

- Cordes, E. H., and Jencks, W. P. (1962), *J. Am. Chem. Soc.* 84, 832.
- Cordes, E. H., and Jencks, W. P. (1963), *J. Am. Chem. Soc.* 85, 2843.
- Dawid, I. B., French, T. C., and Buchanan, J. M. (1963), *J. Biol. Chem.* 238, 2178.
- Dimroth, O., and Zeeppritz, R. (1902), *Ber.*, 35, 984.
- Emerson, M. T., Grunwald, E., and Kromhout, R. A. (1960), *J. Chem. Phys.* 33, 547.
- Fischer, E. H., Forrey, A. W., Hedrick, J. L., Hughes, R. C., Kent, A. B., and Krebs, E. G. (1963), in *Chemical and Biological Aspects of Pyridoxal Catalysis*, Snell, E. E., Braunstein, A. E., Fasella, P., and Rossi-Fannelli, A., eds., New York, Macmillan, p. 556.
- Hammes, G. G., and Fasella, P. (1963), *J. Am. Chem. Soc.* 85, 3029.
- Hantzsch, A., and Kraft, F. (1891), *Ber.* 24, 3521.
- Jencks, W. P. (1959), *J. Am. Chem. Soc.* 81, 475.
- Kastening, B., Holleck, L., and Melkonian, G. A. (1956), *Z. Elektrochem.* 60, 130.
- Lucas, N., King, H. K., and Brown, S. J. (1961), *Biochem. J.* 84, 1962.
- Mosher, H. S. (1950), in *Heterocyclic Compounds*, Vol. I, Elderfield, P. C., ed., New York, Wiley, pp. 408-409.
- Nakamoto, K., and Martell, A. E. (1959), *J. Am. Chem. Soc.* 81, 5857.
- Ölander, A. (1927), *Z. Physik. Chem. (Leipzig)* 129, 1.
- Velick, S. F., and Vavra, J. (1962), *J. Biol. Chem.* 237, 2109.
- Willi, A. V. (1956), *Helv. Chim. Acta* 39, 1193.
- Willi, A. V., and Robertson, R. E. (1953), *Can. J. Chem.* 31, 361.
- Zuman, P. (1950), *Collection Czech. Chem. Commun.* 15, 839.

Carbohydrates in Protein. VIII. The Isolation of 2-Acetamido-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose from Hen's Egg Albumin*

R. D. MARSHALL AND A. NEUBERGER

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

Received April 13, 1964

2-Acetamido-3,4,6-tri-O-acetyl-1-(α -benzyl N-benzyloxycarbonyl-L- β -aspartamido)-1,2-dideoxy- β -D-glucose (II) was synthesized by the condensation of 2-acetamido-3,4,6-tri-O-acetyl-1-amino-1,2-dideoxy- β -D-glucose with α -benzyl N-benzyloxycarbonyl-L-aspartate. The latter was isolated by countercurrent distribution from the products obtained by the condensation of benzyl alcohol with N-benzyloxycarbonyl-L-aspartic anhydride. Compound II was converted to pure 2-acetamido-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose (I) by a catalytic hydrogenation followed by treatment of the compound thus obtained with 0.6 N LiOH at room temperature. In addition a compound (Ia) was isolated by chromatographic and electrophoretic techniques from a partial acid hydrolysate of a glycopeptide from hen's egg albumin, and this was shown to yield aspartic acid, glucosamine, and ammonia in molar amounts when it was hydrolyzed by acid. Some properties of compounds I and Ia were compared and it was shown that they were identical. The nature of the carbohydrate-peptide bond in this protein is thus demonstrated.

A glycopeptide was isolated by a nonchromatographic method from a tryptic hydrolysate of egg albumin by one of us in 1938 (Neuberger, 1938). This material yielded approximately 1 mole of ammonia when it was hydrolyzed with acid (5 N HCl, 3 hours, 100°). As a result of observations on the effect of alkali on this substance it was suggested that part of the glucosamine in the glycopeptide was bound to a nitrogen-containing substance, which was much later identified by a number of workers as aspartic acid (Cunningham *et al.*, 1957; Jevons, 1958; Johansen *et al.*, 1958; Kaverzneva and Bogdanov, 1960). From a study of the properties of similar glycopeptides Johansen *et al.* (1961) and Nuenke and Cunningham (1961) independently suggested that the linkage between aspartic acid and the carbohydrate moiety was formed by the condensation of a β -aspartyl residue with a 1-amino sugar, but the question of whether the latter was derived from mannose or N-acetylglucosamine was left open. Direct evidence was adduced for the participation of the β - rather than the α -carboxyl group of aspartic acid in the linkage by the conversion of a glycopeptide containing aspartic acid as the only amino acid residue to the corresponding derivative in which the aspartic acid appeared as the phenylthiohydantoin, and this was achieved without

splitting the carbohydrate-peptide bond (Fletcher *et al.*, 1963a).

