958. *Synthetic Studies relating to the Carbohydrate–Protein Linkage in Egg Albumin.*

By G. S. MARKS and A. NEUBERGER.

N-(L-β-Aspartyl)-β-D-glucopyranosylamine (Ia) and -D-glucosamine (IIa) have been prepared as models of corresponding derivatives possibly present in egg albumin. α-Benzyl benzyloxycarbonyl-L-aspartate (V) was made to react with tetra-*O*-acetyl-β-D-glucopyranosylamine (III) and -β-D-glucosamine (IV) in the presence of dicyclohexylcarbodi-imide, yielding 2,3,4,6-tetra-*O*-acetyl-*N*-(α-benzyl benzyloxycarbonyl-L-β-aspartyl)-β-D-glucopyranosylamine (Ib) and 1,3,4,6-tetra-*O*-acetyl-*N*-(α-benzyl benzyloxycarbonyl-L-β-aspartyl)-β-D-glucosamine (IIb), respectively. Removal of protecting groups afforded the glucopeptides (Ia) and (IIa). The stability of the *N*-acyl–glucosylamine linkage in (Ia) and of the amide linkage in (IIa) was studied.

THE preparation of a glycopeptide, containing all the sugar residues in crystalline egg albumin has recently been described.[1-3] The nature of the chemical bond linking the carbohydrate to the peptide is of particular interest, and likely structures for this bond were considered. On the basis of the available evidence it was suggested [1] that the most likely structure is that of a β-aspartyl-glycosylamine, although in the absence of direct proof other linkages such as that between an aspartic acid carboxyl group and an amino-group of glucosamine cannot be excluded. It is of interest that aspartic acid is the amino-acid implicated in the linkage of protein to carbohydrate in two other glycoproteins. Gottschalk [4] has obtained suggestive evidence that in ovine submaxillary gland muco-protein the carbohydrate is joined through the reducing end of *N*-acetylglucosamine to the

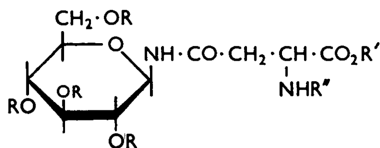
[1] Johansen, Marshall, and Neuberger, *Biochem. J.*, 1960, **77**, 239; 1961, **78**, 518.
[2] Cunningham, Nuenke, and Nuenke, *Biochim. Biophys. Acta*, 1957, **26**, 660.
[3] Jevons, *Nature*, 1958, **181**, 1346.
[4] Gottschalk, *Nature*, 1960, **186**, 949.

β-carboxyl group of aspartic acid by an ester linkage, and Rothfus [5] has evidence that in human serum γ-globulin there is a linkage between aspartic acid and glucosamine. The strength of the bond in the latter protein is indicative of an amide type of linkage which this author suggests might be between the amino-group of glucosamine and the β-carboxyl group of aspartic acid.

The aim of the present work was to synthesize *N*-(L-β-aspartyl)-β-D-glucopyranosylamine (Ia) and *N*-(L-β-aspartyl)-D-glucosamine (IIa) as models of corresponding derivatives possibly present in egg albumin, and to study their properties.

The *N*-acylglycosylamine structure is not novel amongst natural products as it is present in an intermediate in purine biosynthesis, *viz.*, glycine amide ribotide [*N*-glycyl-5′-phosphoribofuranosylamine (VI)].[6,7] Baddiley *et al.*,[8,9] in experiments directed towards the synthesis of this compound, have developed a general method for the synthesis of *N*-glycylglycosylamines. This consists of reaction between an *O*-acylated glycosylamine and benzyloxycarbonylglycyl chloride or benzyloxycarbonylglycyl ethyl carbonate, followed by removal of protecting groups. Thus the benzyloxycarbonyl compound (VII) was obtained by both the acid chloride and the carbonic anhydride route from tetra-*O*-acetyl-β-D-glucopyranosylamine (III) and benzyloxycarbonylglycine.

