Carbohydrates in Protein

6. STUDIES ON THE CARBOHYDRATE-PEPTIDE BOND IN HEN'S-EGG ALBUMIN

By G. S. MARKS,* R. D. MARSHALL AND A. NEUBERGER

Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2

(Received 2 October 1962)

As a result of studies with glycopeptides isolated from hen's-egg albumin, it was suggested that the bond linking carbohydrate to this protein was that of an *N*-(β -aspartyl)glycosylamine (Johansen, Marshall & Neuberger, 1961; Nuenke & Cunningham, 1961). Lee & Montgomery (1962) have indicated that their results are consistent with this idea.

Yamashina & Makino (1962) obtained by partial acid hydrolysis of a similar glycopeptide a complex giving aspartic acid, glucosamine and ammonia in approximately equimolar amounts on further acid hydrolysis. They suggested from this and other studies that the linkage between the aspartic acid and glucosamine is that of an imino ether, the amide oxygen atom of asparagine being directly linked to the reducing carbon atom of this sugar.

In the present paper we present evidence that the sugar moiety involved in the linkage is glucosamine, in agreement with the findings of the Japanese workers. We also include results supporting the idea that the linkage is that of an *N*-(β -aspartyl)glycosylamine. Marks & Neuberger (1961) have described the synthesis and some of the properties of *N*-(*L*- β -aspartyl)- β -D-glucosylamine, i.e. 1- β -(*L*- β -aspartamido)-1-deoxy-D-glucose. The preparation of this compound by a different procedure has also been described by Coutsogeorgopoulos & Zervas (1961). We now report the synthesis of the corresponding *N*-acetyl-D-glucosamine derivative, i.e. 2-acetamido-1- β -(*L*- β -aspartamido)-1,2-dideoxy-D-glucose, and a comparison of some of the properties of this compound with a substance obtained by partial acid hydrolysis of an egg-albumin glycopeptide preparation. Some of this work has been briefly reported (Marks, Marshall & Neuberger, 1962).

EXPERIMENTAL

*Preparation of 2-acetamido-1- β -(*L*- β -aspartamido)-1,2-dideoxy-D-glucose*

2-Acetamido-3,4,6-tri-O-acetyl-1- β -azido-1,2-dideoxy-D-glucose (III). An aqueous suspension of silver azide was prepared from sodium azide (1 g.) and silver nitrate (2.5 g.). The aqueous suspension was washed twice successively with

water, ethanol, ether and chloroform by decantation (Bertho & Révész, 1955; filtration rather than decantation has led to a serious explosion in this Laboratory). The suspension of silver azide in chloroform was refluxed with a solution of acetochloroglucosamine (2 g.) in purified chloroform (20 ml.) for 2 hr., and, after the mixture had been cooled, the salt was removed by filtration and washed with warm chloroform. The chloroform solution and washings were combined and the chloroform was removed by evaporation. After recrystallization of the residue from ethanol, the product (0.70 g.; 35% yield) had m.p. 166–167°. One further recrystallization gave the azide, m.p. 166–168°, $[\alpha]_D^{25} -50^\circ$ (c 0.83 in chloroform) (Found: C, 45.6; H, 5.3. Calc. for $C_{14}H_{20}N_4O_8$: C, 45.2; H, 5.4%). Micheel & Wulff (1956), who prepared this compound by a different route, recorded m.p. 160–161°, $[\alpha]_D^{25} -43^\circ$ (c 1.0 in chloroform).

2-Acetamido-3,4,6-tri-O-acetyl-1- β -amino-1,2-dideoxy-D-glucose (IV). 2-Acetamido-3,4,6-tri-O-acetyl-1- β -azido-1,2-dideoxy-D-glucose (1.07 g.) was hydrogenated in ethyl acetate (65 ml.) with Adams platinum oxide catalyst (0.11 g.) for 4 hr. at atmospheric pressure. The solution was filtered and after removal of the solvent under reduced pressure a crystalline product was obtained (0.7 g.; 71% yield) of m.p. 148–149° (decomp., after darkening from 127°). Two recrystallizations from ethanol gave the product, m.p. 152–153° (decomp., after darkening from 137°), $[\alpha]_D^{25} -13^\circ$ (c 1.0 in chloroform) (Found: C, 48.5; H, 6.3. $C_{14}H_{22}N_2O_8$ requires C, 48.5; H, 6.4%).

