Carbohydrate Research, 63 (1978) 173–181

ISOLATION OF A COLLAGEN FRACTION FROM THE BODY-WALL GLYCOPROTEINS OF THE LEECH (*Hirudo medicinalis*), AND CHARACTERIZATION OF ITS CARBOHYDRATE–AMINO ACID PORTION

TANUSREE BISWAS AND AMAL K. MUKHERJEE

Department of Macromolecules, Indian Association for the Cultivation of Science, Jadavpur, Calcutta 700032 (India)

(Received September 12th, 1977; accepted for publication, October 8th, 1977)

ABSTRACT

Fractionation of the leech (*Hirudo medicinalis*) body-wall glycoproteins yielded a collagen fraction containing only D-glucose and D-galactose as its carbohydrate constituents. Digestion of the collagen with trypsin and pronase, and alkaline degradation of the resulting glycopeptides, gave a product that contained a disaccharide linked to hydroxylysine. Mild, acid hydrolysis of the *N*-acetylated glycopeptides yielded a disaccharide consisting of a D-glucose and a D-galactose residue. Various chemical and enzymic reactions of the disaccharide, the glycosyloxylysine, and the glycopeptide fraction indicated that the disaccharide is 2-*O*-α-D-glucopyranosyl-D-galactose, and that this is β-glycosidically linked to O-5 of the hydroxylysine residue in the collagen.

INTRODUCTION

One of the varieties of the family of leeches is *Hirudo medicinalis*; these animals live on mammalian blood, and at one time were used to suck blood from the heads of typhoid patients. They have highly elastic bodies, and, during locomotion, elongate the body as much as one foot (0.305 m) and, the next moment, shorten it to only about two inches (5.08 cm). The structures of the carbohydrate part of the collagens from various sources, including vertebrates and invertebrates, have been reported[1–7]. In this context, it was of interest to study the structural pattern of the glycoproteins present in the highly elastic body-wall of these leeches (*H. medicinalis*). In this report, we present the results of structural studies on the carbohydrate part of the collagen fraction of glycoproteins present in leech body-wall.

RESULTS AND DISCUSSION

The carbohydrate part of the gelatin fractions isolated from the body wall of these leeches consists mainly of glucose and galactose, along with traces of other sugars. The yields and total hexose contents of the different fractions are summarized

TABLE I

EXTRACTION OF GELATINS FROM LEECH BODY-WALL[a] WITH WATER AT HIGH TEMPERATURE AND PRESSURE

Fractions	*Yield (g)*	*Hexose (%)*
1	0.664	3.9
2	1.056	7.8
3	1.795	8.0
4	0.556	6.5
5	0.036	5.4
6	0.025	5.4
7	0.027	4.6
8	0.019	4.0

[a]Wet weight, 65 g.

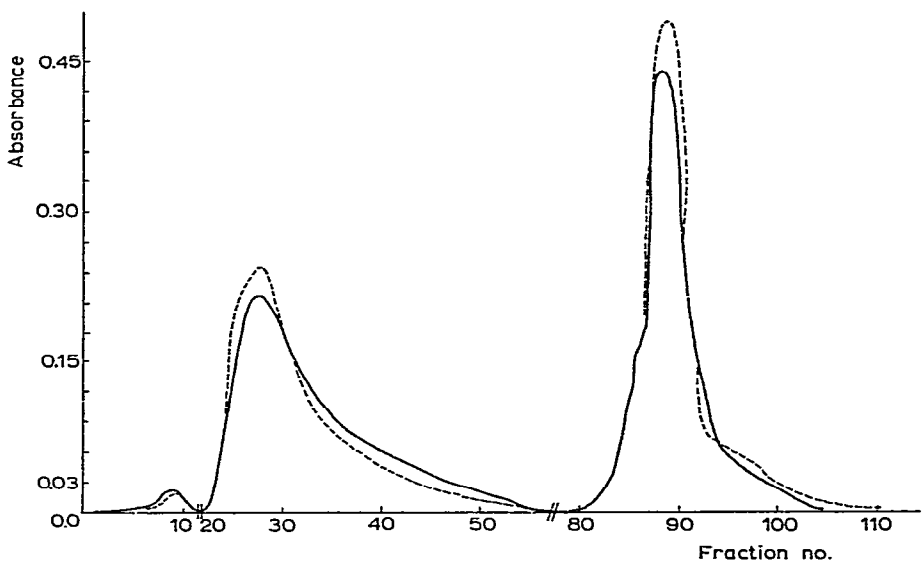

Fig. 1. Fractionation of leech body-wall gelatin (115 mg) on a column of DEAE-cellulose. [Gelatin mixed with DEAE-cellulose was applied on the top of the column (3 cm diam. × 23 cm) and eluted successively with water (tubes 1–20), 0.05M sodium chloride (tubes 21–80), and 0.5M sodium chloride (tubes 81–120). Fractions (5 mL) were collected, and analyzed for protein (280 nm, ——), and hexoses (anthrone method, ——). Appropriate pools of fractions 1, 2, and 3 were dialyzed and then lyophilized. Fractions 1 (very small) and 2 were collagenic, and 3 was noncollagenic.]

in Table I. Fraction 1 was slightly colored, and was not used for the investigations. Fractions 2–4 were used for our studies.

