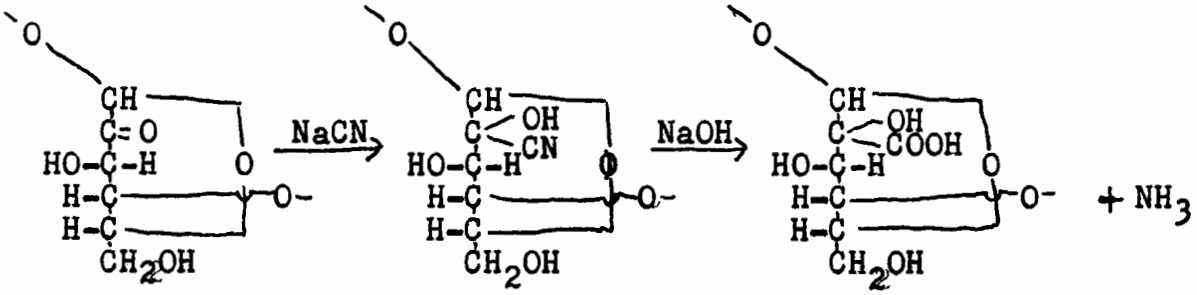
and methoxyl contents were in the same range as those of barium-dimethyl-keto-glucuronate and barium monomethyl-tartrate. However, the authors were not able to identify the individual compounds. Uronic acid determinations were equivalent to 50% glucuronic anhydride, and yields of furfural by distillation with 12% hydrochloric acid were corresponding to that uronic acid content. An oxime of the oxystarch was prepared which had a nitrogen content corresponding to one carbonyl group in 65-75% of the anhydro-glucose units.

Although Ellington and Purves (8, 9) oxidized corn starch with chromium trioxide and not with hypohalite, their work developed indirect methods of establishing the positions of carbonyl and carboxyl groups formed during the oxidation. Free carboxylic acid groups were determined by the addition of aqueous sodium bromide to the oxystarch and titration of the liberated hydrobromic acid, as well as by the standard calcium acetate method (14). Estimation of carbonyl groups was by the condensation of the oxystarch with hydroxylamine hydrochloride and by titration of the liberated hydrochloric acid according to the equation:

$$\rangle C = O + H_2NOH,HCl \longrightarrow \rangle C = NOH + H_2O + HCl$$

The method suffered from inaccuracy caused by the highly buffered system when the amount of carbonyl groups was small. In another method the oxystarch was condensed with

sodium cyanide at pH 9.5 with subsequent hydrolysis of the cyanohydrin and estimation of the ammonia liberated.



III IV V

Differenciation between keto and aldehyde groups was by three independent methods. Aldehyde groups were estimated by reoxidation of the oxystarch with sodium chlorite, or with sodium hypoiodite, followed by the determination of the increase in carboxyl content. In the third method, the oxystarch was allowed to react with sodium bisulfite and the excess bisulfite was back titrated with alkali.

By the above method it was found that 40-60% of the oxydant was consumed for the formation of carboxyl groups and 25-40% was used to produce carbonyl groups. The aldehyde accounted for approximately one third of the carbonyl groups present and the total recovery of oxidant was about 90%. Further evidence as to the location of carbonyl groups was obtained by the preparation of the oxystarch cyanohydrin, followed by reduction with red phosphorus and hydriodic acid. A barium salt of 2-methyl-4-hydroxy-hexanoic acid was isolated in a yield corresponding to one third of the

carbonyl groups present in the oxystarch. The identification of this compound as its crystalline hydrazide established the presence of 2-keto groups in the oxidized starch.

McKillican and Purves (10,11) oxidized gelatinized wheat starch with dilute hypochlorous acid kept at pH 4-4.2 by the addition of calcium acetate. According to Green (15) aqueous hypochlorous acid was in equilibrium with molecular chlorine and hypochlorite ion, according to the hydrogen ion concentration of the solution.

$$HOCl + H^+ + Cl^- \rightleftharpoons H_2O + Cl_2$$

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

At pH 2 the equilibrium mixture contained 20% of molecular chlorine, whereas at pH 4-5, 95% of the mixture was in the form of undissociated hypochlorous acid. In the alkaline range dissociated hypochlorite anion was present almost entirely. McKillican and Purves oxidized the starch to the extent of 0.04-0.33 atoms of oxygen per anhydroglucose unit and followed the progress of the oxidation both by iodometric titrations and by the arsenite method with concordant results. The authors experienced some difficulty in the determination of the extent of oxidation, because blank solutions of hypochlorous acid decreased in titer with time. The error introduced was 10-20% of the total oxidant consumed. The oxidized starch was converted to the calcium salt and separated into the water-soluble

and water-insoluble fractions which together gave a yield of 93-95% by weight. The water-insoluble fraction amounted to one fifth of the total oxystarch.

Since the water-soluble portion was in the form of its calcium salt, the carboxyl groups were calculated from the calcium content. Uronic anhydride determinations gave values exceeding those calculated from the calcium content by 30%. However, the authors did not attach too much importance to this discrepancy since the determination of uronic anhydrides by decarboxylation with 12% hydrochloric acid was likely to yield high results when applied to plant materials. They postulated that all, or nearly all, of the carboxyl groups were of the uronic acid type. The amount of carboxyl groups in the water-insoluble portion was determined by conversion of the calcium salts to the free acids and subsequent addition of calcium acetate solution, the liberated acetic acid then being back titrated with standard alkali.

The carbonyl content of the oxidized starches was determined by condensation with hydroxylamine hydrochloride and back-titration of the liberated hydrochloric acid with standard alkali. Preparation of the cyanohydrin addition product, followed by hydrolysis and measurement of the ammonia liberated, was the method used. Values from both determinations were in good agreement with each other. Reaction of the

oxystarch with alkaline hypoiodite to estimate the aldehydic carbonyl groups resulted in overoxidation. Treatment with sodium bisulfite solution and titration of the "bound" bisulfite showed that 55% of the carbonyl groups were of the aldehyde type. In another experiment, a part of the oxystarch was reoxidized with chlorous acid, which presumably oxidizes the aldehyde to the corresponding carboxyl groups selectively. The remaining carbonyl groups postulated to be of the keto-type were estimated by the usual hydroxylamine hydrochloride method. Seventy-eight per cent of the carbonyl groups were found to be of the aldehyde type by this method.

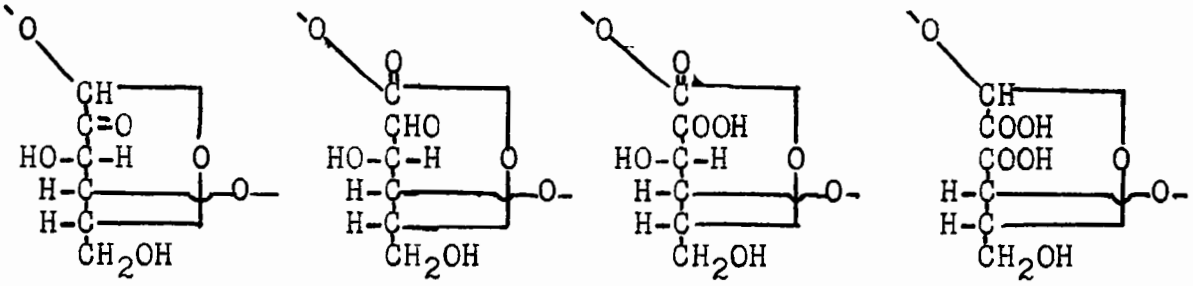
A part of the oxystarch was converted to the cyanohydrin and this non-reducing adduct was hydrolysed with dilute sulphuric acid to glucose and the corresponding sugar acids. The latter were isolated as their barium salts in a yield which was close to that theoretically expected from the content of carboxyl and carbonyl groups in the original oxystarch. Reduction of these acids with red phosphorus and hydroiodic acid, followed by Raney Nickel in caustic soda, yielded an oil which was further purified by fractional distillation under diminished pressure. The physical constants of the product corresponded closely to those of 2-methyl-4-hydroxy-hexanoic lactone and identification was completed by the preparation of the crystalline hydrazide. The yield was equivalent to 30% of

the keto groups. Thus at least one-third of the keto groups were in second positions of the anhydroglucose units.

Kaverzneva, Ivanov and Salova (16) oxidized cellulose with 20 volumes of 0.049 N sodium hypochlorite at pH values of 4.6, 6.8 and 11.0 and found that the rate of oxidation decreased with increasing pH. Total carboxyl groups in the oxycellulose were determined by the calcium acetate method of Meesok and Purves (14) and uronic carboxyl groups by the standard decarboxylation method with 12% hydrochloric acid. Aldehyde groups were determined by reoxidation with sodium hypoiodite at pH 9.2 and total carbonyl groups by the condensation with hydroxylamine hydrochloride according to the method of Gladding and Purves (17). Carbonic acid esters were determined and the method was apparently the same as described by Kaverzneva during a chemical meeting in Stockholm (18). This determination consisted in treating the cellulose sample with 0.025 N sodium hydroxide for one hour at room temperature followed by digestion with 12% hydrochloric acid (19). Small amounts of carbon dioxide were evolved from cellulose oxidized in acidic and neutral medium. Cellulose, and cellulose oxidized in alkaline medium, gave almost no carbon dioxide in this treatment.

Cellulose samples oxidized with hypochlorite at pH 4.6 and 6.8 showed higher uronic carboxyl than total carboxyl. Carboxyl and aldehyde content increased with

the extent of oxidation and small amounts of carbonic acid esters were present. At pH 11 the amount of carboxyl groups increased sharply with the progress of the oxidation and was much higher than the uronic acid content. No carbonic acid esters and almost no aldehyde groups were found. In both cases keto groups were detected. The authors believed that primary attack of the hypochlorite on the cellulose took place at carbon atoms number six and two. Oxidation at carbon six produced an aldehyde which in acidic and neutral solutions was slowly oxidized to carboxyl. In alkaline medium, this aldehyde group was not stable and oxidation proceeded rapidly to carboxyl. Oxidation at the number two carbon produced a 2-keto-anhydroglucose (VI) which in acid solution (pH 3-5) was further partly cleaved with formation of carbonic acid esters (VII) and (VIII).



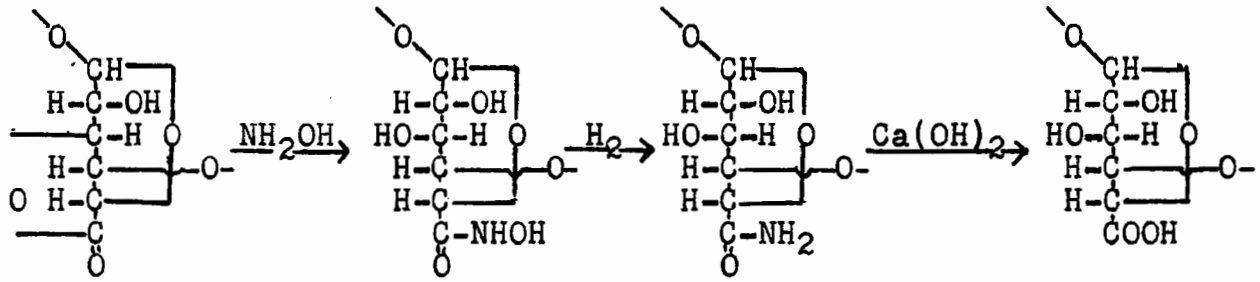
VI VII VIII IX

In alkaline solution anhydro-2-keto-glucose was oxidized in part to 2,3-diketo-anhydroglucose, yielding ultimately a 2,3-dicarboxylic acid fragment (IX).

Kaverzneva (18) postulated that keto groups were introduced into the cellulose by the oxidation with hypochlorite. A sample of the hypochlorite oxycellulose was treated with sodium hypoiodite at pH 9.3 to transform aldehyde groups to carboxyl, and was then condensed with hydroxylamine. Reduction with metallic calcium, and hydrolysis, yielded a product which contained amino groups. These amino groups were determined according to the Van Slyke method and found to be equivalent to 20-30% of the nitrogen present in the hydroxylamine condensation product. The presence of α-hydroxy-keto groups was further confirmed by heating oxycellulose with 10 parts of 10% sodium carbonate solution for one minute at 70° to form an endiol, which formed a colour complex when reduced with arseno-phospho-tungstic acid (Benedict's Reagent). The oxycellulose samples gave a blue colour, but no colouration was obtained with purified cotton cellulose or with cellulose oxidized with periodate and chlorine dioxide.

The presence of ester or lactone units in oxycellulose was demonstrated by the reaction with hydroxylamine to form a hydroxamic acid, followed by the addition of ferric chloride. Hydroxamic acids were known to give a red complex with ferric ions. Purified cotton, periodate oxycellulose and hypochlorite oxycellulose treated with 0.05 N sodium hydroxide gave no

colour, while hypochlorite and hydrogen peroxide oxycelluloses obtained in an acid medium or kept in dilute acid after alkaline oxidation, showed a distinct red colour. Kaverzneva believed that anhydro-l-4-glucuronolactone was responsible for the lactone groups, and since this compound reacted with hydroxylamine, excessively high carbonyl values for oxycelluloses might be obtained with the Meesok and Purves hydroxylamine condensation method (14,17). When such a hydroxylamine condensation product was reduced with metallic calcium and was then hydrolysed, ammonia was liberated in an amount equivalent to 20-40% of the original nitrogen present. The reaction presumably proceeded according to the scheme:



X XI XII XIII

Patel, Mankad and Patel (20) oxidized starches, which were isolated as granules from different plant materials, with aqueous 0.028 M sodium hypochlorite at pH 6.95. Oxidations were carried out over a period of three hours with and without addition of nickel sulphate as catalyst, the concentration of the starch being 0.617 mole of

$C_6H_{10}O_5$ per litre. The consumption of oxidant varied from 0.003 to 0.013 mole per anhydroglucose unit, and the increase in oxidation caused by the nickel sulphate catalyst was in most cases less than 20% of the hypochlorite consumed. Maize (Zea mays) and tuver (Cajanus cajan) starches oxidized under the same conditions but with hydrogen ion concentration varying from pH 5.5 to 9.2 showed a maximum reducing power and a minimum carboxyl content in the range pH 6.4 and 6.9

Mechanism of Alkaline Oxidation of Starch and Cellulose

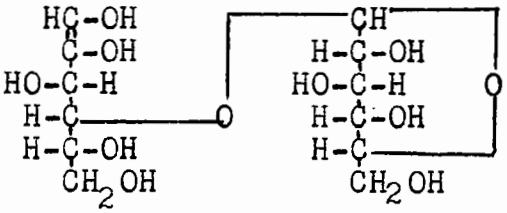
Evans (21) was the first to study systematically the mechanism of carbohydrate oxidation in alkaline solution. Preliminary oxidations of simple sugars such as glucose, fructose, mannose and galactose with potassium permanganate in alkaline solution yielded carbon dioxide and oxalic acid in slightly different amounts. These differences tended to disappear when either the reaction temperature or the alkali concentration was increased. In order to study the mechanism of oxidation in alkaline solution more closely, the oxidant was omitted and the sugars were treated with pure alkali of different normalities over a 48 hour period. Treatment of glucose, galactose (22), fructose (23) and mannose (24) with sodium hydroxide solution of varying concentration yielded formic, acetic, lactic and saccharinic acids as well as pyruvic aldehyde, which was isolated as its osazone. Formic and acetic acids were preferentially

produced at lower alkali concentration; 0.5 N sodium hydroxide producing the maximum yield. Alkali concentrations of 0.5-1.0 N gave the highest recoveries of pyruvic aldehyde. Lactic and saccharinic acids were formed in greatest yield with 9.0 N alkali, the strongest concentration used.

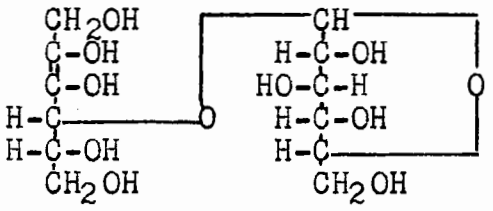
In his studies on the behaviour of alkali toward reducing hexoses, Nef (25) postulated the existence of an equilibrium consisting of 1-2, 2-3 and 3-4 endiols. Evans (21) explained the formation of lactic acid and pyruvic aldehyde by the existence of a hexose 3-4 endiol intermediate and its cleavage at the double bond into two moles of glyceric aldehyde, which in turn was further rearranged to pyruvic aldehyde and lactic acid. Lactic acid could be regarded as a saccharinic acid of the triose series. The breakdown of pyruvic aldehyde might be partly responsible for the formation of acetic and formic acids. When DL-glyceric aldehyde was subjected to alkaline degradation under the same conditions, formic, acetic, lactic acids and pyruvic aldehyde were obtained, their yields being in the same general relationship to alkali concentration as was found to be present with the hexoses (26).

The action of alkali on maltose produced formic acid in higher yields per glucose unit than those normally obtained from glucose itself (27). On the other hand, yields of pyruvic aldehyde and lactic acid were smaller. The authors

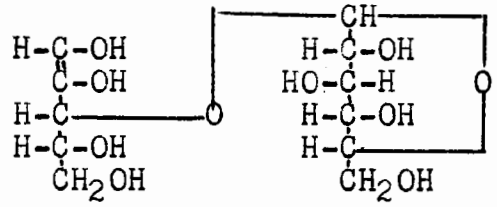
explained these differences by the assumption that maltose formed two endiols, namely 4-glucosido-glucose-1,2-endiol (XIV) and 4-glucosido-glucose-2,3-endiol (XV). Cleavage of the 1-2-endiol would result



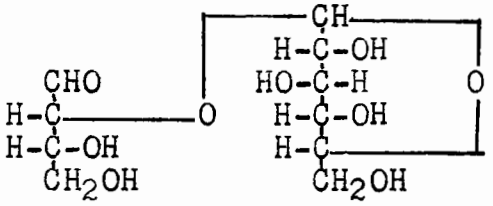
XIV



XV



XVI

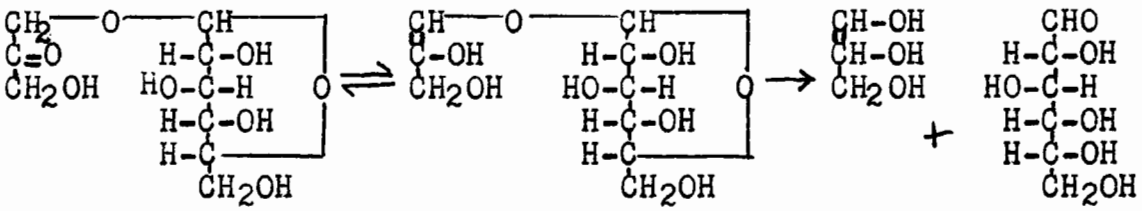


XVII

in formaldehyde and the 3-glucosido-arabinose-1,2-endiol (XVI), which in turn might be further degraded to 2-glucosido-erythrose (XVII) and formaldehyde. The formaldehyde in alkaline solution might then undergo a Canizzaro reaction leading to formic acid. 2-glucosido-erythrose might also be formed in the cleavage of the 4-glucosido-glucose-2,3-endiol with the simultaneous production of glycolic aldehyde. The latter might in turn undergo transformation to acetic acid. Erythrose, glycolic and formic aldehyde in alkaline solution would not give rise to lactic acid (28), thus the formation of glyceric aldehyde would be confined to the second or the non reducing glucose moiety. Yields of

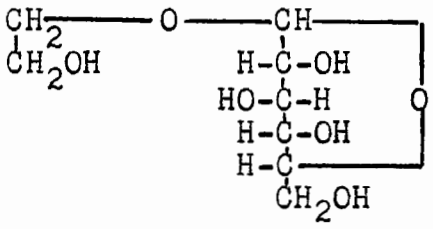
lactic acid per glucose unit from maltose should then be only one-half of those obtained from glucose under identical experimental conditions.

Similar studies on cellobiose and lactose (29) showed that these disaccharides yielded lactic acid only in such amounts as would have been expected from their hexosido (non-reducing) portions. The action of alkali on gentiobiose (6-β-D-glucopyranosido-D-glucose), on the other hand, resulted in lactic acid yields very similar to those obtained from two moles of glucose. Similar yields (30) were obtained from pure β-glucosido-dihydroxy-acetone (XVIII) and from a mixture of equimolar quantities of glucose and dihydroxyacetone. No lactic acid was obtained by the action of alkali on β-hydroxyethyl (XXI) and allyl glucosides (XXII) and on trehalose (1-α-D-glucopyranosido-α-D-glucopyranoside). The authors explained these experimental findings by the assumption that prior to alkaline hydrolysis, an endiol was formed at the carbon atom participating in the glycosidic bond, rendering this linkage unstable to alkali. Thus degradation of glucosido-dihydroxyacetone would proceed as shown:

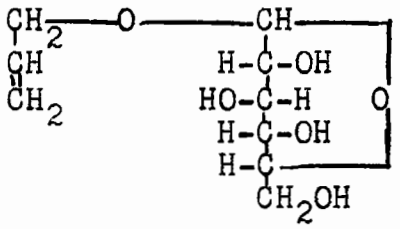


XVIII XIX XX

Allyl glucoside and -hydroxyethyl-glucoside were not hydrolysed by alkali, as there was no possibility for endiol formation.

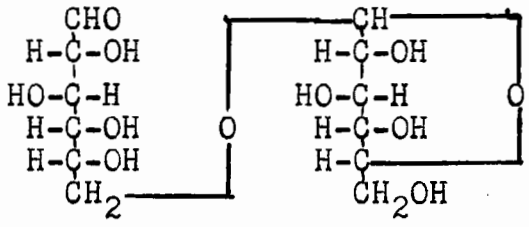


XXI

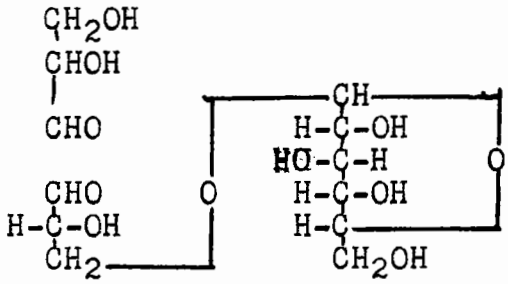


XXII

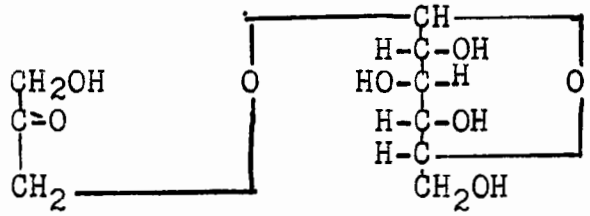
The high yields of lactic acid, obtained upon the action of alkali on gentobiose (XXIII) were explained in a similar manner. Gentobiose was first split into glycolaldehyde and glucosido-dihydroxyacetone (XXIV), which in turn underwent further degradation:



XXIII

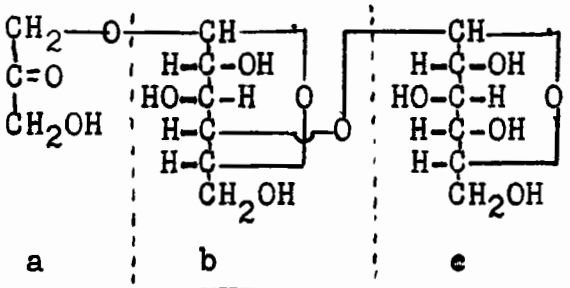


XXIV



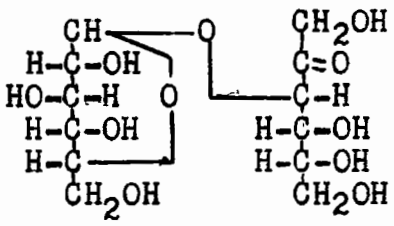
XXV

Identical yields of lactic acid were obtained on degradation of equimolar amounts of cellobiosido-dihydroxyacetone (XXVI) and glucosido-dihydroxyacetone (XVIII). This result had been expected, since only the dihydroxyacetone portion (a) and the second glucose unit (c) of cellobiosido-dihydroxyacetone would contribute to lactic acid formation. The middle glucose unit (b) would be degraded in a different manner. Turanose (3-α-D-glucopyranosido-D-fructose) (XXVII) was readily hydrolysed by alkali (31) in agreement with Evan's concept of glycosidic bond cleavage in alkaline medium via endiol formation.



a b c

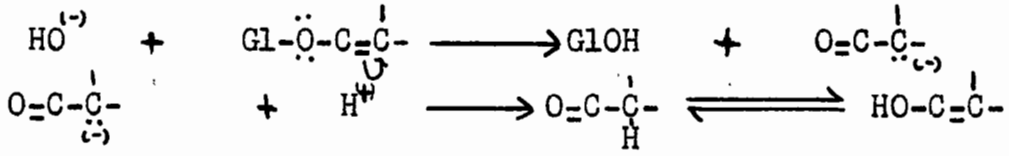
XXVI



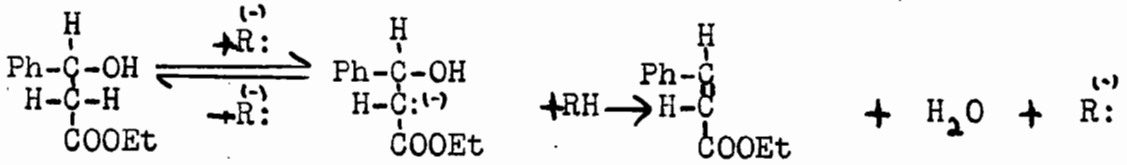
XXVII

Davidson (32) found that periodate oxycellulose was particularly sensitive to alkali. Periodate oxycellulose contained aldehyde groups at the number two and three carbon atoms, which were adjacent to the glycosidic linkages, thus making possible the formation of an endiol. When this oxycellulose was oxidized with chlorine dioxide to convert the aldehyde to carboxyl groups, the alkali sensitivity disappeared (33), as carboxyl groups could not enolize

readily. Isbell (34) believed that this type of alkaline cleavage of the glycosides took place by the addition of hydroxyl groups to the glycosidic half of the endiol, accompanied by an electron shift and subsequent addition of a proton to the other carbon atom of the endiol.



