TRANSFORMATIONS OF SUGARS IN ALKALINE SOLUTIONS
PART II*. PRIMARY RATES OF ENOLIZATION

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ABSTRACT

A method has been developed for studying the enolization of sugars in D_2O, and determining by infrared absorption the DOH formed. The method does not require isotopically labeled substrates and does not involve a primary isotope-effect.It can be applied to reducing sugars in general and to diverse reaction-systems. By use of radioactivity measurements in conjunction with measurements of infrared absorption, the isotope effect k_T/k_H, for the release of hydrogen atoms from deuterated D-glucose-*2-t*, was measured and found to be 0.13.

INTRODUCTION

The transformations of reducing sugars in alkaline solutions have engaged the attention of chemists for many years, but progress was slow until the isotopes of hydrogen become available for study of reaction mechanisms. In 1937 Fredenhagen and Bonhoeffer[2] utilized hydrogen–deuterium exchange in D_2O to test the enediol mechanism first advanced by Wohl and Neuberg[3]. In the interconversion of D-glucose, D-mannose, and D-fructose at low temperatures, Fredenhagen and Bonhoeffer did not observe the expected exchange by deuterium in the products. Contrary to these results, Topper and Stetten[4] in similar experiments found hydrogen–deuterium exchange. Later, Sowden and Schaffer[5] reinvestigated the subject with ^{14}C-labeled substrates in alkaline D_2O, and also found deuterium exchange, in accordance with the classical enediol-mechanism. Recent studies have cast doubt, however, on the assumption that the epimerization and degradation of sugars in alkaline solutions take place only by the enolization mechanism. Rose and O'Connell[6] found that D-fructose-*1-d* 6-phosphate is converted into D-glucose-*2-d* 6-phosphate by phosphoglucose isomerase, without release of deuterium to the solvent. Fodor and Saccheto[7] reported that D-glucose 3-phosphate in slightly basic D_2O is converted into D-fructose 3-phosphate with no incorporation of deuterium, and Gleason and Barker[8] have found recently that a simple enolization scheme cannot satisfactorily account for the distri-

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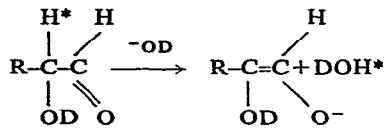
bution of tritium in the rearrangement products from D-ribose-*2-t*. In view of the possible, if not probable, existence of several paths for transformation of sugars in alkaline solutions, Isbell and coworkers undertook several years ago a detailed study of the transformations of sugars in alkaline solutions, using ^{14}C- and tritium-labeled substrates. One of the first tasks was to measure the so-called primary rate of enolization[1,9]. Two methods were developed: (*1*) measurement of tritium uptake by the sugar in alkaline solution; (*2*) measurement of the rate at which hydrogen is released to the solvent from a sugar labeled with tritium at the alpha carbon atom. The latter method involves an isotope effect.

One of the ojectives of the present study was to determine the magnitude of this isotope effect. Another objective was to develop a method, not requiring tritium-labeled sugars as substrates, for measuring the primary rate of enolization. This objective was realized by a procedure in which the alpha hydrogen atom of a deuterated sugar is released by enolization in D_2O (Eq. *1*). The hydrogen released is measured by infrared absorption of the DOH formed[10,11]. In D_2O containing H_2O in low concentration, nearly all of the hydrogen is present as DOH, and a linear relationship is found between the H_2O present and the absorbancy at 2.95 μm.



(*1*)

By rigorous exclusion of extraneous moisture, avoidance of bubbles, close control of the temperature, and standardization of the method under the specific conditions used, highly satisfactory values are obtained for the amount of hydrogen released to the solvent from deuterated sugars.

The ultimate objective of the investigation is to ascertain the rate of release of the hydrogen atoms bound to different carbon atoms under diverse conditions. The reactions may, or may not, consist of simple enolizations. By study of sugars labeled with tritium at various positions, reaction mechanisms may be tested and a more complete understanding of the transformations may be obtained.

