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PERIODATE OXIDATION ANALYSIS OF CARBOHYDRATES

XIII*. SIMULTANEOUS GAS CHROMATOGRAPHIC DETERMINATION OF THE ALDEHYDES IN THE PERIODATE OXIDATION PRODUCTS OF NON-DIALYZABLE URINARY CARBOHYDRATE MATERIALS AS DIETHYL DITHIOACETALS

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SUMMARY

The aldehydes in the periodate oxidation products of non-dialyzable urinary carbohydrate materials were determined simultaneously by gas chromatography of their diethyl dithioacetal derivatives. The yields of aldehydes for normal male urine varied considerably among subjects, but their molar ratios were almost constant. The average values for glyceraldehyde/glyoxal and lactaldehyde/glyoxal molar ratios were 0.41 and 0.27, respectively. The average amount of L-fucose at the non-reducing terminals of carbohydrate chains, as estimated from the yield of lactaldehyde, was about 5 mg/day.

INTRODUCTION

There are a number of diseases associated with the abnormal accumulation of complex carbohydrate materials in urine. Fucosidosis [1] and mannosidosis [2] are the most well-known cases which are characterized by excretion of L-fucose- and D-mannose-rich oligosaccharides due to an inheritable deficiency of α-fucosidase and α-mannosidase, respectively. Aspartylglycosaminuria [3] is noted for an unusual accumulation of N-acetylglucosaminoaspartate induced by lack of the enzyme which catalyzes cleavage of the sugar—amino acid bond. Abnormal accumulation of carbohydrate materials is also found in Hurler's syndrome [4], G_{M1} gangliosidosis [5], and I-cell mucolipidosis [6]. The

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increased amounts of urinary carbohydrate materials in these metabolic disorders is so drastic that they can be easily characterized by conventional determination of monosaccharides. However, we can expect that there might be many other cases in which deviation of the amounts of constituent monosaccharides from normal values is not so great, but significant change of carbohydrate structure is still involved, including monosaccharide sequence and linkage type.

Recent advancements in mechanistic studies of biosynthesis and metabolism of carbohydrate materials suggests that detailed analysis of these materials might provide information reflecting the activity of carbohydrate transferases and hydrases in tissues and body fluids, and hence serve for diagnosis of the physical state. From such viewpoints our project is concerned with elucidating the fine structures of macromolecular carbohydrate materials in body fluids to correlate their variation to disease states.

We have already published a new convenient method [7, 8] for the simultaneous determination of conjugated aldehydes in the periodate oxidation products of carbohydrate materials. This paper aims to establish the conditions for the determination of aldehydes formed by periodate oxidation of non-dialyzable fractions of human urine.

EXPERIMENTAL

Chemicals

All chemicals were of the highest grade commercially available. D-Xylitol (internal standard) was also obtained from a commercial source, and dried in vacuo for 24 h over phosphorus pentoxide. Pyridine was dehydrated by heating it under reflux with barium oxide, and distilled before use.

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a hydrogen flame ionization detector. A glass column (2 m × 3 mm I.D.) packed with 3% silicone OV-1 on Chromosorb W AW DMCS (80–100 mesh) was used at 170°, and the carrier gas (nitrogen) was regulated at a flow-rate of 50 ml/min throughout the work. Peaks were integrated by a Shimadzu Chromatopak E1A integrator.

Urine samples

These were obtained from male volunteers. For the study of daily variation each sample was used immediately after collection, but for other assays samples were pooled in a refrigerator before 24-h composites were obtained.

Pre-treatment of urine samples

A 10-ml portion of each sample was dialyzed in a Visking tube against tap water for 24 h, except for the samples for the study of dialysis efficiency. The latter samples were dialyzed under the conditions indicated in Table II. The non-dialyzable fraction was concentrated below 40°, and the volume was adjusted to 1.00 ml.