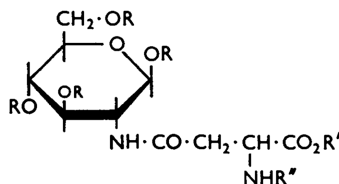
We have synthesized *N*-(L-β-aspartyl)-β-D-glucopyranosylamine (Ia) by a similar method *via* tetra-*O*-acetyl-*N*-(α-benzyl benzyloxycarbonyl-L-β-aspartyl)-β-D-glucopyranosylamine (Ib). The starting point was α-benzyl benzyloxycarbonyl-L-aspartate [10] (V). The preparation of this compound from benzyloxycarbonyl-L-aspartic anhydride calls for some comment since difficulty was experienced in obtaining a pure product by crystallization of the crude material. Le Quesne and Young [11] in a re-investigation of the reaction of benzyloxycarbonyl-L-aspartic anhydride with amino-esters and alcohols demonstrated



(Ia: R = R′ = R″ = H)

(Ib: R = Ac; R′ = CH_2Ph; R″ = O·CO·CH_2Ph)

(Ic: R = R′ = H; R″ = O·CO·CH_2Ph)

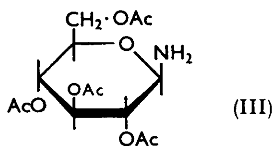


(IIa: R = R′ = R″ = H)

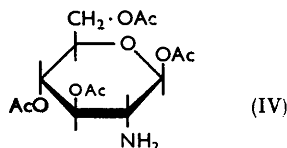
(IIb: R = Ac; R′ = CH_2Ph; R″ = O·CO·CH_2Ph)

(IIc: R = H; R′ = Me; R″ = O·CO·CH_2Ph)

(IId: R = Ac; R′ = R″ = H)



(III)



(IV)

that both α- and β-aspartyl derivatives can be formed. Owing to the closer proximity of the α-carboxyl group than of the β-carboxyl group to the benzyloxycarbonylamino-group, the β-isomer is the stronger acid and Le Quesne and Young were able to utilize this property

[5] Rothfus, *Fed. Proc.*, 1961, **20**, 383.
[6] Peabody, Goldthwait, and Greenberg, *J. Biol. Chem.*, 1956, **221**, 1071.
[7] Hartman, Levenberg, and Buchanan, *J. Biol. Chem.*, 1956, **221**, 1057.
[8] Baddiley, Buchanan, Handschumacher, and Prescott, *J.*, 1956, 2818.
[9] Baddiley, Buchanan, Hodges, and Prescott, *J.*, 1957, 4769.
[10] Bergmann, Zervas, and Salzmann, *Ber.*, 1933, **66**, 1288.
[11] Le Quesne and Young, *J.*, 1952, 24.

to separate some of the isomers by fractional extraction of the mixture from an organic solvent with aqueous sodium carbonate. By utilizing this method of fractionation we were able to obtain pure α-benzyl benzyloxycarbonyl-L-asparate from the mixture of isomers. The rotation of this half-ester was in agreement with that reported by Bryant *et al.*[12] for this compound prepared by an alternative route. Recently, Dr. D. A. Lowther, working in our laboratory, has found a more convenient method of separation by utilizing an ion-exchange chromatographic procedure based on that of Busch, Hurlbert, and Potter [13] for the separation of organic acids. When the mixture of isomers was adsorbed on a strong-base resin in the acetate form, and the isomers were eluted with acetic acid of progressively increasing concentration, the two components were separated and both were recovered crystalline and in good yield.

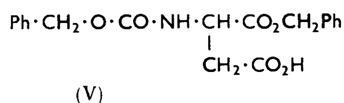
The next step involved the condensation of α-benzyl benzyloxycarbonyl-L-aspartate (V) with tetra-*O*-acetyl-β-D-glucopyranosylamine (III) to give the glucopeptide acetate (Ib). We obtained a good yield by using the carbodi-imide method of Sheehan and Hess [14] for this condensation. Treatment of the product (Ib) with lithium hydroxide in aqueous acetone caused simultaneous hydrolysis of the benzyl ester group and de-*O*-acetylation to the free acid (Ic). Removal of the benzyloxycarbonyl group by catalytic hydrogenation afforded the required *N*-(L-β-aspartyl)-β-D-glucopyranosylamine (Ia). It was of interest that this compound had a pK_2 value of 8·82 which is in close agreement with the pK_2 value of 8·80 reported for the α-amino-group of asparagine.[15] When the glucopeptide (Ia) was heated with the ninhydrin reagent of Moore and Stein [16] a brown colour was obtained identical with that obtained with asparagine.