EXPERIMENTAL

General. — The cells, syringes, and other apparatus used were thoroughly dried in a vacuum desiccator under diminished pressure by using an efficient two-stage vacuum pump, and were stored under dry argon in a desiccator. The desiccator was not opened except in a dry box. The dry box was maintained at 30° under slight positive pressure, with circulation of oxygen-free nitrogen by a centrifugal pump through a column containing Drierite. Before use, the absorption cells were washed successively with aqueous acetic acid, water, and pyridine, and then dried. The cells were filled, and all transfers of D_2O and samples were made, in the dry box. I.r. spectra were measured with a Beckman Model IR-8 spectrophotometer in double-beam operation. The measurements were conducted in sealed cells of 0.2 mm path-

length, having calcium fluoride windows. The cells were held in a water jacket through which water at 30° was circulated. The temperature was measured and controlled by means of a thermocouple inserted into a port in the cell. Four matched cells were used for the measurements. Two were filled with the isomerization reagent, one was filled with the isomerization reagent plus a known amount of water, and the other one was filled with the reaction mixture. Volumes of the solutions were calculated from the total weight of the constituents divided by the experimentally determined densities.

The pH of the reaction mixtures was determined with a Fisher Model 310 pH meter. The pD values[12] are the meter readings plus 0.4. A Packard Tri-Carb Liquid Scintillation Counter was used for the radioactivity measurements. Assays were made by using 0.1-ml aqueous samples, and 10 ml of scintillation fluid containing 7 g of PPO, 0.3 g of dimethyl POPOP, and 100 g of naphthalene per liter of *p*-dioxane solution.

D-*Glucose-2-t.* — The preparation of this sugar is given in ref. 13. The position of the label was established by the lack of radioactivity in the H_2O formed from carbon 1 by oxidation of the sugar with bromine[14], and by lack of radioactivity in the phenylosazone prepared from the labeled sugar.

Oxygen-free D_2O. — Commercial D_2O (99.8%) was boiled in a dry box under vacuum in a flask fitted with a stopcock for removing vapor and a sidearm fitted with a rubber septum. After all oxygen had been removed and a small amount of D_2O had been vaporized, the vacuum was released by introduction of dry argon. The D_2O was stored under argon and portions were withdrawn when needed by means of a hypodermic needle inserted through the rubber septum.

Potassium carbonate (M) *solution in D_2O.* — A 1.38 g quantity of high purity, anhydrous potassium carbonate was placed in a 10-ml graduated, glass-stoppered flask. Traces of water were removed by heating the sample in the flask at 140° over phosphoric anhydride, in an "Abderhalden drying apparatus" connected to an efficient mechanical vacuum-pump. Heating was discontinued after 12 h. The vacuum was relieved with dry argon. The apparatus was transferred to the dry box and oxygen-free D_2O was added to the flask to give a volume of 10.0 ml. The solution was stoppered and kept in the dry box. Solutions containing carbonate–hydrogen carbonate mixtures were prepared by the same procedure except that sufficient dry carbon dioxide was introduced to give the desired proportion of hydrogen carbonate. The carbon dioxide was introduced after most of the D_2O had been added, but before final adjustment of the volume to 10.0 ml.

Water–D_2O standards. — Water in D_2O was determined[10,11] by the characteristic DOH absorption band at 2.95 μm. Thus the difference in the molar concentration of DOH in matched cells is given by the relationship:

$m = f(\log T - \log T_s)$, where f is the standardization constant, T is the transmittance at 2.95 μ when the reference and sample cells are filled with portions of the same D_2O solution, and T_s is the transmittance when the solution in the sample cell contains m moles of DOH per liter more than the solution in the reference cell.

