GLYCOSYL DERIVATIVES OF ANALOGUES OF ASCORBIC ACID AND RELATED COMPOUNDS

By

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I. INTRODUCTION

The prominent part that ascorbic acid (vitamin C) plays in human biology has stimulated a vast amount of research dealing with ascorbic acid, its analogues and derivatives. It has been established that the presence of this substance in the diet is necessary for the well-being of all primates, the guinea pig and some microorganisms (1)[1]. Apparently vitamin C is required by all animals and plants but some are able to synthesize it. A large deficiency of ascorbic acid in man causes scurvy; a small deficiency results in minor disturbances and retards the healing of wounds (2). The latter effect makes important an abundant supply for military forces. The animal body is unable to store large quantities of the vitamin, and thus a deficiency of vitamin C occurs very rapidly when it is not present in the diet.

Presumably the capacity of ascorbic acid to participate in reversible oxidation-reduction processes is responsible for its important biological role (3). Its instability in respect to oxygen in the presence of metal catalysts, however, results in large losses of the vitamin in the canning, storage, and shipment of foods. For this reason, the synthesis of a relatively stable ascorbic acid derivative which could be used for the generation of vitamin C in vivo would be of inestimable value.

The widespread occurrence of glycosides (4,5) and the multifarious reactions in which they are involved suggests their possible use in this connection. Presumably any of the hydroxyl groups of ascorbic

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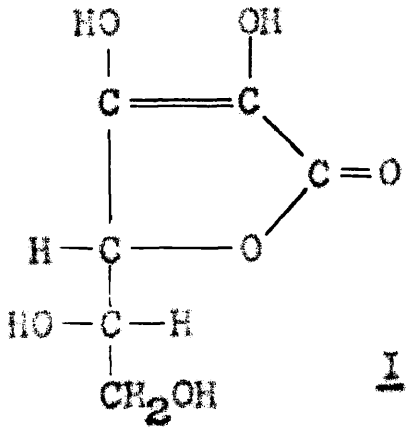
1. Figures in parentheses refer to literature references at the end of the thesis.

acid might be substituted with a glycoside group. If the product were stable to air oxidation, the vitamin could be placed in food in the form of the glycoside; and since the digestive systems of man and animals contain enzymes which are capable of splitting certain glycosides, it is reasonable to suppose that ascorbic acid would be liberated in the body. A β-galactosyl derivative, for example, would be split by the lactase of the intestinal tract to form galactose and ascorbic acid. Thus, a product stabilized by a β-galactosyl group could be added to evaporated milk to eliminate the need for supplementing the diet of infants with orange juice.

Although highly desirable, there is only a small chance for the successful development of a compound suitable for use in foods. The study of the subject, however, is of significance in relation to biological chemistry in general and to the chemistry of the enolic acids in particular.

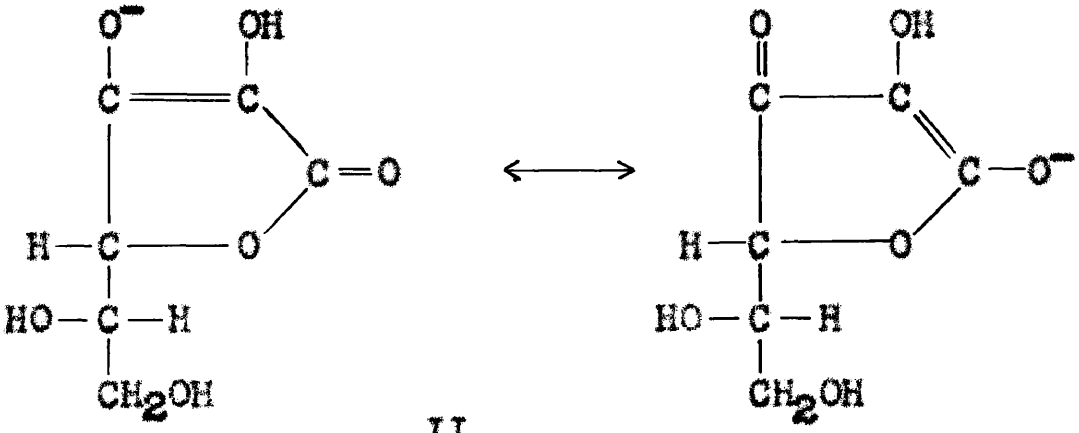
The importance of glycosides in plant and animal life has been recognized for a long time. The detoxification and elimination of phenolic substances and sterols in the form of glucuronic acid glycosides show the capacity of the animal body to synthesize glycosides (6). Consequently, glycosidic derivatives of ascorbic acid might be formed in biological systems and the in vitro preparation of such compounds might prove, at any day, a stepping stone for the elucidation of some puzzling biological product available in micro quantities.

II. DISCUSSION OF POSSIBLE METHODS OF PREPARATION OF GLYCOSYL DERIVATIVES OF ASCORBIC ACID.

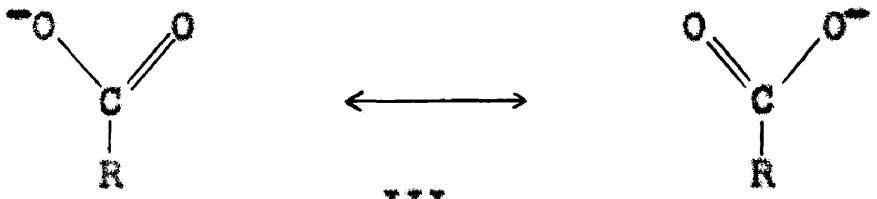


I

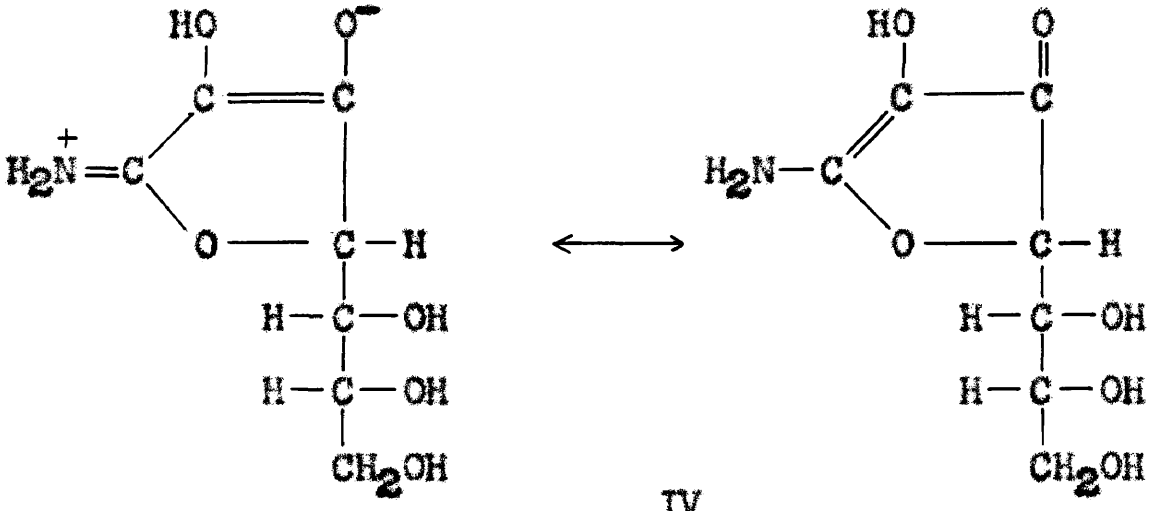
Examination of the formula for ascorbic acid I reveals hydroxyl groups of diverse character. The substance is dibasic with dissociation constants of 6.76×10^{-5} and 2.75×10^{-12} at 25° C (7). The first dissociation constant may be ascribed to the hydroxyl of carbon 3 and the second to that of carbon 2. Ascorbic acid reacts with one mole of diazomethane to give 3-methyl ascorbic acid; with larger quantities to yield 2,3-dimethyl ascorbic acid (8). The hydroxyl of carbon 3 is somewhat analagous to that of a carboxyl group. The relationship may be seen from the resonance forms II and III on page 4. The resonance of the ascorbate ion operates through a conjugated system of double bonds in contrast to that of the carboxylate ion in which the resonance involves a single carbon atom. In both cases the greater resonance of the ion in comparison with the resonance of the unionised acid accounts for the tendency of the acids to ionise. Other substances



II



III



IV

show the mesomerism depicted in II as, for example, imino 6-glucoascorbic acid IV on page 4. The rotatory dispersion of sodium ascorbate differs markedly from that of ascorbic acid, but resembles closely that of imino 6-glucoascorbic acid. The resemblance of the dispersion of the salt and the imino compound may be explained by the similarity of resonance structures II and IV.

The hydroxyl of carbon 2 is similar to one of the hydroxyls of ortho-hydroquinone in that it is attached to a resonance system which includes a hydroxyl on the adjacent carbon. In both compounds the hydrogens of each of the two hydroxyl groups can be ionized and the divalent anions are easily oxidized (9). Blocking of either of the hydroxyl groups destroys the strong reducing power of either ascorbic acid or of ortho-hydroquinone. Hence, 2-methyl ascorbic acid and 3-methyl ascorbic acid, like guaiacol (monomethyl-ortho-hydroquinone), are not powerful reducing agents. Thus it seems possible that introduction of a glycoside group at carbon 2 of ascorbic acid would stabilize the substance toward oxidation. Such a substituted compound would be inert to atmospheric oxidation and might be capable of generation of vitamin C by enzymatic cleavage in vivo.

The hydroxyl groups attached to carbon atoms 5 and 6 are of the alcoholic type, one secondary and one primary. Inasmuch as the constituent group would be in the side chain, we would expect glycosides derived from these hydroxyls to resemble ascorbic acid more closely than the 2-or 3-substituted acids. However, the ascorbic acid molecule has been shown by X-ray measurements to be substantially planar (10). The introduction of a large group into the side chain might disturb the

planar structure, and since this structure is necessary for a high degree of resonance, there would be less resonance in the resulting enolic acid. The decrease in resonance energy would increase the susceptibility of the lactone ring to hydrolysis and otherwise alter the properties of the enolic acid. Hence we might anticipate that the 5-and 6-glycosyl derivatives would be less stable than the unsubstituted ascorbic acid. Nevertheless a study of this type of derivative seemed advisable because glycosides of this character might be obtained from disaccharides by synthetic methods analagous to those used for the synthesis of ascorbic acid from monosaccharides.

Several general methods have been described for the preparation of compounds of the ascorbic acid type from the monosaccharides (11). Aside from the limitations imposed by the glycosidic linkage present in disaccharides, any of these methods might be applied to the preparation of glycosidic derivatives of ascorbic acid from disaccharides.

Natural ascorbic acid, vitamin C, requires raw material not readily obtained, but the configurational analogue, isoascorbic acid, can be prepared from glucose, and the corresponding glycosidic derivatives might be prepared from some of the common disaccharides. For the development of synthetic methods and the ascertainment of the characteristics of the desired compounds, configuration is of no importance. Hence, the selection of derivatives to be studied was based upon the availability of the starting materials rather than their configuration. Lactose, melibiose, maltose and cellobiose were chosen as raw materials.

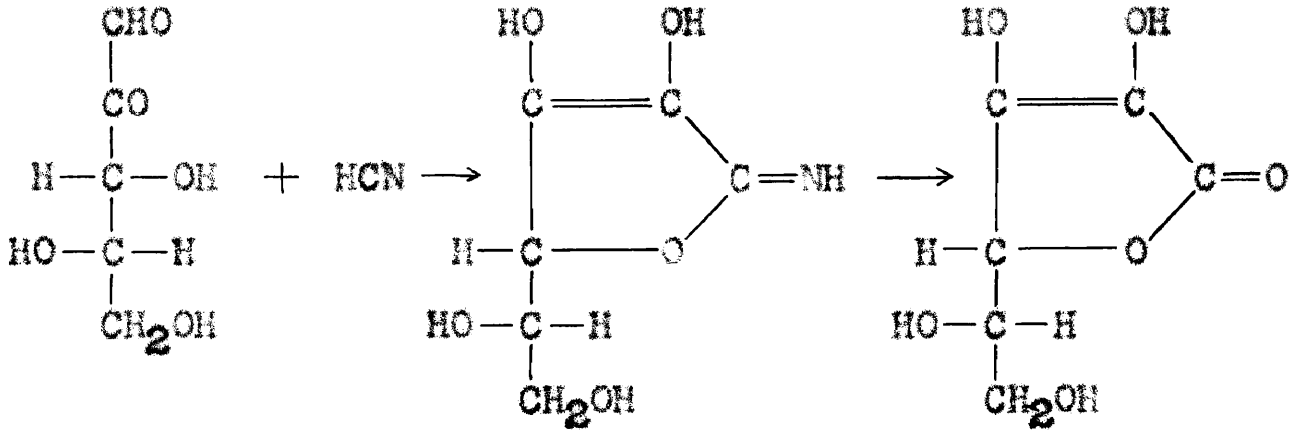
The plan of the present work was to examine the methods which have been used for the preparation of ascorbic acid or its analogues

from monosaccharides and to modify them as necessary for use with di-saccharides.