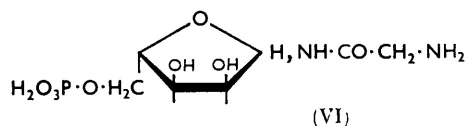
The first synthesis of derivatives of D-glucosamine substituted on the nitrogen atom by acylamino-acid residues was carried out by Bergmann and Zervas.[17] They prepared tetra-*O*-acetyl-*N*-(benzyloxycarbonylglycyl)-β-D-glucosamine (VIIIa) by condensing benzyloxycarbonylglycyl chloride with tetra-*O*-acetyl-β-D-glucosamine (IV); removal of protecting groups afforded *N*-glycyl-D-glucosamine (VIIIb). Link and his co-workers,[18] utilizing this method, have prepared a series of *N*-acyl derivatives of D-glucosamine where the acyl groups are represented by amino-acid residues. They point out that the use of the acid chloride route for the synthesis of these derivatives suffers from the disadvantage that benzyloxycarbonylamino-acid chlorides are notably unstable, decomposing even below room temperature to benzyl chloride and an *N*-carboxy-amino-acid anhydride.[19] To overcome this disadvantage they attempted to acylate tetra-*O*-acetyl-β-D-glucosamine (IV) with acylamino-acylazides. However, the Curtius rearrangement occurred in every case investigated and this route was therefore inapplicable to the synthesis of these compounds. By contrast with these findings, we have found the carbodi-imide method to be particularly useful for the synthesis of these derivatives. Thus tetra-*O*-acetyl-β-D-glucosamine (IV) was condensed with benzyloxycarbonylglycine and α-benzyl benzyloxycarbonyl-L-aspartate (V) to give the glucopeptide acetates (VIIIa) and (IIb) respectively in good yield.

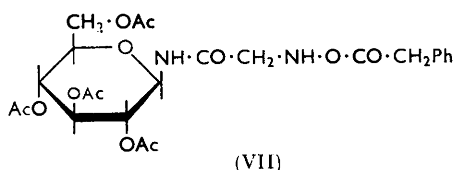
From the glucopeptide acetate (VIIIa) Bergmann and Zervas [17] removed *O*-acetyl groups with sodium methoxide and the benzyloxycarbonyl group by catalytic hydrogenation. Deacetylation with sodium methoxide was, however, inappropriate in the case of the glucopeptide acetate (IIb) since this reagent would be expected to cause transesterification with the formation of an α-methyl ester (IIc). In an attempt to effect simultaneous

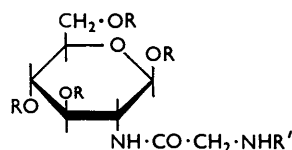
[12] Bryant, Moore, Pimlott, and Young, *J.*, 1959, 3868.
[13] Busch, Hurlbert, and Potter, *J. Biol. Chem.*, 1952, **196**, 717.
[14] Sheehan and Hess, *J. Amer. Chem. Soc.*, 1955, **77**, 1067.
[15] Cohn and Edsall, " Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, 1943 p. 84.
[16] Moore and Stein, *J. Biol. Chem.*, 1948, **176**, 367.
[17] Bergmann and Zervas, *Ber.*, 1932, **65**, 1201.
[18] Doherty, Popenoe, and Link, *J. Amer. Chem. Soc.*, 1953, **75**, 3466; Popenoe, Doherty, and Link *J. Amer. Chem. Soc.*, 1953, **75**, 3469.
[19] Bergmann and Zervas, *Ber.*, 1932, **65**, 1192.

