acetic acid after refluxing one-half hour also gave the unchanged substance. The acid (1.0 g.) was now heated in a sealed tube with 7.0 cc. of hydrochloric acid (1.19) at 160° for two hours. Some pressure developed and hydrogen sulfide was identified among the gases evolved. No definite crystalline product was isolated from this reaction mixture.

5-Alkyl-5-(β-isothiouroniumbromidoethyl)-barbituric Acids.—In a typical experiment 15 g. (0.049 mole) of 5-isoamyl-5-(β-bromoethyl)-barbituric acid and 3.8 g. (0.050 mole) of thiourea were periodically swirled with 50 cc. of alcohol as it heated to the temperature of complete solution (80°). In five minutes at this temperature the rapid precipitation of the product began. When this had subsided the temperature of the bath was raised to 90° and the mixture was heated for five hours. After cooling in ice the solid product, obtained by filtration and concentration of the filtrate, weighed 17.8 g. (95%). It is very soluble in hot water but quite insoluble in cold water and was purified by crystallization from water.

5-Alkyl-5-(β-isothioureidoethyl)-barbituric Acids.—Five grams (0.013 mole) of 5-isoamyl-5-(β-isothiouroniumbromidoethyl)-barbituric acid was dissolved by warming in 65 cc. of water. The solution was filtered immediately and 1 cc. (0.015 mole) of aqua ammonia (0.90) was added gradually to the filtrate with stirring while it was cooled in an ice-bath. The white crystalline product obtained by filtration required no further purification, yield 4.0 g. (80%).

Mercapto Acid from the Isothiouronium Compound.—One gram (0.0026 mole) of 5-*n*-amyl-5-(β-isothiouroniumbromidoethyl)-barbituric acid was dissolved in the minimum amount (5 cc.) of ice-cold 10% sodium hydroxide. The solution was allowed to stand for an hour in an ice-bath. The 5-*n*-amyl-5-(β-mercaptoethyl)-barbituric acid was precipitated by hydrochloric acid, yield 0.6 g. (95%), m.p. 132–133.5°. It was identical with the mercapto acid prepared from the corresponding xanthate.

NEWARK, DELAWARE RECEIVED AUGUST 13, 1951

[CONTRIBUTION FROM THE RADIOCHEMISTRY LABORATORY, DEPARTMENT OF CHEMISTRY, WASHINGTON UNIVERSITY]

The Reaction of D-Glucose, D-Mannose and D-Fructose in 0.035 *N* Sodium Hydroxide at 35°

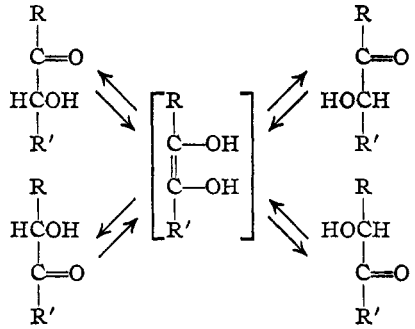
BY JOHN C. SOWDEN AND ROBERT SCHAFFER[1]

The reactions of individual molar solutions of D-glucose, D-mannose and D-fructose in 0.035 *N* sodium hydroxide at 35° have been studied using radioisotopic dilution analysis for D-glucose and D-fructose in the reaction mixtures and a corrected phenylhydrazone precipitation procedure for D-mannose. After reaction times of four to eight weeks, summation of the analyses for these three isomeric sugars accounted in each instance for only 77–80% of the starting carbohydrate. The remainder was shown, by fermentation experiments, to have been converted nearly quantitatively to a mixture of non-fermentable sugar products. Some exploratory experiments were performed concerning the nature of these non-fermentable substances.

The complex reaction sequence promoted by the action of aqueous alkali on reducing sugars includes isomerization, fragmentation and fragment recombination. Two kinds of isomeric products form: carbohydrates[2] and saccharinic acids.[3,4,5] The products of fragmentation are themselves at the oxidation level of carbohydrate and they also may isomerize[6] or recombine to larger molecules.[7] In addition, there are formed colored products of high molecular weight and undetermined structure.

Lobry de Bruyn and Alberda van Ekenstein demonstrated the interconvertibility of D-glucose, D-mannose and D-fructose in aqueous alkali. They also isolated fractions from the reaction which they considered to be, respectively, the 3-epimer of D-fructose ("pseudo-fructose," "psicose," D-ribohexulose) and a mixture of 3-ketohexoses. However, the former material does not conform in its properties to synthetic D-ribohexulose[8,9] and their "3-ketohexose" mixture ("glutose") is now considered to be a complex mixture containing fructosans.[10] Conclusive evidence for the presence of material epimeric with D-glucose at carbon-3 has been obtained recently, however, by characterization of the products obtained by electroreduction of the mixture that results from the interaction of D-glucose and aqueous alkali.[11]

The generally accepted mechanism of the Lobry de Bruyn–Alberda van Ekenstein isomerization reaction postulates enediol intermediates, as illustrated in the scheme



(R = H, CH_2OH, etc.; R′ = H, CH_2OH, etc.)