The standardization constant was checked with each set of measurements by use of a hemetically sealed reference-cell containing the D_2O reagent and a known amount of water. Curve *c* of Fig. 1 shows a typical standardization spectrogram. The standard contained 0.63 mmoles of DOH per ml of the alkaline D_2O reagent; T was 0.90, T_s was 0.24, and f was calculated to be 1.098.

The absorbance of a sealed calcium fluoride cell filled with the alkaline D_2O reagent did not change over a period of several weeks; with freshly filled silica cells the absorbance at 2.95 μm increased slightly for several days and then became constant.

Preparation of deuterated sugars. — A weighed sample (0.5 mmole) of the sugar was placed in a 5 ml serum vial closed with a rubber cap suitable for introduction, or withdrawal, of liquid by means of a hypodermic needle. The hydrogen atoms attached to oxygen in the sugar were replaced by repeated addition and evaporation of small volumes (about 0.5 ml each) of D_2O; the transfers of D_2O were made in a dry box with a hypodermic needle. The needle was left in the stopper as a vent during the evaporation, which was conducted under diminished pressure in a vacuum desiccator over P_2O_5.

In each cycle the vacuum in the desiccator was relieved by introduction of argon, and a new portion of D_2O was added in the dry box through the hypodermic needle. After six additions of D_2O, substantially all of the hydrogen bound to oxygen had been replaced by deuterium, and the hypodermic needle was withdrawn. The solvent-free "deuterated" sugar in the rubber-capped vial was used for measuremen of the rate of enolization, or for other studies.

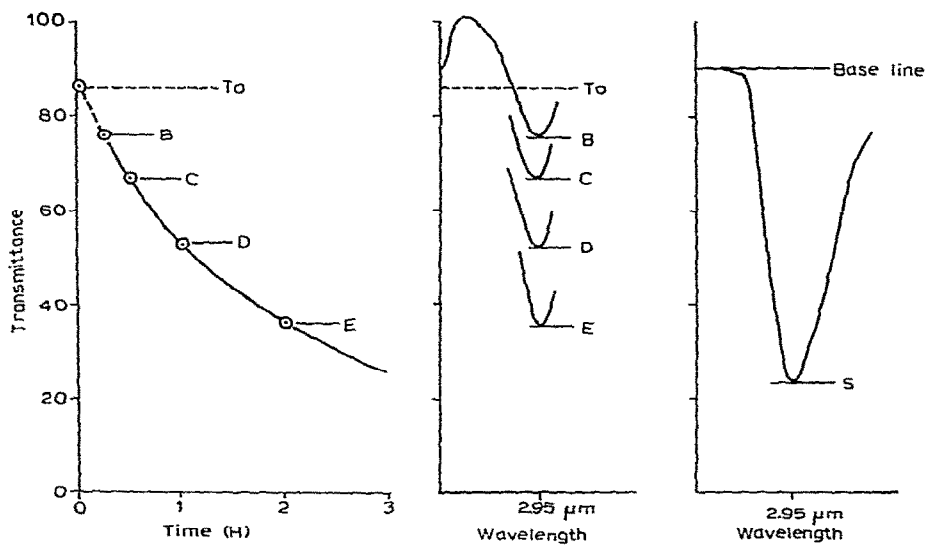


Fig. 1. Typical graphs for measurement of rate of enolization of a deuterated sugar in D_2O. Graph of transmittance at 2.95 μm at times shown in spectrograms given in (*b*); (*c*) spectrogram water standard.

Measurement of the rate of enolization in D_2O. — In a dry box at 30° under nitrogen, 0.5 ml of the D_2O solution used as a reactant (usually M K_2CO_3) was added through a hypodermic needle to the dry, deuterated sugar (0.5 mole) in a 5-ml serum bottle, and the time was recorded. After the sugar had dissolved, a portion of the solution was withdrawn from the serum bottle with a hypodermic needle, and transferred to a previously cleaned and dried i.r. cell (Beckman RIIC-FH-Ol), having a 0.2-mm light path and calcium fluoride windows.

