vessel were transferred to a 125 ml. flask with 15 ml. of water and acidified by addition of 0.1 ml. of 0.5 *N* sulfuric acid. After five minutes, 1.5 g. of sodium bicarbonate, 5 ml. of 0.03 *M* sodium arsenite and 1.0 ml. of 10% potassium iodide were added in succession.[10] Twenty minutes later excess arsenite was titrated with 0.01 *N* iodine. A glycogen sample of 10.8 mg. required 12.9 ml. of iodine, which corresponded to a periodate consumption of 0.97 mole per mole.

The *p*H was measured by transferring the contents of a vessel after equilibration, or at the end of a run, to a *p*H-meter having glass electrodes. A fine stream of carbon dioxide gas was passed into the solution during the measurement making it difficult to obtain an accurate reading. The *p*H values quoted are therefore approximate.

Estimation by Titration of the Formic Acid Produced in Periodate Oxidation (Figs. 2 and 3).—In a typical experiment 100 mg. of glycogen, dissolved in 25 ml. of water, was treated with 0.2 *M* sodium metaperiodate (25 ml.), in the dark at 16.6°. At suitable intervals aliquots (10 ml.) of the solution and of a reagent blank were withdrawn and, 20 minutes after the addition of 0.5 ml. of ethylene glycol and 2.0 ml. of 10% potassium iodide, were titrated with 0.02 *N* sodium thiosulfate.[18]

Acknowledgment.—The technical assistance of Mr. J. Giroux is gratefully acknowledged. The author is indebted to Prof. F. Smith, University of Minnesota, for his kind interest in this work and for the gift of glycogen samples. Sincere gratitude is also expressed to several colleagues in this Division for advice in the use of the Warburg respirometer and for providing apparatus.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, TRINITY COLLEGE]

Chromatographic Adsorption. III. Investigation of the Isomer Distribution during Fischer Methyl D-Galactoside Formation

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RECEIVED JANUARY 4, 1954

The changing distribution of isomers during the formation of methyl D-galactosides by the Fischer method at room and reflux temperatures using 0.5 and 4% hydrogen chloride was studied by means of chromatography and polarimetry. It was found that β-isomers are formed first and change to α-isomers, principally α-D-galactopyranoside, the change being accelerated at higher temperatures or hydrogen chloride concentrations. The change of furanosides to pyranosides which takes place simultaneously is, contrary to the commonly accepted view, a less important reaction, at least in this case. Conditions under which maximum yields of the various isomers may be expected are included.

Introduction

In the past, study of the rearrangements taking place during glycoside formation by the Fischer method has been seriously handicapped by the lack of easy reliable methods by which the various isomers can be quantitatively determined. An early investigation of this reaction by Levene, Raymond and Dillon[3] indicated that furanosides were formed first and changed slowly to pyranosides as the reaction continued. Until very recently no good method for the quantitative determination of α- and β-isomers has been known and therefore changes from α- to β-forms or *vice versa* could not be followed. In 1946 Binkley and Wolfrom[4] reported the chromatographic separation of the anomeric forms of the penta-O-acetyl-D-glucopyranoses. More recently Hough, Jones and Wadman[5] reported the chromatographic separation of methyl α- and β-L-rhamnopyranosides. Very recently Augestad, Berner and Weigner[6] have reported the chromatographic separation of methyl fructosides and methyl galactosides using a powdered cellulose column. It has been found that Florex XXX, a fuller's earth type of adsorbent, can be used for separation of sugar mixtures[7] and that with the proper pretreatment[8] essentially 100% recovery of the individual sugars can be expected. It was thought that if methyl galactosides could be separated by this adsorbent, a quantitative method for their determination might be realized. In addition, it was hoped that data might be obtained for a comparison of the relative capacities of Florex and cellulose columns for the separation of methyl galactosides.

