[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, PURDUE UNIVERSITY]

Isolation of Crystalline D-Glucose and Cellobiose from an Enzymatic Hydrolyzate of Cellulose[1]

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Crystalline D-glucose and cellobiose have been isolated from cell-free enzymatic hydrolyzates of cellulose. While crude enzyme preparations hydrolyze cellulose to D-glucose as the only detectable simple sugar, an enzyme preparation purified by adsorption on and elution from powdered cellulose, reacts with swollen cellulose to produce cellobiose as the major product for the first three hours of hydrolysis. Formation of a disaccharide intermediate in the enzymatic hydrolysis of cellulose is thus proved. Some properties of the purified enzyme preparation are described.

Only meager information is available on the mechanism by which cellulose is hydrolyzed by enzymes. Pringsheim[2] used iodoform to check the rapid growth of an impure bacterial culture on cellulose and separated both D-glucose and cellobiose, as their respective phenylosazones, from the accumulated reducing substances. Hungate[3] reported that cellobiose was the only sugar present in a hydrolyzate six weeks after cessation of active fermentation on cellulose by the protozoan *Endiplodinium neglectum*. However, from these results it cannot be concluded whether cellobiose is an intermediate in the enzymatic hydrolysis of cellulose or is a metabolic product of microörganisms.

In this Laboratory it was observed that a crude extract from *Aspergillus niger*[4] acted on swollen cellulose to produce D-glucose as the only detectable sugar. An enzyme preparation which acted on swollen cellulose to produce detectable amounts of cellobiose was not obtained from the crude extract (1) when it was fractionated by ammonium sulfate or acetone, (2) when it was held for various periods at elevated temperatures, (3) when it was subjected to strong acid or strong alkaline conditions or (4) when it was fractionated by sorption on ion exchange resins, charcoal, wheat starch, Kaolin–Super Cel, bauxite or aluminum hydroxide. However, after sorption of the crude extract at *p*H 3 on a column of powdered cellulose there could be obtained by elution with a borate buffer at *p*H 9, an enzyme preparation which acted on swollen cellulose to produce isolatable amounts of cellobiose and D-glucose. Cellobiose was the principal sugar formed during the first three hours of enzyme action.

Powdered cellulose columns have not been extensively used for the separation of proteins, although cotton has been reported[5] to adsorb proteins and the movements of enzymes on columns of filter paper discs[6] are known to be influenced by variations in salt concentrations or by variation in hydrogen ion concentrations.

Experimental

Materials.—The Rohm and Haas product, 19AP, isolated from *Aspergillus niger* growing on bran, was the source of cellulose-splitting enzymes. Swollen or "reactive" cellulose was prepared according to the phosphoric acid procedure outlined by Walseth.[7] Cellulose used for column adsorption of protein was Standard Grade, Whatman Ashless Powder for Chromatography.

Enzyme Purification.—Ten grams of crude *Aspergillus niger* extract was added to 200 ml. of water, stirred and allowed to stand for several hours in the refrigerator. The insoluble residue (*ca.* 25%) was removed by centrifugation and the supernatant solution was filtered through fine fritted glass. The filtrate at *p*H 3 was passed through a wet cellulose column (50 × 406 mm.) that was packed to deliver not less than 1 ml. per minute when the receiver was connected to the water-pump. As soon as the solution had passed into the column, washing was begun with 1 liter of water adjusted to *p*H 3. These effluent washings were inactive upon both cellulose and cellobiose and were discarded. An eluate obtained by washing the column with 1 liter of a Clark and Lubs borate buffer at *p*H 9 was adjusted to *p*H 6 and made 80% saturated with respect to ammonium sulfate. After 24 hours the precipitate obtained was centrifuged and dissolved in 80 ml. of water. The solution was dialyzed in a collodion sac against running tap water at 15° for 24 hours and then for an equal period against distilled water at 5°. The solution was then freeze dried to yield approximately 0.1 to 0.2 g. of a gray powder.