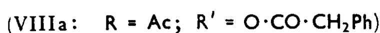
deacetylation and hydrolysis of the benzyl ester group, the glucopeptide acetate (IIb) was treated with lithium hydroxide in aqueous acetone, and after removal of the excess of lithium ions by passage through Zeo-Karb 225 resin (H^+ form) an oil was obtained. This oil was catalytically hydrogenated. The product, thought to be the glucopeptide (IIa), which could not be crystallized, was hydrolysed with N-hydrochloric acid for 2·5 hr., and











the hydrolysate shown by paper chromatography to contain aspartic acid in the expected amount but only a trace of glucosamine. This suggested that dehydration of the glucosamine moiety had occurred since it is known that under the alkaline conditions of the Morgan–Elson procedure (0·05N-sodium carbonate, 100° for 4 min.), *N*-acetylglucosamine is converted into anhydro-chromogenic derivatives.[20] This idea received support from the observation reported in the review by Baer [21] that treatment of penta-acetylglucosamine with barium methoxide or barium hydroxide in cold methanol results in rapid dehydration of approximately half of the compound with the formation of chromogens. To test this possibility the product of alkaline deacetylation and hydrogenation of the glucopeptide acetate (IIb) was treated with Ehrlich reagent. The consequent production of an intense magenta colour confirmed that dehydration of the glucosamine moiety had occurred with the formation of chromogens. These results probably explain the failure of Doherty *et al.*[18] to obtain crystalline products on alkaline deacetylation of *O*-acetyl derivatives of *N*-acylglucosamines, and show that such compounds should not be exposed to alkaline conditions.

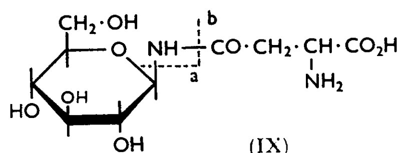
Conditions of acid hydrolysis were then sought which would result in de-*O*-acetylation of the glucopeptide acetate (IIb) without removal of the *N*-acyl group. The hydrolysis of α-glucosamine penta-acetate by 2·5N-sulphuric acid at room temperature was followed polarimetrically; after 36 hr. the specific rotation became constant and equal to that of *N*-acetylglucosamine. After neutralisation of the solution with barium hydroxide and removal of the barium sulphate, the supernatant liquid was shown by paper chromatography to contain *N*-acetylglucosamine together with traces of glucosamine. The residue obtained on evaporation was purified by several crystallisations and shown to be *N*-acetylglucosamine. Accordingly, after removal of the benzyloxycarbonyl and benzyl groups by catalytic hydrogenation, the product (IId) was treated with 2·5N-sulphuric acid at room temperature for 44 hr., and after neutralization *N*-(L-β-aspartyl)-D-glucosamine (IIa) isolated.* It was of interest that when the latter product was heated with the ninhydrin reagent of Moore and Stein [16] a brown colour was obtained identical with that obtained with asparagine. The pK_2 value of 8·82 found for the glucopeptide (IIa) was in close agreement with the pK_2 value of 8·80 found for the α-amino-group of asparagine.[15]

* In a recent brief communication Rothfus [5] reports the preparation of the glucopeptide (IIa); no details are given, however, of the method of preparation, or of the properties of this compound.

[20] Kuhn and Krüger, *Chem. Ber.*, 1956, **89**, 1473; 1957, **90**, 264.
[21] Baer, *Fortschr. Chem. Forsch.*, 1958, **3**, 830.

It has been previously observed [21] that *N*-acyl derivatives of glucosamine, in which the *N*-acyl group is represented by an amino-acid residue, give a positive Morgan–Elson reaction. In accordance with this the glucopeptide (IIa) gave a positive Morgan–Elson test, the coloured complex with *p*-dimethylaminobenzaldehyde showing light absorption with maxima at 538 and 576 mμ, and requiring >4 hr. for the maximum intensity of absorption to be reached at room temperature. These characteristics of the coloured complex enable it to be differentiated from the coloured complex formed by *N*-acetylglucosamine in the Morgan–Elson reaction since in the latter case the coloured complex shows light absorption with maxima at 550 and 590 mμ and the maximum intensity of absorption of both peaks was reached 1·5 hr. after the addition of the *p*-dimethylaminobenzaldehyde reagent.[22] The maximum intensity of the colour given by the glucopeptide (IIa) was 7·5% of that obtained by Aminoff *et al.* with an equal weight of *N*-acetylglucosamine. These observations may be of assistance in establishing the structures of glucopeptide fragments obtained by hydrolysis of glycoproteins.