*2-Acetamido-3,4,6-tri-O-acetyl-1- β -(α -benzyl *N*-benzyloxycarbonyl-*L*- β -aspartamido)-1,2-dideoxy-D-glucose (Ib).* Dicyclohexylcarbodi-imide (0.35 g.) was added to a solution of α -benzyl *N*-benzyloxycarbonyl-*L*-aspartate (0.56 g.) and 2-acetamido-3,4,6-tri-O-acetyl-1- β -amino-1,2-dideoxy-D-glucose (0.54 g.) in anhydrous methylene chloride (11 ml.). The solution was stirred for 6 hr. at room temperature, a few drops of acetic acid were added (to decompose unchanged di-imide) and stirring was continued for a further 30 min. 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. Water was removed from the mixture by standing it over anhydrous sodium sulphate, the solvent was removed under reduced pressure, and, after recrystallization of the residue from ethanol, the product (0.52 g.; 49% yield) had m.p. 219–221°. One further recrystallization gave the *acetylated glycopeptide ester*, m.p. 221–222° (Found: C, 58.3; H, 5.8; N, 5.95. $C_{35}H_{39}N_3O_{13}$ requires C, 57.8; H, 5.7; N, 6.1%).

*2-Acetamido-1- β -(*L*- β -aspartamido)-1,2-dideoxy-D-glucose (Ia).* 2-Acetamido-3,4,6-tri-O-acetyl-1- β -(α -benzyl *N*-

* Present address: Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada.

benzyloxycarbonyl-L- β -aspartamido)-1,2-dideoxy-D-glucose (0.38 g.) was dissolved in acetone-water (4:1, v/v) (56 ml.), and a solution of lithium hydroxide (64 mg.) in water (4 ml.) was added at room temperature during 30 min. with stirring. After being kept at 4° for 2 hr. the solution was concentrated to 20 ml. under reduced pressure at room temperature, and then after the addition of water (30 ml.) was passed through a column of Zeo-Karb 225 resin (H⁺ form). The column was washed with water and the effluent evaporated to dryness. Without further purification, this product (presumably Ic) was dissolved in water (35 ml.) and hydrogenated with 8% palladized charcoal. Evolution of carbon dioxide ceased after 2 hr.; the solution was filtered from the catalyst, which was washed with water. The combined solution and washings were concentrated to 1 ml., and ethanol (15 ml.) was added, whereupon the product (144 mg.; 77% yield) separated. Three further recrystallizations from ethanol-water (3:1, v/v) gave a hydrated glycopeptide, m.p. 220-5° (decomp., darkening from 205°) (Found: C, 40.9; H, 6.7; N, 12.1. Calc. for C₁₂H₂₁N₃O₈·H₂O: C, 40.8; H, 6.5; N, 11.9%).

This material was investigated by paper chromatography (butanol-acetic acid-water; 12:3:5, by vol.), and a small amount of material (blue spot with ninhydrin) was found to contaminate the major product (brown spot with ninhydrin). The mixture was placed on a Dowex 1 (acetate form) column in water, and eluted with a shallow linear gradient of water-acetic acid (two identical vessels containing 80 ml. of water and 0.3N-acetic acid respectively mounted at the same level and connected together). The fractions containing the required compound (brown spot with ninhydrin) emerged first, followed by the contaminating material. Evaporation to dryness of the fractions containing pure material and trituration of the residue with ethanol yielded a solid product. The homogeneity of this compound was shown by column chromatography and high-tension paper electrophoresis. Some of this material (1.08 μ moles) was hydrolysed (4N-hydrochloric acid for 3 hr. at 100°) and the presence of 1.16 μ moles of glucosamine and 1.16 μ moles of aspartic acid was shown by column chromatography [Moore & Stein chromatography with the Technicon Autoanalyser (Technicon Instruments Co. Ltd., Chertsey, Surrey)].

O-Acetylation of the *N*-benzyloxycarbonyl derivative of the egg-albumin glycopeptide. The *N*-benzyloxycarbonyl derivative of the glycopeptide (23 mg. of 'mannose') which contained aspartic acid as the only amino acid component (Fletcher, Marks, Marshall & Neuberger, 1963) was carefully dried *in vacuo* over phosphoric oxide. It was dissolved in 3 ml. of pyridine at 4° and shaken with 2 ml. of acetic anhydride for 24 hr. After the addition of water, the solution was evaporated to dryness under reduced pressure and this procedure repeated twice more. The residue was an oil that was soluble in dry ethyl acetate, dioxan or acetic acid, but insoluble in water.

Experiments with hydrogen bromide in acetic acid or dioxan

The synthetic compounds (about 1 mg.) dissolved in a saturated solution of hydrogen bromide in acetic acid were allowed to interact for about 12 hr. at room temperature. The solution was evaporated to dryness over sodium hydroxide *in vacuo*, and the residue dissolved in water and examined by paper chromatography (butanol-acetic acid-

water; 12:3:5, by vol.) and by high-tension paper electrophoresis (55 v/cm.; buffer: 70 ml. of 98-100% formic acid plus 50 ml. of acetic acid diluted to 2.5 l. with water, pH 1.85). With the *N*-benzyloxycarbonyl-*O*-acetyl derivative of the egg-albumin glycopeptide (see below) the residue after evaporation of the hydrogen bromide-acetic acid was taken up in water and placed on a column of Zeo-Karb 225 (H⁺ form). After the column had been washed with water, the eluate that emerged with aq. N-ammonia (which was shown to elute asparagine in a separate experiment) was evaporated to dryness and subjected to paper chromatography and high-tension paper electrophoresis as described above.

