Determination of 2-Acetamido-2-deoxy-D-galactose and Mechanism of Formation of Chromogens

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A method for the colorimetric determination of 2-acetamido-2-deoxy-D-galactose was developed. The procedure is based on the high reactivity of the aldehyde group of this amidosugar with pentane-2,4-dione in anhydrous alkaline conditions. The product of reaction was crystallized and the structure 1-C-acetonil-2-acetamido-2-deoxy-D-galactitol was deduced from chemical evidence. When the *N*-acetyl group of this compound is split off by hydrolysis, the formation of pyrrole groups ensues by condensation of the free amino group with the carbonyl group of the chain at C-1. 2-Methylpyrrole was isolated by steam distillation after mild alkaline hydrolysis and estimated by reaction with *p*-dimethylaminobenzaldehyde. A more complex pyrrole is formed during acid hydrolysis under the conditions used in the direct Ehrlich reaction.

The mechanism of the reaction of 2-amino-2-deoxy-D-hexoses with pentane-2,4-dione was investigated by Cessi & Serafini-Cessi (1963) and Serafini-Cessi & Cessi (1970). If the condensation, the first step of the Elson–Morgan (1933) reaction for the determination of aminohexoses, is carried out in anhydrous methanol and triethylamine, pentane-2,4-dione reacts with the aldehyde group of the aminohexoses, provided that the amino group is protected. Pentane-2,4-dione itself affords the protecting reaction. When the *N*-substituent is removed the acetonil substituent at C-1 reacts with the amino group and a pyrrole ring is formed, with the possible loss of the polyhydroxylic side chain. 2-Methylpyrrole, which is the main contributor to the colour in the Elson–Morgan reaction (Cornforth & Firth, 1958), is separable by distillation. The overall reaction proceeds more readily with 2-amino-2-deoxy-D-galactose than 2-amino-2-deoxy-D-glucose.

The same principle holds for the 2-acetamido-2-deoxyhexoses, where the amino group is protected and the aldehyde group is available for the reaction with pentane-2,4-dione. Hydrolysis of the acetamido linkage is followed by cyclization and production of pyrroles. The high reactivity of *N*-acetylgalactosamine with pentane-2,4-dione in anhydrous conditions allows specific determination of this sugar. The product of condensation of 2-acetamido-2-deoxy-D-galactose with pentane-2,4-dione has been separated in a crystalline form, and its chemical structure gives further support to the views of García González & Gómez Sánchez (1965) and Ludowieg & Benmaman (1968) on the mechanism of formation of chromogens in the Elson–Morgan reaction.

Experimental

Materials

2-Acetamido-2-deoxy-D-hexoses were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals were reagent grade. 2-Methylpyrrole was prepared as described by Cornforth & Firth (1958). 1-C-(1-Acetylacetonil)-2-deoxy-2-(1-methyl-3-oxobutenyl)amino-D-galactitol was synthesized as described by Serafini-Cessi & Cessi (1970).

Reagents. Pentane-2,4-dione reagent: this consists of (v/v) 25% pentane-2,4-dione and 25% triethylamine in anhydrous methanol and should be prepared freshly. Ehrlich reagent: a solution of *p*-dimethylaminobenzaldehyde (0.8%, w/v) in ethanol containing 3.5% (v/v) conc. HCl was used. Borate buffer, pH8.0: this buffer was prepared as described by Cessi & Serafini-Cessi (1963), and contained 50.2 g of $K_2B_4O_7 \cdot 4H_2O$ and 40.6 g of H_3BO_3 in 1 litre. It was stored at 35°C. When the effect of pH was investigated, this was adjusted with H_3BO_3 or KOH.

Chemical determinations

The apparatus described by Cessi & Serafini-Cessi (1963) was used for the distillation of 2-methylpyrrole. The spectrophotometric determinations were carried out by a Zeiss PMQ II spectrophotometer. The i.r. spectra were obtained by the Infracan Hilger and Watts H 900. The n.m.r. (nuclear-magnetic-resonance) spectra were recorded on a Joel C-60 HL instrument; 2H_2O was used as solvent and sodium 2,2-dimethyl-2-silapentane-5-sulphonate ($\tau = 9.98$).

Uncorrected melting points of the compounds were determined by the sealed capillary-tube method.

Paper chromatography

Ascending chromatography on Whatman no. 1 paper was developed for 8 h with butan-1-ol-ethanol-water (5:1:4, by vol.) and sprayed with alkaline permanganate or Ehrlich reagent (5 min, 110°C).