The cell was sealed quickly with stainless-steel plugs. The hermetically tight cell was then removed from the dry box and inserted in the thermostated, water-jacketed, sample compartment of the spectrophotometer. The spectrophotometer was used in double-beam operation; a matched cell, filled with the alkaline D_2O reagent, was placed in the reference beam. The instrument was set to read zero transmittance when the sample beam was blocked, and 90% transmittance for a sample of the alkaline D_2O reagent. Water at 30° was circulated through the water-jacket and the temperature in the sample cell was measured by a thermocouple. As soon as possible the i.r. spectrum in the region of 2.5 to 3.5 μm was recorded and the time was noted. After suitable time-intervals, additional spectrograms were recorded and the transmittance at the minimum at 2.95 μm was measured. A graph of transmittance against time was prepared for each set of measurements and the value of T_0, the transmittance at zero time, was ascertained. Thus, curve (*a*) of Fig. 1 shows a graph for the transmittance of D-fructose in M K_2CO_3 solution (Experiment No. 4 of Table 1). Curve (*b*) shows the spectrograms at the times marked by letters, and (*c*) shows the spectrogram used for standardization. Extrapolation of transmittance to zero time compensates for changes in absorption arising from replacement of part of the D_2O reagent by the deuterated sugar, and for traces of water that may remain in the sample at the beginning of the measurement.

The amount of DOH formed during time period, t, was calculated by means of the relationship: $m = f(\log T_0 - \log T_t)$, and the rate constant was calculated from the equation

$$k_H = {}^1/t \ln [A/(A-X)] \qquad (2)$$

where A is the equivalents of active hydrogen per liter of solution at the beginning of the measurement, and X is the moles of DOH formed per liter of solution during the reaction time (expressed in h). Satisfactory constants were obtained on the assumption that aldoses have one active hydrogen atom (presumably that at C-2) and ketoses have two (presumably at C-1). Results for D-glucose, D-mannose, and D-fructose are given in Table I.

Determination of k_T/k_H, the isotope effect for the enolization of D-*glucose*-2-t. — D-Glucose-*2-t* was deuterated and treated with M potassium carbonate in D_2O in the manner described for measurement of the rate of enolization. The change in i.r. absorption at 2.95 μm arises almost entirely from release of hydrogen ions to the solvent, because there are relatively few tritium atoms in the labeled sugar. Hence, the rate constant k_H, determined by the change in i.r. absorption arising from DOH,

TABLE I

MEASUREMENT OF RELEASE OF HYDROGEN FROM DEUTERATED SUGARS IN M K_2CO_3 IN D_2O AT 30°

Time (*h*)	*Transmittance* T	*log* T_0-*log*T	*DOH formed*[a] (*mmoles*)	*% Active protons released*[b]	$^cK_H \times 10^2$
Expt. no. 1 D-*Glucose*[d]		(*Solution purged with argon*)			
0.0	0.8850				
0.25	0.8775	0.00369	0.0025	0.5	(1.98)
1.0	0.8850	0.01497	0.0100	2.0	2.01
2.0	0.8333	0.02614	0.0174	3.5	1.77
3.0	0.8066	0.04028	0.0268	5.4	1.84
4.0	0.7800	0.05485	0.0366	7.3	1.85
5.0	0.7566	0.06750	0.0450	9.0	1.85
				Average	1.86
		Average from 7 separate experiments			1.78 ±0.22
Expt. no. 2 D-*Glucose*[e]		(*Solution containing dissolved oxygen*)			
0.0	0.8360				
0.33	0.8225	0.0073	0.0048	1.0	(2.92)
3.0	0.7475	0.0489	0.0324	6.5	2.23
4.25	0.7250	0.0619	0.0410	8.2	2.01
5.0	0.7050	0.0740	0.0491	9.8	2.06
6.0	0.6750	0.0929	0.0616	12.3	1.29
23.4	0.3233	0.4130	0.2739	54.8	(3.39)
				Average	2.12
			Duplicate measurement		2.25
Expt. no. 3 D-*mannose*[f]		(*solution purged with argon*)			
0.0	0.9150	0.0000			
1.00	0.9000	0.0072	0.0051	1.0	0.99
2.00	0.8833	0.0154	0.0108	2.2	1.09
3.00	0.8666	0.0234	0.0165	3.3	1.11
4.00	0.8475	0.0335	0.0236	4.5	1.22
5.00	0.8375	0.0387	0.0273	5.5	1.13
6.00	0.8233	0.0460	0.0324	6.5	1.10
23.75	0.5375	0.2314	0.1631	32.6	(1.66)
				Average	1.11
Expt. no. 4 D-*Fructose*[g]		(*Solution purged with argon*)			
0.00	0.8550	0.0000			
0.25	0.7530	0.0552	0.0362	3.6	14.3
0.50	0.6675	0.1079	0.0708	7.1	14.7
1.00	0.5275	0.2102	0.1379	13.8	14.8
2.00	0.3575	0.3793	0.2489	24.9	14.3
3.00	0.2500	0.5341	0.3504	35.0	14.4
4.00	0.1775	0.6840	0.4488	44.9	14.9
5.00	0.1375	0.1375	0.7953	52.3	14.7
				Average	14.6

