methanolic potassium hydroxide and refluxed for 2 hr., the solution was cooled and neutralized with glacial acetic acid, precipitated in excess ice water, and extracted into ether. The organic layer was washed, dried, and concentrated to give, upon crystallization from isopropyl ether, 45 mg. of testosterone, identical with genuine material by melting point, mixed melting point, and infrared spectrum.

3-Methoxy-$\Delta^{3,5}$-androstadien-17-one (Vb). Δ^4-Androstene-3,17-dione (IVb.) (1.0 g.) was dissolved in 5 ml. of 2,2-dimethoxypropane, and 5 ml. of dimethylformamide *p*-toluenesulfonic acid monohydrate (26 mg.) and 0.2 ml. of methanol were added (resultant *p*H = 6) and the solution was refluxed for 3.5 hr. After cooling, it was neutralized with 152 mg. of sodium bicarbonate and added dropwise to a rapidly stirring mixture of ice water. After 20 min., the resulting oil had solidified and was filtered to give 1.1 g. of product. Paper chromatographic investigation showed it to be mainly enol ether (in the heptane–Methyl Cellosolve system, $R_f = 0.61$) with some starting material ($R_f = 0.16$) and an unidentified non-polar contaminant ($R_f = 0.91$) also present. Recrystallization from acetone-methanol (containing a drop of pyridine) gave 494 mg. of paper-chromatographically homogeneous enol ether, m.p. 141–163°; λ^{Nujol} at 5.78 (17-ketone), 6.06 and 6.15 μ. (enol ether); ϵ_{239} 20,000; $[\alpha]_D^{24} - 84.4$.

Anal. Calcd. for $C_{20}H_{26}O_2$: C, 79.95; H, 9.39. Found: C, 80.28; H, 9.24.

3-Methoxy-$\Delta^{3,5}$-pregnadien 20-one (Vc). Progesterone (IVc) (300 mg.) was dissolved in 2.5 ml. of 2,2-dimethoxypropane and an equal volume of dimethylformamide *p*-toluenesulfonic acid monohydrate (8 mg.) and 0.1 ml. of methanol were added, and the solution refluxed for 3.5 hr. After cooling and neutralization with 45 mg. of sodium bicarbonate, the solution was slowly added to 200 ml. of ice water, stirred for 0.5 hr. and filtered. The enol ether thus obtained (288 mg.) had λ^{Nujol} at 5.90 (C-20-ketone) 6.06 and 6.15 μ (enol ether doublet). Recrystallization from acetone-methanol containing a trace of pyridine gave an analytical sample, m.p. 135–160°; $[\alpha]_D^{23} - 61.4°$; ϵ_{239} 20,000; infrared spectrum identical with the crude product.

Anal. Calcd. for $C_{22}H_{32}O_2$: C, 80.44, H, 9.83. Found: C, 80.76; H, 9.85.

3-Methoxy-$\Delta^{3,5,16}$-pregnatrien-20-one (VII). $\Delta^{4,16}$-Pregnadiene-3,20-dione (VI) (300 mg.) was subjected to enol-etherification conditions as above. A crude yield of 316 mg. was obtained. An analytical sample from acetone-methanol (trace of pyridine) had a m.p. of 152–167°; λ^{Nujol} at 5.99 (20-one), 6.04, 6.13 (enol ether), and 6.30 (Δ^{16}) μ ϵ_{239} 28,000; $[\alpha]_D^{23} - 109.4°$.

Anal. Calcd. for $C_{22}H_{30}O_2$: C, 80.93; H, 9.26. Found: C, 81.05; H, 9.20.

3-Ethoxy-$\Delta^{3,5}$-pregnadien-20β-ol acetate (Vd). 20β-Acetoxy-Δ^4-pregnen-3-one (IVd) (1 g.) was dissolved in 5 ml. of dimethylformamide and an equal volume of 2,2-dimethoxypropane. Ethanol (10 ml.) and 26 mg. of *p*-toluenesulfonic acid monohydrate were added, and the solution refluxed for 4 hr. It was then cooled and poured into an excess of ice water. The oily suspension was extracted with ether, dried, and concentrated. After overnight standing in the refrigerator and trituration with isopropyl ether, the enol ether crystallized and was filtered off. The product had λ^{Nujol} 5.79 (acetate), 6.02 and 6.11 (enol ether), 8.02 (acetate C—O), and 8.54 μ (ether C—O). It was identical with a product prepared *via* the classical ethyl orthoformate method of Serini and Köster: m.p. 102–117°, $[\alpha]_D^{24} - 94.8$, ϵ_{240} 18,900.

