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A METHOD FOR ANALYSIS OF AMINO SUGARS: SPECIFICITY AND MECHANISM OF THE REACTION

JULIO J. LUDOWIEG AND JOSEPH D. BENMAMAN

Department of Orthopaedic Surgery, University of California School of Medicine, San Francisco, California 94122 (U.S.A.)

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ABSTRACT

Two methods for the analysis of amino sugars are presented. Ratios of the colorimetric values obtained with these two methods permit the preliminary characterization of several amino sugars and direct determination when they are in admixture with 2-amino-2-deoxy-D-glucose.

The main features of the mechanism of the color reaction are outlined. Factors affecting the reproducibility of the Elson and Morgan analysis and the role of 2-methylpyrrole in the production of color are discussed.

INTRODUCTION

A valuable method for the analysis of amino sugars is based on their reaction with an alkaline solution of 2,4-pentanedione; the products of this reaction yield, with an acid solution of *p*-dimethylaminobenzaldehyde, a colored solution (Elson and Morgan reaction).

This report describes a modification of a method based on the Elson and Morgan reaction[1]. It considers the possible variables involved in its reproducibility and discusses the mechanism of the color reaction.

RESULTS AND DISCUSSION

Analysis. — The "Hot-Reaction" method*, when the chromogens are formed at 100°, and the "Cold-Reaction" method, when the chromogens are formed at 0° (see Experimental), were used for establishing colorimetric ratios ("Hot-Reaction" to "Cold-Reaction" ratio). These ratios, useful for the preliminary identification of a single amino sugar, are also useful in the direct quantitative analysis of a mixture. As shown in Table I, of all the amino sugars analyzed, a high color intensity ratio was obtained only with 2-amino-2-deoxy-D-glucose. In the "Cold-Reaction" method

*The "Hot-Reaction" and "Cold-Reaction" methods are modifications of previously published "Total-Hexosamine" and "Hexosamine" methods, respectively[1]. The new procedures of analysis are at least twice as sensitive.

this amino sugar gave about 3% of the color intensity produced by 2-amino-2-deoxy-D-galactose

TABLE I

PRODUCTION OF COLOR BY AMINO SUGARS

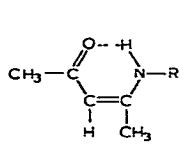
Amino sugar (*0 22 μmole*)	*Absorbance*				
	Cold-Reaction (*525 nm*)		*Hot-Reaction* (*535 nm*)		
		ε^a		ε^a	*Ratio*[b]
2-Amino-2-deoxy-D-glucose	0 020	524	0 598	15700	30 0
2-Amino-2-deoxy-D-allose[c]	0 121	3170	0 616	16350	5 2
2-Amino-2-deoxy-D-galactose	0 600	15750	0 596	15630	1 0
2-Amino-2-deoxy-D-altrose	0 975	25500	0 584	15300	0 60
2-Amino-2-deoxy-D-mannose	0 981	25700	0 492	12900	0 50
2-Amino-2-deoxy-D-gulose	1 073	28200	0 452	11850	0 42
2,6-Diamino-2,6-dideoxy-D-allose	0 152	3980	0 238	6230	1 57
2,6-Diamino-2,6-dideoxy-D-mannose	1 160	30400	0 242	6340	0 21
2,6-Diamino-2,6-dideoxy-D-gulose	1 170	30600	0 334	8750	0 29
2-Amino-2-deoxy-D-glucuronic acid	0 077	2020	0 534	13800	6 83
2-Amino-2-deoxy-D-xylose	0 403	10580	0 664	17400	1 65

[a]Molar absorbance coefficient, based on the concentration of amino sugar (chromogens transformed), in the colored solution [b]"Hot-Reaction" to "Cold-Reaction" [c]Amorphous prepared from a crystalline derivative [P H Gross, K Brendel, and H K Zimmerman Jr *Ann Chem* 683 (1965) 175]