For footnote see p. 325.

represents release of hydrogen atoms in the enolization process. The rate constant for release of tritium atoms (k_T) was determined from radiochemical data obtained from the same reaction mixture. The rate constant for the tritium species of the reaction was calculated from the equation:

$$k_T = 1/t \ln [A^*/(A^* - X^*)] \quad (3)$$

where A^* is the tritium present in the original reaction mixture and X^* is the tritium released during the time t (measured in h). The value of A^* was determined by liquid-scintillation counting of a sample of the reaction mixture after suitable dilution. The value of X^* was determined by diluting a 50-μl sample of the reaction mixture after a suitable time with one ml of ice–water and quickly lyophilyzing the mixture in the apparatus of Fig. 2. The water-t obtained from the lyophilization was lyophilized a

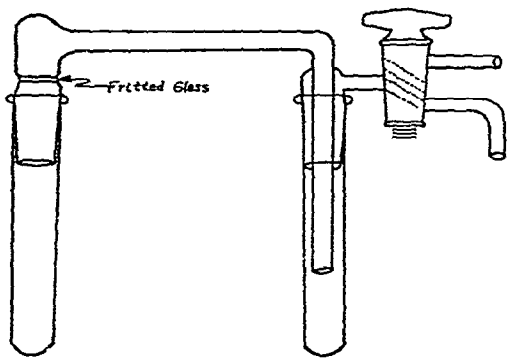


Fig. 2. Apparatus for purification of tritiated water.

second time for further purification, and was then assayed by liquid-scintillation counting. Finally, k_H and k_T, were calculated from equations 2 and 3. Some results are given in Table 2.

DISCUSSION

In our first attempt to determine the isotope effect for the enolization of D-glucose-*2-t*, the reaction mixtures were kept in rubber-capped vials, and samples were taken from time to time for determination of the tritiated water and of the DOH

[a]% DOH formed, conversion factor × volume of reaction mixture × (log T_0 − log T). [b]Percentage of the C-2 protons of an aldose, or of the C-1 protons of a ketose. [c]$k_H = 1/t \ln [A/(A-X)]$, where A is the equivalents of active hydrogen at zero time, and X is the moles of DOH formed during the time period, measured in h. [d]90 mg D-glucose in 601 μl of solution; pD 11.9. [e]90 mg D-glucose in 521 μl of solution; pD 11.9. [f]90 mg D-mannose in 604 μl of solution; pD 11.7. [g]90 mg D-fructose in 597 μl of solution; pD 11.6. [h]The rate constant expressed as atoms of hydrogen released per hour per mole on sugar is twice the value given here because the calculation is based on an equivalent weight of 90 for the sugar.