That the sugar involved in the linkage was indeed N-acetylglucosamine was shown by the isolation from partial acid hydrolysates of glycopeptides of substances which yielded aspartic acid and glucosamine but no mannose on acid hydrolysis (Yamashina and Makino, 1962; Bogdanov *et al.*, 1962; Marks *et al.*, 1962, 1963). The production of approximately 1 mole of ammonia also from these fragments under similar conditions (Yamashina and Makino, 1962; Bogdanov *et al.* 1962) was in accord with its probable identification as 2-acetamido-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose (I).

Two groups of workers (Marks *et al.*, 1963; Bolton and Jeanloz, 1963) have described the synthesis and characterization of 2-acetamido-3,4,6-tri-O-acetyl-1-(α -benzyl N-benzyloxycarbonyl-L- β -aspartamido)-1,2-dideoxy- β -D-glucose (II). One set of conditions used for removal of the protecting groups was shown to be associated with some side reactions (Marks *et al.*, 1963). It is the purpose of the present communication to describe first a procedure whereby these protecting groups may be removed to yield the desired model compound in almost quantitative yield without the occurrence of undesirable rearrangements. Second, the preparation from egg albumin of crystalline material which yields glucosamine, ammonia, and aspartic acid on acid

* The financial assistance of the United States Public Health Service is gratefully acknowledged. Paper VII of this series is Neuberger and Papkoff (1963).

hydrolysis is described. Finally the properties of this material are compared with those of the synthetic model substance and their identity is thus established. Some similar studies have also been briefly reported by other workers (Yamashina *et al.*, 1963; Bogdanov *et al.*, 1964).

SYNTHESIS OF MODEL COMPOUND

All melting points reported here were done on the Kofler block and are given without stem correction. The model compound I was synthesized by a procedure which involved the condensation of α -benzyl *N*-benzyloxycarbonyl-L-aspartate with 2-acetamido-3,4,6-tri-*O*-acetyl-1-amino-1,2-dideoxy- β -D-glucose in a similar manner to that previously described (Marks *et al.*, 1963), but some features are new and will be described.

α -Benzyl *N*-benzyloxycarbonyl-L-aspartate was isolated from the products obtained by condensing 5 g *N*-benzyloxycarbonyl-L-aspartic anhydride (III) (20 mmoles) with 3 ml benzyl alcohol (30 mmoles) in a sealed tube at 100° for 4 hours. After cooling, 5.5 g of the products was dissolved in 10 ml of ether and the substance was subjected to countercurrent distribution (101 transfers) between ether and 0.1 M sodium phosphate buffer, pH 8.0 (Gomori, 1955), using 10-ml. volumes of each solvent. By determining the optical densities of the aqueous phases of the various fractions at $\lambda = 258 \text{ m}\mu$ the distribution represented in Figure 1 was obtained. The substances contained in the fractions indicated by A, B, and C were obtained by extracting them into ether after adjusting the pH of the aqueous layer to 1.7 with 3 N HCl, when precipitates formed. Substance D was similarly extracted into ether but in this instance directly from the phosphate buffer. In each case the ether extracts were dried over anhydrous sodium sulfate for 1 hour, filtered, and evaporated to dryness. On standing overnight in the cold room, the substances crystallized. They were each recrystallized by dissolving in a minimum volume of ethyl acetate and adding light petroleum (bp 40–60°) to incipient turbidity. The purified fraction A (needles, 0.34 g) had mp 114° and a mixed mp with authentic *N*-benzyloxycarbonyl-L-aspartic acid of 114–115°. Bergmann and Zervas (1932) record mp 116° for this compound. Substance B (needles, 0.49 g) had mp 108–110° and $[\alpha]_D^{25} = +11.6$ (c 5, CH_3COOH), in agreement with recorded values of mp 108° and $[\alpha]_D^{25} = +12.1$ (c 10, CH_3COOH) (Berger and Katchalski, 1951; Bryant *et al.*, 1959) for β -benzyl *N*-benzyloxycarbonyl-L-aspartate. Substance C (rectangular blades, 1.31 g) was the corresponding α -benzyl derivative with mp 84–85°, $[\alpha]_D^{25} = -10.4$ (c 5, CH_3COOH), and values of mp 84–85°, $[\alpha]_D^{25} = -9.7$ (c 5.59, CH_3COOH) have been reported (Bergmann *et al.*, 1933; Bryant *et al.*, 1959). The last compound, D (rectangular blades, 0.9 g), had mp 63–64° and a mixed mp with authentic $\alpha\beta$ -dibenzyl *N*-benzyloxycarbonyl-L-aspartate of 64°, $[\alpha]_D^{25} = -2$ (c 5, CH_3COOH). This compound has a mp recorded of 66.5 and $[\alpha]_D^{25} = -2.5$ (c 10, CH_3COOH) (Berger and Katchalski, 1951).