Observations, based on the measurement of deuterium exchange when the reaction was conducted in heavy water, apparently in conflict with the enediol mechanism have been recorded.[12,13] These objections to the mechanism, however, have not been sustained in more recent studies with heavy

(1) Abstracted from the thesis of Robert Schaffer presented in partial fulfillment for the degree Doctor of Philosophy, Washington University, October, 1950.

(2) C. A. Lobry de Bruyn and W. Alberda van Ekenstein, *Rec. trav. chim.*, **14**, 203 (1895); **15**, 92 (1896); **16**, 257, 262, 274, 282 (1897); **18**, 147 (1899); **19**, 5 (1900).

(3) E. Peligot, *Ann. chim. et phys.*, [2] **67**, 154 (1839).

(4) C. Schiebler, *Ber.*, **13**, 2212 (1880).

(5) H. Kiliani, *ibid.*, **15**, 701, 2953 (1882).

(6) J. U. Nef, *Ann.*, **357**, 294 (1907); **376**, 1 (1910); **403**, 204 (1913).

(7) E. Fischer and J. Tafel, *Ber.*, **20**, 2566 (1887).

(8) Marguerite Steiger and T. Reichstein, *Helv. Chim. Acta*, **19**, 187 (1936).

(9) M. L. Wolfrom and E. F. Evans, THIS JOURNAL, **67**, 1793 (1945).

(10) L. Sattler and F. W. Zerban, *Ind. Eng. Chem.*, **37**, 1133 (1945).

(11) M. L. Wolfrom and co-workers, THIS JOURNAL, **68**, 122, 578, 1443, 2342 (1946).

(12) H. Fredenhagen and K. F. Bonhoeffer, *Z. physik. Chem.*, **A181**, 392 (1938).

(13) K. Goto, *J. Chem. Soc. Japan*, **63**, 217 (1942).

water.[14,15] It also has been reported[16] that the mechanism of isomerization shows cationic dependence in that different types of enolic intermediates are involved when alkalies containing, respectively, mono- or divalent cations are employed. In the present work we have confirmed qualitatively Kusin's observation that the initial course of the reaction is different in mono- and divalent bases of 0.5 *N* concentration. However, at lower concentrations (0.035 *N*) of alkali, we could detect no difference in the catalysis of the isomerization by calcium hydroxide or sodium hydroxide.

Previous quantitative studies of the course of the isomerization reaction have been handicapped by a lack of specific analytical methods for individual sugars other than D-mannose in complex mixtures. Analyses for D-glucose and D-fructose in the isomerization mixture previously were based on methods generally applicable to the aldose and ketose classes of sugars.[17] With the advent of isotopic labeling, it has been possible to apply the method of radioisotopic dilution analysis to the course of the isomerization reaction. In the present work, the isomerization of molar solutions of D-glucose, D-mannose and D-fructose in 0.035 *N* sodium hydroxide at 35° has been studied. The growth or disappearance of D-glucose and D-fructose was followed by radioisotopic dilution analysis while variations in the D-mannose content were measured by a corrected phenylhydrazone precipitation procedure. In this manner, by summation of the progressive concentrations of D-glucose, D-mannose and D-fructose, there was detected in the reaction mixtures the rapid growth of a sugar fraction containing none of these three hexoses. Fermentation experiments then showed that this fraction consisted nearly quantitatively of non-fermentable, non-acidic sugar material. Similar non-fermentable substances have been obtained previously[2] as products of considerably more drastic treatment with alkali. Preliminary experiments with this non-fermentable fraction have shown it to be a complex mixture, and a detailed study of its nature is contemplated.

A quantitative study of the isomerization of D-fructose by aqueous alkali apparently has not been recorded previously. Its inclusion here seems especially appropriate since, according to the enediol mechanism, it is the precursor of the 2,3-enediol which in turn may give rise to non-fermentable aldo- and ketohexoses.

Experimental

Materials. D-Glucose, D-Mannose and D-Fructose.—Commercial preparations of the sugars were recrystallized until they possessed physical properties in accord with the accepted values.

1-C^{14}-D-Glucose.—The labeled sugar (*ca.* 0.05 μc./mg.) was prepared from D-arabinose *via* the nitromethane synthesis with C^{14}-nitromethane.[18]

C^{14}-D-Fructose.—Uniformly labeled C^{14}-D-fructose (*ca.* 1.2 μc./mg.), prepared photosynthetically from $C^{14}O_2$ and purified by paper chromatography, was kindly supplied by Drs. M. Gibbs and S. Udenfriend.[19]

Amberlite I.R.-100-A.G.—This product of Rohm and Haas Co., Philadelphia, Pa., was put through several sodium hydroxide–hydrochloric acid cycles and then washed free of chloride ions prior to use.

