Periodate Oxidation of Glycopeptides from Ovalbumin*

Rex Montgomery, Ya-Chen Wu, and Yuan Chuan Lee†

ABSTRACT: The glycopeptides of ovalbumin were oxidized by sodium periodate with the liberation of 2 moles of formic acid. Correlation of the consumption of the oxidant and the destruction of the sugar residues is complicated by the slow oxidation of a 1,2-linked D-mannopyranose unit. Identification of this reaction was made using classical methylation techniques before oxidation and after 2.5 and 3.0 residues of the 5 D-mannose in the asparaginyl carbohydrate glycopeptide had been oxidized. A further insight into the structure of the carbohydrate in ovalbumin was gained by application of three consecutive Smith degradations to the asparaginyl carbohydrate. The structure contains a terminal nonreducing 2-acetamido-2-deoxy-D-glucopyranosyl residue and 2 nonreducing D-mannopyranosyl residues which are part of a branched tetramannosyl side chain.

Periodate oxidation of carbohydrates has been extensively applied to the elucidation of their structures, based upon the consumption of the oxidant, the concomitant formation of formic acid and formaldehyde, and the properties of the oxidized carbohydrate after its subsequent reduction to a polyalcohol (Smith and Montgomery, 1959). However, the information requires careful evaluation, particularly in the case of glycoproteins, where determination of the periodate consumption has little meaning and the formic acid production, at least as measured titrimetrically, is confused by the changed pK_a values of the oxidized protein. Information concerning the fate of the sugar residues does lead to an insight into the types of linkages between the carbohydrate residues in glycoproteins (Chatterjee *et al.*, 1961; Lee and Montgomery, 1961; Chatterjee and Montgomery, 1962; Bragg and Hough, 1961; Spiro, 1964) but in this general procedure there exist severe limitations. Owing to the different kinds of oxidation taking place in the glycoprotein it is difficult to ascertain the point of complete Malepradian-oxidation of the carbohydrate groups, and overoxidation can occur. In part this reflects the problems of carbohydrate determination in the continuously changing mixture that is represented by a glycoprotein undergoing periodate oxidation.

An extension of the periodate oxidation techniques to include reduction of the oxidized product and preferential hydrolysis of the sensitive acetal bonds so produced, followed by additional oxidation, reduction, and mild acid hydrolysis of the resistant oligosaccharides formed at each stage, results in a stepwise degradation of the molecule. This procedure, now called the Smith degradation, has been extensively applied by Smith and co-workers to the study of the fine structure of polysaccharides (Abdel-Akher *et al.*, 1952; Hamilton and Smith, 1956; Goldstein *et al.*, 1957; Smith and Unrau, 1959). By this series of reactions a chain of sugar residues is hydrolyzed at the points where periodate oxidation takes place. It is evident that in the case of the glycoproteins the Smith degradation procedure may release at each step some small oligosaccharide or monosaccharide units and these appear to have been oxidized if only the amino acid–containing residues are analyzed for the residual sugar content. Furthermore the peptide chain may be cleaved during the degradative reactions with the result that no residue in the molecule can be considered constant in relation to the original protein. These problems were encountered with the glycohexapeptide of γ-globulin (Rothfus and Smith, 1963) and with ovomucoid (Chatterjee and Montgomery, 1962). In the latter case the results were further complicated by the existence in the molecule of three oligosaccharide groups (Montgomery and Wu, 1963) of similar composition but unknown structure.

In view of these problems in working with the whole glycoprotein, the periodate oxidation and Smith degradation of the asparaginyl carbohydrate from ovalbumin, Asp(NH·carbohydrate), was studied. The simplest of the glycopeptides has been obtained in pure form (Montgomery *et al.*, 1965) and is composed of 3 residues of 2-acetamido-2-deoxy-D-glucose and 5 residues of D-mannose, with one of the hexosamine residues linked through the hemiacetal carbon to L-asparagine. However, periodate oxidation of this simple octasaccharide resulted in the destruction of 2.5 moles of D-mannose (Lee and Montgomery, 1961; Fletcher *et al.*, 1963; Clamp and Hough, 1963) which was difficult to explain. The present paper presents a detailed study of this periodate oxidation reaction, with conclusions supported by methylation studies.

* From the Department of Biochemistry, University of Iowa, Iowa City. *Received November 3, 1964.* This investigation was supported by a U.S. Public Health Service research grant (HE-06717) from the National Heart Institute. A brief report was presented at the 148th Meeting of the American Chemical Society in Chicago, September 1964 (Montgomery, 1964).

† Present address: Department of Biochemistry, University of California, Berkeley, Calif.

Materials

The glycopeptides Tyr-Asp(NH·carbohydrate), Glu-Lys-Tyr-Asp(NH·carbohydrate), and Asp(NH·carbohydrate) were prepared from ovalbumin as described previously (Lee and Montgomery, 1962; Montgomery *et al.*, 1965).

In some instances the glycopeptide mixture that resulted from the digestion of ovalbumin twice with pronase was used without separation into Asp(NH·carbohydrate) and Asp(NH·carbohydrate)-Leu by zone electrophoresis.

Glycyl-L-tyrosylglycinamide, chromatographically pure, was obtained from Mann Research Laboratories and used without further purification.

Methods

Paper Chromatography. Chromatographic analyses were carried out in a descending manner on Whatman No. 1 paper in the following solvent systems: (1) ethyl acetate–acetic acid–formic acid–water (18:3:1:4, v/v); (2) 1-butanol–acetic acid–water (4:1:1, v/v); (3) butanone–water azeotrope; (4) 1-butanol–ethanol–water (40:11:19, v/v). Sugars were located on the chromatograms by a silver nitrate–sodium hydroxide dipping reagent (Trevelyan *et al.*, 1950).

Two-dimensional Paper Electrophoresis and Paper Chromatography. The separation of the methyl ethers of D-mannose was achieved by paper chromatography in solvent systems (3) or (4) followed by paper electrophoresis at right angles in 0.17 M borate buffer, *p*H 10.2, at 360 v for 4.5 hours. Sugars were detected with aniline phthalate reagent (Hough *et al.*, 1950), which had been acidified with 1.5 volume % of 85% phosphoric acid.

Determination of Sugars. The carbohydrate analyses were performed exactly as described previously (Lee and Montgomery, 1962).