The proportions in which these four substances were formed was assessed by calculating the areas under the peaks in Figure 1, and calculating the amounts present on the basis of the observation that a benzyl ester and a benzyloxycarbonyl group exhibit equal "molar" absorption at this wavelength. Thus *N*-benzyloxycarbonylglucosamine was found to have a molar extinction at 257 $\text{m}\mu$ in 30% (ml per 100 ml solvent) ethanol in water of 207 (see also Fletcher *et al.*, 1963b) and α -benzyl *N*-benzyloxycarbonyl-L-aspartate one of 428. Moreover the spectra were identical. The values thus

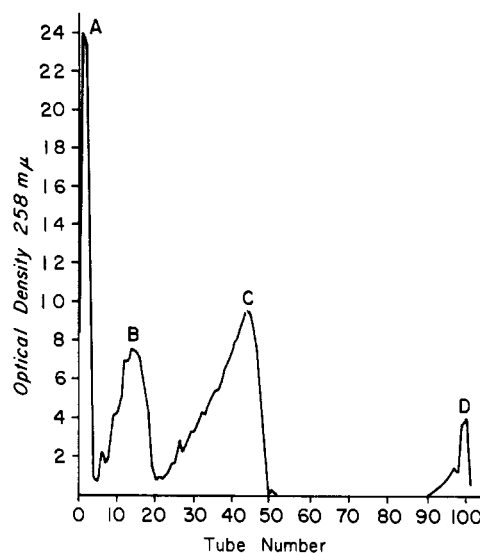


FIG. 1.—A separation by countercurrent distribution of the products obtained by condensing benzyl alcohol with *N*-benzyloxycarbonyl-L-aspartic anhydride. The solvents were ether and 0.1 M sodium phosphate buffer, pH 8.0. The ultraviolet absorption of the phosphate buffer layer was read at 258 $\text{m}\mu$.

obtained were used in conjunction with the distribution coefficients of the substance between ether and buffer to show that fraction A consisted of 2.63 mmoles (0.70 g, 19% theoretical based on the amount of *N*-benzyloxycarbonyl-L-aspartic anhydride used) of *N*-benzyloxycarbonyl-L-aspartic acid. Fractions B, C, and D consisted, respectively, of 1.63 mmoles (0.58 g, 12%) of the β -benzyl ester, 4.72 mmoles (1.68 g, 34% of the α -benzyl ester, and 2.54 mmoles (1.13 g, 18%) of the dibenzyl ester of *N*-benzyloxycarbonyl-L-aspartic acid. The excess benzyl alcohol appeared in the ether layers of tubes 100 and 101.

The condensation of benzyl alcohol with *N*-benzyloxycarbonyl-L-aspartic anhydride yields therefore not only both the α - and β -benzyl esters as noted by LeQuene and Young (1952), but also some of the $\alpha\beta$ -dibenzyl ester. This might be formed by transesterification between either or both of the two primary reaction products, the α - and β -monobenzyl esters. The presence of the $\alpha\beta$ -dibenzyl ester and of the free *N*-benzyloxycarbonyl aspartic acid in equimolar amounts is compatible with this explanation.