In further experiments the stability of the *N*-acylglucosylamine linkage in the glucopeptide (Ia) to alkaline treatment was studied. For the conversion of the *N*-acylglucosylamine (Ia) to a nitrogen-free monosaccharide, ammonia, and aspartic acid, two bonds are required to be cleaved, *viz.*, the glucosylamine and the amide bond. It was most convenient to follow the hydrolysis of this compound by measuring the release of ammonia, bearing in mind that the formation of the latter is the result of two consecutive reactions, *i.e.*, fission of bond a or b (see IX) and subsequent hydrolysis of the glucosyl-



(IX)

amine or the asparagine formed. The compound was dissolved in 0·2N-sodium hydroxide and a stream of nitrogen gas passed through the boiling solution to drive the ammonia liberated into a 2% boric acid solution which was titrated at intervals with standard acid. The rate of liberation of ammonia followed first-order kinetics and gave a rate constant of $5{\cdot}5 \times 10^{-3}$ min.$^{-1}$ at 100°. In 0·5N-sodium hydroxide at room temperature the linkage was found by optical-rotation measurement to be stable for 7 hr.

In order to rupture the postulated *N*-acylglucosylamine link in egg albumin and thus separate carbohydrate from peptide, the fission of either bond a or b (see IX) would suffice. From this point of view it was important to know how the rate of liberation of ammonia from the *N*-acylglucosylamine linkage compared with that from the carbon–nitrogen link in both asparagine and glucosylamine, since it was possible that a rapid fission of either link a or b was followed by slow hydrolysis of glucosylamine or asparagine. In such a case conditions milder than those required for the release of ammonia from the *N*-acylglucosylamine link might suffice for the cleavage of either the a or b link. Accordingly, the liberation of ammonia from D-(+)-glucosylamine and L-(+)-asparagine in 0·2N-sodium hydroxide at 100° was measured by the method used for the glucopeptide (Ia) and the rate of liberation of ammonia found to follow first-order kinetics in both cases. A rate constant of $42{\cdot}2 \times 10^{-3}$ min.$^{-1}$ was found for the former compound and $14{\cdot}5 \times 10^{-3}$ min.$^{-1}$ for the latter under these conditions of hydrolysis. These results reveal that the carbon–nitrogen bonds in D-(+)-glucosylamine and L-(+)-asparagine are cleaved considerably more readily than are either of the two carbon–nitrogen bonds (*a*, *b*) in the *N*-acylglucosylamine linkage. Similar findings were obtained with *N*-acetyl-D-glucosylamine, prepared by the method of Brigl and Keppler.[23] The first-order rate constant for the liberation of ammonia from this

[22] Aminoff, Morgan, and Watkins, *Biochem. J.*, 1952, **51**, 379.
[23] Brigl and Keppler, *Z. physiol. Chem.*, 1929, **180**, 38.

linkage by 0·2N-sodium hydroxide at 100° was $2{\cdot}7 \times 10^{-3}$ min.$^{-1}$ compared with $41{\cdot}0 \times 10^{-3}$ and $42{\cdot}2 \times 10^{-3}$ min.$^{-1}$ found for acetamide and D-(+)-glucosylamine respectively under the same conditions. These results rule out the possibility of rapid fission of either link *a* or *b* of the *N*-acylglucosylamine followed by slow hydrolysis of glucosylamine or asparagine.

The stability to acid of the *N*-acylglucosylamine linkage in the glucopeptide (Ia) was next studied. After treatment with N-hydrochloric acid at 100° for 2 hr. the compound was found by paper chromatography to be partially hydrolysed to aspartic acid and glucose. It was most convenient to follow this hydrolysis by measuring the release of glucose by Hoffman's method,[24] adapted for the " Autoanalyser," bearing in mind that we were only measuring the fission of bond *a*. The rate of liberation of glucose followed first-order kinetics and gave a rate constant $4{\cdot}9 \times 10^{-3}$ min.$^{-1}$ at 100° in N-hydrochloric acid, similar to the value ($7{\cdot}8 \times 10^{-3}$ min.$^{-1}$) found for the liberation of glucose, under the same conditions of hydrolysis from *N*-glycyl-β-D-glucosylamine hydrogen oxalate (kindly made available to us by Professor J. Baddiley, F.R.S.).

