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Preparation and properties of hexitol-lysyl conjugates[1]

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Summary. The facile preparation of hexitol-lysyl derivatives is reported. Some of the properties of these compounds, particularly those relevant to protein structural studies, are described.

The occurrence in several proteins of glycosyl derivatives in which the linkage is an aldimine bond between a reducing sugar and an amino group (either the N-terminal α-amino or the ε-amino of a lysyl residue) of the protein has recently been reported. Proteins that are substrates for this nonenzymatic derivatization include albumin[4-6], haemoglobin[7,8], collagen[9-12], and crystallins[13]. The function of these is unknown but changes in their concentrations have been reported in pathological conditions, most notably diabetes[4,8,11,12]. The failure to detect these earlier and the lack of knowledge concerning their function is probably the result of their instability under normal protein hydrolysis conditions.

One method of detection is the conversion of the carbohydrate moiety to the 5-hydroxymethylfurfural derivative and the colorimetric assay of this with thiobarbituric acid[7]. The disadvantage of this assay is that it does not distinguish the isomeric form of the sugar attached to the lysyl or hydroxylysyl residue. An alternative technique is the reduction of the aldimine linkage, so forming the more stable hexitol-lysyl. The use of these derivatives in quantifying Schiff bases in foodstuffs has been described[14]. It is the preparation of N^{ε}-hexitol-lysyls and some of their characteristics, particularly under conditions used in protein structural studies, that is described in this communication.

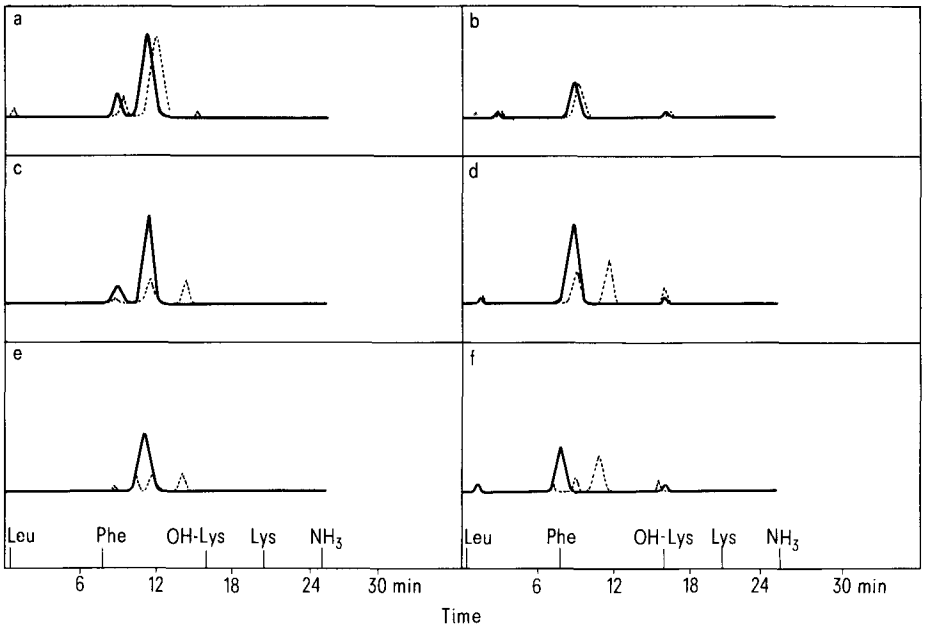
Although both lysine and hydroxylysine have been successfully conjugated to galactose, glucose and mannose, the combination of galactose with these 2 amino acids will be presented here as an example of the procedure. The amino acid (10 µmoles) and the hexose (20 µmoles) are each dissolved in buffer and mixed (final volume of 4 ml) and stirred at room temperature for 1 h. 140 µl of freshly prepared sodium borohydride (50 mg/ml) is added and stirred for another h. The reduction reaction is then stopped by lowering the pH to approximately 3.5 with acetic acid and the mixture lyophilized. After redissolving, the reactants and products are separated by preparative high voltage paper electrophoresis at pH 2.1 and 3000 V. After ninhydrin and/or radioactivity scanning of markers, the hexitol-lysine can be eluted with 2% acetic acid.