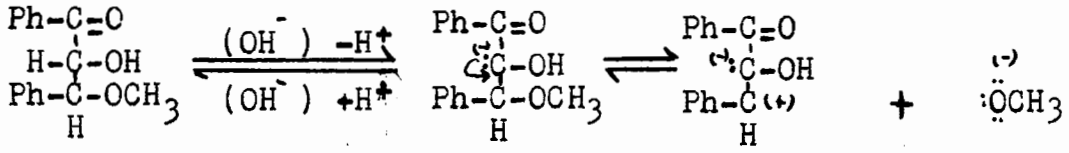
Helferich (35) studied the behaviour of various glycosides toward alkali and found that nitroethanol glucosides and related compounds which had the nitro group in the β-position to the glycosidic group were easily cleaved. Hauser and Breslow (36) noted that ethyl-β-phenyl-β-hydroxypropionate was readily dehydrated in the presence of bases such as sodium ethoxide and sodium triphenylmethyl. These authors proposed the following mechanism for this "aldol dehydration":



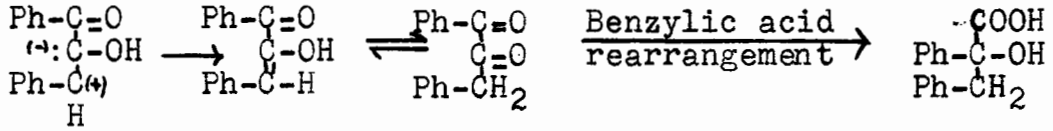
XXVIII XXIX XXX

The strongly negative carboxyethyl group had an inductive effect, which made the hydrogen atom on the α-carbon atom acidic enough to be removed by a strong base. A carbanion was thus formed which reacted with elimination of a hydroxyl

group to form an unsaturated compound. Haskin and Hogsed (37) used the same mechanism to explain the results of Nicolet (38), who found that α-hydroxy-β-methoxy-β-phenyl-propiophenone was rearranged in the presence of alkali to α,β-diphenyl-lactic acid (XXXI).



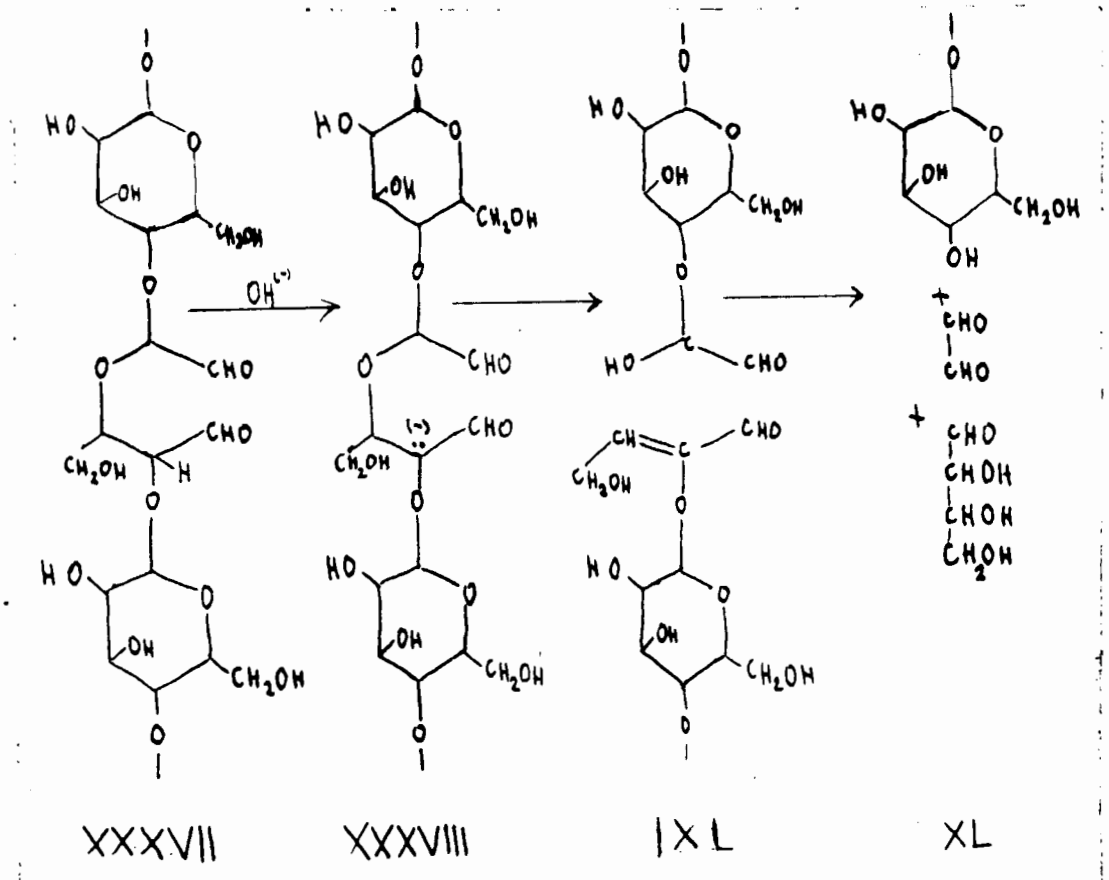
XXXI XXXII XXXIII



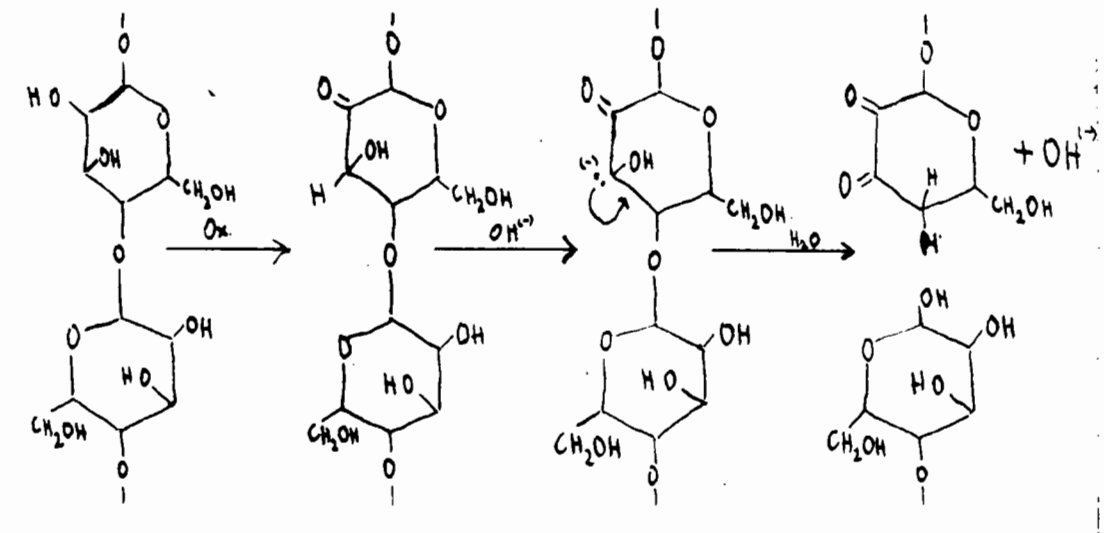
XXXIV XXXV XXXVI

In this particular case, the carbonyl group had the required inductive effect on the hydrogen at the α-carbon atom. It was necessary that the electro-negative group and the ether linkage be separated by exactly two carbon atoms, for scission to occur. Thus, alkaline cleavage of ether linkages might take place if the ether linkage was separated from the negative group by two carbon atoms, and if the α—carbon atom carried at least one hydrogen atom which could be split off as a proton.

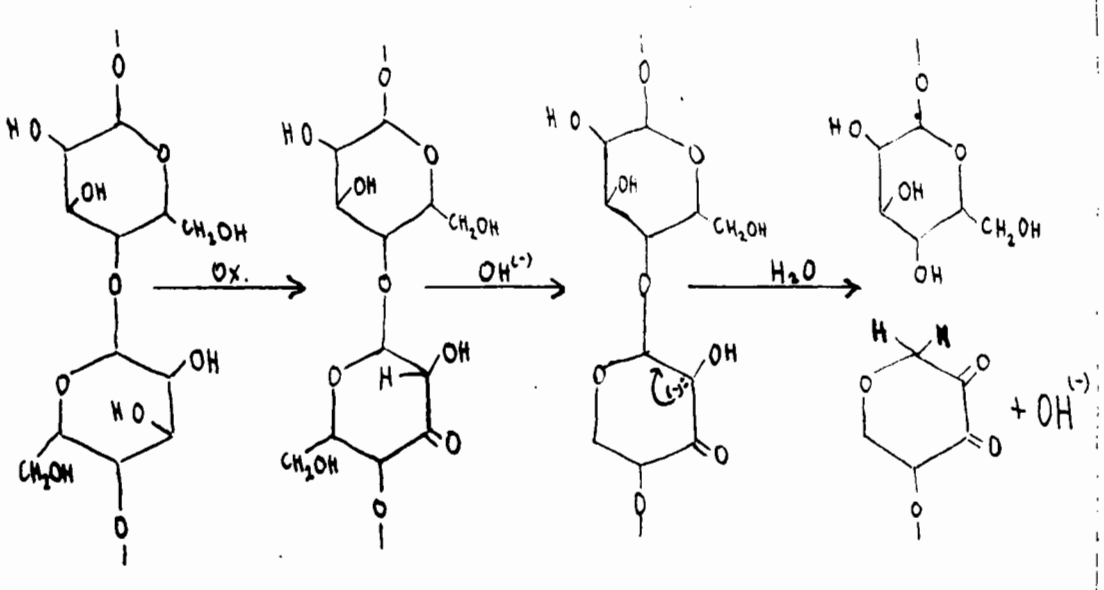
The alkali sensitivity of periodate oxycellulose was explained by Haskin and Hogsed (37) by the same "aldol dehydration" mechanism.



The same authors also advanced a reaction mechanism in which oxidation in alkaline solution at the number two and number three carbon atoms yielded a product which could readily undergo aldol dehydration.



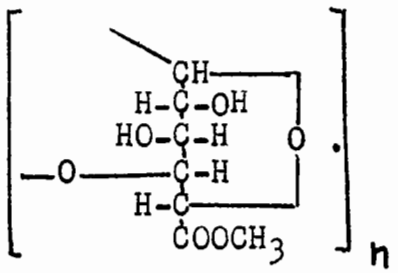
XLI XLII XLIII XLIV



XLV XLVI XLVII XLVIII

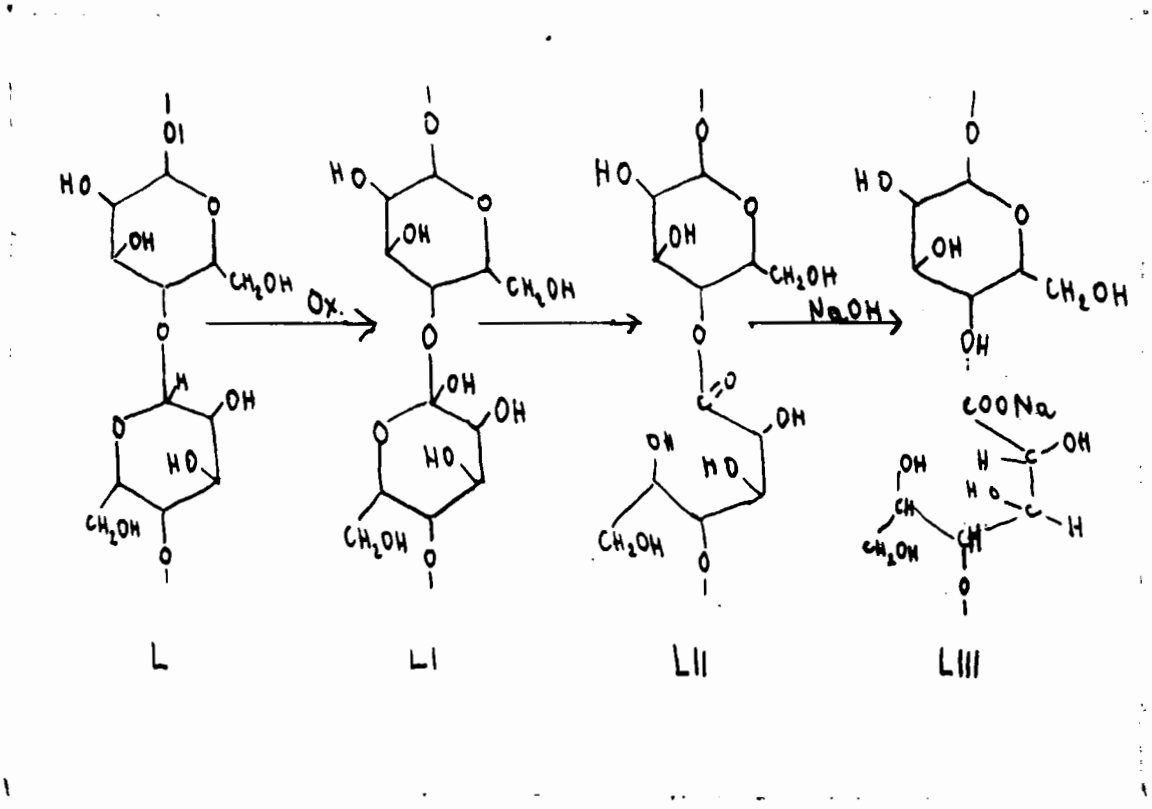
Under the influence of alkali, the hydrogen atom in the α-position to the carbonyl group was split off as a proton with formation of a carbanion. In the subsequent reaction the glycosidic bond was cleaved, and an endiol formed which was in a tautomeric equilibrium with the corresponding diketone.

The stability to alkali of periodate oxycellulose further oxidized with chlorine dioxide was explained by McBurney (39) on the basis that the carboxyl groups of the polyanhydroglucose at C_2 and C_3 underwent complete ionization in an alkaline medium and became negatively charged. The inductive effect of these groups was then reversed and the hydrogen atom in the α- position was non-acidic and not removable as a proton by the presence of a base. These considerations were in line with the result of Vollmert (40), who found that methyl pectate (IL), underwent in alkaline solution a simultaneous de-esterification and depolymerization, but that the depolymerization stopped as soon as all ester groups had been hydrolysed. When the product was re-esterified and again treated with alkali, the depolymerization continued.



IL

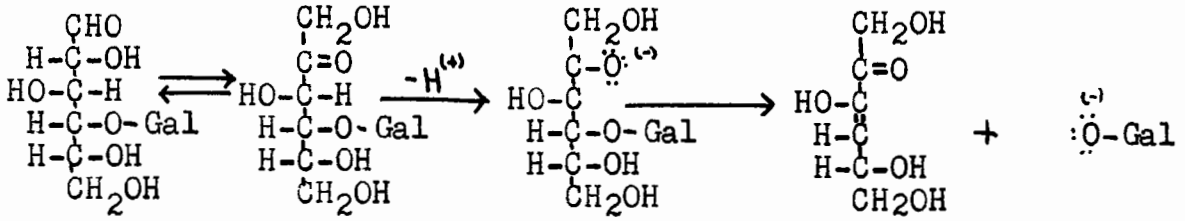
Heuser (41), explained the degradation of cellulose by oxidation in an alkaline medium by assuming a primary oxidation at the number one carbon atom of the anhydroglucose unit. Oxidation of this kind would result in a saponifiable ester linkage.



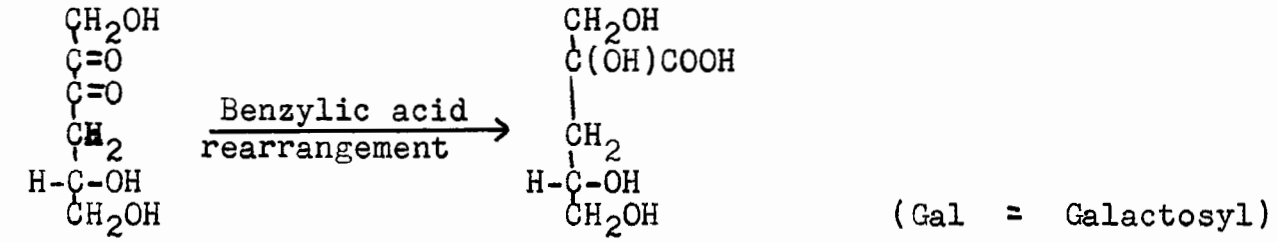
D-gluconic acid should be obtained upon hydrolysis of the oxidized material.

More recently Corbett and Kenner (42), treated lactose

(LIV), with saturated aqueous calcium hydroxide at room temperature under exclusion of atmospheric oxygen. After 24 and 96 hours, 33.9 and 78.2%, respectively, of the lactose was decomposed. Analyses by paper chromatography showed that lactulose (LV), tagatose, galactose and isosaccharinic acid (LIX), were produced. The last two compounds were isolated from the reaction products and identified as their crystalline derivatives. The authors postulated for this degradation the following mechanism:



LIV LV LVI LVII

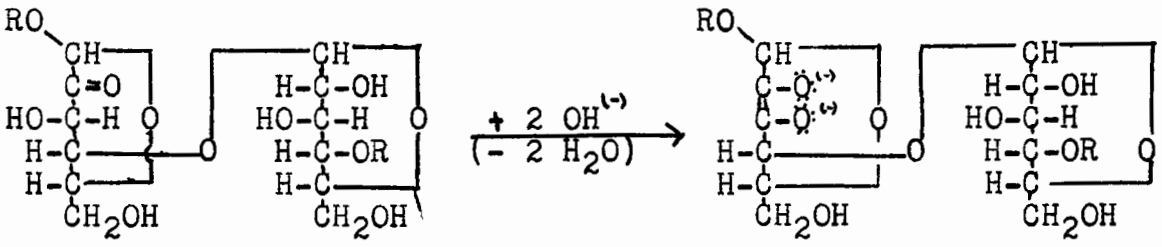


LVIII LIX

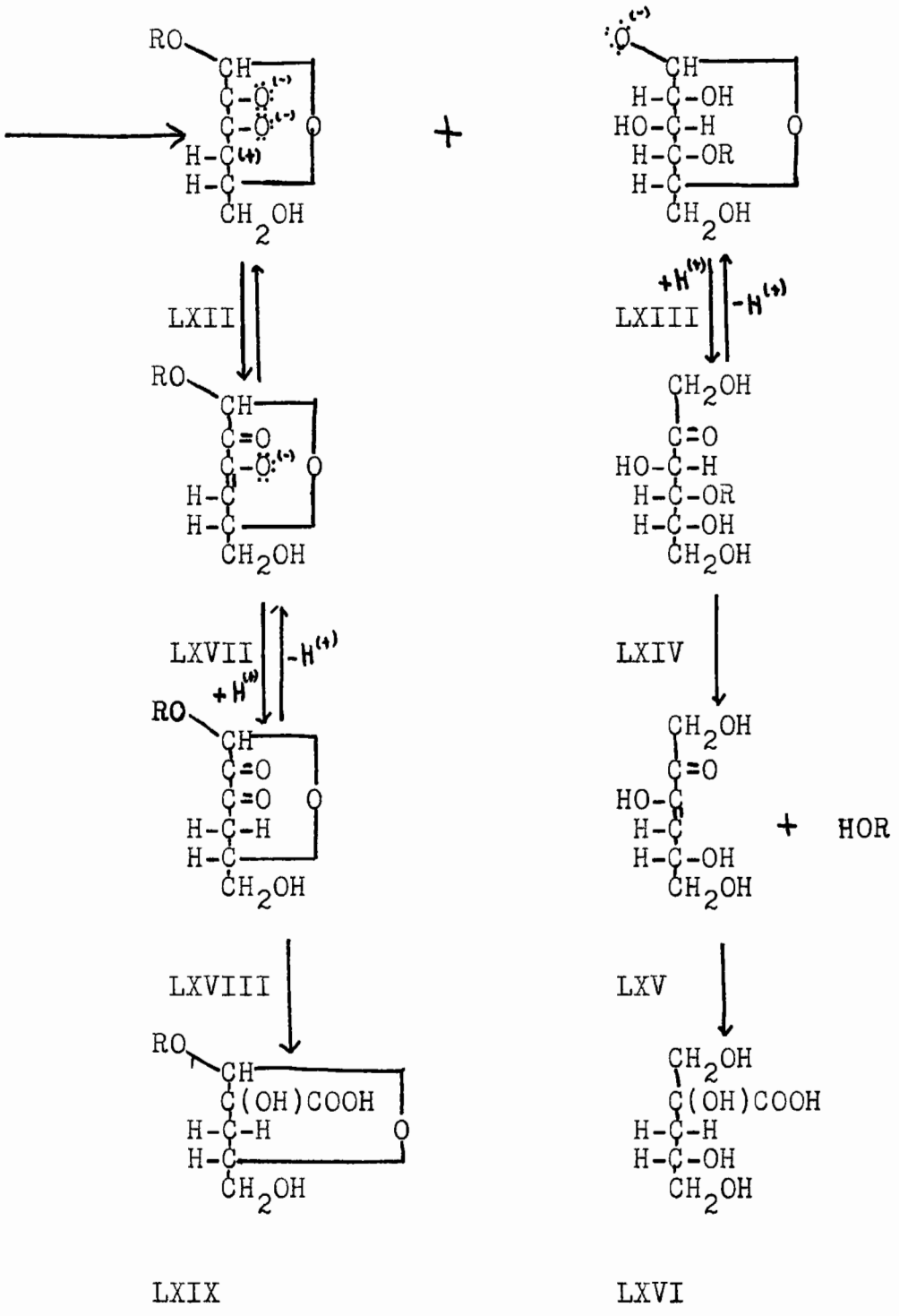
(Gal = Galactosyl)

First lactose was transformed into the keto form (lactulose), which enolized in alkaline solution. The resulting anion was transformed to a neutral product by extrusion of the

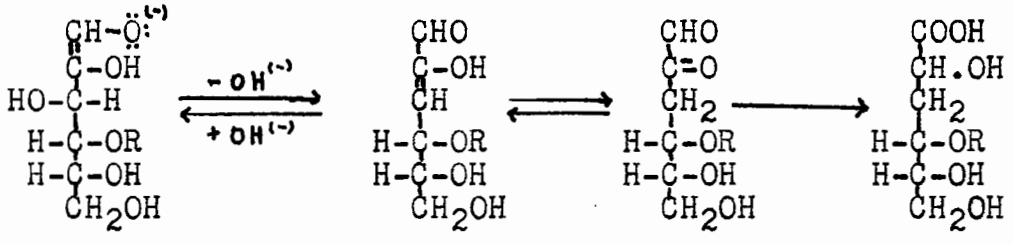
galactosyl group in its anionic form. This endiol (LVII), was in a tautomeric equilibrium with the α,β-diketone (LVIII), which in turn yielded iso-saccharinic acid (LIX), by benzilic acid rearrangement. Kenner (43) interpreted the alkaline decomposition of carbonyl oxycellulose along similar lines. Thus for 2- or 3- carbonyl oxycellulose (LX) the following mechanism was postulated:



LX LXI


LXII
LXVII
LXVIII
LXIII
LXIV
LXV
+ HOR
LXIX
LXVI

The anhydroglucose unit containing the carbonyl group on C_2 or C_3 was transformed to a doubly negatively charged anion (LXI). Scission of the cellulose chain took place, exposing the reducing end-group of the next glucose unit (LXIII), which partly rearranged to fructose (LXIV), by the Lobry de Bruyn transformation. The glucosidic linkage at C_4 was then in the β-position to the carbonyl group and this linkage was again cleaved, exposing the next glucose unit. Thus a step by step degradation resulted. Such a degradation would have proceeded indefinitely, until all glucose units were successively peeled off. It was known, however, that alkaline depolymerization of oxycellulose did not proceed to completion. Quite recently Machell and Richards (44) have pointed out that the anion (LXIII) was in equilibrium with a second anion (LXX), which could undergo aldol dehydration and benzilic acid rearrangement to yield a 4-substituted metasaccharinic acid (LXXIII). This reaction would have terminated the depolymerization as the substituted metasaccharinic acid was resistent to alkali.



LXX LXXI LXXII LXXIII

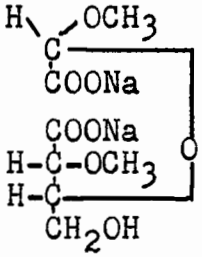
Treatment of maltose and maltulose with lime water under the same experimental conditions resulted in α- and

β-isosaccharinic acids which were isolated as their calcium salts, and also in the formation of glucose and fructose which were detected by paper chromatography (45). Maltulose was formed during the degradation of maltose, but its detection was quite difficult because of its much faster rate of decomposition. The corresponding rates of degradation were 7.5 and 50.6% after 2 hours and 83.4% and 95.5% after 24 hours for maltose and maltulose, respectively. In another experiment, cellobiose and cellotetraose were treated with lime water under the same conditions (46). The corresponding degradations were 39% and 21% after 24 hours; 83.5% and 46.5% after 70-80 hours, respectively. Cellotriose, cellotriulose, cellobiose, cellobiulose, glucose, fructose and saccharinic acids were detected by paper chromatography during the decomposition of cellotetraose. These results were in excellent agreement with the reaction mechanism postulated by the authors, which has been described above.

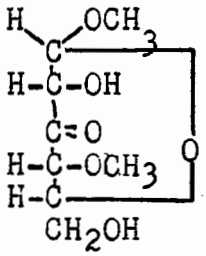
A similar degradation of 4-O-methyl-D-fructose and of 4-O-methyl-D-glucose resulted in the formation of α- and β-isosaccharinic acids and of methanol (47).

Since the above was written, a more comprehensive review of the highly scattered literature dealing with the alkaline degradation of polysaccharides was published by Whistler and Be Miller (48).

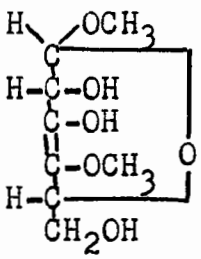
Two or three years previously, Whistler and other collaborators (49) published detailed information about the products formed when methyl-α and β-4-O-methyl-D-glucopyranoside were oxidized with 10 equivalents of sodium hypochlorite at pH 9. After separation of the products on a cellulose column, the disodium salt of 2-O-methyl-3-O-(glyoxylic acid methyl acetal)-D-erythronic acid (LXXIV) was obtained in 35% yield by weight. Glyoxylic acid, 0.33 to 0.43 mole, was isolated as the 2,4-dinitrophenylhydrazone directly from the hydrolysate, but the bis-dinitrophenylhydrazone of glyoxal was also recovered, the amount being 0.1 mole throughout the oxidation.



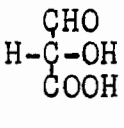
LXXIV



LXXV



LXXVI



LXXVII

The authors explained the presence of glyoxal among the degradation products by assuming that the D-glucopyranoside was first oxidized to a 3-keto-sugar (LXXV) which enolized in the alkaline medium at the 3-4 positions (LXXVI). Oxidation of the endiol produced 2-carboxyglycolic aldehyde (LXXVII), which was known to yield glyoxal-bis-phenylhydrazone with phenylhydrazine (50). By analogy 2,4-dinitrophenylhydrazine should have behaved in the same way.

The presence of the methyl group at the 4-position would impose a definite limitation to oxidation of this carbon atom to a carbonyl group. When methyl-2-O-methyl-α- D-glucopyranoside was oxidized with 10.6 and 34.5 equivalents of hypochlorite at pH 9 (51), the yields of gloxal bis-2,4-dinitrophenylhydrazone increased to 0.42 and 0.54 moles, respectively. In this case, the methyl group in the 2-position of the methylglucoside left the 3- and 4-positions more susceptible to oxidation, and an increased formation of the 3-4 endiol was to be expected.

Whistler's recent work on the oxidation of starch with alkaline hypochlorite (49,52), will be discussed later.

