or to water acceptors. The former reaction results in the synthesis of new oligosaccharides while the latter in hydrolysis of allolactose. Evidence for reversibility of these reactions has been presented.

Experimental

Enzyme Material.—An enzyme preparation from *Saccharomyces fragilis* was kindly provided by Dr. W. M. Connors, National Dairy Research Laboratories, Long Island, New York. Solutions of the enzyme material exhibited hydrolytic and transferring activities. A measure of both types of activities was obtained by assaying the enzyme solution on lactose-1-C^{14}. In the assay procedure, 0.05 ml. of 0.6 *M* solution of lactose-1-C^{14} (0.36 μc./mg.) were treated with 0.05 ml. of the enzyme solution in 0.1 *M* phosphate buffer of *p*H 6.7. Samples of 5 microliters were analyzed at 0 hr. and after incubation at 30° for 1 hr. by the multiple ascent paper chromatographic method.[10] The radioactivities of the lactose and the glucose in the samples were measured directly on the paper chromatograms as previously described.[11] The rate of lactose disappearance is a measure of both hydrolytic and transferring activity while the rate of glucose production is a measure of the hydrolytic activity only. Under the conditions of the assay procedure, 0.065 mmole of lactose were converted to glucose and galactose and 0.026 mmole of lactose were converted to new oligosaccharides in the 1 hr. period by 1 mg. of enzyme preparation. This amount of activity has been defined as one unit of enzyme activity.

Carbohydrates.—Allolactose was prepared as previously described. The specific rotation of the compound was +25° and on paper chromatography in several solvent systems only one reducing spot was detectable in the preparation. Chromatographically pure *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-D-glucose has been previously prepared in this Laboratory[10] and was used in this study. Glucose-1-C^{14}, galactose-1-C^{14} and lactose-1-C^{14} were purchased from the National Bureau of Standards.

Enzymatic Digest of Allolactose.—To 0.08 ml. of 0.15 *M* allolactose solution were added 0.04 ml. of enzyme solution (total activity, 1.2 units) buffered to *p*H 6.7 with 0.1 *M* phosphate buffer. Samples of 5 microliters were placed on paper chromatograms at 0, 0.5, 1, 2, 4 and 8 hr. and immediately inactivated by heating at 100° for 5 minutes. The compounds in the samples were separated on the paper and located by spraying with copper sulfate and molybdic acid reagents. Glucose and galactose appeared in the digest in 0.5 hr. and increased in concentration up to 8 hr. A new compound with chromatographic mobility of a trisaccharide appeared in 0.5 hr., increased in concentration at 1 and 2 hr., decreased at 4 hr. and disappeared at 8 hr. Allolactose decreased progressively in concentration at 0.5, 1, 2, 4 and 8 hr.

For isolation of the trisaccharide, a digest of 0.8 ml. of allolactose (0.15 *M*) and 0.4 ml. of enzyme (12 units) was prepared and inactivated after reaction time of 1.5 hr. The reducing compounds in the digest were separated chromatographically and isolated as previously described.[10] Partial acid hydrolysis of the trisaccharide yielded allolactose and galactobiose while complete hydrolysis yielded glucose and galactose in molar ratio of 1:2. This information and the chromatographic behavior of the compound indicate that the trisaccharide is probably *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→6)-D-glucose.

Enzymatic Digests of Allolactose and Glucose-1-C^{14}.—To 0.05 ml. of a solution of allolactose (0.3 *M*) and glucose-1-C^{14} (0.3 *M* and with specific activity 1.92 μc./mg.) were added 0.05 ml. of enzyme solution (1.5 units). Samples of 5 microliters were placed on a chromatogram after 0, 1, 2, 4 and 8 hr. reaction time and treated as above. The compounds in these aliquots were located on the chromatogram with copper sulfate and molybdic acid sprays and the radioactive products were detected by radioautography. Radioactivity values for glucose, allolactose and the trisaccharide were obtained with a conventional counting apparatus and are recorded in Table I.

Enzymatic Digest of Galactose and Glucose-1-C^{14}.—To 0.05 ml. of solution of galactose (0.3 *M*) and glucose-1-C^{14} (0.3 *M*) was added 0.05 ml. of enzyme solution (1.5 units). Analyses for reducing and radioactive products were performed on aliquots of the digest after 0, 0.5, 1, 2 and 4 hr. as described above. The results are contained in Table II.