Anal. Calcd. for $C_{25}H_{38}O_3$: C, 77.67; H, 9.91. Found: C, 77.68; H, 9.79.

3,11β,17α,21-Tetrahydroxy-$\Delta^{3,5}$-pregnadien-20-one 17,21-acetonide 3-methyl ether (IX). Hydrocortisone (VIII) (15 g.) was dissolved in 225 ml. of 2,2-dimethoxypropane and 75 ml. of dimethylformamide. *p*-Toluenesulfonic acid monohydrate was added, and the resulting solution was refluxed for 3 hr. After cooling, 2.3 g. of sodium bicarbonate was added, and the suspension shaken and filtered. Vacuum concentration of the filtrate gave an oil that crystallized after overnight standing with methanol. Filtration gave 8.2 g. of the acetonide enol ether, m.p. 147–195° (homogeneous by paper chromatography). Recrystallization from acetone-methanol gave an analytical sample, m.p. 152–183°; λ^{Nujol} at 2.81 (OH), 5.86 (C-20-ketone), 6.02 and 6.14 μ (enol ether), ϵ_{238} 19,800.

Anal. Calcd. for $C_{25}H_{36}O_5$: C, 72.08; H, 8.71. Found: C, 71.87; H, 8.71.

BLOOMFIELD, N. J.

[CONTRIBUTION FROM THE INSTITUTE OF PAPER CHEMISTRY]

Acid Epimerization of D-Glucose[1a]

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Received October 13, 1960

The formation of small amounts of fructose from glucose by heating with dilute sulfuric acid under the conditions of cellulose hydrolysis has been corroborated, but not that of mannose. The fructose was isolated by paper chromatography and definitely identified by means of infrared spectrography. Ost's early experiments on the acid epimerization of glucose were similarly corroborated. The possibility of such conversions must be considered in interpreting the analyses of plant carbohydrates.

Shortly after paper chromatography began to prove useful in the analysis of plant polysaccharides, it was pointed out[2] that great care had to be taken during neutralization of the hydrolyzates as isomerization of glucose to fructose could occur at a *p*H greater than 4.0. Since the epimer mannose can also be formed,[3] this becomes a matter of some importance in the analysis of the sugars in the hydrolyzates from plant materials.[4,5]

(1)(a) Presented at the 138th National Meeting of the American Chemical Society, New York, N.Y., Sept. 11–16, 1960. (b) Research Fellow from Tokyo University of Agriculture and Technology, Tokyo, Japan.

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(3) J. C. Speck, Jr., *Advances in Carbohydrate Chemistry*, Vol. 13, Academic Press, New York, 1958, pp. 63–103.

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TABLE I

PRODUCTS FROM GLUCOSE AND 2.5% SULFURIC ACID AT 120°

Glucose Concentration, %	0.17			0.34			10.00
Time of Treatment, hours	1	5	10	1	5	10	10
Monosaccharides							
Glucose	+	+	+	+	+	+	+
Fructose	+ (faint)	+	+	+ (faint)	+	+	+
Mannose	−	−	−	−	−	−	−
Reversion products							
Cellobiose and β-Sophorose	−	+	+	−	+	+	+
β-Nigerose	−	+	+	−	+	+	+
Maltose	−	+	+	−	+	+	+
Isomaltose and Gentiobiose	−	+	+	−	+	+	+
Higher oligosaccharides	−	+	+	−	+	+	+

Although acid epimerization of sugars has long been recognized,[6] its application to this problem was apparently not noted at first. However, Saeman, Moore, Mitchell, and Millett[7] noted two spots appearing on the chromatogram when glucose was subjected to a simulated pulp hydrolysis (one hour with 72% sulfuric acid at 30° followed by one hour with about 2.5–3% sulfuric acid at 15 p.s.i. steam pressure). One of these, moving at about the rate of mannose, was eluted and separated by rechromatographing with ethyl acetate–acetic acid–water to give a faster and a slower spot than mannose. Neither component was identified and both were present in very small amounts. The authors recommend a correction of the apparent mannose content of pulps by 0.15% to take the effect into consideration.

Later, Matsuzaki, Ward, and Murray[8] studied the effect of hot dilute sulfuric acid on glucose, using the techniques of Adams and Bishop[9] to determine very small amounts of nonglucose sugars in the presence of large amounts of glucose. After destroying the glucose with glucose oxidase, spots corresponding to fructose and mannose were obtained, but the sugars were not definitely identified. The authors concluded that these two sugars might have been formed from glucose by the acid Lobry de Bruyn reaction and it was the aim of the present work to corroborate this by positive identification of the two sugars.