RESULTS AND DISCUSSION

In the course of this work, it was necessary to prepare several known compounds in order to compare their chromatographic flow rates and infra-red spectra with the compounds isolated from oxidized starch. These preparations consumed a considerable amount of time, and led to substantial improvements in method in case of glyoxylic and D-glucuronic acids.

Goebel and Babers (53) converted D-1,4-glucuronolactone to the neutral barium salt, which was isolated and decomposed with sulphuric acid. This method was simplified in that the lactone was directly converted to the acid by adsorbtion on a strong anion-exchange resin. The adsorbed free acid was then eluted with dilute acetic acid and isolated in the usual way.

Glyoxylic acid was prepared by oxidizing L-tartaric acid with about 1 molar equivalent of periodate according to the method of Weissbach and Sprinson (54). These authors extracted glyoxylic acid from the reaction mixture with ether, neutralized the ether extract with sodium hydroxide and isolated the crystalline sodium glyoxylate in 80% yield. We preferred to remove all iodate and periodate ions from the reaction mixture as the insoluble lead salts prior to

neutralization of the filtrate and isolation of the desired glyoxylic acid. A new salt, calcium sodium glyoxylate $CaNa_2(OOCCHO)_4.4H_2O$ was prepared in 87% yield. In another run, the acid was isolated in 87% yield as its barium salt $Ba(OOCCHO)_2.2H_2O$, which was quite readily soluble in water, Hatcher and Holden (55) finding only the very limited solubility of 0.005% at 18° and 0.08% at 65°. The salt was freed from barium by treatment with a cation-exchange resin and the resulting solution eventually yielded crystalline, anhydrous glyoxylic acid melting at 104-107°. Only Hatcher and Holden previously crystallized this compound and then found a melting point of 98°. All attempts by other workers to crystallize anhydrous glyoxylic acid appeared to have failed (56).

D-Erythronolactone was prepared by oxidizing starch first with periodate and then with bromine and hydrolysing the product as described by Jayme and Maris (57). It was found necessary to isolate the crude product first as brucine salt, if the lactone was to be obtained in a pure crystalline form.

Preliminary Oxidations with Calcium Hypochlorite

The wheat starch used in this work was purified by extraction with 97% acetic acid according to the method of Murray and Purves (58) to remove fats, waxes and most of

the ash and nitrogeneous compounds. This treatment was also known to change thick-boiling into thin-boiling starch. The starch was then gelatinized by heating with water and oxidized with calcium hypochlorite at room temperature. The alkalinity of the mixture was maintained at pH 12 during the entire reaction by suitable additions of calcium hydroxide.

In oxidations 1 and 2 the initial amount of the hypochlorite corresponded to 1.06 and 0.53 moles per mole of anhydroglucose respectively, and the consumption of oxidant was followed by titrations both with sodium thiosulphate and with sodium arsenite-iodine. Table I shows that both methods gave practically identical results. Davidson (59) determined that the dissociation constant of hypochlorous acid was given by the equation

$$\frac{[H^+]\ [ClO^-]}{[HClO]} = 3.7\times10^{-8}$$

so that at pH 12

$$\frac{[ClO^-]}{[HClO]} = \frac{3.7\times10^{-8}}{10^{-12}} = 3.7\times10^{4}$$

The concentration of undissociated hypochlorous acid at pH 12 was thus only 0.0027% of the hypochlorite ion concentration. Hypochlorous acid might react with hypochlorite ion to yield chlorate ion: $2\ HClO + ClO^- = 2\ HCl + ClO_3^-$ (60), but owing to the very low concentration of hypochlorous acid, this reaction would be insignificant at pH 12. Quite recently, Whistler and Schweiger (52) found that only 0.5% of the hypochlorite was converted to chlorate during the oxidation of amylopectin with 0.5-3.0 mole of hypochlorite per anhydroglucose

TABLE I

PRELIMINARY OXIDATIONS OF STARCH WITH HYPOCHLORITE

Experiment 1

Initial concentration: oxidant 0.1171 mole hypochlorite/l
starch 0.1102 mole $C_6H_{10}O_5$/l

Oxidation Hours	Moles Hypochlorite per $C_6H_{10}O_5$ by $Na_2S_2O_3$	by $Na_3AsO_3 - I_2$	pH
0.0	0.000	0.000	12.25
0.4	0.008	-	-
0.8	-	0.012	-
2.0	0.035	0.030	11.95
14.0	0.248	0.243	11.40(1)
22.0	0.409	0.408	11.80(1)
44.0	0.731	0.747	11.80

Experiment 2

Initial concentration: oxidant 0.0598 mole hypochlorite/l
starch 0.1125 mole $C_6H_{10}O_5$/l

0.0	0.000	-	12.20
1.4	0.000	0.004	12.10
13.1	0.099	0.124	11.80(1)
21.5	0.166	0.177	12.30
44.0	0.310	0.327	12.30

(1) Powdered calcium hydroxide, 0.5 g, added, causing a decrease in hydrogen ion concentration to pH 12 within a few minutes.

Blank

Oxidation Hours	Moles Hypochlorite per $C_6H_{10}O_5$/l by $Na_2S_2O_3$	by $Na_3AsO_3 - I_2$	pH
0.0	0.1177	-	11.80
1.5	0.1172	-	12.40
14.3	0.1177	0.1180	12.25
22.5	0.1177	-	12.30
44.0	0.1177	-	12.05

unit at pH 12. In the present work a hypochlorite blank remained unchanged for over 44 hours at pH 12. Any conversion to chlorate would have caused lower titration values with the sodium thiosulphate and arsenite-iodine methods, as chlorate was reduced by neither of these reagents.

Oxidations 1 and 2 proceeded slowly and after 44 hours only 69.5% and 59.8%, respectively, of the hypochlorite initially present was consumed.

Oxidation 3 was accordingly carried out at a much higher concentration of hypochlorite and with a hypochlorite starch ratio of 5.5 to 1, the other conditions being the same. This large ratio was expected to increase the reaction rate, and to simplify the shape of the corresponding rate plot. The data in columns 1 and 3 of Table II gave a smooth plot, (Fig. 1a) but a plot of $\log \frac{[\text{Initial } ClO^-]}{[\text{Remaining } ClO^-]}$ against time became linear after 76 hours (Fig. 1b), as a first order or pseudo first order

Figure 1a

Oxidation of 0.183 base molar starch solution with 1.33 volumes of 0.756 M calcium hypochlorite.

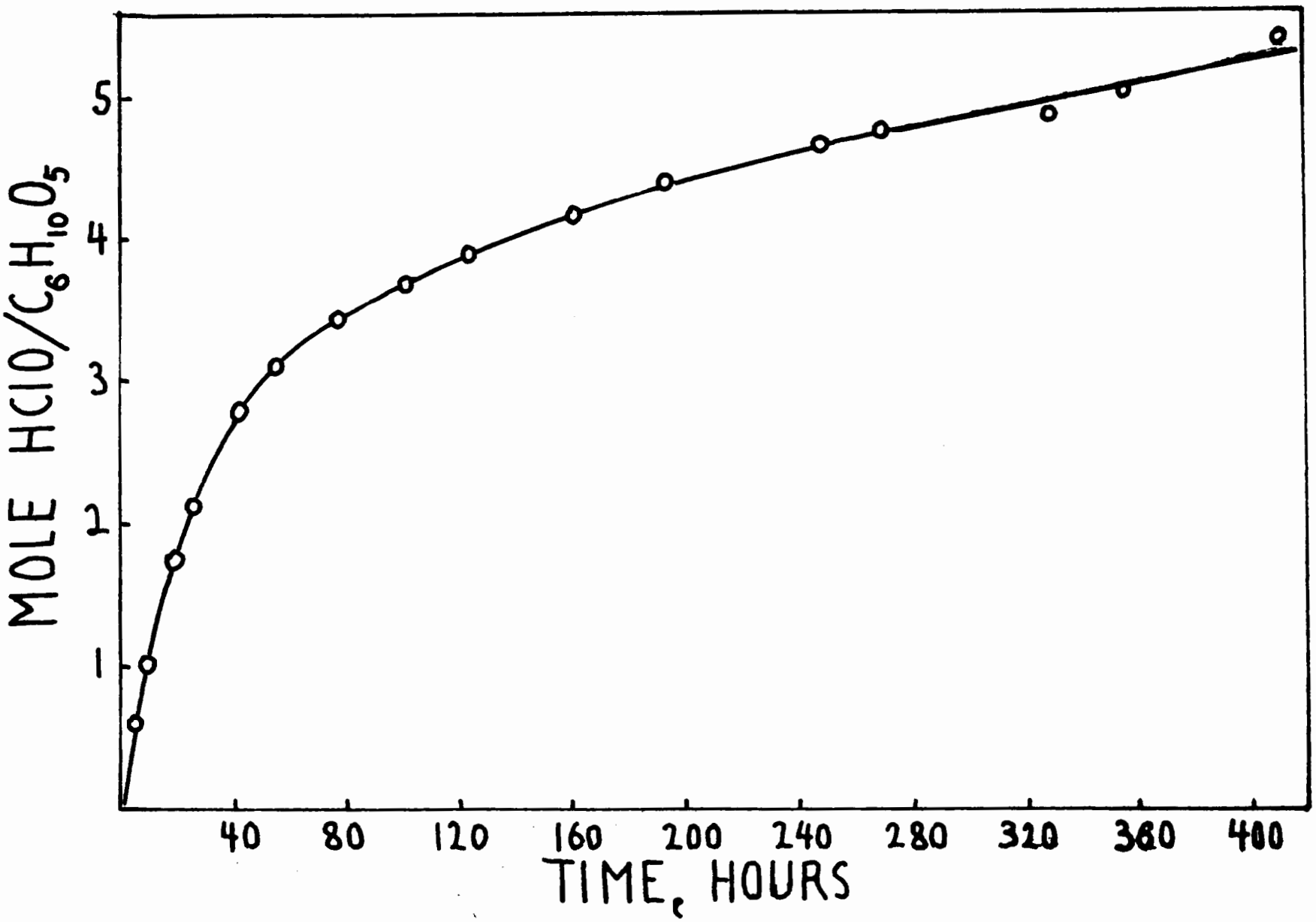

MOLE HClO/C6H10O5
1
2
3
4
5
40
80
120
160
200
240
280
320
360
400
TIME, HOURS

Figure 1b

Oxidation of 0.183 base molar starch solution with 1.33 volumes of 0.756 M calcium hypochlorite. Ordinates, log of ratio of initial to remaining concentration of hypochlorite.

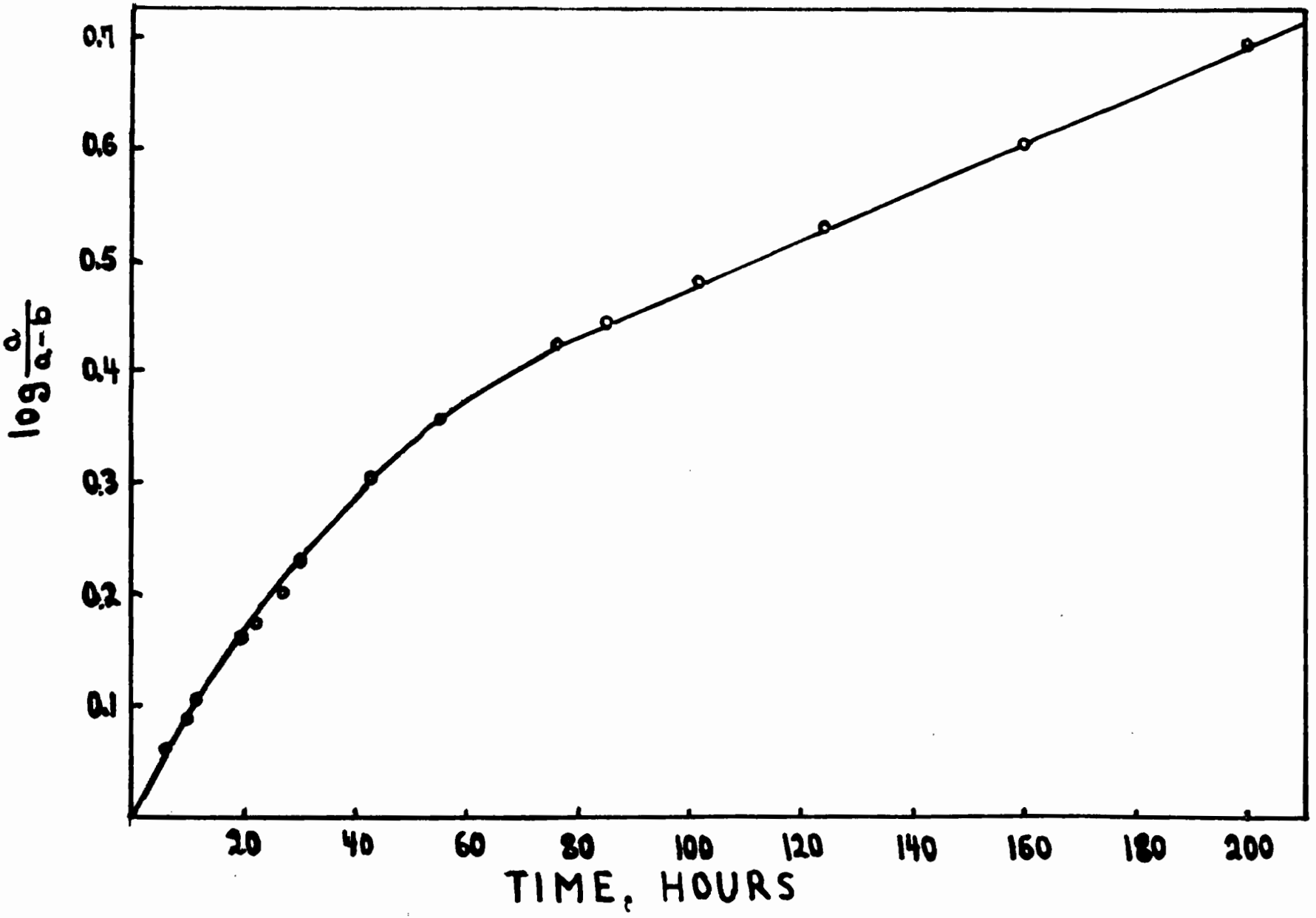

0.7
0.6
0.5
0.4
0.3
0.2
0.1
$\log \frac{a}{a-b}$
20
40
60
80
100
120
140
160
180
200
TIME, HOURS

TABLE II

OXIDATION OF STARCH WITH 5.5 MOLAR RATIO OF HYPOCHLORITE

Initial concentration; oxidant 0.433 mole hypochlorite/l
starch 0.0787 mole $C_6H_{10}O_5$/l

Oxidation Hours	Hypochlorite Concentration M	Moles HClO consumed per Mole $C_6H_{10}O_5$	$\log \frac{a}{a-b}$ [1]	pH
0.0	0.433	0.000	0.000	11.9
0.9	0.425	0.097	0.008	-
4.5	0.385	0.615	0.051	12.0
5.5	0.379	0.692	0.058	-
6.5	0.372	0.772	0.066	-
9.0	0.352	1.030	0.090	11.85
10.7	0.339	1.190	0.109	11.95
18.5	0.296	1.737	0.165	11.55[2]
20.3	0.289	1.834	0.176	11.9
22.0	0.283	1.908	0.185	-
26.0	0.266	2.124	0.212	11.85[2]
28.7	0.253	2.284	0.233	12.05
41.7	0.215	2.767	0.304	12.05
54.0	0.190	3.089	0.358	-
76.0	0.162	3.443	0.427	-
101.3	0.142	3.700	0.484	-
124.0	0.127	3.893	0.533	-
159.5	0.106	4.157	0.611	-
192.5	0.086	4.408	0.702	-

Oxidation Hours	Hypochlorite Concentration M	Moles HClO consumed per Mole $C_6H_{10}O_5$	$\log \frac{a}{a-b}$ (1)	pH
248.5	0.065	4.681	0.824	-
267.5	0.058	4.762	0.873	-
329.0	0.048	4.891	0.955	11.6
354.5	0.035	5.051	1.086	-
412.5	0.023	5.212	1.260	-

(1) a = Initial concentration of hypochlorite;

a - b = concentration remaining at time of sampling

(2) One gram of calcium hydroxide added

reaction would require. The bent shape of the plot from zero to 76 hours suggested that there might be a second, faster reaction, superimposed on the slower first order reaction, and that this faster reaction was completed after 76 hours. Mathematical treatment of the data according to Mahoney and Purves (61) showed that the straight part of the plot could be expressed by the equation

$$\log \frac{a}{a-b} = \log \frac{a}{a_2} + k_2 t \qquad (1)$$

a, b and t having their customary significance and the suscript$_2$ applying to the slower of the two reactions, so that a_2 was the relevant initial concentration of hypochlorite and k_2 the reaction rate. Since a had the value 0.433 mole/l., equation (1) became

$$\log \frac{0.433}{0.433-b} = \log \frac{0.433}{a_2} + k_2 t$$

The values of a_2 and k_2 were determined by substitution of known vales for b and t for the straight part of the plot, and were found to be: $a_2 = 0.239$ mole/l. and $k_2 = 2.21 \times 10^{-3}$ mole/l./hours. As the slower reaction proceeded according to the equation

$$\log \frac{a_2}{a_2 - b_2} = k_2 t$$

substitution of the known values for a_2 and k_2 gave the expression

$$\log \frac{0.239}{0.239 - b_2} = 2.21 \times 10^{-3} t \qquad (2)$$

If the faster reaction was also of the first order it would follow the equation

$$\log \frac{a_1}{a_1 - b_1} = k_1 t \qquad (3)$$

The value for a_1 was obtained by substraction of the initial concentration of the hypochlorite required for the slower reaction from the total initial concentration of hypochlorite, thus $a_1 = a - a_2$ and by substitution $a_1 = 0.194$ moles/l.

Values for b_1 were found from the relationship $b_1 + b_2 = b$; b_2 was calculated from equation (2) for reaction times of 5.5, 10.7 and 41.7 hours. The corresponding b_2 values were 0.0068, 0.0123 and 0.0456 moles/l., respectively. When these values were substituted into equation (3) k_1 was found to be 2.21×10^{-2}, 2.22×10^{-2} and 2.28×10^{-2}. These calculations, and the close agreement of the k_1 values, showed

that the faster oxidation also behaved as a reaction of the first order according to the equation

$$\log \frac{0.194}{0.194-b_1} = 2.24\times10^{-2}t \qquad (4)$$

The above calculations were not inconsistent with the view that there were two different hypochlorite molecules reacting with the starch at a different rate. However, the hypochlorite-hypochlorous acid equilibrium must be very mobile and this possibility appeared most unlikely. On the other hand the data also suggested that the hypochlorite attacked the starch in two different ways, the faster reaction being terminated after (0.194 x 5.50 / 0.433) = 2.46 moles of hypochlorite per mole of anhydroglucose unit were consumed. The appearance of both reactions as first order might be due to the large excess of hypochlorite used. Second order would appear as first order reactions when $b \gg a$ and hence $b - x \approx b$; $b - a \approx b$, where x was the amount reduced at anytime.

Hence $k = \frac{1}{t(a-b)} \ln \frac{b(a-x)}{a(b-x)}$ became $k = -\frac{1}{tb} \ln \frac{a-x}{a}$

and $k = \frac{1}{tb} \ln \frac{a}{a-x}$, where b was a constant

The fourth preliminary experiment was with sodium hypochlorite at pH 12 and with a lower concentration of both reactants. The initial ratio of hypochlorite to anhydroglucose units was 6.08 : 1, but the oxidation proceeded so

slowly that after 76 hours only 0.68 mole of hypochlorite per anhydroglucose unit was consumed (Table III). A plot of log $\frac{a}{a-b}$ against the reaction time failed to give a straight line and work with sodium hypochlorite was discontinued.

Preliminary Investigation of the Products

Oxidations of the starch with calcium hypochlorite were repeated under exactly the same conditions as in Table II. (Oxidation 3). After 3.0 mole of hypochlorite per anhydroglucose unit had been consumed, the excess oxidant was destroyed by the addition of acetone, and the insoluble reaction products, contaminated with large amounts of calcium hydroxide and calcium carbonate, were isolated by filtration. In oxidation 5 these insoluble products corresponded to 143% by weight of the original starch.

Found: Ca, 20.1%.

Treatment with 0.1 M oxalic acid, in an amount necessary to convert all of the calcium into calcium oxalate, caused much of the solids to dissolve. A part of the oxalic acid, however, was not used at all, and had to be reprecipitated from the filtrate with calcium acetate solution. Analyses of the calcium oxalate fractions by titration with acid permanganate revealed an excess of permanganate reducing material which corresponded to 10% of oxalic acid based on the starch. Thus it appeared likely that calcium oxalate had already been present among the insoluble reaction products.

TABLE III

OXIDATION OF STARCH WITH SODIUM HYPOCHLORITE

Initial concentrations; oxidant: 0.259 mole hypochlorite/l
starch : 0.0426 mole $C_6H_{10}O_5$/l

Oxidation Hours	Moles HClO consumed per Mole $C_6H_{10}O_5$(1)	pH
0.0	0.000	-
0.9	0.023	12.0
4.5	0.023	12.1
5.7	0.047	-
6.7	0.070	12.2
9.0	0.106	12.3
10.8	0.106	12.25
18.6	0.211	12.0
26.5	0.258	12.1
42.3	0.446	12.1
54.0	0.493	-
76.0	0.681	12.0

(1) Determined iodometrically with standard sodium thiosulphate solution.

A material balance of the calcium oxalate fraction with the amount of calcium oxalate expected from the addition of the 0.1 M oxalic acid, showed the presence of other insoluble products in an amount equivalent to 33.4% by weight of the original starch.

Evaporation of the filtrates from the calcium oxalate fractions yielded 61% by weight of carbohydrate acids with a copper reducing power of 2.9% as glucose. Their specific rotation $[\alpha]_D^{22}$ 93.5°, was greatly different from that of starch $[\alpha]_D^{20}$ 203°. At this point the experiment was abandoned, because a part of the oxidation products remained in the calcium oxalate fractions and was difficult to separate. Also no possibility was seen of establishing the possible presence of oxalic acid among the degradation products.

Removal of the calcium with dilute alcoholic hydrochloric acid was tried next. Starch oxidation 6 produced the insoluble reaction product in 225% yield by weight; found: Ca, 31.3%. The higher yield and calcium content were due to the presence of an increased amount of inorganic salts, such as calcium hydroxide and calcium carbonate. The product was then digested at -10°for one hour with 0.1 N hydrochloric acid containing 70% and 90%, respectively, of alcohol. Under these conditions no appreciable hydrolysis of the glucosidic linkages of the oxidized starch would be expected. The amount of acid used in the first digestion was 50% in excess of that theoretically required to convert all of the calcium into calcium chloride, which was readily soluble in aqueous alcohol. In the second digestion, a fourfold excess of the acid was used. Both treatments failed to reduce the calcium content of the product below 13.6% but dissolved most of the carbohydrate

material. The yields of the insoluble product were 18.8 and 55.8%, respectively. Since the starch and cellulose of higher molecular weight were insoluble in 70 and 90% aqueous alcohol, the high solubility of the product indicated that the alkaline hypochlorite oxidation had resulted in extensive degradation. The digestion with alcoholic hydrochloric acid was discarded.

Better results were obtained by neutralization of the insoluble reaction product to pH 7, followed by precipitation with aqueous alcohol. In a preliminary test, a part of the product from oxidation 6 was dispersed in water and the mixture was adjusted to pH 7 with hydrochloric acid. In this way, all of the calcium hydroxide was converted to the water-soluble calcium chloride, and on filtration the water-insoluble calcium salts of the sugar acids were obtained, together with calcium carbonate in 83% yield by weight. (Fraction I). Found: Ca, 25.0%. Concentration of the filtrate and addition of alcohol yielded the water-soluble calcium salts of the sugar acids as fractions IIb and IIc, the combined yield being 39.7%. Found: Ca, 14.7%, 10.9%, respectively. A part of these water-soluble calcium salts was freed from calcium ions by treatment with a cation-exchange resin. Hydrolysis of this material and analysis for reducing sugars by paper chromatography revealed the presence of glucose and glyoxylic acid. Thus it became probable that the oxidative attack was random; some of the glucose units not being attacked at all. The presence of

glyoxylic acid confirmed the results of Whistler, Linke and Kazeniac (49) and suggested that oxidation of the secondary alcohol unit on carbon number 2 to a carboxyl group had occurred.

Neutralization with carbon dioxide of the soluble products from starch oxidation 6 precipitated calcium hydroxide as the carbonate. On concentration of the filtrate crystals separated, which consisted of calcium acetate monohydrate, contaminated with calcium chloride and calcium carbonate. Calcium acetate and chloroform had been formed when acetone was added to destroy the residual hypochlorite at the end of the oxidation. This reaction proceeded according to the equation:

$$2\ CH_3COCH_3 + 3\ Ca(ClO)_2 = 2\ HCCl_3 + (CH_3COO)_2Ca + 2\ Ca(OH)_2$$

No other material was precipitated from the concentrated, soluble part of the reaction mixture, even after additions of large volumes of ethanol. In subsequent experiments this soluble portion was not further investigated, and all attention was placed on the insoluble fraction.

Recovery and Identification of the Oxidation Products

Preparation of the Calcium Salts

The isolation of reaction products by fractionation at neutral pH showed promise and further work was continued along

this line. Two more oxidations of starch under standard reaction conditions were carried out. Oxidation 7 gave the neutral water-insoluble fraction I in 68.8% yield, (found: Ca, 22.4%) and the water-soluble fraction II in 49.5% yield (found: Ca, 8.7%). In oxidation 8 a still higher yield of the neutral, water-soluble fraction II was obtained owing to a prolonged fractionation from aqueous alcohol of the mother liquors. Yield fraction I, 37.5% (found: Ca, 23.2%) fraction II, 87.0% (found: Ca, 11.0%). All these yields, which were based on the weight of the original starch, were crude and were not corrected for calcium carbonate, calcium chloride and moisture.

A part of fraction II from oxidation 8, which consisted mostly of the water-soluble calcium salts of sugar acids, was freed from calcium by treatment with cation-exchange resin. On evaporation of the eluate the free sugar acids were obtained in 70.5% yield by weight of fraction II. This yield was in good agreement with the value of 73.6%, calculated from the moisture, calcium chloride and inorganic carbonate content of fraction II. The free sugar acids in that fraction represented thus 61.3% and 64.0%, respectively, by weight of the starch.

The sugar acids were 84% dialysable, so that an extensive degradation of the starch must have taken place during the alkaline hypochlorite oxidation. The neutralization equivalent, 173, showed excellent agreement with the value

of 173, calculated from the calcium content of fraction II and corrected for moisture, calcium chloride and calcium carbonate. These neutralization equivalents corresponded to 740, 772 millimoles of carboxyl, respectively, in fraction II.