Enzymatic Digest of Trisaccharide and Glucose-1-C^{14}.—To 0.05 ml. of solution of trisaccharide (0.07 *M*) and glucose-1-C^{14} (0.3 *M*) were added to 0.05 ml. of enzyme (1.5 units). Samples of 5 microliters were obtained at 0, 0.5, 1, 2 and 4 hr. and were analyzed for reducing and radioactive products by the methods described above. The radioactivities of the products are recorded in Table III.

Enzymatic Digest of Glucose and Galactose-1-C^{14}.—A digest of 0.1 ml. was prepared containing glucose (0.15 *M*), galactose-1-C^{14} (0.15 *M* and specific activity, 2.24 μc./mg.) and 1.5 units of enzyme. Samples of 5 microliters were analyzed at 0, 0.5, 1, 2 and 4 hr. Results of radioactivity measurements on the products are recorded in Table IV.

LINCOLN, NEBRASKA

(11) J. H. Pazur, THIS JOURNAL, **75**, 6323 (1953).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, WASHINGTON UNIVERSITY]

The Isomerization of D-Glucose-1-C^{14} to D- and L-Sorbose-C^{14} by a Strong Base Resin[1]

BY JOHN C. SOWDEN AND ROBERT R. THOMPSON

RECEIVED NOVEMBER 4, 1957

The D- and L-sorbose-C^{14} formed by the action of a strong base resin on D-glucose-1-C^{14} contain over 90% of the label in carbons 1 and 6. This fact, as well as the relative distributions of the label between C-1 and C-6 of the enantiomorphs, indicates that the racemic ketose is formed by the enolization–ketonization route rather than by fragment recombination.

(DL + D)-Sorbose recently has been established as a product of the action of potassium hydroxide on D-fructose[2] and of a strong base resin on D-glucose.[3] Since the cleavage of hexose to triose[4] and the condensation of DL-glycerose to racemic sorbose and fructose[5] are known to occur in alkaline solution, it seemed likely[2,3] that the D-hexoses formed racemic sorbose in the above two instances by the fragmentation–racemization–recombination route. However, the results reported herein with D-glucose-1-C^{14} indicate that the racemic ketose is formed almost exclusively by the enolization–ketonization mechanism, and that fragment recombination plays only a minor role in the isomerization.

D-Glucose-1-C^{14} was isomerized by the action of Amberlite IRA-400[6] at 50–60°, and the resulting (DL + D)-sorbose was isolated, converted

(1) Abstracted from a thesis submitted by Robert R. Thompson in partial fulfillment of the requirements for the Ph.D. degree, Washington University, January, 1957.

(2) M. L. Wolfrom and J. N. Schumacher, *El Crisol*, **6**, 67 (1952); THIS JOURNAL, **77**, 3318 (1955).

(3) M. Grace Blair and J. C. Sowden, *ibid.*, **77**, 3323 (1955).

(4) *Cf.* W. L. Evans, *Chem. Revs.*, **31**, 537 (1942).

(5) E. Fischer, *Ber.*, **23**, 2114 (1890); E. Schmitz, *ibid.*, **46**, 2327 (1913).

(6) A product of Rohmand Haas Co., Philadelphia, Pa.

to the osotriazole, and the latter resolved into the pure enantiomorphs as described previously.[3] The enantiomorphs and the racemate were then individually degraded by the method of Bishop[7] to obtain fragments containing, respectively, C-1 + C-2, C-1 + C-2 + C-3, C-4 + C-5, and C-6 of the original sorboses. Further degradation of the 2-phenyl-4-formylosotriazole from C-1 + C-2 + C-3, by the method of von Pechmann,[8] gave *sym*-phenylhydrazinoacetic acid from C-2 + C-3. Combinations of the radioassay data for the various fragments then provided radioactivity values for C-1, C-2, C-3, C-4 + C-5, and C-6 of the sorboses. The complete degradation scheme is shown in Fig. 1.