TABLE II.

ENOLIZATION OF D-GLUCOSE-2-*t*- IN D_2O AT 30° AND pD 11.9 (90 mg D-GLUCOSE-2-*t* + 500 μl M K_2CO_3)

Expt. No.	*Reaction time (h)*	*Active protons released (%)*	*Radioactivity in*		*Rate constants*		k_T/k_H
			Orig. sugar A*	H_2O-t X*	[a]$k_H \times 10^3$	[b]$k_T \times 10^3$	
6	5.0	9.0	23,700	242	1.89	0.205	0.108
7	5.0	9.0	18,010	210	1.85	0.234	0.126
8	5.0	7.3	12,063	146	1.58	0.244	0.154
9	6.0	8.9	4,386	63	1.56	0.241	0.154
10	22.9	34.9	17,851	907	1.85	0.228	0.123
						av.	0.133

[a]See footnote *c*, Table 1. [b]$k^* = 1/t \ln [A^*/(A^*-X^*)]$; where A^* is the counts per second of the D-glucose-2-*t* used in the reaction mixture, and X^* is the disintegrations per sec. of the water-*t* formed in the specified time, expressed in h.

formed. The values obtained for k^* and k_H vaired widely, and the ratios were of the order of 1:50. The difference in the values was too great to be explained by an isotope effect. Apparently, the values of k_H were high because of absorption of extraneous moisture. This complication was eliminated by keeping the sample in a hermetically sealed cell, and measuring k_H by i.r. absorption and k_T by radioactivity assay of the freeze-dried solvent, at the end of a suitable reaction time. The results from seven measurements reported in Table II give an average value of 0.13 for k_T/k_H. This value compares favorably with isotope effects (0.12 to 0.17) found for oxidation of aldoses-1-*t* with iodine[15]. The precision of the measurements is not high. Substantial variations arise from experimental errors and from poorly controlled factors, such as traces of oxygen in the reaction medium. Experiments 1 and 2 of Table 1 show the accelerating effect of oxygen on the release of hydrogen from deuterated D-glucose in alkaline solutions.

It is noted that the rate of hydrogen release for aldoses usually increases as the reactions proceed. Presumably, the increase arises from accumulation, in the reaction mixture, of products more reactive than the original sugar. Thus, D-glucose, by reversible enolization, yields D-fructose more rapidly than D-glucose (See experiments 1 and 4 of Table I). The rate of hydrogen release at any time during the reaction period depends on all the constituents of the solution. Thus, the rate of hydrogen release is characteristic of the original sugar only during the initial stages of the reaction, when the contribution of the reaction products to release of hydrogen may be neglected. To avoid complications from reaction products, Isbell and coworkers[8] restricted their measurments of hydrogen–tritium exchange to experiments involving less than 2% of enolization. Short reaction-periods are subject to larger measurement errors than longer periods, but they are kinetically more significant.

The rates of hydrogen release in D_2O are not directly comparable to the rates previously reported for tritium uptake in water-*t* solutions. The rate of hydrogen

release for D-glucose reported in Table I is about 40 times the rate previously reported for uptake of tritium (0.000460 equivalent.mole^{-1}.h^{-1}). The difference may be ascribed to differences in base strength and temperature (11.9 pD and 30° for the measurement of hydrogen release, and 11.2 pH and 25° for the measurement of tritium uptake). Differences may also arise from isotope effects that have not been evaluated.

The relative rates of tritium uptake previously reported (1.0, 0.5, and 10.7 for D-glucose, D-mannose, and D-fructose, respectively), show marked correlation with the relative rates of hydrogen release (1.0, 0.6 and 29) found in the present study. The differences are not surprising, because the enolization–de-enolization process is complicated by secondary reactions. The reaction systems are complex and little understood. Further study with isotopically labeled substrates will undoubtedly lead to a better understanding of the subject.

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