Gas-Liquid Partition Chromatography. The analyses by gas-liquid partition chromatography were made using an Aerograph "Hy-Fi" Model A-600-B (Wilkens Instrument and Research, Inc.) fitted with a hydrogen-flame detector and employing a stainless steel column 152 cm × 3 mm o.d. (5 ft × 1/8 in.) packed with 2% (w/w) neopentyl glycol succinate polyester (Applied Science Laboratories) on 100–110 mesh ABS Anakrom (Analabs Inc.) at 177° with flow rates of nitrogen and hydrogen of about 25 ml/min.

Amino Acid Analyses. Amino acid analyses were carried out with ion-exchange resin columns at 50° (Hirs *et al.*, 1954; Moore *et al.*, 1958) using an amino acid analyzer (Model K-5000, Phoenix Precision Instrument Co., Philadelphia).

Determination of Amino Groups. Amino compounds were determined by a modification of the ninhydrin method (Rosen, 1957).

Periodate Oxidation of Glycopeptides. Periodate oxidation was carried out with 0.01–0.1 M sodium periodate at 5° in the dark, using at least a 5-fold excess of oxidant. The rate of periodate consumption was determined by a microtitrimetric procedure based on the arsenite method of Fleury and Lange (1933) or spectrophotometrically at 260 mμ (Dixon and Lipkin, 1954; Grado and Ballou, 1961). In the titrimetric method, 100-μl aliquots of the reaction mixture (containing about 0.4 μmole of sample and 10 μmoles of periodate) were added to an Erlenmeyer flask containing 5 ml of saturated sodium bicarbonate, 1 ml of 0.02 N sodium arsenite, and 0.5 ml of 20% potassium iodide. The mixture stood for 15–30 minutes at room temperature in the dark. Starch indicator (1%, 1 ml) was added and the residual arsenite was titrated with 0.0786 N iodine solution from a syringe-type microburet (0.5 ml/inch use/inch of linear movement).

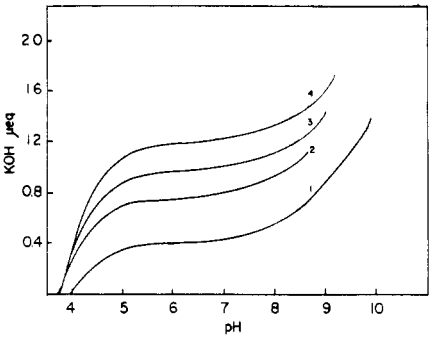


FIGURE 1: The titration curves of formic acid produced during the oxidation of 38.8 μmoles of Asp(NH·carbohydrate) with 0.1 M sodium periodate (10 ml) at 5°. Curves 1, 2, and 3 were obtained at zero, 1, and 18 hours; curve 4 for both 54 and 66 hours. Titration of 100-μl aliquots with 0.01 N potassium hydroxide was performed as described in the text.

The increase in acidity during oxidation owing to the liberation of formic acid was followed both titrimetrically and enzymically using formyl tetrahydrofolate synthetase (Rammler and Rabinowitz, 1962). For the titrimetric procedure, 100-μl aliquots of the periodate reaction mixture, containing about 0.4 μmole of glycopeptide, were each added to 25 μl of 50% aqueous ethylene glycol and 1 ml of water in 15 × 40 mm glass vials, which were stoppered. After standing for 15–30 minutes at room temperature, 1 ml of water was added and the solution was titrated with 0.01 N potassium hydroxide in an atmosphere of nitrogen. In each case titration curves between *p*H 3.8 and 9.5 were obtained with a Radiometer Model SBR2c/SBVla Titrigraph. A blank titration curve was obtained by treating the corresponding amount of periodate solution with ethylene glycol as described and then adding the appropriate amount of pure glycopeptide. From the family of titration curves (for example, see Figure 1) the titration values at *p*H 6.25 were corrected for the blank value and the moles of formic acid formed per mole of glycopeptide were calculated.

In order to analyze for the sugars that were unoxidized, aliquots of the periodate reaction mixture were treated with excess ethylene glycol and desalted by passing through a column (1.8 × 33 cm) of Sephadex

TABLE I: Periodate Oxidation of Tyr-Asp(NH·Carbohydrate), Glu-Lys-Tyr-Asp(NH·Carbohydrate), and Gly-Tyr-Gly(NH_2).

	Time (hours)							
	10	21	32	45	69	100	117	165
	Moles IO_4 consumed per mole compound							
Tyr-Asp(NH·carbohydrate)		5.00		5.52	5.58	5.78		
Glu-Lys-Tyr-Asp(NH·carbohydrate)	3.84		5.02		5.43		6.43	7.34
Gly-Tyr-Gly(NH_2)		0.06		0.08	0.11	0.31	0.55	0.57
	Moles residual sugar/mole Tyr-Asp(NH·carbohydrate)							
D-Mannose	2.58			2.34	2.28			
2-Amino-2-deoxy-D-glucose	1.95			1.74	1.61			

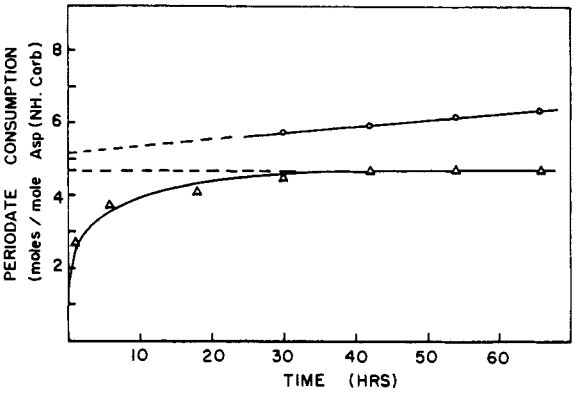


FIGURE 2: Rate of periodate consumption by Asp(NH·carbohydrate). The conditions of oxidation are exactly as given in Figure 1. For the titrimetric (O—O) determination, 100, μl aliquots were titrated as described in the text. Spectrophotometric (Δ—Δ) determinations were made at 260 mμ (1-cm light path) on aliquots (30.2 μl) of the reaction solution diluted with water (3.0 ml).

G-25 (medium), developing with 0.1 M trimethylamine acetate, *p*H 4.5. The carbohydrate-containing fractions appeared at 30–55 ml effluent volume and the iodate after 60–75 ml. The carbohydrate fractions were combined, concentrated *in vacuo* at 40° to approximately 5 ml, and treated with 200 mg of sodium borohydride at 5° overnight. The alkaline solution was then neutralized with acetic acid and analyzed for 2-amino-2-deoxy-D-glucose and D-mannose.