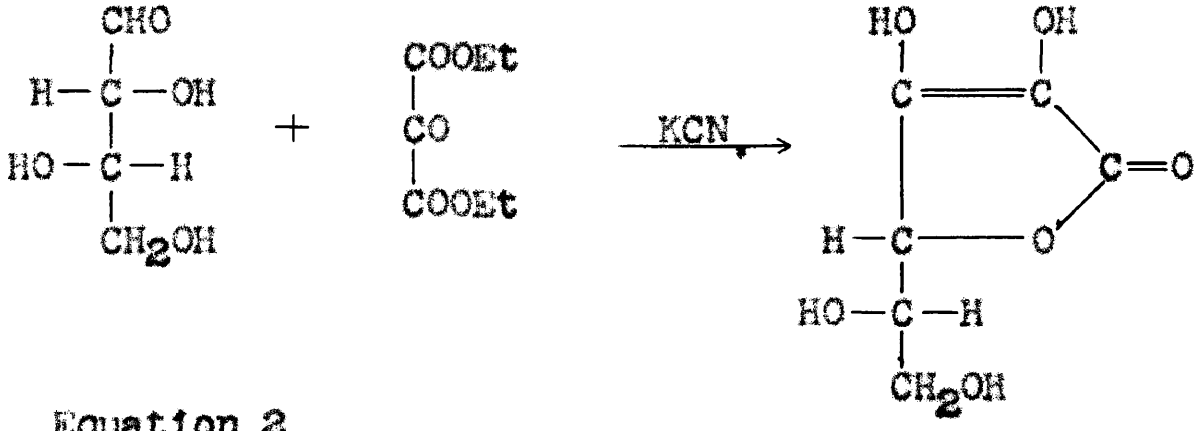
The following methods have been used for the synthesis of ascorbic acid and its analogues from monosaccharides:

1. Addition of hydrogen cyanide to the osone followed by hydrolysis (12 to 22 inclusive). Equation 1 on page 8.
2. Condensation of aldoses with ethyl mesoxalate or ethyl glyoxalate (23, 24). Equations 2 and 3 on page 8.
3. Isomerization and lactonization of 2-keto-acids or esters (25 to 49 inclusive). Equation 4 on page 9.
4. Condensation of esters of hydroxy acids (50, 51). Equation 5 on page 9.
5. Reaction between an acyl bromide and potassium cyanide followed by hydrolysis, then through method 1 (52, 53). Equation 6 on page 9.

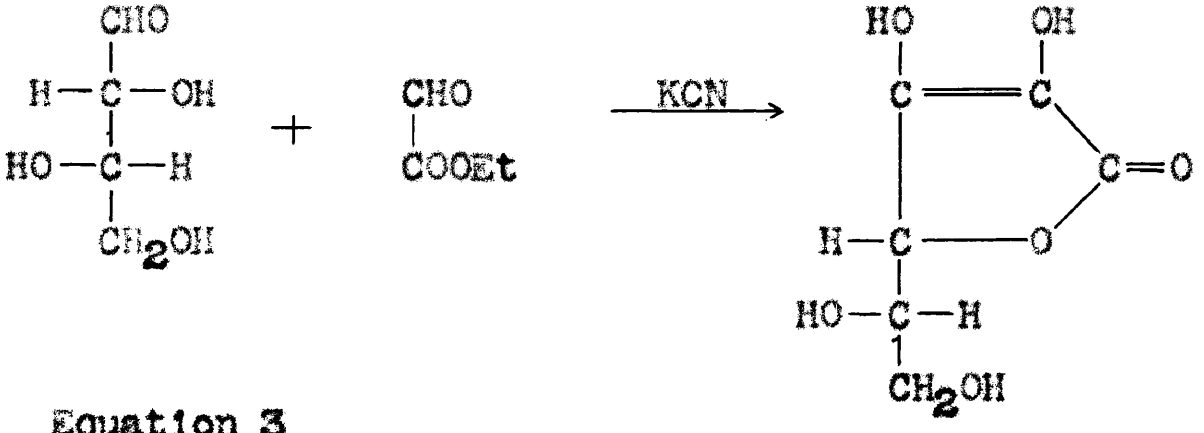
The first and fifth of these methods yield an ascorbic acid with one more carbon than the parent sugar; thus one would obtain 5-β-galactosyl-L-gluconoascorbic acid V from lactose, and 7-α-galactosyl-L-gluconoascorbic acid VI from melibiose. The second and fourth methods add two carbon atoms to the chain and 6-β-galactosyl-gluconoheptonoascorbic acid VII would be obtained from lactose and 6-α-galactosyl-gluconoheptonoascorbic acid VIII from melibiose. The third method would give an ascorbic acid having the same number of carbon atoms as the parent 2-keto-acid. Thus 6-α-galactosyl-L-isoascorbic acid IX would be obtained from 2-keto-melibionic acid. The method would not be applicable to 2-keto-lactobionic acid because ring formation at carbon 4 is blocked by the galactosyl group. The possibility of ring formation in this



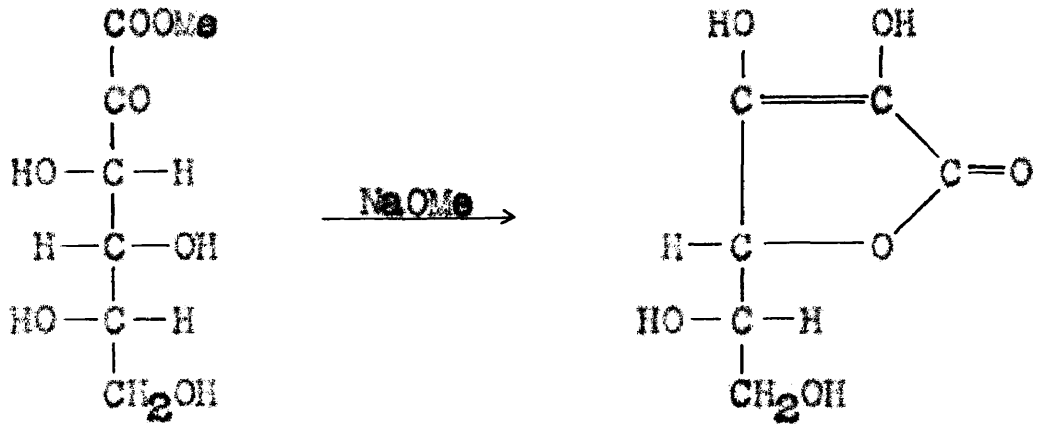
Equation 1



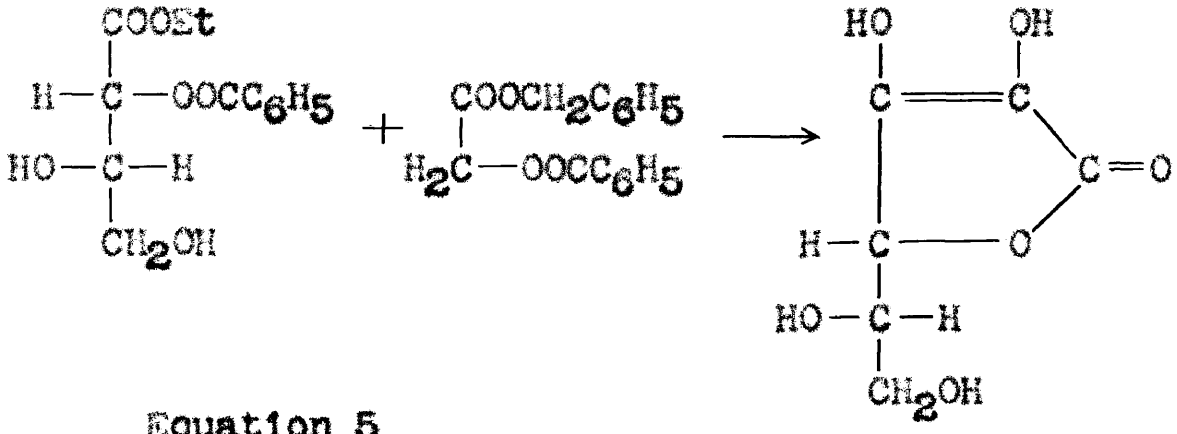
Equation 2



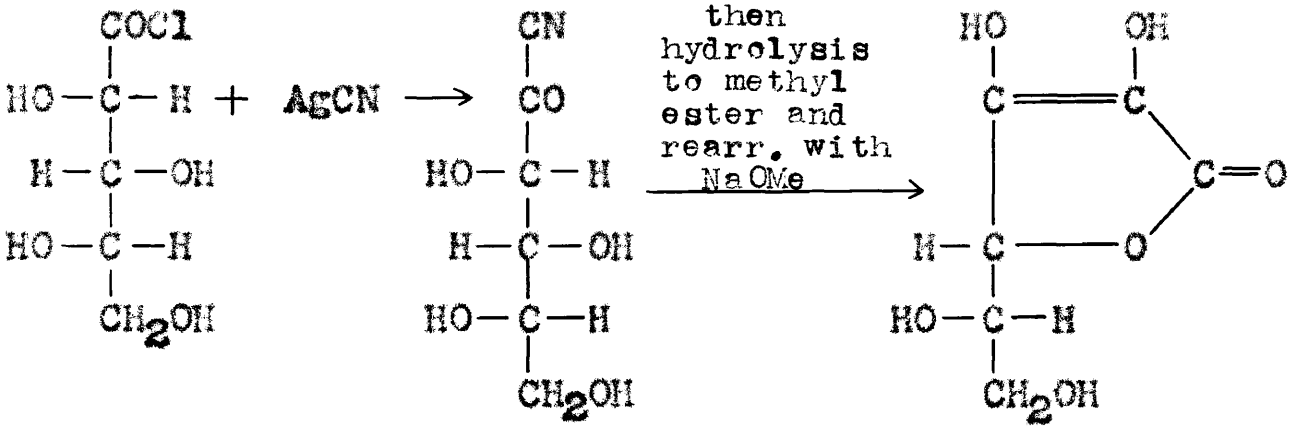
Equation 3



Equation 4



Equation 5

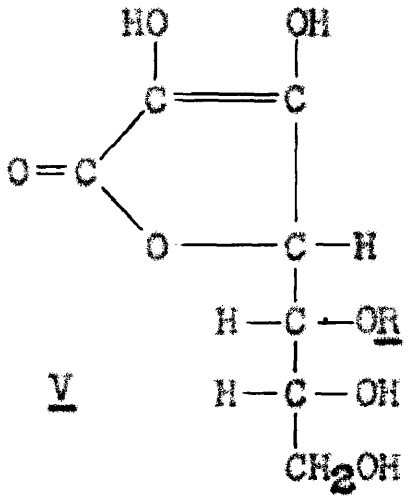


Equation 6

compound is discussed on pages 25 and 24.

A search of the literature very surprisingly disclosed only one reference to an attempt to prepare an ascorbic acid analogue from a disaccharide. Baird, Haworth, Herbert, Hirst, Smith, and Stacey (16) describe the action of potassium cyanide in the presence of calcium chloride, on lactosone and maltosone (method 1). The cyclic imino-compound which was formed could not be isolated; and when it was hydrolysed, the biose linkage was split simultaneously with the elimination of the imino group. The final product was l-glucoascorbic acid.

These results clearly show that glycosidic ascorbic acid derivatives cannot be obtained by methods involving a drastic acid hydrolysis. Since Haworth and coworkers were unable to obtain a glycosidic derivative of an ascorbic acid analogue by the cyanohydrin synthesis, attention was directed to the other methods.



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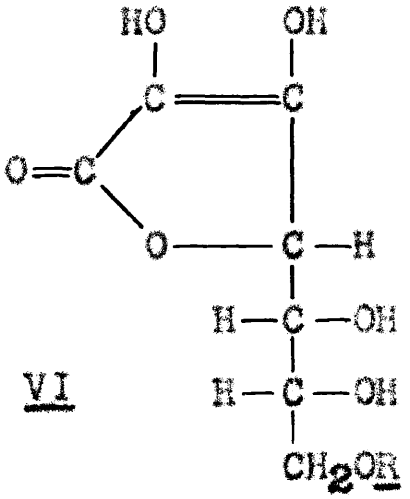

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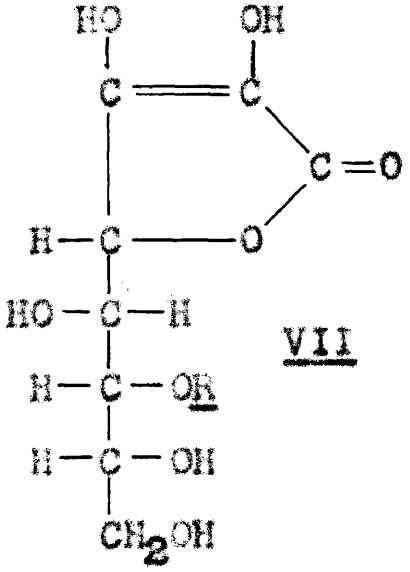
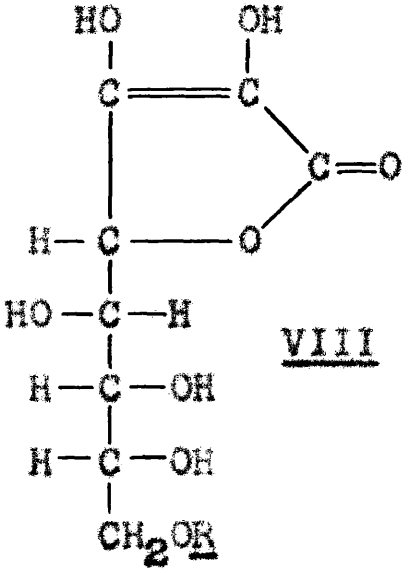


VII



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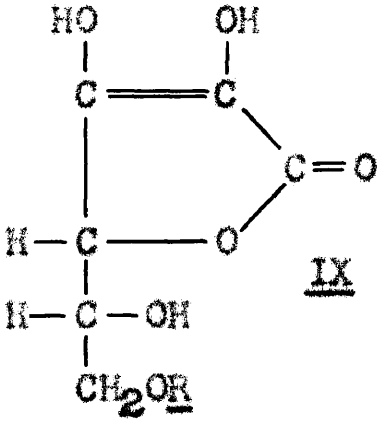


IX

R= Galactosyl group

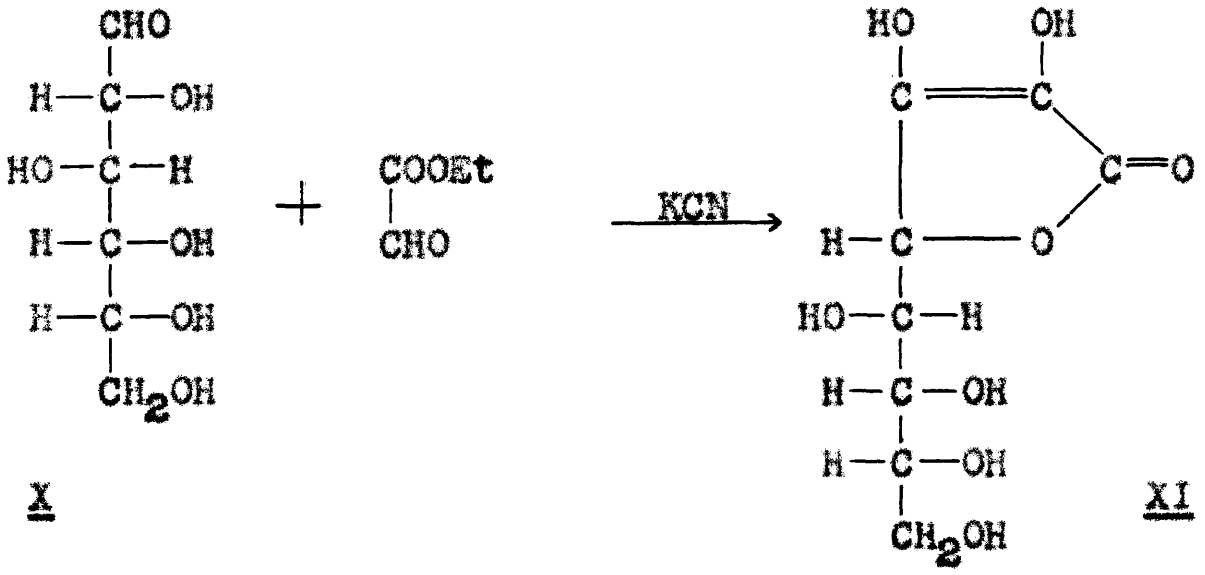
III. PREPARATION OF GLYCOSYL DERIVATIVES OF ANALOGUES OF ASCORBIC ACID BY THE MESOXALATE ESTER METHOD

The acyloin condensation, which takes place with aldehydes that have no hydrogen on the alpha carbon atom, has been known for many years and is exemplified by the well-known condensation of benzaldehyde in the presence of potassium cyanide to yield benzoin. The mechanism of this reaction (54), for which the cyanide ion is a specific catalyst, is probably as shown in equation 7 on page 14.

