ISOLATION AND IDENTIFICATION OF 2-ACETAMIDO-1-β-(L-β-ASPARTAMIDO)-1,2-DIDEOXY-D-GLUCOSE FROM PARTIAL HYDROLYSATE OF OVALBUMIN GLYCOPEPTIDE PREPARATION

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In recent years some evidence has accumulated that the polysaccharide moiety of ovalbumin glycopeptide is attached to an aspartyl residue of peptide chain through N-acetylglucosamine (Jevons,1958; Nuenke,Cunningham,1961; Yamashina,Makino,1962; Clamp,Hough,1963; Marks et al.,1963; Micheel et al.,1963).

Concerning the chemical nature of the aspartic acid-glucosamine linkage, there are following three possibilities: (1) iminoether type, the amide group of asparagine linked to C-1 of the glucosamine not through C-N-C but C-O-C bonds (Yamashina,Makino,1962); (2) glucosaminylamine type, an N-glucosaminide, involving the amide group of asparagine (Jevons,1958; Nuenke,Cunningham,1961; Marks et al.,1963), analogous to glycinamide ribotide (Peabody,1956); (3) N-acyl-glucosamine type, peptide linkage of the β-carboxyl group of aspartic acid and the amino group of glucosamine (Micheel et al.,1963).

Marks et al. (1963) have succeeded recently in the synthesis of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose(I) and also in the isolation of a substance

behaving electrophoretically and chromatographycally like compound (I) from partial hydrolysate of ovalbumin glycopeptide. However, they failed to isolate the product in pure, crystalline form.

In this paper we present the evidence of identity of chemically synthesized compound (I) with the crystalline compound resulting from the partial acid hydrolysis of ovalbumin glycopeptide, which was obtained by the enzymatical degradation (Pronase-P) of ovalbumin.

Preparation of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose(I) - During the cource of this study*, a method for the preparation of (I) was developed by Marks et al. (1963). The scheme of its preparation is shown in Fig.1. Acetochloroglucosamine(II) in formamide was heated with NaN_3 at 80° to yield 1-azide-2,3,4,6-tetraacetyl-β-D-glucosamine (III), which was then hydrogenated in dioxane over Adams' catalyst. The resulting 1-amino-2,3,4,6-tetraacetyl-β-D-

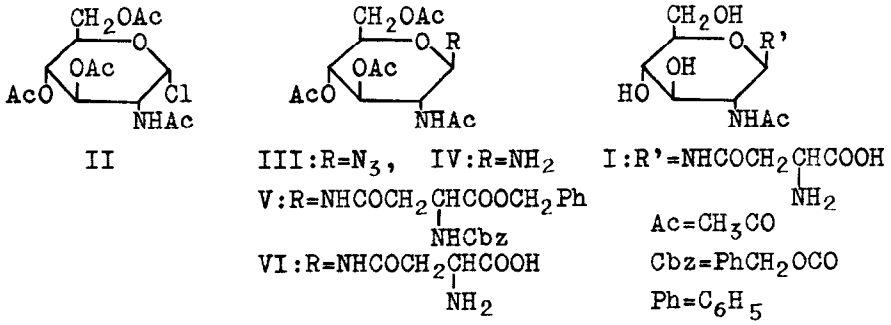

Fig.1 Synthesis of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose

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glucosamine(IV) in tetrahydrofuran was condensed in the presence of dicyclohexylcarbodiimide with α-benzyloxycarbonyl-L-aspartate. Catalytic hydrogenation of this condensation product, 1-(α-benzyloxycarbonyl-L-β-aspartamido)-2,3,4,6-tetraacetyl-β-D-glucosamine(V) in MeOH over 8% Pd-C was followed by deacetylation with 1% Mg methoxide at 0°. The resulting mixture was purified through Dowex 50(H^+) and Amberlite IRC-50(H^+) column. Ninhydrin-positive fractions were evaporated to dryness *in vacuo* to give crystalline compound. After three recrystallization from cold aqueous EtOH, it gave colorless plates(Ia), m.p. 215-222°(decomp.)**, $[\alpha]_D^{24}$ + 23.2(c=1.5 in H_2O), while from hot aqueous EtOH, it gave colorless needles(Ib), m.p. 255-258°(decomp.), $[\alpha]_D^{22}$ + 23.6(c=1.0 in H_2O). The latter (Ib) could be readily reconverted to original compound (Ia) by recrystallization from cold aqueous EtOH. *Anal.*Calcd. for $C_{12}H_{21}N_3O_8 \cdot 3H_2O$: C,37.01; H,6.94; N,10.79. Found: C,37.12; H,7.30; N,10.69. *Anal.*Calcd. for $C_{12}H_{21}N_3O_8$: C,42.98; H,6.31; N,12.53. Found: C,42.66; H,6.45; N,12.16.

From the results of elemental analyses, it was suggested that (Ia) was trihydrate and (Ib) was anhydrous form, however, no dehydration of (Ia) occurred on heating *in vacuo* at 130° for 12 hr. over P_2O_5. Furthermore, both compounds are similar in its behavior on paper electrophoresis (5 N AcOH, Fig.2) and paper chromatography (BuOH:AcOH:H_2O=12:3:5, Rf. 0.14), in spite of considerable differences in the infrared absorption spectra (Fig.3). Finally, it was observed that the 2,4-dinitrophenyl derivatives of two com-

** Marks *et al.* (1963) recorded m.p. 220.5°(decomp.) as monohydrate.

pounds were hydrolysed to DNP-aspartic acid and free glucosamine.