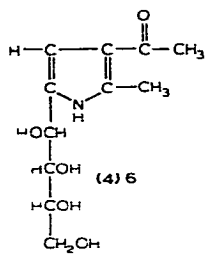
Since amino acids and neutral carbohydrates are known to interfere with the Elson and Morgan reaction[2], colorimetric ratios may be obtained which could, mistakenly, be attributed to an amino sugar. This can be avoided by purification of the hydrolyzed sample[3] When an ion-exchange column is used for the separation and analysis, artifacts of this nature are usually found in elution volumes just following the void volume of the column[4] The hydrolysis and analysis (by the ion-exchange procedure of Gardell[4]) of a protein–polysaccharide (15% protein) from the *nucleus pulposus* of whale intervertebral discs is given as example After this sample was hydrolyzed with 4M hydrochloric acid in a nitrogen atmosphere for 14 h at 100°, three elution peaks were obtained; the second and third peaks corresponded to 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose, respectively The first elution peak was a complex mixture in which several amino acids and galactose were identified by paper chromatography The "Hot-Reaction" to "Cold-Reaction" ratio of this elution volume was 10 However, the color of the solution was anomalous and similar to the color produced by mixtures of amino acids and neutral carbohydrates[5]. The same sample, when hydrolyzed with 6M hydrochloric acid in evacuated, sealed tubes for 21 h at 110°, gave only two peaks, corresponding to 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose

Reaction intermediates — 2-Methylpyrrole[6,7], 3-acetyl-2-methylpyrrole[7],

3-acetyl-5-(D-*arabino*-tetrahydroxybutyl)-2-methylpyrrole[8–10] (**4**) and 2-deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-glucose[11] (**1**) have been isolated from the reaction product of 2-amino-2-deoxy-D-glucose with 2,4-pentanedione The enamine derivatives **2** and **3**, products obtained from the condensation of 2-amino-2-deoxy-D-galactose and



1 R = 2-deoxy-D-glucose
2 R = 2-deoxy-D-galactose
3 R = 2-deoxy-D-mannose



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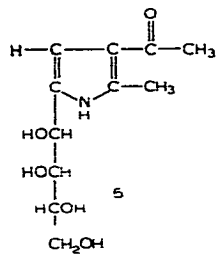

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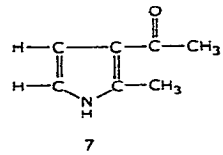

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2-amino-2-deoxy-D-mannose, respectively, with 2,4-pentanedione and 3-acetyl-5-(D-*lyxo*-tetrahydroxybutyl)-2-methylpyrrole (**5**), obtained from 2-amino-2-deoxy-D-galactose, have not been reported before

Role of 2-methylpyrrole — A high color-yield derivative, isolated from the reaction product of a hexosamine with an alkaline solution of 2,4-pentanedione, has been identified as 2-methylpyrrole, this was suggested to be the most important chromogen of the Elson and Morgan reaction[7] This view was challenged by García González *et al*[10], who pointed out that the color produced in the Elson and Morgan reaction and several of its modifications showed an absorption curve with maximum at 530 nm, whereas 2-methylpyrrole gives a maximum at 548 nm with the Ehrlich reagent

Two possible mechanisms have been suggested for the formation of 2-methylpyrrole[7 12 13] Although both appear valid and seem to operate, the role of 2-methylpyrrole in the color produced by the reaction is not clear as yet

The absorption spectra, observed by using the "Hot-Reaction" and "Cold-Reaction" methods, suggest that smaller amounts of 2-methylpyrrole are formed when the chromogens are produced at a lower temperature (Fig 1) The formation of 2-methylpyrrole was demonstrated by lyophilization of the reaction mixtures prior to the development of color, and by analysis of the water of lyophilization with the Ehrlich reagent. Whereas only a trace amount of a volatile chromogen (2-methylpyrrole)[7] was found when the conditions of incubation of the "Cold-Reaction" method were used, considerably higher amounts of 2-methylpyrrole were obtained when the conditions of the "Hot-Reaction" method were used. Thus, when the "Hot-Reaction" method was applied to 2-amino-2-deoxy-D-galactose (one of the amino sugars giving the higher yields of 2-methylpyrrole), the color intensity developed in the water of lyophilization by addition of the Ehrlich reagent was about two-thirds of the total color intensity of the non-lyophilized solution When the "Cold-Reaction" method was applied to 2-amino-2-deoxy-D-galactose, the contribution to the color of the solution by 2-methylpyrrole was found to be negligible (less than 1%) From these results, it is clear that, whatever mechanisms are postulated for the formation of

chromogens by the "Cold-Reaction" method, the formation of 2-methylpyrrole cannot be considered important. However, in the "Hot-Reaction" method, 2-methylpyrrole seems to be one of the predominant factors responsible for the color of the solution.