Fraction II had a very low copper reducing value, 2.7%, 2.9% as glucose, thus indicating a very small amount of carbonyl groups. This result was in agreement with expectation, as carbonyl groups were unstable in alkaline hypochlorite and would be further oxidized to carboxylic acids.

Whistler, Martin and Harris (62), measured the rate of evolution of carbon dioxide from uronic acids, and uronic anhydride-containing carbohydrates, when boiled with 12% hydrochloric acid under conditions commonly used for the determination of uronic anhydrides. They found that the carbon dioxide evolution from uronic acids was about complete after three hours, but that some non-uronic carbohydrates evolved traces of carbon dioxide at a constant rate during the total measured period of eight hours. The authors suggested that determinations of carbon dioxide evolution after three and five hours refluxing with 12% hydrochloric acid, and extrapolation to zero time, would correct for the non-uronic material and give the real or "intrinsic" uronic anhydride content. This extrapolation is used by the Pulp and Paper Research Institute of Canada in their standard testing procedure for uronic acids in wood, pulp and hemicelluloses.

The "intrinsic" uronic anhydride content of fraction II (starch oxidation 8) was 17.1, 17.6% and the pentosan content, determined by distilling fraction II with 12% hydrochloric acid, was 3.7%. It is well known that under these conditions polyuronic acids were decarboxylated and partially transformed into furfuraldehyde. Norris and Resch (63), found that the yield of furfuraldehyde from pure glucuronic acid was 39.4% of theory, corresponding to a ratio of uronic anhydride to furfural of 4.66 : 1. Thus pentosan values might be transformed into uronic anhydride by the simple relationship:
% pentosan x 4.66 x 0.727 x 0.88 = % uronic anhydride, where 0.727 and 0.88 were correction factors used by the Pulp and Paper Research Institute of Canada in their standard method to convert furfuraldehyde into pentosan. The pentosan content of fraction II corresponded to 11.0% of uronic anhydride, whereas 17.3% was found by the uronic anhydride determination. The true uronic anhydride content was probably between these two values.

A calculation from the calcium, inorganic carbonate, chloride and moisture content of fraction I (starch oxidation 8) showed that 79.1% of this product consisted of neutral calcium salts of organic acids, and that these salts contained 22.9% of calcium, a value which corresponded to a neutralization equivalent of 68.3. The free sugar acids were equivalent to 60.1% by weight of fraction I, and thus corresponded to 22.5% by weight of the original starch. It was not possible to verify these results, experimentally, as not all of the

calcium was removable with cation-exchange resin. The calculated neutralization equivalent corresponded to 688 millimoles of carboxyl in fraction I (starch oxidation 8).

The "intrinsic" uronic anhydride of fraction I was 6.6, 7.1% by direct determination. Calculation from the pentosan content of 1.14% gave an uronic anhydride value of 3.4%.

Hydrolysis and Separation of the Water-Soluble Calcium Salts

Samples of fraction II were hydrolysed by heating under reflux with N sulphuric acid and the increase in reducing value was followed by Somogyi's copper titration method. Results are shown in figures 2 and 3 in which the time scales differ. After three hours hydrolysis the reducing power was equivalent to 35.9% as glucose. From then on only a slight increase took place, so that after thirty hours the reducing power was 43.2%. This behaviour again suggested the presence of uronic acid groups in fraction II since polyuronides were known to hydrolyse only with considerable difficulty. A part of fraction II from starch oxidation 7 was converted to the free acid, and after hydrolysis, the sulphate ions were removed as barium sulphate and the filtrate was evaporated to a syrup. Several solvents were investigated as to their suitability for the separation of this syrup by paper chromatography. A solvent consisting of the organic phase of an equilibrated mixture of 115 parts of 85% formic acid, 385 parts of water and 500 parts of n-butanol was

Figure 2

Hydrolysis of oxystarch with boiling N sulphuric acid.

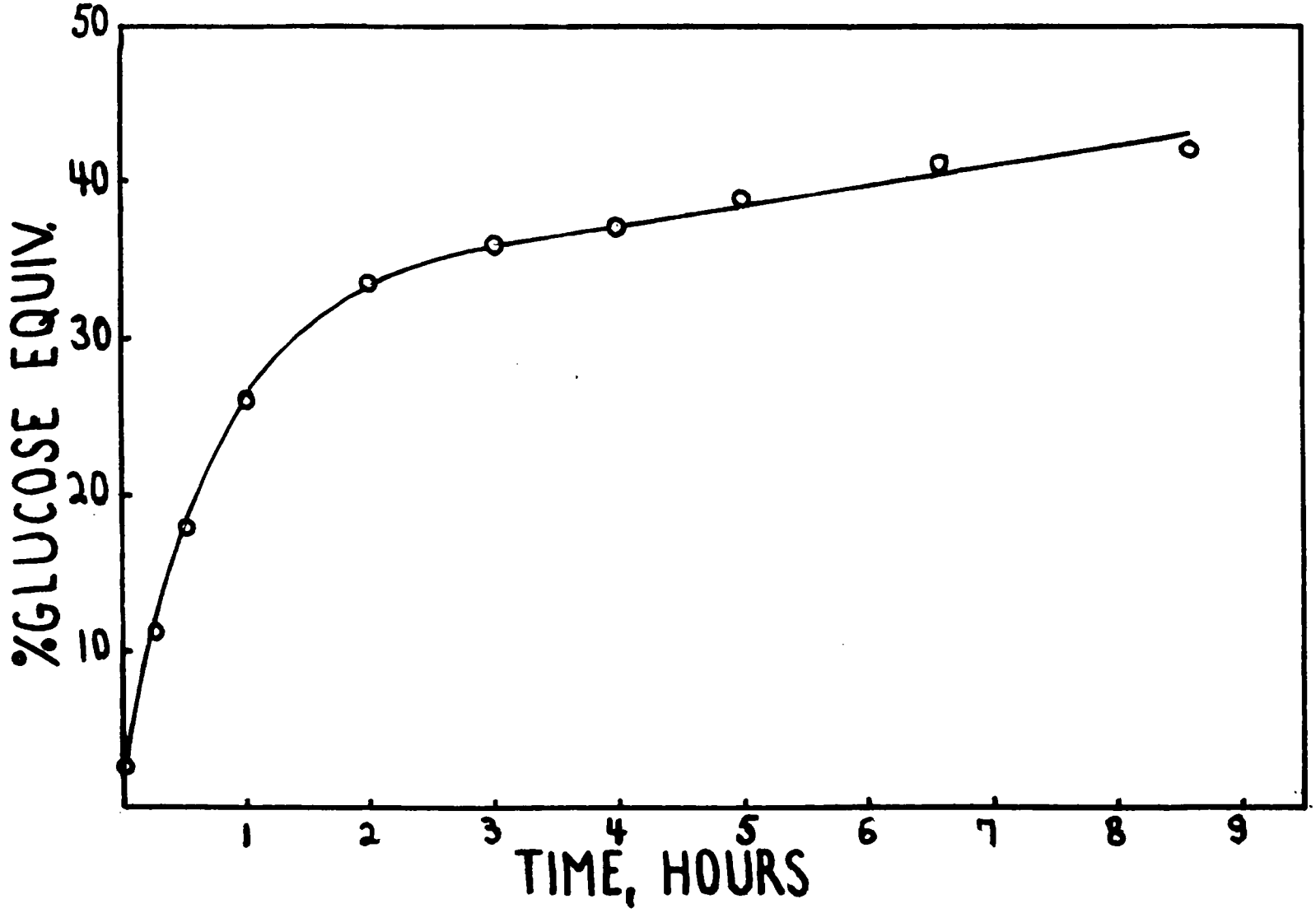
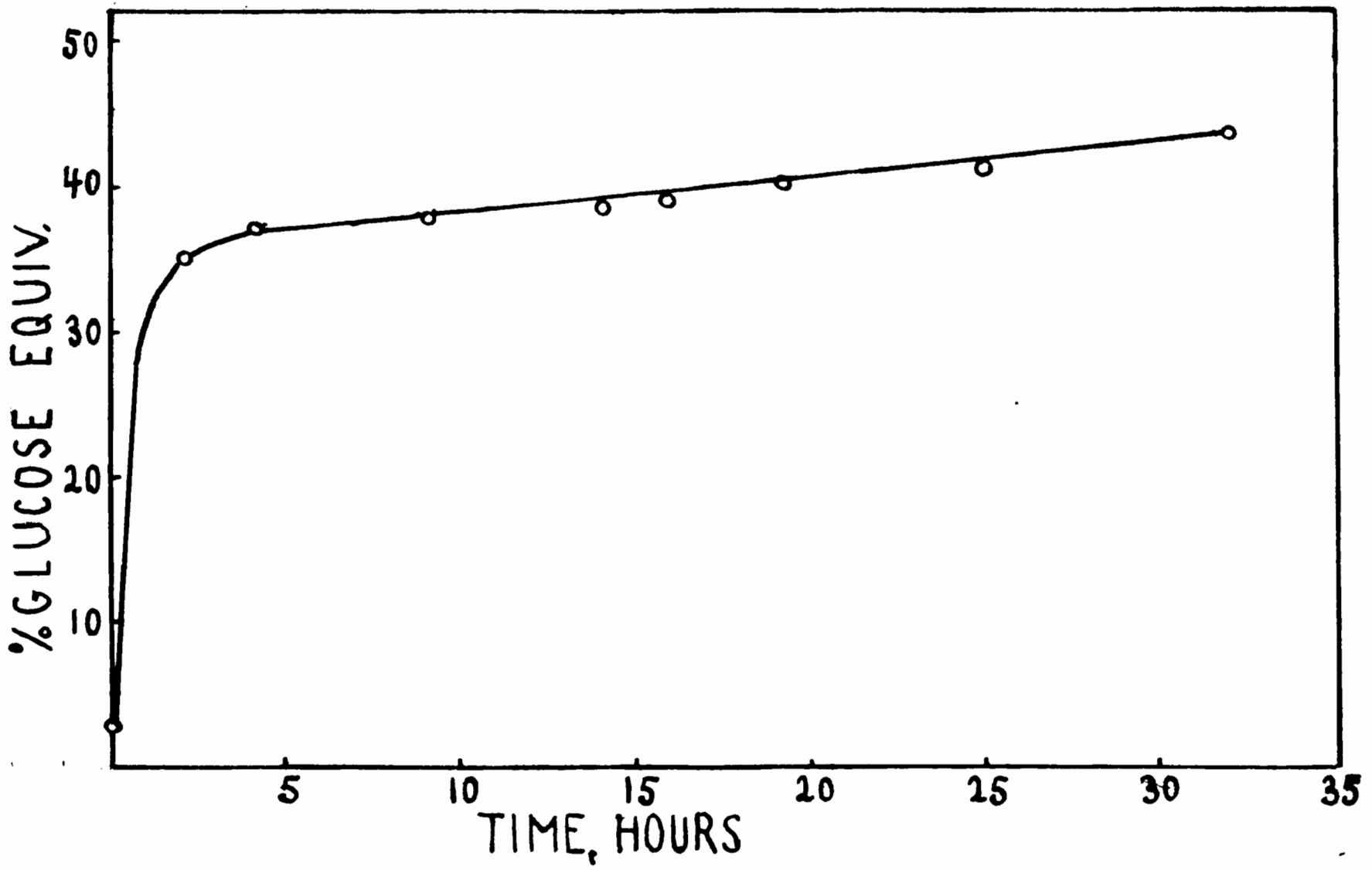

50
40
30
20
10
%GLUCOSE EQUIV.
1
2
3
4
5
6
7
8
9
TIME, HOURS

Figure 3

Hydrolysis of oxystarch with boiling N sulphuric acid over a prolonged period.


50
40
30
20
10
% GLUCOSE EQUIV.
5
10
15
20
25
30
35
TIME, HOURS

most satisfactory. The acidic components were made visible by drying the paper chromatogram under vacuum at 110° for several hours, to remove the last traces of formic acid and then spraying with a neutral alcoholic solution of bromphenol blue. Separations on a large scale were made on a cellulose column using the same solvent system. The individual effluent fractions were analyzed by paper chromatography with the results shown in Table 4. Four main acidic compounds were present, of which three had the same chromatographic flow rate as glyoxylic, D-erythronic and optically active tartaric acid. Fractions containing the same compounds were combined and evaporated to a syrup, but attempts to isolate crystalline derivatives failed. The cellulose column was finally eluted with water, and concentration of the aqueous effluent yielded 46.2% of a partially crystalline syrup, consisting nearly entirely of D-glucose. D-glucose was identified by the preparation of the phenylglucosazone and its conversion to the phenylglucotriazole.

In the subsequent work, fraction II from the oxidation 8 was used. Calcium ions were removed, the mixture was evaporated to a syrup and was hydrolysed by heating under reflux with N sulphuric acid, for two and one half hours. These conditions were chosen because previous hydrolyses showed that the reducing power of the hydrolysate reached a nearly constant value after that time. The amount of

TABLE IV

CHROMATOGRAPHY OF HYDROLYSATE FROM FRACTION II (a)

Fraction #	Bromphenol Blue R_f Spot	Spray Intensity	Weight of Combined Fractions	Possible Compounds	Neutralization Equivalent
10	-	-			
20	-	-			
24	.61-.70 (b)	v.weak	#24-37, 30 mg (d)	Glyoxylic acid	
27	.60-.72 (b)	fair			
30	.62-.73 (c)	fair			
33	.63-.69 (b)	weak			
40	-	-			
50	-	-			
60	-	-	#68-80, 77.7 mg		120
70	.29-.32	fair			
80	.30-.33	fair	#80-89, 51.6 mg		
83	.30-.33	fair			
	.22-.26	fair			
87	.30-.33	weak			
	.22-.26	fair			
90	.22-.26	fair	#90-110, 54.6 mg	Erythronic acid	124
100	.22-.26	fair			
110	-	-	#111-159, 91.3 mg		
120	.17-.20	fair		Optically-active Tartaric acid	139
130	.17-.20	fair			

TABLE IV (cont'd).

Fraction #	Bromphenol Blue R_f	Spray Spot Intensity	Weight of combined Fractions	Possible Compounds	Neutralization Equivalent
140	.17-.20	fair			
150	.12-.20	fair			
160	-	-			
170	-	-			
190	-	-			
210	-	-			
230	-	-			
250	-	-			
Water Elution			675 mg	D-Glucose	
Total Recovery			928.6 mg or 60%		

(a) Using a cellulose column and an equilibrated organic phase of a mixture of 500 parts n-butanol, 385 parts of water and 115 parts of 85% formic acid as eluent.
(b) With o-aminodiphenyl as spray.
(c) With o-aminodiphenyl and bromphenol blue as sprays.
(d) Weight of the 2,4-dinitrophenylhydrazone.

carbon dioxide evolved during this hydrolysis was 23.4 mg per gram of fraction II, being equivalent to the destruction of 13.0% carboxyl groups in fraction II. Sulphate ions in the hydrolysate were removed by precipitation in the usual way, and most of the glucose in the filtrate was destroyed by fermentation with yeast. The resulting solution was then concentrated to a syrup. Great effort and a considerable amount of time were spent on the separation of this syrup by paper sheet chromatography but all attempts to isolate crystalline derivatives were unsuccessful.

Attention was then placed on other methods of separation. In 1952 Khym and Doherty (64) separated galacturonic and glucuronic acids in the presence of arabinose and galactose by ion-exchange chromatography. The acidic components of this mixture were adsorbed on a column containing Dowex-1, a strong anion-exchange resin, and on elution of the column with 0.15 M acetic acid the glucuronic and galacturonic acids were obtained in separate fractions. Quite recently, Timell and Gillham (65) isolated by the same method 4-O-methyl-glucuronic, aldobiuronic, glucuronic and galacturonic acids from partially hydrolysed hemicellulose of white elm. In ion-exchange chromatography the acidic components are adsorbed at the top of the resin column, then gradually displaced by an acidic eluant, and slowly moved toward the bottom of the column. The rate of this displacement would be dependent upon the dissociation constant of the acidic

component and the dissociation constant as well as the concentration of the acid in the eluate.

To investigate this method a separation on a small scale was carried out. A sample of fraction II, oxidation 8, was freed from calcium ions, hydrolysed and freed of sulphate ions in the usual way. The acidic components of the hydrolysate were adsorbed on a micro-column of only 2 cm length containing Dowex-1-x-4 anion-exchange resin in its free hydroxy form. This column was then eluted with 2 N acetic acid, followed by 1 N formic acid and 0.5 N trifluoroacetic acid. Analyses of the individual effluent fractions by paper chromatography showed a group-wise separation of the acidic components.

This preliminary separation was repeated on a large scale. A resin column of much larger diameter and 110 cm in length was chosen. Hydrolysed fraction II, from oxidation 8, free of calcium and sulphate ions, was prepared in the usual way, but no attempt was made to remove glucose by fermentation, since neutral compounds were not adsorbed on the resin. It had been pointed out by Pascu and Rebenfeld (66) that some epimerization might occur when aqueous solutions of reducing sugars were exposed to a strongly basic anion-exchange resin for a prolonged period of time. Care therefore was taken to pass the aqueous solution of the hydrolysate through the column as quickly as possible. The resin was then immediately reconverted to the acetate form with acetic acid. Chromatographic analyses of the neutral glucose effluent

was made for other reducing sugars, but none were detected. The column was then eluted with 2 N acetic acid, followed by 1 N formic and 0.5 N trifluoroacetic acid. The effluent was collected in 3594 fractions of 20 and 25 ml volume. Every twentieth or thirtieth fraction was analyzed by paper chromatography as summarized in Table 5. A graph representing the concentration of the individual effluent fractions against the volume of the acetic and formic acid effluents is shown in figure 4, compounds eventually identified being marked by shading.

Evaporation of the effluent fractions containing erythronolactone and erythronic acid yielded 8.4% (based on the starch) of a syrup, which crystallized in part after addition of a seed crystal. The pure compound was identified by its melting point, neutralization equivalent and specific rotation. A mixed melting point with D-erythronolactone prepared from periodate starch was undepressed. Infra-red spectra of the two samples were identical.

Fractions containing glucuronic acid, when purified, yielded 0.4% of a syrup which crystallized on addition of alcohol and a few seed crystals. The pure compound had a melting point and specific rotation in good agreement with values reported in the literature. Chromatographic flow rate and infra-red spectrum were identical with those of D-glucuronic acid prepared from D-glucuronolactone.

Fractions containing glyoxylic acid yielded a syrup,

TABLE V

COLUMN CHROMATOGRAPHY OF OXYSTARCH HYDROLYSATE ON DOWEX 1-x-4 (ACETATE FORM)

Eluent 2 N Acetic Acid

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
20	-	-	-	-	-	-		
40	-	-	-	-	-	-		
60	-	-	-	Trace	-	-	Glucose	
80	-	-	-	Fair	-	-	Glucose	
98	-	-	-	Trace	-	-	Glucose	
120	-	-	-	-	.37-.42	Weak		
140	-	-	-	-	.07-.09	Weak		
					.37-.42	Trace		
160	-	-	-	-	.07-.09	Fair		
	-	-	-	-	.37-.42	Trace		
180	-	-	-	-	.07-.09	Fair		#151-#225
	.12-.17	Trace	-	-				0.327 g
200			.00-.05	Weak				
					.07-.09	Fair		
	.12-.17	V.Weak						
220			.00-.05	Weak				
					.07-.09	Fair		
	.12-.17	Weak						
240			.00-.05	Weak				#226-#340
					.07-.09	Fair		0.845 g
	.12-.17	Fair						
260			.00-.05	Weak				
					.07-.09	Fair		
	.11-.18	Strong						
280			.00-.05	Weak				
					.07-.09	Weak		
	.11-.18	Strong						

TABLE V (cont'd).

Fraction #	Sprays				Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride	
	R_f	Intensity	R_f	Intensity	R_f Intensity	
300			.00	Trace		
			.00-.05	Fair		
					.07-.09 Weak	
			.07-.11	Trace		
	.12-.17	Strong				
320			.00	Fair		
			.00-.05	Fair		
			.07-.09	Weak		
					.07-.09 V.Weak	
	.12-.17	Strong				
			.13-.15	Fair		
	.19-.23	Trace				
340			.00	Fair		#341-#375
			.00-.05	Fair		0.426 g
					.07-.09 Fair	
			.07-.11	Weak		
	.12-.15	Fair				
			.13-.15	Fair		
	.12-.23	V.Weak				
	.72-.74	Weak				
360			.00	Fair		
			.00-.05	Fair		
					.07-.09 Trace	
			.07-.11	V.Weak		
	.12-.15	Fair				
			.13-.15	Fair		
	.19-.23	Fair				
	.72-.74	Fair				

TABLE V (cont'd).

Fraction #	Sprays: Bromphenol Blue R_f	Intensity	Sprays: o-Aminodiphenyl R_f	Intensity	Sprays: Hydroxylamine-Ferric Chloride R_f	Intensity	Compound	Weight of combined Fractions
380			.00	V.Weak				
	.23-.28	Weak			.22-.30	Fair	Erythronic acid	#376-#509
	.56-.61	Fair						4.825 g
	.72-.74	Weak						
400	.23-.28	Strong	-	-	.22-.30	Strong	Erythronic acid	
	.40-.45	Strong			.37-.42	Strong	Erythrono-lactone	
	.58-.61	Weak						
420	.23-.28	Strong			.22-.30	Strong	Erythronic acid	
	.40-.45	Strong			.37-.42	Strong	Erythrono-lactone	
	.58-.61	Weak						
435	.23-.28	Strong			.22-.30	Strong	Erythronic acid	
	.40-.45	Strong			.37-.42	Strong	Erythrono-lactone	
450	.23-.28	Strong			.22-.30	Strong	Erythronic acid	
	.40-.45	Strong			.37-.42	Strong	Erythrono-lactone	
470	.23-.28	Strong			.22-.30	Strong	Erythronic acid	
	.40-.45	Strong			.37-.42	Strong	Erythrono-lactone	

TABLE V (cont'd).

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
490	.01-.03	Trace	.01-.03	Weak				
	.23-.28	Fair			.22-.30	Trace	Erythronic acid	
	.40-.45	Strong			.37-.42	Fair	Erythrono-lactone	
510	.01-.03	V.Weak	.01-.03	Fair	-	-		#511-#525
	.23-.28	Trace			-	-	Erythronic acid	0.035 g
	.40-.45	Fair			-	-	Erythrono-lactone	
530	.01-.03	Weak	.01-.03	Strong	-	-		#525-#600
550	.01-.03	Weak	.01-.03	Strong	-	-		0.130 g
570	.01-.03	Weak	.01-.03	Fair	-	-		
590	.01-.03	V.Weak	.01-.03	Weak	-	-		
610	.00-.03	Weak	.00-.03	Fair	.00-.03	Weak		
630	.00-.03	Fair	.00-.03	Strong	.00-.03	Weak		
650	.00-.03	Strong	.00-.03	Strong	.00-.03	Weak		
670	.00-.03	Strong	.00-.03	Strong	.00-.03	Weak		#601-#880
			.05-.07	Trace				
			.13-.17	Trace				0.650 g
690	.00-.03	Strong	.00-.03	Strong	.00-.03	V.Weak		
			.05-.07	Trace				
	.14-.17	Trace	.13-.17	Weak	.15-.19	Trace		
710	.00-.03	Weak	.00-.03	Weak	.00-.03	V.Weak		
	.05-.07	Trace	.05-.07	Trace				
	.14-.17	Weak	.13-.17	Weak	.15-.19	Trace		
730			.00-.03	Trace				
			.05-.07	Trace				
	.14-.17	Trace	.13-.17	Weak				

TABLE V (cont'd).

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
750	-	-	.05-.07	Trace				
			.13-.17	Trace	.15-.19	Trace		
770	-	-	.05-.07	Trace				
					.15-.19	Trace		
790			.05-.07	Trace				
					.13-.17	Trace		
810	-	-	-	-	.13-.17	Trace		
830	-	-	-	-	.13-.17	Trace		
850	-	-	-	-	.13-.17	Trace		
870	-	-	-	-	-	-		
890	-	-	.06-.09	Trace	-	-	Glucuronic acid	#880-#924
910	.06-.08	Trace	.06-.09	Weak	-	-	Glucuronic acid	0.015 g
924	.06-.08	Weak	.06-.09	Strong	-	-	Glucuronic acid	
			.24-.27	Trace				
940	.05-.07	Weak	.04-.07	Strong	.04-.07	Weak	Glucuronic acid	
960	.05-.07	Weak	.04-.07	Strong	.04-.07	Weak	Glucuronic acid	
980	.04-.07	Weak	.04-.07	Strong	.04-.07	Weak	Glucuronic acid	#924-#1105
1000	.04-.07	Weak	.04-.07	Strong	.04-.07	Weak	Glucuronic acid	
1020	.04-.07	Weak	.04-.07	Strong	.04-.07	V.Weak	Glucuronic acid	0.322 g
1 040	.04-.07	V.Weak	.04-.07	Strong	.04-.07	Trace	Glucuronic acid	

TABLE V (cont'd).

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
1060	.05-.07	V.Weak	.04-.07	Strong	.04-.07	Trace	Glucuronic acid	
1080	.05-.07	Trace	.04-.07	Fair	-	-	Glucuronic acid	
1100	-	-	.04-.07	Trace	-	-	Glucuronic acid	
1120 to 1230	-	-	-	-	-	-		#1150-#1300 0.094 g
1260	-	-	.61-.70	V.Weak	-	-	Glyoxylic acid	
1290	-	-	.61-.70	Weak	-	-	Glyoxylic acid	
1320	-	-	.00	V.Weak	.00	Trace		
	.66-.74	Fair	.61-.70	Strong	.61-.72	Fair	Glyoxylic acid	
1350			.00	Weak	.00	V.Weak		#1301-#1500
	.66-.74	Fair	.61-.70	Strong	.61-.72	Fair	Glyoxylic acid	0.917 g
1380			.00	Weak	.00	V.Weak		
	.66-.74	Fair	.61-.70	Strong	.61-.72	Fair	Glyoxylic acid	
1410			.00	Weak	.00	V.Weak		
	.07-.11	V.Weak	.07-.11	V.Weak	.05-.11	V.Weak		
	.66-.74	Weak	.61-.70	Strong	.61-.72	Fair	Glyoxylic acid	
1440			.00	Weak	.00	V.Weak		
	.07-.11	V.Weak	.07-.11	V.Weak	.05-.11	V.Weak		
	.66-.74	Trace	.61-.70	Strong	.61-.72	Fair		
1470			.00	Weak	.00	V.Weak		

TABLE V (cont'd).