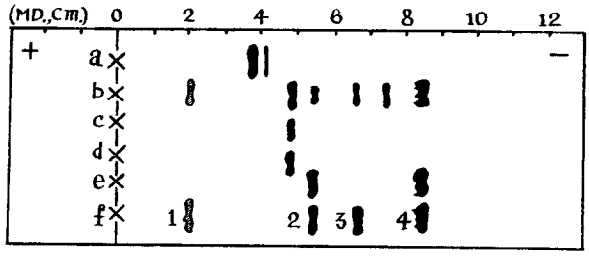

Fig.2 Paper electrophoresis (5 N AcOH, 800 V, 30 min.) a:Glycopeptide(Complex-P); b:Hydrolysate of Complex-P(2 N HCl, 100°, 25 min.); c:Natural (Ia), (Ib); d:Synthetic (Ia), (Ib); e:Hydrolysate of natural (Ib)(2 N HCl, 100°, 3 hr.); f:Authentic compounds (1:N-acetylglucosamine and mannose; 2:aspartic acid; 3:leucine; 4:glucosamine HCl)

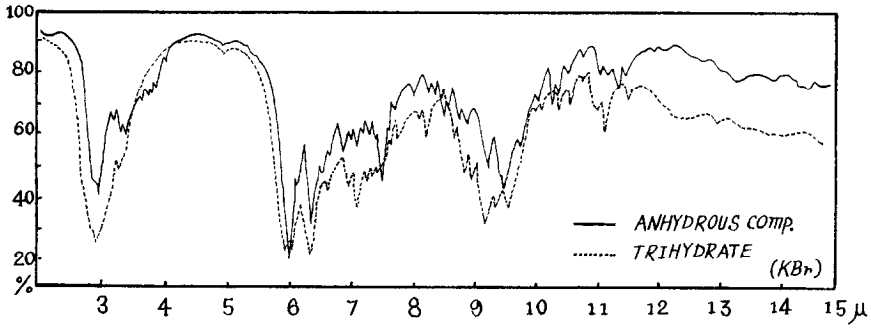

Fig.3 Infrared absorption spectra of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose and its trihydrate

Therefore the conclusion that can be drawn from above findings is that above conversion can be attributed to the presence of transition point at some temperature bellow which (I) crystallizes as the hydrate and above which it crystallizes as the anhydrous form.

Isolation and identification of (I) from the ovalbumin glycopeptide - A salt free suspension of 94 g. of heat-denatured ovalbumin in 4 L. of water containing 0.01 M $CaCl_2$, 200 ml.

of EtOH was treated with 0.4 g. of Pronase-P at pH 8. After two days incubation at 37°, the mixture was filtered, passed through Dowex 50×8(H^+) column and eluted with desalted water. The orcinol-positive fractions containing mannose were collected and followed by immediate neutralization with Amberlite IRA-410(HCO_3^-). The filtrate and washing were combined, and evaporated to 150 ml. in vacuo. After addition of EtOH to this concentrate, it was allowed to stand overnight in the refrigerater and the slightly hygroscopic precipitate was collected by centrifugation. This substance was submitted to above procedure three more times. Orcinol-positive fractions of final procedure were applied to Sephadex G-25 column to remove further impurities, and eluted with water. The desired fractions were collected, reduced, precipitated with EtOH and dried in vacuo to give 2.14 g. of colorless glycopeptide (Complex-P). The mannose content of this preparation was 55.1%. Quantitative analyses of hydrolysate revealed the precence of glucosamine, aspartic acid and leucine in molar ratio of 2.8:1.1:0.3 per five molecules of mannose.

For the isolation of (I), Complex-P was used without further purification. A solution of 1.9 g. of Complex-P in 114 ml. of 2 N HCl was hydrolysed for 25 min. in a boiling water bath. After ice-cooling the mixture was added with 400 ml. of ice-water, neutralized to pH 5.6 with Amberlite IR-4B(OH^-). The filtrate and washing were combined, reduced to 50 ml. in vacuo and passed through Dowex 50(H^+) column. The column was thoroughly washed with water to remove neutral sugars and followed by the displacement elution with 0.15 N ammonia. The desired material stained a blue-violet color, distinguishable from amino acids and

glucosamine, by ninhydrin spray reagent on paper chromatogram. The desired fractions were placed on a Amberlite IR-45(Cl^-) column to remove acidic substances. Finally, the aqueous elute was passed through Amberlite IRC-50 (acetate buffer, pH 4.7, Winters, Kunin, 1949) column to remove basic substances. The elute was collected, evaporated *in vacuo* to a colorless clear syrup, which crystallized from aqueous EtOH in a refrigerater to yield 75 mg. of colorless plates, m.p. 212°(decomp.). Recrystallization from cold aqueous EtOH gave fine plates m.p. 215-222°(decomp.). Recrystallization from hot aqueous EtOH, analogous to synthetic compound (Ia), raised the m.p. to 254-256°(decomp.). $[\alpha]_D^{24}$ + 24.6(c=1.9 in H_2O). *Anal.* Calcd. for $C_{12}H_{21}N_3O_8$: C,42.98; H,6.31; N,12.53. Found: C,42.66; H,6.31; N,12.05.

Both compounds showed no depression of mixed m.p. with synthetic compound (Ia) and (Ib), respectively. Elemental analyses, infrared absorption spectra, paper chromatography and paper electrophoresis also indicated the identity of natural compound with synthetic one.

From these observations it was concluded that asparagine is attached through its amide group to reducing end of N-acetylglucosamine at the connecting point of carbohydrate and peptide in ovalbumin.

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