Periodate Oxidation of Tyrosine-containing Glycopeptides. Tyr-Asp(NH·carbohydrate), and Glu-Lys-Tyr-Asp(NH·carbohydrate) containing the equivalent of 10.48 and 10.25 mg, respectively, of D-mannose, were oxidized at 5° with 0.01 M sodium periodate, and the consumption of periodate was followed by the arsenite method. In an attempt to correct for the periodate consumption due to the tyrosyl residue (Lee and Montgomery, 1961), 8.08 μmoles of Glu-Tyr-Gly(NH_2) were similarly treated. The results, summarized in Table I, show the small but significant slow oxidation of the tripeptide. Using these values to correct those found for the glycopeptides it is seen that the Tyr-Asp(NH·carbohydrate) reaches a maximum and constant degree of oxidation at 5.47 moles of periodate per mole. This contrasts with the Glu-Lys-Tyr-Asp(NH·carbohydrate) oxidation, which continues to slowly oxidize, with an extrapolated zero time value at 4.7 moles of periodate consumed per mole.

The fate of the sugar residues during the oxidation of Tyr-Asp(NH·carbohydrate) is similar to that found for the whole protein. An extrapolation to zero time indicates an oxidation of 30% of the 2-amino-2-deoxy-D-glucose and 50% of the D-mannose.

Periodate Oxidation of Asp(NH·Carbohydrate). Asp(NH·carbohydrate) was oxidized with 0.1 M sodium periodate at 5°. The periodate consumption, followed titrimetrically and spectrophotometrically, extrapolated at zero times to 5.2 and 4.7 moles/mole, respectively (Figure 2). These results were similar to those obtained for the glycopeptides containing tyrosine and to those reported by other workers (Clamp and Hough, 1963; Fletcher *et al.*, 1963).

The concurrent production of formic acid corresponded to 2.07 moles/mole of Asp(NH·carbohydrate) when determined either titrimetrically or enzymically using formyl tetrahydrofolate synthetase. However, when the concentration of periodate was reduced to 0.05 M the second mole of formic acid was liberated at a significantly slower rate (Figure 3).

At room temperature (25°) in the dark, the oxidation of 12.1 mg of Asp(NH·carbohydrate) with 2 ml of 0.0684 M sodium periodate is much more rapid and overoxidation is excessive. The periodate consumption was followed spectrophotometrically and the concomitant formation of formic acid was measured enzymically. The results are summarized in Table II.

Periodate Oxidation of Methyl α-D-Mannopyranoside. Methyl α-D-mannopyranoside (10.8 mg) was oxidized at 5° in the dark with 5 ml of 0.1 M, 10 ml of 0.05 M, and 50 ml of 0.01 M sodium periodate. The formic acid

TABLE II: Periodate Oxidation of Asp(NH·Carbohydrate) at Room Temperature (25°) with 0.068 M Sodium Periodate.

Time (hours)	Moles/Mole of Asp(NH·Carbohydrate)	
	Periodate Consumption	Formic Acid Production
9	6.15	3.06
20	6.88	3.67
44	8.38	4.07
70	9.48	4.90
105	11.0	
145	12.9	6.56

TABLE III: Periodate Oxidation of Methyl α-D-Mannopyranoside.

Time (hours)	Moles Formic Acid/Mole Glycoside		
	0.1 M IO_4^-	0.05 M IO_4^-	0.01 M IO_4^-
0.3	0.34	0.36	0.29
24	0.90	0.74	0.47
48	1.00	0.90	0.60
137	1.01	1.00	
200	1.05	1.03	0.93

production was followed titrimetrically in each reaction and the results are summarized in Table III.

Smith Degradation of Asp(NH·*Carbohydrate*). EXPERIMENT 1. Asp(NH·carbohydrate) (19.2 mg, 12.4 μmoles) was oxidized with 0.08 M sodium periodate (3.6 ml) in the dark at 4–5° for 136 hours. The excess periodate was destroyed with 50% aqueous ethylene glycol (1 ml) at room temperature for 3 hours and, after the material had cooled to 4–5°, sodium borohydride (250 mg) was added. The reduction proceeded overnight in the refrigerator and the excess borohydride was decomposed with glacial acetic acid (1 ml). The resulting solution was desalted by gel filtration on a column (28 × 1.8 cm) of Sephadex G-25 in 1 M acetic acid at a flow rate of 25 ml/hour. The eluate (between volumes 20 and 45.5 ml) was collected and contained the equivalent of 5.3 mg (29.4 μmoles) of D-mannose, as determined by the phenol–sulfuric acid procedure. One-half of the D-mannose in Asp(NH·carbohydrate) thus resisted oxidation. The effluent was concentrated *in vacuo* to a sirup, which was dissolved in 0.1 N hydrochloric acid (4 ml). After 1 day at room temperature, the solution was filtered through a column of Sephadex G-25 as before and the corresponding effluent volume was collected (4.13 mg equivalent of D-mannose), together with the tailing portion (between volumes 46 and 71

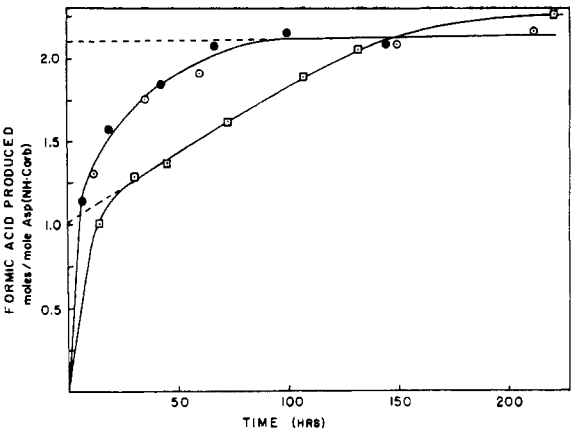


FIGURE 3: Rate of formic acid formation from the periodate oxidation of 15.1 μmoles of Asp(NH·carbohydrate) with 0.05 M (⊡—⊡) and 0.1 M (●—● and O—O) concentrations of sodium periodate (3 ml) at 5°.

ml) which contained 0.4 mg equivalent of D-mannose. The total recovery of D-mannose from this gel filtration was thus 85%. The molar ratio of 2-amino-2-deoxy-D-glucose, determined by the method of Boas (1953), to D-mannose, determined by phenol–sulfuric acid reagents, was 2.0:2.3 and 2.0:2.2 in duplicate analysis. For comparison and correlation with the procedure used in experiment 2, the molar ratio was also determined by a submicro procedure in which a sample was hydrolyzed in an evacuated sealed ampule with 0.03 N hydrochloric acid for 25 hours at 110°. Paper chromatography (solvent system 1) of the hydrolysate separated the two sugars, which were quantitatively eluted from the paper and analyzed by the procedure of Park and Johnson (1949). Duplicate analyses gave the molar ratio of 2-amino-2-deoxy-D-glucose to D-mannose as 2.0:2.7 and 2.0:2.6.