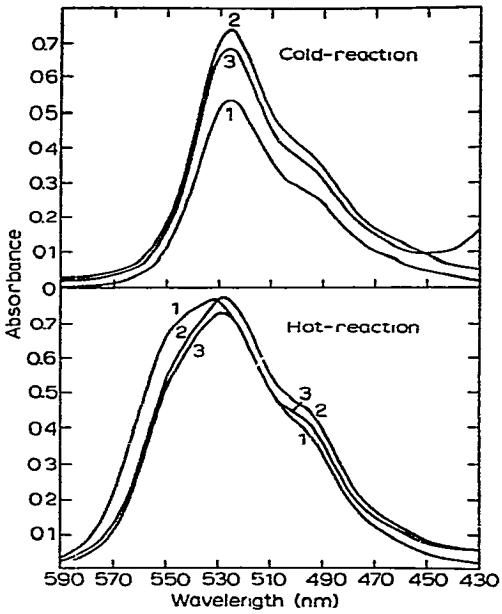

Fig 1 Absorption spectra produced by hexosamines 1 2-amino-2-deoxy-D-galactose, 2 2-amino-2-deoxy-D-mannose, and 3 2-amino-2-deoxy-D-glucose

Transformations of 2-methylpyrrole catalyzed by oxygen and light were observed A fresh alkaline solution of 2-methylpyrrole gave a higher color intensity with the Ehrlich reagent than an equivalent solution that had been previously heated in a glass stoppered tube at 100° However, if a solution containing 2-methylpyrrole is heated in the dark and in the absence of oxygen (in presence of nitrogen), the color intensity of the solution produced with the Ehrlich reagent remains essentially the same In fact, if the "Hot-Reaction" method is used for the analysis of 2-amino-2-deoxy-D-galactose, the color yield increases by 20% when the reaction is carried out in the dark and in the presence of nitrogen gas In a previous study, 2-methylpyrrole could not be identified by paper chromatography[7]. A possible explanation for this was that the compound may have been lost by evaporation from the paper before it was sprayed with the Ehrlich reagent, or partly lost during the heating of analytical samples with an alkaline solution of 2,4-pentanedione[7]. Both evaporation and decomposition of 2-methylpyrrole are variables which may affect the reproducibility of the "Hot-Reaction" method

On the basis of qualitative observations, a possible mechanism of color formation by the "Cold-Reaction" method was proposed[1] (see footnote on Page 1) which would involve the formation of different amounts of enamine derivatives *vs*

chromogens. We have found, however, by quantitative paper-chromatographic analysis, that 2-amino-2-deoxy-D-glucose produced about 20% of **1** and 10% of 3-acetyl-2-methylpyrrole (7), 2-amino-2-deoxy-D-galactose produced about 5% **2** and 20% of 3-acetyl-2-methylpyrrole (7); and 2-amino-2-deoxy-D-mannose produced trace amounts of **3** and about 25% of 3-acetyl-2 methylpyrrole (7). The remaining product(s) of the reaction from 2-amino-2-deoxy-D-glucose was a component having a lower paper chromatographic mobility (R_F 0.12) than the original hexosamines, which gave no color with the Ehrlich reagent and a pink color with the Elson and Morgan reagents An analogous paper-chromatographic component was also formed, but in smaller amount, from 2-amino-2-deoxy-D-galactose, none of this product could be detected when 2-amino-2-deoxy-D-mannose was used. The compound(s) with R_F 0 12 was presumably produced by degradation, since the hexosamines incubated for 18 h at 0° in an alkaline solution (pH 9.5) containing no 2,4-pentanedione produced a similar paper, chromatographic component Apparently 2-amino-2-deoxy-D-glucose is less reactive with 2,4-pentanedione and, as a free hexosamine, is subjected to the deteriorating conditions of an alkaline incubation for a longer period of time than other amino sugars

The low reactivity of 2-amino-2-deoxy-D-glucose with the reagent and the formation of predominantly low color-yield chromogens derived from its enamine derivative **1** seems to be, then, the cause for the negligible color produced by this hexosamine in the "Cold-Reaction" method

In addition to the enamine derivatives and the 3-acetyl-2-methylpyrrole which were produced by the hexosamines, strong color-yield chromogens were also observed on paper chromatograms when 2-amino-2-deoxy-D-galactose and 2-amino-2-deoxy-D-mannose were used in the reaction mixture ("Cold Reaction" method). It was, however, difficult to detect these chromogens (presumably because of their low concentrations in this reaction mixture) when 2-amino-2-deoxy-D-glucose was used as the reactant

EXPERIMENTAL

Ehrlich reagent — A solution of *p*-dimethylaminobenzaldehyde (1 g) in 3 8M perchloric acid (50 ml) was diluted to 100 ml with 95% ethanol