In the present investigation, methyl galactoside mixtures formed under various conditions of hydrogen chloride concentration and temperature were analyzed by passage in methanol through a Florex XXX column by a procedure previously[8] described. The β-isomers, with negative rotations in methanol, were eluted from the column first, followed by the α-isomers, with positive rotations. Although separation of furanosides from pyranosides was not possible with this adsorbent, a complete separation of α- and β-isomers was obtained. The distribution of furanoside and pyranoside in each fraction can be calculated from the specific rotations of the two components, the weight of the fraction, and the ml.-degree area under the elution curve provided only that no more than traces of other substances are present. This is believed to be the case, as the most important by-products of the reaction, D-galactose dimethyl acetal and the methyl galactoseptanosides would be expected in very small amounts only. D-Galactose dimethyl acetal, for

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(3) P. A. Levene, A. L. Raymond and R. T. Dillon, *J. Biol. Chem.*, **95**, 699 (1952).

(4) W. W. Binkley and M. L. Wolfrom, THIS JOURNAL, **68**, 1720 (1946).

(5) L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.*, 1702 (1950).

(6) I. Augestad, E. Berner and E. Weigner, *Chemistry & Industry*, 376 (1953).

(7) B. W. Lew, M. L. Wolfrom and R. M. Goepp, Jr., THIS JOURNAL, **68**, 1449 (1946).

(8) D. F. Mowery, Jr., *ibid.*, **73**, 5047, 5049 (1951).

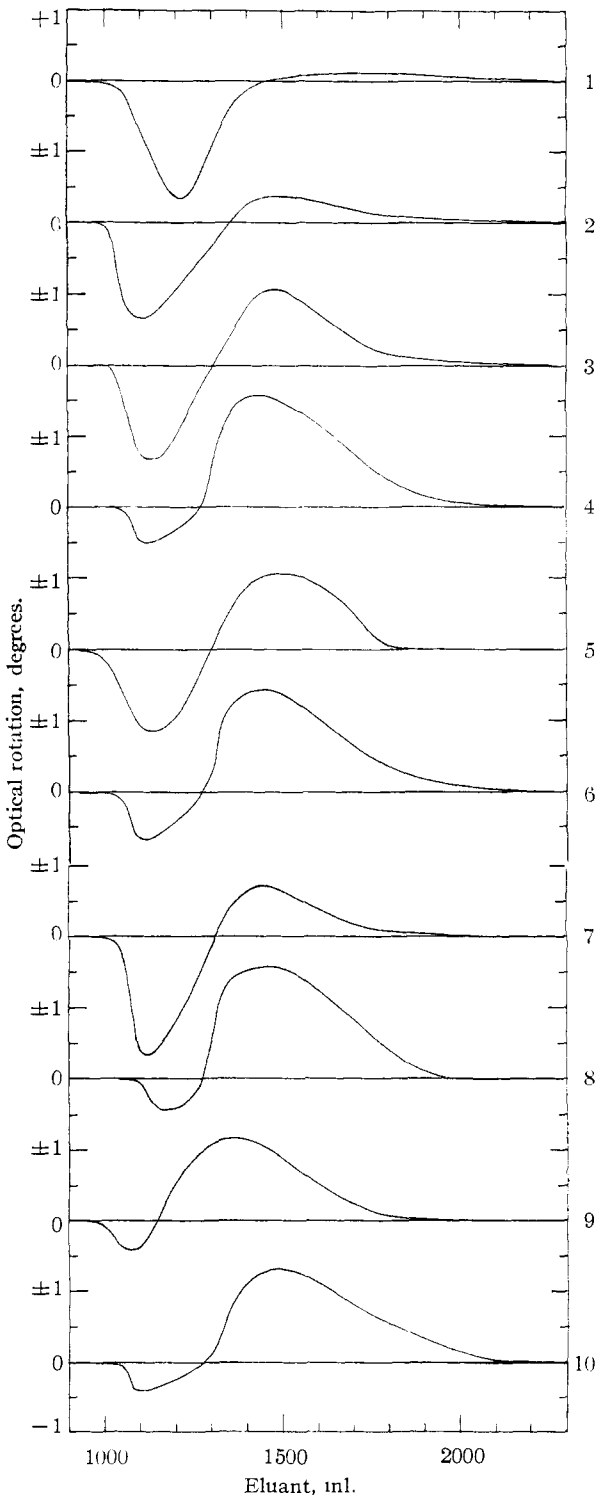

Fig. 1.—Polarimetric curves showing elution of methyl α- and β-D-galactosides from the Florex XXX Column.