Since our measurement of the extent of acid hydrolysis of this compound was confined to the measurement of the fission of bond *a*, it was possible that a rapid fission of link *b* is followed by a slow cleavage of the liberated glucosylamine. This is unlikely for two reasons: (*a*) *NN*-dimethylglucosylamine is very rapidly hydrolysed by acid;[25] (*b*) milder conditions of acid hydrolysis used by Baddiley *et al.*,[8] *viz.*, 0·1N-hydrochloric acid at 100° for 4 hr., were without effect on *N*-glycyl-β-D-glucosylamine hydrogen oxalate.

It is of considerable interest that hydrolysis of the *N*-acylglucosylamine linkage in the glucopeptide (Ia) proceeds with approximately the same speed in 0·2N-sodium hydroxide as in N-hydrochloric acid. That hydrolysis is considerably more rapid in alkali than in acid of equivalent strength is a characteristic of amide hydrolysis [26] and suggests that fission of the amide link b is the rate-determining step in both acid and alkaline hydrolysis, followed by a rapid hydrolysis of the glucosylamine linkage a. This idea is supported for the case of acid hydrolysis by the following considerations. Isbell and Frush [27] have studied the mechanism of hydrolysis of L-arabinosylamine and have concluded that the iminium ion, $>C=NH_2^+$, formed by rupture of the ring after addition of a proton to the ring oxygen is a key intermediate. However, resonance in the amide structure of the *N*-acylglycosylamines will result in partial sharing of the lone pair of electrons of the nitrogen atom with the adjacent oxygen-bearing carbon atom. The resultant fractional positive charge on the nitrogen atom would inhibit the formation of the iminium ion, and hydrolysis of the glucosylamine bond would be suppressed. This is in accord with the view that fission of the amide linkage b is the rate-determining step in the hydrolysis of the *N*-acylglucosylamine linkage.

The stability of the amide linkage in the glucopeptide (IIa) to acid hydrolysis was the next subject of study. It was most convenient to follow this hydrolysis by means of the Morgan–Elson reaction. The rate of hydrolysis followed first-order kinetics and gave a rate constant $3{\cdot}4 \times 10^{-3}$ min.$^{-1}$ at 100° in N-hydrochloric acid, similar to the first-order rate constant of $4{\cdot}9 \times 10^{-3}$ min.$^{-1}$ for hydrolysis of *N*-(L-β-aspartyl)-β-D-glucosylamine (Ia) in the same conditions.

Experimental

Potentiometric Titration.—Solutions (1 ml.) of the glucopeptides (Ia) and (IIa) in 0·1N-potassium chloride were titrated. The pH was varied from 2·5 to 11 by using hydrochloric acid and sodium hydroxide of 0·2N-concentration in 0·1N-potassium chloride. A microglass

[24] Hoffman, *J. Biol. Chem.*, 1937, **120**, 51.
[25] Kenner, in " Ciba Foundation Symposium on Chemistry and Biology of Purines," ed. Wolstenholme and Millar, Churchill Ltd., London, 1957, p. 312.
[26] Taylor and Baker, " Sidgwick's Organic Chemistry of Nitrogen," Oxford University Press, 1945, p. 145.
[27] Isbell and Frush, *J. Res. Nat. Bur. Stand.*, 1951, **46**, 132.

electrode was used in conjunction with a potassium chloride salt bridge. Appropriate corrections were made by adding acid or alkali to volumes of 0·1N-potassium chloride similar to those used in the experiments. From the end-points equivalent weights and pK values were determined.

