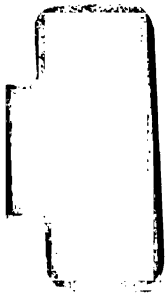
A METHOD FOR THE DEGRADATION OF HAMAMELOSE

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

Barry L. Rhinehart

1967



ABSTRACT

A METHOD FOR THE DEGRADATION
OF HAMAMELOSE

by Barry L. Rhinehart

A degradation procedure for hamamelose (2-C-hydroxymethyl-D-ribose) has been developed whereby the free sugar is first oxidized to the potassium aldonate and this degraded by periodate oxidation to formaldehyde, formate, and oxalate. The products of the periodate oxidation are separated by anion-exchange chromatography on Dowex 1 (Cl^-) resin.

A METHOD FOR THE DEGRADATION OF HAMAMELOSE

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1967

VITA

Barry L. Rhinehart was born on July 28, 1942 in Midland County, Michigan. He was graduated from Breckenridge High School in Breckenridge, Michigan in June of 1960. In 1964, he received the Bachelor of Science degree in chemistry from Alma College in Alma, Michigan. Graduate studies were started at Michigan State University in the Department of Biochemistry in September of 1964 with the aid of a graduate assistantship. He will complete the requirements for the degree of Master of Science in the winter of 1968.

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Paul K. Kindel for his guidance, encouragement, and constructive criticism throughout this investigation.

I would also like to thank Dr. Richard L. Anderson and Dr. Loran L. Bieber for serving on my guidance committee. I am also grateful to Miss Roswitha Blohm for her technical assistance in the enzymatic determinations. Much thanks is also due to many graduate students for their advice and assistance.

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INTRODUCTION

In 1898 a crystalline tannin was isolated from the bark of witch hazel (Hamamelis virginiana) (1). This tannin proved to be peculiar in that it contained a new and unusual sugar. The first evidence of the unusual nature of the sugar component of this tannin was given in 1912 by Fischer and Freudenberg (2). This sugar was named "hamamelose" in 1929 by Schmidt (3) who later proved hamamelose to be a branched-chain sugar having the structure 2-C-hydroxymethyl-D-ribose (4,5).

More recently the hamamelose-containing tannin has been isolated from the bark of chestnut (Castanea sativa) (6) and oak (Quercus rubra) (7). Hamamelose has also been found as the free sugar in the leaves of a variety of plants suggesting that the sugar may be widely distributed throughout the plant kingdom (8).

The pathway for biosynthesis of hamamelose is not known. A logical approach to this problem would involve the administration of ^{14}C-labeled precursors, the isolation of the hamamelose, and the degradation of this hamamelose to determine the distribution of ^{14}C. Such experiments would require a method for degrading hamamelose. This thesis describes such a method.

EXPERIMENTAL PROCEDURE

Materials

Preparation of Hamamelose--Hamamelose was synthesized from D-arabinose by the method of Overend and Williams (9). The product was obtained as a syrup.

Preparation of Potassium Hamamelonate--Hamamelose was oxidized to potassium hamamelonate following the general procedure of Moore and Link (10) for the oxidation of aldoses to aldonates. A 500 ml, 3-neck flask was fitted with a thermometer and dropping funnel. To the flask were added 10.5 g of iodine and 150 ml of methanol. The vessel was stoppered and the contents stirred by means of a magnetic stirrer until all the iodine had gone into solution. Using a water bath the iodine solution was heated to 40° and a solution of 3.7 g of hamamelose in 45 ml of methanol was introduced. No further heat was applied. With stirring, 120 ml of 4% KOH in methanol was added over a period of 20 minutes by means of the dropping funnel. The solution was allowed to stir for an additional 10 minutes during which time a white, crystalline precipitate began to form. While stirring an additional 92.5 ml of 4% methanolic KOH was added over 15 minutes. The stirring was continued for another 10 minutes. Most of the remaining color in the

solution was removed by the addition of 5 ml more of the 4% methanolic KOH solution. The mixture was cooled and filtered yielding 3.5 g of crude potassium hamamelonate. This represents a yield of 71.5%. The crude product was recrystallized twice from 80% methanol, ground to a fine powder, and suspended in absolute methanol overnight. Filtration of this methanol suspension yielded 2.7 g of white product. Dried to constant weight in vacuum over P_2O_5, this purified product had a melting point of 173-176°.