Helferich and Peters (23, 24) saw the possibility of applying this reaction to the synthesis of ascorbic acid and its analogues. They found that an aldose, such as D-glucose X, despite the presence of hydrogen on the alpha carbon atom, would undergo an analogous reaction with ethyl glyoxalate to yield, through concomitant lactonization, L-glucoheptonascorbic acid XI. Helferich discovered later, that ethyl glyoxalate could be replaced by ethyl mesoxalate (24). D-arabinose XII, for example, yields L-gluconascorbic acid VIII with this reagent. The similarity of this reaction and the benzoin condensation can be seen by a comparison of equations 7 and 8 on page 14.

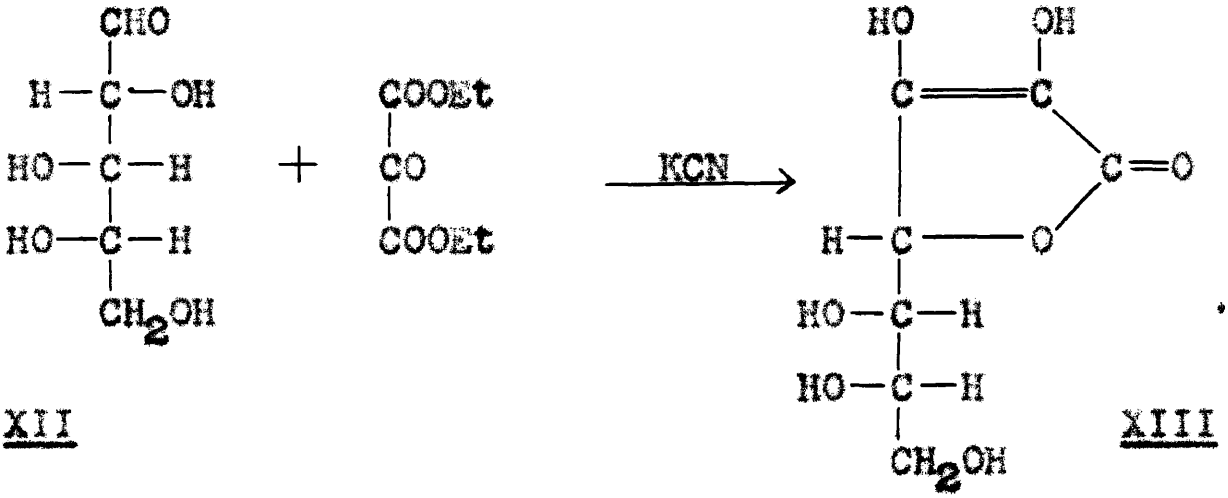
The aldose can be replaced by the acetylated nitrile of the next higher sugar acid in either reaction because the acetyl groups are removed in the alkaline solution and the resulting deacetylated nitrile readily undergoes transformation to yield one of the intermediates A postulated in the mechanism of the condensation as shown on page 14, equation 9.

It is obvious from the mechanism of the reaction that the aldehyde form of the sugar, or a derivative such as a sugar acid nitrile,



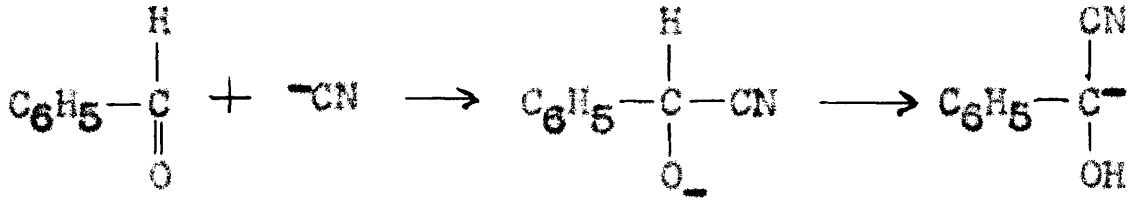
X

XI



XII

XIII



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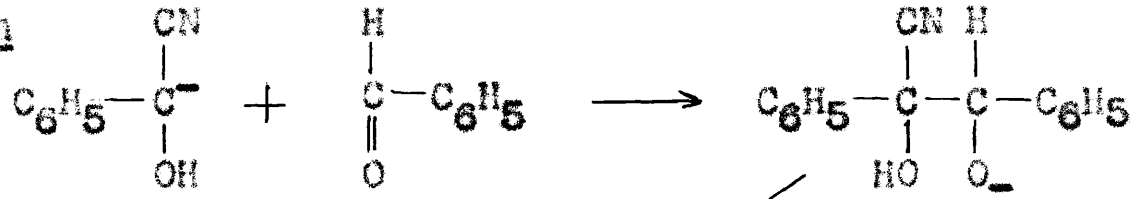
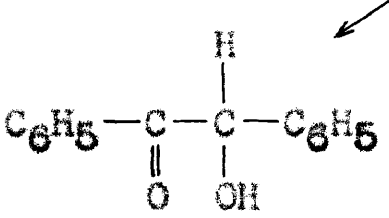
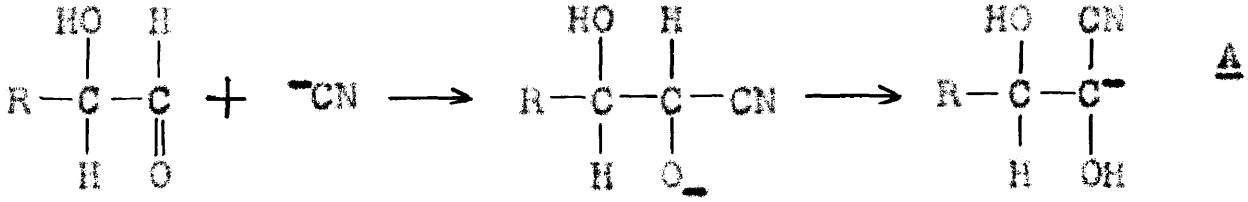




Equation 7



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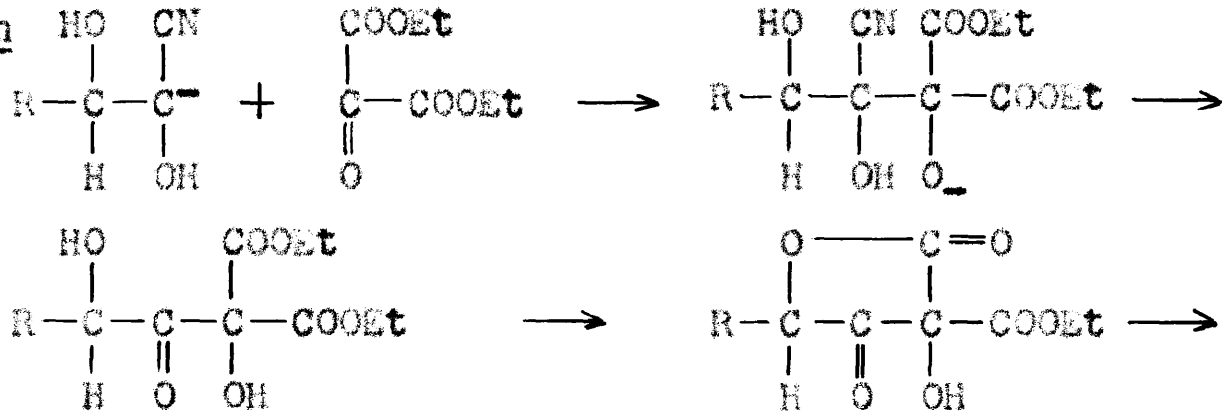
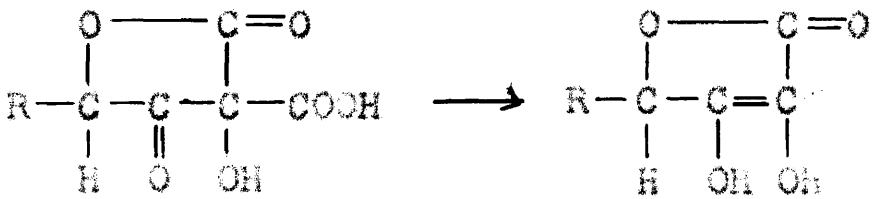
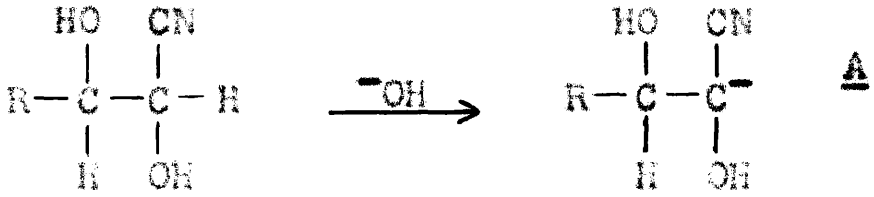




Equation 8



Equation 9

which can yield directly one of the intermediate compounds through which the reaction proceeds is essential for the condensation. It was found by Helferich that higher yields were obtained with the acid nitriles than with the sugars and that the yield varies for different sugars.

For the preparation of vitamin C by this method, L-threose or tetraacetyl-L-xylonenitrile is required. Despite the inaccessibility of these compounds, the method has been used in Germany for the commercial preparation of vitamin C. The method is of general applicability for the synthesis of ascorbic acid analogues. Its simplicity, when the necessary starting materials are available and the fact that the reaction takes place under conditions that do not split the biose linkage, recommend its use in the present study.

The application of this method to lactose, melibiose and other glucose derivatives is of particular interest because the resulting products have the configuration for carbons 4 and 5 which is characteristic of vitamin C. Hence the product might be expected to be physiologically active.[2] Melibiose would be expected by this method to yield 6-α-galactosyl-glucoheptoascorbic acid XIV and maltose to yield 6-α-glucosyl-glucoheptoascorbic acid XV. The structure of the portion of the molecules above the dotted line in the formulas XIV and XV corresponds to that of ascorbic acid which is shown again in XVI.

It seemed advisable, before extending the reaction to the disaccharides, to repeat the work of Helferich with the monosaccharides.

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2. The physiological activity of numerous analogues of ascorbic acid has been reported (55). It has been shown that L-glucoheptoascorbic acid has 1/100 the activity of the natural vitamin.

Condensation of arabinose with diethyl mesoxalate and titration of the resulting solution with iodine[5] indicated a yield of 41 percent compared to a value of 20 percent reported by Kelferich. The condensation was then carried out with melibiose, maltose, lactose and cellobiose. It was found that melibiose and maltose yielded a precipitate of a yellow sodium salt similar to the precipitate of sodium ascorbate formed from monosaccharides. However, lactose and cellobiose did not dissolve very readily in the methanol solution and the precipitate which formed was nearly white and appeared to be different from the products obtained with melibiose and maltose. The precipitates were dried in vacuo at room temperature; weighed samples were dissolved in water and the acidified solutions were titrated with iodine. Determination by iodine titration of the sodium glycosyl-ascorbate in the product obtained from melibiose showed a purity of 21 percent. The yield of the glycosyl-ascorbate calculated from the weight of the salt and its purity was 9.8 percent of the theoretical. The sodium glycosyl-ascorbate obtained from maltose showed a purity of only 17.8 percent, but the yield was the same as above, namely 9.2 percent.

The precipitates obtained with lactose and cellobiose, in contrast to the above sugars, did not reduce an appreciable quantity of iodine, and it was apparent that little or no material of the ascorbic type was formed. It was found, however, that the original filtrates from the precipitates of these two sugars, as well as those from melibiose and maltose consumed some iodine. The source of this reducing

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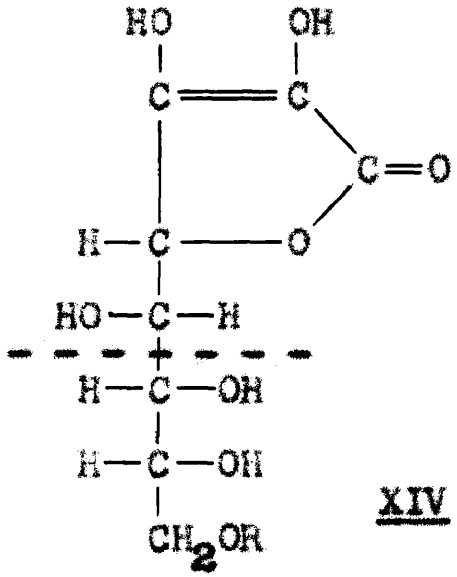
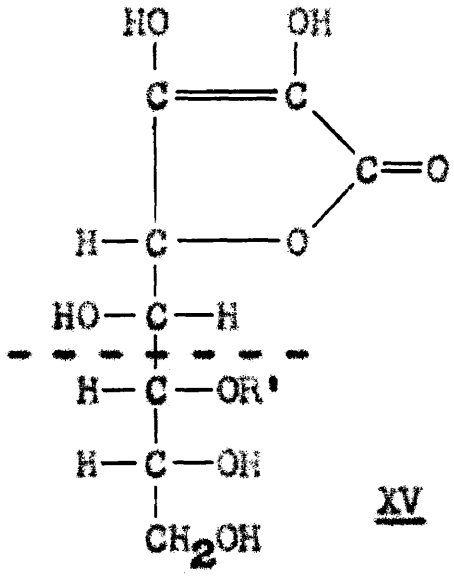
5. One mole of ascorbic acid reduces two equivalents of iodine in acid solution. The reaction provides a convenient method for estimation of ascorbic acid in the absence of other reducing material. The ascorbic acid is oxidized to dehydroascorbic acid.