2-Acetamido-3,4,6-tri-*O*-acetyl-1-azido-1,2-dideoxy- β -D-glucose (IV) was prepared as described earlier (Marks *et al.*, 1963) by refluxing the corresponding α -chloro compound (2.3 g, 6.3 mmoles; Leaback and Walker, 1957) with silver azide (15 mmoles; Bertho and Révész, 1955) in about 30–40 ml dry chloroform for various periods of time from 45 minutes to 3 hours. The samples were crystallized twice from ethyl alcohol. The mp of the substance was invariably 167–168° but the rotation was somewhat variable, the best samples having $[\alpha]_D^{25} = -55^\circ$ (c 0.84, CHCl_3). The yield was not related to the time of refluxing, the best being about 37%. Bolton and Jeanloz (1963) have synthesized the substance from the corresponding α -bromo compound by a similar technique with a yield of 68% and the properties described by Micheel and Wulff (1956; mp 160–161°, $[\alpha]_D^{25} = -43^\circ$ [c 1.0, CHCl_3]).

Because of the low yields obtained by us with the chloro compound the ethanolic mother liquors from the crystallization of the compound were taken to dryness *in vacuo* at 40°. The residual oil was dried over H_2SO_4 *in vacuo* at room temperature, and it slowly became

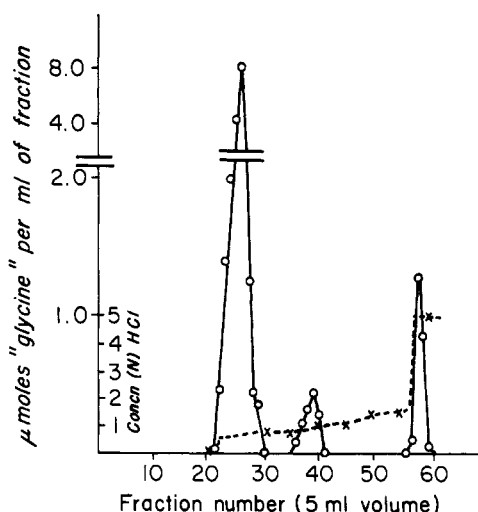


FIG. 2.—Chromatography on Dowex-50 of a partial acid hydrolysate of a glycopeptide from egg albumin. —○—○— = ninhydrin-positive materials, —x—x— = acid concentration (HCl) in the gradient employed.

solid. This material exhibited an absorption in the infrared at 2120 cm^{-1} , which is characteristic of the $\text{N}\equiv\text{N}$ asymmetric stretching vibration. Moreover, its intensity was about the same as that given by the pure β -azido compound. The material had an $[\alpha]_D$ value of $+26.6$ ($c\ 2.4$, CHCl_3) and probably consists of a mixture of the α - and β -azido anomers. The use of Hudson's (1909) isorotation rule suggests that the $[\alpha]_D$ value for the pure α -azido compound is about 160° , and this probably indicates that about 40% of the material in the mother liquors is composed of this α anomer.

2-Acetamido-3,4,6-tri-O-acetyl-1-amino-1,2-dideoxy- β -D-glucose (V) was prepared by reducing compound IV in a manner similar to that previously described, but by examining the infrared absorption at about 2120 cm^{-1} after various periods of time it was found that reduction was complete in 15 minutes. We earlier reported the mp of this compound as $152\text{--}153^\circ$, but of eight recent preparations seven melted at $237\text{--}238^\circ$ (decomp) but usually with indications of less definite crystal structure at about 145° . The eighth sample melted at 151° and reset almost immediately, finally melting at $233\text{--}234^\circ$ (decomp). The $[\alpha]_D^{25}$ values of the eight preparations were all between -19.6 and -23.3 ($c\ 1.5$, CHCl_3). We earlier reported $[\alpha]_D = -13$ ($c\ 1.0$, CHCl_3 ; Marks *et al.*, 1963). Bolton and Jeanloz (1963) reported mp $225\text{--}230^\circ$ (decomp) and $[\alpha]_D^{25} = -5.2$ ($c\ 1.27$, CHCl_3).

2-Acetamido-3,4,6-tri-O-acetyl-1-(α -benzyl *N*-benzyloxycarbonyl-L- β -aspartamido-1,2-dideoxy- β -D-glucose (II) was prepared, as described, by condensing compound V with α -benzyl *N*-benzyloxycarbonyl-L-aspartate (Marks *et al.*, 1963). The mp was $220\text{--}221^\circ$ (decomp) and $[\alpha]_D^{25}$ values of $+8.2$ ($c\ 0.98$, CHCl_3), $+6.8$ ($c\ 1.12$, CHCl_3), and $+9$ ($c\ 1.0$, CHCl_3) were found for three different preparations. The mp has also been reported as $214\text{--}217^\circ$ decomp and $[\alpha]_D^{25} = +28^\circ$ ($c\ 1.41$, CHCl_3) (Bolton and Jeanloz, 1963).