Procedure for the assay of aldehydes

The procedure was essentially the same as that described in the previous paper [8]. A 0.1 *M* sodium metaperiodate solution (100 μl) contained in a small sample tube (5 cm × 5 mm I.D.) was evaporated in vacuo in a desiccator containing sodium hydroxide. To the residue was added an aliquot of the concentrate of the non-dialyzable fraction of urine. The volume of the aliquot added corresponded to that resulting from 0.1% of a 24-h composite sample. Water was added to make the total volume to 200 μl, and the mixture was heated for 3 h at 50° in the dark. The reaction mixture was cooled on an ice-bath, and 0.1 *M* silver nitrate solution (120 μl) was added with vigorous shaking. For the study of the oxidation course, an aliquot of 10% of the reaction mixture obtained from the ten-fold scale reactants was removed after appropriate reaction time, and worked up in the same manner. The mixture was evaporated in vacuo in a desiccator, and to the residue was added a 2:1 (v/v) mixture (20 μl) of ethanethiol and trifluoroacetic acid. The whole was shaken gently for 10 min, closed tightly with a polyethylene stopper. Then a 0.02 *M* solution of D-xylitol (internal standard) in pyridine (50 μl) was added, followed by hexamethyldisilazane (100 μl) and trimethylchlorosilane (50 μl), and the mixture was incubated for 30 min at 50° with occasional shaking. The mixture was centrifuged, and a 1-μl sample of the supernatant was injected into the gas chromatography column. The retention time and molar response factor of each aldehyde derivative, both relative to D-xylitol trimethylsilylate, were, in the following order: glyoxal, 1.49, 0.52; glyceraldehyde, 0.68, 0.64; lactaldehyde, 0.29, 0.51; erythrose, 1.81, 0.77; hydroxymalonaldehyde, 3.12, 0.75.

RESULTS AND DISCUSSION

The aldehydes in the periodate oxidation products were determined by a modification [8] of the procedure [7] established in this laboratory. All kinds of aldehyde except for glyoxal were quantitatively derived to diethyl dithioacetal trimethylsilylates by a simple procedure of treating oxidized samples with a mixture of ethanethiol and trifluoroacetic acid, followed by hexamethyldisilazane and trimethylchlorosilane. Glyoxal was converted into its bis(dithioacetal). Gas chromatography of the derivatized products made it possible to determine all the aldehydes simultaneously. The accompanying periodate and iodate ions in the oxidation reaction mixtures interfered with the determination of aldehydes, but they were conveniently removed by addition of silver nitrate.

Fig. 1 depicts a typical gas chromatogram of dithioacetal derivatives obtained from the non-dialyzable fraction of human urine. There are three major peaks of lactaldehyde (peak 1), glyceraldehyde (peak 2), and glyoxal (peak 4) derivatives, together with minor peaks of erythrose (peak 5) and hydroxymalonaldehyde (peak 6) derivatives. Lactaldehyde is exclusively derived from the methylpentose at the non-reducing terminal. Since L-fucose is the sole methylpentose constituent found in urinary carbohydrate materials, the amount of lactaldehyde corresponds to that of the terminal L-fucose. Glyceraldehyde arises mainly from the hexose residues at the non-reducing ends. The non-reducing hexose residues substituted at C-2, C-6, and both C-2