This was partially achieved in that fructose was definitely identified by the infrared spectra, although mannose was not found. Fructose, but not mannose, was also found in a repetition of Ost's work,[3] in which a solution of glucose in cold 8*N* sulfuric was let stand for several months. There are several possible explanations for the absence of mannose, which is formed much more slowly than frutose in the usual epimerization.[10] One likely reason is the fact that the enzyme used in the present experiments was a different batch from that used by Matsuzaki and there may have been somewhat more action on mannose with the later enzyme. Another possible explanation is that the mannose may have been used up in reversion or other chemical conversions as rapidly as formed, since its rate of ring opening is somewhat faster than that of glucose, but this still leaves unexplained its occurrence in previous work.

In both cases, the simulated hydrolysis and the verification of Ost's experiments, small amounts of reversion products are formed. Besides the isomaltose observed by Ost,[3] these products included β-sophorose, β-nigerose, maltose, gentiobiose, cellobiose, and a number of more slowly moving spots, probably higher oligosaccharides. The sugars named were simply indicated by comparison chromatographically with known reference sugars. The techniques used did not permit good separation of isomaltose and gentiobiose or by β-sophorose and cellobiose, but since the results are incidential to the main purpose and are moreover in accord with well known data on acid reversion,[11,12] it was not considered necessary to refine the techniques. The results are tabulated in Tables I and II.

Epimerization occurs before reversion (see Table I) and the disaccharides, as would be expected, before the higher oligosaccharides (see Table II). Because our interest was centered on the epimerization as it related to polysaccharide analysis, its relationship to reversion was purely incidental, but it may be worth considering briefly Mora's hypothesis of condensation[12a] of glucose. This supposes the formation from glucose and a hydrogen ion of a carbonium ion (carbon atom one), which reacts with another sugar molecule to form reversion products. This same carbonium ion has been postulated as an intermediate in other reactions, being con-

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TABLE II

PRODUCTS FROM GLUCOSE (21.18%) AND 8*N* SULFURIC ACID AT ROOM TEMPERATURE

Time of Treatment	10 Days	4 Months	8 Months	1 Year
Monosaccharides				
Glucose	+	+	+	+
Fructose	+	+	+	+
Mannose	−	−	−	−
Reversion products				
Cellobiose and β-Sophorose	+	+	+	+
β-Nigerose	+	+	+	+
Maltose	+	+	+	+
Isomaltose and Gentiobiose	+	+	+	+
Higher oligosaccharides	−	+	+	+

verted by regeneration of the hydrogen ion, to the open-chain aldehyde. The aldehyde form is the intermediate in most theories of base-catalyzed epimerization.[3] We thus have a possible mechanism for the acid epimerization of such a nature that it would explain why this occurs faster than reversion, for the latter requires collision with another sugar molecule whereas the former is simply an internal break-down.

The amounts of epimer formed are too small to be of much practical importance in the analysis of plant polysaccharides, except in the cases where trace amounts are important, as in the work of Adams and Bishop,[9] but in such cases the authors feel that blank runs on glucose should be used to control the conditions of hydrolysis and chromatography.

That the fructose is not formed by the action of the glucose oxidase was shown by running blanks at 120° without oxidase. The fructose spot was observed on the chromatogram, but, in this case, was not examined by the infrared technique.

In this work, the chromatograms were run close to the end of the paper strip to afford maximum separation and we therefore do not know whether faster-moving compounds in small amounts were present, for these would have run off the paper. One might have expected furan derivatives or sugar anhydrides,[12b] for example.

EXPERIMENTAL

Acid treatment of glucose. For the treatment at 120°, various amounts of D-glucose[13] (Run 1, 0.3 g.; Run 2, 0.6 g.) were added to 173.7 ml. of 2.5% sulfuric acid and heated in pressure bottles for 1 hr., 5 hr., and 10 hr. Run 3 was made with 17.28 g. of D-glucose and 170 ml. of 2.5% sulfuric acid for 10 hr. A glycerin bath at 120° ± 0.5° was used for the environment. The acid concentration corresponds to those used in the secondary hydrolysis of cellulosic pulps.

In Run 4, a set of experiments to verify the experiments of Ost, 1000 g. of D-glucose were dissolved in a mixture of 2 l. of water and 1 l. of concd. sulfuric acid (sp. gr. 1.835) at room temperature. The solution was kept in the dark at 22.8° and 25-ml. samples were taken for investigation after 10 days, 4 months, 8 months, and 12 months. Tests for bacterial contamination were negative.