$KC_6H_{11}O_7$

Calculated: C, 30.76; H, 4.73

Found: C, 30.7; H, 4.8

Because of the hygroscopic nature of the product, solutions of potassium hamamelonate were prepared from weighed samples dried to constant weight directly in volumetric flasks.

Purification of the Dowex 1 Resin--Prior to its use in the degradation procedure, the Dowex 1-X8 (chloride form, 100-200 mesh) was washed, in sucession, with 3 M NaOH, 10 M acetic acid, and glass-distilled water. The resin was reconverted to the chloride form with 3 M NaCl and finally rinsed with glass-distilled water.

Other Materials--The sodium meta-periodate used was Mallinckrodt, analytical reagent. The tetrahydrofolate formylase was a gift from Dr. J. C. Rabinowitz. The

^{14}C-formaldehyde and ^{14}C-formate were purchased from New England Nuclear Corporation. ^{14}C-Oxalate was purchased from Nuclear Chicago Corporation. All other chemicals were the commercial preparations.

Methods

General Methods--Periodate consumption was determined using the method of Fleury and Lange (11). Formaldehyde was determined using chromotropic acid reagent (12). Formate was determined in two ways: Method A, the enzymatic procedure of Rabinowitz (13) using tetrahydrofolate formylase; and Method B, the colorimetric procedure of Grant (14) involving the reduction of formic acid to formaldehyde. Oxalate was determined by the colorimetric procedure of Calkins (15) involving the reduction of oxalic acid to glycolic acid.

Standard Degradation Procedure for Potassium Hamamelonate--To a small test tube were added in order approximately 22 µmoles of potassium hamamelonate and 190 µmoles of sodium periodate. The resulting 2 ml of solution were mixed and allowed to react for 5 minutes after which time 0.07 ml of 1 N NaOH was added to adjust the pH to 5-6. After an additional 10 minutes, the sample was applied to a 0.6 x 10 cm Dowex 1 (Cl^-) column at the rate of 0.1 ml per minute. The collection of 5 ml fractions was started with the first addition of the sample solution. All further solutions were

passed through the resin at the same rate of 0.1 ml per minute.

The sample tube was washed with two 1-ml portions of water. These washings were applied to the column, each time allowing the liquid above the resin to drop to the resin bed before application. Additional glass-distilled water was washed through the resin until six 5-ml fractions were collected. Following the water wash, the column was treated with 0.125 M sodium chloride until 25 additional fractions were collected. To determine elution volumes of the degradation products all fractions were analyzed individually. Fractions 7 through 31 were analyzed for both formate and oxalate.

After it had been determined in which fractions the three substances appeared, the following fractions were routinely combined and analyzed: fractions 1-6, for formaldehyde; fractions 7-14, for formate; and fractions 21-28, for oxalate. The actual quantity of each substance recovered from the column was determined by taking the value obtained from the analysis and subtracting the value of the corresponding blank (see below).

Identification of the Degradation Products--A dimedon derivative (16) was prepared of the compound occuring in the combined fractions 1-6. The melting point of this derivative was determined and compared with the melting point of the authentic formaldimethone.

Fractions 7-14 were combined, concentrated to a volume of about 5 ml, acidified by the addition of 0.5 ml of 10 M

H_2SO_4, and subjected to liquid-liquid extraction for 10 hours with diethyl ether. The ether-extracted material was neutralized by the addition of 1 ml of diethyl amine and concentrated to a volume of about 1 ml. A sample of this concentrate was spotted on Whatman No. 1 paper opposite known diethylammonium formate. The chromatogram was developed by ascending chromatography in n-butanol-water-diethyl amine (100:15:1) (17). Spots were made visible by spraying the air-dried chromatogram with 0.05% bromophenol blue in 0.2% citric acid. The R_f's of the two compounds were compared.

The combined fractions 21-28 were concentrated to about 5 ml, acidified, and extracted with ether for 8 hours. The ether extract was concentrated to a volume of about 1 ml and applied to a sheet of Whatman No. 1 paper opposite known oxalic acid. The chromatogram was developed by ascending chromatography in n-butanol-pyridine-water (6:4:3) (18). Spots were made visible by spraying the air-dried chromatogram with the above bromophenol blue indicator. The R_f's of the two compounds were compared.