Similar experiments were carried out with the acylated egg-albumin glycopeptide (400 μ g. of mannose) by using hydrogen bromide in anhydrous dioxan (0.1 ml.; 2.73N with respect to hydrogen bromide) at 4° for 1 hr. or 10 hr. After evaporation to dryness *in vacuo* the water-soluble residue was examined by paper electrophoresis (18 v/cm.; 0.5N-acetic acid); the insoluble residue was discarded.

Rates of hydrolysis in acid and alkali

The rate of liberation of ammonia from the glycopeptide after acid hydrolysis was measured by determining the ammonia after suitable periods of time (Marshall & Neuberger, 1960). The rate of hydrolysis in the presence of alkali was determined by a method described by Marks & Neuberger (1961).

Partial acid hydrolysis studies

In a typical experiment, a solution of egg-albumin glycopeptide (equivalent to 2 mg. of mannose) in 2N-hydrochloric acid (0.2 ml.) was hydrolysed for 18 min. in a boiling-water bath. After being cooled, the solution was neutralized with 8 ml. of 0.05M-silver acetate (freshly recrystallized) solution. Silver chloride was removed by centrifuging, and the supernatant was evaporated to dryness under reduced pressure at 40°. The residue was dissolved in water and subjected to high-voltage paper electrophoresis or column chromatography.

RESULTS AND DISCUSSION

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

The starting point for the synthesis of 2-acetamido-1-(L- β -aspartamido)-1,2-dideoxy-D-glucose (Ia) was acetochloroglucosamine (II) (Leaback & Walker, 1957). Treatment of this chloro derivative with silver azide yielded 2-acetamido-3,4,6-tri-*O*-acetyl-1- β -azido-1,2-dideoxy-D-glucose (III), which was hydrogenated with Adams platinum oxide catalyst to give 2-acetamido-3,4,6-tri-*O*-acetyl-1- β -amino-1,2-dideoxy-D-glucose (IV). The next step involved the condensation, by the carbodi-imide method of Sheehan & Hess (1955), of this glucosaminylamine derivative (IV) with α -benzyl *N*-benzyloxycarbonyl-L-aspartate (V). The product (Ib) was treated with lithium hydroxide in aqueous acetone to cause simultaneous hydrolysis of the

O-acetyl and the benzyl ester groups to give the free acid (Ic). The latter compound was not isolated but was converted by catalytic hydrogenation into a compound which gave the correct analysis for the hydrate of 2-acetamido-1-(L-β-aspartamido)-1,2-dideoxy-D-glucose (Ia). This compound is believed to have the β-configuration in the reducing carbon atom of the sugar moiety for the following reasons. The acetochloroglucosamine used almost certainly has the α-configuration (Baker, Joseph, Schaub & Williams, 1954; Bertho & Révész, 1955; Leaback & Walker, 1957), and substitution of the chloro by the azido group generally occurs by a Walden inversion. Reduction of the azide and further substitution on the nitrogen are unlikely to cause either inversion or racemization of the asymmetric centre under consideration (Bertho & Révész, 1955).

When the synthetic glucopeptide (Ia) was investigated by paper chromatography a small amount of contaminant (blue spot with ninhydrin) was found

together with the major product (brown spot with ninhydrin). The latter substance, from its colour with ninhydrin (see Fletcher *et al.* 1963), was considered to be the desired β-aspartyl compound (Ia). The contaminant was removed by chromatography of the product on Dowex 1 (acetate form), and the purified material shown to yield equimolar amounts of aspartic acid and glucosamine after acid hydrolysis (4*N*-hydrochloric acid for 3 hr. at 100°).

That a rearrangement from a β-aspartyl (brown spot with ninhydrin) to an α-aspartyl derivative (blue spot with ninhydrin) may have occurred is possible (Battersby & Robinson, 1955), but we cannot be sure that the material giving the blue spot with ninhydrin is in fact the α-aspartyl peptide. If the contaminant had this structure it would have been expected to emerge before the β-aspartyl compound from a Dowex 1 column (Buchanan, Haley & Markiw, 1962), whereas the reverse order of emergence was observed in our experiment.