Duolite A-4.—This product of Chemical Process Co., Redwood City, Cal., was put through several hydrochloric acid–sodium hydroxide cycles and then washed thoroughly. Before use, the resin was treated with several volumes of 5% sodium chloride solution and then washed free of chloride ions.

The Isomerization Reaction.—Samples (180.00 g.) of each of the three sugars were dissolved in freshly distilled water in tared 1-l. volumetric flasks. Sufficient 1 *N* sodium hydroxide solution (35.0 ml.) was added to each to give a final concentration of 0.035 *N* and the flasks were filled to the mark with water. After weighing, the resulting solutions were transferred, together with 0.1 ml. of xylene, to 2-l. round-bottomed flasks, previously blackened to avoid photochemical effects[20] and designed to deliver samples by application of nitrogen pressure. The flasks were thermostated at 35° and their air content was replaced with nitrogen by repeated alternate evacuation (water-aspirator) and refilling with washed nitrogen. Periodically thereafter, samples of the reaction mixtures were removed under nitrogen pressure and aliquots of each sample were used for determinations of D-glucose, D-mannose and D-fructose, acid–base titration, *p*H determination and measurement of optical activity. Amounts of solution below 5 ml. were weighed; the remainder pipetted.

Standardization of Radioactive Sugar Solutions. 1-C^{14}-D-Glucose.—To a series of weights (15–80 mg.) of D-glucose were added 0.050-ml. aliquots of an aqueous stock solution of 1-C^{14}-D-glucose containing about 2.5 mg./ml. of radioactive sugar. After solution in water and mixing, the resulting diluted 1-C^{14}-D-glucose samples were crystallized, washed and dried as described below. Accurately weighed amounts (*ca.* 2.5 mg.) of each sugar sample were dissolved in water on individual stainless steel dishes (12.6 sq. cm. in area) and 2 drops of a 0.1% solution of the detergent "Santomerse" (a product of Monsanto Chemical Co., St. Louis, Mo.) added to each. By manipulation under an infrared lamp, the solutions were evaporated to produce evenly distributed, thin layers of the sugar. The specific radioactivities then were determined by timing 10,000 counts from each sample in an R. C. L. nucleometer (a product of Radiation Counter Laboratories, Chicago, Ill.). A single check determination was made similarly with known D-glucose at the conclusion of the analyses of the isomerization mixtures to demonstrate the stability of the stock 1-C^{14}-D-glucose solution. The results of the standardization are shown in Table I.

TABLE I

STANDARDIZATION OF STOCK 1-C^{14}-D-GLUCOSE SOLUTION

Sample no.	D-Glucose, mg.	Specific radioactivity, cts./min./mg.	Total radioactivity, cts./min.
1	23.27	391.1	9100
2	29.96	308.1	9230
3	40.20	230.6	9270
4	51.06	179.6	9170
5	64.64	142.0	9180
6	78.26	115.5	9040
Check	44.12	210.2	9270
		Average	9180 ± 70

It is apparent from Table I that the total radioactivity found for each sample was essentially constant. Thus, the principle of isotopic dilution analysis is applicable and quantitative analysis for the amount of the sugar in an unknown mixture to which the tracer has been added is given by the quotient of the appropriate constant with the observed specific radioactivity of a sample of the sugar isolated from the mixture.

(14) Y. J. Topper and DeWitt Stetten, Jr., *J. Biol. Chem.*, **189**, 191 (1951).

(15) J. C. Sowden and R. Schaffer, THIS JOURNAL, **74**, 505 (1952).

(16) A. Kusin, *Ber.*, **69**, 1041 (1936).

(17) M. L. Wolfrom and W. L. Lewis, THIS JOURNAL, **50**, 837 (1928); R. D. Greene and W. L. Lewis, *ibid.*, **50**, 2813 (1928).

(18) J. C. Sowden, *J. Biol. Chem.*, **180**, 55 (1949).

(19) S. Udenfriend and M. Gibbs, *Science*, **110**, 708 (1949).

(20) A. L. Bernouli and R. Cantieni, *Helv. Chim. Acta*, **15**, 119 (1932).

C^{14}-D-Fructose.—A similar standardization to that described above for 1-C^{14}-D-glucose was made for a stock solution of C^{14}-D-fructose (*ca.* 0.033 mg./ml.) with known D-fructose. The results are shown in Table II.