EXPERIMENT 2. Asp(NH·carbohydrate) (23 mg, 14.9 μmoles) was oxidized with 0.05 M sodium periodate (3 ml) in the dark at 4–5° for 94 hours. The excess periodate was reduced with 50% ethylene glycol (1 ml) and after the material had stood at room temperature (25°) for 30 minutes, sodium borohydride (250 mg) was added. The reduction proceeded overnight at 5° and the alkaline solution (*p*H 10) was then neutralized with glacial acetic acid (0.2 ml). The resulting solution was desalted by gel filtration on a column (29 × 1.8 cm) of Sephadex G-25 in water at a flow rate of 10 ml/hour. The eluate (16–46 ml, equivalent to 7.5 mg mannose) contained 94% of the D-mannose equivalent put on the column and 55% of the D-mannose equivalent of the starting material. The solution of oxidized and reduced Asp(NH·carbohydrate), designated ACO,[1] was freed

[1] Abbreviations used in this work: ACO, solution of oxidized and reduced Asp(NH carbohydrate); ACOO, oxidized and reduced residue of a mild acid treated ACO; ACOOO, final degradation product of ACO.

from any contaminating cellulose lint by filtration through glass wool and evaporation to dryness *in vacuo*. The residue was dissolved in exactly 5 ml of water and the solution was stored at −20° and used as the stock solution of ACO. Duplicate analyses gave L-aspartic acid–2-amino-2-deoxy-D-glucose–D-mannose molar ratios of 1.0:2.0:2.9 and 1.0:2.0:3.0 using the submicro procedure outlined in experiment 1.

Paper chromatography (13 hours in solvent 1 by descending flow) of the hydrolysate of ACO showed only glycerol (R_{man} 2.71), mannose, and 2-amino-2-deoxy-D-glucosamine (R_{man} 0.42). Similar results were obtained when the hydrolysate was chromatographed in solvent 2 for 13 hours: glycerol (R_F 0.39), mannose (R_F 0.17), and 2-amino-2-deoxy-D-glucose (R_F 0.09). There was no evidence of erythritol.

To an aliquot (4 ml) of the stock solution of ACO containing the equivalent of 6.1 mg of D-mannose was added water (2 ml) and constant-boiling hydrochloric acid (0.1 ml). After 25 hours at room temperature (25°), in a closed container, the solution was evaporated to dryness *in vacuo* and the residue dissolved in water (100 μl). Aliquots (5 μl) were chromatographed in solvent system 2 for 12 hours; one heavy spot of glycerol (R_F 0.36) and two weak spots (R_F 0.53 and 0.63) were revealed by the silver reagent. When the paper was sprayed with ninhydrin solution and kept overnight at room temperature there was only one intense purple spot at the origin, which corresponded to the periodate-resistant nucleus of Asp(NH·carbohydrate). Duplicate analyses by the submicro procedure showed a molar ratio of 2:2.9 and 2:2.5 for 2-amino-2-deoxy-D-glucose and D-mannose.

The mild acid treated ACO solution (containing the equivalent of 6 mg of D-mannose) was purified by gel filtration on a column (29 × 1.8 cm) of Sephadex G-25 in 1 M acetic acid at a flow rate of 30 ml/hour. The eluate from 20 to 50 ml was collected (equivalent to 3 mg mannose, 50% recovery) and evaporated to dryness *in vacuo*. The residue was oxidized with 0.1 M sodium periodate (2 ml) in the dark for 72 hours. The oxidized material was treated with ethylene glycol, reduced with sodium borohydride, and desalted with Sephadex G-25 in 1 M acetic acid as described previously. The eluate from 15 to 45 ml was collected (equivalent to 0.5 mg mannose) and evaporated to dryness *in vacuo*, and the residue (designated ACOO) was made up to a standard stock solution in 5 ml of water. An aliquot showed a molar ratio of 1.0:2.0:1.1 for L-aspartic acid, 2-amino-2-deoxy-D-glucose, and D-mannose, respectively.

EXPERIMENT 3. Asp(NH·carbohydrate) (20 mg) was subjected to three consecutive Smith degradations by the procedures given in experiment 2. The final product (designated ACOOO) was partially desalted by gel filtration on a column (29 × 1.8 cm) of Sephadex G-25 in 1 M acetic acid. Fractions (5 ml) were collected. Aliquots (0.5 ml) from alternate fractions were mixed with water (0.5 ml) and used for the ninhydrin reaction (Rosen, 1957). The eluate containing ACOOO (35–60 ml) overlapped considerably with that containing the inorganic salts (45–94 ml). A desalted portion (35–45 ml, containing about 0.5 μmole of hexosamine) was evaporated to dryness, and the residue was hydrolyzed as usual for the purpose of paper chromatography. There was a heavy spot of 2-amino-2-deoxy-D-glucose but only a trace amount of mannose as revealed with the silver reagent. A second quantitative paper chromatographic analysis using solvent system 1 separated aspartic acid from 2-amino-2-deoxy-D-glucose. These spots were extracted from the paper with water (5 ml), and aliquots (1 ml) were analyzed by the Rosen ninhydrin reaction. Although the absorbance at 570 mμ, corrected for the paper blank, for L-aspartic acid (0.54) was higher than that of 2-amino-2-deoxy-D-glucose (0.43), it is most likely that there were approximately equimolar amounts of these residues in ACOOO.