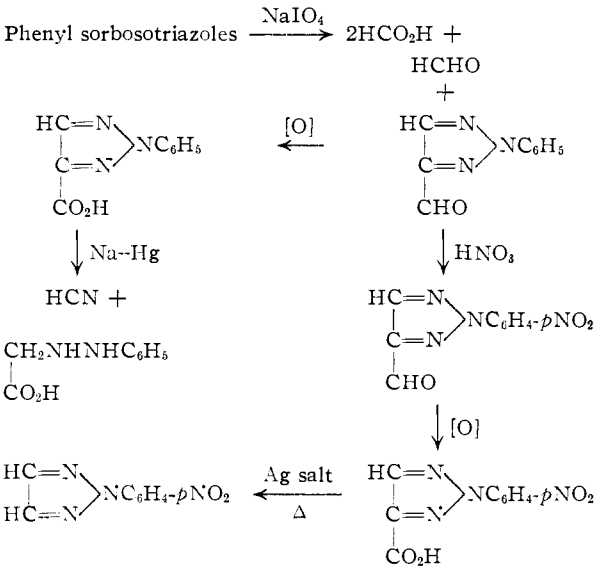

Fig. 1.

The observed pattern of radioactivity in the enantiomorphs, shown in Fig. 2, indicates that the sorboses were formed primarily by an intramolecular isomerization with a resulting distribution of the label between C-1 and C-6. The low degree of labeling at other than the terminal carbon atoms may be taken as a measure of the extent to which fragment recombination is involved.

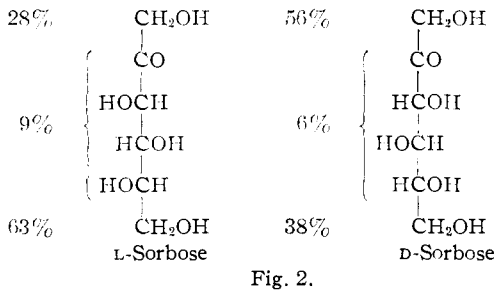

Fig. 2.

The relative degrees of labeling in C-1 and C-6 of the respective ketoses also are in agreement with their formation from hexose 3,4-enediol by the enolization–ketonization route. The 3,4-enediol formed directly from a D-hexose is restricted to one or other of the structures I and II (Fig. 3). Structure I is a *meso* compound when formed from *unlabeled* D-hexose, and hence can give only racemates by further isomerization to sugars. However, when structure I is formed directly from D-hexose-1-C^{14}, asymmetry is maintained by the presence of the labeled carbon atom and the enediol can isomerize further to equal amounts of D-hexose-1-C^{14} and L-hexose-6-C^{14}. Structure II, in which carbons 2 and 5 both have the D-configuration, when formed from D-hexose-1-C^{14} can isomerize further to an equal mixture of D-hexose-1-C^{14} and D-hexose-6-C^{14}. Thus, if it is assumed that 3,4-enediols of both structures I and II are formed in the reaction, the enolization–ketonization mechanism predicts that (1) starting from D-glucose-1-C^{14}, more D- than L-sorbose will be formed, (2) that the L-sorbose will be labeled principally in C-6, and (3) that the D-sorbose will be labeled more heavily in C-1 than in C-6. All of these predictions are in agreement with our experimental results. The appreciable amount of label in C-1 of the L-sorbose presumably comes from secondary isomerizations (for example, of D- or L-sorbose-6-C^{14}) and reflects the fact that the reaction mixture remains dynamic as long as reducing sugars are present.

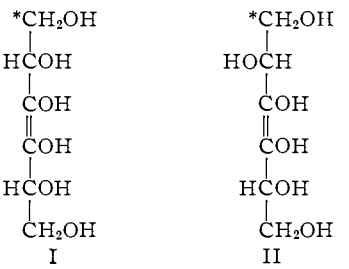

Fig. 3.

Examination of the mother liquors from the crystallization of the (DL + D)-sorbose described above revealed the presence therein of L-fructose, identified through its phenylosotriazole. Thus the experimental evidence that any hexose in alkaline solution gives a mixture containing all possible hexoses is completed.[9]

The present work appears to constitute the first firm experimental evidence that the 3,4-enediol is a significant intermediate in the alkaline isomerization of hexoses and, as such, it has an important bearing on the classical "parasaccharinic acid" problem.[10] Although Nef[11] showed rather conclusively that Kiliani's[12] "parasaccharinic acid" (obtained by the action of lime-water on D-galactose) was in fact "*β*"-D-galactometasaccharinic acid, nevertheless the generally-accepted Nef–Isbell mechanism for saccharinic acid formation[13] predicts that the "parasaccharinic acid" structure could be formed from hexose 3,4-enediol in alkaline solution. However, due to the symmetry properties of the hexose 3,4-enediols discussed above, any "parasaccharinic acid" thus formed would