TABLE II

STANDARDIZATION OF STOCK C^{14}-D-FRUCTOSE SOLUTION

Sample no.	D-Fructose, mg.	Specific radioactivity, cts./min./mg.	Total radioactivity, cts./min.
1	15.44	409.5	6320
2	23.19	277.5	6440
3	41.92	153.8	6450
4	50.25	126.9	6380
5	64.86	100.1	6490
6	79.86	79.6	6360
Check	44.12	146.4	6460
		Average	6410 ± 50

Isolation of D-Glucose for Isotopic Dilution Analysis. From the D-Glucose Isomerization Reaction.—To each aliquot of the reaction mixture, estimated to contain 100–400 mg. of D-glucose, was added 0.250 ml. of the stock radioactive D-glucose solution. The solution was passed, with washing, through Amberlite I.R.-100-H (0.5 g./ml. of reaction mixture) and the effluent was evaporated at reduced pressure. The resulting sirup was dissolved in 10 ml. of 95% ethanol and, after standing overnight, the solution was filtered, concentrated to 3–4 ml., and seeded with a few minute crystals of D-glucose. After crystallization was complete, the supernatant liquid was decanted and the residual D-glucose was washed, ground and rewashed with 95% ethanol. Recrystallization was performed from 3–4 ml. of 95% ethanol. Before assay, the sugar was dried at 105° in high vacuum over phosphorus pentoxide.

From the D-Fructose and D-Mannose Isomerization Reactions.—Each alkaline reaction aliquot, estimated to contain 100–400 mg. of D-glucose, was treated with 0.250 ml. of the stock radioactive D-glucose solution and then neutralized with acetic acid. D-Mannose phenylhydrazone was precipitated as described below and filtered off. The filtrate, after addition of 0.5 ml. of benzaldehyde and 0.5 g. of benzoic acid, was refluxed for 30 minutes, cooled, extracted three times with chloroform, and concentrated at reduced pressure to a volume of 4–5 ml. Two grams of sodium bisulfite was dissolved in the concentrate and 20 ml. of absolute ethanol then added gradually. After several hours, the precipitated bisulfite addition compound[21] was centrifuged off, dissolved in 25 ml. of water, and the solution de-ionized by successive passage through 30 ml. of Amberlite I.R.-100-H and 40 ml. of Duolite A-4. A total of 100 ml. of effluent was collected. If the optical rotation of the effluent was negative, bisulfite precipitation and de-ionization were repeated. D-Glucose then was isolated and purified for assay as described above for the D-glucose isomerization reaction.

Isolation of D-Fructose for Isotopic Dilution Analysis.—To each aliquot of reaction mixture, estimated to contain 100–400 mg. of D-fructose, was added 0.250 ml. of the stock radioactive D-fructose solution. For each 10 ml. of reaction mixture, there then was added 65 ml. of water, 6 g. of barium benzoate, 1 g. of benzoic acid and 0.6 ml. of bromine.[22] Oxidation was allowed to proceed at room temperature in the dark for a minimum of 40 hours. Following removal of barium by precipitation with sulfuric acid and benzoic acid by extraction with chloroform, the solution then was passed through 60 ml. of Duolite A-4. The residual sugar acids and lactones in the effluent were titrated at 0° with 1 *N* sodium hydroxide to the phenolphthalein end-point, acidity was just restored with 1 *N* hydrochloric acid, and the solution then was concentrated at reduced pressure to a volume of 25 ml. Following de-ionization with Amberlite I.R.-100-H and Duolite A-4, the titration with sodium hydroxide at 0° was repeated, the solution was again made just acid with hydrochloric acid and the concentration continued to a volume of 5 ml. Alkalinity was just restored with sodium hydroxide at 0° and the solution immediately de-ionized again by ion exchange. After observation of the optical rotation, the solution was concentrated at reduced pressure to a sirup. The latter was dissolved in absolute ethanol (1 ml./100 mg. of D-fructose indicated by optical rotation), the solution allowed to stand for two days, filtered if necessary, and then seeded with a minute amount of D-fructose. The crystallized sugar was washed by decantation with absolute ethanol, recrystallized from the same solvent, and dried for assay at 65° in high vacuum over phosphorus pentoxide.

(21) P. A. Ashmarin and A. D. Braun, *Byull. Eksptl. Biol. Med.*, **4**, 374 (1937).

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

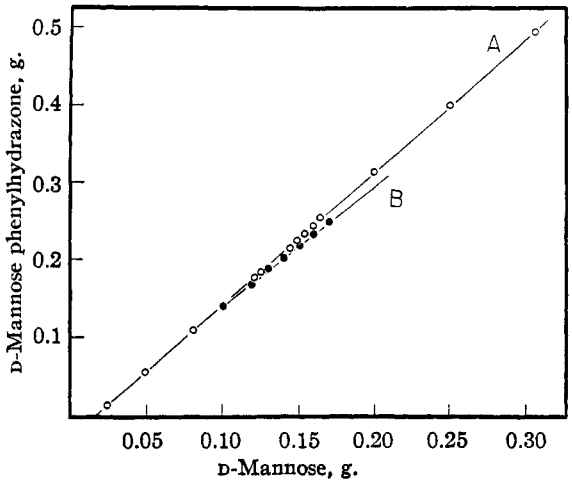


Fig. 1.—Yields of D-mannose phenylhydrazone from D-mannose in the presence of D-glucose and D-fructose, total sugar, 1.8 g. in 25 ml. of solution: A, D-mannose and D-glucose; B, D-mannose and D-fructose.