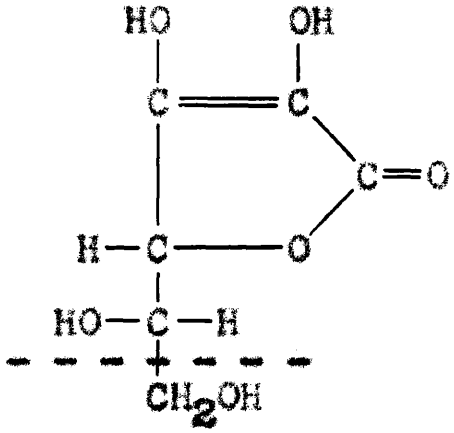
XIV

R = Galactosyl



XV

R' = Glucosyl



XVI

material was discovered by heating the reagents in the absence of any sugar. Under these conditions, the resulting solution reduced the same amount of iodine as the above filtrates. This experiment proved the necessity of the separation of the sodium salt as the first step in the purification of the product. The results obtained with lactose and cellobiose demonstrated the effectiveness of the filtration procedure as a means of separating the desired product from other reducing material.

The lack of the formation of an appreciable quantity of an ascorbic acid derivative from lactose and cellobiose is surprising. The inertness of these sugars in this synthesis may arise from the fact that they have little tendency to exist in the aldehyde form. Presumably the aldehyde form is a prerequisite for the condensation reaction.

Ascorbic acid and related compounds show strong absorption in the ultraviolet region, a property which has been widely used for identification and for analytical purposes (56, 57). To establish the character of the substances obtained from the condensation of diethyl oxalate with melibiose and maltose, a study was made of the absorption spectra of the products. The results are shown in graphs 1 and 2 on page 20. The logarithm of the molar absorbancy index,[4] a_M, of ascorbic acid and its analogues is reported to be approximately 4.0. The peak of the absorption band is at 265 mμ in neutral solution and at 245 mμ in acid solution.

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4. The term "molar absorbancy index" has been recommended by the National Bureau of Standards in place of the term "extinction coefficient", and is designated a_M. The equations involved in its calculation are as follows:

$$a_M = \frac{A}{b \times M} \cdot \quad A_s = -\log 10\, T_s \cdot \quad T_s = \frac{\text{transmittancy of solution}}{\text{transmittancy of solvent}}$$

where b = length in centimeters of absorbing path between the boundary layers of the solution, A_s = absorbancy of sample, T_s = transmittancy of the sample, M = molecular weight of sample.

Although the present compounds were impure, their molar absorbancy indices were calculated from the absorption data and are shown in Table 1. The values reported for ascorbic acid (16) are included for comparison.

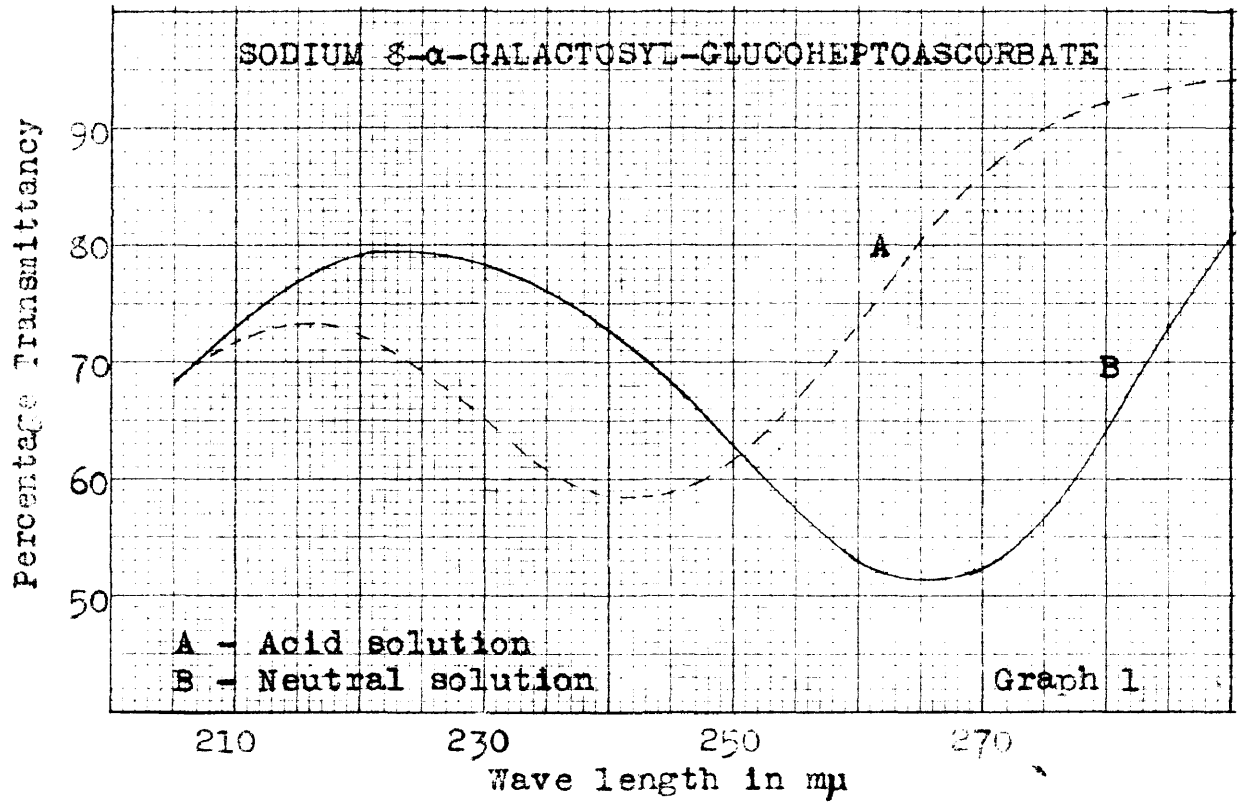
Table 1.

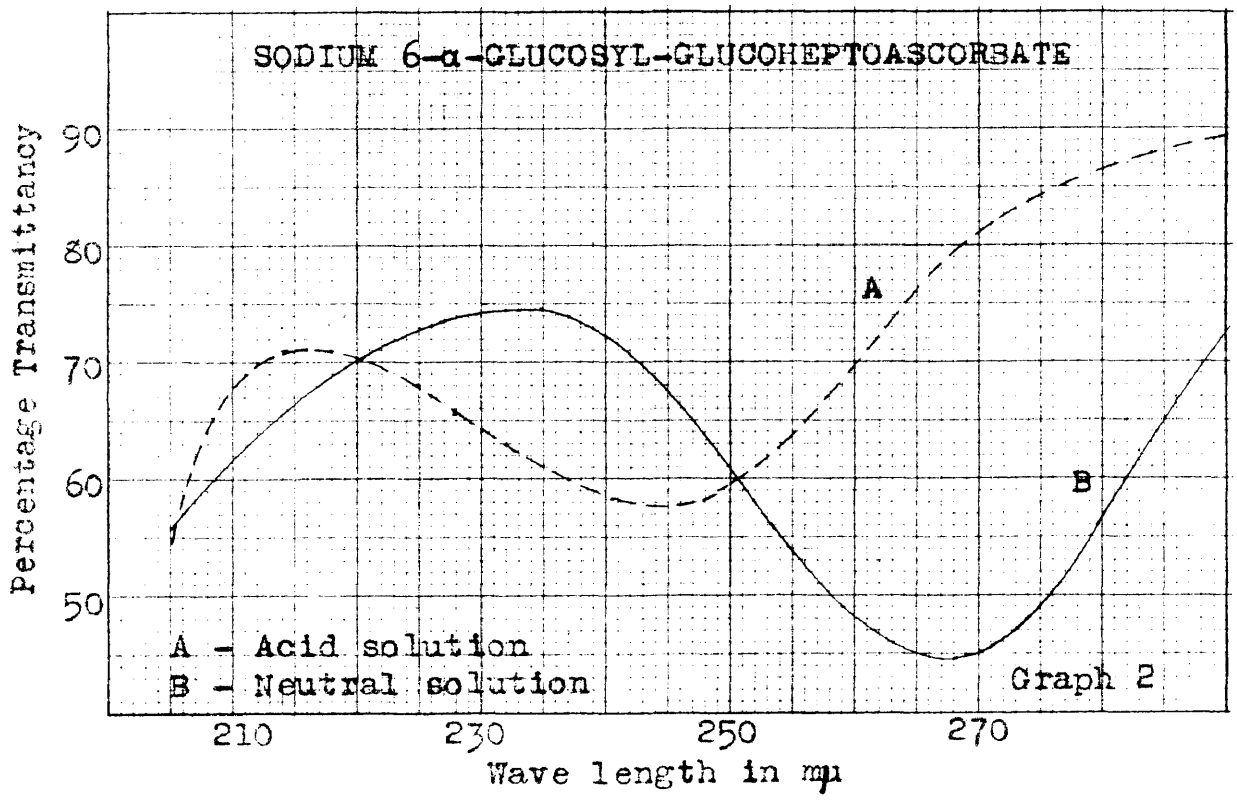
Substance	$\log_{10}$ of molar absorbancy index		Wave length in mμ at max. absorption	
	neutral	acid	neutral	acid
ascorbic acid	4.0	4.0	265	245
product from melibiose	3.7	3.6	265	242
product from maltose	3.9	3.8	260	245

Thus in acid solution both products show a maximum absorption at wave lengths near the maximum for ascorbic acid and in neutral or alkaline solution at wave lengths near the maximum for sodium ascorbate. The intensity of the absorption (molar absorbancy index) of the new compounds is of the same order as that of ascorbic acid and the peak of maximum absorption undergoes the same shift in wave length with change in pH as that of ascorbic acid.

The absorption spectra clearly prove that the products from melibiose and maltose are of the ascorbic acid type. Their method of formation would yield 6-α-galactosyl-glucoheptoascorbic acid VIV from melibiose and 6-α-glucosyl-glucoheptoascorbic acid IV from maltose. Attempts to separate the pure acids or their salts, however, were unsuccessful.

ABSORPTION SPECTRA





IV. PREPARATION OF 2-KETO-ALDOBIONIC ACIDS AND RELATED COMPOUNDS

The commercial method for the production of ascorbic acid involves the formation of diacetone-2-keto-l-gulonic acid followed by esterification and conversion to ascorbic acid by treatment with either an acid or basic catalyst. The conversion of methyl 2-keto-l-gulonate by sodium methylate to a salt of ascorbic acid is substantially quantitative. Since the conversion is effected in alkaline solution, the method seems applicable to the present problem. The only drawback to the process is the difficulty of obtaining the ester of the 2-keto-bionic acid, which arises primarily from the sensitivity of the glycosidic linkage to acid hydrolysis.

Two routes for obtaining methyl 2-keto-aldobionic esters seem feasible. One of these is oxidation of the aldobionic acid in methanol with chlorates in the presence of vanadium pentoxide (41). Fair yields of methyl 2-keto-aldonates have been obtained by this method with aldonic acid derived from monosaccharides. Ordinarily, the reaction is conducted in the presence of phosphoric or sulfuric acid. Attempts to apply this method to melibionic acid and to lactobionic delta lactone revealed that the reaction is very slow in the absence of appreciable quantities of phosphoric or other strong acids, and conditions could not be found for conducting the oxidation without extensive hydrolysis of the aldobionic acid. In the course of the work an excellent method for the preparation of calcium melibionate was developed. This will be described in a later section.

The second route for the preparation of esters of 2-keto acids consists of formation of the osone from the sugar followed by oxidation of this to the acid and finally esterification.

Two methods are available for preparing the necessary osones from the sugars. The first method is by oxidation with copper acetate (59). The method is not generally applicable to the preparation of osones from disaccharides, but a modification of this method has been developed and used in the preparation of melibiosone (see page 27).

The classical method for obtaining osones consists of the formation of the phenylosazones and cleavage with strong hydrochloric acid (60). The method is not applicable to the present problem because of hydrolysis of the biose linkage, but it is possible to use the phenylosazone if its conversion to the osone is accomplished with benzaldehyde, a procedure originated by Herzfeld (61) and extended by Fischer (62). By application of this method, Fitzgato (63) prepared maltosone from which he obtained 2-keto-maltobionic acid in solution. He separated the latter compound as the amorphous barium salt.

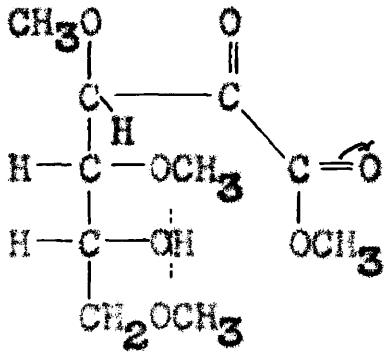
In the present investigation, bromine oxidation of lactosone, prepared from lactose phenylosazone by treatment with benzaldehyde gave 2-keto-lactobionic acid which was separated as a crystalline barium salt. This is the first crystalline metallic salt of a 2-keto-bionic-acid to be reported. The structure of the new salt was proved by enzymatic hydrolysis with lactase followed by identification, first of the galactose fragment by fermentation with a galactose-fermenting yeast, and second of the 2-keto-gluconic acid fragment through the formation of the phenylhydrazine salt of 2-keto-gluconic acid phenylhydrazone.