The combined fractions 21-28 were also tested for oxalate using the aniline blue test (19). Without concentrating, the combined fractions were treated with 1 ml of 1% $CaCl_2 \cdot 2H_2O$. The resulting cloudy suspension was mixed and allowed to stand for 30 minutes. The white precipitate was collected by centrifugation and washed, successively, with 1 M acetic acid, glass-distilled water, and 95% ethanol. Five drops of syrupy phosphoric acid were added to the dried

precipitate together with about 1-2 mg of diphenylamine. The mixture was heated over a flame until it began to bubble. The green solution was cooled and diluted with about 2 ml of 95% ethanol. The color produced was compared with the color produced by known calcium oxalate. The blank was conducted using calcium chloride.

To further identify the degradation products a mixture of known compounds was prepared similar in composition to that expected from the degradation of potassium hamamelonate. For details concerning the composition of this mixture see Table 2. The solution was applied to a Dowex 1 column, eluted, and analyzed as described in the degradation procedure. The elution volumes of the knowns and the products of the degradation were compared.

Determination of Blank Values for Formaldehyde, Formate, and Oxalate Fractions--A blank value was determined for each of the three compounds isolated from the degradation. For each compound a blank solution was prepared. This solution consisted of a mixture of known compounds in the quantities and concentrations expected following degradation of potassium hamamelonate with the exclusion of that one substance--either formaldehyde, formate, or oxalate--for which the blank value was being determined. Each blank solution was neutralized to pH 5-6 with NaOH when necessary, applied to a column, eluted, and the combined fractions normally containing the missing substance analyzed for that substance. The procedure followed

was exactly as described above in the degradation procedure. For example, the formaldehyde blank solution was prepared from 45.00 μmoles of sodium formate, 22.50 μmoles of oxalic acid, 110 μmoles sodium periodate, and 90 μmoles sodium iodate. The solution was neutralized to pH 5-6, applied to a column, and eluted. Combined fractions 1-6 were then analyzed for formaldehyde.

The blank values from these determinations were zero within the experimental error of the determinations with one exception: the formate blank by colorimetric procedure gave a value, averaged from two determinations, of 4.05 μmoles of "formate."

Extent of Cross-Contamination of the Three Degradation Products Using Radioactive Compounds--To determine to what extent each degradation product contaminates the other two following their separation, three degradation experiments were conducted on potassium hamamelonate--one in the presence of ^{14}C-formaldehyde; one in the presence of ^{14}C-formate; and one in the presence of ^{14}C-oxalate and the products separated as above. The experimental details are described in Table 4.

RESULTS

From the periodate number of 4.0 and the production of 2.0 moles of formaldehyde (Table I), the other degradation products of potassium hamamelonate were predicted to be 2.0 moles of formate and 1.0 mole of oxalate. This is what was found (Table II).

Formaldehyde was identified as a product of the degradation by its positive reaction with chromotropic acid reagent (Table I), by its elution as a neutral compound from the Dowex 1 column (Figure 2), and by the preparation of its dimedon derivative. The dimedon derivative had a melting point of 189-191°C compared with a melting point of 190-192° for the authentic formaldimethone.

Formate was identified by its position of elution from the Dowex 1 column compared with known formate (Figures 1 and 2), but more so by its positive reaction with the tetrahydrofolate formylase (20), and its identical R_f with known formate after paper chromatography.

Oxalate was identified by its position of elution from the Dowex 1 column compared with the elution of known oxalate (Figures 1 and 2), by its identical R_f with known oxalate after paper chromatography, and by its positive aniline blue test.

The data in Table III show the isolation procedure

described results in the quantitative recovery of formaldehyde, formate, and oxalate from the Dowex 1 column. Furthermore, the data in Table IV show that these compounds are isolated with very little cross-contamination. At least part of the contamination that is observed in the formate fraction from labeled formaldehyde may be due to formate produced by the spontaneous oxidation of the ^{14}C-formaldehyde prior to its use. Part of the contamination of the ^{14}C-oxalate into the formate fraction may be due to a small amount of residual ^{14}C-formate present in the ^{14}C-oxalate since the preparation of the ^{14}C-oxalate was by the alkaline fusion of ^{14}C-sodium formate.