instance, has been found[9] to be very rapidly converted to methyl galactosides in acidified methanol and a seven-membered ring has considerably less tendency to form than a five- or six-membered ring[10] and is as sensitive to acid hydrolysis as a five-membered ring.[11] None of these substances have ever been isolated from reactions producing glycosides by the Fischer method.

(9) H. A. Campbell and K. P. Link, *J. Biol. Chem.*, **122**, 635 (1938); M. L. Wolfrom and S. W. Waisbrot, THIS JOURNAL, **61**, 1408 (1939).

Experimental

Reagents.—The D-galactose was Pfanstiehl C.P. material. Hydrogen chloride was obtained from a cylinder and dried by passage through a column of calcium chloride. The weakly basic ion-exchange resin, Deacidite, was obtained from the Permutit Company.

Apparatus.—The chromatographic column was a 4.8 × 122 cm. column of air blown Florex XXX constructed and operated in a manner previously[8] described. With absolute methanol as developer, pressures of about 1 atm. were sufficient to produce an effluent of 20–25 ml. per minute from this size column. With pressures of this magnitude a 3.5-gallon Pyrex bottle provided a convenient solvent reservoir.

Determination of Column Capacity.—Twenty-seven grams of D-galactose, suspended in one liter of methanol containing 0.5% of dry hydrogen chloride, was stirred at room or reflux temperature for about 70 hours. The clear solution was neutralized with silver carbonate, filtered and evaporated to a thick sirup. The sirup was divided into five portions of 2, 3, 5, 7 and 10 g. Each portion was run through the chromatographic column and the usual plot of optical activity *vs.* ml. of effluent was made. Assuming complete separation into positively and negatively rotating fractions for the 2 g. charges, the areas to be expected upon complete separation of the other charges should be proportionately larger and the % separation of the larger charges can therefore be calculated as 100 × observed area/calculated area. It had previously been determined that the specific rotation of methyl α-D-galactopyranoside in methanol changes very little with concentration or small temperature changes and it was assumed that the other methyl galactosides would behave similarly. The results of these experiments are summarized in Table I and indicate that 5 g. of a predominantly negative mixture of methyl galactosides and 3 g. of a predominantly positive mixture can be completely separated by the chromatographic column used in this work.

TABLE I

DETERMINATION OF COLUMN CAPACITY

Conditions (0.5% HCl)	Charge, g.	Area, sq. in. Obsd.	Area, sq. in. Calcd.	Separation, %
Room temp.	2	0.81		
	3	1.22	1.21	101
	5	2.01	2.03	100
	7	2.79	2.85	98
	10	3.76	4.07	92
Reflux temp.	2	0.92		
	3	1.41	1.38	102
	5	2.10	2.30	91
	7	2.92	3.24	90
	10	4.03	4.60	88

Preparation of Methyl Galactoside Mixtures.—Methyl galactoside mixtures were made by dissolving 3.0 g. of D-galactose in 400 ml. of absolute methanol at room temperature or 200 ml. at reflux temperature. One-half or 4% of dry hydrogen chloride was then introduced and the mixture allowed to stand or refluxed until the reducing sugar analysis[12] indicated that only traces of galactose remained. Some of the mixtures were worked up at this point while others were allowed to continue for longer periods of time. The reaction was stopped by neutralization of the hydrochloric acid by means of silver carbonate or passage through a Deacidite column. The clear solution was evaporated under vacuum at 50° to a thick sirup, which was weighed to the nearest 0.1 g. The sirup was then transferred quantitatively to the top of the chromatographic column by means of absolute methanol and run down into the adsorbent under

(10) G. W. Wheland, "Advanced Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1949, pp. 372.