EXPERIMENT 4. A mixture of glycopeptides (80.9 mg, containing the equivalent of 29.0 mg of D-mannose) from the digestion of ovalbumin with pronase was oxidized with 0.1 M sodium periodate (10 ml) at *p*H 3.5, initially, and 5° in the dark for 42 hours. The resulting oxidation mixture was reduced with sodium borohydride and gel-filtered through Sephadex G-25 as described previously. The product contained the equivalent of 13.0 mg of D-mannose, indicating that 45% of the D-mannose resisted periodate oxidation, and the submicro procedure described that there were equimolar amounts of 2-amino-2-deoxy-D-glucose and D-mannose present. The material was subjected to mild hydrolysis with 0.1 N hydrochloric acid for 25 hours at room temperature and the periodate-resistant nucleus, isolated by gel filtration as before, was methylated as described later.

Preliminary Studies of Asp(NH·Carbohydrate) and Its Periodate-oxidized Derivatives by Methylation. The general procedure for methylation and subsequent methanolysis is illustrated for the case of Asp(NH·carbohydrate), 47 mg of which was thoroughly dried and dissolved in dimethyl sulfoxide (2 ml). To this solution were added dimethylformamide (2 ml), methyl iodide (1 ml), and powdered barium oxide (3 g). The reaction mixture was sealed in an ampule under an atmosphere of nitrogen and shaken in the dark at room temperature for 2 days. The resulting mixture was dissolved in 80% aqueous acetic acid (60 ml) and gel-filtered on a column (64 × 4 cm) of Sephadex G-25 and eluted with 1 M acetic acid at 100 ml/hour. The fractions between effluent volumes 250 and 550 ml, containing all the carbohydrate material, were combined and evaporated to dryness *in vacuo*. The residue, now soluble in dimethylformamide (4 ml) without the addition of dimethyl sulfoxide, was methylated twice more as before. The final product (47 mg) was a pale-yellow glass. The infrared spectrum of the three-times-methylated Asp(NH·carbohydrate), 1.88 mg in potassium bromide (280 mg), showed no OH absorption in the bending region at 1560 cm^{-1}; any OH-stretching frequencies would be masked by the NH-stretching frequencies at about 3300 cm^{-1}.

A portion (9.4 mg) of methylated Asp(NH·carbohydrate) was treated with 1 N methanolic hydrogen chlo-

ride (2 ml) at 100° for 16 hours and the resulting solution was evaporated to dryness *in vacuo*. The residue was treated with a 50% aqueous suspension (2 ml) of Dowex 50 X8 (H^+ form). The mixture was filtered and the resin was washed several times with water until free from carbohydrate. The combined filtrates were evaporated to dryness *in vacuo* and the residue was dissolved in methanol (2 ml). The solution was analyzed by gas-liquid chromatography (Figure 4a).

The analysis in Figure 4a is compared with that (Figure 4b) from the methanolysate of methylated ACO, which was obtained by periodate oxidation of Asp(NH·carbohydrate) according to the procedure of experiment 2, followed by reduction and methylation.

The Smith degradation product obtained in experiment 4 was methylated and then methanolyzed. The mixture of methyl glycosides was hydrolyzed with 1 N hydrochloric acid at 100° for 6 hours and the resulting free sugars were analyzed qualitatively by two-dimensional paper chromatography and paper electrophoresis as described earlier. The two major components were 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-mannose, with a smaller amount of di-*O*-methyl-D-mannose, possibly the 3,6-isomer.

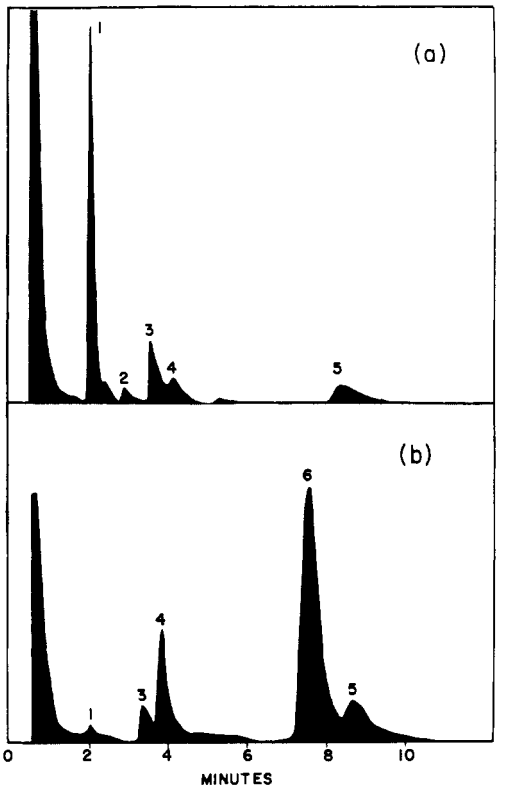


FIGURE 4: Gas-liquid partition chromatography of the cleavage fragments from the methanolysis of methylated Asp(NH·carbohydrate) (a) and its periodate-oxidized and reduced derivative (ACO) (b). (1) Methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside; (2) methyl 2,3,-4,6-tetra-*O*-methyl-β-D-mannoside; (3) methyl 3,4,6-tri-*O*-methyl-α-D-mannoside; (4) methyl 2,4,6-tri-*O*-methyl-α-D-mannoside; (5) methyl 3,6(?)-di-*O*-methyl D-mannoside; (6) not identified.

Discussion

The ovalbumin glycopeptides Tyr-Asp(NH·carbohydrate) and Glu-Lys-Tyr-Asp(NH·carbohydrate), where the carbohydrate is an octasaccharide composed of 3 residues of 2-acetamido-2-deoxy-D-glucose and 5 residues of D-mannose, were oxidized with 0.01 M sodium periodate, and the results were similar to those reported earlier (Fletcher *et al.*, 1963; Clamp and Hough, 1963) for similar materials. The periodate consumption extrapolated to approximately 5 moles/mole at zero time when corrected for oxidation of the tyrosyl residue by following the consumption of periodate by the tripeptide Gly-Tyr-Gly(NH_2). Even with this correction the two glycopeptides did not behave the same with respect to the degree of "overoxidation" (Table I). The analyses for the surviving sugar residues during the oxidation of Tyr-Asp(NH·carbohydrate) gave results that agreed with the study of the whole protein (Lee and Montgomery, 1961). Again, extrapolation to zero time suggested that one of the three 2-acetamido-2-deoxy-D-glucose residues and 2.5 residues of D-mannose were oxidized. Since the latter figure defied a simple explanation and could not be used to choose between 2 or 3 moles of D-mannoses being oxidized, both of which were possible from the amount of periodate consumed, a more extensive study was made of the simplest glycopeptide from ovalbumin.