TABLE I

Periodate Consumption and Formaldehyde Production of Potassium Hamamelonate

Periodate numbers were determined using two different periodate concentrations, 0.13 M and 0.017 M. The first number in parenthesis was obtained from the reaction conducted at a beginning periodate concentrations of 0.13 M. The second number was obtained when the beginning periodate concentration was 0.017 M. The number in front of the parenthesis is the numerical average of these two numbers. Each determination was conducted on a separate reaction mixture containing 15.75 μmoles of potassium hamamelonate and 190 μmoles of sodium periodate.

The quantity of formaldehyde produced from the periodate oxidation of potassium hamamelonate was measured using a reaction mixture of 1 ml of 0.19 M sodium periodate and 0.5 ml of 0.0315 M potassium hamamelonate. A reaction blank of 1 ml of 0.19 M sodium periodate and 0.5 ml water was also prepared. At the indicated time periods, 0.25 ml of the reaction mixture and the reaction blank were quickly transferred to tubes containing 1 ml of 1 M sodium arsenite, 1 ml sulfuric acid, and 0.25 ml water. The resulting solutions were mixed immediately and allowed to stand for at least 10 minutes before analyzing them for formaldehyde.

Reaction Time	Periodate Number	Formaldehyde Produced per Mole of Potassium Hamamelonate
(min)		(moles)
5	4.03 (4.02, 4.03)	1.94
15	4.02 (4.03, 4.00)	1.97
30	4.03 (4.05, 4.00)	1.98
60	4.07 (4.10, 4.03)	1.96

Figure 1. Elution profile of formaldehyde, formate, and oxalate from a known mixture. To a small test tube were added, in order, 43.50 μmoles of formaldehyde, 45.00 μmoles of sodium formate, 22.50 μmoles of oxalic acid, 110 μmoles of sodium periodate, and 90 μmoles of sodium iodate. The solution was neutralized to pH 5-6 with NaOH, applied to the Dowex 1 column, eluted, and analyzed exactly as described in the "Methods" for the degradation of potassium hamamelonate. Each fraction contains 5 ml. The elution profile shown corresponds to the first experiment in Table III.