(Ia) $R = R' = R'' = H$ (Ib) $R = Ac; R' = CH_2 \cdot Ph$
 $R'' = O \cdot CO \cdot CH_2 \cdot Ph$ (Ic) $R = R' = H$
 $R'' = O \cdot CO \cdot CH_2 \cdot Ph$

(II)

(III)

(IV)

(V)

(VIa) $R = R' = R'' = H$ (VIb) $R = Ac; R' = CH_2 \cdot Ph$
 $R'' = O \cdot CO \cdot CH_2 \cdot Ph$ (VIc) $R = R' = H$
 $R'' = O \cdot CO \cdot CH_2 \cdot Ph$

Experiments with hydrogen bromide in acetic acid or dioxan

Marks & Neuberger (1961) showed that when *N*-(*L*- β -aspartyl)- β -D-glucosylamine (VIa) was treated with *N*-hydrochloric acid at 100° for 2 hr. the compound was partially hydrolysed to aspartic acid, glucose and ammonia, i.e. both bond *a* and bond *b* are split under these conditions (see below). We have investigated the possibility of bringing about a preferential splitting of bond *a* in these compounds. If this were possible the identification

of asparagine would constitute a good diagnostic test for linkages of this type in naturally occurring glycopeptides. We have tried to bring about this kind of splitting with a saturated solution of hydrogen bromide in acetic acid. This idea was suggested to us by Dr P. Gallop, who reasoned that asparagine and a 1-bromo sugar would be formed by analogy with the reaction of penta-acetylglucose with hydrogen bromide in acetic acid. In the latter case a C-O bond cleavage occurs at the reducing carbon atom with the formation of 1-bromopenta-acetylglucose and acetic acid.

When the glucose-containing glycopeptides (VIa) and (VIb) were treated with hydrogen bromide-acetic acid at room temperature for about 12 hr., asparagine was formed and identified by paper chromatography and high-tension paper electrophoresis. However, neither of the *N*-acetylglucosamine-containing glycopeptides (Ia) or (Ib) gave asparagine under these conditions. Our egg-albumin glycopeptide, containing aspartic acid as the only amino acid, was converted into the *N*-benzyloxycarbonyl-*O*-acetyl derivative before treatment with this reagent in order to enhance its solubility in acetic acid or dioxan. After treatment with this reagent the product was subjected to a preliminary purification on Zeo-Karb 225, before examination by paper chromatography and by paper electrophoresis. However, asparagine could not be detected in this experiment. Since a glycopeptide containing mannose would be expected to behave more like one containing glucose (VIa) than like one containing *N*-acetylglucosamine (Ia), it is probable that, if our assumption of a β -aspartyl-

glycosylamine linkage is correct, then the sugar involved in the linkage is *N*-acetylglucosamine and not mannose.

The effect of hydrogen bromide in dioxan on the *N*-benzyloxycarbonyl-*O*-acetyl derivative of the egg-albumin glycopeptide was also studied. After this treatment for either 1 hr. or 10 hr., the mixture was subjected directly to paper electrophoresis at pH 2.7. On staining the paper with ninhydrin as described by Fletcher *et al.* (1963), two brown spots appeared, which had moved 1.7 and 7.2 cm. respectively towards the cathode. Under these conditions aspartic acid had moved 5.0 cm. and asparagine 9.2 cm. Thus, although asparagine was not formed by this procedure, two compounds staining brown with ninhydrin under our conditions were produced. From the results of subsequent experiments, it is probable that these compounds were *N*-(β -aspartyl)glycosylamine derivatives containing one or more hexose molecules. It may be safely assumed that both the α -benzyl and the benzyloxycarbonyl groups had been removed by the action of hydrogen bromide in acetic acid or anhydrous dioxan (Ben-Ishai & Berger, 1952).

Stability of the glycopeptide and of model compounds

Marks & Neuberger (1961) measured the stability of *N*-(*L*- β -aspartyl)- β -D-glucosylamine (VIa) to acid and alkali and found the first-order rate constant for the liberation of glucose in *N*-hydrochloric acid at 100° to be $4.9 \times 10^{-3} \text{ min.}^{-1}$. The rate constant for the liberation of glucose from the closely related *N*-glycyl-D-glucosylamine under the same conditions was $7.8 \times 10^{-3} \text{ min.}^{-1}$. Under the same conditions also the liberation of ammonia from the latter compound proceeds with a rate represented by $8.2 \times 10^{-3} \text{ min.}^{-1}$. Since the rates of production of glucose and ammonia are the same for this substance, it is reasonable to assume that this will also apply to *N*-(*L*- β -aspartyl)-D-glucosylamine. Therefore we have compared the rate of liberation of ammonia from the egg-albumin glycopeptide with the previously determined rate of liberation of glucose from *N*-(*L*- β -aspartyl)- β -D-glucosylamine. First-order rate constants of $8.2 \times 10^{-3} \text{ min.}^{-1}$ in *N*-hydrochloric acid and of $20 \times 10^{-3} \text{ min.}^{-1}$ in 2*N*-hydrochloric acid were found for this glycopeptide. This preparation of glycopeptide contained approximately a molar quantity of leucine and smaller amounts of other amino acids as well as aspartic acid ('fraction A'; Fletcher *et al.* 1963). That substitution of the α -carboxyl group of aspartic acid (as it is in this preparation of glycopeptide) does not affect the rate of liberation of ammonia very markedly is indicated by the work of Leach & Lindley (1953).