(7) C. T. Bishop, *Science*, **117**, 715 (1953).
(8) H. von Pechmann, *Ann.*, **262**, 265 (1891).
(9) *Cf.* W. Pigman, "The Carbohydrates," Academic Press, Inc., New York, N. Y., 1957, p. 64.
(10) *Cf.* J. C. Sowden, *Adv. in Carbohydrate Chem.*, **12**, 35 (1957).
(11) J. U. Nef, *Ann.*, **376**, 1 (1910).
(12) H. Kiliani and H. Sanda, *Ber.*, **26**, 1649 (1893).
(13) H. S. Isbell, *J. Research Natl. Bur. Standards*, **32**, 45 (1944).

consist of two racemates each admixed with an excess of one of its component enantiomorphs.

Experimental

Preparation of D-, L- and DL-Sorbose-C^{14} Phenylosotriazoles.—A solution of D-glucose-1-C^{14} (~1.5×10^5 cts./min./mM.) in 600 ml. of water was added to 105 g. of moist Amberlite IRA-400 resin and the mixture was heated at 50° for 24 hours. The resin was removed by filtration, re-suspended in 500 ml. of water, treated with a slow stream of carbon dioxide for 3 hours, and again filtered. The combined filtrate was subjected to two successive fermentations with bakers' yeast, clarified by filtration with Celite,[14] de-ionized, and concentrated to yield 26 g. of unfermentable sugars. Chromatography[3] of the latter on carbon–Celite yielded crystalline (DL + D)-sorbose. This was converted to the phenylosotriazoles and the latter were resolved by fractional crystallization, as described previously,[3] into pure samples of the phenylosotriazoles of D-sorbose (m.p. 157–158°, $[\alpha]^{29}D$ +48° (*c* 0.7 in pyridine)), L-sorbose (m.p. 158–159°, $[\alpha]^{20}D$ −47° (c 0.4 in pyridine)), and DL-sorbose (m.p. 140–141°, $[\alpha]^{29}D$ 0° (*c* 0.7 in pyridine)).

Degradation of the Phenylosotriazoles. (A). Method of Bishop.—Certain minor modifications of Bishop's method[7] were found to give improved yields of the degradation products from the sorbose phenylosotriazoles. The following degradation of D-sorbose phenylosotriazole is typical of the procedures used in the present work.

The osotriazole (30 mg.) was shaken mechanically for 24 hours with a solution containing 1.1 ml. of 0.4 *M* sodium metaperiodate and 3.7 ml. of water. After cooling to 0°, the precipitate was filtered, washed with water and air-dried to yield 18 mg. of 2-phenyl-4-formylosotriazole, m.p. 68–69°.[8,15] The combined filtrate and washings were diluted to 15 ml., and 24 ml. of 0.4% dimedon in water was added. After 12 hours, the precipitate was filtered off and recrystallized from methanol–water to yield 9 mg. of formaldehyde dimedon, m.p. 190–191°.

The 2-phenyl-4-formylosotriazole (18 mg.) was added to 0.05 ml. of *ice-cold* fuming nitric acid. After standing 1 hour at room temperature, the solution was diluted to 1.5 ml. with ice-water. Recrystallization of the resulting precipitate from 0.2 ml. of 50% ethanol yielded 11 mg. of 2-(*p*-nitrophenyl)-4-formylosotriazole, m.p. 135–136°.[7] The latter (10 mg.) was added to a mixture of 0.20 ml. of 5% sodium hydroxide and 0.3 ml. of 3% hydrogen peroxide at 65–70°. After solution was complete (15–20 min.) the mixture was cooled, acidified with 10% sulfuric acid, and filtered. The resulting 2-(*p*-nitrophenyl)-osotriazole-4-carboxylic acid showed m.p. 243–244°.[8] The acid (8 mg.) was suspended in a solution containing 0.8 ml. of 50% ethanol and 0.032 ml. of 2.8% ammonium hydroxide. The mixture was warmed to effect solution and then treated with 0.01 ml. of 4.7 *M* silver nitrate. The resulting precipitate of the silver salt, after filtration and drying at 100° in high vacuum over phosphorus pentoxide, weighed 10 mg. Pyrolysis[7] of the salt yielded 2-(*p*-nitrophenyl)-osotriazole, m.p. (after recrystallization from 0.1 ml. of 70% ethanol) 182–183°.[8]