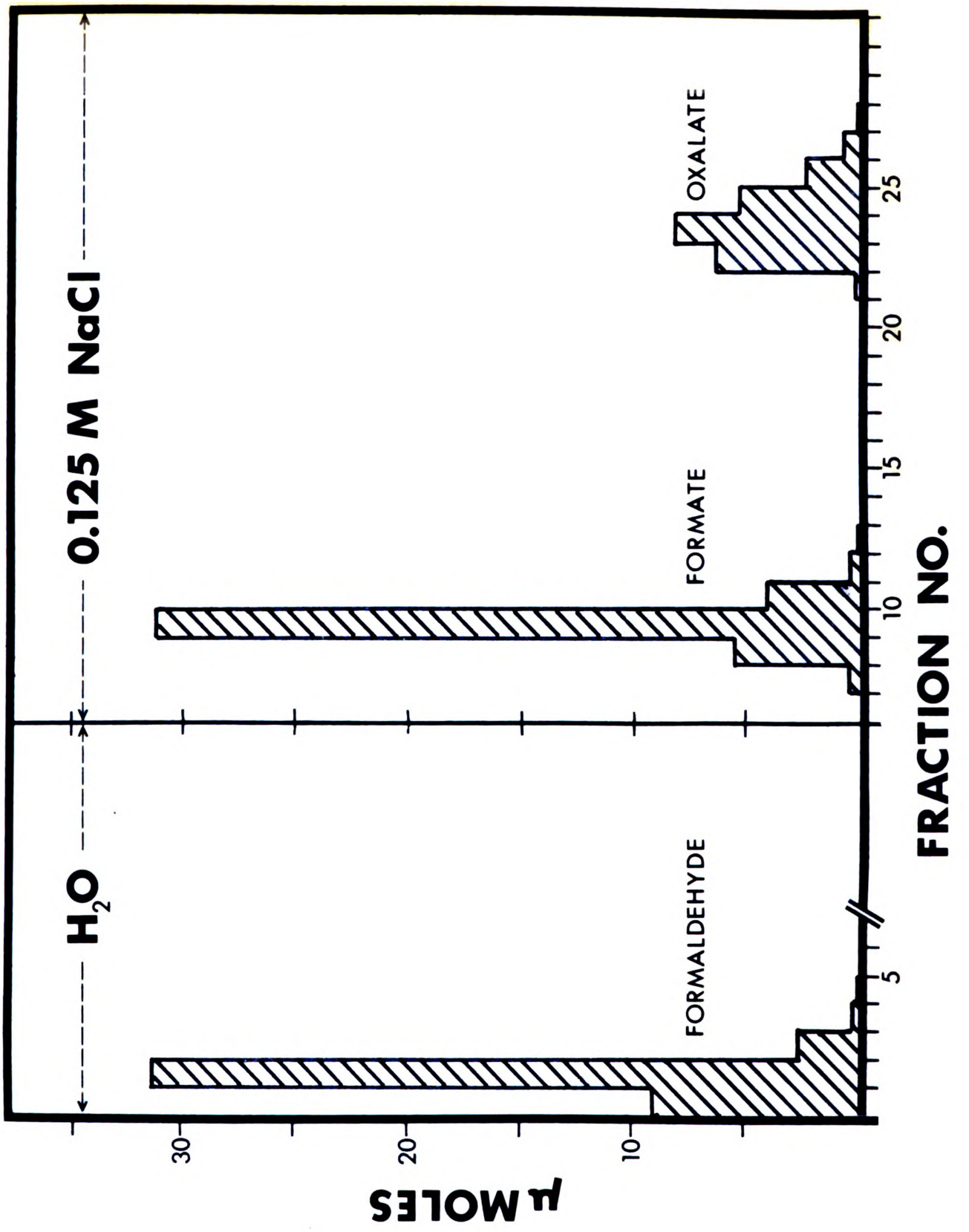

H2O
0.125 M NaCl
FORMALDEHYDE
FORMATE
OXALATE
μ MOLES
30
20
10
5
10
15
20
25
FRACTION NO.

Figure 2. Elution profile of the products from the periodate degradation of potassium hamamelonate. To a small test tube were added, in order, 22.05 μmoles of potassium hamamelonate and 190 μmoles of sodium periodate. This reaction mixture was neutralized, applied to the Dowex 1 column, and analyzed exactly as described in the "Methods" for the degradation of potassium hamamelonate. Each fraction contains 5 ml. The elution profile shown corresponds to the first experiment in Table II.