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
1470	.07-.11	V.Weak	.07-.11	Weak	.05-.11	Weak		
			.61-.70	Fair	.61-.72	Weak	Glyoxylic acid	
1500			.00	V.Weak	.00	Trace		
	.07-.11	V.Weak	.07-.11	V.Weak	.05-.11	V.Weak		
			.61-.70	Fair	.61-.72	Weak	Glyoxylic acid	
1530			.00	Trace	.00	Trace		#1500-#1620
	.07-.11	V.Weak	.07-.11	V.Weak	.05-.11	V.Weak		0.182 g
			.61-.70	Weak	.61-.72	Weak	Glyoxylic acid	
1560			.00	Trace				
	.07-.11	Trace	.07-.11	V.Weak	.05-.11	Trace		
			.61-.70	V.Weak	.61-.72	Trace	Glyoxylic acid	
1590	.07-.11	Trace	.07-.11	V.Weak	.05-.11	Trace		
			.61-.70	Trace			Glyoxylic acid	
1620	-	-	.07-.11	Trace	.05-.11	Trace		
1650 to 1849	-	-	-	-	-	-		#1621-#1849 0.112 g
			Eluent I N Formic Acid					
1880			.01-.04	Trace				
	.05-.07	Trace						#1851-#1910
1910	.05-.07	Trace	.00-.01	Trace				0.033 g

TABLE V (cont'd).

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
1940 to 2060	-	-	-	-	-	-		#1911-#2060 0.054 g
2090	-	-	.00	Fair	.00	Weak		
			.01-.13	Fair	.01-.08	Weak		
	.04-.07	V.Weak						
2120	.00	Weak	.00	Fair	.00	Weak		
	.01-.03	Fair						
			.01-.16	Fair	.01-.12	Weak		#2061-#2175 0.379 g
	.38-.45	Trace						
2150	.00	V.Weak	.00	Fair	.00	Fair		
	.01-.03	Fair						
					.01-.13	Fair		
	.04-.06	Weak						
	.38-.45	Trace						
2180	.00	V.Weak	.00	Fair				
	.01-.03	Fair						
					.01-.09	Weak		
	.10-.21	Fair					Meso-Tartaric acid	
2210	.00	Weak	.00	Fair	.00	Weak		#2176-#2300 1.226 g
	.00-.03	Weak						
					.01-.08	Weak		
	.07-.31	Strong			.11-.30	Fair	Meso-Tartaric acid	
2240	.00	V.Weak	.00	Weak	.00	Fair		
					.01-.09	Weak		
	.05-.31	Strong			.11-.30	Fair	Meso-Tartaric acid	

TABLE V (cont'd).

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
2270	.00	Trace	.00	Weak	.00	Fair		
					.01-.18	Weak		
	.05-.21						Meso Tartaric acid	
2300	.00	Trace	.00	Weak	.00	V.Weak		
					.01-.13	Weak		
	.05-.14	Weak					Meso-Tartaric acid	
2330	.00	Weak	.00	Weak	.00	Fair		
					.01-.15	Weak		
	.05-.10	Weak					Meso-Tartaric acid	#2301-#2375 0.357 g
2360	.00	V.Weak	.00	V.Weak	.00	V.Weak		
					.01-.10	V.Weak		
	.04-.08	V.Weak					Meso-Tartaric acid	
2390	.00	Trace	.00	V.Weak	.00	V.Weak		
2420	-	-	.00	V.Weak	.00	Trace		#2376-#2465 0.180 g
2450	-	-	.00	V.Weak	.00	Trace		
2480	-	-	.00	V.Weak	.00	Trace		
			.02-.07	Trace				
	.11-.21	Weak			.14-.22	V.Weak	L-and DL-Tartaric acid	
2510			.00	V.Weak	.00	Trace		#2466-#2600 1.092 g
			.02-.07	Trace				
	.10-.29	Strong			.13-.30	Strong	L-and DL-Tartaric acid	

TABLE V (cont'd).

Fraction #	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
2540			.00	V.Weak	.00	Trace		
	.05-.12	Weak						
	.12-.28	Strong			.14-.30	Strong	L-and DL-Tartaric acid	
2570			.00	V.Weak	.00	Trace		
	.05-.12	Weak						
	.12-.28	Strong			.14-.28	Strong	D- and DL-Tartaric acid	
2600	.00	Trace	.00	V.Weak				
	.05-.14	Weak						
	.14-.24	Strong			.16-.24	Fair	D- and DL-Tartaric acid	
2630	.00	Trace	.00	V.Weak				#2601-#2650 0.180 g
	.02-.04	Trace						
	.05-.13	V.Weak						
	.13-.20	Weak			.15-.23	Trace	D- and DL-Tartaric acid	
2660	.00	Trace	.00	V.Weak	-	-		
	.02-.04	Trace						
	.05-.08	Trace						
2690	.00	Trace	.00	Trace				
	.02-.04	Trace						
	.05-.08	Trace						

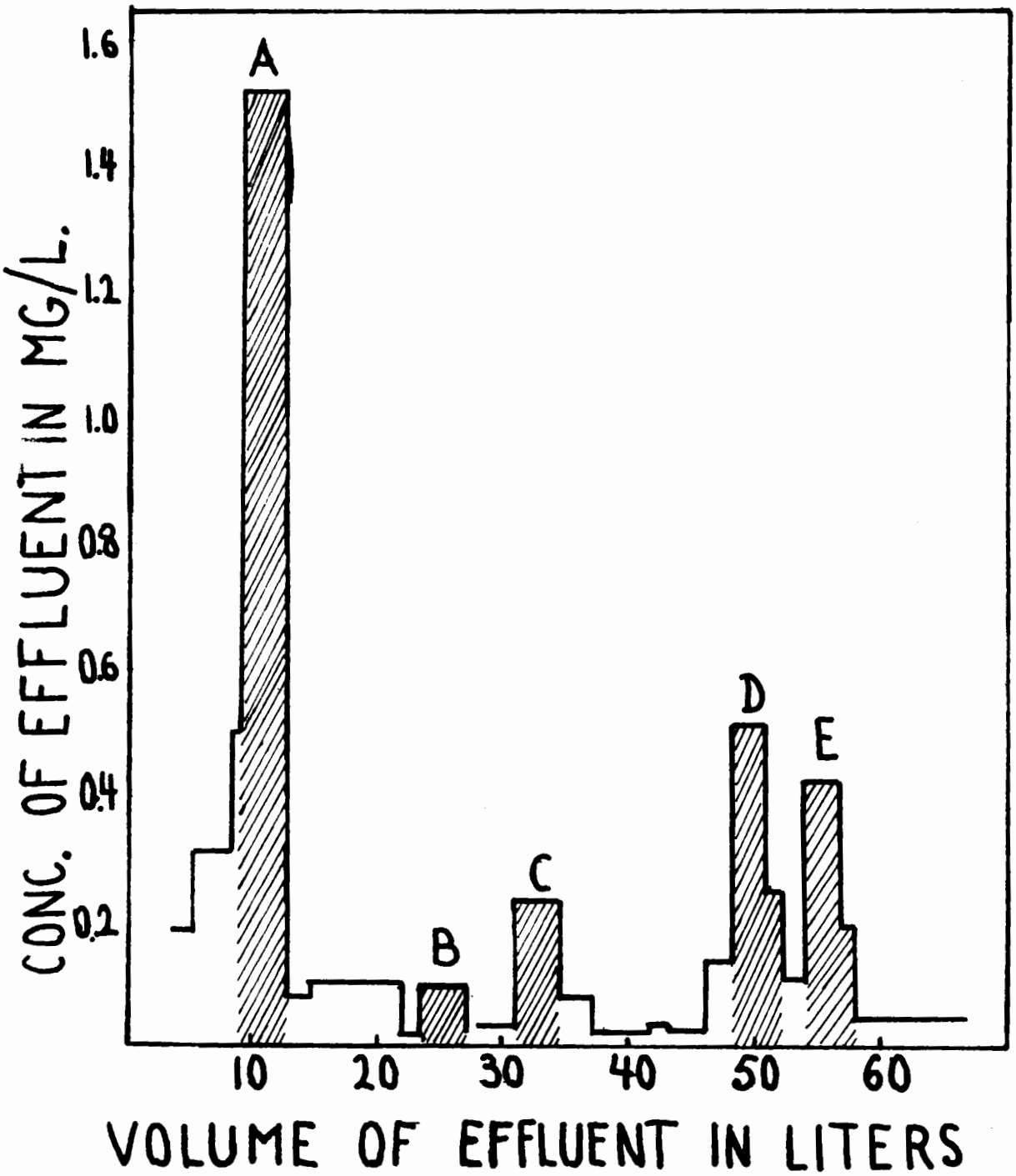
TABLE V (cont'd).

Fraction	Sprays						Compound	Weight of combined Fractions
	Bromphenol Blue		o-Aminodiphenyl		Hydroxylamine-Ferric Chloride			
	R_f	Intensity	R_f	Intensity	R_f	Intensity		
2720 to 3125	.00	Trace	-	-	-	-		#2651-#3125 0.346 g
	.02-.04	Trace	-	-	-	-		
			Eluent 0.5 N Trifluoroacetic acid					
3155 to 3515	-	-	-	-	-	-		
3545	.00	V.Strong	.00	Fair				
			.00-.02	Fair				
			.00-.10	Weak				
	.00-.22	V.Strong			.00-.35	Fair	Oxalic acid (a)	
	.32-.39	Strong						
					.40-.47	Weak		
	.52-.61	Strong						
					.72-.76	Weak		
	.81-.85	V.Weak						#3516-#3553
3554	.00	V.Strong	.00-.02	Fair	.00	Fair		1.158 g (b)
			.04-.10	Trace				
	.04-.24	V.Strong			.02-.30	Trace		
3575	.00	Trace	.00	Trace	-	-		
	.04-.07	Trace						
3594	.00	Trace						
	.04-.05	Trace						

(a) 213 mg
(b) ether extract

Figure 4

Separation of sugar acids from hydrolysis of oxystarch by ion exchange chromatography. A, D-erythronic, B, D-glucuronic, C, glyoxylic, D, meso-tartaric and E, D - and DL yartaric acids.


A
B
C
D
E
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
CONC. OF EFFLUENT IN MG/L.
10
20
30
40
50
60
VOLUME OF EFFLUENT IN LITERS

which partly crystallized after prolonged standing in a desiccator over phosphorus pentoxide. Yield 1.6% based on the starch. Chromatographic flow rates on paper and the colour reaction with o-aminodiphenyl were identical to those of glyoxylic acid prepared by oxidizing tartaric acid with aqueous periodate. A part of this syrup was converted to the crystalline 2,4-dinitrophenylhydrazone, which had the correct nitrogen content and melting point. The infra red spectrum of the 2,4-dinitrophenylhydrazone was identical to that of the authentic sample.

The meso-tartaric acid crystallized in part after the addition of a few seed crystals. Yield 2.1% based on the starch. After purification via the barium salt, the crystalline mono-potassium salt was prepared, which had the calculated ash content. Removal of potassium with cation-exchange resin yielded the free acid, which was optically inactive and melted in the correct range.

Both DL- and D-tartaric acids were present in the same effluent fractions. This result was expected, as both compounds would have the same dissociation constant. Experience with ion-exchange chromatography showed that the rate at which an acidic compound was eluted was primarily dependent on its dissociation constant. The constants for glyoxylic, meso-tartaric, D- and DL-tartaric acids, for example, were reported (67,68,69) as given by $10^4K = 4.74$,

6.3 and 9.7, respectively, and the acids were isolated in that order. D- and DL-tartaric acids, however, differed considerably in their solubilities. At 20°, 139.4 g of the optically active, but only 20.6 g of the racemic acid, dissolved in 100 ml of water (70). Thus the mixture of the two acids was evaporated to approximately 70% solids, where a partial crystallization took place.

The crystalline part (yield 1.1% based on the starch), when recrystallized, was optically inactive, and its melting point and neutralization equivalent were in good agreement with values reported for DL-tartaric acid. This identification was confirmed by a precipitation test with a saturated aqueous calcium sulphate solution, and by the preparation of the crystalline mono-potassium salt, which had the correct ash content. The mother liquor containing D-tartaric acid was reconcentrated to a syrup. Yield 0.8% based on the starch. The addition of a calculated amount of potassium hydroxide yielded the crystalline mono-potassium salt, which contained the calculated amount of ash. On removal of the potassium ions with a cation-exchange resin, the free acid was obtained with the correct melting point and specific rotation.

Tests were carried out to establish whether the DL-tartaric acid could have been artifact. An aqueous solution of L-tartaric acid was kept at pH 12 for 48 hours, but no decrease in optical rotation was observed. It was therefore unlikely that racemization occurred during the oxidation of the

starch at that pH. The optical rotation of aqueous L-tartaric acid remained similarly unchanged upon heating under reflux with N sulphuric acid for two and one half hours, or in conditions similar to those used in the hydrolysis of the oxidized starch.

Final elution of the column was with 0.5 N trifluoroacetic acid. This acid was as strong as hydrochloric acid and might therefore be expected to displace all acids of lesser strength from the resin. Several acids were obtained by this elution, but they were not separated from each other. The bulk of the trifluoroacetic acid in those effluents was removed by evaporation under reduced pressure and the remaining solution was contineously extracted with ether. A small amount of oxalic acid, corresponding to 0.5% of the original starch, crystallized upon evaporation of the ether and was identified as the dihydrate by mixed melting point with an authentic sample and by titration with acid permanganate. The rest of the ether extract, 1.6% by weight of the starch, was not identified. Considerable effort was expended to purify this product, but no crystalline derivatives were obtained. The chromatoghaphic flow rate was similar to that of tartronic acid.

Fractionation and Hydrolysis of the Water-insoluble Calcium Salts

A slurry in water of the insoluble fraction I from starch

oxidation 8 was acidified to pH 3.1, the insoluble part being recovered and treated with cation exchange resin. On evaporation of the calcium-free solution, oxalic acid dihydrate crystallized and was identified by mixed melting point with an authentic sample and by titration with acid permanganate.

Another part of fraction I was extracted with 5% acetic acid and the extract was treated with cation-exchange resin to remove calcium ions. A small amount of the chloride ions was removed as the silver salt, and the filtrate yielded 13.6% of fraction I as free sugar acids (5.1% of the original starch; fraction Ia). These acids were isolated as a colourless glass, of which 69% passed through a cellophane membrane when dialysed against water. Hydrolysis and paper chromatography revealed the presence of glucose, glyoxylic acid and also of non-reducing compounds, which had R_f values close to those of meso-tartaric, L-tartaric, D-erythronic and tartronic acids and of D-erythronolactone. The results of those paper chromatograms are in table VI.

The portion of fraction I, insoluble in 5% acetic acid, was dissolved in 20% hydrochloric acid and the solution was continuously extracted with ether for several hours, according to the method of Logan (71). After the evaporation of the ether and crystallization from water oxalic acid dihydrate was recovered in 41.6% yield based on fraction I, or 15.6% by weight of the original starch. Titration with acid permanganate showed the product to be pure.

TABLE VI

PAPER CHROMATOGRAPHY OF HYDROLYSED FRACTION Ia (1)

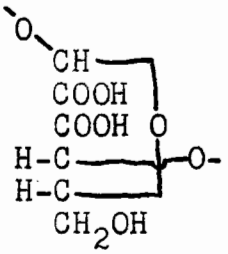
Sprays						Possible Compounds (2)
Bromphenol Blue		o-Amino-diphenyl		Hydroxylamine Ferric Chloride		
R_f	Intensity	R_f	Intensity	R_f	Intensity	
		.00	fair (brown)			
.00-.20	fair					DL-Tartaric, Meso-Tartaric Acids
		.04-.09	strong (brown)			D-Glucose
.21-.27	strong					D-Erythronic Acid
.29-.34	strong					D-Tartaric Acid
.38-.43	fair			.34-.39		D-Erythronolactone
.47-.51	fair					Tartronic Acid
.66-.72	weak	.64-.71	fair (yellow)			Glyoxylic Acid

(1) An equilibrated organic phase of a mixture of 500 parts n-butanol, 385 parts of water and 115 parts of 85% formic acid was used as eluent.

(2) These compounds had R_f values close to those observed in the hydrolysate of fraction II.

Significance of the Results and Possible Oxidation Mechanism

After the present research was well under way, Whistler and his collaborators published articles in September 1956 and December 1957, which covered some similar ground. Whistler, Linke and Kazeniac (49) oxidized corn amylose at pH 9 and pH 11 with 0.5, 1.0 and 2.0 equivalents of hypochlorite per anhydroglucose unit. The yield of the non-dialysable oxidized amylose was 75-88%, and after hydrolysis and separation of the individual constituents by paper partition chromatography the authors were able to isolate glyoxylic and D-erythronic acids. This result showed for the first time that alkaline hypochlorite oxidized and cleaved the starch in the 2- and 3-position of the anhydroglucose units with the formation of carboxyl groups at these positions (LXXVIII). Up to



LXXVIII

27% of the hypochlorite was consumed in bringing about this oxidation. The authors examined the hydrolysate for gluconic, glucaric, tartaric and glucuronic acid by paper chromatography. None of these acids was detected.

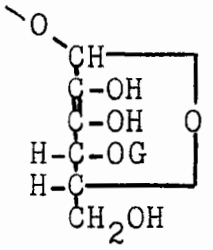
Whistler and Schweiger (52) oxidized corn amylopectin

with 3 mole of hypochlorite per anhydroglucose unit at pH 3-13. Reaction was most rapid at pH 7 and proceeded very slowly at pH 11 and pH 13. D-erythronic and glyoxylic acids were again isolated from the hydrolysed non-dialysable portion of the product. The yields of glyoxylic acid at pH 7 and pH 9 were equivalent to 0.24 and 0.22 mole, respectively, but at pH 3 and 12 the values were 0.02 and 0.015 mole, respectively. Aldehyde groups in the hydrolysate, determined by the hypoiodite method of Willstaetter and Strudel, decreased with increasing pH of the oxidation. The carbon dioxide evolved in oxidations at pH values from 3 to 9 ranged from 0.84 to 0.59 mole per anhydroglucose unit.

In the present work, the yield of glyoxylic acid after hydrolysis of the oxidized starch was 0.03 mole per anhydroglucose unit, in good agreement with the value of 0.015 obtained by Whistler and co-workers at pH 12. On the other hand, the present yield of D-erythronolactone at 0.11 mole was considerably higher than that of glyoxylic acid. The isolation of D-glucuronic acid and of meso-tartaric acid, to the extent of 0.004 and 0.023 mole, respectively, indicated oxidation to carboxyl at the 6-position of the anhydroglucose units. Although the yield of D-glucuronic acid was low, polyglucuronic acids hydrolysed with difficulty and were easily decarboxylated. The decarboxylation of the neutral calcium salts of the oxidized starch measured under the conditions of hydrolysis was equivalent to 0.075 mole of carbon dioxide

per anhydroglucose unit. An upper estimate of the amount of glucuronic acid units originally present was given by the 17.3% of "intrinsic" uronic anhydride in the water soluble-calcium salts of the oxidized starch (fraction II) and by the 6.9% in the water-insoluble calcium salts (fraction I). These values corresponded to 0.16 mole glucuronic anhydride per anhydroglucose unit of the starch.

The oxidative attack of the alkaline hypochlorite on the starch will probably start by the formation of a carbonyl group at either carbon atom two, three or six of the anhydroglucose units of the starch. Oxidation at the 2- or 3-position would produce an equilibrium with the endiol (LXXIX), which would easily be further oxidized to the dicarboxyl compound (LXXVIII). The presence of a carbonyl group in the 2-position (LXX) may also render the molecule susceptible to alkaline

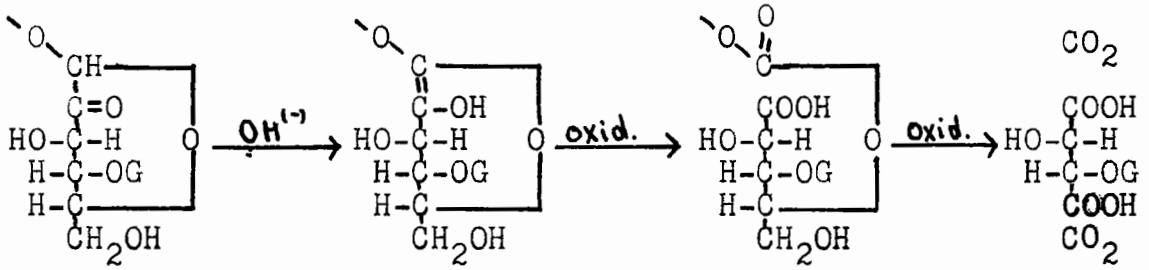


LXXIX

cleavage according to the reaction mechanism put forward by Kenner (43), Machell and Richards (44), involving the scission of the glycosidic bond in a β-position to an electronegative group. This mechanism was outlined

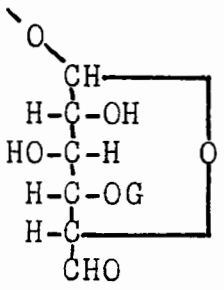
previously, and would result in the formation of saccharinic acids. Since no saccharinic acids or other dehydro-sugar compounds were detected upon hydrolysis of the alkaline hypochlorite oxidized starch, this type of cleavage apparently was not an important reaction.

An initial oxidation to 2-keto-anhydroglucose (LXXX) would permit the formation of either a 1,2- (LXXXI), or a 2,3-endiol (LXXIX). Further oxidation of the 1,2-endiol might produce a fragment containing a carbonic acid ester (LXXXII). The formation of carbonic esters of this type in the oxidation of cellulose by hypochlorite in acidic and neutral media was postulated by Kaverzneva (18) on the basis of indirect evidence. In the present work, D- and DL-tartaric acids were isolated from the hydrolysate of the oxy-starch in yields of 0.009 and 0.011 mole, respectively. Thus Kaverzneva's mechanism of chain cleavage between the 1- and 2-positions appeared to be very probable, since D-tartaric acid could only originate from carbon atoms 2-5 of the anhydroglucose units. The reaction might be as follows:

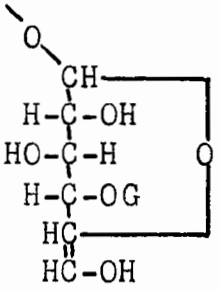


LXXX LXXXI LXXXII LXXXIII

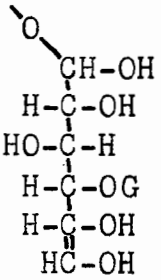
If the oxidative attack took place at the number six carbon atom to form the aldehyde (LXXXIV), the corresponding endiol (LXXXV) would render the pyranose ring structure susceptible to alkaline hydrolysis (30), although the rate at which this reaction actually occurred would depend on the rate of further oxidation to carboxyl groups. Cleavage of the pyranose ring would result in a hemiacetal structure (LXXXVI), which might be further hydrolysed either to isomeric tetra-hydroxy-adipic aldehydes (LXXXVII), (LXXXVIII), still linked to the next glucose unit in the 4-position, or to a 4-substituted 5-keto-D-glucose (IXC). The latter derivative again might



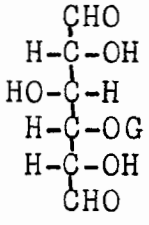
LXXXIV



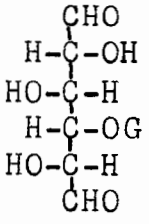
LXXXV



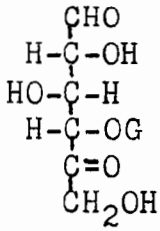
LXXXVI



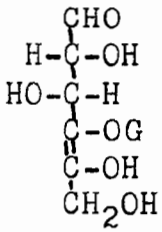
LXXXVII



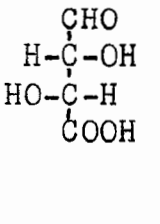
LXXXVIII



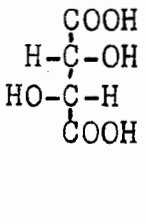
IXC



XC



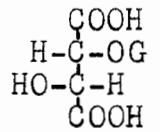
XCI



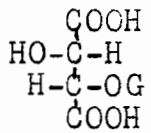
XCII

form a 4,5-endiol (XC), the glycosidic bond becoming unstable and being cleaved. Further oxidation would then produce L-threonic (XCI) and L-tartaric acid (XCII), together with glycolic acid, which in turn might yield oxalic acid. Further oxidation of the structures (LXXXVII) and(LXXXVIII) would also yield L-tartaric

(XCIII) D-tartaric (XCIV), or meso-tartaric (XCV) acids, still linked to the glucose chain. The isolation of 0.006 mole of L-tartaric acid in the form of the DL-tartaric acid and of 0.023 mole of meso-tartaric acid, after hydrolysis of the oxidized starch, suggested that some such mechanism actually occurred. The yield of oxalic acid was 0.208 mole.



XCIII



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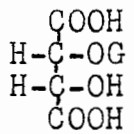

XCV

Different molar quantities of hypochlorite were necessary for the formation of a carboxyl group in an anhydroglucose unit of the starch, depending upon the carbon atom oxidized. Oxidation of a primary hydroxyl group consumed 2 mole of hypochlorite, whereas for the oxidation of an aldehyde group only 1 mole was required. If the oxidation took place on a secondary hydroxyl position 1.5 mole was necessary. In starch oxidation 8, 1.29 base mole was oxidized with 3.48 mole of hypochlorite. The products included 0.69 mole of carboxyl group in fraction I and 0.76 mole in fraction II, a total of 1.45 mole. Assuming an average of 1.70 mole of hypochlorite for the oxidation to a carboxyl group and no formation of carbonyl groups (which was nearly the case), this value corresponded to 2.47 mole of hypochlorite, or to 70.9% of the amount used. The calcium carbonate in fractions I and II was 0.174 mole. Allowing 1.5 mole of hypochlorite for the formation of carbon dioxide from a

secondary hydroxyl and 2 mole from a primary hydroxyl group, again 1.7 mole could be assumed as near the average. On this assumption 0.174 mole carbon dioxide corresponded to 0.296 mole hypochlorite. The total amount of oxidant accounted for in this way was thus 2.77 mole or 79.7% of the total.

EXPERIMENTAL

Preparations

Wheat starch

The starch used in this work was a commercial, unbleached sample, supplied through the courtesy of Ogilvie Flour Mills Co. Ltd., Montreal.

The starch was purified by the method of Murray and Purves (72), which involved steeping in 97% aqueous acetic acid over a period of five days with occasional shaking. After being isolated by filtration, the starch was again steeped with fresh acetic acid for five more days. The purified starch was freed from acetic acid by repeated repulping with distilled water until the supernatant remained neutral on standing with the starch overnight. This treatment was known to change thick boiling into thin boiling starch, removing simultaneously any fats and alkaline ash. The increase in copper reducing power, however, was inappreciable.

Brucine D-Erythronate

Starch was oxidized with periodate according to Michell and Purves (73). Sodium metaperiodate, 32.18 g (0.150 mole), was dissolved in 300 ml of water, acetic acid, 50 ml, was added and the solution adjusted with sodium hydroxide to pH 4.2. Anhydrous wheat starch, 24.00 g (0.148 mole),

freshly dried at 105° for several hours, was added and the mixture mechanically stirred at room temperature for 24 hours. Titration of an aliquot, taken from the reaction mixture showed that 99% of the periodate had been reduced. The oxidized starch granules were isolated by filtration, washed with water until free of iodate and dried under vacuum. Yield 23.6 g (99.6%).