The coupling and reduction can be performed in non-amino containing volatile buffers such as pyridine-acetate with yields of up to 50% for the lysyl and 70% for the hydroxylysyl derivatives. The use of such buffers is advantageous for the electrophoretic step. The pH optimum is between pH 6.0 and 6.5 and the number of aliquots in which the sodium borohydride is added does not significantly influence the yield. That the Schiff base does involve the ε-amino rather than the α-amino group of the amino acid is confirmed by performing the synthesis using α-tBOC-lysine[14] (Fluka) and ε-tBOC-lysine (Sigma). After the reduction, the blocking group is removed with 98% formic acid[15]. Only the α-tBOC-lysine yields the same hexitol lysine as when lysine is the substrate.

Frequently these hexitol-lysyl products have to be examined in protein hydrolyzates. So their behavior when subjected to hydrolysis in constant boiling hydrochloric acid in vacuo at 110 °C for 24 h and in 2N sodium hydroxide at 110 °C for 24 h was examined. The figure illustrates the multiple peaks that appear in amino acid chromatograms of acid hydrolyzed hexitol-lysines. There is a close similarity to the pattern reported by Robins and Bailey[9] although their hydrolyses were only for 12 h. They suggested that the formation of acid anhydrides was the cause of the multiplicity of peaks. This increase in the number of ninhydrin-positive species is confirmed by high voltage paper elctrophoresis. The alkaline hydrolyzates yield no ninhydrin-positive material that is detectable either on the amino acid analyzer or by paper chromatography or electrophoresis.

The relative mobilities of these products with respect to serine when electrophoresed at pH 2.1 in a Savant high electrophoresis enclosure are presented in the table. As might be expected at pH 6.4, these behave as neutral amino acids. R_f-values for descending paper chromatography in butanol-acetic acid-water solvents (with or without pyridine) are all less than 0.1 as are lysine and hydroxylysine. However, in phenol-water solvents, better separations are achieved and are detailed in the table. The sugar isomers are not separable by these paper techniques, but can be identified by amino acid analysis.

Thus, although the detection of Schiff bases involving amino groups of the protein and reducing moieties of sugar is difficult due to the lability of these bonds, stabilization



Amino acid analyses of hexitol-lysyls (solid lines) and their acid hydrolysates (dashed lines) on a Beckman 119CL amino acid analyzer. Relative peak sizes are indicated. *a* Galactitol-lysine; *b* galactitol-hydroxylysine; *c* glucitol-lysine; *d* glucitol-hydroxylysine; *e* mannitol-lysine; *f* mannitol-hydroxylysine.

Relative electrophoretic and chromatographic mobilities of lysyl- and hydroxylysyl-galactitol

	Lysine	Lysyl-galactitol	Lysyl-galactitol (acid hydrolyzed)	Glycine	Proline	Hydroxylysine	Hydroxylysyl-galactitol	Hydroxylysyl-galactitol (acid hydrolyzed)
Electrophoresis pH 2.1 (relative to serine)	1.55	1.24	1.63 1.35	1.29	0.83	1.63	1.24	1.50
Chromatography								
a Phenol: water 40 :12 (R_{front})	0.58	0.63	0.44	0.40	0.86	0.33	0.42	0.34
b Phenol: water 30 :8 (R_{front})	0.55	0.54	0.34	0.35	0.89	0.21	0.42	0.27

by reduction with borohydride prior to acid hydrolysis is essential to achieve this purpose. The use of tritiated reductant assists the determination of the sugar isomer. The disadvantage due to the production of multiple peaks from each derivative by acid hydrolysis of the protein is countered by the increased sensitivity of detection gained by the use of a radioactive label and the ability to distinguish the different sugar isomers by amino acid analysis.

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