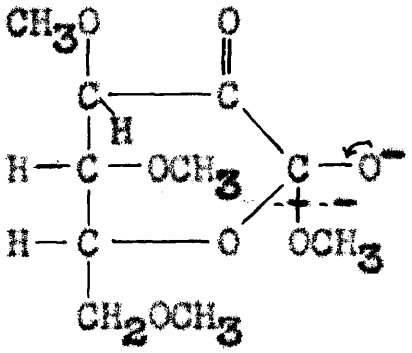
When pure, barium 2-keto-lactobionate crystallizes readily, but when mixed with impurities as in the product from the oxidation of lactosone, it does not separate satisfactorily. It was found, however, that 2-keto-lactobionic acid forms a barium bromide double salt which crystallizes more readily. This appears to be somewhat analogous to the calcium bromide double salt of lactobionic acid (64), but so far as known, this is the first crystalline salt of a sugar acid to contain barium bromide. The double salt is particularly suitable for the separation of 2-keto-lactobionic acid from the mixture obtained by oxidation of lactosone with bromine. In this case, the barium bromide necessary for the salt is a by-product of the reaction. The new salt should be very useful for the identification of 2-keto-lactobionic acid.

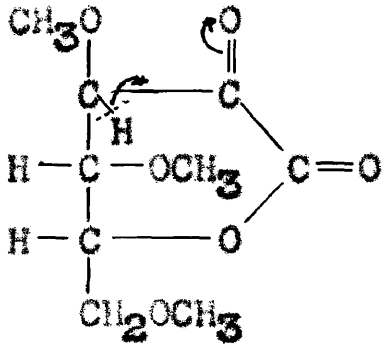
As previously pointed out, the galactosyl group at carbon four of 2-keto-lactobionic acid precludes the use of this substance for obtaining an ascorbic acid derivative. Nevertheless, a study of the behavior of methyl 2-keto-lactobionate on treatment with basic catalysts seemed desirable.

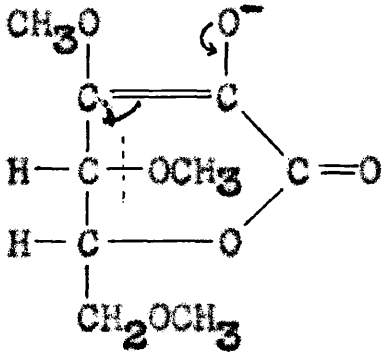
Haworth, Hirst, and Jones found that when methyl 3,4,6-trimethyl-2-keto-gluconate is treated with sodium methylate, the methyl group on the fourth carbon atom is eliminated and an analogue of ascorbic acid containing a six-membered ring is obtained (40). The mechanism for the conversion of methyl 3,4,6-trimethyl-2-keto-gluconate to a derivative of α-pyrone may be represented as shown in equation 10 on page 24.

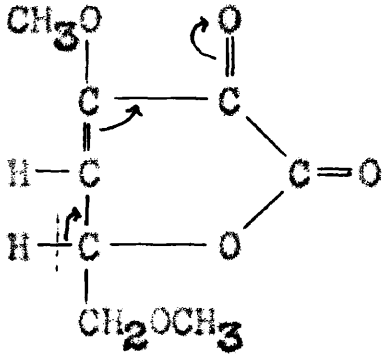
In consideration of this mechanism it seemed possible that methyl 4-galactosyl-2-keto-gluconate (methyl 2-keto-lactobionate) on treatment with sodium methylate might rearrange with the elimination of the galactosyl group and the formation of a six-membered ring analogue

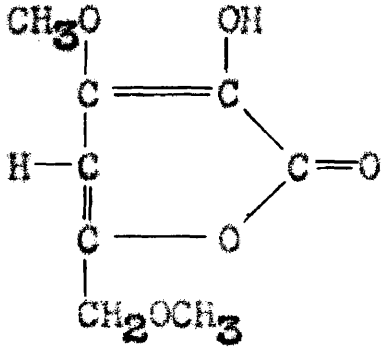










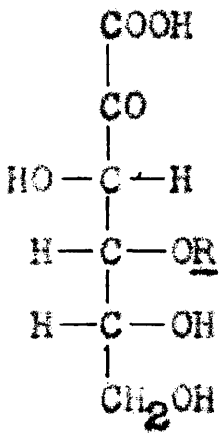


Equation 10

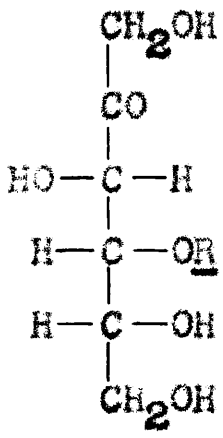
of ascorbic acid. This would provide a means for obtaining the six-membered ring compounds for study.

Methyl 2-keto-lactobionate was prepared by treatment of 2-keto-lactobionic acid with diazomethane (65). Conditions could not be found, however, to effect the lactonization and enolization of the ester by treatment with sodium methylate or other basic agents that have been used to bring about this type of rearrangement. Micheel and Brassert (3) found that although 2-keto-L-gulonic acid did not rearrange to give ascorbic acid with basic catalysts, it did rearrange in aqueous solutions or in the presence of mineral acids. (The methyl ester, of course, rearranges with basic catalysts). A final attempt to obtain enolization and lactonization of the 2-keto-lactobionic acid was therefore made by heating the free acid in aqueous solution, but titration with iodine proved that no rearrangement had occurred.

The 4-galactosyl derivative differs from the 3,6,4-orthodiol compound in that the keto group can form a relatively stable pyranose ring. Possibly for this reason there is little tendency to form a lactone ring and to undergo further alterations. 2-Keto-lactobionic acid still has much the same structure as lactulose XVIII, a sugar which is unique in that it crystallizes in the furanose form (66). The keto acid differs from lactulose merely in having a carboxyl group in place of a CHOH group. The keto acid and its salts could exist in pyranose, furanose and keto forms. Hence a complex mutarotation might be manifested. Measurement of the optical rotation of barium 2-keto-lactobionate at 20° C., however, revealed only a small, slow mutarotation with an equilibrium value of $(\alpha)_D^{20} = -37.2°$. At 0.6° C. the mutarotation was also small and the equilibrium rotation was $(\alpha)_D^{0.6} = -42.3°$. A comparison of these rotations with those obtained by Isbell and Pigman (66)



XVII



XVIII

R = Galactosyl group

for lactulose, levulose, and turanose is shown in Table 2.

Table 2

Substance	Equilibrium rotation 0° C	20° C
barium 2-keto-lactobionate	-42.5°	-37.9°
lactulose	-24.8°	-23.0°
levulose	-46.6°	-42.7°
turanose	+31.6°	+35.5°

Thus as with lactulose, levulose and turanose, the optical rotation is shifted in the levo direction by a decrease in temperature. Since the mutarotation is small in comparison to that of lactulose or levulose, one cannot draw any conclusions as to the character of the change, and the determination of the ring structure of the sugar entity must await further study.

Although the methyl 2-keto-lactobionate was thoroughly investigated it was recognised from the beginning that it could not yield a glycosyl derivative of isoascorbic acid. A parallel study was conducted, however, with melibiose. By application of the procedure used for the preparation of methyl 2-keto-lactobionate, one should obtain from melibiose, methyl 2-keto-melibionate. On enolisation this substance would yield 6-α-galactosyl-isoascorbic acid, a compound of the desired type. Although considerable progress has been made it has not been possible to prepare 2-keto-melibionic acid. The principal difficulty is the preparation of the melibiose osone. When melibiose phenylosazone was treated with benzaldehyde to convert the osazone to the osone, large quantities of tarry material were obtained but very little osone.

In order to determine whether there was any fault in the procedure or technique which was used with lactose and melibiose, the

preparation of D-isoascorbic acid from calcium 2-keto-gluconate was carried out by the same procedure. D-isoascorbic acid was obtained and identified by a mixed melting point with authentic D-isoascorbic acid.

Since the results by the osazone method were not satisfactory with melibiose, attention was directed to the preparation of melibiosone by the oxidation of melibiose with copper acetate. (See page 22). The copper acetate method is not generally applicable to the preparation of osones from disaccharides because these sugars react very slowly. It was found in the study, however, that a fairly rapid reaction could be obtained even with disaccharides by using ethylene glycol as a solvent in place of water. Under these conditions the product showed considerable osone by analysis. However, oxidation of the crude melibiosone with bromine followed by esterification with diazomethane and enolization yielded no ascorbic acid derivative. Possibly sufficient copper remained in the crude osone to effect complete oxidation of the desired product.

V. EXPERIMENTAL

1. Preparation of 2-Keto-aldobionic Acids by the Vanadium Pentoxide-Chlorate Oxidation of Aldobionic Acids.

Since oxidation of aldonic acids in methanol with chlorates in the presence of vanadium pentoxide yields methyl 2-keto-aldonates directly, this reagent was investigated first in the hope of obtaining a methyl ester of the 2-keto-aldobionic acid without troublesome intermediate steps. Although the reaction takes place in an acid medium, it seemed possible that conditions might be found to effect the reaction without hydrolysis of the glycosidic linkage. It was found, however, that in the absence of strong mineral acid the reaction is very slow. Nevertheless the results show some of the limitations of this highly selective method for oxidising carbohydrates.

A supply of calcium lactobionate-calcium bromide was available for the preparation of 2-keto-lactobionic acid. The galactosyl group in this compound is on carbon 4 and this prevents the formation of the 1,4-ring characteristic of ascorbic acid. It seemed possible that ring closure might take place at carbon 5 and hence the substance was investigated, even though an ascorbic acid derivative was not anticipated.

None of the common disaccharides can yield a 2-keto acid suitable for the preparation of a glycosyl-ascorbic acid. However, the 6-substituted disaccharides, melibiose and gentiobiose, are suitable because the glycosyl group would not interfere with ring closure. Attention was directed to the study of melibiose, 6-α-galactosyl-glucose, because it could be obtained from raffinose, a material which

was at hand.

A. Preparation of melibiose.

Although several authors (67, 68, 69) have described the preparation of melibiose from raffinose, none of the methods are entirely satisfactory. Raffinose,[5] a trisaccharide which occurs in small quantities in sugar beets, consists of galactose, glucose, and fructose residues in glycosidic combination. After hydrolysis of the melibiose-fructose linkage and fermentation of the fructose with top yeast, melibiose is recovered from the solution by crystallization.

$$\text{raffinose} \xrightarrow{\text{invertase}} \text{melibiose} + \text{fructose} \xrightarrow{\text{yeast}} \text{melibiose} + CO_2 + C_2H_5OH$$

Unfortunately the process is not dependable and sometimes the melibiose fails to crystallize from the sirup. Under these conditions it is necessary to purify the sugar further by conversion to the crystalline acetate, which is then de-acetylated. This procedure requires expensive chemicals and much labor. According to Harding (69), the difficulty in crystallization can be alleviated by use of acetic acid as a solvent. Even with this reagent crystallization does not always take place, possibly because of a small amount of acid hydrolysis.

In the present investigation it has been found that if a concentrated aqueous solution of melibiose is saturated with dioxane, crystallization occurs consistently, and dependably. In comparative

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5. Raffinose is a by-product in the recovery of sucrose from beet molasses, and may be obtained from the Great Western Sugar Company, Denver, Colorado.

tests with a number of solvents, including acetic acid, the dioxane method proved to be superior to any which are recommended in the literature. The following method for the preparation and crystallization of melibiose gives consistently satisfactory results:

Five moles (2,975 grams) of raffinose was dissolved in 15 liters of water and the optical rotation was found to be 91.1° S. when observed in a 2-dm. tube at 20° C. Sixty milliliters of commercial fermentation solution and 300 ml. of a nutrient medium,[6] were added to the solution of raffinose and the mixture was allowed to stand at room temperature for two days. The optical rotation was measured as before and found to be 64.7° S. Assuming complete hydrolysis of the raffinose to melibiose and fructose, the optical rotation in degrees S in a 2-dm. tube should have changed from 91.1° S. to 47.1° S. Hence the hydrolysis was only partially complete. Six moles of yeast were added and the mixture was allowed to stand for two days for completion of hydrolysis and fermentation of the fructose. The optical rotation was then found to be 74.9° S. (2-dm. tube) whereas that calculated for complete conversion to melibiose and elimination of the fructose is 71.5° S. The solution was treated with 150 ml. of decolorizing carbon, filtered, and concentrated under reduced pressure to a sirup having a refractive index of 1.456 at 20° C. The sirup was mixed with 500 ml. of dioxane, seeded with melibiose dihydrate and rotated in a crystallizer for three days at room temperature. The massecuite was placed in a refrigerator for several days. The crystals which formed were then

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6. The nutrient medium was made by dissolving 2.5 g. of NH_4Cl, 0.3 g. of KH_2PO_4, and 2.5 g. of $MgSO_4.7H_2O$ in one liter of water.

collected on a filter and washed with aqueous dioxane which was just miscible with the mother liquor. After drying in air the product weighed 1295 grams and the specific rotation was found to be $(\alpha)_D^{20}$ = 126.5° (water c = 4). The mother liquor was concentrated, treated with additional dioxane, seeded and allowed to stand for several weeks in a refrigerator to give a second crop of 143 grams with a specific rotation $(\alpha)_D^{20}$ = 125.6° (water c = 4). The accepted value is $(\alpha)_D^{20}$ = 129.5° (70).

The use of aqueous dioxane also proved advantageous in the recrystallization of the sugar. Eight hundred grams of crude melibiose was dissolved in 400 ml. of hot water, 600 ml. of dioxane was added, and the mixture, after seeding, was rotated slowly for several days. The resulting crystals, when separated and air-dried, weighed 500 grams. By concentration of the mother liquor, nearly all of the sugar was recovered.

B. Preparation of calcium melibionate.

For the preparation of 2-keto-melibionic acid, a supply of melibionic acid was required. Prior to the present investigation, melibionic acid had been obtained by oxidation of melibiose with bromine for four days, followed by separation of the resulting hydrobromic acid through precipitation with silver carbonate. The acid was purified by repeated precipitation of the calcium salt with methanol (71). This process, as well as other processes which have been used (72, 73), requires a laborious method of purification involving the use of large quantities of silver carbonate or lead acetate and hydrogen sulfide.