Cellobiose.—To a solution of 0.2 g. of purified enzyme in 100 ml. of water there was added 2 g. of swollen cotton and 1 mg. of phenylmercuric acetate. After stirring the mixture for two hours at 45° and *p*H 4.5, the insoluble cellulose was filtered off and the filtrate was boiled for five minutes. The precipitate was removed by centrifugation and the supernatant was concentrated *in vacuo* to a brown solid. This material was dissolved in 2 ml. of water, filtered and applied 3 inches from the top of eight paper strips each 7 inches in width. After development with a 3:6:4 mixture of water, butanol-1 and pyridine for 20 hours, the strips were dried and sample ribbons were cut lengthwise from each side and down the center of each strip. The ribbons were sprayed with ammoniacal silver nitrate solution and the cellobiose region on each strip determined. This unsprayed portion of the paper which the reference strip indicated to contain cellobiose was cut out and extracted with water for 1 hour in a Soxhlet extractor. The extract was concentrated to about 1 ml. and 50 ml. each of methanol and butanol-1 was added. Heating on the steam-bath resulted in the formation of a trace of flocculent material which was removed by filtration. After evaporation of the filtrate to dryness, the sugar was dissolved in about 0.5 ml. of water and 25 ml. of acetone was added. On cooling, needle-like crystals were obtained. The crystals were dried at 70°. The melting point was 230°, and was not depressed when the sample was mixed with an authentic specimen of cellobiose. The X-ray diffraction pattern was also identical with that of an authentic specimen of cellobiose.

Larger amounts of cellobiose were isolated by charcoal: Celite chromatography[8] and characterized as the phenylosazone. This compound showed a melting point of 194–197°, which was not depressed when a sample was mixed with an authentic specimen of cellobiosazone. The X-ray diffraction pattern of the sample was also identical with that of an authentic specimen.

The possibility that cellobiose is a reversion product was negated by showing that when 0.01 g. of purified enzyme

(1) Journal Paper No. 659 of the Purdue Agricultural Experiment Station.

(2) H. Pringsheim, *Z. physiol. Chem.*, **78**, 266 (1912).

(3) R. E. Hungate, *J. Bact.*, **48**, 499 (1944).

(4) A product designated 19AP of the Rohm and Haas Company, Philadelphia 5, Penna.

(5) H. Tauber, *J. Biol. Chem.*, **113**, 753 (1936).

(6) H. K. Mitchell and F. A. Haskins, *Science*, **110**, 278 (1949); H. K. Mitchell, M. Gordon and F. A. Haskins, *J. Biol. Chem.*, **180**, 1071 (1949).

(7) C. S. Walseth, Dissertation, Lawrence College, 1948.

(8) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

was incubated for 5 hours with 150 mg. of D-glucose in 60 ml. of water at 45°, the only detectable reducing sugar was D-glucose.

D-Glucose.—The isolation of crystalline D-glucose was accomplished in essentially the same manner as described above for cellobiose. Recrystallization from water–ethanol gave a product which melted at 145°. The same melting point was obtained when the sample was mixed with known D-glucose.

Quantitative Analysis.—Cellobiose appeared, by visual inspection of paper chromatograms, to be the major sugar roduced during the first several hours of the action of the purified cellulase preparation on cellulose, while D-glucose appeared to be the major sugar produced after five hours. Confirmation of this observation was obtained by quantitative analysis of the hydrolyzate at intervals during the reaction. The quantitative microanalytical procedure used was a modification[9] of that of Deuel and co-workers[10] wherein the sugars are separated by paper chromatography. One milliliter aliquots of the reaction mixture were taken periodically during a typical hydrolysis. After evaporating an aliquot to 0.1 ml., several 20 λ portions were applied to the starting line of a paper strip. At the end of 36 hours of descending development with water:butanol-1:pyridine in the ratio 3:6:4 the paper was dried and definite marker strips removed and sprayed with ammoniacal silver nitrate solution. The sugar-containing patches were cut from the corresponding locations on the unsprayed paper and each was then extracted in a Wiley-type extractor. In the case of D-glucose, 4 ml. of water was used for extraction while for cellobiose 4 ml. of 0.1 *N* hydrochloric acid was used in the receiver to hydrolyze the extracted sugar to D-glucose. After 1 hour of extraction the 4 ml. of sugar solution was mixed successively with 3 ml. of 3,5-dinitrosalicylic acid solution, 2 ml. of 6 *N* sodium hydroxide solution and 1 ml. of 50% Rochelle salt solution. The sample was then heated in boiling water for ten minutes, cooled and the color measured with a Beckman DU spectrophotometer at 543 mμ *versus* a reagent blank. From a standard curve of D-glucose concentration *versus* per cent. transmission, the weight of D-glucose was obtained. In the case of cellobiose this value is multiplied by 0.95. The results are summarized in Fig. 1.