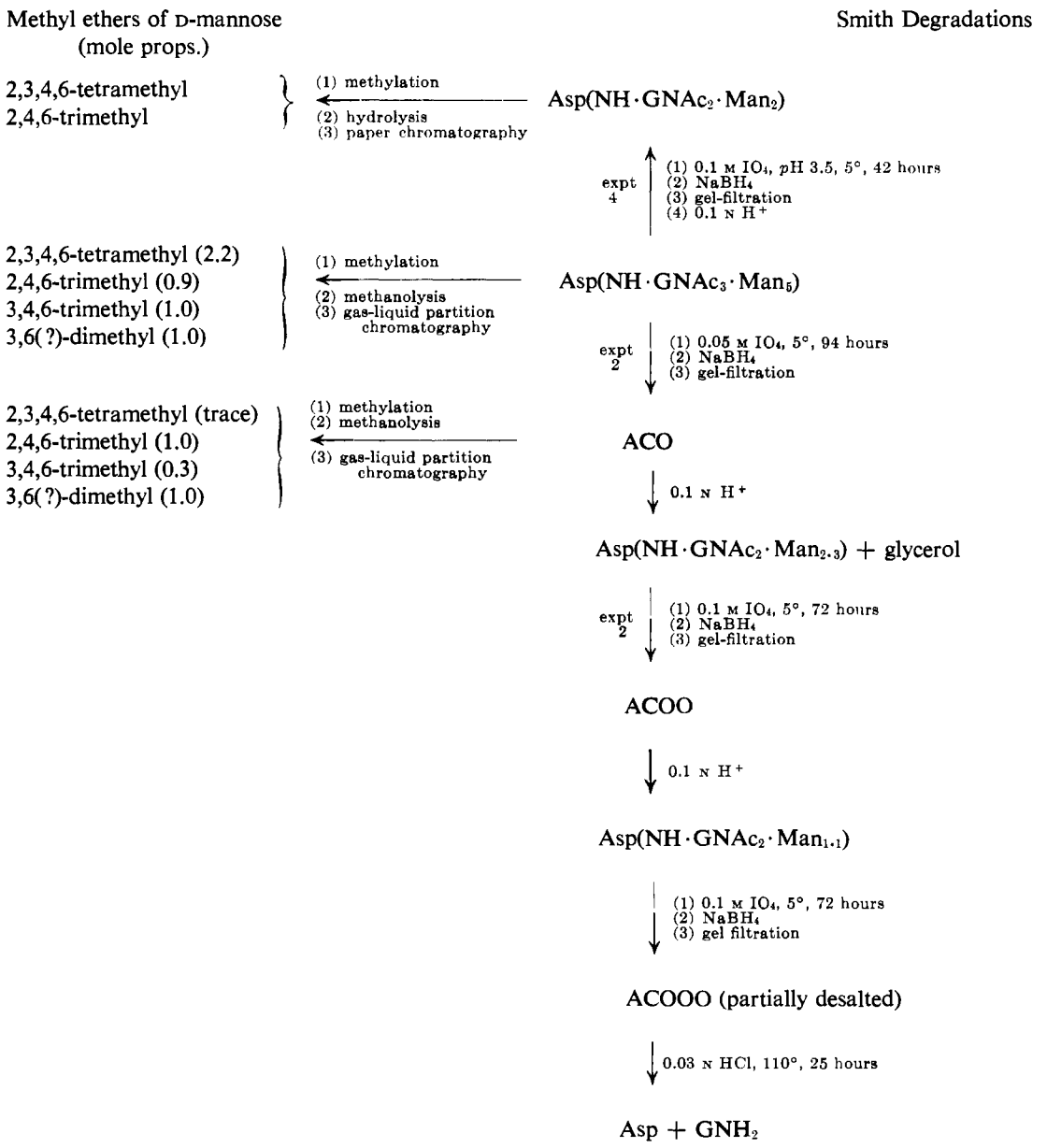
The asparaginyl carbohydrate (Asp[NH·carbohydrate]) was oxidized under several conditions and the reaction was followed by various analytical methods. At room temperature there was extensive overoxidation (Table II), but with 0.10 M periodate at 5° the consumption of oxidant, followed both titrimetrically (Fleury and Lange, 1933) and spectrophotometrically (Dixon and Lipkin, 1954; Grado and Ballou, 1961), extrapolated to 5.2 and 4.7 moles/mole of Asp(NH·carbohydrate), respectively (Figure 2), in agreement with other workers (Fletcher *et al.*, 1963; Clamp and Hough, 1963). Contrary to the results of these investigators, who reported 0.7–1.0 mole of formic acid formed per mole of glycopeptide, the results of the present studies corresponded to 2.07 moles/mole of Asp(NH·carbohydrate), when determined either titrimetrically or by a procedure involving the specific enzyme, formyl tetrahydrofolate synthetase (Rammler and Rabinowitz, 1962). The identity of the results by the two methods (Figure 3) confirmed that the increased acidity was due to formic acid and not a change in the *pK* values of the asparaginyl residue.

The second mole of formic acid was liberated from Asp(NH·carbohydrate) at a significantly slower rate when 0.05 M periodate was used (Figure 3), approaching the even slower rate that was reported previously with 0.01 M oxidant (Fletcher *et al.*, 1963). Similar rates for the production of formic acid were observed with methyl α-D-mannopyranoside when it was oxidized

with 0.01–0.10 M periodate (Table III). As in the case of hyaluronic acid (Montgomery and Nag, 1963), the rates of reaction of several oxidizable groups in glycopeptides and other carbohydrates may differ enough to cause a primary rate to be interpreted as "overoxidation." Thus an extrapolation of the essentially linear portion of the curve showing formic acid formation from Asp(NH·carbohydrate) with 0.05 M periodate between 30 and 100 hours (Figure 3), on the assumption that this is due to overoxidation, would erroneously suggest that the molecule oxidizes with the formation of 1 mole of formic acid per mole.

A further insight into the reactions of the carbohydrate group with periodate was gained by an application of the Smith degradation, together with methylation studies of some of the products. Since several experimental conditions and products were involved, the procedures and results are outlined as shown in Scheme I.