(B) Method of von Pechmann.—2-Phenylosotriazole-4-carboxylic acid (m.p. 193–194°)[8] was prepared by oxidation of 2-phenyl-4-formylosotriazole as described above for the corresponding nitrated derivatives. The acid (10 mg.) was dissolved in 0.134 ml. of 0.39 *N* sodium hydroxide and treated with 280 mg. of 4% sodium amalgam. After 10 minutes at room temperature, the mixture was heated at approximately constant volume on the steam-bath for 30 minutes, cooled, decanted from mercury, and acidified to ~*p*H 3 with 10% hydrochloric acid. Sodium acetate (10 mg.) then was added and the solution was cooled to 0°. The precipitate (2 mg.), after recrystallization from methanol, gave *sym*-phenylhydrazinoacetic acid of m.p. 159–160° dec.[8]

Anal. Calcd. for $C_8H_{10}N_2O_2$: C, 57.8; H, 6.06. Found: C, 57.6; H, 5.98.

Radioactivity Determinations.—The various fragmentation products from the D-, L- and DL-sorbose-C^{14} phenylosotriazoles were mounted as thin layers (<50 μg./sq. cm.) on stainless steel planchettes, by spreading with appropriate solvents, and radioassayed in an RCL Nucleometer.[16] The recorded values, shown in Table I, are averages of several successive determinations agreeing within ±2%.

TABLE I

RADIOACTIVITY OF THE SORBOSE PHENYLOSOTRIAZOLES AND THEIR DEGRADATION PRODUCTS

Sample	Carbon atoms	Radioactivity, cts./min./mM. × 10^{-3}
D-Sorbose phenylosotriazole	All	154
2-(*p*-Nitrophenyl)-4-formylosotriazole	1–3	90
2-(*p*-Nitrophenyl)-osotriazole	1, 2	84
Phenylhydrazinoacetic acid	2, 3	4
Formaldehyde dimedon	6	58
DL-Sorbose phenylosotriazole	All	154
2-(*p*-Nitrophenyl)-4-formylosotriazole	1–3	64
Formaldehyde dimedon	6	76
L-Sorbose phenylosotriazole	..	..
2-Phenylosotriazole-4-carboxylic acid	1–3	46
Phenylhydrazinoacetic acid	2,3	3
Formaldehyde dimedon	6	97

Identification of L-Fructose.—The mother liquors of the chromatographic fractions that had yielded crystalline (DL + D)-sorbose were combined and concentrated to a sirup. Two grams of the latter was subjected to two successive oxidations with bromine in the presence of barium benzoate,[17] followed each time by removal of the resulting aldonic acids with Duolite A-4. After the first oxidation with bromine, the residual ketose fraction weighed 1.7 g. and, after the second oxidation, 1.6 g. Treatment of the latter with phenylhydrazine and acetic acid in the usual manner yielded 2.5 g. of phenylosazones, m.p. 105–115°. Conversion to the phenylosotriazoles[15] and crystallization from water then yielded 60 mg. of crude phenylosotriazole, m.p. 165–175°. Recrystallization from water gave pure L-fructose phenylosotriazole,[15] m.p. 194–195° and $[\alpha]^{22}D$ +80.4° (*c* 0.5 in pyridine). For confirmation, the tetraacetate[15] was prepared, m.p. 79–80° and $[\alpha]^{21}D$ +24° (*c* 0.4 in chloroform).

Acknowledgment.—The authors are pleased to acknowledge the generous support of the Corn Industries Research Foundation, Washington, D. C., during the course of this work.

ST. LOUIS 5, MO.

(14) A product of Johns-Manville Co., New York, N. Y.

(15) R. M. Hann and C. S. Hudson, THIS JOURNAL, **66**, 735 (1944).

(16) A product of Radiation Counter Laboratories, Chicago, Ill.

(17) C. S. Hudson and H. S. Isbell, THIS JOURNAL, **51**, 2225 (1929).