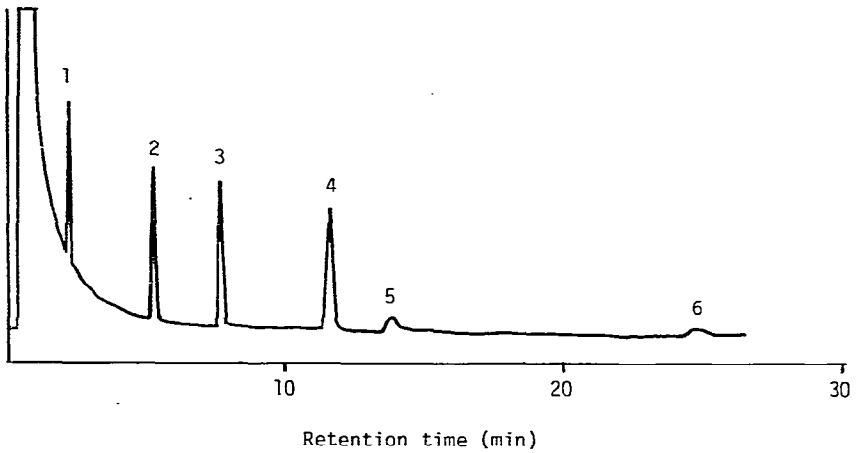

Fig. 1. Gas chromatographic separation of aldehydes in the periodate oxidation products of non-dialyzable urinary carbohydrate materials as diethyl dithioacetal derivatives. Peak assignment: 1, Lactaldehyde; 2, glyceraldehyde; 3, D-xylitol (internal standard); 4, glyoxal; 5, erythrose; 6, hydroxymalonaldehyde.

and C-6 are also possible sources of glyceraldehyde. The D-xylose residue, in which the C-4 hydroxyl group is substituted but the C-2 and C-3 hydroxyl groups are free, can also produce glyceraldehyde, but this portion of glyceraldehyde is small considering the content of D-xylose. Other pentoses can be disregarded since they are not usually present in urinary carbohydrate materials. Glyoxal is a universal aldehyde liberated from the C-1—C-2 part of every non-reducing monosaccharide residue whose C-2 and C-3 hydroxyl groups are not substituted. Erythrose and hydroxymalonaldehyde are unique components formed, respectively, from the C-3—C-4—C-5—C-6 part of the non-reducing hexose residues, in which the C-4 hydroxyl group is substituted but the C-2 and C-3 hydroxyl groups are unsubstituted, and from the C-1—C-2—C-3 part of the non-reducing hexose residues whose C-2 hydroxyl group is occupied but whose C-3 and C-4 hydroxyl groups are free. Oxidizable N-acylhexosamine residues may form dialdehydes, but the component aldehydes are not detected in the gas chromatogram, as they are not derivatized to dithioacetal derivatives under the conditions used for the present assay [9].

Fig. 2 shows the course of oxidation, as observed from the yields of main aldehydes. It also includes the changes in their molar ratios. The yields of all the aldehydes were rapidly increased in the initial 30 min when the non-dialyzable fraction was oxidized at 50°. The rate of increase slowed down thereafter for all the aldehydes. It is noticed that both the lactaldehyde/glyoxal and glyceraldehyde/glyoxal molar ratios remained almost constant throughout oxidation for at least 6 h.

Table I gives the precision data obtained for the products of 3-h oxidation. It is indicated that each aldehyde could be determined with high reproducibility. Efficiency of urine dialysis should not be ignored, since incomplete removal of dialyzable substances caused underestimation of aldehydes for an unknown reason, as seen from Table II. Dialysis should be performed against tap water for at least 24 h. Partial enzymic hydrolysis of some monosaccharide residues