Following the method given for periodate-oxycellulose by Jayme and Maris (57), periodate-oxystarch, 12.3 g, was dispersed in 100 ml of water and gelatinized by heating on the steam bath for one and a half hours. Bromine, 15 g, was added and the mixture allowed to stand in a closed flask for 24 hours with occasional shaking. Additional bromine, 18 g, in two portions of 9 g each, was added at intervals of two days. The flask was kept well closed and was occasionally shaken. After two more days, the brown solution was evaporated under reduced pressure to a small volume, fresh water was added and the solution re-evaporated. This procedure was repeated until all bromine had been removed. The reaction mixture was neutralized to pH 7 with 800 ml of a saturated barium hydroxide solution, and the barium salts which precipitated were separated, washed with water, with ethanol, with ether and were then dried in a vacuum oven. Yield 18.2 g.

The barium salts, 22.7 g, from two of the above preparations were hydrolysed by heating under reflux with

0.2 N sulphuric acid, 1100 ml, for twelve hours and the hydrolysate was neutralized with barium hydroxide to pH 7. Several hours were necessary for this neutralization to convert all of the lactones into the corresponding barium salts. Barium sulphate was removed by filtration, the remaining barium ions by treatment with "Amberlite IR 120" ion-exchange resin, and the filtrate was concentrated under reduced pressure. An excess of brucine was added and the mixture heated for one hour on the steam bath. Excess brucine was removed by filtration and the filtrate was extracted with chloroform. The extracted aqueous layer was concentrated under reduced pressure to a small volume; ethanol was added until the solution became cloudy, and the mixture was placed in the refrigerator to crystallize overnight. The crude brucine salt, 8 g, was removed by filtration and twice recrystallized from 95% aqueous ethanol. Yield 4.2 g, m.p. 201° (decomposition). $[\alpha]_D^{20} = -23.9°$ (water, c = 10). Jayme and Maris (57) reported a melting point of 210-212° and an optical rotation $[\alpha]_D^{20} = -23.8°$ (water, c = 10).

D-Erythronolactone

The brucine D-erythronate was dissolved in water, an excess of barium hydroxide solution was added and the mixture extracted with chloroform. Barium ions were then removed by passing the solutions through a small "Amberlite IR 120" ion-exchange resin column. The acidic effluent was decolorized

with activated charcoal ("Norite") and evaporated to dryness under reduced pressure. The last traces of moisture were removed by the addition of toluene and distillation of the mixture. The product crystallized during this distillation and was purified by recrystallization from ethyl acetate. The colourless crystals melted at 102.5-103° and had the specific rotation $[\alpha]_D^{23} = -72.0° \pm 1.2°$. Jensen and Upson (74) reported m.p. 102° and specific rotation $[\alpha]_D^{20} = -72.8°$.

Sodium Calcium Glyoxylate

Fifteen grams or 0.1 mole of L-tartaric acid was dissolved in 300 ml of water. An aqueous solution, 200 ml, containing 22.00 g or 0.103 mole of sodium metaperiodate was added and the mixture allowed to react in the dark at room temperature for 36 hours. After this time an aliquot was removed and titrated with sodium-arsenite, iodine. No periodate remained. A solution of 19.0 g (0.501 mole) of lead acetate trihydrate was then added to the reaction mixture and the precipitate of lead iodate was removed. An iodometric determination of the residual iodate in the filtrate showed that 99.6% of the amount originally present had been eliminated.

The filtrate was evaporated to 50 ml under reduced pressure and neutralized with 2 N sodium hydroxide to pH 8. Addition of 130 ml of an aqueous solution containing 17.6 g or 0.100 mole of calcium acetate monohydrate produced crystallization. The mixture was placed in the refrigerator

for a period of 24 hours, the crystals were removed by filtration, washed with a small volume of water and vacuum dried. Yield 19.6 g or 87.1%.

Found: Ash, 45.5, 45.6%; Ca in sample 9.27, 9.21, in ash 9.27%; Na, 9.65, 9.58%. Calcd. for sodium calcium glyoxylate $CaNa_2C_8H_4O_{12}.4H_2O$: Ash, 45.8; Ca, 8.32; Na, 10.22%. Calcium was determined as the oxalate, and sodium by the uranyl acetate (75) method.

Glyoxylic acid in the salt was determined manganometrically according to the method of Hatcher and Holden (55). The sample, 0.10-0.20 g, was dispersed in 60 ml of water, concentrated sulphuric acid, 3 ml, was added and the solution titrated with 0.101 N potassium permanganate until the pink colour, produced by the addition of one drop of permanganate remained stable for one minute. Samples 0.1338, 0.2038, and 0.1024 g, reduced 24.6, 37.7, and 19.0 ml of the permanganate, the volumes calculated for the sodium calcium glyoxylate being 23.6, 35.9 and 18.1 ml, respectively.

Five grams of the sodium calcium glyoxylate was dissolved in 250 ml of water at 60° and 0.275 g of residue was removed by filtration. The filtrate was evaporated under reduced pressure to 50 ml, crystallization occurring, during this evaporation. The mixture was placed overnight in the refrigerator, filtered and repulped with 5 ml of water, with ethanol and ether. Yield 4.34 g. Another recrystallization left 4.02 g.

Found: Na, 8.98, 8.97; Ca 9.54%. Calcd. for $Na_2CaC_8H_4O_{12}.4H_2O$ Na, 10.32, Ca 8.92%.

Barium Glyoxylate

L-Tartaric acid, 15.0 g (0.100 mole), in concentrated aqueous solution was mixed with a concentrated aqueous solution of paraperiodic acid, 25.08 g, (0.110 mole), and the mixture diluted with water to a volume of 300 ml. An exothermic reaction took place immediately, and the mixture had to be cooled for the first 30 minutes in order to keep it at 23°. The mixture was then allowed to stand at room temperature for an additional ten hours. An aliquot was then removed and titrated with sodium arsenite-iodine solution. No periodic acid was found. A 0.521 molar solution of lead acetate, 110 ml, was added. Lead iodate crystallized and the mixture was mechanically stirred for three more hours in order to complete crystallization. The lead iodate was removed by filtration and repulped with 100 ml of water. The excess lead ions in the filtrate and washings were removed as lead sulphide by the addition of the necessary amount of an aqueous hydrogen sulphide solution and the resulting mixture evaporated under reduced pressure to a syrup. This syrup was evacuated under 0.5 mm Hg pressure for four more hours to remove traces of acetic and formic acids, the last being formed during the periodic acid oxidation. The product, 17.4 g, was dissolved in 200 ml

of water and neutralized to pH 7 with 525 ml. of 0.345 N barium hydroxide, the barium hydroxide being dropwise added under continuous stirring. The resulting clear solution was evaporated under reduced pressure to 200 ml and allowed to crystallize overnight. The crystalline product was removed by filtration, pressed out well and dried under vacuum over phosphorus pentoxide. Yield 23.8 g, (Fraction I). Partial evaporation of the mother liquor yielded 3.9 g. of crystalline material. (Fraction II). Combined yield (fraction I and II) 27.8 g, or 86.8%. On evaporation of the remaining mother liquor to dryness 0.6 g. material was obtained. (Fraction II).

Fraction I. Found: Ash, 60.9, 61.2%; Calcd. for $BaC_4H_2O_6.2H_2O$; 61.8%. Samples, 0.2248 g. and 0.1817 g, reduced 29.5 and 23.8 ml 0.1003 N permanganate. Calcd. for $BaC_4H_2O_6.2H_2O$; 28.1 and 22.7 ml.

Fraction II. Found: Ash, 60.9%. Calcd. for $BaC_4H_2O_6.2H_2O$; 61.8%. Sample, 0.2015 g, reduced 27.1 ml 0.1003 N permanganate. Calcd. for $BaC_4H_2O_6.2H_2O$; 25.1 ml.

A part of fraction I, 20.0 g, was recrystallized from 120 ml of hot water. The crystalline product was removed by filtration, repulped in 25 ml of 50% aqueous ethanol, and dried <u>in vacuo</u> over phosphorus pentoxide. Yield 15.2 g. (Fraction I_1). Partial evaporation of the mother liquor

yielded 2.8 g of additional crystalline product. (Fraction I_2).

Fraction I_1. Found: Ash, 61.5, 61.7%. Calcd. for $BaC_4H_2O_6.2H_2O$; 61.8%. Samples 0.258 g, and 0.2577 g, reduced 32.5 and 32.2 ml, of 0.1044 N permanganate. Calcd. for $BaC_4H_2O_6.2H_2O$; 30.9 and 30.9 ml.

Glyoxylic acid

a) A portion of the sodium calcium glyoxylate was dissolved in water and liberated from calcium and sodium ions by treatment with "Amberlite IR 120" ion-exchange resin. The resulting cation-free solution was evaporated under reduced pressure to a syrup, which was dried in a vacuum desiccator over phosphorus pentoxide. After four weeks the material crystallized in part.

Found: Samples 0.0639 g and 0.0650 g consumed 15.85 ml and 15.95 ml of 0.101 N potassium permanganate. Calcd. for $C_2H_2O_3$, 17.05 ml and 17.4 ml, respectively. A 0.2887 g sample neutralized 9.20 ml of 0.369 N barium hydroxide. Calcd. for $C_2H_2O_3$, 10.55 ml.

These analyses showed that 40.1 and 85.0 g, of the crude glyoxylic acid were equivalent to 1 liter of N potassium permanganate and N barium hydroxide, respectively. Hatcher and Holden (55) found that the first of these equivalents was exactly one-half of the second for pure glyoxylic acid.

The present sample contained about 85% of anhydrous glyoxylic acid. When the crystals were freed from the mother liquor by pressing them between filter paper, they melted at 55-75°.

b) Barium glyoxylate, 8.00 g of fraction I_1, was dissolved in 80 ml of water. An insoluble residue, 0.06 g, was removed by filtration. The filtrate was passed through a 21 x 18 cm column containing "Amberlite IR 120" ion-exchange resin and the column washed with distilled water until the effluent reached pH 4. No barium ions could be detected in the effluent. Evaporation under reduced pressure yielded a syrup, which was dried in a vacuum desiccator over phosphorus pentoxide for several months. The last water of crystallization was very hard to remove. After two weeks of drying the syrup weighed 3.965 g. (Calcd. for anhydrous glyoxylic acid from 8.00 g, barium glyoxylate dihydrate, 3.70 g). The syrup crystallized on prolonged standing and after eight months the completely crystalline product weighed 3.68 g, and melted at 104-107°, after softening at 94°.

Glyoxylic Acid 2,4-Dinitrophenylhydrazone

Preparation was made according to the method of Whistler, Linke and Kazeniac (49). A sample of the sodium calcium glyoxylate, 0.0365 g, was dispersed in water and dissolved by the addition of a few drops of hydrochloric acid. A freshly filtered solution, 15 ml, of 0.4% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid was added, the mixture diluted with

30 ml of water and allowed to crystallize in the refrigerator overnight. The yellow crystalline precipitate was removed by filtration, washed with 12 ml of water and dried to constant weight at 105 °. Yield 0.0658 g, (78.8%), m.p. 191-191.5° (decomposition). The melting point recorded by Whistler, Linke and Kazeniac was 190-191°.

D-Glucuronic Acid

Commercial D-1,4-glucuronolactone, 1.00 g, m.p. 171-172°, was dissolved in 10 ml of water and adsorbed on a small column containing Dowex 1-x-4 anion-exchange resin in the free base form. The column was washed with 25 ml of water and the washings discarded. This operation was accomplished within eight minutes, as exposure of the material to the strongly alkaline resin might produce epimerization (66). The resin column was eluted with 120 ml of 2 N acetic acid and the effluent evaporated under reduced pressure at 40° to a syrup, which crystallized in part. After crystallization had been completed by the addition of 20 ml of ethanol, the product was collected by filtration and washed with ethanol. Yield 0.797 g, (73%); m.p. 147-148 (decomposition). Recrystallization from 9 ml of 85% aqueous ethanol yielded the crystalline product, 0.326 g, m.p. 158-160° (decomposition).

The specific rotation of the product was determined using an aqueous solution. Mutarotation was rapid, equilibrium being reached after a few hours. After fourteen minutes,

the solution had $[\alpha]_D^{20} = +25.85^\circ$ (c = 8.8 and after three hours a constant value of $[\alpha]_D^{20} = +36.6^\circ, \pm 0.4^\circ$ was reached. Goebel and Babers (53) reported m.p. 165°C, $[\alpha]_D^{24} = +36^\circ$ (c = 2, H_2O, equil.).

Analytical Methods

Hypochlorite

Solutions of calcium hypochlorite were prepared from Reagent-Grade material, and were filtered through sintered glass prior to being standardized. Those of sodium hypochlorite were purchased from Fisher Scientific Co. Ltd., Montreal. a) An aliquot of the sample was acidified with hydrochloric acid; potassium iodide, 2 g, was added and the liberated iodine titrated with 0.1 N sodium thiosulphate. Several drops of a freshly prepared 1% starch solution were used as indicator. b) An aliquot of the sample was neutralized by the addition of excess sodium bicarbonate. A measured excess of 0.1 N sodium arsenite was added and the mixture titrated with 0.1 N iodine solution. Starch was used as indicator (76).

Carbonated Ash (for calcium, barium, sodium and potassium salts)

The sample was ashed at 475 - 525° until constant weight was obtained. This temperature range was required to convert calcium salts of organic compounds into the corresponding calcium carbonate.

Sulphated Ash (for calcium, barium and sodium salts)

The sample was treated with an excess of sulphuric acid and the latter was evaporated over a free flame. The residue was ignited at 900° until constant weight was obtained.

Calcium

The sample was dissolved in dilute hydrochloric acid, the calcium precipitated with ammonium oxalate and determined manganometrically. The well washed calcium oxalate was dissolved in warm, dilute sulphuric acid and titrated with 0.1 N potassium permanganate (77).

Sodium

Sodium was determined gravimetrically as sodium uranyl zinc acetate hexahydrate (78).

Sodium Periodate and Periodic Acid

a) Determination of periodate in the absence of iodate.

An aliquot of the sample was acidified with hydrochloric acid. Potassium iodide, 2 g, was added and the liberated iodine was titrated with 0.1 N sodium thiosulphate. Several drops of a 1% starch solution were used as an indicator (79).

$$\% \text{ periodate} = \frac{\text{mol wt. periodate} \times \text{ml thiosulphate} \times N \times 100}{\text{wt. sample} \times 8}$$

b) Determination of periodate in the presence of iodate.

An aliquot of the sample was neutralized by the addition of excess sodium bicarbonate. A measured excess of 0.1 N

sodium arsenite and 1 ml of 20% potassium iodide were added. After five minutes at room temperature the excess of arsenite was titrated using a standard solution of 0.1 N iodine. A few drops of a 1% starch solution were used as indicator. Iodic acid and iodates were known not to interfere in this determination (79).

$$\% \text{ periodate} = \frac{\text{mol wt. periodate}(N_{\text{arsenite}} \times \text{ml arsenite} - N_{\text{iodine}} \times \text{ml iodine}) \times 100}{\text{wt. sample} \times 2}$$

Iodic Acid and Iodates in the Absence of Periodate

The same method as for determination of periodate in the absence of iodate was used.

$$\% \text{ iodate} = \frac{\text{mol wt. iodate} \times N \times \text{ml thiosulphate} \times 100}{\text{wt. sample} \times 6}$$

Inorganic Chlorides

The sample containing 0.01-0.1 g, of chloride was dispersed in water, 50 ml, and dissolved by the addition of reagent grade concentrated nitric acid, 3 ml. The solution was mechanically stirred and titrated potentiometrically with standard 0.1 N silver nitrate, using silver and calomel electrodes. The end point was reached at 275 millivolts (80).

Inorganic Carbonates

A 3-necked flask, 200 ml, was equipped with a separatory funnel, gas inlet tube reaching to the bottom of the flask

and connected to a adsorbtion system, described by Browning (81). This system consisted of a moisture adsorbtion tube filled with anhydrous magnesium perchlorate, and of a carbon dioxide adsorbtion tube filled with "Ascarite" and anhydrous magnesium perchlorate. A second such adsorbtion tube was used as guard to protect the first tube from atmospheric moisture and carbon dioxide. The sample, 1-2 g, was placed in the flask and 10 ml of freshly boiled distilled water was added. The gas inlet tube was connected and carbon dioxide free nitrogen passed through the apparatus at a rate of 30-60 ml per minute for one hour to displace all air from the apparatus. The inorganic carbonates in the sample were then decomposed by the addition of 60 ml of a 12% aqueous hydrochloric acid through the separatory funnel. Carbon dioxide was adsorbed in the first adsorbtion tube, which had been weighed previously. The determination was complete after this tube had reached a constant weight.

Uronic Anhydride

These analyses were carried out by the analytical section of the Pulp and Paper Research Institute of Canada (courtesy Mr. J.E. Tasman) according to their standard procedure (81). Inorganic carbonates were first decomposed by the addition of 12% aqueous hydrochloric acid to the sample and slow heating of the mixture to 70°. The mixture was then heated under reflux for three and five hours. The

carbon dioxide evolved during these refluxing periods was adsorbed and determined gravimetrically.

Pentosan

The pentosan determinations were also carried out by the analytical section of the Pulp and Paper Research Institute of Canada (courtesy Mr. J.E. Tasman) according to their standard procedure. The sample was distilled with 12% hydrochloric acid, and the distillate collected. A measured volume of 0.2 N bromate-bromide solution was then added to this distillate and the solution back titrated with 0.1 N thiosulphate. A blank determination was made using all the reagents except the sample. The pentosan content was calculated from the following formula:

$$\% \text{ pentosan} = \frac{100 \times 0.048 \ (V_2 - V_1) \ N}{0.727 \times 0.88 \times W}$$

where, 0.048 was the weight of furfural in gram corresponding to 1 ml of N thiosulphate solution,

0.727 the theoretical conversion factor of pentosan to furfural,

0.88 a factor to compensate for the incomplete conversion of pentosan to furfural,

N the normality of the thiosulphate,

V_1 the volume of thiosulphate used for the determination,

V_2 the volume of thiosulphate used for the blank,

W the sample weight.

Permanganate Oxidation Equivalent

Warm dilute sulphuric acid was added to the sample and the mixture titrated under the same conditions as commonly used for the determination of oxalic acid (82). Results were reported as % calcium oxalate monohydrate.

Copper Reducing Sugars

Copper reducing sugars were determined by the method of Somogyi (83), (84). An aliquot was placed in a large test tube and diluted with distilled water to 5 ml. Five milliliters of Somogyi Copper Reagent (containing approximately 25 ml of 1 N potassium iodate per liter, was added. The test tube was placed in a boiling water bath for a period of 15 minutes and then rapidly cooled to room temperature. One milliliter of 5% potassium iodide solution and 3 ml of 2 N sulphuric acid were added, the mixture was well shaken and titrated with 0.005 N sodium thiosulphate. A few drops of starch solution were used as an indicator. The Somogyi solution was standardized with D-glucose. The following values were obtained:

mg D-glucose	ml 0.0048 N thiosulphate consumed	ml 0.0048 N thiosulphate consumed per mg D-glucose
0.50	3.63	7.26
	3.58	7.16
1.00	7.13	7.13
1.50	11.03	7.35
	10.48	6.99
	average	7.18

Neutralization Equivalent

A measured excess of 0.05 N sodium hydroxide was added to the sample and after a waiting period, the mixture was back-titrated to neutrality with 0.05 N hydrochloric acid. The titration was followed potentiometrically with a Beckman Model H 2 pH meter. In other cases phenolphtalein indicator was used. Neutralization equivalent was reported as grams sample to neutralize 1 litre of N alkali.

Paper Chromatography

Descending paper chromatography was used throughout this work.

a) Preparation of eluents

Solvent A. A mixture of water, 500 ml, n-butanol, 400 ml and ethanol, 100 ml, was equilibrated by mechanical shaking and the organic phase was isolated for use.

Solvent B. A mixture of n-butanol, 500 ml, water, 385 ml, and 85% formic acid, 115 ml, was equilibrated by boiling under reflux for one hour. After cooling the organic phase was isolated for use (85).

Solvent C. n-butanol, 1000 ml, pyridine, 300 ml, and water, 300 ml, were mixed together.

b) Spray reagents

o-Amino-diphenyl. This compound, 3 g, was dissolved in glacial acetic acid, 100 ml, and 85% phosphoric acid, 1.3 ml, was added (86). The dried chromatograms were developed by spraying with this solution and then heating the paper for

five minutes at 105°. Glucose gave brown, glucuronic acid and ∫-glucuronolactone purple and glyoxylic acid yellow spots.

Hydroxylamine-ferric chloride. The dried chromatograms were sprayed with a freshly prepared alkaline solution of hydroxylamine, prepared by mixing equal volumes of 1 N methanolic hydroxylamine hydrochloride and 1.1 N methanolic potassium hydroxide. The papers were allowed to dry for ten minutes and then were sprayed again with an aqueous solution of 1.5% ferric chloride, which contained 1% of hydrochloric acid (87). Lactones appeared as brown spots. This method was, however, not entirely specific, as acids gave also spots with that reagent.

Bromphenol blue. Fifty milligrams of bromphenol blue was dispersed in 5 ml of water and the mixture neutralized to pH 6.5 with sodium hydroxide. The resulting solution was filtered and diluted with 95 ml of ethanol. The air-dried chromatograms were placed in a vacuum oven and dried at 110° and 3 mm Hg pressure for eight hours. This drastic procedure was necessary to remove the last traces of formic acid from the paper. The chromatograms were then sprayed with the bromphenol blue reagent. Acids gave well defined yellow spots on a blue background (88).

Preliminary Oxidations with Calcium and Sodium Hypochlorite

Oxidation 1. Purified, thin-boiling starch, 5.00 g, (10.7% moisture), slurried with 25 ml of cold water, was

gelatinized by the addition of 75 ml of boiling water and the mixture was then heated for ten more minutes by immersion in a boiling water bath. After cooling to room temperature 100 ml, of calcium hypochlorite solution, containing 0.292 mole hypochlorite per litre, water, 50 ml, and powdered calcium hydroxide, 0.5 g, were added. The mixture was mechanically stirred at room temperature and the flask covered with aluminum foil to protect the contents from light. The hydrogen ion concentration was measured at intervals, and the mixture was kept at pH 12 by the addition of more powdered calcium hydroxide, whenever a drop in pH occurred. Aliquots of the reaction mixture were periodically removed to determine the concentration of hypochlorite iodometrically by titration with sodium thiosulphate, or with sodium arsenite-iodine solutions.

Oxidation 2. A second batch of starch, 5.00 g, (10.7% moisture), was gelatinized and oxidized under the conditions described above with the exception that only one-half of the hypochlorite solution, 50 ml, was used. The other half of this solution was replaced with water, so that the final volumes of both reaction mixtures were identical.

Simultaneously a blank with identical composition, but containing no starch was prepared. Periodically the pH of the mixture was checked and aliquots removed and titrated with sodium thiosulphate or sodium arsenite-iodine solutions to determine the residual concentration of hypochlorite. Results are in table I.

Oxidation 3. Thin-boiling starch, 2.50 g, (10.7% moisture), was dispersed in 12.5 ml, of water and the mixture gelatinized by the addition of 37.5 ml, of boiling water. The resulting solution was immersed in a boiling water bath for a period of ten minutes and then cooled to room temperature. Calcium hypochlorite solution, 100 ml, containing 0.756 moles of hypochlorite per litre, water, 25 ml, and powdered calcium hydroxide, 1.0 g, were added. The mixture was mechanically stirred and the reaction allowed to proceed at room temperature. The flask was covered with aluminum foil in order to protect the contents from light. Hydrogen ion concentration was periodically checked as before; the reaction mixture being kept at pH 12 by the addition when necessary of calcium hydroxide powder. Aliquots of the mixture were removed at intervals and the concentration of hypochlorite determined iodometrically by titration with sodium hypochlorite solution. The results are in table II and in figures 1a and 1b.

Oxidation 4. Thin boiling starch, 2.50 g, (10.7% moisture), was dispersed in 12.5 ml of cold water and gelatinized as before by the addition of 37.5 ml of boiling water. Sodium hypochlorite solution, 250 ml, containing 0.337 mole per litre of hypochlorite, and water, 25 ml, were added. The reaction flask was covered with aluminum foil and the mixture was mechanically agitated at room temperature. Periodically the pH of the reaction mixture was checked and aliquots were removed for the determination of residual hypochlorite by the

iodometric thiosulphate method. (Table III).

Preliminary Investigation of the Products

Oxidation 5. Thin boiling starch, 5.00 g (10.7% moisture), was oxidized with calcium hypochlorite as outlined above. Powdered calcium hydroxide, 2.0 g, was added at the beginning of the reaction. The hydrogen ion concentration was kept at pH 12 throughout the oxidation by the addition of 1.0 g of calcium hydroxide. Initial concentration of oxidant: 0.457 mole hypochlorite/l.; concentration of starch: 0.0919 mole $C_6H_{10}O_5$/l. The progress of the oxidation was followed by periodical titration of aliquots by the sodium thiosulphate method.

After 3.02 mole of hypochlorite per anhydroglucose unit of the starch had been consumed, the reaction was stopped by the addition of 4.5 ml of acetone. The excess hypochlorite was destroyed in this way with the simultaneous formation of calcium acetate and chloroform. The reaction mixture was filtered and the insoluble material well washed with 100 ml of cold water. The filtrate plus washings was adjusted to pH 6.7 by the addition of solid carbon dioxide and a white crystalline precipitate formed, which was presumably calcium carbonate. This material, 0.97 g, was removed by filtration. The filtrate was evaporated to 40 ml volume and 80 ml of ethanol was added, but no more precipitate was obtained. The insoluble material was vacuum dried. Yield 6.42 g, or 143% by weight of the starch. Found: Ca, 20.1%.

To 2.186 g of this fraction 110 ml of 0.100 M oxalic acid was added. The resulting mixture was mechanically stirred for several hours and was then allowed to stand for 24 hours in the refrigerator in order to convert all of the calcium into the insoluble calcium oxalate. The insoluble material was filtered, well washed with distilled water and dried under vacuum at 60°. Weight 1.837 g, (calcd. for calcium oxalate monohydrate 1.602 g). A permanganate titration in dilute sulphuric acid was equivalent to 83.8, 82.9% of calcium oxalate monohydrate (corresponding to 1.539, 1.523 g, respectively, of calcium oxalate monohydrate). Decimolar calcium acetate solution, 19.1 ml, was added to the filtrate in two portions in order to precipitate the excess oxalic acid. The resulting mixture was evaporated to 50 ml and the insoluble material was removed in two fractions by filtration. These fractions were vacuum dried at 60° and weighed 0.152 g and 0.129 g. Permanganate titration corresponded to 80.0%, 80.6%, respectively, of calcium oxalate monohydrate (0.122 g and 0.104 g $CaC_2O_4.H_2O$). Two drops of oxalic acid and calcium acetate solutions were added to the filtrate, but no further precipitation was obtained. Thus both reagents had been eliminated.