A convenient method for the preparation of aldonic acids from aldoses by electrolytic oxidation has been developed (74). In the case of glucose, crude solutions can be oxidised and the acid separated directly from the electrolyte as the calcium salt. It seemed possible that calcium melibionate might be prepared by this method directly from the liquor which remained after hydrolysis of raffinose and fermentation of the resulting fructose. Application of the electrolytic process to the preparation of calcium melibionate was made as follows:

The hydrolytic liquor derived from 2975 g. of raffinose by the procedure described on page 30 was concentrated in vacuo to a volume of 8 liters and transferred to a large crock. One hundred grams of calcium bromide and 250 g. of calcium carbonate were added. Four anodes and four cathodes (graphite rods 12 inches long and 1 inch in diameter) were immersed in the solution to a depth of 9 inches, and the solution was stirred mechanically while a current of 5 amperes at 6.5 volts was passed through it. Electrolysis was continued for 300.0 ampere hours (1.15 times the theoretical). At that time, titration of an aliquot of the solution with iodine indicated that 88 percent of the melibiose was oxidised. The crude calcium melibionate that had precipitated during the electrolysis was separated by filtration; it weighed 1250 grams after drying in air. The calcium melibionate in the filtrate was recovered in part by concentrating the solution and cooling it to a low temperature. The crude calcium melibionate was recrystallised from hot water to give 1669 grams of calcium melibionate trihydrate, corresponding to 83 percent of the theoretical yield. Analysis after air-drying:

Calculated for $Ca(C_{12}H_{21}O_{12})_2 \cdot 5H_2O$: C, 35.64; H, 5.88; Ca, 4.96

Found: C, 35.61; H, 6.22; Ca, 4.83

By drying at 70° C. in vacuo for 18 hours, the water of hydration was removed. Calculated for a trihydrate: water, 6.68%. Found: (loss in weight), 6.59%.

The trihydrate obtained in the present investigation represents a new compound with exceptionally good crystallizing properties. It is readily obtained pure and has a specific rotation of $(\alpha)_D^{20} = 99.3°$ (water, c = 2). The calcium melibionate reported in the literature contained from 11 percent to 15 percent of water, corresponding to at least 5 moles of water of crystallization. The specific rotation reported on the anhydrous basis was $(\alpha)_D^{17} = 88.6°$, but this appears to be somewhat low.

C. Preparation of basic calcium melibionate.

It has been found previously that aldonic acids in general form difficultly soluble basic calcium salts (75). Those derived from monosaccharides are of the type $Ca(acid)_2 \cdot 2CaO$. Two have been prepared from the disaccharide acids, namely maltobionic and lactobionic acids. These have the formula $Ca(acid)_2 \cdot 4CaO$. It seemed desirable to ascertain whether melibionic acid likewise forms a basic calcium salt, and if so, whether it conforms to one of the two known types. Preparation of a basic calcium salt of melibionic acid for analysis was therefore carried out as follows:

Ten grams of calcium melibionate was dissolved in 200 ml. of water and the solution was cooled to 0° C. A suspension of 3.5 grams of calcium oxide in 200 ml. of water at 0° C. was added, and

the mixture was shaken for several minutes. It was then filtered into a flask through which a current of carbon dioxide-free air was passed. The clear solution was concentrated in vacuo to 150 ml. and finally heated for a few minutes on the steam bath to complete the precipitation of the basic salt. The salt was separated by filtration in the absence of carbon dioxide, and dried at 60° C. in vacuo. Calculated for $Ca(melibionic)_2 \cdot 4CaO$: total Ca, 20.47; CaO, 22.81. Found: Ca, 20.45; CaO, 22.56.

The new basic salt can be used to advantage for the separation of calcium melibionate from the mother liquors which remain after separation of the normal salt. The solution containing the calcium melibionate was merely added with stirring to a suspension of calcium oxide in water, containing at least 4 moles of calcium oxide per mole of calcium melibionate. The basic salt precipitated as a granular solid which was separated by filtration and washed with lime water. The purified normal salt was regenerated by passing a stream of carbon dioxide through a suspension of the basic salt in water until the mixture was neutral to phenolphthalein. The mixture was heated to dissolve any normal calcium melibionate which sometimes crystallized. After filtration to remove the precipitated calcium carbonate, the solution was concentrated and cooled. Crystalline calcium melibionate separated in high yield. The electrolytic oxidation of melibiose, together with the separation of the basic calcium melibionate provide a cheap and convenient method for preparing the comparatively rare calcium melibionate from commercial raffinose.

E. Oxidation of melibionic acid with vanadium pentoxide and a chlorate.

Melibionic acid was prepared by mixing 0.1 equivalent of calcium melibionate with 0.1 equivalent of finely powdered oxalic acid in the dry state and slowly adding this mixture to 75 ml. of water while stirring the solution vigorously. The precipitated calcium oxalate was removed by filtration and the aqueous solution of melibionic acid was dehydrated by repeated evaporation with methanol in vacuo. This step results in the formation of a mixture of gamma and delta lactones of melibionic acid. Treatment of these with phosphoric anhydride in methanol would yield the methyl ester. The mixture of melibionic acid lactones was diluted to 100 ml. with methanol, transferred to a glass-stoppered flask, and 5.7 g. of sodium chlorate, 2.0 g. of vanadium pentoxide and 0.5 g. of phosphoric anhydride were added. The flask was closed with a glass stopper and shaken on a shaking machine until the color of the solution changed from yellow to a bluish-green. Oxidations were also carried out in which the phosphoric anhydride was replaced with 1.0 ml. of phosphoric acid, and in which neither the anhydride nor the acid was present. The oxidation was completed in two or three days with phosphoric anhydride, in four days with phosphoric acid, and in two weeks when neither was present.

When the oxidation was complete as shown by the color change, the solution was filtered, and the insoluble material was discarded. The solution was then evaporated to a sirup which was mixed with 10 volumes of acetone to precipitate the inorganic material. The acetone-soluble material was separated, the solution was concentrated, and an effort made to crystallize methyl 2-keto-melibionate. The material

failed to crystallise and was found to contain a considerable quantity of inorganic salts. Consequently the oxidation was repeated a number of times, using different proportions of acetone, and also various other organic solvents and mixtures of solvents, in an effort to effect a clean-cut separation of the inorganic and organic constituents. Nevertheless, it was not possible to obtain pure methyl 2-keto-melibionate.

2. Oxidation of lactobionic acid with vanadium pentoxide and a chlorate.

Lactobionic acid was prepared from calcium lactobionate by treatment with an equivalent quantity of oxalic acid. The lactobionic acid was oxidised in the same manner as melibionic acid and the same difficulty was experienced in the separation of the product from the inorganic matter. No pure methyl 2-keto-lactobionate was obtained.

2. Preparation of 2-Keto-aldobionic Acids Through the Intermediate Preparation of the Phenylosazone and Osone.

A number of 2-keto-aldonic acids have been obtained by the intermediate preparation of the phenylosazone and osone, followed by bromine oxidation of the 2-keto-acid (76). It is generally assumed that osazone formation occurs readily with the sugars, and in high yield. The osazones of disaccharides, however, have a marked tendency to form anhydrides, from which the osone cannot be obtained. Considerable work was therefore necessary to obtain phenylosazones sufficiently free from anhydrides to give good yields of osones. As previously noted, the process is complicated further by the difficulty

of hydrolyzing the osazone to the osone without hydrolyzing the biose linkage at the same time. The oxidation of the osone to the β-keto-acid takes place smoothly in the presence of an alkaline earth carbonate without hydrolysis.

A. Preparation of lactose phenylosazone.

Although lactose phenylosazone was prepared in 1887 by Fischer (77), and has been used by all students of carbohydrate chemistry for identification purposes, there is still no satisfactory method for obtaining the compound in pure condition and high yield. The product ordinarily contains a substantial proportion of an anhydride (78) which may even increase upon standing. The purification of lactosazone is complicated further by the existence of several isomeric forms. Evidence for the existence of these isomers is found in the mutarotation, which involves a change from $(\alpha)_D^{20} = -25.4°$ to $-7.9°$ (79). In this investigation the isomer presumably responsible for the change in the dextrorotation has been separated in the crystalline state for the first time. Altogether, about 166 g. of dextrorotatory material, having specific rotations from +40° to +50° was obtained. The purest material had a specific rotation of +50.2°. Purification of the isomer has been difficult and uncertain, and at this time it can be stated that the dextrorotatory substance has a rotation at least as great as +51°.

Various methods for the preparation of the lactosazone were tried (80), but best results were obtained by the following procedure.

One hundred and eighty grams of lactose was dissolved in one liter of water and 164 ml. of phenylhydrazine and 328 ml. of a 10-per-cent aqueous solution of acetic acid were added. The mixture was heated on the steam bath for two hours at 80° C., cooled quickly and

stored in a refrigerator overnight. The osazone was removed by filtration, washed with dilute acetic acid, water and finally with ether. The product was recrystallized from a 50-percent aqueous alcohol solution. The yield was 104 grams.

B. Preparation of lactose osone.

In 1908, Fischer (31) prepared solutions of osones of a number of disaccharides as well as monosaccharides by treating the corresponding phenylosazones with concentrated hydrochloric acid. The yields of the disaccharide osones were small, since extensive hydrolysis occurred. A more convenient method of disaccharide osone preparation which largely avoids hydrolysis of the biose linkage was found by Fischer and Armstrong (32) who showed that the phenylosazones of maltose and cellobiose, on boiling with benzaldehyde and water yield the corresponding osones. This method was applied in the present investigation, but since the osones were to be used for several successive reactions, the amount of starting material (osazone) was increased to 70 g. from the previous maximum of 20 g. Best results were obtained by lengthening the reaction time from 30 minutes to four hours. The amount of osone formed was estimated by the method of Fischer and Armstrong, i.e., by re-formation of the osazone by the addition of phenyl hydrazine acetate in the cold or with slight warming. The yield of lactose osone was of the order of 65 percent of the theoretical[7] in several preparations. The preparation of lactose osone was carried out as follows:

Seventy grams of lactosazone was dissolved in 6 liters of hot water, 56 g. of benzaldehyde was added, and the mixture was heated

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7. A yield of 70% was reported by Fischer and Armstrong on a preparation of maltose osone from 5 g. of maltose osazone.

on the steam bath under vigorous stirring and in an atmosphere of nitrogen for four hours. The solution was cooled and filtered, and the filtrate was extracted several times with ether to remove excess benzaldehyde. The water solution was then treated with a decolorizing carbon, filtered and concentrated in vacuo to a volume of 250 ml. The yield of osone was estimated by adding to an aliquot of the solution sufficient phenylhydrazine acetate to convert the theoretical yield of osone to osazone, and warming the mixture for ten minutes on the steam bath. The osazone which formed was collected on a sintered glass crucible, washed with ether and air-dried. The osone was oxidized with bromine as described in the next section.

C. Oxidation of lactose osone and preparation of crystalline barium 2-keto-lactobionate-barium bromide.

Oxidation of lactosone to 2-keto-lactobionic acid was accomplished conveniently by treatment with bromine in the presence of barium carbonate. In this medium there is no danger of hydrolysis of the disaccharide. The acid can be separated in the form of a new crystalline normal barium salt, or as a unique crystalline double salt of barium 2-keto-lactobionate and barium bromide. The latter compound has exceptionally good crystallizing properties. It was prepared as follows:

The aqueous solution of lactosone obtained above, (volume 200 ml.), was saturated with carbon dioxide and cooled in an ice bath. Thirty-five grams of barium carbonate and 12 ml. of bromine were added and the mixture was stirred. After five minutes it was removed from the ice bath and stirred for half an hour at room temperature. The major part of the excess bromine was removed with a rapid stream of

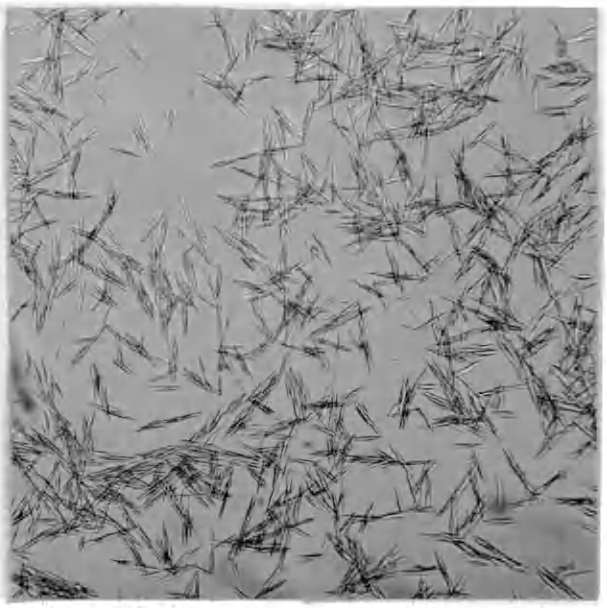
carbon dioxide and the last traces by reaction with linseed oil. After separation from the oil, the mixture was filtered; the solution was treated with 10 g. of a decolorising carbon and re-filtered. The colourless filtrate was concentrated in vacuo to a volume of approximately 50 ml. and was then transferred to a beaker, together with about 20 to 25 ml. of washings. In one preparation crystals began to separate at this point; in others the sirup was allowed to stand in a vacuum desiccator over sulfuric acid for a period of a day or two before a satisfactory crystallisation had taken place. The yield in different preparations varied widely; the highest yield was 28.8 g. and the lowest was 4 g. The uncertain step appears to be the formation of the osone.

The compound is soluble at room temperature to the extent of about 6 g. in 100 ml. of water, but it is practically insoluble in methyl alcohol or ether. It may be recrystallised from a concentrated aqueous solution, from which it separates slowly in fragile plates. A photomicrograph of crystals obtained by the slow evaporation of a saturated aqueous solution is shown in figure 1. Analysis: Calculated for $Ba(C_{12}H_{19}O_{12})_2 \cdot BaBr_2 \cdot 6H_2O$: C, 25.7; H, 3.8; Ba, 22.6; Br, 13.1. Found: C, 25.9; H, 3.8; Ba, 22.6; Br, 13.2. At equilibrium, the specific rotation $(\alpha)_D^{20} = -28.2°$ (water, c = 2).