2-Acetamido-3,4,6-tri-O-acetyl-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose (Va) was prepared by a catalytic reduction of II. A 490-mg portion of compound II was dissolved in 60 ml 90:10 (v/v) acetic acid-water. Reduction with hydrogen, using palladium black (81 mg), was effected at room temperature for 70 minutes. The catalyst was removed by filtration and the solution was evaporated to dryness *in vacuo* at 40° . The compound was dissolved in acetic acid and precipitated

with light petroleum (bp $60\text{--}80^\circ$). To the precipitate 20 ml of water was added and the mixture was evaporated to dryness again. The substance was dried *in vacuo* at room temperature over P_2O_5 and KOH pellets. The compound had mp $212\text{--}214^\circ$; $[\alpha]_D^{25} = +6^\circ$ ($c\ 1.0$, H_2O), $+22.4^\circ$ ($c\ 0.64$, 2 N HCl). Found: C, 45.9; H, 6.2; N, 9.2. $\text{C}_{18}\text{H}_{27}\text{N}_3\text{O}_{11}$ requires C, 46.8; H, 5.9; N, 9.2%.

2-Acetamido-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose (I) was obtained by de-O-acetylating compound Va. The latter (120 mg) was dissolved in 2.4 ml of aqueous 0.6 N LiOH. The solution was kept at room temperature for 1 hour after which it was placed in an Amberlight CG-50(H^+) column ($4.5 \times 1.0\text{ cm}$). Elution with water resulted in the emergence of compound I from the column in the first five 5-ml fractions. These were combined and evaporated to dryness *in vacuo*. The residue was taken up in 0.2 ml of water, 10 ml ethanol was added slowly with stirring, and the substance was allowed to crystallize in the cold room. A further recrystallization was effected giving the monohydrated compound; yield 83 mg (93% theoretical); mp = $222\text{--}223$ with decomp, $[\alpha]_D^{25} = +18.6^\circ$ ($c\ 1$, H_2O), $+25.7^\circ$ ($c\ 0.66$, 2 N HCl). We earlier reported a mp of 220.5° (decomp), darkening from 205° , of similar material in which a contaminant was present as indicated by paper chromatography. The present compound was homogeneous by chromatography in three solvents and by electrophoresis (*vide infra*). Found: C, 40.8; H, 6.5; N, 11.8. $\text{C}_{12}\text{H}_{21}\text{N}_3\text{O}_8$, H_2O requires C, 40.8; H, 6.5; N, 11.9.

THE ISOLATION OF A GLUCOSAMINE-ASPARTIC ACID COMPLEX (IA) FROM EGG ALBUMIN

Glycopeptide which contained aspartic acid as the only amino acid residue was prepared by a procedure already described (Fletcher *et al.*, 1963c; method 2). This material was degraded to give an aspartic acid-glucosamine complex in the following way.

An amount of glycopeptide containing 110 mg mannose was dissolved in 25 ml 2 N HCl (prepared from constant-boiling HCl) and heated at 100° for 12 minutes. After cooling in running tap water, the solution was placed on an Amberlite IR-4B (150-g) column in the OH^- form. The pH of the aqueous effluent (500 ml) was adjusted to 5.6 with 3 N HCl, the solution was evaporated to dryness in a rotary evaporator at 40° , and the residue was taken up in about 0.5 ml water. The solution was placed on a Dowex-50 $\times 4$ (200–400 mesh) in the H^+ form (2 g, $3 \times 1\text{ cm}$) together with $2 \times 0.5\text{ ml}$ water washings, and the column was eluted with water. No ninhydrin-positive material (spot-tested on paper with 1% ninhydrin in acetone containing 1% pyridine) emerged in twenty 5-ml fractions. Elution with an aqueous-HCl gradient (two identical vessels containing 100 ml of water and 1.95 N HCl mounted at the same level and connected together) and collection of the effluent in 5-ml fractions yielded ninhydrin-positive (brown) material in fractions 21–29 (Fig. 2) and also (purple) in fractions 37–41. Elution with 5 N HCl yielded further ninhydrin-positive (purple) material (fractions 56–60). Fractions 21–29 were combined and placed on a Dowex-2 $\times 8$ (200–400 mesh) in the acetate form (40 g, $43 \times 1.5\text{ cm}$) and eluted with water. A bulk fraction of 45 ml was collected, followed by five 5-ml fractions. All these fractions were combined, evaporated to a small volume, and subjected to electrophoresis on a $110 \times 2.7\text{-cm}$ Porath (1956) column which contained 250 g Munktell cellulose. The buffer used was a formic acid-acetic acid mixture (70 ml, 98% formic acid, and 50 ml acetic acid