For both the 120° and the 22.8° experiments, the acid solution at the end of the reaction period was diluted to 0.02*N* concentration with deionized water and neutralized by passing through a column of IR-45 (acetate form). The column was washed with deionized water until no sugar could be detected by the Molisch test. The final volume of neutral solution plus washing was about 7 l. for the runs at 120° and 15 l. for the runs at room temperature.

For a few runs at 120° no treatment with glucose oxidase was used. In these cases, the solution was concentrated to about 6 ml., 2 ml. of absolute ethanol was added, and enough deionized water to make up 10 ml. This solution was stored in the refrigerator and used for chromatographing. In most cases the bulk of the glucose was first destroyed by glucose oxidase.

Treatment with glucose oxidase.[9] The neutralized solution was concentrated to about 100 ml. and oxidized with 200 mg. (for Runs 1 and 2) or 1500 mg. (Run 3) of glucose oxidase,[14] for 2 days at room temperature (73° F.). Run 4 was oxidized with 75 mg. of glucose oxidase for 5 days. In all cases, the gluconic acid formed was neutralized with barium hydroxide solution using the Beckman *p*H meter. After filtration, 100 ml. of ethanol was added to precipitate an additional amount of barium gluconate, which was removed by filtration. The filtrate was concentrated and treated again with ethanol. This procedure was repeated until no further precipitate formed. Then, alcohol was removed at 40° *in vacuo* and the aqueous solution deionized with Amberlite IR-45 (carbonate form) and IR-120 (acid form). The solution was concentrated to about 6 ml. with an evaporator. Two milliliters of absolute ethanol was added to the solution and deionized water was then added to bring the volume of the solution to 10 ml. The concentrate was stored in the refrigerator. It was spotted on a Whatman No. 1 or 3 MM filter paper and sugars were detected chromatographically.

Separation of sugars. Whatman 1 and Whatman 3 MM papers were used for this separation. The paper was washed twice with water before use. The hydrolyzates were spotted on the same sheet as known reference sugars[15] and developed with 8:2:1 ethyl acetate–pyridine–water or with 9:2:2 ethyl acetate–acetic acid–water.

Three sprays were used. Chromatograms were usually run in triplicate and one sprayed with each of the sprays. The first two are particularly effective for the aldohexoses, the last is a specific spray for fructose.

A. *o-Aminodiphenyl reagent.* This was a modification of Timell's reagent.[16] *o*-Aminodiphenyl (0.4 g.) was dissolved in 75 ml. of glacial acetic acid. Sixteen milliliters of distilled water was added and the solution made up to 100 cc. with glacial acetic acid. The solution was shaken well and stored in the refrigerator.

B. *p-Anisidine hydrochloride reagent.* This was a modification of Pridham's reagent.[17] One gram of recrystallized *p*-anisidine hydrochloride in 10 ml. of water was added to 20 ml. of 95% ethanol and this to 170 ml. of *n*-butyl alcohol.

C. *Urea reagent*, as prepared by Wise, *et al.*[18]

Infrared microtechniques for the identification of fructose. Infrared absorption spectra were recorded with a Perkin-

(13) Anhydrous dextrose, Analytical Reagent, Mallinckrodt.

(14) Dee O: manufactured by the Takamine Laboratory, N. J.

(15) D-Glucose–anhydrous dextrose, Analytical Reagent, Mallinckrodt. D-Mannose—Pfanstiehl Laboratories. D-Levulose—Pfanstiehl Laboratories.

(16) T. E. Timell, C. P. J. Glaudemans, and A. L. Currie, *Anal. Chem.*, **28**, 1916 (1956).

(17) J. B. Pridham, *Anal. Chem.*, **28**, 1967 (1956).

(18) C. S. Wise, R. J. Dimler, H. A. Davis, and C. E. Rist, *Anal. Chem.*, **27**, 33 (1955).