The rate of liberation of ammonia from this egg-albumin glycopeptide at 100° in excess of 0.2N-sodium hydroxide was measured by the technique described by Marks & Neuberger (1961). A first-order rate constant ($7.2 \times 10^{-3} \text{ min.}^{-1}$) has been calculated for the initial part of the reaction by assuming that only 1 mole of ammonia is liberated/mole of glycopeptide. This assumption is incorrect, however, since any glucosamine liberated will rapidly evolve its ammonia in the presence of the hot alkali (Morgan, 1936; Tracey, 1952), and this is also shown in our results (Fig. 1). The rate constant quoted above therefore represents a maximum value for the rate of cleavage of the carbohydrate-peptide bond, if it is assumed that this is the source of the labile ammonia. This value may be compared with that obtained by Marks & Neuberger (1961) for *N*-(L-β-aspartyl)-β-D-glucosylamine under identical conditions ($5.5 \times 10^{-3} \text{ min.}^{-1}$). However, since we have concluded (see below) that in the egg-albumin glycopeptide the sugar involved in the linkage is *N*-acetylglucosamine, it would have been preferable to compare the rates of evolution of ammonia in acid and alkali from the egg-albumin glycopeptide with those from 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose.

Yamashina & Makino (1962) indicated that about 60% of the labile ammonia is liberated on heating their preparation of glycopeptide in 2N-hydrochloric acid at 100° for 1 hr., and our results agree with this (Fig. 1). We have found that a similar amount of ammonia is liberated in 0.2N-sodium hydroxide at 100° in 2 hr., assuming only 1 mole of ammonia/mole of glycopeptide. Yamashina & Makino (1962) reported that a concentration of 0.01N-sodium hydroxide at 100° gives a similar degree of hydro-

lysis, but this is contrary to the earlier report of Neuberger (1938) and our present results.

Marks & Neuberger (1961) have shown that the rates of hydrolysis at 100° in 0.2N-sodium hydroxide and in N-hydrochloric acid are in a ratio of 1.1:1 for *N*-(L-β-aspartyl)-β-D-glucosylamine. Our present results show a ratio for these rates of 0.9:1 for the egg-albumin glycopeptide. These values show that both substances are more stable to acid than to alkali of equivalent concentration, as is generally observed for amide hydrolysis (Taylor & Baker, 1945). The two ratios of rates are in fair agreement and the values for the absolute rates are similar. Yamashina & Makino (1962) showed that the stability in acid of 2-acetamido-1-anilido-1,2-dideoxyglucose is much less than that of the egg-albumin glycopeptide and that their model compound is less stable in acid than in alkali. Because of the difference in stability to acid and alkali between their model compound and the egg-albumin glycopeptide they concluded that the linkage between the sugar and aspartic acid in egg albumin is not that of a glucosylamine but is probably that of an imino ether. However, such a comparison is erroneous, for the Japanese workers' model compound is a glucosylamine formed by condensation of an unsubstituted aromatic amine with a sugar, whereas the linkage envisaged in egg albumin is that of an *N*-acylated non-aromatic glucosylamine. Moreover, acylation of a glucosylamine markedly increases its stability to acid and alkali (Marks & Neuberger, 1961).

Johansen *et al.* (1961) obtained preparations of glycopeptide that gave 1.7 moles of ammonia/mole after hydrolysis in 2N-hydrochloric acid for 3 hr. Our more recent preparations of glycopeptide containing fewer amino acid residues yielded 1.0 mole of ammonia/mole under these and other conditions of acid hydrolysis (see Fig. 1). Other workers have also found that 1 mole of ammonia/mole is liberated from glycopeptide on acid hydrolysis (Nuenke & Cunningham, 1961; Lee & Montgomery, 1962; Yamashina & Makino, 1962).

Partial acid hydrolysis

For these experiments the glycopeptide containing aspartic acid as the only amino acid constituent ('fraction B'; Fletcher *et al.* 1963) was used. The conditions of hydrolysis chosen (2N-hydrochloric acid for 18 min. at 100°) were based on considerations of the relative stability of the carbohydrate-amino acid bond and the glycosidic bonds within the polysaccharide. Such a partial acid hydrolysis should leave about 70% of the postulated glucosylamine bond intact. The results of Neuberger (1938) and of Nuenke & Cunningham (1961) indicate that in 1.5N-hydrochloric acid at 100° about 60% of all the glycosidic bonds are cleaved in 18 min. If it is

Fig. 1. Rate of liberation of ammonia at 100° from egg-albumin glycopeptide by: ▲, N-HCl; ○, 2N-HCl; ●, 0.2N-NaOH.

assumed that this rate is approximately equal to the actual rate of hydrolysis of the glycosidic bond(s) linking the sugar attached to aspartic acid to its neighbour(s), then 1 μ mole of glycopeptide should yield about 0.4 μ mole of *N*-(L - β -aspartyl)hexose on hydrolysis under these conditions.