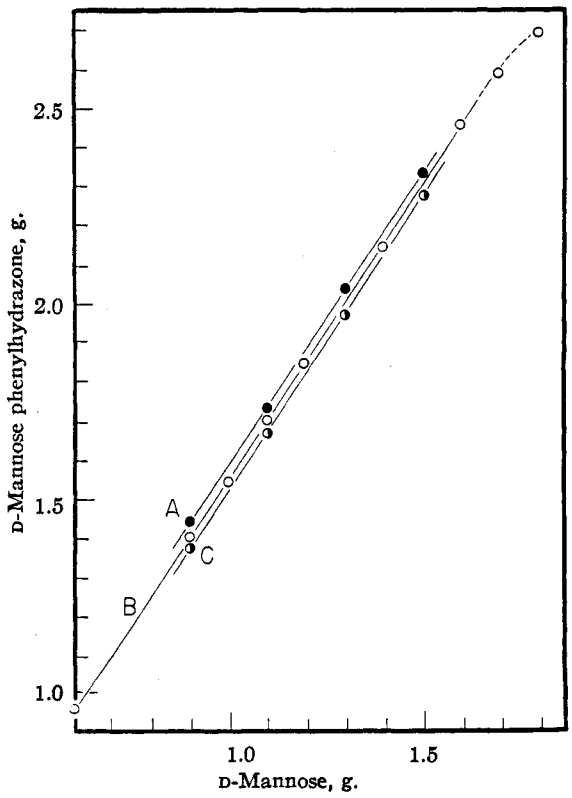


Fig. 2.—Yields of D-mannose phenylhydrazone from D-mannose in the presence of D-glucose and D-fructose, total sugar, 1.8 g. in 25 ml. of solution: A, D-mannose and D-glucose; B, D-mannose and equal amounts of D-glucose and D-fructose; C, D-mannose and D-fructose.

TABLE III

MOLAR D-GLUCOSE AND 0.035 *N* NaOH AT 35°

Time, hr.	D-Glucose, %	D-Glucose, $[\alpha]^{30}D$	D-Fructose, %	D-Fructose, $[\alpha]^{20}D$	D-Mannose, %	Sum: D-Glucose, D-fructose, D-mannose, %	$[\alpha]^{35}D$	*p*H	Acid formed, equiv.
4	95.0	...	..	.....	0.2	..	.....	...	....
11	88.8	52.7°[b]	8.5	−92.1°	.3	97.6	40.2°	10.46	0.0010
20	82.3	...	13.0	.....	.4	95.7	31.8	10.46	.0018
			13.7						
50	68.8	...	20.1	.....	1.4	90.3	17.1	10.34	.0070
102	57.2[a]	...	28.4	−91.8	2.6		8.25	10.12	.0170
	57.0					88.2			
188	51.7	52.0	29.0	.....	3.8	84.5	4.61	9.66	.0281
477	46.3		28.9	.....	4.2		Opaque	8.40	.0350
	46.6	52.1	28.1			78.9			
646	45.4	...	27.7	.....	4.3	77.2	.....	7.50	.0360
1484	45.3	...	27.5	.....	3.9	76.5	.....	6.00	.0370

Sample calculations

Total sugar in sample (based on amount of reaction mixture taken), g.	0.187	Total sugar in sample (based on amount of reaction mixture taken), g.	1.80
Specific radioactivity of D-glucose, cts./min./mg.	297.5	Specific radioactivity of D-fructose, cts./min./mg.	137.1
D-Glucose, g. $= \frac{5 \times 9180 \times 1000}{297.5}$	0.154	D-Fructose, g. $= \frac{5 \times 6410 \times 1000}{137.1}$	0.234
D-Glucose, %	82.3	D-Fructose, %	13.0

[a] Where duplicate values are given, the second value was observed after recrystallization and re-assay of the sugar. [b] Specific optical rotation of the radio-assayed sample.