The periodate-oxidized Asp(NH·carbohydrate), when submitted to the Smith degradation procedure, afforded a product which was isolated by gel filtration. Its analysis showed L-aspartic acid and 2-amino-2-deoxy-D-glucose in the molar ratio of 1.0:2.0, with the amount of D-mannose varying somewhat with the conditions of the oxidation. Glycerol but no erythritol was found in the hydrolysate of the ACO, which excludes the existence of any D-mannose units linked through positions 1 and 4 or 1, 4, and 6. Furthermore, since the aspartyl residue is still linked to the periodate-resistant carbohydrate nucleus after the Smith degradation, it follows that the oxidized 2-acetamido-2-deoxy-D-glucose residue is not that to which the amino acid is



SCHEME I

attached. A second Smith degradation afforded a further product in which the aspartic acid–2-amino-2-deoxy-D-glucose–D-mannose molar ratios were 1.0:2.0:1.1, showing that the first Smith degradation exposed between one and two D-mannose units to periodate oxidation that were previously resistant in the parent Asp(NH·carbohydrate). The 2-acetamido-2-deoxy-D-glucose content was unchanged. For technical reasons the third Smith degradation product could not be analyzed quantitatively, but there was a good indication that the product was Asp(NH·GNAc).

oxidation. This would require the consumption of 4 moles of periodate. The 2 D-mannopyranose residues that gave rise to the tri-*O*-methyl fragments are linked one through positions 1,3 and the other through positions 1,2, the latter also being open to oxidation. However, when one takes into account that one residue of 2-acetamido-2-deoxy-D-glucose was oxidized by periodate, then the consumption of oxidant is theoretically 6.0 moles per mole of Asp(NH·carbohydrate) with the destruction of 3 of the D-mannose residues. It seemed probable therefore that the 1,2-linked D-man-

TABLE IV: Oligosaccharide Fragments from the Hydrolyses of Asp(NH·$GNAc_3$. Man_5) and Its DNP Derivative.

Fragment	Ratio of			DNP-Asp(NH·Carb)		Asp(NH·Carb)
	Asp	GNAc	Man	Acid	Emulsin	Emulsin
A	1	1	0	0.9:0.0[a] (13)	1.0:0.0 (6)	
B	1	1	1	0.8:1.1 (0.7)		
C	1	1	2	1.4:2.1 (0.3)		
D	1	2	0	2.0:0.1 (2.2)	+[b] (1)	
E	1	2	2	2.2:2:3 (0.4)		
F	1	2	3	2.3:3.0 (0.3)		
G	1	2	4	2.2:4.1 (0.1)		
H	0	1	1		1.2:1.0 (4)	
I	0	2	2			2.2:2.0 (1)
J	0	2	3			1.9:3.0 (2)
K	0	2	4			1.8:4.0 (27)
L	0	3	4			3.2:4.0 (12)

[a] The molar ratios of GNAc/Man obtained experimentally are given with reference to 1 mole of aspartic acid. The weight per cent recovered is given in parenthesis. [b] Too small an amount for analysis.

The nonstoichiometric oxidation of the D-mannose residues by periodate under the various conditions investigated made any structural interpretation of the data ambiguous. The problem was therefore approached by the classical technique of methylation by which it was demonstrated using gas-liquid chromatography (Figure 4a) that the methylated D-mannose cleavage fragments of methylated Asp(NH·carbohydrate) consisted of 2,3,4,6-tetra-*O*-methyl-D-mannose (2 moles), 2,4,6-tri-*O*-methyl-D-mannose (1 mole), 3,4,6-tri-*O*-methyl-D-mannose (1 mole), and 3,6-di-*O*-methyl-D-mannose (1 mole). It should be stressed that the identification of the tetra- and trimethyl ethers of D-mannose are based at this stage of the investigation upon a comparison of behaviors on the gas chromatograph with authentic samples. Work is in progress for their further identification. The di-*O*-methyl ether is provisionally identified in the absence of a standard sample on the basis of its electrophoretic migration in borate buffer and the fact that it does not behave like the 3,4- and 2,3-di-*O*-methyl ethers on gas-liquid chromatography. It follows that there are two nonreducing end groups of D-mannopyranose units, which is consistent with the formation of 2 moles of formic acid upon periodate

nose residue was being slowly oxidized because of a *trans* arrangement of its *vic*-glycol group and that the extrapolation of the periodate oxidation curve was again a case of confusing "overoxidation" with a primary reaction. The oxidized and reduced product from Asp(NH·carbohydrate) with about 50% of D-mannose oxidized was methylated without hydrolyzing the acid-sensitive polyhydric residues. Analyses of the methylated mannose cleavage fragments showed a negligible amount of 2,3,4,6-tetra-*O*-methyl-D-mannose, which indicated that all the nonreducing D-mannose end groups were oxidized but that the acid-sensitive acetal linkages in the reduced product ACO were not hydrolyzed before methylation. There was, however, a reduction in the amount of 3,4,6-tri-*O*-methyl-D-mannose by about 70% which, together with the nonterminal end groups, represents a total destruction of 54% of D-mannose, a value close to that demonstrated by analysis of the unmethylated starting material.

Several structures can be assigned to the carbohydrate group of ovalbumin from the results obtained by periodate oxidation and Smith degradations. These are limited, however, by the results from partial hydrolyses of Asp(NH·carbohydrate) (Montgomery *et al.*,