α-Benzyl Benzyloxycarbonyl-L-aspartate.—Benzyloxycarbonyl-L-aspartic anhydride (8·5 g.) and benzyl alcohol (5·4 g.) were heated in a sealed tube at 100° for 4 hr. The resulting syrup was dissolved in ether and extracted with successive portions of aqueous sodium carbonate; the later fractions crystallized on acidification, to give α-benzyl benzyloxycarbonyl-L-aspartate which recrystallised from ethyl acetate–light petroleum, then having m. p. 84—85° (2·5 g.), $[\alpha]_D^{23}$ −14·2° (c 2·5 in acetone). Bergmann, Zervas, and Salzmann [10] record m. p. 84—85°; Bryant *et al.*[12] record $[\alpha]_D^{17}$ −14·8° (c 5·0 in acetone). The earlier fractions were combined and additional quantities of the pure α-benzyl ester were obtained by repetition of the extraction procedure.

*2,3,4,6-Tetra-*O*-acetyl-*N*-(α-benzyl Benzyloxycarbonyl-L-β-aspartyl)-β-D-glucopyranosylamine.*—Dicyclohexylcarbodi-imide (0·27 g.) was added to a solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamine (0·43 g.; obtained by reduction of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl azide [28]) and α-benzyl benzyloxycarbonyl-L-aspartate (0·45 g.) in dry methylene chloride (15 ml.). The solution was stirred for 6 hr. at room temperature, a few drops of glacial acetic acid were added (to decompose unchanged di-imide), and stirring was continued for a further ½ hr. Dicyclohexylurea, which had separated, was filtered off and the solvent was removed under reduced pressure. The residue was extracted into ethyl acetate, and washed successively with N-hydrochloric acid, water, aqueous sodium hydrogen carbonate, and water. After drying (Na_2SO_4), the solvent was removed under reduced pressure, and, after recrystallization of the residue from absolute ethanol, the product (0·56 g., 66%) had m. p. 139—141°. One further recrystallization afforded the *glucopeptide acetate* as needles, m. p. 142·5—143·5°, $[\alpha]_D^{22}$ +19·0° (c 2·01 in $CHCl_3$) (Found: C, 58·1; H, 5·4; N, 4·1. $C_{33}H_{38}N_2O_{14}$ requires C, 57·7; H, 5·6; N, 4·1%).

N-(Benzyloxycarbonyl-L-β-aspartyl)-β-D-glucopyranosylamine.—2,3,4,6-Tetra-*O*-acetyl-*N*-(α-benzyl benzyloxycarbonyl-L-β-aspartyl)-β-D-glucopyranosylamine (0·69 g.) was dissolved in aqueous acetone (1 : 4, 100 ml.), and a solution of lithium hydroxide (0·13 g.) in water (8 ml.) was added during ¾ hr. with stirring at room temperature. After being kept at 4° for 2 hr. the solution was concentrated to 40 ml. under reduced pressure at room temperature, and then after addition of water (60 ml.) was passed slowly through a column of Zeo-Karb 225 resin (H^+ form; 2 × 9 cm.). The column was washed with water until the effluent had a negative Molisch reaction, and the combined effluents were evaporated to 2 ml. On addition of methanol (25 ml.) and cooling overnight, needles separated, having m. p. 125—130° (0·20 g.), and the mother liquors yielded a further 0·04 g., m. p. 120—125° (total yield, 54%). Recrystallization from methanol afforded the *product*, m. p. 125—130°, $[\alpha]_D^{20}$ −8·3° (c 0·603 in H_2O) (Found: N, 6·3; equiv., 446. $C_{18}H_{24}N_2O_{10},H_2O$ requires N, 6·3%; equiv., 446).