Results

Reactions of 2-acetamido-2-deoxy-D-hexoses with pentane-2,4-dione

The condensation of 2-acetamido-2-deoxy-D-galactose with pentane-2,4-dione was carried out with pentane-2,4-dione reagent at 55°C for 20 h. After evaporation of most of the reagent under reduced pressure, the residue was washed with light petroleum (b.p. 40–60°C) and dried. The residue was dissolved in anhydrous methanol and crystallized twice at 5°C. The crystallization of the compound was slow, taking over 1 month. A single spot (R_F 0.30, purple with Ehrlich reagent) was observed on paper chromatography. The structure 1-C-acetyl-2-acetamido-2-deoxy-D-galactitol (I) was proposed for this compound (Found: C, 47.1; H, 7.7; N, 5.1; $C_{11}H_{21}O_7N$ requires: C, 47.3; H, 7.6; N, 5.0%). The compound had m.p. 171–173°C. The u.v. spectrum failed to show any typical absorption. The i.r. spectrum in Nujol showed bands at 1610 and 1550 cm^{-1} typical of the monosubstituted amides. The following relevant signals were seen by n.m.r., in addition to the singlet at $\tau=8.0$ due to the acetamido methyl group of the parent amido sugar: a singlet at $\tau=8.6$, attributable to a methyl group, and a doublet (2H) centred at $\tau=7.8$, due to a methylene group, both belonging to the new acetyl residue. The shift of the signal at $\tau=8.6$ due to the methyl group does not indicate an adjacent carbonyl group, and the possibility of a semiacetalic structure of the molecule must be considered.

A second non-crystalline compound was present in the mother liquors. It gave a spot by paper chromatography (R_F 0.5), which became deep-purple with Ehrlich reagent.

When 2-acetamido-2-deoxy-D-glucose was allowed to react with pentane-2,4-dione under the above conditions, 75% of the amido sugar was collected unchanged in crystalline form. From the mother liquors only an amorphous derivative was traced by paper chromatography (R_F 0.52), which gave colour with the Ehrlich reagent.

Transformation of compound (I) into pyrroles

Compound (I) is converted into 2-methylpyrrole by heating in 0.25 M- Na_2CO_3 buffer (100°C, 60 min).

Fig. 1. Yields of 2-methylpyrrole from 0.2 μ mol of compound (I)

Values are in E_{548} units after hydrolysis (100°C, 1 h): ●, in borate buffer at different pH values; ○, in 0.25 M- Na_2CO_3 , adjusted to different pH values with conc. HCl.

2-Methylpyrrole was separated by steam-distillation; the u.v. spectrum and R_F value on paper chromatography were identical with those of an authentic sample of the compound prepared as described by Cornforth & Firth (1958). 2-Methylpyrrole could be easily estimated by Ehrlich reagent at 548 nm. Fig. 1 shows conversion of compound (I) into 2-methylpyrrole in two different buffers at various pH values.

When compound (I) was heated (70°C, 1 h) directly with Ehrlich reagent, a pyrrole was derived which gave a pigment with *p*-dimethylaminobenzaldehyde with an absorption maximum at 525 nm. The absorption of the pigment from compound (I) in the direct Ehrlich reaction was much higher than that obtained by alkaline hydrolysis and steam distillation. For reasons discussed below, the assumed structure of the chromogen is 2-methyl-5-tetrahydroxybutylpyrrole. Table 1 summarizes the colorimetric properties of the products of reaction of 2-amino-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-galactose with pentane-2,4-dione.

Analytical procedure

The reactions described were developed into an analytical procedure suitable for the differential estimation of 2-acetamido-2-deoxy-D-galactose in the presence of the corresponding D-glucose derivative. Two methods are described that differ in sensitivity and specificity.

Procedure 1. Dried samples containing up to 0.2 μ mol of 2-acetamido-2-deoxy-D-galactose and up to 2 μ mol of the interfering compounds (2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose

Table 1. Formation of pyrroles from derivatives of 2-acetamido-2-deoxy-D-galactose and 2-amino-2-deoxy-D-galactose by direct Ehrlich reaction and after mild alkaline hydrolysis and distillation

The product of condensation of 2-acetamido-2-deoxy-D-galactose with pentane-2,4-dione was compound (I), that derived from 2-amino-2-deoxy-D-galactose was 1-C-(1-acetyl)-2-deoxy-2-(1-methyl-3-oxobut-1-enyl)amino-D-galactitol. Borate buffer, pH 8.0, was used for the alkaline hydrolysis (100°C, 1 h) before the distillation of 2-methylpyrrole. The yield of 2-methylpyrrole was calculated by assuming that this compound after the reaction with Ehrlich reagent had an $\epsilon_{\text{max}, 548} = 82000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Other details are given in the text.

Derivative of	Direct Ehrlich reaction		Distillation of 2-methylpyrrole		
	$\lambda_{\text{max.}}$	ϵ	$\lambda_{\text{max.}}$	ϵ	Yield (%)
2-Acetamido-2-deoxy-D-galactose	525	38000	548	9000	11
2-Amino-2-deoxy-D-galactose	525	37000	548	30000	37

Table 2. Extinctions obtained with different compounds by procedures 1 and 2

The experimental conditions are as described in the text.