TABLE IV

MOLAR D-FRUCTOSE AND 0.035 *N* NaOH AT 35°

Time, hr.	D-Fructose, %	D-Fructose, $[\alpha]^{22}D$	D-Glucose, %	D-Glucose, $[\alpha]^{20}D$	D-Mannose, %	Sum: D-Fructose, D-glucose, D-mannose, %	$[\alpha]^{35}D$	*p*H	Acid formed, equiv.
4.5	...	.....	..	...	0.7	..	−79.2°	10.47	0.0022
20	80.8[a]		11.9		1.8		−59.8	10.36	.0086
	80.8	−92.2°[b]	11.3	51.4°		93.9			
50	...	.....	..	...	3.4	..	−41.1	10.10	.0188
72	57.0	.....	23.4	...	4.1	84.5	−33.3	9.93	.0237
			23.4						
100	52.0	.....	..	...	4.6	..	−29.0	9.60	.0278
140	50.2	.....	30.0		4.9		−25.4	9.35	.0307
	49.9		28.5	51.2		83.3			
260	44.7	.....	29.5	...	5.3		Opaque	8.70	.0345
			29.4			79.4			
477	43.0	.....	28.4		5.3		.....	7.50	.0370
	42.8		28.0	50.9		76.1			
1437	43.3	.....	29.8		5.1		.....	5.85	.0374
			28.9	48.8		77.2			

[a] Where duplicate values are given, the second value was observed after recrystallization and re-assay of the sugar. [b] Specific optical rotation of the radio-assayed sample.

Determination of D-Mannose as the Phenylhydrazone.—The determination of D-mannose as the phenylhydrazone[23] is not applicable to low concentrations of the sugar because of a slight solubility of the derivative. A study of the yields of phenylhydrazone obtained, under specified conditions, from known mixtures of D-mannose with D-glucose and D-fructose (Figs. 1 and 2) showed that amounts above 100 mg. of D-mannose were readily determined. Accordingly, smaller amounts were made measurable by the addition of 100 mg. of D-mannose to the samples before hydrazone precipitation and the appropriate correction was made to the value for D-mannose thus obtained. Curve A, Fig. 1, was used for determinations of D-mannose in the D-glucose isomerization mixture and curve B, Fig. 1, in the D-fructose isomerization mixture. Curve B, Fig. 2, was employed for the D-mannose isomerization mixture.

To each 10-ml. aliquot of reaction mixture was added, at 0°, 2 drops of glacial acetic acid, 10 ml. of water and, to samples of the D-glucose and D-fructose isomerization mixtures, 100 mg. of D-mannose. No additional sugar was added to aliquots of the D-mannose isomerization mixture. A 5-ml. portion of a solution, prepared by combination of 1.2 g. of freshly distilled phenylhydrazine, 6 drops of glacial acetic acid and 4.2 g. of water, then was added. After standing overnight at 0°, the hydrazone was filtered onto a tared, porous-bottomed crucible and washed with 10 ml. of ice-water, 10 ml. of absolute ethanol at 0° and 10 ml. of ether. Drying was performed at 110° for two hours. The observed weight of D-mannose phenylhydrazone then was converted to weight of D-mannose by reference to Figs. 1 and 2.

Determination of Total Acidic Products.—Periodically, 10-ml. aliquots of the reaction mixtures were titrated to the phenolphthalein end-point with 0.1 *N* hydrochloric acid. In the later stages of the reaction, when the reaction mixtures were acidic to the indicator, titration was performed with 0.1 *N* sodium hydroxide. The *p*H of the reactions also was followed by use of a Beckman *p*H meter.

(23) E. Bourquelot and H. Herissey, *Compt. rend.*, **129**, 339 (1899).

TABLE V

MOLAR D-MANNOSE AND 0.035 *N* NaOH AT 35°

Time, hr.	D-Mannose, %	D-Fructose, %	D-Fructose, $[\alpha]^{20}D$	D-Glucose, %	D-Glucose, $[\alpha]^{20}D$	Sum: D-Mannose, D-fructose, D-glucose, %	$[\alpha]^{25}D$	*p*H	Acid formed, equiv.
5	95.8	...	.....	..	...	..	12.1°	10.36	0.0007
19	92.1	5.9[a]		..	...	..	8.22	10.35	.0016
		5.7	−91.8°[b]						
52	82.2	12.1	.....	..	...	..	2.67	10.32	.0035
73	73.7	14.2	.....	6.4	...		1.03	10.23	.0057
		14.8		5.9	48.0°	94.4			
142	64.7	18.5	.....	10.1	...		−1.36	10.15	.0169
				9.9	49.1	93.1			
260	55.3	20.1	.....	12.7			−2.20	9.76	.0263
				11.6	50.5	87.0			
382	52.3	20.5	−91.1	..	...	..	−2.25	9.32	.0323
549	51.0	19.3	.....	11.1			Opaque	8.45	.0350
		19.7		10.5	50.0	81.2			
765	51.0	...	.....	..	...	..	.....	6.90	....

[a] Where duplicate values are given, the second value was observed after recrystallization and re-assay of the sugar. [b] Specific optical rotation of the radio-assayed sample.

Tabulation of Results.—The specific radioactivities of the isolated D-glucose and D-fructose samples were determined as described above for the standardization of the stock radioactive sugar solutions. Representative data for the individual isomerization reactions are shown in Tables III, IV and V.