Treatment of the glycopeptide by this procedure was followed, after neutralization, by high-tension paper electrophoresis (on Whatman 3 MM paper at 35 v/cm. for 1.25 hr. at pH 1.85). On staining the paper with ninhydrin, a substance was seen to be present with the same mobility (15 cm. towards the cathode) as *N*-(L - β -aspartyl)- β -D-glucosylamine, *N*-(L - β -aspartyl)-D-glucosamine (Marks & Neuberger, 1961) and 2-acetamido-1- β -(L - β -aspartamido)-1,2-dideoxy- β -D-glucose. All these spots were characteristically stained brown, as was an asparagine marker under the conditions employed for the ninhydrin reaction (Fletcher *et al.* 1963).

Column chromatography of a similar partial acid hydrolysate was carried out and the eluate analysed in the Technicon Autoanalyser. Two ninhydrin-

positive peaks appeared fairly early in the chromatogram. One of these peaks corresponded in position to that of 2-acetamido-1-(L - β -aspartamido)-1,2-dideoxy-D-glucose and differed from that of *N*-(L - β -aspartyl)-D-glucosamine (Fig. 2).

A further partial acid hydrolysate was subjected to column chromatography, but the eluate was not examined in the Autoanalyser but was diverted to a fraction collector. We examined the fractions emerging in the region expected for 2-acetamido-1-(L - β -aspartamido)-1,2-dideoxy-D-glucose. Three overlapping peaks emerged at this point and analysis of the largest of these, after further acid hydrolysis (4N-hydrochloric acid for 3 hr.), revealed the following molar composition: aspartic acid, 1.0, glucosamine, 0.75, and mannose, less than 0.06. In addition the following amounts of other amino acids were present: threonine, 0.03; serine, 0.05; glutamic acid, 0.19; valine, 0.09 and leucine 0.04. An ammonia analysis would have been highly desirable, but this was not possible, owing to the appearance of extraneous ammonia from the chromatogram at the ammonia position. This result indicates that glucosamine is the sugar linked to aspartic acid, but we do not know why the aspartic acid:glucosamine ratio was not exactly 1.0. Certainly free aspartic acid could not be present in this fraction since it would have been eluted much later from the column (Fig. 2).

Carbohydrate-peptide linkage

The suggestion that the linkage between carbohydrate and protein in hen's egg albumin was that of a β -aspartylglycosylamine (Johansen *et al.* 1961; Nuenke & Cunningham, 1961) was followed by the synthesis of a model compound, *N*-(L - β -aspartyl)- β -D-glucosylamine (Marks & Neuberger, 1961). The stability of this compound has now been compared with that of the egg-albumin glycopeptide under both acid and alkaline conditions of hydrolysis. The rates of release of ammonia from these two substances were indeed very similar.

Column chromatography of a partial acid hydrolysate of the glycopeptide, containing aspartic acid as the only amino acid constituent, resulted in the separation of a small amount of material that contained aspartic acid and glucosamine as the main components and that was almost completely free of mannose. From these observations it was deduced that glucosamine is the sugar molecule attached to aspartic acid. This result agrees with the findings of Yamashina & Makino (1962), who in addition have shown that 1 mole of ammonia/mole is released on acid hydrolysis. As indicated above, however, we do not agree with their suggestion that the linkage is that of an imino ether.

From our comparison of the rate of liberation of ammonia from the egg-albumin glycopeptide with

Fig. 2. Column chromatography on Dowex 50 of (a) 2-acetamido-1- β -(L - β -aspartamido)-1,2-dideoxy-D-glucose (marked A) and *N*-(L - β -aspartyl)-D-glucosamine (marked B); (b) *N*-(L - β -aspartyl)-D-glucosamine (marked B); (c) partial acid hydrolysate of egg-albumin glycopeptide. The elution volume for aspartic acid is 195 ml.