(11) F. Micheel and F. Suckfüll, *Ber.*, **66B**, 1957 (1953).

(12) L. J. Heidt and F. W. Southam, THIS JOURNAL, **72**, 599 (1950).

TABLE II

DISTRIBUTION OF METHYL GALACTOSIDE ISOMERS PRODUCED UNDER VARIOUS CONDITIONS

Run no.	Conditions	Pos. fract. Wt.[a]	Pos. fract. Area[b]	α-Isomers, % Total	α-Isomers, % Fur.	α-Isomers, % Pyr.	Neg. fract. Wt.[a]	Neg. fract. Area[b]	β-Isomers, % Total	β-Isomers, % Fur.	β-Isomers, % Pyr.	Total, % Fur.	Total, % Pyr.
	0.5% HCl r.t.												
1	6 hr.	0.5	60	17	17	0	2.5	320	83	33	50	50	50
2	48	0.9	126	29	29	0	2.2	270	71	26	45	55	45
3	240	1.7	344	49	33	16	1.8	220	51	19	32	52	48
4	940	2.3	606	79	30	49	0.6	66	21	7	14	37	63
	0.5% HCl refl.												
5	3 hr.	1.6	354	52	30	22	1.5	220	48	23	25	54	46
6	87	2.3	564	70	33	37	1.0	94	30	7	23	40	60
	4% HCl r.t.												
7	5 hr.	1.2	214	38	30	8	2.0	258	62	25	37	55	45
8	75	2.6	624	87	43	44	0.4	54	13	5	8	48	52
	4% HCl refl.												
9	3 hr.	2.8	440	82	73	9	0.6	44	18	4	14	77	23
10	20	2.3	554	79	39	40	0.6	54	21	5	16	44	56

[a] Wt. of fraction in g. after evaporation in vacuum. [b] Area in ml.-degrees = 200 + area in sq. in. obtained by planimeter from plot.

1 atm. pressure. Methanol was then run through the column and the rotation of the effluent, determined at 50-ml. intervals, was plotted in Fig. 1. The effluent was separated into positively and negatively rotating fractions and each fraction evaporated under vacuum and weighed. Recovery of material from the column was 97% or better.

Compositions of Chromatographic Fractions.—Methyl β-D-galactopyranoside[13] of specific rotation 0° (*c* 0.69)[14] in water and −17° (*c* 1.52) in methanol was crystallized from the negatively rotating fractions of the effluent. Methyl α-D-galactopyranoside monohydrate[15] of specific rotation +178° (*c* 0.73) in water and +171° (*c* 1.20) in methanol was crystallized from the positively rotating fractions. Methyl β-D-galactofuranoside[16] of specific rotation −110° (*c* 0.68) in water and −137° (*c* 0.71) in methanol was crystallized from a negatively rotating fraction from the chromatographic column using as charge a furanoside mixture prepared from galactose diethyl mercaptal by the method of Pacsu and Green.[16] The fraction from which this isomer was crystallized appeared at the same point in the effluent as the negative fractions from the Fischer reactions and it is therefore assumed that in all cases the negative fractions consist of the two negatively rotating methyl β-D-galactosides. In addition, the specific rotations of all the sirups obtained from the negative fractions indicated the presence of an isomer having a larger negative rotation than the methyl β-D-galactopyranoside actually isolated. The above crystalline methyl galactosides were obtained from absolute ethanol or ethyl acetate solutions after removal of methanol and water by several vacuum evaporations with absolute ethanol. The specific rotations of the positive fractions indicated the presence of an isomer of specific rotation less than +70° in methanol. This was assumed to be the methyl α-D-galactofuranoside which, although not isolated, could be calculated by means of Hudson's isorotation rules to have a specific rotation of +87° in water and +68° in methanol. It has been found that Hudson's rules hold very exactly for the methyl glucosides, ethyl glucosides and ethyl galactosides and they should therefore hold also for the methyl galactosides. A specific rotation of +104° has recently been reported[6] for methyl α-D-galactofuranoside in water, but in view of the divergence of this figure from that predicted by Hudson's rules its validity is questionable until further substantiated.