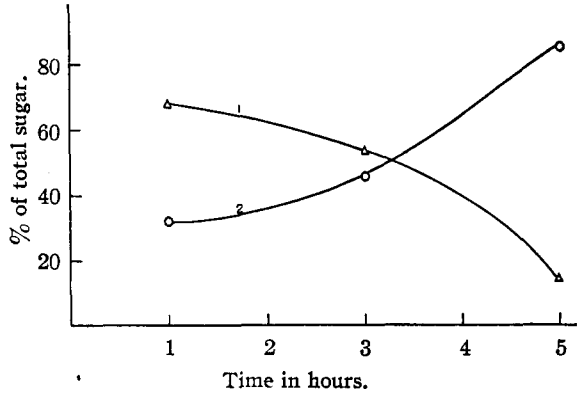

Fig. 1.—Variation in amounts present of cellobiose (1) and D-glucose (2) during enzymatic hydrolysis of cellulose with cellulase preparation.

Optimum *p*H of Purified Enzyme.—A 0.1-g. sample of the enzyme preparation purified by powdered cellulose was dissolved in 100 ml. of water. Ten milliliter aliquots were mixed with 50-ml. portions of eight different Clark and Lubs phthalate buffer mixtures and each of the resulting solutions was added to 1 g. of swollen cotton and 1 mg. of phenylmercuric acetate. The mixtures were allowed to stand at 45° for 5 hours and the reducing power (calculated as D-glucose) was determined by the Shaffer–Hartman modification of the Munson–Walker copper reduction method. The results are shown in Fig. 2.

Optimum Temperature.—To 0.1 g. of purified enzyme there was added 50 ml. of water, and the *p*H was adjusted

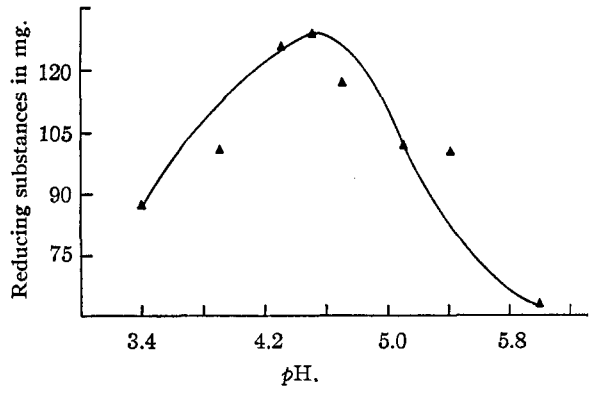

Fig. 2.—Effect of *p*H on yield of sugars produced by cellulase acting on cellulose.

to 4.5 with concentrated acetic acid and a 0.6 *N* solution of sodium hydroxide. The solution was made to 100 ml. and 10-ml. aliquots were each added to 50 ml. of water, 1 g. of swollen cotton and 1 mg. of phenylmercuric acetate. The mixture was then stirred at one of the temperatures shown on the abscissa of Fig. 3. Reducing substances (calculated as D-glucose) present at the end of 5 hours are shown on the ordinate. The formation of D-glucose is accentuated at higher temperatures as seen from paper chromatographic analyses.