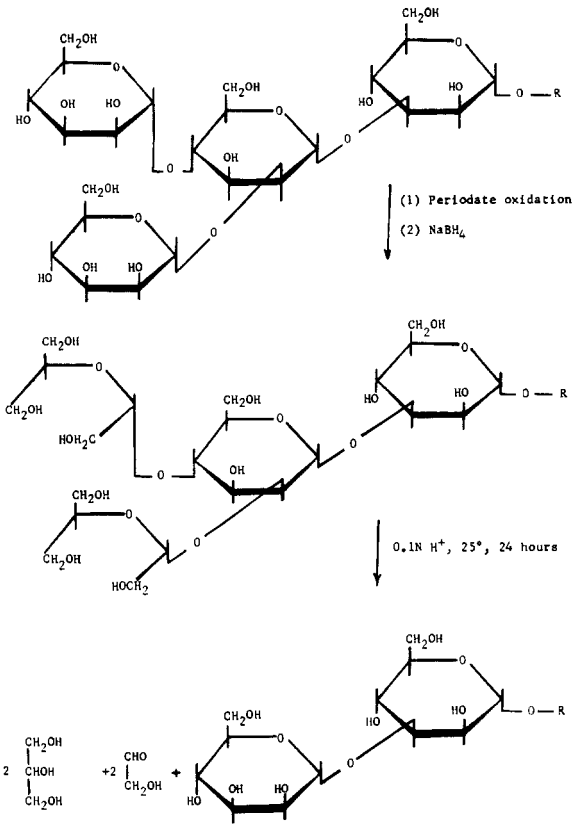
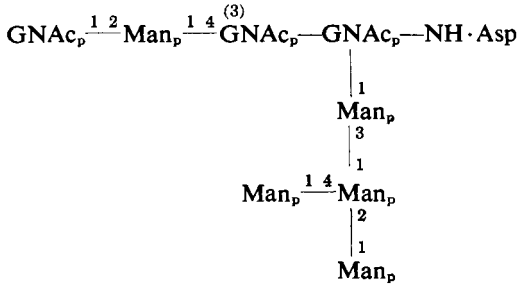


FIGURE 5: Reaction scheme of the Smith degradation of a possible tetramannosyl side chain in Asp(NH·carbohydrate), assuming α-D-anomeric linkages.

1965), which are summarized in Table IV. On the basis of the combined information one structure for the carbohydrate group might be written



The presence of a nonreducing terminal 2-acetamido-2-deoxy-D-glucose residue in Asp(NH·carbohydrate) is supported by the liberation of 1 mole of the sugar with *N*-acetyl-β-D-glucosaminidase (Clamp and Hough, 1963; Kaufman and Marshall, 1964). The remaining 2 hexosamine residues must be linked together to account for fragment D in Table IV, but the positions and nature of these glycosidic linkages must await further study.

The separation of the two 2-acetamido-2-deoxy-D-glucose residues by a 1,2-linked D-mannopyranose residue, rather than the presence of this residue in the tetramannose side chain, is indicated by the methylation study of the Smith degradation product of experiment 4, in which periodate oxidation resulted in the destruction of nearly three D-mannose residues. The methylated cleavage fragments of this product did not show by paper electrophoresis any 1,2-linked D-mannopyranose residue, but only the presence of a terminal and a 1,3-linked D-mannopyranose. These results are not consistent with the 1,3-linked D-mannopyranose unit's being adjacent to the nonreducing terminal hexosamine in Asp(NH·carbohydrate). Furthermore, the structure of the tetramannose side chain is supported by the same result, in that any other position of the nonreducing mannopyranose units would result in a different tri-*O*-methyl-D-mannose fragment in the Smith degradation product of experiment 4.

The tetramannosyl side chain is illustrated in Figure 5 with the α-anomeric structure being assumed for each residue. If the branch point were adjacent to the R group then the residual dimannosyl chain in the degraded product would be linked to the new nonreducing terminal end (derived in this case from a 1,3-linked D-mannopyranosyl unit) through either position 2 or 4, neither of which would give rise to the 2,3,6-trimethyl ether described before.

Further study is required to propose a unique structure for the carbohydrate prosthetic group of ovalbumin. The present work serves not only to limit the possibilities but to caution further against the interpretation of periodate oxidation studies in terms of structural assignment from the amount of oxidant consumed or the formic acid produced without other supporting evidence.

Acknowledgment

The authors are indebted to Dr. J. C. Rabinowitz for the formyl tetrahydrofolate synthetase.

References

Abdel-Akher, M., Hamilton, J. K., Montgomery, R., and Smith F. (1952), *J. Am. Chem. Soc.*, *74*, 4970.

Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.

Bragg, P. D., and Hough, L. (1961), *Biochem. J. 78*, 11.

Chatterjee, A. K., Durant, G. J., Hendrickson, H., Lee, Y. C., and Montgomery, R. (1961), *Biochem. Biophys. Res. Commun. 4*, 425.

Chatterjee, A. K., and Montgomery, R. (1962), *Arch. Biochem. Biophys. 99*, 426.

Clamp, J. R., and Hough, L. (1963), *Chem. Ind. (London)*, 82.

Dixon, J. S., and Lipkin, D. (1954), *Anal. Chem. 24*, 1092.

Fletcher, A. P., Marks, G. S., Marshall, R. D., and Neuberger, A. (1963), *Biochem. J. 87*, 265.

Fleury, P., and Lange, J. (1933), *J. Pharm. Chim. 17*, 107.

Goldstein, I. J., Hamilton, J. K., Montgomery, R., and Smith, F. (1957), *J. Am. Chem. Soc. 79*, 6469.

Grado, C., and Ballou, C. E. (1961), *J. Biol. Chem. 236*, 54.

Hamilton, J. K., and Smith, F. (1956), *J. Am. Chem. Soc. 78*, 5907, 5910.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 941.

Hough, L., Jones, J. K. N., and Wadman, W. H. (1950), *J. Chem. Soc.*, 1702.

Kaufman, H. H., and Marshall, R. D. (1964), Abstracts of the Sixth International Congress of Biochemistry, New York, Vol. II, p. 92.

Lee, Y. C., and Montgomery, R. (1961), *Arch. Biochem. Biophys. 95*, 263.

Lee, Y. C., and Montgomery, R. (1962), *Arch. Biochem. Biophys. 97*, 9.

Montgomery, R. (1964), Abstracts of Papers, 148th Meeting, American Chemical Society, Chicago, Ill., August–September 1964, p. 11C.

Montgomery, R., Lee, Y. C., and Wu, Y. C. (1965), *Biochemistry 4*, 566 (this issue; preceding paper).

Montgomery, R., and Nag, S. (1963), *Biochim. Biophys. Acta 74*, 300.

Montgomery, R., and Wu, Y. C. (1963), *J. Biol. Chem. 238*, 3547.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.

Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.

Rammler, D. H., and Rabinowitz, J. C. (1962), *Anal. Biochem. 4*, 116.

Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.

Rothfus, J. A., and Smith, E. L. (1963), *J. Biol. Chem. 238*, 1402.

Smith, F., and Montgomery, R. (1959), Chemistry of Plant Gums and Mucilages, New York, Reinhold, pp. 194–222.

Smith, F., and Unrau, A. M. (1959), *Chem. Ind. (London)*, 881.

Spiro, R. G. (1964), *J. Biol. Chem. 239*, 567.

Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.