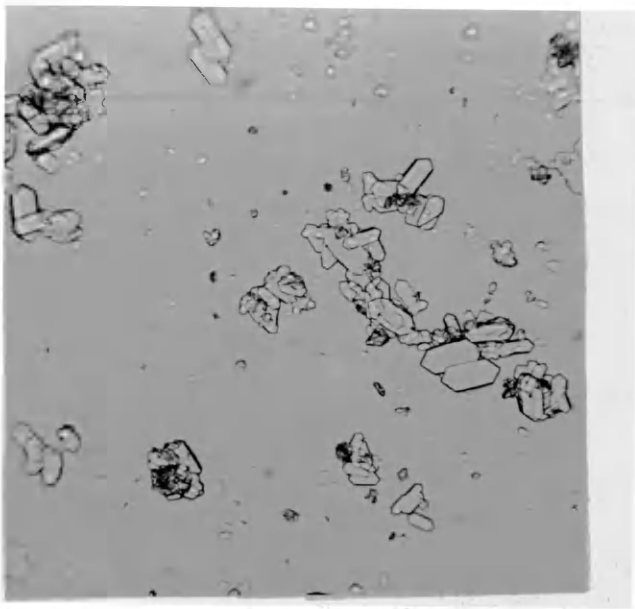
F. Crystalline barium 2-keto-lactobionate.

(1) Preparation from lactosone.

A solution of lactosone was oxidised and treated as described in section C above to remove the excess bromine. The barium bromide was removed by treatment with silver sulfate until the solution gave no test for bromide or silver ions. It was then filtered

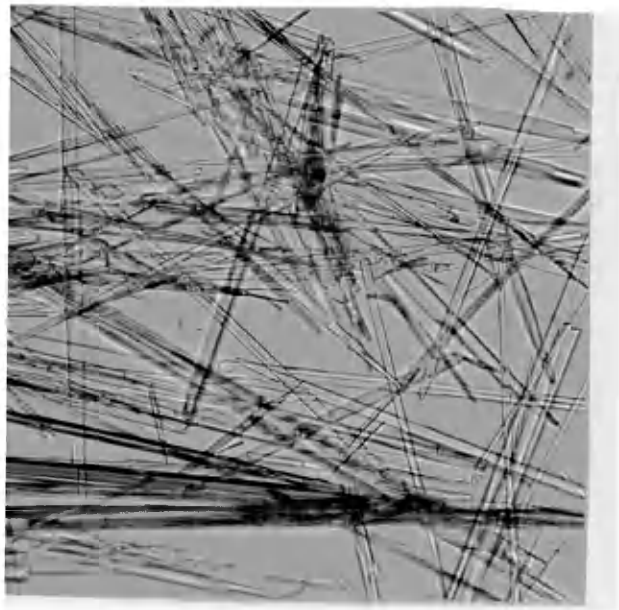


Barium 2-keto-lactobionate dihydrate



Barium 2-keto-lactobionate-barium bromide tetrahydrate

A



B

Phenylhydrazine salt of the phenylhydrazone of 2-keto-gluconic acid

A Prepared from hydrolyzate of barium 2-keto-lactobionate

B Prepared from calcium 2-keto-gluconate

and concentrated to about 15 ml. Methanol was cautiously added until a faint, permanent turbidity was produced, which was then discharged with a drop of water. Some difficulty was experienced in bringing the sirup to crystallization. However, a few seed crystals were finally discovered, and by seeding with these a fairly complete crystallization could be attained within a day. The crystals which formed were collected on a filter, washed with methanol containing sufficient water to be miscible with the mother liquor, and finally air-dried.

Barium 2-keto-lactobionate is considerably more soluble in water than the barium bromide double salt. It is practically insoluble in ether and methanol. It crystallizes from water in slender needles, a photomicrograph of which is shown in figure 1, page 41. Analysis: Calculated for $Ba(C_{12}H_{19}O_{12})_2 \cdot 2H_2O$: C, 32.6; H, 4.8; Ba, 15.5. Found: C, 32.7; H, 4.8; Ba, 15.7. At equilibrium, the specific rotation $(\ )_D^{20} = -37.9°$ (water c = 2).

(2) Preparation of barium 2-keto-lactobionate from the barium bromide double salt.

To establish that the normal barium 2-keto-lactobionate and the barium bromide double salt are derivatives of the same 2-keto-aldobionic acid, the normal salt was prepared from the double salt by removal of the barium bromide. Ten grams of barium 2-keto-lactobionate-barium bromide in 275 ml. of water was mixed with a quantity of silver sulfate exactly equivalent to the bromide ion (2.56 g.). The precipitate was removed by filtration, and the filtrate was evaporated to a sirup which was brought to crystallization by the addition of methyl alcohol. The crystals, when separated and dried, had the same optical rotation, reducing power and appearance as the normal salt

obtained directly from the oxidation process.

Furthermore, by the addition of a mole of barium bromide, the double salt was regenerated from the normal salt. Thus the two new crystalline salts differ only in that the one contains a molecule of barium bromide.

(3) Proof of the structure of barium 2-keto-lactobionate.

Since the synthesis began with lactose, (4-β-galactosylglucose), in order to establish the structure of the new compound it was necessary (1) to prove that the biose linkage was still present and (2) to establish the identity of the products of hydrolysis as galactose and 2-keto-gluconic acid. Enzymatic hydrolysis of the substance with a sample of lactase[8] established the existence of the biose linkage. The presence of galactose after hydrolysis was shown by the action of a galactose-fermenting yeast, and that of 2-keto-gluconic acid by separation of the characteristic phenylhydrazine salt of the phenylhydrazone.

(a) Enzymatic hydrolysis of the disaccharide constituent of $Ba(2\text{-keto-lactobionate})_2$ by means of a lactase.

A 1.196 gram sample of barium 2-keto-lactobionate was treated with an equivalent amount of 0.1 N sulfuric acid and the solution was filtered and diluted to 50 ml. The optical rotation in degrees S was -3.69 (2-dm. tube). Twenty-five hundredths gram of calcium carbonate and 0.150 g. of lactase were added and the pH was raised to 7.0 by removal of the dissolved carbon dioxide with a stream

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8. The lactase was obtained from the research laboratories of Rohm and Haas, Philadelphia, Pa.

of nitrogen. Toluene was added to prevent mold formation and the solution was stored at 50° C. for seven days. Hydrolysis was followed by means of optical rotation, which was read from time to time. Additional lactase was added at the end of one, three, and six days. After the seventh day the observed optical rotation in degrees S was -1.62 (2-dm. tube). At the given dilution, the rotation of an equimolecular mixture of calcium 2-keto-gluconate and galactose would have been -0.80° S. Thus the degree of hydrolysis produced by the lactase is calculated to be 71.6 percent. The solution was separated from the toluene and filtered; the filtrate was used for the work described in sections (b) and (c).

(b) Fermentation of the aldose constituent by means of a galactose-fermenting yeast.

A galactose-fermenting yeast was prepared by the method of Kirby and Atkin (65) and its efficiency was tested upon a solution of pure galactose. A small sample of the yeast was added to a solution of one gram of galactose in 25 ml. of water containing 1 ml. of nutrient solution.[9] After standing 48 hours at 37° C., the galactose solution showed no further evolution of carbon dioxide. The optical rotation, following filtration was practically zero.

The yeast was then used for fermentation of the hydrolysate of barium 2-keto-lactobionate prepared as described in section (a). Before fermentation, the solution was filtered through a column of decolorizing carbon. A small quantity of the yeast and 1 ml. of the

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9. The nutrient solution contained 2.5 g. of NH_4NO_3, 0.5 g. of KH_2PO_4 and 0.25 g. of $MgSO_4 \cdot 7H_2O$ per 100 ml. of water.

solution were added to 14 ml. of the solution and the mixture was kept at 37° C. for one day, after which time the evolution of carbon dioxide was marked. The material was then filtered and the rotation (expressed on a volume of 14 ml. instead of 15 ml.) was found to be -2.23°Z (2-dm. tube). The change in rotation was in the direction which would result from a loss of galactose, and corresponds to a fermentation of 15.6 percent of the aldose freed from the disaccharide by lactase. Although an active galactose yeast was used, the percentage of galactose fermented was low, probably because the action of the yeast was inhibited by a trace of toluene in the solution. The danger of mold formation made it advisable to discontinue the fermentation after the destruction of galactose had been demonstrated by the change in optical rotation.

(c). Identification of 2-keto-gluconic acid.

The presence of 2-keto-gluconic acid in the solution which remained after fermentation of the hydrolysate was shown by the separation of the crystalline phenylhydrazine salt of 2-keto-gluconic acid phenylhydrazone. Before attempting the preparation of the phenylhydrazine derivative from the hydrolysate, the derivative was prepared from authentic calcium 2-keto-gluconate by the method of Ohle (26). After two crystallizations from water, the substance melted at 96° to 98° C. with decomposition.

The same substance was then prepared from the solution derived from barium 2-keto-lactobionate as described in sections (a) and (b). The solution of section (b) was concentrated in air to 4 ml. and five drops of acetic acid and five drops of phenylhydrazine were added. The solution was then seeded with the previously prepared phenylhydrazine salt of the phenylhydrazone of 2-keto-gluconic acid. Crystals

formed readily, and were separated by filtration, washed with ether, and dried in air. The melting point was 95° to 97° C. with decomposition. A mixed melting point of the two preparations was likewise 95° to 97° C. Microphotographs in figure 1, page 41 show the similarity in appearance of crystals of the derivative prepared from 2-keto-gluconic acid and from the fermented hydrolysate of the disaccharide.

This same preparation was carried out on a portion of the unfermented hydrolysate of the disaccharide. Crystals formed more slowly and in smaller quantity, but were identified by mixed melting point with those of the known phenylhydrazine salt of the phenylhydrazone of 2-keto-gluconic acid.

5. 2-Keto-lactobionic acid and methyl 2-keto-lactobionate.

Ten grams of barium 2-keto-lactobionate-barium bromide was dissolved in 275 ml. of water and 2.80 g. of silver sulfate was added. The solution was stirred for two hours, treated with several grams of a decolorizing carbon and filtered with suction through a layer of carbon. The filtrate was adjusted with either silver sulfate or barium bromide so that it gave a negative test for both bromide and silver ions. After filtration, the solution was concentrated in vacuo to 50 ml. and was then passed through a suitable column of cation exchange resin.[10] The column was washed four times with 5 to 10-ml. portions of water. The treated solution, combined with the washings, was evaporated in vacuo to a thick sirup. Dehydration of the 2-keto-lactobionic acid was accomplished by dissolving it in 50 ml. of methanol and re-evaporating the solution in vacuo. This process was repeated

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10. The resin used was Amberlite I-R-100-H Ag, a product of the Resinous Products Company, Philadelphia, Pa.

four times. The anhydrous acid, in the form of a thick sirup, was diluted to 100 ml. with methanol, and the solution was cooled to 0° C. in an ice-bath. An ethereal solution of diazomethane[11] was added slowly with constant stirring until the solution retained a yellow color. At this point the pH of the solution changed from acid to neutral. The ether and excess diazomethane were removed by vacuum distillation.

The crude product reacted neutral to acid-base indicators. A methoxyl determination by the Zeisel method (64) showed the presence of approximately one equivalent of methoxyl per mole of 2-keto-lactobionate methylated. A part of the material was used for the determination of a copper-reducing value by the Scales method (65). Based on the amount of the 2-keto acid methylated, the reducing power of the methyl ester was 98 percent of that of an equivalent quantity of barium 2-keto-lactobionate. The methoxyl content and the reducing power of the product are evidence that diazomethane did not appreciably methylate the keto group or the neighboring hydroxyl groups. Although the product was undoubtedly methyl 2-keto-lactobionate, it could not be brought to crystallization.

Inasmuch as it seemed possible that methyl 2-keto-lactobionate might undergo the reaction outlined on page 24, attempts were made to effect a lactonization and enolization reaction. A quantity of the

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11. The diazomethane was prepared by treating nitrosomethylurea with 40% KOH in the presence of ether at 5° C. The ether layer, containing the diazomethane, was separated from the aqueous layer and dried over solid KOH. The method is described on page 50 of Organic Reactions, Vol. 1.
The nitrosomethyl urea was prepared from methyl amine hydrochloride, urea, and sodium nitrite by the method described in Organic Syntheses Vol. II, page 461.

methyl ester, prepared as described above from 10 g. of barium 2-keto-lactobionate-barium bromide was diluted with methanol to a volume of 100 ml. Based on the amount of double salt used, each millilitre of this solution contained 0.0001644 moles of the methyl ester. Aliquots of the solution were used for a number of lactonisation and enolisation experiments such as those described below:

(1) Rearrangement with sodium methylate.

The most frequently used agent for the rearrangement of the esters of keto-acids to ascorbic acid analogues is sodium methylate. Haworth (40) has used the method for the rearrangement of methyl 5,4,6-trimethyl-2-keto-gluconate to a compound containing a six-membered ring. A rearrangement of the methyl ester of 2-keto-lactobionate was carried out under the conditions described for the substituted 2-keto-gluconate. A 2-ml. aliquot was transferred to a micromethoxyl flask fitted with a reflux condenser, a stream of nitrogen was introduced through the side arm and 0.5 ml. of 1.4 N sodium methylate was added. The mixture was refluxed for 45 minutes, cooled, diluted with water, acidified with sulfuric acid and titrated with 0.01 N iodine. The titration was only 0.4 ml., whereas for 100 percent conversion, the solution should have consumed 66 ml.

In order to find suitable conditions for the reaction, it was repeated a number of times but with considerable variation in the temperature and period of heating. Thus temperatures as low as 0° C. and as high as 70° C. were used; the period of heating was varied from 10 to 60 minutes. The iodine consumed was in all cases less than 1 ml.

(2) Rearrangement with metallic magnesium.