The solution was evaporated to dryness and diluted with a small volume of water. A small, insoluble residue, 0.017 g, believed to be calcium oxalate was removed by filtration. The filtrate was again evaporated to a syrup, which was dried in

a vacuum desiccator over phosphorus pentoxide to a brittle glass, weighing 0.921 g. Yield 61.1% based on the original anhydrous starch. The product was ground to a yellow powder, which had a specific rotation in water $[\alpha]_D^{22} = +93.5^\circ$.

Found: Carbonated ash, 2.50%; neutralization equivalent 216; copper reducing power 2.9% as D-glucose.

Comparison of the oxalic acid added, 0.0110 mole, with the amount found in the different fractions by permanganate titration, 0.0127 mole, indicated strongly, that during the oxidation of the starch with hypochlorite some oxalic acid was produced. The combined weight of the insoluble fractions was 2.118 g, versus an expected 1.607 g of calcium oxalate monohydrate from 0.0110 mole oxalic acid. This difference of 0.511 g was equivalent to 33.4% by weight of the original starch. For these reasons the method of removal of calcium from the reaction products by oxalic acid was considered unsuitable and discarded.

Oxidation 6. Twenty-five grams of starch of 8.6% moisture was oxidized with calcium hypochlorite in the presence of 10 g of calcium hydroxide in the usual way. The concentration of oxidant was 0.440 mole hypochlorite/l., and that of the starch 0.082 mole $C_6H_{10}O_5$/l. The pH throughout the reaction was maintained between 11.8-12.1 by the addition of 20 g of calcium hydroxide. After 3.02 mole of hypochlorite per anhydroglucose unit of the starch had been consumed, the oxidation was stopped by the addition of 27 g of acetone.

After recovery, washing with 60 ml of water and vacuum drying at 60° the product weighed 51.6 g, or 225% by weight of the starch. Fraction A. Found: Ca, 31.3, 31.3, 31.2%; Cl, 1.6%.

A part of this fraction, 3.00 g, was stirred at - 10° for one hour with 800 ml of 0.1 N hydrochloric acid, which contained 70% ethanol. The excess of acid was 100%. The insoluble material was recovered and washed free of chloride with 150 ml of 90% aqueous ethanol. The product was vacuum dried at 60° and weighed 1.37 g, (103% yield from the anhydrous starch). Found: Carbonated ash, 39.4%; Cl, 0.4%. The ash was equivalent to 15.8% calcium.

One gram of this product was again digested under identical conditions with 300 ml or a four fold excess of 0.1 N hydrochloric acid containing 70% ethanol. Only 0.182 g (19% yield from anhydrous starch) of insoluble material was obtained.

In a second experiment 1.50 g, of fraction A was digested at -10° with 400 ml of 0.1 N hydrochloric acid containing 90% ethanol. The residue weighed 0.614 g, (93% yield from anhydrous starch). Found: Carbonated ash, 34.0%, being equivalent to Ca, 13.6%.

One-half gram of this material yielded on redigestion with 120 ml of 0.1 N hydrochloric acid in 90% ethanol 0.293 g, (54% yield from anhydrous starch), of insoluble residue. Found Sulphated ash, 54.5, 55.8%, corresponding to Ca, 16.0, 16.4%.

The method was abandoned at this point, since an appreciable amount of the oxy-starch was apparently soluble in 70 and 90% aqueous ethanol.

Fifteen grams of fraction A was dispersed in 200 ml of water and the mixture was adjusted to pH 7 by the addition of 112 ml of dilute hydrochloric acid (1:10). The insoluble material, named fraction I, was separated by filtration, well washed with 150 ml of water and vacuum dried. Weight 5.55g, or 83.5% by weight of the starch. Found: Sulphated ash, 84.6, 85.4%; Cl 0.8% (corresponding to: Ca, 24.9, 25.15%; $CaCl_2$, 1.2%).

Five grams of fraction I was re-dispersed in 100 ml of water and the mixture adjusted to pH 3.5 by the addition of 32.5 ml of dilute hydrochloric acid. The insoluble material was again separated by filtration, washed with 70 ml of water and vacuum dried. Weight of fraction Ia, 1.307 g (21.7% of the starch). Found: Sulphated ash, 79.5%, 79.5% Cl, 0.33% (corresponding to: Ca, 23.4, 23.4%; $CaCl_2$, 0.5%)

Fraction Ia was fractionally extracted with dilute hydrochloric acid. The soluble portion was liberated from calcium ions by passing through a column containing "Amberlite IR 120" cation-exchange resin. On slow evaporation of the effluent in vacuum over solid sodium hydroxide a crude, crystalline material was obtained. This product was dissolved in a fairly large volume of diethyl-ether. On evaporation of the ether colourless crystals formed, which melted at 101-102°.

Mixed melting points with oxalic acid dihydrate were undepressed.

An aliquot of the filtrate from fraction Ia, that is the portion insoluble at pH 7, but soluble at pH 3, was liberated from calcium ions by passing through a column containing "Amberlite IR 120" and the resulting effluent slowly evaporated in a vacuum desiccator over solid sodium hydroxide. The dry residue, named fraction Ib, was equivalent to 1.37 g or 22.8% by weight of the starch.

The filtrate from fraction I, that is the portion water-soluble at pH 7, was evaporated to 30 ml. Some insoluble material separated during this evaporation and was removed by filtration. This residue, named fraction IIa, weighed after vacuum drying 0.357 g or 6.0% by weight of the starch. Found: Sulphated ash, 76.4, 76.6%; Cl, 1.7% (corresponding to Ca,22.4, 22.5%; $CaCl_2$, 2.7%). For the $CaCl_2$ free product calcd. Ca, 22.1%.

To the filtrate of fraction IIa two equal volumes of ethanol were added and the calcium salts of the sugar acids precipitated in two fractions.

Fraction IIb; weight 1.92 g, or 32.0% of the starch. Found: Sulphated ash, 49.9, 50.4%; Cl, 9.9, 10.4% (corresponding to Ca, 14.7, 14.8%; $CaCl_2$, 15.5, 16.3%). For the $CaCl_2$ free product calcd. Ca, 10.7%.

Fraction IIc; weight 0.67 g, or 11.1% of the starch. Found: Sulphated ash, 36.9, 36.8%; Cl, 1.1% (corresponding to Ca,10.9, 10.8%; $CaCl_2$, 1.7%). For the $CaCl_2$ free product calcd. Ca, 10.5%.

A part of fraction IIb was liberated from calcium with "Amberlite IR 120" cation-exchange resin. Chloride ions were removed as the insoluble silver salt by the addition of silver acetate. The resulting demineralized solution was evaporated to dryness and hydrolysed by heating for 4.5 hours on a steam bath with 2% sulphuric acid. The sulphate ions were then precipitated by the addition of a calculated amount of barium hydroxide solution. The resulting solution was chromatographed using solvent A as eluent and o-amino-diphenyl as spray. Two well defined spots were obtained. The first one, brown in colour, corresponded to glucose. The second yellow spot corresponded to glyoxylic acid. Paper chromatography of the demineralized, but not hydrolysed solution of fraction IIb, using the same spray and solvent, gave a brown spot on the starting line. Hence fraction IIb consisted of polymeric sugar acids containing unoxidized glucose units.

The soluble part of the reaction mixture from oxidation 6 was neutralized with solid carbon dioxide to pH 6 and the precipitated calcium carbonate removed by filtration. The solution was then evaporated to 150 ml volume. On cooling crystals separated, which were removed and vacuum dried. Weight 8.32 g. Found: Ca, 27.3, 27.1%; Cl, 20.0, 20.2%; water-insolubles, 7.3%. The insoluble material gave vigorous gas evolution with dilute hydrochloric acid and was presumably calcium carbonate. A part of the 8.32 g fraction was treated with "Amberlite IR 120" cation-exchange

resin to remove calcium ions, and the deionized liquor was subjected to a slow evaporation in a vacuum desiccator over solid sodium hydroxide. A solid residue of only 2.2% remained. An other part of the fraction was recrystallized from 80% aqueous ethanol yielding the chloride free product. Found: Sulphated ash, 77.1%. Calcd. for calcium acetate monohydrate, $CaC_4H_6O_4.H_2O$, 77.3%.

The above data suggested that the crystalline material obtained upon the concentration of the filtrate was calcium acetate monohydrate containing some calcium chloride and calcium carbonate. $CaCl_2$ (calcd. from Cl analysis, 31.4%; $CaCO_3$ (detn. as water insoluble material), 7.3%; unknown material, 2.2%; $CaC_4H_6O_4.H_2O$ (calcd. by difference), 59.1% These values corresponded to the following contribution to the calcium content: $CaCl_2$, 11.34%, $CaCO_3$, 2.92%, $CaC_4H_6O_4.H_2O$, 13.44. Calcd. for the crude product 27.66%. Found: Ca, 27.3, 27.1%.

After the above crystals had been separated from the original oxidation liquor at pH 6, the filtrate, about 150 ml, was diluted with 25 ml of ethanol. A second crop of crystals, 3.94 g, separated, and the addition of more alcohol did not produce any further precipitation.

Recovery and Identification of the Oxidation Products

Preparation of the Calcium Salts

Oxidation 7. Fifty grams of starch with 7.1% moisture was oxidized with calcium hypochlorite solution at pH 12 in the usual way. A total of 50 g of calcium hydroxide was added during the reaction in order to maintain alkalinity at pH 11.8-12.1. The concentration of oxidant was 0.444 mole hypochlorite/l., and that of the starch 0.0836 mole $C_6H_{10}O_5$/l. After the consumption of 2.99 mole of hypochlorite per anhydroglucose unit the reaction was stopped by the addition of 130 ml of acetone. The mixture was filtered and the insoluble material, named fraction A, was air dried. Weight, 156.0 g. Moisture, 46.1%.

Some of this material, 140 g, was dispersed in 200 ml of water and the slurry was adjusted to pH 7 by the addition of 52.5 ml of concentrated hydrochloric acid. The mixture was filtered and the insoluble material dried. The filtrate was evaporated to 200 ml and allowed to stand for several days. Some material separated which was removed by filtration, well washed with water and dried. Weight 2.6 g. This material was combined with the rest of the product insoluble at pH 7. That combined insoluble portion weighed 31.98 g and was named fraction I. Yield 68.8%.

Found: Sulphated ash, 76.2%; Cl, 0.6, 0.6% (corresponding to Ca, 22.4%; $CaCl_2$ 0.9%).

The calcium salts of the sugar acids were obtained from the filtrate by precipitation with two volumes of ethyl alcohol. Two more reprecipitations from aqueous ethanol freed them from most of the calcium chloride and the alcoholic mother liquors were discarded. The salts were air dried and named fraction II. Weight 23.01 g, yield 49.5%.

Found: Moisture 13.9%, sulphated ash, 30.0, 29.3%; Cl 0.7, 0.7% (corresponding to Ca, 8.8, 8.7%; $CaCl_2$, 1.1, 1.1%). Calcd. for the anhydrous, $CaCl_2$ free product Ca, 9.7%.

Oxidation 8. Thin boiling starch, 225 g, with 7.2% moisture, was oxidized with calcium hypochlorite solution in the usual way. Powdered calcium hydroxide, 210 g, was added during the reaction to maintain alkalinity at pH 11.8-12.1. The concentration of oxidant was 0.443 mole hypochlorite/l., and that of the starch 0.0860 mole $C_6H_{10}O_5$/l. After 2.98 mole of hypochlorite per anhydroglucose had been consumed the reaction was stopped by the addition of 500 ml of acetone. The reaction mixture was filtered and the insoluble material repulped with water, 1000 ml, all filtrates and washings being discarded. After the insoluble product had been dispersed in fresh water, 2000 ml, the slurry was neutralized to pH 7.0 by the addition of 300 ml of concentrated hydrochloric acid. Neutralization was considered to be complete after the pH remained unchanged for 24 hours. The insoluble residue was removed by filtration, slurried

again with 1000 ml of water and recovered. After the particle size had been reduced with the help of a Waring Blendor and after two more extractions with a total of 1100 ml of water the product (Fraction I) was air dried. Yield 78.2 g or 37.5% by weight of the anhydrous starch.

Found: Ash, 32.4, 32.4%, (the sample was ashed at 1200°, so that all of the calcium was converted to calcium oxide); Cl, 0.25, 0.22% (corresponding to Ca, 23.2, 23.2%; $CaCl_2$ 0.39, 0.34%); moisture, 8.1%; pentosan, 1.14, 1.13, 1.15%. Uronic anhydride, (three hours refluxing) 9.84, 9.67%; (five hours refluxing) 11.95, 11.35%. Inorganic CO_2, 5.62, 5.37%. Calcd. for the anhydrous $CaCl_2$ and $CaCO_3$ free product, Ca, 22.9%. Samples, 0.1394, 0.2042 and 0.2003 g, reduced 9.7, 14.2 and 14.1 ml of 0.101 N potassium permanganate under conditions suitable for the determination of oxalic acid. Reduction equivalent to 51.3, 51.3, 51.9% of calcium oxalate monohydrate.

All filtrates and washings from fraction I were combined and concentrated to 700 ml by evaporation under reduced pressure. The oxystarch in the form of its calcium salt was precipitated by the addition of two volumes of ethanol. This precipitation was repeated two more times to remove calcium chloride, which was readily soluble in aqueous alcohol. The product was air dried. All alcoholic filtrates were combined and concentrated by evaporation under reduced pressure.

More of the oxystarch calcium salts were precipitated in aqueous ethanol, purified by fractional precipitation with the same solvent, air dried, (14.0 g) and combined with the main fraction of the water soluble oxystarch. The combined weight of the product was 181.7 g, or 87.0% by weight of the anhydrous starch. Fraction II.

Found: Sulphated ash, 37.4, 37.1, 37.3%; Cl 1.4, 1.5% (corresponding to Ca, 11.0, 10.9, 11.0%; $CaCl_2$, 2.2, 2.3%); moisture, 11.9%; pentosan, 3.63, 3.54, 3.84%. Uronic anhydride (three hours refluxing), 21.48, 21.64%; (five hours refluxing), 24.42, 24.32%. Inorganic CO_2, 1.83, 1.85%. Calcd. for the anhydrous, $CaCl_2$ and $CaCO_3$ free product, Ca, 10.4%.

Neutralization equivalent of fraction II was determined in the following way:

1) By assuming y for the equivalent weight of the free acids and 10.4 as the correct average calcium content of their neutral salts, the value of y could be calculated from the obvious relationship $10.4 = (40 \times 100)/(2y + 38)$. Hence $y = 173$.

2) An independent method of determining y was to prepare the free acids and titrate them directly. A sample of fraction II, 2.000 g, was dispersed in water, 15 ml, and heated on the steam bath for a half hour with 10 g of "Amberlite IR 120". This mixture was transferred on top of a column, (1.2 cm diameter) containing 10 g of "Amberlite IR 120", which was

then eluted with water until the effluent became neutral. The acidic effluent was diluted with freshly boiled distilled water to 100 ml. Aliquots of the solution, 20 ml, corresponding to 0.400 g of fraction II were removed. An excess of 0.103 N sodium hydroxide solution and a few drops of phenolphtalein solution were added to samples (a) and (b) and the mixtures allowed to stand overnight. The excess sodium hydroxide was back titrated the next day with 0.100 N hydrochloric acid.

Sample (a) Back titration of 25 ml of alkali required 7.90 ml of acid or 4.46 milliequivalents per gram of fraction II.

Sample (b) Back titration of 30 ml of alkali required 12.80 ml of acid or 4.52 milliequivalents per gram of fraction II.

Sample (c) Kept in vacuum desiccator over solid sodium hydroxide until weight became constant at 0.2821 g or 70.5% of fraction II (0.705 g per gram of fraction II).

As each gram of fraction II contained 0.0145 g or 0.41 milliequivalent of chloride, the alkali required to neutralize the sugar acids was 4.49 - 0.41 = 4.08 milliequivalents. The neutralization equivalent of the sugar acids was thus 705/4.08 or 173.

The extent to which fraction II was dialysable was determined in the following way. A sample of fraction II, 1.317 g, was dispersed in 7 ml of water, "Amberlite IR 120" was added and the mixture stirred. The supernatent liquor was decanted and the resin extracted with fresh water until

the mixture became neutral. The combined extracts were dialyzed through collodion against two 4 litre volumes of 0.01 N hydrochloric acid over a period of 36 hours. The solution was then further dialyzed against two 4 litre volumes of distilled water for 48 hours to remove all hydrochloric acid. Evaporation of the contents of the dialysis sac yielded 0.153 g. Hence 11.6% of fraction II was not dialysable.

The amount of carbon dioxide evolved during hydrolysis of fraction II with 1 N sulphuric acid was determined with the help of an uronic acid analysis apparatus (81). The sample of fraction II, 1.017 g, was placed in the reaction flask and 60 ml of 1 N sulphuric acid was added. Nitrogen was passed through the solution for one hour; after this time all inorganic carbonates were decomposed and all air from the apparatus displaced. A carbon dioxide adsorption tube was then connected to the apparatus and the mixture was heated under reflux for a further two and one half hours (bath temperature 145°), and the carbon dioxide evolved during this period was adsorbed and determined gravimetrically.

Found: CO_2, 0.0238 g or 2.34% (corresponding to 0.53 milliequivalents per gram of fraction II).

Hydrolysis and Separation of the Water-Soluble Calcium Salts

The copper reducing power of fraction II was determined with the method of Somogyi (83), (84). Samples of 0.0166 g and 0.0256 g in 5 ml volumes of water required 3.12, 5.12 ml

of 0.00503 N sodium thiosulphate. Hence the reduction was 2.7, 2.9%, respectively as glucose (see analytical part).

In another experiment a sample of fraction II, oxidation 7, 0.598 g, was dissolved in N sulphuric acid, 20.0 ml, and the solution was boiled under reflux for 8.6 hours. Aliquots were periodically removed and neutralized with sodium hydroxide solution. The amount of copper reducing sugars was determined as glucose by Somogyi's method. The results are shown in Table VII.

A second hydrolysis of fraction II, 0.2998 g, was carried out by refluxing with 20 ml of 1 N sulphuric acid for 31.8 hours. The results are shown in Table VIII.

An aqueous solution of 2.00 g of fraction II, oxidation 7, was freed of calcium by treatment with "Amberlite IR 120". The resulting calcium free solution was evaporated to near dryness and hydrolysed by refluxing with 40 ml of N sulphuric acid. The hydrolysate was diluted with water to 500 ml and barium hydroxide solution, equivalent to the amount of sulphuric acid used was added dropwise. The resulting mixture was stirred overnight in order to convert all of the sulphate ions into insoluble barium salts. The filtered solution was evaporated to a syrup weighing 1.86 g, and a part of this syrup, 1.58 g, chromatographed on a cellulose column using solvent B as eluent. The effluent was collected in 250 fractions of 15 ml each. Several of these fractions were separately evaporated to 0.3 ml volume and were chromatographed

TABLE VII

HYDROLYSIS OF FRACTION II WITH N SULPHURIC ACID

Hours Hydrolysis	ml 0.0048 N thiosulphate for 0.20 ml.	for 1 mg	Reduction as Glucose %
0			2.8
0.25	11.85 (a)	0.79	11.0
	4.65	0.78	
0.5	7.65	1.27	17.8
	7.60	1.28	
1.0	11.05	1.85	26.0
	11.25	1.88	
2.0	14.55	2.43	33.5
	14.25	2.38	
3.0	15.20	2.54	35.9
	15.75	2.63	
	15.45	2.58	
4.0	15.95	2.67	37.0
	15.80	2.64	
5.0	16.75	2.80	38.7
	16.25	2.72	
	16.85	2.82	
6.57	17.65	2.95	41.2
	17.65	2.95	
8.57	17.75	2.97	42.0
	18.20	3.04	

blank: 5 ml Somogyi's copper reagent consumed 24.60, 24.70 ml 0.0048 N sodium thiosulphate.

(a) 0.5 ml aliquot.

TABLE VIII

HYDROLYSIS OF FRACTION II WITH N SULPHURIC ACID OVER A PROLONGED PERIOD

Hours Hydrolysis	ml 0.0048 N thiosulphate for 0.20 ml	for 1 mg	Reduction as Glucose %
0			2.8
2.0	7.4 7.6	2.47 2.54	34.9
4.0	8.0 7.9	2.67 2.64	37.0
9.0	8.2 8.0 8.0	2.74 2.67 2.67	37.5
14.0	8.2 8.2	2.74 2.74	38.2
15.8	8.6 8.1	2.87 2.70	38.8
19.1	8.6 8.7	2.87 2.90	40.2
24.8	8.8 8.9	2.94 2.97	41.2
31.8	9.3 9.4	3.10 3.14	43.5

blank: 5 ml Somogyi's copper reagent consumed 39.4, 39.4 ml 0.0048 N thiosulphate.

on paper using the same solvent in order to obtain qualitative information about the composition. o-Amino-diphenyl and bromphenol blue were used as sprays. The results are summarized in table IV. The column was washed with 1500 ml of solvent B in order to remove other small fragments and then eluted with 5000 ml of water. The fractions containing compounds with identical R_f values were combined and evaporated to a syrup which was dried in vacuum over solid sodium hydroxide and phosphorus pentoxide.

Neutralization equivalents of the individual fractions were obtained by titration with 0.365 N barium hydroxide using phenolphtalein as indicator. In each case rapid titration did not give definite end points, indicating the presence of lactones. The final titration was carried out by a dropwise addition of the barium hydroxide solution until the pink colour of phenolphtalein remained unchanged over a period of ten hours. No insoluble barium salts formed during these titrations. Attempts to isolate crystalline derivatives by different methods were not successful.

The fraction believed to be glyoxylic acid was evaporated to a small volume. A solution of 2,4-dinitrophenylhydrazine, 0.4% in 2 N hydrochloric acid was added and after some time a crude, crystalline product of orange colour, separated in very small yield. Attempts at further purification of this crystalline material were not successful.

The aqueous eluate from the column, 5000 ml, was evaporated under reduced pressure to a syrup. This syrup, 0.675 g, crystallized in part on standing in a vacuum desiccator over solid sodium hydroxide. A portion of this syrup, 0.120 g, was used to prepare the phenylosazone according to the method of Taketoni and Miura (89). Anhydrous sodium acetate, 0.386 g, phenylhydrazine hydrochloride, 0.240 g, and water, 2.4 ml, were added, and the resulting solution heated for two hours under reflux. The mixture was then allowed to cool; the yellow crystalline product was separated by filtration and washed with a little acetone. Yield 0.127 g, m.p. 198-199° (decomposition). According to Taketoni and Miura the reaction was carried out with only 74.6% of the theoretically required amount of the phenylhydrazine hydrochloride.

This supposed phenylosazone, 0.100 g, was added to a mixture of methanol, 6 ml, water, 9 ml, cupric sulphate pentahydrate, 0.3 g, and 0.25 ml of 1 N sulphuric acid to prepare the osotriazole as described by Hudson and Hann (90). The mixture was heated under reflux on a steam bath, whereby the yellow glucosazone dissolved and after two hours the solution became light blue in colour. When evaporated under reduced pressure to 3 ml and placed in a refrigerator for two days, the solution deposited crystals which were separated by filtration and washed with 1 ml of water. The crude product was decolorized with charcoal and recrystallized from 8 ml of hot water. Yield 0.0137 g (18.5%) m.p. 195.5°.

The melting point was not depressed by the addition of an authentic sample of glucophenyltriazole.

A control experiment with 0.100 g of D-glucose, 0.200 g of phenylhydrazine hydrochloride, 0.332 g of anhydrous sodium acetate and 2.0 ml of water yielded 0.110 g of phenylosazone, m.p. 204° (decomposition). Conversion of this phenylosazone, 0.100 g, with cupric sulphate under conditions identical to those described above yielded 0.0108 g (14.6%) of the glucophenyltriazole, m.p. 195.5-196°.

In another experiment 20.0 g of fraction II from oxidation 8 was freed from calcium with "Amberlite IR 120", in the way previously described. The resulting acidic solution was concentrated under reduced pressure and then made up to 100 ml volume and 1 ml removed for analysis. Qualitative paper partition chromatography using solvent B as eluent and bromphenol blue as spray showed acidic material with R_f 0.00-0.11. The remaining solution, 99 ml, was evaporated under reduced pressure to yield 19.2 g of a syrup, which was hydrolysed by boiling under reflux with 0.989 N sulphuric acid, 100 ml, for two and one half hours. A calculated amount of 0.369 N barium hydroxide, 270 ml, was added and the precipitate of barium sulphate removed by filtration. The filtrate was neutralized to pH 4.1 with sodium hydroxide solution and baker's yeast, 0.7 g, was added. The mixture was then allowed to ferment in a warm place in order to destroy the bulk of the glucose. The fermented solution, however, still contained

glucose, which was estimated by paper chromatography to be of the order of 1 g. The yeast was separated by centrifuging and the clear solution decolorized with activated carbon. Sodium ions were removed by passing the solution again through an "Amberlite IR 120" cation-exchange resin column, and evaporation of the acidic effluent yielded 10 g of a syrup.

Portions of this syrup, 0.75-1.5 g, were chromatographed on Whatman No 1 and No 3 MM paper sheets (46 x 57 cm.), using solvent B as eluent, over periods ranging from 40 to 50 hours. Side strips of the papers were cut off and sprayed with bromphenol blue to establish the locations of the individual acids. Paper bands containing these acids were cut out and eluted with water according to the method of Dent (91). Solutions of compounds believed to be identical were combined and evaporated to syrups under reduced pressure. Analyses of these syrups by paper chromatography showed that they still consisted of mixtures of the individual acids. Attempts to prepare crystalline barium, calcium or brucine salts failed.

Another part of the syrup, 0.25 g was dissolved in 1.5 ml of water and adsorbed on a micro column, 4.0 x 0.9 cm., containing Dowex 1-x-4 anion-exchange resin (free base form). The column was washed with water and eluted first with 65 ml of 2 N acetic acid, then with 32 ml of 1 N formic acid and with 4 ml of 2 N trifluoroacetic acid. The effluent was collected in 4 ml portions, the flow rate being 2 ml/min. Chromatography of the individual fractions showed that a

group-wise separation of the acids was obtained which was in the order of their dissociation constants.