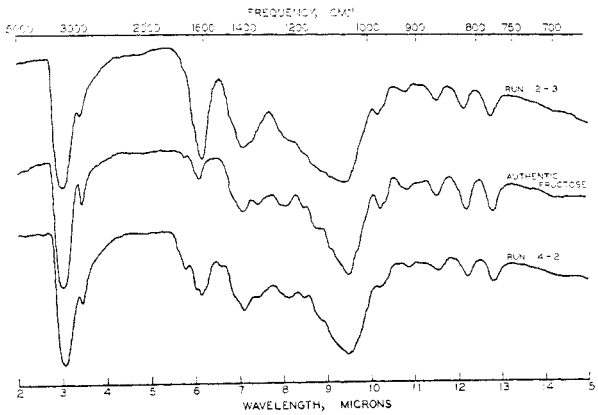

Fig. 1. Identification of fructose by infrared spectra. The middle tracing (authentic fructose) is to be compared with the top tracing (action of hot acid on glucose) and the bottom tracing (action of acid at room temperature on glucose)

Elmer Model 21, double-beam spectrophotometer equipped with a sodium chloride prism.

In Run 2–3, 0.6 g. of D-glucose was treated with 173.7 ml. of 2.5% sulfuric acid at 120° for 10 hr. After removal of the glucose from the reaction mixture by means of glucose oxidase, the solution was concentrated. The concentrate was applied by streaking on Whatman 3 MM paper, developed with 8:2:1 ethyl acetate–pyridine–water, and guide strips sprayed with urea spray reagent. The fructose spots, detected by the guide strips, were cut out and eluted with deionized water. The eluates were collected by means of a capillary elution pipet from eighty sheets of Whatman 3 MM and concentrated to about 5 ml. The concentrate contained a small amount of what appeared to be fibers which were readily removed by centrifuging. The sample was then air-dried overnight at room temperature. The resulting sirup was further dried for 15 hr. in a vacuum desiccator containing phosphorus pentoxide. After drying in the desiccator, the sample had still not crystallized. Rather than to attempt any further drying techniques, it was decided to obtain the spectrum of the sirupy sample which was, therefore, warmed in a 60°-oven for 0.5 hr. and spread on a potassium bromide disk, and its infrared spectrum was recorded. For comparison, a small amount of authentic crystalline fructose (C.P. Special) was wetted, allowed to air dry, and further dried in the same manner as the sample. The resulting sirup was also spread on a potassium bromide disk and its infrared spectrum recorded.

The fructose spots from a chromatogram of Run 4–2(4 months treatment of glucose with 8*N* sulfuric acid at room temperature) were similarly examined. In this case, the eluates from 35 sheets were collected. The moist sample was treated with alcohol and washed with alcohol by centrifuging. Both the alcohol-soluble and the alcohol-insoluble (granular) fractions were incorporated into potassium bromide pellets and the spectra recorded.

RESULTS

Tables I and II show the results of chromatographic separation of the glucose isomerization and reversion products. No effort was made to resolve the overlapping spots of β-sophorose and cellobiose or of isomaltose and gentiobiose. From the findings of previous investigators,[11,12] all four sugars can be expected to occur. Particularly heavy spots were formed for maltose and for the isomaltose-gentiobiose combination.

Both at 120° and at room temperature, the higher oligosaccharides do not appear at short periods of time. The fructose spot is also fainter after 1 hr. of treatment at 120° than after longer treatments. Neither effect is unexpected.

The absence of mannose formation is somewhat unexpected, in view of previous work.[7,8] There are several differences in the details of the experiments, however, which may account for divergent results.

The color and R_f or R_g values of a sugar spot are not positive identification; but identical infrared spectra are conclusive. The eluted fructose spots were, therefore, compared with an authentic fructose sample over the range 2 to 15 microns. Figure 1 compares the spectra.

The top spectrum is the eluted material from Run 2–3 and the middle spectrum is authentic fructose. They are clearly similar, but not identical, the only major difference being a strong absorption at 6.18 μ for the sample to be identified. Absorption in this region may be associated with bound water or metallic salts. It can be concluded that the sample is largely fructose, probably with a contaminating salt.

This conclusion is borne out by a study of the material from Run 4–2. This, too, appeared to be an impure fructose. When separated into an alcohol-soluble and an alcohol-insoluble fraction, however, the comparison of the spectra showed practical identity of the spectra of the alcohol-soluble fraction and fructose except for very slight differences in the 6-μ region. Since the alcohol-insoluble fraction proved to be a metallic sulfate, we can identify our organic constituent as fructose with a contaminating sulfate which was not removed by the neutralization procedures. This positive identification (compare the bottom line, Run 4–2, which is the alcohol-soluble portion with the line above) makes it clear that the Lobry de Bruyn reaction can occur during the hydrolysis as well as during the neutralization in the characterization of pulps and that the interpretation of small amounts of fructose must be viewed with caution. Although we failed to find it in the present experiments, mannose is known to be formed in very small amounts by this reaction and may well occur in detectable amounts under certain conditions.

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