Calculations of Isomer Distributions.—The distributions of methyl α- and β-D-galactofuranosides and pyranosides produced by 0.5 and 4% hydrogen chloride in methanol solutions at room and reflux temperatures in various times are presented in Table II. The total percentage of α-isomers was calculated as 100 (wt. of positive fractions)/(wt. of positive fraction + wt. of negative fraction). Using +68° and 171° as specific rotations of methyl α-D-galactofuranoside and pyranoside monohydrate, respectively, in methanol, the proportion of furanoside in this fraction can be calculated as (171 − ml. deg. area/2 × fract. wt.)/103. The percentage α-furanoside and α-pyranoside can then be determined. In exactly comparable manner the percentage β-furanoside and β-pyranoside can be calculated. In this case specific rotations of −137° and −17° for the β-furanoside and pyranoside, respectively, lead to the following proportion of furanoside: (ml. deg. area/2 × fract. wt. − 17)/120. The last two columns in Table II give the total % furanoside and pyranoside, calculated as the sum of the α- and β-isomers in each of these ring forms.

Reducing Sugar and Furanoside Analyses.—Two methods of analysis were employed in following the course of the glycoside formation. Both of these involved the determination of reducing sugar and were the hypoiodite method used by Levene, *et al.*,[3] and the cupritartrate method recently refined by Heidt and Southam.[12] The latter method was found to be more reliable than the hypoiodite procedure and was therefore used in most cases. A heating time of one hour was found most satisfactory for galactose analyses. Total furanoside was determined by the method of Levene, *et al.*[3]

Discussion of Results

General Course of the Reaction.—A study of Table II and the graphs of the corresponding runs, reproduced in Fig. 1, shows that in the formation of methyl galactosides by the action of acidified methanol upon galactose, β-isomers are formed first. These may amount to as much as 83% for short reaction times under mild conditions (run 1). As the reaction continues β-isomers change to α, the change being more rapid the higher the temperature or the greater the hydrogen chloride concentration. For example, with 0.5% hydrogen chloride at room temperature β-isomers drop from 83 to 21% in about 3 weeks time (runs 1–4), whereas the change requires less than 3 hours with 4% hydrogen chloride at reflux temperature (runs 9 and 10). Intermediate conditions such as 0.5% hydrogen chloride at reflux temperature or 4% hydrogen chloride at room temperature require intermediate times (runs 5–8). At the same time that β-isomers are changing to α a smaller change of furanosides to pyrano-

(13) C. N. Riiber, J. Minsaas and R. T. Lyche, *J. Chem. Soc.*, 2173 (1929).

(14) All specific rotations in this paper are given for the D-line of sodium at 20°; *c* = g. of sugar per 100 ml. of solution.

(15) M. Voss, *Ann.*, **485**, 297 (1931).

(16) E. Pacsu and J. W. Green, THIS JOURNAL, **59**, 1205 (1937); **60**, 2056 (1938).

sides is taking place, this change reducing the furanoside content from 50 to 37% in 3 weeks with 0.5% hydrogen chloride at room temperature and to 44% in 20 hours with hydrogen chloride at reflux temperature. About 4 days are required for a similar change at the intermediate conditions of 0.5% hydrogen chloride at reflux temperature or 4% hydrogen chloride at room temperature. The α-pyranoside content is seen to increase from 0 to 49% in 3 weeks at room temperature with 0.5% hydrogen chloride, accounting for most of the β-isomer decrease. The α-pyranoside increase is faster under the more drastic conditions of temperature or acid concentration. The formation of α-furanoside seems to be somewhat erratic, first increasing and then decreasing in 0.5% hydrogen chloride at room temperature, remaining almost constant for 87 hours in 0.5% hydrogen chloride at reflux temperature, increasing in 4% hydrogen chloride at room temperature, and decreasing in 4% hydrogen chloride at reflux temperature. The data here are too incomplete to prove a definite relationship but seem to indicate the α-furanoside content may pass through a maximum and then decrease, the maximum being attained faster under more drastic conditions such as 4% hydrogen chloride at reflux temperature.