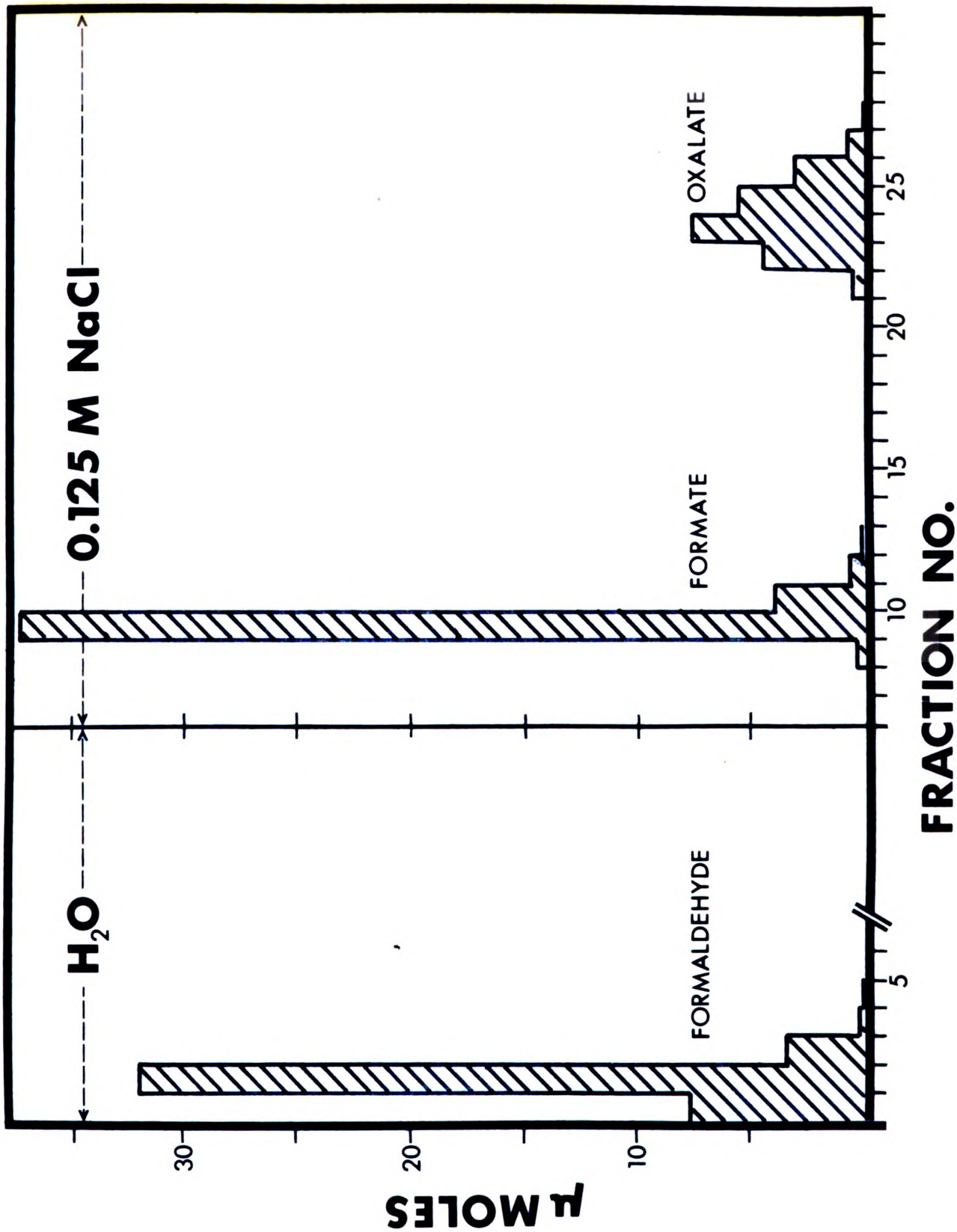

H2O
0.125 M NaCl
FORMALDEHYDE
FORMATE
OXALATE
μ MOLES
30
20
10
5
10
15
20
25
FRACTION NO.

TABLE II

Isolation and Determination of the Degradation Products

Obtained from Periodate Oxidation of Potassium Hamamelonate

These experiments were conducted exactly as described in Figure 2.

Exp. No.	Hamamelonate Used	Degradation Products							
		Formaldehyde		Formate Method A		Formate Method B		Oxalate	
	µmole	µmole	molar ratio*	µmole	molar ratio*	µmole	molar ratio*	µmole	molar ratio*
1	22.05	43.9	1.99	42.9	1.95			22.1	1.00
2	22.05	42.0	1.90	41.1	1.86	44.5	2.02	19.6	0.89
3	22.27	42.5	1.90	45.1	2.02	45.0	2.02	21.3	0.96
Molar ratio averages:			1.93		1.97				0.95

*Moles per mole hamamelonate.

TABLE III

Recovery of Known Compounds from a Dowex 1 Column

The known mixtures were prepared, separated, and analyzed exactly as described in Figure 1.

Exp. No.	Formaldehyde Recovered		Formate Recovered				Oxalate Recovered	
			Method A		Method B			
	µmole	%	µmole	%	µmole	%	µmole	%
1	43.7	100	41.8	93			23.8	106
2	43.8	101	42.2, 52.0*	105	42.5	94	22.1	98
3	43.0	99	41.5, 47.7*	99	46.8	102	21.9	97
Average recoveries:		100%		98.6%				100%

*These two values represent results obtained from two separate determinations of formate. The reason for the difference between the two values is not known.

TABLE IV

Extent of Cross-Contamination of Degradation Products

To a test tube were added 22.27 μmoles of potassium hamamelonate and either ^{14}C-formaldehyde, ^{14}C-formate, or ^{14}C-oxalate. To this was added 190 μmoles of sodium periodate. The solution was neutralized, applied to a column, and eluted exactly as described in the degradation procedure. Fractions 1-6, 7-14, 15-20, and 21-28 were combined and analyzed for radioactivity using Bray's solution (21) and a Packard liquid scintillation counter.