Rearrangement of the methyl esters of 2-keto-acids in aqueous solution has been accomplished in some cases by the use of metallic magnesium (Pasternak, U. S. Patent 2,155,184). The procedure was applied to the rearrangement of methyl 2-keto-lactobionate in the following manner:

A 2-ml. aliquot of the methanol solution of the ester was transferred to a micromethoxyl flask and the methanol removed under reduced pressure. Five milliliters of water and 4 mg. of magnesium were added and a stream of nitrogen was passed through the flask. The mixture was boiled for 50 minutes, cooled, and titrated as above in acid solution. The solution required only 0.1 ml. of 0.01 N iodine and the theoretical value, as above was 66 ml. The period of heating was varied in a number of similar experiments, but in no case was an appreciable amount of iodine consumed.

(3) Rearrangement of 2-keto-acids in boiling water.

Reichstein and Grüssner (50) found that 2-keto-l-gulonic acid could be converted to ascorbic acid in an inert atmosphere. Although methyl 2-keto-lactobionate did not undergo lactonization and enolization, it was possible that 2-keto-lactobionic acid might exist in solution in a form that would enable it to undergo this transformation. Therefore a rearrangement of 2-keto-lactobionic acid was carried out as follows:

One gram of barium 2-keto-lactobionate-barium bromide was dissolved in 50 ml. of water and the barium bromide was removed with an equivalent quantity of silver sulfate. The 2-keto-lactobionic acid was obtained by treating the solution of the barium salt with cation

exchange resin. The acid was then heated for two hours in 30 ml. of water in an inert atmosphere. The solution was cooled, acidified with sulfuric acid and titrated with 0.01 N iodine. Less than 0.1 ml. of iodine was required and the theoretical value for 100 percent conversion was over 500 ml.

(4) Preparation of L-isoascorbic acid from calcium 2-keto-gluconate.

In order to ascertain whether the methylation and rearrangement reactions were being carried out in a manner adequate to give a known ascorbic acid, calcium 2-keto-gluconate was converted to 2-keto-gluconic acid and methylated with diazomethane by the process described on page 45. A sodium methylate rearrangement was then made, the mixture was treated with an exactly equivalent quantity of sulfuric acid in isopropanol, and after removal of sodium sulfate, the solution was concentrated to a sirup. On seeding with L-isoascorbic acid, a crystalline product formed, which, when purified by recrystallization, melted at 167° C. The melting point of the substance, when mixed with pure L-isoascorbic acid was unchanged.

F. Preparation of melibiose phenylosazone and attempts to convert it to melibiose osone.

Low yields of melibiosazone were obtained by the method reported in the literature for the preparation of this osazone (86). The procedure finally adopted gave a lighter colored, and more readily separated product.

Fifty grams of melibiose, 240 ml. of water, 24 ml. of acetic acid and 50 ml. of phenylhydrazine were placed in a flask and heated in a water bath at 60° C. for four hours. The mixture was then allowed

to cool slowly. After four hours at room temperature and 18 hours in the refrigerator, the crystals which separated were collected on a filter and washed with ether. The crude product was dissolved in 750 ml. of hot water, filtered, and the filtrate was cooled very rapidly by ice water. This procedure resulted in a crystalline product which was separated readily by filtration.[12] The crystals were washed first with ice water and then with ether. On drying, the product turned dark brown.

Application to melibiosazone of the method used for the conversion of lactosazone to lactosone gave a tarry precipitate but apparently very little osone. Variations in the method were tried but a satisfactory method for obtaining the osone was not found.

5. Preparation of Ascorbic Acid Derivatives by Condensation of Diethyl Mesoxalate with Sugars.

As already mentioned, this method is analagous to the well-known acyloin condensation which takes place between aromatic aldehydes in the presence of alkali cyanides. This type of reaction was first applied to the preparation of ascorbic acids from the sugars by condensation with ethyl glyoxalate (24a), or better with ethyl mesoxalate (24b). Although this method has been applied to several of the simple sugars, prior to the present investigation, condensation reactions of this character had not been studied with the disaccharides. It seemed probable that the condensation should yield glycosidic derivatives of glucoheptoascorbic acid. The necessary diethyl mesoxalate was prepared as

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12. Slow cooling gave a gelatinous product which held up a large quantity of the mother liquor.

described in the following section.

A. Preparation of diethyl mesoxalate.

A combination of the methods of Müller (67) and Battin, Newman, and Riley (68) were used. A mixture of 55 g. of selenium dioxide[15] and 76 ml. of ethyl malonate was stirred and heated at 135° C. for three hours. The mixture was cooled to room temperature and the selenium separated by filtration and washed with ether. The filtrate and washings were combined, diluted to 500 ml. with ether and dried over anhydrous calcium sulfate. The ether was removed by distillation at atmospheric pressure and the residue fractionally distilled in a Podd fractionating column. This column had 50 theoretical plates at atmospheric pressure and enabled the separation of the product in a pure condition with a single distillation. The fraction distilling between 93° and 95° C. was collected, and measured 22.5 ml.

B. Condensation of diethyl mesoxalate with sugars.

Condensation with diethyl mesoxalate is a reaction which involves the carbonyl group of the sugars. These substances exist for the most part in the form of cyclic hemi-acetals, and reactions of the carbonyl group take place only by virtue of the rapid re-establishment of the equilibrium state. Considerable differences exist in the equilibrium portion of the free carbonyl form of the various sugars and the rapidity with which equilibrium is established. Hence wide variations were to be expected in the reactivity of the sugars with diethyl mesoxalate.

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15. The selenium dioxide was prepared from metallic selenium by the method described on page 119 of Inorganic Syntheses, Volume I.

Before applying the reaction to the disaccharides, condensation of L-arabinose with diethyl mesoxalate by the method of Helferich was repeated (24b). The results were essentially as described in the reference. Application of this procedure to melibiose and maltose gave satisfactory yields of compounds of the ascorbic type. Lactose and cellobiose, however, did not yield the desired products. The applications of the condensation method to the individual sugars are described below.

(1) Melibiose.

Eighty milliliters of methanol was added to 5 g. of melibiose in a small flask which was fitted with a reflux condenser and a side arm through which a slow stream of nitrogen was passing. To this material were added 1.84 g. of sodium cyanide and 4.10 g. of diethyl mesoxalate, and the mixture was boiled for 10 minutes. A yellow precipitate, presumably containing the sodium salt of 6-α-galactosyl-glucoheptonascorbic acid, began to form shortly after heating was started. The solution was cooled and the yellow precipitate was separated by filtration and dried over calcium chloride, in vacuo, at room temperature. The precipitate weighed 2.45 g. and when an aliquot was titrated with iodine, the titration indicated that 21 per cent of the precipitate, or 0.5145 g., was the desired product. This corresponds to a yield of 9.2 per cent of the theoretical. The presence of a substance of the ascorbic acid type is shown conclusively by the ultraviolet absorption spectra reported and discussed on page 78.

In an endeavor to separate the 6-α-galactosyl-glucoheptonascorbic acid, the crude sodium salt was dissolved in oxygen-free water

and treated with a cation exchange resin.[14] The solution of the acid was concentrated in vacuo and various solvents were tried in an effort to crystallise the acid. No crystalline material was obtained. The material was fractionally precipitated by organic solvents but none of the fractions showed any concentration of the glucoheptoseascorbic acid derivative.

In another preparation purification of the material was tried by use of the basic lead salt, which has been used for the purification of ascorbic acid. Neutral lead acetate was added to an aqueous solution of the sodium salt and the precipitate which formed was removed by centrifuging, and was discarded. The clear solution was adjusted to a pH of 7.6 by the addition of ammonium hydroxide.[15] A precipitate formed which was separated and washed in the centrifuge. It was redissolved in acetic acid, separated from the insoluble matter by centrifuging, and reprecipitated by again adjusting the solution to a pH of 7.6 with ammonium hydroxide. This precipitate did not contain a higher concentration of the product than the original material. Addition of more ammonium hydroxide caused the precipitation of more material, but titration of the various fractions with iodine showed that the ascorbic acid derivative was distributed in all of the fractions.

(2) Maltose.

Five grams of maltose was condensed with diethyl oxalacetate by the procedure used with melibiose. The crude sodium salt

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14. An experiment with this resin and ascorbic acid proved that it did not cause any appreciable oxidation of ascorbic acid.

15. Basic lead ascorbate precipitates at this pH and it seemed probable that the basic lead salt of the analogue might behave similarly.

weighed 2.85 g., and titration with iodine indicated the presence of 17.8 percent or 0.507 g. of sodium 6-α-glucosyl-glucoheptonascorbate. This corresponds to a yield of 9.2 percent of the theoretical. The ultraviolet absorption spectra of the material are given on page 20.

(5) Lactose and cellobiose.

Condensation of lactose and cellobiose with diethyl mesoxalate was tried by the method used with melibiose and maltose, but the precipitates that were obtained did not react with iodine. A number of variations in the condensation procedure were tried in an effort to effect the condensation. The period of heating was increased from 10 to 30 minutes, the amount of sodium cyanide was doubled and finally, since the cyanide ion is responsible for the condensation, a little water was added to the mixture, but no ascorbic acid derivative was formed.

C. Absorption spectra of glycosyl glucoheptoascorbic acids.

A characteristic property of ascorbic acid and its analogues is the strong absorption shown by their solutions in the ultraviolet region (16). The similarity of the absorption spectra of ascorbic and dihydroxymaleic acid provided an important link in the elucidation of the structure of vitamin C (60). The molar absorbancy index[16] of ascorbic acid has been used for the quantitative determination of this compound (56, 57, 58).

Measurements of the absorption in the ultra-violet region of solutions prepared from the products obtained by condensation of diethyl mesoxalate with melibiose and maltose therefore offered an

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16. The term "molar absorbancy index" is discussed in a footnote on page 18.

excellent means for their identification as analogues of ascorbic acid, and were made as follows:

Based on the purity of the product as determined by titration with iodine (see page 16) a 0.0001 molar solution of the sodium salt was prepared with conductivity water. Twenty-five milliliters of this solution was diluted with conductivity water to make a 0.000025 molar solution for the measurements in neutral solution, and another 25 ml. of the 0.0001 molar solution was diluted with conductivity water and standard hydrochloric acid to make a 0.000025 molar solution in 0.1 N acid for the measurements in acid solution. The transmittancies of the solutions were measured over the range from 200 to 400 mμ with a Beckman quartz photoelectric spectrophotometer (90). The absorption cells (91) were one centimeter in length and consisted of Pyrex tubing, 3.8 cm. in diameter, with polished ends, and fitted with removable quartz plates. These plates were held in place by a metal holder. Corrections were made for slight differences in the transmittancies of the cells containing solvent and solution. The light source was a water-jacketed hydrogen arc. All measurements were made in a room maintained at 25° C. The transmittancies of the solutions are shown in graphs 1 and 2 on page 20. The molar absorbancy indices, and the wave lengths at which maximum absorption occurred are shown in Table 1, page 17. The values reported for ascorbic acid (16) are included for comparison.

VI. SUMMARY

This investigation was undertaken to study methods of preparation of analogues of ascorbic acid containing a glycosyl group. The object of the study was the preparation of certain representatives of this class of compounds, none of which have been obtained by other investigators. The presence of the acid sensitive glycosidic group of the disaccharide limited the use of or necessitated the modification of known methods of ascorbic acid preparation.

Analogues and derivatives of ascorbic acid can be prepared most readily by enolization and lactonization of esters of 2-keto-acids, but heretofore, no esters of 2-keto-gluconic acids have been reported. To develop the technique for the preparation of the desired esters, a study was made with lactose, even though the corresponding ester is not suitable for the formation of a glycosyl ascorbic acid. The sugar was converted first to the phenylosazone, then to the osone and finally to the 2-keto acid by oxidation with bromine in the presence of barium carbonate. In the course of the work a new dextrorotatory form of lactose phenylosazone was prepared. Furthermore, two new crystalline salts, barium 2-keto-lactobionate dihydrate and barium 2-keto-lactobionate-barium bromide tetrahydrate were prepared and their structures were proved by hydrolysis with lactase and identification of the resulting galactose and 2-keto-gluconic acid. Barium 2-keto-lactobionate is the first crystalline metallic salt of a 2-keto-aldobionic acid that has been reported. Barium 2-keto-lactobionate-barium bromide is unique in that it is the only salt of a sugar acid thus far isolated which

containing barium bromide. It has exceptionally good crystallizing properties and provides a suitable means for the separation and identification of 2-keto-lactobionic acid.

The methyl ester of 2-keto-lactobionic acid was prepared by the action of diazomethane on the acid freed from the barium salt. On treatment with sodium methylate in methanol the 2-keto-ester did not decompose and rearrange to form a six-membered ring analogue of ascorbic acid.

Attempts to prepare 2-keto-melibionic acid through the intermediate preparation of the phenylosazone and osone of melibiose led to non-crystalline products which failed to yield an ascorbic analogue. It was found in the course of this work that the use of dioxane in the preparation of crystalline melibiose gives consistently good results and represents an improvement over previous methods of crystallizing this sugar.

Preparation of 2-keto-melibionic acid by catalytic oxidation of melibionic acid was also tried, but the methyl ester of the compound could not be separated from the inorganic reagents present in the reaction mixture. In connection with this investigation a convenient method was developed for the preparation of the comparatively rare calcium melibionate from raffinose. The method involves hydrolysis of the raffinose, removal of the fructose by yeast fermentation and electrolytic oxidation of the crude melibiose in the presence of calcium bromide and calcium carbonate. The resulting calcium melibionate is separated directly from the electrolyte in the form of a new stable crystalline trihydrate.

A new amorphous basic salt of calcium melibionate was prepared and found to be useful for the separation of calcium melibionate remaining in the mother liquor from the electrolytic oxidation process. The salt contains four moles of calcium oxide per mole of calcium melibionate.

The method of preparing ascorbic acid and its analogues by condensation of sugars with diethyl mesoxalate was found to be applicable to the disaccharides. Two new glycosyl derivatives of glucoheptoascorbic acid were prepared by this method, but they could not be isolated in the pure state. Sodium 6-α-galactosyl-glucoheptoascorbate was prepared by the condensation of diethyl mesoxalate with melibiose and sodium 6-α-glucosyl-glucoheptoascorbate was prepared by a similar condensation with maltose. The nature of the products was established by measurements of their ultra-violet absorption spectra and their reaction with iodine in acid solution.

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