diluted to 2.5 liters) and the pH was 1.85. Electrophoresis was continued for 8 hours at a potential of 1000 v. The column was eluted with the same buffer and the eluent was collected in 10-ml fractions. Fractions 34-38 were ninhydrin positive, giving a brown color when tested under the particular conditions earlier described (Fletcher *et al.*, 1963c). They were evaporated to dryness *in vacuo* at 40° and crystallized from water-ethanol. Yield was about 12 mg (28% of theoretical).

A COMPARISON OF SYNTHETIC MODEL COMPOUND (I) WITH THE GLUCOSAMINE-ASPARTIC ACID (Ia) COMPLEX FROM THE PROTEIN

The physical constants of compounds I and Ia were first compared. The melting points were found to be 222-223° (decomp) and 220-223° (decomp), respectively. The $(\alpha)_D$ values in water were +18.6° (c 1.0) and +21.4° (c 0.45) for the two substances and neither substance underwent mutarotation. In this latter respect the compounds differ from 2-L- β -aspartamido-2-deoxy-D-glucose (VI) which had an $[\alpha]_D$ value of +29° to +22° (Marks and Neuberger, 1961; Liefänder, 1962). Bogdanov *et al.* (1964) have recently isolated a similar substance from egg albumin which had $[\alpha]_D$ in water of +23.7° (c 6.17).

The reducing power of the substances was assessed. Aqueous solutions of compounds I, Ia, and VI were prepared (1 mg/ml). Amounts of 5 μ l were applied to paper and treated with alkaline silver nitrate (Smith, 1960) or with aniline hydrogen phthalate (Partridge, 1949). Neither compound I nor Ia exhibited any reducing power, but compound VI, as expected, did.

The infrared spectra of compounds I and Ia were measured. Solid-state spectra were obtained with 1 mg of substance in 100 mg KCl. The spectra observed were similar. The interpretation of data in the 2000-3000 cm^{-1} region is made difficult by the fact that the substance is known to exist as a monohydrate. The peaks occurring at 1650 and 1548 cm^{-1} may be assigned to the amide compounds I and II bands, respectively. Peaks due to esters would occur at about 1740 and 1200 cm^{-1} and these were absent. They were observed, however, in the spectra of compounds IV and V at 1740 and 1235 cm^{-1} in each case, and this was expected because of the presence of O-acetyl groups in the molecules. Similar spectra have been reported by Yamashina *et al.* (1963) and by Bogdanov *et al.* (1964). In the case of our synthetic compound the definition of the peaks occurring at the stated wavelengths was much sharper than that observed with compound Ia and also than that previously reported by the other workers. We do not know the reason for this difference but it may be that the crystal forms differ slightly. It might be mentioned that the infrared spectrum given by 2-L- β -aspartamido-2-deoxy-D-glucose is almost identical qualitatively with that given by compound I, so that this test does not conclusively identify compound I with Ia.

The substances were analyzed after hydrolysis in 4 N HCl at 100° for 3 hours and removal of the HCl *in vacuo* over KOH and H_2SO_4 at room temperature. The residue was applied to an automatic amino acid analyzer. The synthetic compound gave rise to 1.05 moles aspartic acid, 1.21 moles ammonia, and 0.90 mole glucosamine. Values of 0.98 mole aspartic acid, 1.12 moles ammonia, and 0.93 mole glucosamine were found for the material from the protein.