that from *N*-(*L*- β -aspartyl)- β -D-glucosylamine in acid and alkali, and also the chromatographic behaviour of a partial acid hydrolysate of the glycopeptide (Fig. 2), we do not believe that the bond is formed by linking the β -carboxyl group of aspartic acid with the amino group of glucosamine, the presence of which in human γ -globulin has been suggested by Rothfus (1961). This was discussed as a possible but unlikely linkage by Johansen *et al.* (1961). At that time the possibility of a fourth glucosamine residue could not be excluded and, because the glycopeptide contained three acetyl residues, it was conceivable that the amino group of the undetected glucosamine residue was available for combination with the β -carboxyl group of aspartic acid. As amino acid and nitrogen analyses of our recent preparations of glycopeptide make it certain that there are only three glucosamine residues (Fletcher *et al.* 1963), the β -aspartyl-glucosamine linkage can be confidently ruled out, with one reservation that is discussed below. Our conclusion that the linkage is formed by combination of the β -carboxyl group of aspartic acid with the 1-amino group of 2-acetamido-1-amino-1,2-dideoxyglucose is based on the following considerations: (a) glucosamine and not mannose is involved in the linkage to aspartic acid; (b) the production of 1 mole of ammonia/mole on relatively mild acid hydrolysis of the whole glycopeptide; further, the source of this ammonia has been shown by Yamashina & Makino (1962) to reside in a partial acid hydrolysis product of a similar glycopeptide that yielded aspartic acid, glucosamine and ammonia in equimolar amounts on further acid hydrolysis; (c) the stability to acid and alkali of the glycopeptide and one of our model compounds is similar; (d) that the β -carboxyl group of aspartic acid is involved in the linkage is indicated by the production of a brown colour with ninhydrin produced under conditions specified by Fletcher *et al.* (1963) by both the whole glycopeptide and by partial acid hydrolysis products thereof; (e) the identity of behaviour on electrophoresis and on column chromatography of a partial acid hydrolysis product of the glycopeptide and of 2-acetamido-1- β -(*L*- β -aspartamido)-1,2-dideoxy-D-glucose, the preparation of which is described in the present paper. However, we have not excluded the possibility of a linkage involving a similar type of compound in which the β -aspartyl and acetyl groups are interchanged, i.e. of the form 1-acetamido-2-(*L*- β -aspartamido)-1,2-dideoxyglucose, but we believe that such a linkage is less likely.

Marshall (1958) suggested that the type of bond between carbohydrate and protein in egg albumin might be of fairly widespread occurrence. Indeed Tanaka (1961) obtained a substance, from a glycopeptide from ovomucoid, that contained aspartic

acid and glucosamine only, and that was fairly stable to alkaline conditions. Masamune (1955) isolated a carbohydrate-peptide complex from a mucopolysaccharide from pig-stomach mucus in which he believed aspartic acid was linked to the reducing carbon atom of *N*-acetylhexosamine. The compound was fairly stable to alkali and he suggested an *N*-glycosidic bond to the α -amino group of aspartic acid. Rothfus (1961) has proposed that the aspartic acid-glucosamine linkage occurring in γ -globulin is that of an *N*-(β -aspartyl)glucosamine, which has approximately the same stability to acid as *N*-(*L*- β -aspartyl)- β -D-glucosylamine (Marks & Neuberger, 1961). The results obtained in all these studies are compatible with the occurrence of *N*-glycosidic linkages involving the amide group of asparagine and the reducing carbon atom of a glucosamine residue. Such a linkage might also be present in orosomucoid as suggested in the studies of Eylar (1962).

That a carbohydrate-peptide bond of this type is not universal in animal tissues is clear from the elegant studies in which glycosidic ester bonds (between *N*-acetylgalactosamine and the β - and γ -carboxyl groups of aspartic acid and glutamic acid respectively) have been shown to exist in the submaxillary-gland mucoprotein of sheep (Gottschalk & Murphy, 1961) and of ox (Murphy & Gottschalk, 1961). These bonds are about 100 times less stable to alkali than the *N*-acylglycosylamine linkage, and this provides a ready means of differentiation.

SUMMARY

1. 2-Acetamido-3,4,6-tri-*O*-acetyl-1- β -(α -benzyl *N*-benzyloxycarbonyl-*L*- β -aspartamido)-1,2-dideoxy-D-glucose (Ib) has been prepared. Removal of the protective groups gave 2-acetamido-1- β -(*L*- β -aspartamido)-1,2-dideoxy-D-glucose (Ia) contaminated with another substance.

2. The cleavage of the *N*-glycosidic bond in *N*-(*L*- β -aspartyl)-D-glucosylamine with hydrogen bromide in acetic acid to give asparagine is described. Neither the analogous compound where the sugar moiety was *N*-acetyl-D-glucosamine nor the egg-albumin glycopeptide gave asparagine with this reagent.

3. The stability of the glycopeptide was compared with that of *N*-(*L*- β -aspartyl)-D-glucosylamine under acid and alkaline conditions and shown to be similar.

4. Partial acid hydrolysis yielded material behaving electrophoretically and chromatographically like 2-acetamido-1- β -(*L*- β -aspartamido)-1,2-dideoxy-D-glucose. Analysis has indicated that the compound contains aspartic acid and glucosamine and negligible amounts of mannose.

5. Evidence is presented showing that the carbohydrate-peptide bond in egg albumin consists of a linkage of the β -carboxyl group of an aspartic acid residue with the 1-amino group of 2-acetamido-1-amino-1,2-dideoxyglucose. This has been compared and contrasted with carbohydrate-peptide bonds in other glycoproteins.

We thank Professor J. Baddiley for his gift of 1-glycinamido-1-deoxyglucose and Mr V. Bhoyroo for technical assistance. Acknowledgement is made for grants from the British Empire Cancer Campaign (to G.S.M.) and the U.S. Public Health Service (to R.D.M.).