N-(L-β-Aspartyl)-β-D-glucopyranosylamine.—*N*-(Benzyloxycarbonyl-L-β-aspartyl)-β-D-glucopyranosylamine (0·086 g.) was hydrogenolysed in water (25 ml.) with 8% palladized charcoal. Evolution of carbon dioxide had ceased after 1 hr. and the solution was then filtered from the catalyst, which was washed with water. The combined solution and washings were concentrated to 1 ml., and methanol (20 ml.) was added, whereupon the product, m. p. 200—203° (decomp.) (58 mg., 84%), separated. Two further recrystallizations from water–methanol afforded the hydrated *glucopeptide*, m. p. 203—204° (decomp.), $[\alpha]_D^{20}$ −17·3° (c 0·55 in H_2O), pK_2 8·82 (Found: C, 39·6; H, 6·4; N, 9·0. $C_{10}H_{18}N_2O_8,\frac{1}{2}H_2O$ requires C, 39·6; H, 6·3; N, 9·2%). Very recently Coutsogeorgopoulos and Zervas [29] reported the preparation of the glucopeptide (Ia) by a novel route. They record $[\alpha]_D^{16}$ −16·5° (in H_2O) and m. p. 253° (decomp.) for their glucopeptide (obtained as the anhydrous form). While their specific rotation is in agreement with our value, their m. p. is higher than that found for our glucopeptide (obtained as the hemihydrate). We therefore recrystallized our glucopeptide according to their directions and after drying the compound at 105° for 4 hr. obtained the anhydrous form (Found: C, 40·4; H, 6·5; N, 9·4. Calc. for $C_{10}H_{18}N_2O_8$: C, 40·8; H, 6·2; N, 9·5%). The melting behaviour was examined on a Kofler micro heating stage in polarized light; the material did not melt sharply but sintered at 208° and birefringence disappeared completely at 240°.

[28] Berthoe and Maier, *Annalen*, 1932, **498**, 50.
[29] Coutsogeorgopoulos and Zervas, *J. Amer. Chem. Soc.*, 1961, **83**, 1885.

1,3,4,6-Tetra-O-acetyl-N-(benzyloxycarbonylglycyl)-β-D-glucosamine.—Dicyclohexylcarbodi-imide (0·45 g.) was added to a solution of benzyloxycarbonylglycine (0·41 g.) and 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine (0·67 g.) in dry methylene chloride (45 ml.), and the reaction carried out as described above. Crystallization of the crude product from ethanol afforded needles, m. p. 166—168° (0·75 g., 72%). Bergmann and Zervas [17] record m. p. 165°.

1,3,4,6-Tetra-O-acetyl-N-(α-benzyl Benzyloxycarbonyl-L-β-aspartyl)-β-D-glucosamine.—Dicyclohexylcarbodi-imide (0·29 g.) was added to a solution of 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine (0·44 g.) and α-benzyl benzyloxycarbonyl-L-aspartate (0·45 g.) in dry methylene chloride (15 ml.) and the reaction carried out as described above. Crystallization of the crude product from ethanol gave needles of m. p. 208—209° (0·65 g., 76%). After recrystallization from ethanol the *glucopeptide acetate* had m. p. 209·5—210·5°, $[\alpha]_D^{22}$ +6·3° (*c* 1·27 in acetone) (Found: C, 57·7; H, 5·4; N, 4·0. $C_{33}H_{38}N_2O_{14}$ requires C, 57·7; H, 5·6; N, 4·1%).

N-(L-β-Aspartyl)-D-glucosamine.—1,3,4,6-Tetra-*O*-acetyl-*N*-(α-benzyl benzyloxycarbonyl-L-β-aspartyl)-β-D-glucosamine (0·62 g.) was hydrogenolysed in glacial acetic acid (30 ml.) with 8% palladized charcoal. Evolution of carbon dioxide had ceased after 22 hr. and the solution was then filtered from the catalyst, which was washed with acetic acid. After evaporation of the solvent (the last traces *in vacuo* over potassium hydroxide), the residue was triturated with methanol–ether (1 : 1), whereupon crystals separated (0·33 g., 79%). Without further purification, this product (0·23 g.) was dissolved in 2·5N-sulphuric acid (10 ml.), and the hydrolysis followed polarimetrically at room temperature. After 44 hr. the solution was brought to pH 4 by the addition of saturated barium hydroxide and, after removal of the barium sulphate by centrifugation, the solution was evaporated to dryness under reduced pressure. The solid residue was dissolved in water (5 ml.); on addition of methanol (30 ml.) and cooling, crystals separated (0·125 g., 87%). After three recrystallizations from water–methanol the *glucopeptide* had m. p. >260° after darkening from 200°, $[\alpha]_D$ +29° ⟶ +22° (*c* 0·28 in H_2O), pK_2 8·82 (Found: C, 37·6; H, 6·3; N, 8·4; equiv., 318. $C_{10}H_{18}N_2O_8,1\frac{1}{2}H_2O$ requires C, 37·4; H, 6·6; N, 8·7%; equiv., 321).

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