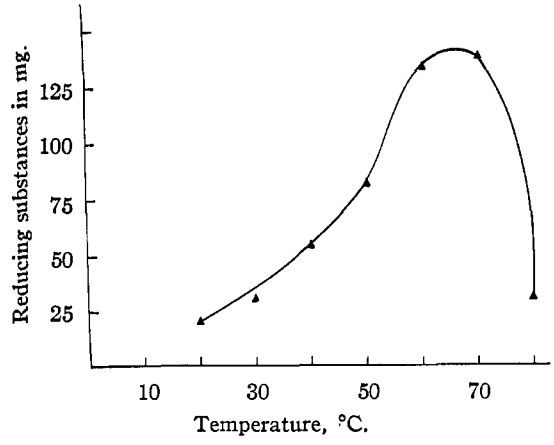

Fig. 3.—Effect of temperature on the yield of sugars produced by the action of cellulase on cellulose.

Search for Other Oligosaccharide Intermediates.—Paper chromatographic analyses of a hydrolyzate at various stages did not reveal the presence of oligosaccharides other than cellobiose. Yet there exists the possibility that oligosaccharide fragments, possessing only one reducing end group, might be present in small amounts. In an attempt to detect such oligosaccharide intermediates aliquots taken during various periods of the hydrolysis of cellulose were developed on paper strips and sprayed with a 0.5% solution of hydrochloric acid. After the strips were heated in an oven at 60° for 10 minutes, they were sprayed with ammoniacal silver nitrate solution and heated at 120° for 5 minutes. No reducing substances other than D-glucose and cellobiose could be detected.

Search for Phosphorylated Sugars.—Phosphorylated sugars may readily be detected on a paper chromatogram by any of a variety of color tests for sugars but the R_F values of D-glucose 1-phosphate and D-fructose 1,6-diphosphate are so low that a modification of the usual procedure was made to permit simultaneous identification and differentiation of reducing sugars or substances hydrolyzable to reducing sugars.

A procedure satisfactory for the simultaneous identification of known D-glucose, cellobiose, D-glucose 1-phosphate,

(9) R. L. Whistler and J. L. Hickson, unpublished work.

(10) E. Borel, F. Hostetter and H. Deuel, *Helv. Chim. Acta*, **35**, 115 (1952).

D-fructose 1,6-diphosphate and inorganic phosphate was a modification of Gutzeit's method.[11]

Herein the dried paper chromatogram is sprayed with a 1% hydrochloric acid solution which contains 2% molybdic acid. After drying, the paper is sprayed thoroughly with a 1% solution of benzidine in acetic acid. At this point phosphate ion gives a bright blue spot, phosphate ion with reducing sugar gives a yellow-green spot and reducing sugar alone gives a brown spot. To bring out the position of minute amounts of sugars, the dried paper is next sprayed with ammonium hydroxide solution and heated at 100° for 5 minutes. Phosphate ion is now seen as a green spot, phosphate ion with reducing sugar as a gray-green spot and reducing sugar alone as a dark brown spot. The test, sensitive enough to detect 0.3 microgram of D-glucose 1-phosphate, has been, with some modifications, previously used[12] for the detection of phosphorylated sugars but not for the simultaneous detection of phosphorylated and non-phosphorylated sugars.

These techniques did not indicate the presence of phosphorylated sugars as either intermediates or products in the hydrolysis of cellulose with cellobiose.

Acknowledgment.—The authors take pleasure in acknowledging their indebtedness to Professor A. K. Balls of this Department for helpful suggestions.

(11) G. Gutzeit, *Helv. Chim. Acta*, **12**, 829 (1929), quoted by F. Feigl, "Qualitative Analysis by Spot Tests," Third English Edition, Elsevier Publishing Co., New York, N. Y., 1946, p. 284.

(12) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, THIS JOURNAL, **72**, 1718 (1950).

LAFAYETTE, INDIANA

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

"Enamine" Derivatives of Steroidal Carbonyl Compounds. II

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Steroidal C_3-ketones have been shown to condense readily with pyrrolidine to form 3-(N-pyrrolidyl) enamines. In those polyketonic steroids having carbonyl groups in the C_3-position and elsewhere in the molecule the reaction was selective on the three position leaving the other functional groups unprotected and available for further study. Testosterone was readily prepared by the lithium aluminum hydride reduction of the C_3-(N-pyrrolidyl)-3,5-androstadien-17-one obtained thus from 4-androstene-3,17-dione.