The paper chromatographic behavior of the materials was studied. Descending chromatography was employed with Whatman No. 1 paper. When isobutyric

acid-0.5 N aqueous ammonia (5:3, v/v) was used as solvent, R_F (expressed as percentage, Smith, 1960) values of about 7 were found for compounds I and Ia, and of about 3 for compound VI. The mobility of all three substances in butanol-acetic acid-water (12:3:5, v/v) was also low. Each of the substances I, Ia, and VI exhibited an R_F value of about 4. With a solvent of phenol (or phenol-ammonia) as described by Smith (1960) compounds I and Ia on the one hand were clearly differentiated from compound VI. The R_F value of the first two compounds was 39, that of the last mentioned was 4. In all cases the position of the substances was revealed with ninhydrin in acetone containing pyridine. Each gave a brown color with this reagent provided the particular conditions earlier described were employed (Fletcher *et al.*, 1963c). If different conditions are used, other colors are obtained (see Bryant *et al.*, 1959).

High-voltage paper electrophoresis in a formic acid-acetic acid buffer at pH 1.85 (Marks *et al.*, 1963) indicated that compounds I, Ia, and VI behaved in an identical manner. With a potential gradient of 62 v/cm the substances moved on Whatman 3MM paper a distance of 15 cm toward the cathode in 1 hour. Aspartic acid and asparagine moved 18 and 22 cm, respectively, under these conditions.

GENERAL CONCLUSIONS

The linkage between the carbohydrate and peptide moieties of egg albumin is known to consist of N-acetyl-D-glucosamine linked to the β -carboxyl group of L-aspartic acid as discussed in the introduction. Two or three possible ways of attachment of the latter to the sugar residue have been considered. Micheel *et al.* (1963) suggested on the basis of chromatographic evidence that the linkage was formed through 2-L- β -aspartamido-2-deoxy-D-glucose (VI), but only one set of conditions was used in their comparison of the behavior of natural and synthetic products, namely, a Dowex-50 column with a uniform citrate buffer, pH 3.25 \pm 0.02, as eluting agent. However it seems likely that compounds I and VI would have the same mobility under these conditions, since even when chromatographed on Dowex-50 with both a pH (beginning at 2.91) and salt gradient these two substances do not show marked differences in behavior. However the properties of the natural material are more in keeping with those of the acylglucosaminylamine (I) than with those of the acylglucosamine derivative (VI; Marks *et al.*, 1963). The electrophoretic "fingerprint" obtained with a partial acid hydrolysate of the whole glycopeptide also make it likely that compound I constitutes the linkage (Fletcher *et al.*, 1963b). The evidence presented here in which some properties of a glucosamine-aspartic acid complex prepared from the protein are compared with those of compounds I and VI further substantiates this view. Thus compounds I and VI are differentiated in the present studies by the following: the behavior of solutions of the substances as observed with polarized light, the differences in reducing behavior, the production of ammonia from compound I but not from VI on acid hydrolysis, and the mobility on paper with phenol as solvent. In all these properties compounds I and Ia behaved in the same manner and differed from compound VI. Other properties such as the infrared spectra in the solid state, mobility on paper with either butanol-acetic acid-water (12:3:5, v/v) or isobutyric acid-0.5 N aqueous ammonia (5:3, v/v) as solvents and the electrophoretic behavior at pH 1.85 also support this conclusion but are not helpful in differentiating compound Ia

from VI. Furthermore the similarity in optical rotation of compounds I and Ia indicates that they have the same anomeric configuration, and this must be β if one considers the method of preparation of the model compound. Yamashina and Makino (1962) at one time suggested that the linking moiety might be the tautomeric imino ether, but this proposal has been withdrawn (Yamashina *et al.*, 1963). All the present results as well as those of others (Yamashina *et al.*, 1963; Bogdanov *et al.*, 1964) are in accord with the presence of 2-acetamido-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose as the oligosaccharide-peptide-linking moiety in hen's egg albumin.

We thank Dr. E. D. Kaverzneva of the Academy of Sciences, Moscow, for a sample of glucosamine-aspartic acid complex which had been isolated from egg albumin. The infrared spectrum in the solid state and mobility in the three solvents we used were identical with those obtained with similar material which we isolated.