Maximum Yields of Various Isomers.—Conditions under which maximum yields of the various isomers may be expected are listed in Table III.

TABLE III

CONDITIONS FOR MAXIMUM[a] YIELDS OF ISOMERS

Isomer	Time, hr.	HCl, %	Temp.	Yield, %
Methyl α-D-galactofuranoside	3	4.0	Reflux	73
Methyl α-D-galactopyranoside	20	4.0	Reflux[b]	40+
Methyl β-D-galactofuranoside	6	0.5	R.t.	33
Methyl β-D-galactopyranoside	6	0.5	R.t.	50

[a] For conditions investigated. [b] Yield of this isomer appears to increase with time and although all conditions eventually produce methyl α-D-galactopyranoside, these conditions produce a faster reaction and larger yields would be expected with longer reaction times than 20 hours.

Agreement of Results with Previous Work.—The results obtained are in agreement with an early observation by Jungius[17] that methyl β-D-galactoside in methanol containing hydrogen chloride changes partially into methyl α-D-galactoside. There is also agreement with the work of Levene, Raymond and Dillon,[3] who showed that with 0.5% hydrogen chloride at room temperature the total galactofuranoside passes through a maximum between 3 and 24 hours. Although the furanoside analysis by means of acid hydrolysis under carefully standardized conditions as used by Levene, *et al.*, undoubtedly provides a satisfactory indication of the position of a furanoside maximum, the absolute values for furanoside content are questionable. If the acid treatment time is increased from 10 to 20 minutes the furanoside analysis (48-hr. samples in Table IV) increases from 39 to 55% by the hypoiodite method and from 38 to 49% by the cupritartrate method. Apparently the hydrolysis time is so critical the method cannot be relied upon to give an accurate absolute furanoside estimation. Galactose determinations of Levene, *et al.*, could not be duplicated either by the hypoiodite method, which they used, or the cupritartrate method, used in the present work, although these two methods were themselves in good agreement (columns 2, 3, and 4 of Table IV). The discrepancy may lie in the initial concentration of galactose which Levene, *et al.*, state to be 0.344 molal, whereas at room temperature the solubility of galactose in methanol is about 1/4 of this concentration, indicating that they may have had some undissolved galactose at the start of the reaction, which could account for their higher free sugar analyses.

TABLE IV

METHYL GALACTOSIDE FORMATION USING 0.5% HYDROGEN CHLORIDE AT 25°

Time, hr.	Galactose, % Levene, *et al.*	Hypoiodite	Cupritartrate	Furanosides, % Levene, *et al.*	Hypoiodite	Cupritartrate
0	101	100	98	7	6	0
1	75	58	59	23	25	15
3	46	25	27	38	49	42
7	21	8	6	48	52	50
24	6	4	4	40	48	44
48	1	3	3	30	39	38
48[a]					55	49
400			0			19

[a] 20-minute hydrolysis instead of standard 10 minute time.

Less satisfactory agreement is evident upon comparison of the results of the present work with a very recently published note by Augestad, Berner and Weigner,[6] who used 0.017% hydrogen chloride in methanol for 6 hours at reflux temperature. They reported 15% methyl α-D-galactofuranoside, 20% methyl α-D-galactopyranoside, 50% methyl β-D-galactofuranoside and only a trace of methyl β-D-galactopyranoside. No run was made under exactly these conditions, run 5 using 0.5% hydrogen chloride at reflux temperature for 3 hours being the most similar. The greatest discrepancy appears to be in the distribution of the β-isomers, the present work indicating an approximately equal distribution between furanoside and pyranoside and the work of the above authors indicating principally furanoside with very little pyranoside, a very unusual situation in view of the present work. Since the above prepublication note lacks experimental detail and does not account for 15% of the glycoside mixture, a satisfactory reconciliation of the two methods cannot be made at present.

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(17) C. L. Jungius, *Z. physik. Chem.*, **52**, 97 (1905).