Isolation and Partial Characterization of Acidic Products.—A 100-ml. sample taken 43 days after initiation of the D-glucose reaction, containing 3.6 meq. of acid by titration, was passed successively through 10 ml. of Amberlite I.R.-100-H and 15 ml. of Duolite A-4. The resulting effluent was colorless. After washing free of reducing substances, the Duolite A-4 column was connected to deliver into a column containing 50 ml. of Amberlite I.R.-100-H, and 15 ml. of 1 *N* sodium hydroxide was washed through. Effluent from the Duolite A-4 column showed the original dark color of the reaction mixture while the effluent from the Amberlite I.R.-100 was yellow.

Volatile acid, amounting to 1.46 meq. by titration, was distilled by concentration of the effluent at reduced pressure with repeated additions of water. Following titration with sodium hydroxide, the distillate was concentrated to dryness. The equivalent weight of the residual salt, determined by its conversion to sodium sulfate, coincided with that of sodium acetate. The presence also of traces of formic acid in the volatile acid fraction was indicated by the incipient ability of the latter to decolorize dilute acidified potassium permanganate solution. (In preliminary experiments, when air was not rigidly excluded from the alkaline isomerization reaction mixtures, the equivalent weight of the volatile acid produced was intermediate between that of formic and acetic acids.)

The volume of the non-volatile acid solution was reduced to 0.5 ml. and lactic acid then was separated by continuous ether extraction followed by steam distillation of the ether extract.[24] Titration indicated 0.40 meq. of lactic acid.

Titration, with sodium hydroxide, of the acids not extracted by ether produced considerable color in the solution. Accordingly, the titration was followed with a *p*H-meter. About one-half of these acids, 0.65 meq., was rapidly neutralized to *p*H 7. The remaining acids, 0.7 meq., required stepwise additions of alkali over a long period of time before a constant *p*H in the alkaline range was attained.

Isolation and Partial Characterization of Non-acid, Non-fermentable Products.—Samples of the reaction mixtures were de-ionized by ion-exchange and then slurried with fresh baker's yeast equal in weight to the initial amounts of sugar in each sample. Evolution of carbon dioxide was complete after seven hours and the yeast was filtered off after 20 hours. The filtrates were titrated to the phenolphthalein end-point with dilute alkali and then de-ionized by ion-exchange. Following evaporation at reduced pressure, finally with addition of absolute ethanol, the residual sirups were dried at room temperature in high vacuum over phosphorus pentoxide. The resulting material, on solution in water and addition of fresh yeast, did not ferment further.

Attempted phenylosazone formation gave no precipitate.

Aldose sugars were removed from aliquots of the non-fermentable sirups by bromine oxidation followed by ion-exchange as described above. The resulting unoxidized, non-fermentable material gave a positive Fehling test without heating. When treated with acetone, anhydrous cupric sulfate and sulfuric acid according to the directions of Steiger and Reichstein,[8] this material failed to yield 1,2;3,4-diisopropylidene-D-ribohexulose although indications of its production in small amount were obtained by fractionation and polarimetric observations.

Table VI lists observations of fermentation and oxidation reaction yields.

TABLE VI

NON-ACIDIC, NON-FERMENTABLE PRODUCTS FROM D-GLUCOSE AND D-FRUCTOSE

Sugar	Reaction time, days	Vol. of soln. fermented, ml.	Product, g.	$[\alpha]D$ in water	Product unoxidized by bromine, g.	$[\alpha]D$ in water
D-Glucose	43	100	3.90	7.7°	2.52	8.1°
D-Fructose	60	36	1.48	8.5	1.05	7.0

The summation of the above respective amounts of non-acidic, non-fermentable materials and the corresponding analytically observed amounts of D-glucose, D-fructose and D-mannose accounts for 100 ± 1% of the initial amounts of the sugars employed in the isomerization reactions.

Cationic Dependence of the Isomerization in 0.5 *N* Alkali.—Kusin[16] reported a difference in the initial course of the isomerizations of D-glucose and D-fructose, respectively, in 0.5 *N* sodium and calcium hydroxides. A similar effect is demonstrated readily with D-mannose by observation of its mutarotation and changes in its concentration in the two alkaline systems. The early mutarotations in sodium and calcium hydroxides, recorded in Table VII, are in different *directions*. The effect of barium hydroxide appears to be intermediate between those of sodium and calcium hydroxides. Experiments conducted at lower alkali concentrations (*e.g.*, D-glucose with 0.035 *N* sodium and calcium hydroxides at 35°) showed no detectable difference in the course of the isomerization.

Discussion

Accuracy of Analytical Results—Standardization of the stock radioactive sugar solutions by isotopic dilution with pure sugars gave results

(24) W. L. Evans and H. B. Hass, THIS JOURNAL, 48, 2703 (1926).