Addition		DPM Recovered				
		Fractions				Total Recovery
		1-6	7-14	15-20	21-28	
	dpm	%	%	%	%	%
^{14}C-Formaldehyde	1.07×10^6	94.4	1.1	0.3	0.5	96.3
^{14}C-Formate	1.36×10^6	0.0	97.7	0.07	0.3	98.1
^{14}C-Oxalate	9.54×10^5	0.3	1.9	0.6	93.1	95.9

DISCUSSION

The oxidation of sugars or their derivatives by periodate has long been one of the more useful degradation procedures in determination of isotopic carbon distribution. The sugar derivatives commonly used include osazones, osotriazoles, and methyl acetals.

When the project of developing a degradation procedure for hamamelose was undertaken, the first objective was to determine in what form the sugar could be most efficiently degraded by periodate. The logical first choice was to try to degrade the free sugar. The free sugar proved not to be oxidized easily, reproducibly, or in a potentially useful manner. The degradation always resulted in a slow oxidation of the sugar with a final formaldehyde production of between 1 and 2 moles per mole of hamamelose and a periodate number that varied between 2.5 and 3.5.

Because of the structure of hamamelose neither the osazone nor the osotriazole derivatives can be prepared. The hydrazones of hamamelose, however, can be prepared. Freudenberg (22) reported the preparation of two hydrazone derivatives of hamamelose, the p-toluenesulfonhydrazone and the p-nitrophenylhydrazone. Experiments were conducted to determine if either of these derivatives would be useful in the degradation of hamamelose. The toluenesulfonhydrazone

derivatives of xylose, glucose, and hamamelose were prepared. The theoretical periodate numbers for these three derivatives are, respectively, 3.0, 4.0, and 4.0. Conditions that provided the theoretical periodate number of 4.0 for the hamamelose tosyl hydrazone gave periodate numbers of 5.0 and 7.0 for the corresponding xylose and glucose derivatives. Conditions that provided the theoretical periodate number of 3.0 for the xylose tosyl hydrazone gave a periodate number of 2.0 for the hamamelose derivative. The p-toluenesulfonhydrazone was, therefore, not considered a useful means of degradation for hamamelose.

Likewise, the use of the p-nitrophenylhydrazone was discarded when it was found that one of the theoretical degradation products of the hamamelose derivative, the glyoxylic acid nitrophenylhydrazone, could not be obtained in a pure state.

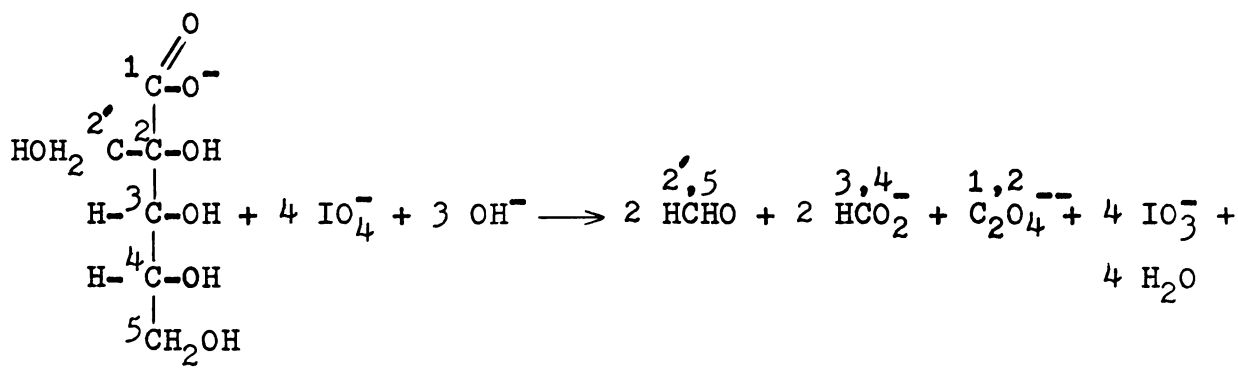
Kandler (23) mentioned the use of the N-benzyl-N-phenylhydrazone derivative in the degradation of another branched-chain sugar, apiose. However, conditions could not be found to reproducibly prepare the benzylphenylhydrazone of hamamelose.

The β-methyl hamamelopyranoside of hamamelose was also tried in the attempt to obtain a degradable derivative. This derivative gave the expected periodate number and appeared to give the expected degradation products. The source of this derivative was as an intermediate in the synthesis of hamamelose described earlier. Unfortunately, conditions could not

be found to convert free hamamelose to the crystalline β-methyl hamameloside.