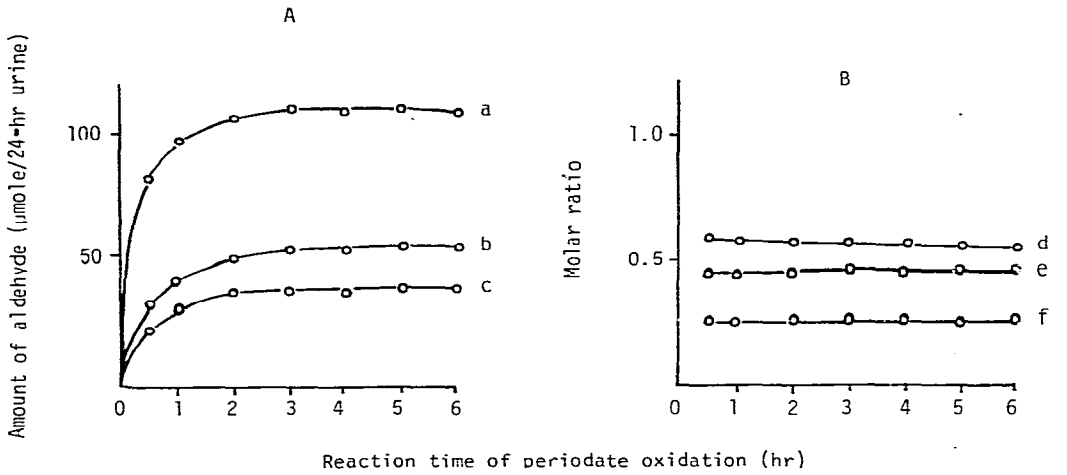

Fig. 2. Course of periodate oxidation of the non-dialyzable fraction of urine, as observed by the yields of aldehydes (A) and their molar ratios (B). a, Glyoxal; b, glyceraldehyde; c, lactaldehyde; d, lactaldehyde/glyceraldehyde; e, glyceraldehyde/glyoxal; f, lactaldehyde/glyoxal.

TABLE I

PRECISION OF THE DETERMINATION OF MAIN ALDEHYDES IN PERIODATE OXIDATION PRODUCTS OF NON-DIALYZABLE URINARY CARBOHYDRATE MATERIALS

No.	Aldehyde* found (μmole/24-h urine)			Molar ratio		
	GlycerA	LactA	GO	$\frac{\text{GlycerA}}{\text{GO}}$	$\frac{\text{LactA}}{\text{GO}}$	$\frac{\text{LactA}}{\text{GlycerA}}$
1	45	31	118	0.38	0.26	0.69
2	47	29	111	0.42	0.26	0.62
3	43	30	107	0.40	0.28	0.70
4	42	29	102	0.41	0.28	0.69
5	48	29	112	0.43	0.26	0.60
6	43	28	103	0.42	0.27	0.65
7	50	30	115	0.43	0.26	0.60
Average	45	29	110	0.41	0.27	0.65
CV (%)	7.1	3.3	5.5	4.4	3.5	6.8

*GlycerA, glyceraldehyde; LactA, lactaldehyde; GO, glyoxal.

during dialysis may be possible, but this is unlikely to be the case from comparing the amounts of aldehydes for 3-day dialysis in pooled water with 1-day dialysis against tap water. The difference in dialysis time did not influence the yield of aldehyde.

Table III shows the distribution of the main aldehydes for several normal male subjects. The yields of aldehydes varied considerably among subjects, but the molar ratios of lactaldehyde/glyoxal and glyceraldehyde/glyoxal were almost constant, giving average values of 0.41 and 0.27, respectively. The lactaldehyde/glyceraldehyde molar ratio was distributed over a wider range

TABLE II

EFFECT OF DIALYSIS EFFICIENCY ON THE DETERMINATION OF ALDEHYDES

Mode of dialysis	Duration of dialysis (days)	Aldehyde* found (μmole/24-h urine)			Molar ratio		
		GlycerA	LactA	GO	GlycerA / GO	LactA / GO	LactA / GlycerA
Pooled water** (1.1 ml of urine)	1	11	19	67	0.16	0.28	1.73
	2	35	25	91	0.38	0.27	0.71
	3	39	25	94	0.41	0.27	0.64
Tap water (10 l/h)	1	39	26	96	0.41	0.27	0.67
	2	41	25	98	0.42	0.26	0.61

*Abbreviations as in Table I.
**Pooled water was changed every day.

TABLE III

DISTRIBUTION OF MAIN ALDEHYDES FOR NORMAL MALE URINE

Subject No.	Age	Blood group	Aldehyde* found (μmole/24-h urine)			Molar ratio			Amount of L-fucose (mg/day)
			GlycerA	LactA	GO	GlycerA / GO	LactA / GO	LactA / GlycerA	
1	26	A	29	19	71	0.41	0.27	0.66	3.1
2	30	A	48	28	120	0.40	0.23	0.58	4.6
3	42	A	32	30	94	0.34	0.32	0.94	4.9
4	11	A	67	39	150	0.45	0.26	0.58	6.4
5	22	B	43	23	93	0.46	0.25	0.53	3.8
6	22	O	38	23	90	0.42	0.26	0.61	3.8
7	7	O	46	35	112	0.41	0.31	0.76	5.7
8	21	AB	66	37	160	0.41	0.23	0.56	6.1
Average			46	29	111	0.41	0.27	0.65	4.8
CV (%)			30	25	28	9	13	21	25