Alkaline 2,4-pentanedione reagent — 2,4-Pentanedione (3 ml) was diluted to 100 ml with 0 7M sodium carbonate The pH of this solution was 9 8 A solution of this reagent (0 5 ml) with 0 3M hydrochloric acid (0 25 ml) had a pH 9 5 When the solution was heated for 20 min at 100°, the pH was 9 4

Cold-Reaction method — Samples (0 25 ml) containing less than 80 μg of amino sugar hydrochloride in 0 3M hydrochloric acid were introduced into tubes refrigerated with ice; alkaline 2,4-pentanedione reagent (0.5 ml) was added to each tube The tubes were glass stoppered, submerged in crushed ice, and incubated in a refrigerator at 0° After 18 h, 90% ethanol (4 ml) was added while the tubes were under refrigeration This was followed by the addition of the Ehrlich reagent (1 ml) The mixtures were shaken after each addition and finally incubated for 1 h at 70°.

After the incubation period, the solutions were cooled for 1 h in a water bath (20–25°), and absorbance measurements were made at 525 nm

Hot-Reaction method — To samples (0 25 ml) containing less than 80 μg of amino sugar hydrochloride in 0 3M hydrochloric acid was added alkaline 2,4-pentanedione reagent (0 5 ml). The solutions were heated in glass stoppered tubes for 20 min at 100°. After cooling, first 90% ethanol (4 ml) and then the Ehrlich reagent (1 ml) were added. The mixtures were shaken after each addition The solutions were then cooled for 1 h in a water bath (20–25°) and absorbance measurements were made at 535 nm

All absorbance readings were made in rectangular cuvettes of 1-cm light-path length with a Gilford spectrophotometer, model 2000

Color intensity — The "Cold-Reaction" method showed a range of linearity of the color up to 3 mg for 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucuronic acid For the remaining amino sugars, the color-linearity range extended to about 80 μg When the "Hot-Reaction" method was used, all the amino sugars analyzed showed a color linearity range up to about 80 μg Intercepts at the absorbance axis were from 0 01 to about 0 04 for both methods of analysis Relative standard deviations (σ/mean) × 100 of the "Hot-Reaction" to "Cold-Reaction" ratios were from 3 to 6%.

Identification of chromogens and intermediates — This identification was made by descending paper chromatography on Whatman Paper No. 1 with propyl alcohol–water (89 28) for 16 h at 5°. Reaction mixtures containing hexosamine (500 μg) in alkaline 2,4-pentanedione reagent (0 25 ml) were incubated by the "Hot-Reaction" and "Cold-Reaction" methods described above Purple spots of the same chromatographic mobility—(R_F 0 71) corresponding to **1**, **2**, and **3**—appeared immediately on the paper after it was sprayed with a solution of 5% $FeCl_3 \cdot 5\,H_2O$ in 50% ethanol–water (v/v) The 5-tetrahydroxybutylpyrrole derivatives **4** and **5** were identified by spraying the paper with the Ehrlich reagent. Both compounds **4** and **5** showed the same chromatographic mobility (R_F 0 65), **4**, however, showed initially a yellow spot which turned blue after 24 h, whereas **5** showed initially a yellow spot, which turned purple after 24 h, and 3-acetyl-2-methylpyrrole (R_F 0 90), initially a pink spot, which turned violet after 24 h. The relative color intensity of the spots produced by the enamine intermediates was estimated with the aid of a paper-scanner densitometer 30 min after spraying The color intensity of the substituted pyrrole derivatives was estimated after 24 h The Ehrlich reagent and the Elson and Morgan reagents for paper spraying were prepared according to Partridge and Westall[14]

Analysis of 2-methylpyrrole — To a solution of an amino sugar (800 μg) in 0 3M hydrochloric acid (0.5 ml) was added alkaline 2,4-pentanedione reagent (1 ml) The mixture was incubated according to the "Cold-Reaction" and "Hot-Reaction" methods At the end of the incubation period the reaction mixture was frozen and lyophilized. From the water of lyophilization, collected in a tube refrigerated with a solid carbon dioxide and acetone bath, an aliquot (0 25 ml) was assayed for 2-methylpyrrole with the Ehrlich reagent. Recovery of 2-methylpyrrole was higher than 95%

The enamine **1** and the (tetrahydroxybutyl)pyrrole derivative of 2-amino-2-deoxy-D-glucose (**5**) were prepared by the methods of García González *et al.*[10], and 3-acetyl-2-methylpyrrole and 2-methylpyrrole by the method of Cornforth and Firth[7]. Several attempts were made to prepare **6** from 2-amino-2-deoxy-D-mannose, none of them succeeded It is of interest to note, however, that cyclization of **1** and **3** should give the same (tetrahydroxybutyl)pyrrole derivative **4**. Compounds **4** and **6** are, therefore, identical