The potassium aldonate derivative was tried next. It now appears that this derivative is probably the best single choice for a degradable derivative of hamamelose. The data show that hamamelose, as potassium hamamelonate, can be efficiently degraded by periodate and that the resulting degradation products can be easily isolated in quantitative yields by anion-exchange chromatography.

From structural considerations the data support the following reaction of potassium hamamelonate with sodium periodate:



This degradation procedure will have utility in the determination of isotopic carbon distribution in hamamelose since carbon atoms 1 and 2 will be isolated as oxalate, carbon atoms 3 and 4 as formate, and carbon atoms 2' and 5 as formaldehyde. This degradation procedure should be very useful in determination of the biosynthetic pathway for hamamelose. It should be pointed out that the degradation procedure using this derivative does not distinguish carbon atom 1 from 2,

3 from 4, nor 5 from 2'. However such a distinction may not be necessary for the determination of the biosynthetic pathway for hamamelose.

There are at least three different pathways that can be proposed for the biosynthesis of hamamelose: the addition of some one-carbon unit onto a pre-existing pentose; the condensation of two triose molecules; and the rearrangement of some existing hexose.

Two mechanisms can be proposed for the addition reaction: a) the addition of a one-carbon unit to form the C-1 of hamamelose through a photosynthetic intermediate like that proposed by Calvin (24); and b) the addition of a one-carbon unit to form the C-2' of hamamelose. One mechanism that can be proposed for the condensation of two triose molecules involves the aldol condensation of two molecules of glyceraldehyde phosphate postulated by Hough and Jones (25). Four rearrangement mechanisms can be proposed: a) with the C-1 of the hexose forming the C-2' of hamamelose; b) with the C-2 of the hexose forming the C-2' of hamamelose; c) with C-3 forming C-2'; and d) with C-6 forming C-2'. The first three of these rearrangements could utilize a nucleotide-bound hexose intermediate (26,27). The last rearrangement is more likely to proceed through a cyclic intermediate of the type postulated by Shemyakin *et al.* (28).

The first experiment could involve a short-term photosynthesis experiment using $^{14}CO_2$. If the majority of the label were to occur in the oxalate fraction then, theoretically,

hamamelose could have been synthesized by the addition mechanism type "a." However, Kandler (8) has shown that hamamelose is not an early product of photosynthesis and hence would probably not be labeled during a short-term experiment. He has also shown that hamamelose is not labeled when methyl-^{14}C methionine is administered. This suggests that hamamelose is not synthesized by either addition reactions.

The administration of ^{14}C-glucose labeled in various carbon atoms would be a good choice for the elucidation of the mechanism of biosynthesis of hamamelose. In Table V are listed the different mechanisms proposed above and the expected distribution of C^{14} in hamamelose following administration of various C^{14}-labeled glucoses. The table assumes that, in the condensation mechanism, hexose would cleave to triose <u>via</u> aldolase and directly condense to form hamamelose.

As indicated in Table V the degradation procedure described in this thesis will distinguish which postulated mechanism is operative for the biosynthesis of hamamelose.

TABLE V

Theoretical Distribution of ^{14}C in Hamamelose Following Administration of Various ^{14}C-Labeled Glucoses

Mechanism	Carbon Atom Labeled		Fraction Containing ^{14}C After Degradation		
	in Glucose	in Hamamelose	Formald.	Formate	Oxalate
			%	%	%
Condensation	1	2',5	100		
	2	2,4		50	50
	3,4	1,3		50	50
	6	2',5	100		
Rearrangement a) (1 2')	1	2'	100		
	2	1			100
	3,4	2,3		50	50
	6	5	100		
Rearrangement b) (2 2')	1	1			100
	2	2'	100		
	3,4	2,3		50	50
	6	5	100		
Rearrangement c) (3 2')	1	1			100
	2	2			100
	3,4	2',3	50	50	
	6	5	100		
Rearrangement d) (6 2')	1	1			100
	2	2			100
	3,4	3,4		100	
	6	2'	100		

SUMMARY

A degradation procedure for hamamelose (2-C-hydroxymethyl ribose) has been developed whereby the free sugar is first oxidized to the potassium aldonate and this degraded by periodate oxidation to formaldehyde, formate, and oxalate. The products of the periodate oxidation are separated by anion-exchange chromatography.

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