Compound	(μmol)	Procedure 1 (525 nm)	Procedure 2 (548 nm)
2-Acetamido-2-deoxy-D-galactose	(0.2)	0.625	0.110
2-Acetamido-2-deoxy-D-glucose	(2)	0.750	0.085
2-Acetamido-2-deoxy-D-mannose	(2)	0.905	0.100
N-Acetylneuraminic acid	(2)	0.120	0

and N-acetylneuraminic acid) were incubated with 1 ml of the pentane-2,4-dione reagent for 16 h at 55°C. Excess of the reagent was evaporated under reduced pressure and washed with light petroleum. Ehrlich reagent (10 ml) was added to the dried samples, which were again incubated for 1 h at 70°C, and the extinctions read at 525 nm.

Procedure 2. The condensation step with pentane-2,4-dione was the same as in procedure 1. The dried samples were heated with 10 ml of borate buffer, pH 8.0, in a boiling-water bath for 1 h. 2-Methylpyrrole was distilled (first 2 ml) and condensed with 8 ml of Ehrlich reagent. The colour was read at 548 nm. The use of borate for the conversion into 2-methylpyrrole improved the differentiation between the derivatives of D-glucose and D-galactose, further depressing the colour from 2-acetamido-2-deoxy-D-glucose. Extinction was linear with concentrations of 0.2–2 μmol of 2-acetamido-2-deoxy-D-galactose. Table 2 reports the values of the extinctions obtained by both methods with various acetamidohehexoses.

Discussion

The product of condensation of 2-acetamido-2-deoxy-D-galactose with pentane-2,4-dione is a

5-acetamidodeoxynonulose. The proposed structure 1-C-acetyl-2-acetamido-2-deoxy-D-galactitol is supported by the following evidence: five H in excess of those present in 2-acetamido-2-deoxy-D-galactose were observed by n.m.r. accounting for the CH_3COCH_2 -substituent; neither u.v. absorption nor the i.r. band at 1700 cm^{-1} typical of the diketone side chain (Rasmussen *et al.*, 1949) was recorded. It is inferred that one acetyl group is engaged in a semi-acetalic ring of the new nonulose and the other is lost at some stage of the condensation. Scheme 1 summarizes the hypothetical reaction. The compound was obtained only from 2-acetamido-2-deoxy-D-galactose and not from the corresponding D-glucose isomer. The result confirms that the *trans* configuration of C-3, C-4 of the sugar decreases the reactivity of the aldehyde group towards pentane-2,4-dione. The low reactivity of 2-amino-2-deoxy-D-glucose was pointed out by Serafini-Cessi & Cessi (1970) for the enamino derivative of D-glucose.

Different pyrrole compounds were obtained from compound (I) depending on the conditions of hydrolysis. The heterocyclic ring is formed after the hydrolysis of the amide linkage. In the direct Ehrlich reaction the acid conditions favour the hydrolysis of the acetamido group. As Gottschalk (1960) mentioned, the amino ketones with the amino group in

Scheme 1. Reaction of 2-acetamido-2-deoxy-D-galactose (a) with pentane-2,4-dione (b)

Nu is a nucleophilic species originating from triethylamine; (I), 1-C-acetyl-2-acetamido-2-deoxy-D-galactitol.

the γ -position to the carbonyl are known to form pyrroles by intramolecular condensation of the Schiff's base type even in an acidic medium. The formed pyrrole condenses with *p*-dimethylamino-benzaldehyde and is protected in this way from destruction by hot HCl. A 2-methyl-5-tetrahydroxy-butylpyrrole (III) would be generated by compound (I) by this route in the direct Ehrlich reaction. The pigment shows a strong maximum of absorption at 525nm. The structure of 2-methylpyrrole (II) was demonstrated for the pyrrole formed *after* alkaline hydrolysis.

It is suggested that the transformation of compound (I) occurs via hydrolysis of the *N*-acetyl group and the splitting of the polyhydroxy side chain by a retroaldol reaction. The weak alkaline conditions that allow the retroaldol reaction promote to a limited extent the

hydrolysis of the amide linkage and subsequent cyclization. The low yield of 2-methylpyrrole from the derivative of 2-acetamido-2-deoxy-D-galactose (11%) compared with the yield from the enamine derivative of 2-amino-2-deoxy-D-galactose (37%) would depend on the different lability of the acetamide and enamine linkages under the weak alkaline conditions. Borate buffer also decreases the yield of 2-methylpyrrole, compared with carbonate buffer. The interference of borate is a possible consequence of the interaction of the ion with the polyhydroxy chain of aminosugars (Tracey, 1955). The course of the retroaldol reaction is affected by the structure of C-3 and C-4 of the sugar molecule, as Johansen *et al.* (1960) pointed out in the extreme case of C-3 substituted aminosugars. The pattern of chromogen formation from compound (I) is shown in Scheme 2.

Scheme 2. Cyclization of 1-C-acetyl-2-acetamido-2-deoxy-D-galactitol

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