ADDED IN PROOF

Very recently H. Tsukamoto, A. Yamamoto, and C. Miyoshita (1964, *Biochem. Biophys. Res. Commun.* 15, 151) have described some similar work. They believe that 2-acetamido-1-(L- β -aspartamido)-1,2-dideoxy- β -D-glucose (I) exists in two forms, one the trihydrate and one the anhydrous form with melting points 215–222° (decomp) and 255–258° (decomp), respectively, and that these two forms have slightly different infrared spectra. Our synthetic material has usually been obtained with mp 222–223° and analysis indicates that it is a monohydrate. This has been confirmed by titration in 0.1 M KCl. An equivalent weight of 350 and apparent pK_a values of 2.05 and 8.6 were found. We have also obtained a sample of this substance with mp 257–259° on one occasion. This latter compound had identical chromatographic and electrophoretic properties to the substance with lower mp and stained brown with ninhydrin under the conditions we used. Both our preparations of synthetic compound I had identical infrared spectra, and were in fact identical with that given by Tsukamoto *et al.* (1964) for his lower-melting-point material.

REFERENCES

- Berger, A., and Katchalski, E. (1951), *J. Am. Chem. Soc.* 73, 4084.
 Bergmann, M., and Zervas, L. (1932), *Ber.* 65, 1192.
 Bergmann, M., Zervas, L., and Salzmann, L. (1933), *Ber.* 66, 1288.
 Bertho, A., and Révész, A. (1955), *Ann.* 581, 161.
 Bogdanov, V. P., Kaverzneva, E. D., and Andrejeva, A. P. (1962), *Biochim. Biophys. Acta* 65, 168.
 Bogdanov, V. P., Kaverzneva, E. D., and Andrejeva, A. P. (1964), *Biochim. Biophys. Acta* 83, 69.
 Bolton, C. H., and Jeanloz, R. W. (1963), *J. Org. Chem.* 28, 3228.
 Bryant, P. M., Moore, R. H., Pimlott, P. J., and Young, G. T. (1959), *J. Chem. Soc.*, 3868.
 Cunningham, L. W., Nuenke, B. J., and Nuenke, R. B. (1957), *Biochim. Biophys. Acta* 26, 660.
 Fletcher, A. P., Marks, G. S., Marshall, R. D., and Neuberger, A. (1963c), *Biochem. J.* 87, 265.
 Fletcher, A. P., Marshall, R. D., and Neuberger, A. (1963a), *Biochem. J.* 88, 37P.
 Fletcher, A. P., Marshall, R. D., and Neuberger, A. (1963b), *Biochim. Biophys. Acta* 71, 505.
 Gomori, G. (1955), *Methods Enzymol.* 1, 143.
 Hudson, C. S. (1909), *J. Am. Chem. Soc.* 31, 66.
 Jevons, F. R. (1958), *Nature* 181, 1346.
 Johansen, P. G., Marshall, R. D., and Neuberger, A. (1958), *Nature* 181, 1345.
 Johansen, P. G., Marshall, R. D., and Neuberger, A. (1961), *Biochem. J.* 78, 518.
 Kaverzneva, E. D., and Bogdanov, V. P. (1960), *Biochim. Biophys. Acta* 26, 105.
 Leaback, D. H., and Walker, P. G. (1957), *J. Chem. Soc.*, 4754.
 LeQuesne, W. J., and Young, G. T. (1952), *J. Chem. Soc.*, 24.
 Liefänder, M. (1962), *Naturwiss.* 49, 394.
 Marks, G. S., Marshall, R. D., and Neuberger, A. (1962), *Biochem. J.* 85, 15P.
 Marks, G. S., Marshall, R. D., and Neuberger, A. (1963), *Biochem. J.* 87, 274.
 Marks, G. S., and Neuberger, A. (1961), *J. Chem. Soc.*, 4872.
 Micheel, F., and Wulff, H. (1956), *Ber.* 89, 1521.
 Micheel, F., Ostmann, E.-A., and Pielmeier, G. (1963), *Tetrahedron Letters*, 115.
 Neuberger, A. (1938), *Biochem. J.* 32, 1435.
 Neuberger, A., and Papkoff, H. (1963), *Biochem. J.* 87, 581.
 Nuenke, R. H., and Cunningham, L. W. (1961), *J. Biol. Chem.* 236, 2452.
 Partridge, S. M. (1949), *Nature* 164, 443.
 Porath, J. (1956), *Biochim. Biophys. Acta* 22, 151.
 Smith, I. (1960), *Chromatographic and Electrophoretic Techniques*, Vol. I, London, Heinemann.
 Yamashina, I., and Makino, M. (1962), *J. Biochem. (Tokyo)* 51, 359.
 Yamashina, I., Ban-I, K., and Makino, M. (1963), *Biochim. Biophys. Acta* 78, 382.