TABLE VII

REACTION OF 0.555 MOLAR D-MANNOSE IN 0.5 *N* ALKALIES AT 25°

Sodium hydroxide			Calcium hydroxide			Barium hydroxide		
Time, hr.	$[\alpha]^{25}D$	D-Mannose, %	Time, hr.	$[\alpha]^{25}D$	D-Mannose, %	Time, hr.	$[\alpha]^{25}D$	D-Mannose, %
1.0	14.5°	..	1.2	−6.2°	..	1.0	5.8°	..
2.0	13.7	..	2.0	−4.3	..	2.0	4.75	..
2.7	12.5	..	3.3	−1.85	..	3.0	3.9	..
4.0	..	89.1	4.5	2.4	72.0	4.0	3.05	..
5.3	11.6	..	6.5	6.45	..	4.5	2.55	..
9.0	9.6	..	9.0	7.95	..	5.5	1.8	..
						22.5	−1.85	..
11.0	6.8	..	11.0	8.45	..	26.5	−2.3	..
24.0	1.95	71.9	24.0	4.95	20.9	27.0	....	60.0
Initial *p*H: 12.45 Acid formed, 24 hr.: 0.025 meq./ml.			Initial *p*H: 11.85 Acid formed, 24 hr.: 0.125 meq./ml.			Initial *p*H: 12.35 Acid formed, 24 hr.: 0.070 meq./ml.		

with average deviations of less than 1%. Isotopic dilution analyses of samples of the reaction mixtures, however, were subject to greater error due to impurity—an error that leads to reduced counting rates and, therefore, increased calculated concentrations. Recrystallizations of sugar samples obtained from the reaction mixtures and redeterminations of their radioactivities indicated unchanged specific radioactivities for all D-fructose samples and for D-glucose samples from the D-glucose and alkali reaction. The optical rotations of these products were in good agreement with accepted values. D-Glucose samples from the other two reaction mixtures could not be brought to constant specific radioactivities even after three crystallizations and the observed specific optical rotations were low by as much as 8% in these instances. Accordingly, these latter values for D-glucose are the least accurate of the analyses reported.

Determinations of D-mannose as the phenylhydrazone are affected by the nature and amounts of other materials present, as shown in Figs. 1 and 2. The substances other than D-glucose and D-fructose formed in the reactions may add to the variance in yields of D-mannose phenylhydrazone observed with the known sugar mixtures. The melting points of the D-mannose phenylhydrazone analytical samples from the reaction mixtures were consistently in the range 193–196°, which is quite satisfactory for this derivative on initial precipitation.

The Course of the Reaction.—The rigorous exclusion of conditions favoring side reactions, *e.g.*, air oxidation and photochemical decomposition which lead to acidic products, results in increased duration and extent of the isomerization reaction.

The comparative rate of reaction as well as the preferred order of formation of the three sugars is: D-fructose > D-glucose > D-mannose. Thus, the reactivity of D-fructose in alkaline solution is much greater than observations of its rate of disappearance from its reaction mixture would indicate.

The initial rate of formation of acidic products is greatest in the D-fructose reaction and decreases regularly in this system while, in the D-glucose and D-mannose systems, the rates at first increase and then decrease. D-Fructose, therefore, appears to act as precursor for initial acid formation. From analysis of the acids formed, the major source of this acid, which eventually stops the isomerization reaction by neutralization of alkali, is the isomerization of fragmentation products. In this connection, it may be noted that acetic and lactic acids are, respectively, the saccharinic acids related to glycolaldehyde and glyceraldehyde.

The amounts of non-acidic, non-fermentable products isolated correspond to the amounts of sugar unaccounted for by direct analyses. Again, the D-fructose system provides the most rapid growth as well as the greatest amounts of these products. The nature of this material remains unknown. Conclusive evidence for the production of sugars epimeric with the reagent carbohydrates at carbon-3 was not obtained, although the enediol mechanism predicts the formation of such substances. Indeed, the isomerization of D-galactose by alkali is known to produce not only D-tagatose but also the epimeric D-sorbose,[2] while D-manno-D-*gala*-heptose produces D-glucoheptulose as well as D-mannoheptulose.[25] A significant portion of the non-fermentable substances was oxidized by bromine under buffered acid conditions and the oxidation products are a promising source of further information concerning the nature of the complex mixture. Reaction of the unoxidized fraction with cold Fehling solution suggests the presence of ketotetroses among the products.

Acknowledgment.—The authors are pleased to acknowledge the generous support of Anheuser-Busch, Inc., Saint Louis, throughout the course of this research.

ST. LOUIS, MISSOURI RECEIVED JULY 13, 1951

(25) Edna M. Montgomery and C. S. Hudson, THIS JOURNAL, **61**, 1654 (1939).