A large scale separation by ion exchange chromatography was carried out by stirring an aqueous suspension of fraction II (from oxidation 8), 50.0 g, with 70 g of "Amberlite IR 120" until the white product dissolved. The resulting mixture was transferred to the top of a column containing the same resin. The column was then eluted with water and the effluent evaporated under reduced pressure to a calcium-free acidic syrup, weighing 51.0 g. This syrup was hydrolysed by heating under reflux with 250 ml of 0.989 N sulphuric acid for two and one half hours. The hydrolysate was diluted with 1000 ml of water and the sulphate ions were precipitated by the addition of 717.1 ml of 0.345 N barium hydroxide solution. After the mixture had been mechanically agitated overnight, the barium sulphate was removed by filtration and extracted with three 400 ml volumes of hot water. The filtrates were concentrated under reduced pressure to 1500 ml and were passed through a 25 x 3.5 cm., column containing "Dowex 1-x-4" anion-exchange resin (free base form) at a rate of 17 ml/min. It had been pointed out by Pacsu and Rebenfeld (66) that some epimerization might occur when aqueous solutions of reducing sugars were exposed to a strongly basic anion-exchange resin. The effluent therefore was chromatographed on paper using solvent D and o-amino-diphenyl as the spray. No other sugar, except glucose, was detected. Evaporation of the effluent

yielded 13.3 g of crystalline glucose, but unfortunately the washings were lost and the recovery was incomplete. The resin containing the adsorbed sugar acids, but no more than traces of glucose, was immediately transferred to the top of a column (110 x 5.6 cm.) containing "Dowex 1-x-4" anion-exchange resin in the acetate form. The contact time of the hydrolysate with the "Dowex 1-x-4" in its free base form had been kept at the minimum, one and one half hours, to prevent possible epimerization. Elution of the column was with 2 N acetic acid at a flow rate of 1.5-3 ml/min., and the effluent was collected in 25 ml and 20 ml fractions. Every twentieth or thirtieth fraction was evaporated under reduced pressure to 0.3 ml and chromatographed on paper. Solvent B was used as the eluent with bromphenol blue, o-amino-diphenyl and hydroxylamine-ferric chloride as the sprays. After 1880 fractions had been collected the eluate was changed from 2 N acetic to 1 N formic acid. After 3125 fractions, the 1 N formic acid was replaced by 0.5 N trifluoroacetic acid. The results are shown in table V. Fractions containing the same compounds were combined and concentrated.

Fractions #226-340:- These fractions yielded on evaporation 0.845 g of a syrup. A part of this syrup was dissolved in 10 ml of water and freed from any cations by passage through "Amberlite IR 120". The acidic effluent was decolorized and neutralized with saturated calcium hydroxide solution. Each time the solution was adjusted to pH 7.0, the pH decreased

within a few minutes, presumably because of the presence of lactones. Neutralization was regarded as complete when no more change in hydrogen ion concentration took place within several hours at pH 8.3. The solution was concentrated by evaporation and several attempts were made to crystallize the calcium salts by gradual addition of ethanol. No crystals were obtained and in all cases an oil formed.

The mixture was again evaporated to dryness, dissolved in water and passed through "Amberlite IR 120" to remove calcium ions. The acidic effluent was concentrated by evaporation and chromatographed on paper at elution times of several days. Solvent B was the eluent and bromphenol blue the spray. Several spots were obtained and no further attempts were made to isolate the individual compounds from the mixture.

D-Erythronolactone:- Fractions #376-510 yielded on evaporation 4.825 g of a syrup. To 4.501 g of this syrup 2 mg of crystalline D-erythronolactone was added and the mixture allowed to crystallize for two days at 5°; 1.099 g of crude crystals m.p. 85-95° was separated. Recrystallization from boiling ethyl acetate and subsequent evaporation of the mother liquor yielded fraction 1, 0.706 g, m.p. 102.5-103°, fraction 2, 0.078 g, m.p. 101-102.5° and fraction 3, 0.252 g; the last fraction being obtained by the evaporation of the mother liquor to dryness. The total recovery of 1.036 g was 94.4% of the crude crystalline material.

Fraction 1 had a specific optical rotation in water of $[\alpha]_D^{21}$ - 72.9° ± 0.8° (c = 4.57). This value agreed with those of $[\alpha]_D$ - 72.8 and 72.9° reported by Jensen and Upson (74) and by Glattfeld and Farbrich (92), respectively.

The neutralization equivalent of fraction 1 was determined by dissolving a sample of 0.0478 g in 20 ml of 0.0684 N barium hydroxide and letting it stand overnight. The solution was then back titrated with 20.0 ml of 0.4805 N hydrochloric acid using phenolphtalein as indicator. Found: neutr. equiv. 117.4. Calcd. for $C_4H_6O_4$, 118.

A mixed melting point with pure crystalline D-erythronolactone was undepressed and the infrared spectra of both compounds were identical.

D-Glucuronic Acid:- Fractions #924-1105 yielded on evaporation a syrup, which was dissolved in a small volume of water. A small insoluble residue was removed by filtration. The filtrate was then further purified by adsorbtion of the acidic portion on a 4.5 x 0.9 cm., micro column of Dowex 1-x-4 anion exchange resin (free base form). The yellow coloured effluent from this column was alkaline and was discarded. It was believed that the alkalinity of this effluent was due to the presence of contaminating cations. The resin column was then washed with water until the effluent became neutral. Care was taken to accomplish this operation in a minimum time of 18 min. since glucuronic acid had a reducing group. The column was immediately eluted with 2 N acetic acid

and evaporation of the first 85 ml effluent at 30° yielded 0.249 g of syrup. By this method any lactone present was converted to the free acid.

The bulk of this fraction, 0.241 g, was dissolved in a small volume of water and decolourized with a little activated carbon before being evaporated to a syrup. The addition of several millilitres of ethanol produced a precipitation. The resulting mixture was again evaporated to near dryness keeping the temperature during evaporation below 30° to prevent reformation of glucuronolactone (53). On addition of 0.3 mg of D-glucuronic acid crystallization occurred, which was brought to completion by placing the material for twenty four hours in the refrigerator.

The main portion of the adhering mother liquor was adsorbed on filter paper and the crystals placed between filter papers overnight to remove additional mother liquor. The crude, crystalline fraction, obtained in this way, weighed 0.106 g (Fraction 1). The mother liquor adsorbed on filter paper was extracted with water according to the method of Dent (91). Evaporation of the extract yielded a syrup weighing 0.105 g (Fraction 2). This syrup was heated for one hour on the steam bath to convert glucuronic acid into the lactone and seeded with a small crystal of D-glucurono-lactone. The sample was placed in a desiccator for several months, but no crystallization occurred.

A part of fraction 1, 0.0891 g, was dissolved in 0.5 ml of water and decolourized by stirring with activated carbon

at room temperature. The carbon was removed by filtration and the resulting colourless filtrate evaporated to 0.1 ml volume. Addition of 1.1 ml of n-propanol produced a partially crystalline syrup. The alcoholic mother liquor was decanted and partially evaporated to yield 0.0084 g of crystals, (fraction 1a); m.p. 153-157° (decomposition). The partially crystalline syrup remaining after the decantation was re-dissolved in 0.15 ml of water. Addition of 1.5 ml of ethanol produced a precipitate, apparently consisting of an inorganic impurity. Weight, 0.0048 g, m.p. > 230°. The filtrate from the above fraction was placed in an open vial. Seeding with 0.2 mg of D-glucuronic acid started crystallization. The vial was left open and the aqueous alcohol allowed to evaporate slowly. After twenty-four hours the volume was reduced to 0.5 ml. The separated crystals were isolated by filtration and washed with a little ethanol. Weight 0.0339 g (fraction 1b), m.p. 154.5-155° (decomposition). The mother liquor yielded 0.007 g of crystals (fraction 1c); m.p. 144-149° (decomposition).

Fraction 1b had a specific optical rotation in water of $[\alpha]_D^{22}$ + 35.6 ± 1.1 (c = 2.02). Mutarotation was rapid and the specific rotation was determined after three hours, when equilibrium was reached. Goebel and Babers (53), reported for D-glucuronic acid $[\alpha]_D^{24}$ + 36° (c = 2, H_2O equil.) and m.p. 154°. The infrared spectrum was identical to that of crystalline D-glucuronic acid, m.p. 158-160°, $[\alpha]_D^{22}$ + 36.6°,

which had been prepared from the commercially available D-glucuronolactone.

Glyoxylic acid:- Fractions #1301-1500 yielded on evaporation 0.917 g of syrup. On paper chromatography using solvent B as the eluent and o-amino-diphenyl as spray, this material showed as a yellow spot of R_f 0.61-0.70. When bromphenol blue was used as spray an R_f value of 0.66-0.74 was obtained. Control chromatograms with authentic glyoxylic acid gave identical values.

The syrup, 0.035 g was dissolved in 30 ml of water and 15 ml of a 0.4% solution of 2.4-dinitrophenylhydrazine in 2 N hydrochloric acid was added. The mixture was left overnight in the refrigerator, the crystals washed with water and dried at 105° to constant weight. The hydrazone crystallized in yellow needles. Yield, 0.0568 g, or 59% of theory from glyoxylic acid hydrate; m.p. 189-191° (decomposition), in good agreement with the value reported by Whistler and co-workers (49).

Found: N, 22.06: Calcd. for $C_8H_6N_4O_6$, 22.05% (a)

Meso-tartaric acid:- Evaporation of fractions #2176-2300 yielded 1.226 g of a syrup which partially crystallized on addition of 0.3 mg of meso-tartaric acid. Several days

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(a) Nitrogen analyses made by Schwartzkopf, Microanalytical Laboratory, N.Y.

were necessary for the crystallization. The chromatographic flow rate on paper was determined with reference to L-tartaric acid using solvent B as eluent and bromphenol blue as spray. The material had $R_{L\text{-Tartaric}}$ 0.78-0.85. Control chromatograms with authentic meso-tartaric acid gave identical values.

Some of this syrup, 0.410 g, was neutralized with 11.25 ml of 0.345 N barium hydroxide to pH 8. The crystals which separated, 0.401 g, were purified by two more recrystallizations. As the crystalline material was difficult to dissolve in water, it was deionized with "Amberlite IR 120", then again converted to the barium salt by the addition of barium hydroxide solution and recovered by partial evaporation. Finally 0.225 g of barium salt (fraction 1) was obtained.

Found: Carbonated ash, 63.7, 62.0, 63.7% (micro method).

Calcd. for barium tartrate monohydrate

$BaC_4H_4O_6.H_2O$: 65.1%; for the dihydrate, 61.1%.

The combined mother liquors on evaporation to 2 ml and addition of an equal volume of ethanol yielded 0.253 g (fraction 2). On evaporation of the last mother liquor to dryness 0.088 g of material was obtained (fraction 3).

A portion of fraction 1, 0.206 g, was deionized with "Amberlite IR 120". Evaporation of the acidic solution yielded 0.100 g of crystals, m.p. 130-137°. These crystals were again dissolved in a little water, and neutralized with 2.4 ml of 0.25 N potassium hydroxide solution to pH 3.5.

The solution was then evaporated to 2 ml volume and two volumes of ethanol were added. The mixture was seeded with 0.3 mg of mono-potassium meso-tartrate and 10 ml of ethanol was slowly added over a period of several hours. The crystals which separated were purified by extraction with ethanol. Yield 0.0821 g.

Found: Carbonated ash, 35.9, 37.2%, (micro method).

Calcd. for mono-potassium meso tartrate, $KC_4H_5O_6$: 36.7%.

A portion of this potassium salt, 0.0739 g was deionized by passing the aqueous solution through an "Amberlite IR 120" cation-exchange resin column. The acidic effluent yielded on evaporation 0.0585 g of colourless crystals which melted at 138.5-140°. The melting point of meso-tartaric acid had been reported as 140° (93). The product, as expected, was optically inactive.

DL-Tartaric and D-Tartaric acids:- Fractions #2466-2600 yielded on evaporation 1.571 g of solution, which crystallized in part. From this liquor 0.612 g of crude crystals (fraction 1) and 0.480 g of residual mother liquor (fraction 2) were obtained.

Recrystallization of a part of fraction 1 from acetone yielded an acid, m.p. 202.5-203.5° (decomposition), with a neutralization equivalent of 76.1. Calcd. for tartaric acid: neutr. equiv. 75.

A much better purification was obtained by the preparation of the mono-potassium salt. Fraction 1, 0.315 g was dissolved in 1.5 ml of water and 8.4 ml of 0.25 N potassium hydroxide was added. Crystallization started after a few minutes. The solubility of the potassium salt was further reduced by the addition of an equal volume of ethanol and the mixture was placed in the refrigerator for twenty four hours. The crystals were washed by successive repulping with 0.7 ml of water, 2 ml of ethanol and 2 ml of ether. Weight 0.298 g. After decolourization and two recrystallizations from hot water 0.181 g of colourless crystals (fraction 1a) was obtained.

Found: Carbonated ash, 36.74, 36.60, 36.74%. Calcd. for mono-potassium tartrate, $KC_4H_5O_6$: 36.74%.

The mother liquors were combined and yielded 0.140 g more of crystalline salt, bringing the total to 0.321 g (84.5%).

A part of fraction 1a, 0.138 g, was dissolved in water and freed from potassium by passing through an "Amberlite IR 120" cation-exchange resin column. Evaporation of the acidic effluent yielded 0.114 g of crystals, which melted at 197.5-200.5° (decomposition). After recrystallization from tetrahydrofurane-chloroform the melting point increased to 203.5-204° (decomposition). The melting point recorded for DL-tartaric acid was 204-206°, (94). The product, as expected, was optically inactive. The addition of a saturated aqueous calcium sulphate solution to

an aqueous solution of the product gave a precipitate. DL-tartaric acid behaved in the same way. This test was used to differentiate between DL-tartaric and optically active tartaric acids (95).

Paper chromatography of fraction 1 with solvent B as the eluate and bromphenol blue as spray showed that it was identical to DL-tartaric acid. The chromatographic flow rate was determined with reference to L-tartaric acid, $R_{L\text{-Tartaric}}$ 0.32-0.62.

Tests were carried out to establish whether DL-tartaric acid could have been artifact. A solution of L-tartaric acid in N sulphuric acid was heated under reflux for two and one half hours, but the optical rotation of the solution remained unchanged. Similarly no change in optical rotation resulted upon allowing an aqueous solution of L-tartaric acid to stand three days at pH 12.

In a preliminary experiment 0.0525 g of fraction 2 was liberated from any contaminating cations by passing an aqueous solution through "Amberlite IR 120". The acidic effluent was evaporated to 0.5 ml volume and adjusted to pH 3.3 by the addition of 1.25 ml of 0.25 N potassium hydroxide. Crystallization was started by seeding with a trace of mono-potassium D-tartrate. An equal volume of ethanol was added, and the mixture placed in the refrigerator for twenty-four hours. The crystals which separated were macerated in 1 ml of aqueous ethanol and dried.

Yield of the acid potassium salt 0.0361 g. This product was freed from potassium with "Amberlite IR 120" in the same manner as outlined above. Evaporation of the acidic effluent yielded 0.0317 g of crystals, m.p. 165-166°. Yield 60% from fraction 2.

A large portion of fraction 2, 0.405 g was then purified as the potassium salt in exactly the same way yielding 0.301 g (60%). The crystals were again dissolved in water, decolourized and twice recrystallized from hot water. Finally 0.116 g (fraction 2a) was obtained. The mother liquors were combined and yielded 0.172 g of crystalline potassium salt. Fraction 2a.

Found: Carbonated ash, 36.94, 36.80%. Calcd. for mono-potassium tartrate $KC_4H_5O_6$; 36.74%.

A portion of the recrystallized potassium salt, 0.0805 g (fraction 2a), was dissolved in water and freed from potassium with "Amberlite IR 120" in the usual way. Evaporation of the acidic effluent yielded 0.0665 g of the crystalline acid, which melted at 167.5-169.5°, and had a specific rotation in water of $[\alpha]_D^{20}$ -14.0 ± 0.6° (c = 5.5). Bischoff and Walden (94), reported m.p. 170° for D-tartaric acid. The specific rotation was in good agreement with the equation given by Landolt (96)

$$[\alpha]_D^{20} = -15.06 + 0.131c;$$

where c was the concentration in grams of D-tartaric acid per 100 ml solution.

Calcd. for c = 5.5; $[\alpha]_D^{20} = -14.3°$.

Paper chromatography showed that the compound was identical with optically active tartaric acid. Solvent B was used as the eluent and bromphenol blue as the spray. The compound had a flow rate of $R_{L\text{-Tartaric}}$ 1.00.

Fractions #3516-3553:- These fractions were evaporated under reduced pressure to 15 ml. Fresh water, 150 ml, was added and the solution again evaporated to a small volume. This procedure was repeated several times until almost all of the trifluoroacetic acid was removed. The resulting solution was continuously extracted with ether which was changed at intervals of four, five and eight hours. After evaporation of the ether the three extracts weighed 0.928 g, 0.085 g and 0.145 g, respectively.

On partial evaporation of the first ether extract crystals separated which were isolated by filtration and dried several hours at 1 mm pressure over phosphorus pentoxide. Yield 0.213 g (fraction 1). A part of this crystalline material, 0.1678 g, was dissolved in 3 ml of water and decolourized with a small amount of activated charcoal. The decolourized filtrate was placed in a closed vacuum desiccator at 30 mm Hg pressure over sodium hydroxide. Trial showed that oxalic acid dihydrate was stable under these conditions. When more vigorous drying agents were used, the dihydrate was dehydrated to the anhydrous form. The solution yielded 0.2108 g of transparent crystals, some of which, 0.0906 g, were

recrystallized from hot water, yielding 0.0532 g of the pure product, m.p. 99.5-101°. The melting point was not depressed by the addition of an authentic sample of oxalic acid dihydrate. A sample 0.0160 g, consumed 9.74 ml of 0.0261 N potassium permanganate. Calcd. for oxalic acid dihydrate 9.73 ml.

Evaporation of the mother liquor from fraction 1 yielded a syrup 0.714 g, (fraction 2). Attempts were made to purify this material, both via the calcium salt and by a chromatic method. A part of fraction 2, 0.2053 g, was dissolved in water and freed from any contaminating cations by "Amberlite IR 120". The acidic solution was evaporated under reduced pressure to 20 ml and neutralized with 0.044 N calcium hydroxide to pH 5.9. The solution was again concentrated to 10 ml under reduced pressure and 0.014 g of calcium oxalate, which separated, was removed by filtration. After the filtrate had again been concentrated to 5 ml under reduced pressure 10 ml of ethanol was gradually added in small portions. The precipitated calcium salt was isolated by filtration, washed with 75% aqueous ethanol and dried under vacuum over phosphorus pentoxide. Yield 0.155 g (fraction 2a). Evaporation of the mother liquor yielded 0.0856 g (fraction 2b).

Samples of fraction 2a and 2b were dissolved in small volumes of water and freed from calcium ions by treatment with "Amberlite IR 120". The resulting filtrates were

chromatographed on paper together with a sample of tartronic (hydroxymalonic) acid. Again solvent B was used as the eluent and bromphenol blue as spray. At low concentrations the R_f values for tartronic acid, fraction 2a, and fraction 2b, were 0.30-0.34, 0.27-0.31 and 0.32-0.34. At higher concentrations the values were 0.30-0.47, 0.40-0.48 and 0.40-0.47, respectively. The similar variations in R_f with concentration made it probable, but failed to prove, that fractions 2a and 2b consisted of tartronic acid.

Fraction 2a, 0.1538 g, was dissolved in 1.5 ml of water and deionized as usual by "Amberlite IR 120". The acidic effluent was adsorbed on a micro column, 3.5 x 0.9 cm., containing Dowex 1-x-4 anion exchange resin (free hydroxy form). The resin column was washed with 10 ml of water, the washings being discarded and then eluted with 15 ml of 1 N acetic acid. The acetic acid effluent was evaporated to dryness, but only 2 mg of residue was found. The resin column was finally eluted with 30 ml of 25% aqueous formic acid. Evaporation of the effluent under reduced pressure yielded a syrup 0.1029 g. Tartronic acid crystals 1 mg, were added as seed, but the material failed to crystallize. The syrup was again dissolved in 1.5 ml of water and neutralized to pH 3.6 with 2.75 ml of 0.25 N potassium hydroxide.

Ethanol, 15 ml, was added, the solution seeded with a crystal of potassium acid tartronate and placed in the refrigerator

for several days. No crystallization took place. The ethanol was finally removed by evaporation under reduced pressure and the solution neutralized to pH 7.0 with 1.45 ml of 0.25 N potassium hydroxide. Addition of an equivalent amount of calcium acetate solution failed to produce a precipitation. Even on evaporation to dryness no crystallization took place.

Control experiments were made using authentic crystalline tartronic acid (a). The crystalline product, 0.0416 g,was dissolved in 0.5 ml of water, 0.25 N potassium hydroxide, 1.40 ml,and ethanol, 2 ml,were added and on cooling crystallization took place. Additional 1.5 ml of ethanol was added, the mixture left in the refrigerator for twenty four hours and the acid potassium salt, 0.0411 g, recovered by filtration.

Experiments with ion-exchange chromatography showed that tartronic acid adsorbed on a small column of Dowex 1-x-4 could be effectively displaced from the resin with 1 N formic acid.

In another experiment a sample of authentic tartronic acid,0.12 g, was dissolved in 10 ml of water, and the solution was neutralized to pH 7 with 0.25 N potassium hydroxide. Addition of an equivalent amount of calcium

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(a) Supplied by Fluka A.G, Basel, Switzerland.

acetate produced a voluminous precipitate of the calcium salt.

Fractionation and Hydrolyses of the Water Insoluble Calcium Salts

Oxalic acid:- Five grams of fraction I from oxidation 8 was dispersed in 500 ml of water and the mixture adjusted to pH 3.1 with 1.1 ml of concentrated hydrochloric acid. The undissolved material was recovered by filtration and air dried. This material, 2.99 g, was dispersed in hot water, "Amberlite IR 120" cation exchange resin, 30 g, was added and the mixture was heated on a steam bath for twenty minutes. The clear solution was decanted and the solids extracted several times with boiling water, all extracts then being combined and evaporated under reduced pressure to 10 ml volume. Crystals, 0.910 g, separated from the solution and recrystallization from hot water yielded 0.65 g of the colourless product which was identified as oxalic acid dihydrate, m.p.100-100.5°. The melting point was not depressed by the addition of an authentic sample of oxalic acid dihydrate. In titrations with acid 0.101 N potassium permanganate 0.0553, 0.0559 and 0.0613 g samples reduced 8.78, 8.66 and 9.65 ml. Calcd. for $(COOH)_2.2H_2O$, 8.68, 8.78 and 9.63 ml, respectively.

The oxalic acid was quantitatively isolated in the following way. Fraction I, 5.00 g, was dispersed in 5% aqueous acetic acid solution and the mixture was agitated for several hours with the help of a mechanical shaker.

The insoluble portion was removed by filtration and again subjected to the same procedure. It was finally washed with water and dried over phosphorus pentoxide under vacuum. Yield 3.04 g with 3.14 g in a second preparation corresponding to 60.8, 62.8% by weight of fraction I.
Found: Sulphated ash, 77.9%, (corresponding to: Ca, 22.9%)
Calcd. for $(COO)_2Ca.H_2O$: Ca, 27.4%

The insoluble product was dissolved in 40 ml of 20% hydrochloric acid and extracted with diethyl-ether using a continuous liquid - liquid extraction apparatus. After five and one half, four and four hours the ether was changed, the solvent removed by evaporation and the extracted material weighed. These weights were 2.067 g, 0.073 g and 0.008 g, respectively. The first two extracts were combined and dissolved in a small volume of water. Traces of insoluble material were removed by filtration. The resulting solution was dried to constant weight in a vacuum desiccator over solid sodium hydroxide. Oxalic acid dihydrate separated in large colourless crystals. Yield 2.078 g, (equivalent to 48.2% of calcium oxalate monohydrate in fraction I). Anal. 0.1937 g consumed 29.22 ml of 0.1044 N acid potassium permanganate. Calcd. for $(COOH)_2.2H_2O$, 29.45 ml.

Calcium Salts Insoluble in Water but Soluble in 5% Acetic Acid:- The liquors obtained by extracting 5.00 g of fraction I with 5% acetic acid (see preceeding preparation) were combined and freed from calcium ion by passage through an "Amberlite IR 120" ion-exchange resin column. The column was washed with water, until the effluent reached pH 4.3 After reducing the volume of the acidic effluent by evaporation under reduced pressure, chloride ions were removed by treatment with a slight excess of freshly prepared silver carbonate. The silver salts were removed by filtration and the resulting solution evaporated to dryness under reduced pressure. A brittle, glass like material was obtained. Yield 0.674 g, (5.0% by weight of the original anhydrous starch), fraction Ia.

A part of this material was hydrolysed by heating under reflux with N sulphuric acid for two and one half hours. Sulphate ions were removed by the addition of a calculated amount of barium hydroxide solution. The filtered hydrolysate was chromatographed on three individual paper sheets with solvent B as eluent. Bromphenol blue, o-amino-diphenyl and hydroxylamine-ferric chloride were the sprays. The results are shown in table VI.

In another experiment, the portion from 5.00 g of fraction I, soluble in 5% acetic acid, was placed in a cellophane membrane. The solution, 250 ml, was dialyzed against distilled water, 3000 ml, for 48 hours, the water

being changed every twelve hours. The dialyzed solution yielded on evaporation to dryness under reduced pressure 0.207 g of residue (1.5% by weight of the original, anhydrous starch). Hence not all of this fraction was dialysable.

SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

1. The oxidation of starch with a large excess of aqueous calcium hypochlorite at pH 12 and 20° was followed by determining the unreacted hypochlorite at various times. A semi-logarithmic plot of the ratio of initial to remaining hypochlorite versus the reaction time was found to correspond to two reactions proceeding at different rates, which were superimposed on each other and behaved as reactions of the first order.

2. The oxidation of starch with 3 mole of aqueous calcium hypochlorite per anhydroglucose unit was found to degrade the polymer greatly. Carboxyl groups, 1.13 mole, and carbon dioxide 0.136 mole, per mole of anhydroglucose, were formed and accounted for about 80% of the oxidant. The amount of carbonyl groups was negligible. Oxalic acid formed, 0.208 mole, accounted for about two fifths of the carboxyl groups.

3. Hydrolysis of the oxidized starch and separation of the sugar acids by ion-exchange chromatography led to the isolation of glyoxylic acid, 0.028 mole, D-erythronolactone, 0.11 mole, D-glucuronic acid, 0.004 mole, meso-tartaric acid,

0.023 mole, DL-tartaric acid, 0.011 mole and D-tartaric acid, 0.009 mole per mole of anhydroglucose. Meso-tartaric DL-tartaric and D-tartaric acids were isolated for the first time from the hydrolysate of starch oxidized with alkaline hypochlorite.

4. An upper estimate of the formation of carboxyl at the 6-position of the anhydroglucose units was given by the determination of uronic anhydride and was 0.16 mole per mole.

5. An estimate of the extent of cleavage of the glucose residues at the 2,3-positions, given by the isolation of D-erythronolactone and meso-tartaric acid, was 0.13 mole per mole.

6. Support for Kaverzeneva's theory that the 1,2-position of the glucose unit was cleaved by the action of alkaline hypochlorite on cellulose was provided by the isolation of 0.009 mole of D-tartaric acid per base mole of starch.

7. Wolf and Weijlard's method (97) for the preparation of butyl glyoxylate from dibutyl tartrate and lead tetracetate was modified to produce free glyoxylic acid from tartaric acid and periodate. Anhydrous glyoxylic acid was obtained in a crystalline state for the second time on record, and a new, crystalline sodium-calcium salt was described.

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