In an earlier paper[1] it was shown that α,β-unsaturated amines—"enamines"—were readily prepared by the reaction of piperidine with representative steroidal aldehydes. Although Mannich and Davidsen[2] converted not only aldehydes but also several ketones, *e.g.*, phenylacetone and cyclohexanone, into enamines, the reaction when conducted as previously described[1] on 3-ketobisnor-4-cholenaldehyde, was remarkably selective on the aldehyde group, thus forming an intermediate which was readily ozonized to progesterone. Likewise, significant yields of enamines were not obtained by the reaction of piperidine with stigmastadienone, cholestan-3-one and 4-cholesten-3-one under the conditions reported here.

Continued studies, however, indicated that C_3-carbonyl groups readily condensed with the secondary amine, pyrrolidine, in such a manner that the formation of 3-(N-pyrrolidyl) enamines, with the elimination of water, appeared to be a characteristic reaction of C_3-steroidal ketones. Pyrrolidine, however, failed to react with the C_{17}- and C_{20}-keto groups of dehydroepiandrosterone and 5-pregnen-3β-ol-20-one, respectively. This observed selectivity led to the ready preparation of 3-(N-pyrrolidyl)-3,5-androstadien-17-one (IX) and 3-(N-pyrrolidyl)-3,5-pregnadien-20-one (I).

To study further the characteristic nature and selective aspects of this reaction the following C_3-pyrrolidyl enamines were prepared in addition to those mentioned above: 3-(N-pyrrolidyl)-2-(or 3)-cholestene (IV), 3-(N-pyrrolidyl)-3,5-cholestadiene (II), 3-(N-pyrrolidyl)-3,5,22-stigmastatriene (III), 3-(N-pyrrolidyl)-3,5-pregnadien-11,20-dione (V), 3-(N-pyrrolidyl)-3,5-pregnadien-11α-ol-20-one (VI), methyl-3-(N-pyrrolidyl)-7,12-diketo-3(or 2)-cholenate (VI), 3-(N-pyrrolidyl)-3,5-androstadien-17β-ol (X) and 3-(N-pyrrolidyl)-17α-methyl-3,5-androstadien-17β-ol (VIII).

These C_3-enamines, when prepared from 3-keto-Δ^4-steroids, were in all instances light yellow highly crystalline compounds which gradually decomposed and darkened upon melting. Even after all traces of solvent had been removed they continued to have a characteristic amine odor unusual for steroidal compounds. In those few cases where apparently pyrrolidine did not react readily with C_3-carbonyl groups, *e.g.*, progesterone, the addition of a catalytic amount of *p*-toluenesulfonic acid caused the reaction to proceed smoothly.

The ease of hydrolysis of the enamines to regenerate the ketones varied considerably between compounds of the single ethylenic double bond type IV and the conjugated ethylenic dienes type I, II and III. The enamine (IV) prepared from cholestan-3-one readily reverted to cholestan-3-one upon heating at reflux for 5 minutes in 95% ethanol whereas it was found better to heat compounds of the type prepared from stigmastadienone for 4 hours in a sodium acetate–acetic acid buffered solution in order to regenerate the ketone. Upon treatment with methanolic semicarbazide acetate, enamines were converted to semicarbazones.

The ultraviolet absorption spectra of the pyrrolidine enamines IV and VII, prepared from cholestanone and methyl dehydrocholate are in agreement with data reported by Bowden, *et al.*[3] Whether the position of the double bond is Δ^2 or Δ^3 has not been established.

(1) M. E. Herr and F. W. Heyl, THIS JOURNAL, **74**, 3627 (1952).

(2) C. Mannich and H. Davidsen, *Ber.*, **69**, 2106 (1936).

(3) K. Bowden, E. A. Braude, E. R. H. Jones and B. C. L. Weedon, *J. Chem. Soc.*, 45 (1946). They report that the conjugation of a single ethylenic bond with an amino group results in light absorption equivalent to that exhibited by classical conjugated systems such as butadiene; thus for C_2H_5·CH=CH·NC_5H_{10} (piperidyl) they report λ_{max}^{hexane} 228 mμ.