*Abbreviations as in Table I.

centering at 0.65. Relatively higher values were observed for subjects 3 and 7, who belong to the same family. It is also noticeable that the type of blood group did not significantly affect the yield of aldehyde.

Periodate oxidizes a monosaccharide residue to yield a pair of hydroxyaldehyde (glycolaldehyde, glyceraldehyde, lactaldehyde, or erythrose) and dicarbonyl (glyoxal or hydroxymalonaldehyde) compounds in equimolar amounts. Since glycolaldehyde was not detected and the amounts of erythrose and hydroxymalonaldehyde were very small, the difference between the amount of glyoxal and the total amount of glyceraldehyde plus lactaldehyde is approximately equal to the total amount of the monosaccharide residues that are attached by unoxidizable monosaccharide residues but which possess free hydroxyl groups at C-2 and C-3. These structures will give rise to glyoxal but no unsubstituted hydroxyaldehyde. The proportion of the total amount of such monosaccharide residues relative to that of all monosaccharide residues cleaved amounted to $1 - (0.41 + 0.27) = 0.32$, which is approximately one third. The low yields of erythrose imply that there were only small amounts of hexose residues whose C-4 hydroxyl group is substituted but whose C-2 and C-3 hydroxyl groups are unoccupied. Similarly, the poor yields of hydroxymalonaldehyde indicate a

TABLE IV

DAILY VARIATION OF ALDEHYDES

Fraction No.	Sampling time	Volume (ml)	Aldehyde* found						Molar ratio		
			nmole/ml of urine			μmole/fraction			GlycerA / GO	LactA / GO	LactA / GlycerA
			GycerA	LactA	GO	GlycerA	LactA	GO			
1	11 a.m.	200	89	55	212	17.7	11.0	42.3	0.42	0.26	0.62
2	3 p.m.	120	48	28	110	5.8	3.3	13.2	0.44	0.25	0.58
3	7 p.m.	195	62	36	128	12.1	7.0	25.0	0.48	0.28	0.58
4	11 p.m.	135	55	31	124	7.4	4.2	16.8	0.44	0.25	0.56
Composite		650	67	39	150	43.6	25.4	97.5	0.45	0.26	0.58

*Abbreviations as in Table I.

low abundance of hexose residues having substituted C-2 and free C-3 and C-4 hydroxyl groups.

The data in Table III also allow the estimation of the amount of L-fucose located at the non-reducing terminal. The average value calculated from the amount of lactaldehyde was approximately 5 mg/day. This value was slightly lower than that obtained by a colorimetric procedure [10] (about 7.5 mg/day). The dithioacetal method tends to underestimate aldehydes, especially for small amounts of samples; the losses of both glyoxal and glyceraldehyde for 10^{-8} mole of methyl α-D-glucopyranoside were approximately 25% [8]. On the other hand, the colorimetric procedure is presumed to overestimate them due to interaction with concomitant hexoses and amino sugars.

Table IV gives the aldehyde distribution for urine samples collected from a healthy young man at various times in 24 h. Although the yields of aldehydes per unit volume, i.e. the concentrations of carbohydrate materials, varied among samples, all the molar ratios were again approximately equal independent of collecting time. The non-dialyzable fraction of urine contains a variety of carbohydrate materials including oligosaccharides, acid mucopolysaccharides, and glycoproteins. The constancy of the molar ratios of aldehydes suggests that the mixture ratios of these materials are almost unchanged during 24 h.

The present study of the products of periodate oxidation provides information on the structures of urinary carbohydrate materials. Our program is now to obtain data for pathologically abnormal conditions by this procedure.

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