REFERENCES

- Baker, B. R., Joseph, J. P., Schaub, R. E. & Williams, J. H. (1954). *J. org. Chem.* **19**, 1786.
 Battersby, A. R. & Robinson, J. C. (1955). *J. chem. Soc.* p. 259.
 Ben-Ishai, D. & Berger, A. (1952). *J. org. Chem.* **17**, 1564.
 Bertho, A. & Révész, A. (1955). *Liebigs Ann.* **581**, 161.
 Buchanan, D. L., Haley, E. H. & Markiw, R. T. (1962). *Biochemistry*, **1**, 612.
 Coutsogeorgopoulos, C. & Zervas, L. (1961). *J. Amer. chem. Soc.* **83**, 1885.
 Eylar, E. H. (1962). *Biochem. Biophys. Res. Commun.* **8**, 195.
 Fletcher, A. P., Marks, G. S., Marshall, R. D. & Neuberger, A. (1963). *Biochem. J.* **87**, 265.
 Gottschalk, A. & Murphy, W. H. (1961). *Biochim. biophys. Acta*, **46**, 81.
 Johansen, P. G., Marshall, R. D. & Neuberger, A. (1961). *Biochem. J.* **78**, 518.
 Leaback, D. H. & Walker, P. G. (1957). *J. chem. Soc.* p. 4754.
 Leach, S. J. & Lindley, H. (1953). *Trans. Faraday Soc.* **49**, 915.
 Lee, Y. C. & Montgomery, R. (1962). *Arch. Biochem. Biophys.* **97**, 9.
 Marks, G. S., Marshall, R. D. & Neuberger, A. (1962). *Biochem. J.* **85**, 15 p.
 Marks, G. S. & Neuberger, A. (1961). *J. chem. Soc.* p. 4872.
 Marshall, R. D. (1958). *Annu. Rep. Brit. Emp. Cancer Campgn, Lond.*, no. 36, 569.
 Marshall, R. D. & Neuberger, A. (1960). *Nature, Lond.*, **186**, 311.
 Masamune, H. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 72.
 Micheel, F. & Wulff, H. (1956). *Chem. Ber.* **89**, 1521.
 Morgan, W. T. J. (1936). *Biochem. J.* **30**, 909.
 Murphy, W. H. & Gottschalk, A. (1961). *Biochim. biophys. Acta*, **52**, 349.
 Neuberger, A. (1938). *Biochem. J.* **32**, 1435.
 Nuenke, R. H. & Cunningham, L. W. (1961). *J. biol. Chem.* **236**, 2452.
 Rothfus, J. A. (1961). *Fed. Proc.* **20**, 383.
 Sheehan, J. C. & Hess, G. P. (1955). *J. Amer. chem. Soc.* **77**, 1067.
 Tanaka, M. (1961). *J. pharm. Soc. Japan*, **81**, 1460.
 Taylor, T. W. J. & Baker, W. (1945). *Sidgwick's Organic Chemistry of Nitrogen*, 3rd ed., p. 145. Oxford: Clarendon Press.
 Tracey, M. V. (1952). *Biochem. J.* **52**, 265.
 Yamashina, I. & Makino, M. (1962). *J. Biochem., Tokyo*, **51**, 359.

Biochem. J. (1963) **87**, 281

A Pure Trypsin Inhibitor from Soya Beans

BY YEHUDITH BIRK, A. GERTLER AND SHULAMITH KHALEF

Faculty of Agriculture, Hebrew University, Rehovot, Israel

(Received 2 August 1962)

An acetone-insoluble trypsin inhibitor (Bowman, 1946) has been separated from soya beans and purified by dialysis, freeze-drying and fractionation on a diethylaminoethylcellulose column (Birk, 1961*a, b*). Rackis, Sasame, Mann, Anderson & Smith (1961) reported the isolation of a similar inhibitor from soya-bean-whey proteins. Applebaum, Janković & Birk (1961) have shown that the freeze-dried preparation exhibits amylase activity. The latter could not be separated completely from the trypsin inhibitor on diethylaminoethylcellulose columns. Preliminary experiments indicate that the complex formed between the inhibitor purified on diethylaminoethylcellulose and trypsin shows neither trypsin nor trypsin-inhibiting activity, but exhibits all the amylase activity of the inhibitor.

These findings suggest that the inhibitor and amylase are two different materials.

This paper is devoted to further fractionation of the purified inhibitor on a carboxymethylcellulose column and to the characterization of the pure inhibitor. A rapid method for preparing the pure inhibitor on this cation-exchanger directly from the dialysed preparation, omitting purification on diethylaminoethylcellulose, is also given.

EXPERIMENTAL

Abbreviations and designations. CM-cellulose, carboxymethylcellulose; DEAE-cellulose, diethylaminoethylcellulose. 'Crude inhibitor', Bowman's (1946) crude, acetone-insoluble inhibitor. 'Non-diffusible inhibitor', dialysed and