Compounds **2** and **3** showed properties in common with those exhibited[10,11] by **1**, and in neutral or alkaline solutions (pH 9 5) underwent ring closure reactions to produce 3-acetyl-2-methylpyrrole and the corresponding (tetrahydroxybutyl)pyrrole derivatives **5** and **4** (**6**) respectively With the exception of 2-methylpyrrole, the remaining intermediates gave, under analytical conditions, colored solutions of lower intensity than those produced by the corresponding hexosamines[10,11]

2-Deoxy-[2-(4-oxo-2-pentenyl)amino]-D-galactose (**2**) — A mixture of triethylamine (25 ml), 2,4-pentanedione (25 ml), and pyridine (0 5 ml) in abs methanol (50 ml) at 0° was added to solid 2-amino-2-deoxy-D-galactose hydrochloride (1 g) The reaction mixture was stirred for 2 h at 0° under nitrogen and concentrated in a flash evaporator below 15° to about 25 ml To this solution was added 100 ml of 50% (v/v) petroleum ether in ethyl ether, an amorphous precipitate separated, which was dried in vacuum, dissolved in abs methanol, and precipitated again by the addition of a large excess of ethyl acetate Compound **2** separated first as an oil which crystallized after being kept for four months at 5°, 0 15 g, m p 115° with softening at 100°; $[\alpha]_D^{20}$ −133 5° (*c* 0 12, water); $\lambda_{max}^{H_2O}$ 312 nm (ε 18,000), λ_{max}^{KBr} 6 16 μm (> C=O), weak band at 3 16 μm for intramolecularly bonded > NH[15,16]

Anal Calc for $C_{11}H_{19}NO_6$· C, 50 56, H, 7 33, N, 5.36 Found C, 49.91, H, 7.32, N, 5 80

2-Deoxy-2-[2-(4-oxo-2-pentenyl)amino]-D-mannose (**3**) — To a solution of 2-amino-2-deoxy-D-mannose hydrochloride (1 g) in 0.8M sodium carbonate (3 ml) at 0° was added 1 ml of 45% (v/v) of 2,4-pentanedione in acetone The reaction mixture (pH 7 6) was stirred for 3 days at 0° and lyophilized The residue of lyophilization was extracted with two portions of 10 ml each of abs methanol and filtered Addition to the methanol solution of an excess of ethyl acetate (120 ml) precipitated a yellow hygroscopic material which was rapidly removed by filtration Compound **3** crystallized out (0 25 g) from the filtrate after being kept overnight at 5°; m p 127–29°; $[\alpha]_D^{20}$ −197° (*c* 0 3, water), $\lambda_{max}^{H_2O}$ 312 nm (ε 21,400), λ_{max}^{KBr} 6 18 μm (> C=O) weak band at 3.16 μm for intramolecularly bonded > N-H[15,16]

Anal Calc for $C_{11}H_{19}NO_6$ C, 50.56; H, 7 33, N, 5 36 Found C, 50.63, H, 7.53, N, 5.30

The yellow hygroscopic material was shown by paper chromatography to consist of several compounds, among them **3** and a second major component having the same paper-chromatographic mobility and color behavior (with the Ehrlich reagent) as **4**

3-Acetyl-5-(D-lyxo-tetrahydroxybutyl)-2-methylpyrrole (**5**). — A solution of

0 8M sodium carbonate (3 ml) at 0° was added to 2-amino-2-deoxy-D-galactose hydrochloride (1 0 g), 1 ml of 45% (v/v) 2,4-pentanedione in acetone was then added (pH of final mixture 7 6) and the mixture was kept for 3 days at 0° This solution was then lyophilized and extracted with two portions of 10 ml each of abs methanol. After filtration an excess of ethyl acetate (120 ml) was added, the mixture was filtered again, and the solution was kept overnight in the refrigerator, compound **5** crystallized out (0 1 g); m p 134–35°, $\lambda_{max}^{H_2O}$ 212, 247, 290 nm (ε 18,400, 7460, 625), λ_{max}^{KBr} 3 08 μm (> NH), 6 12 μm (>C=O); $[\alpha]_D^{20}$ +20° (*c* 1.0, water)

Anal Calc for $C_{11}H_{17}NO_5 \cdot H_2O$ C, 50 56; H, 7 33; N, 5 36 Found C, 50 80, H, 7 12, N, 5 34

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