These gelatin fractions could be further fractionated[8] into collagenic and non-collagenic fractions on a column of DEAE-cellulose. The major, collagen fraction contained only glucose and galactose, whereas the noncollagenic fraction also

contained small proportions of neutral and acidic sugars. The resolution is shown in Fig. 1.

On treatment with trypsin and pronase the pure, collagen fraction yielded a glycopeptide that could not, however, be obtained in an electrophoretically homogeneous state, even after repeated column chromatography on CM-celluose and on Sephadex G-15. Similar glycopeptide could more conveniently be obtained by treating the gelatin with trypsin, removing the undesired heteropolysaccharides by precipitation with cetylpyridinium chloride, and then further digesting the supernatant liquor with pronase; the product was highly enriched in hexose (hexose, 16.5%; glucose:galactose = 1:1). From Sephadex G-15, one of the fractions, which was not electrophoretically homogeneous, contained the following amino acids: glycine, alanine, glutamic acid, aspartic acid, hydroxylysine, and an unidentified amino acid. However, when the whole glycopeptide fraction was treated in a sealed tube, in an oxygen-free atmosphere with 2M sodium hydroxide for 24 h at 105°, it yielded a glycosyloxylysine that, after the usual treatment of the solution, could be isolated in the pure state (from thick papers).

A pure disaccharide containing glucose and galactose could also be obtained by mild treatment of the *N*-acetylated glycopeptide with acid. For structural studies of the carbohydrate part of the collagen fraction, these three materials, *viz.*, the disaccharide, the glycosyloxylysine, and the glycopeptide fraction, were used.

Acid hydrolysis, and analysis of the product, indicated that the glycosyloxylysine contained glucose:galactose:hydroxylysine residues in the ratios of 1:1:1. To determine the point of linkage of the disaccharide to the hydroxylysine, the glycosyloxylysine was treated with 1-fluoro-2,4-dinitrobenzene in the presence of triethylamine, and the product hydrolyzed with acid to remove the sugars. The product was found to move identically with 2,6-bis-*N*-(2,4-dinitrophenyl)-5-hydroxy-L-lysine, prepared according to the method of Sanger and Thompson[9]. This showed that the carbohydrate part is linked glycosidically to O-5 of the hydroxylysine residue, as an ester linkage would not be stable enough to survive the alkaline treatment used for the isolation of the compound.

Partial, acid hydrolysis of the glycopeptide fraction (as also of the glycosyloxylysine) indicated that the glucosyl residue was released preferentially, and at a much higher rate, than the galactosyl residue. This indicated that the glucosyl group is at the nonreducing end of the carbohydrate chain. Isolation of a galactosyloxylysine from the partial, acid hydrolysis product of the glycosyloxylysine proved that the galactosyl group is linked to the hydroxylysine. Partial, acid hydrolysis of the *N*-acetylated glycopeptide released a disaccharide (together with glucose and galactose). The disaccharide was isolated by resolving the mixture on paper.

On hydrolysis, the disaccharide gave equimolecular amounts of glucose and galactose. Reduction of the disaccharide with sodium borohydride, and hydrolysis of the product, yielded only glucose as the reducing sugar, the galactose being reduced to galactitol; this indicated that the disaccharide is a glucosylgalactose and that the glycosyloxylysine is an *O*-glucosylgalactosyloxylysine.

The disaccharide was oxidized with sodium metaperiodate, and the oxidation product was reduced with sodium borohydride. From the hydrolyzate of the reduced product, only glycerol could be detected (but no erythritol or threitol or any sugars could be found by gas–liquid chromatography when analyzed as their alditol acetates). This result showed that the glucosyl residue is linked to either O-2 or O-6 of the galactosyl residue in the *O*-glucosylgalactosyloxylysine, a matter settled by methylation of the disaccharide with methyl iodide and silver oxide in *N*,*N*-dimethylformamide by Kuhn's procedure[10], and remethylating the product by Purdie's method[11]. On hydrolysis, the fully methylated product yielded 2,3,4,6-tetra-*O*-methylglucose and 3,4,6-tri-*O*-methylgalactose (RRT 1.00 and 2.09, column *b*), and neither 2,3,4-tri-*O*-methylgalactose (RRT 2.60) nor 2,4,6-tri-*O*-methylgalactose (RRT 1.89) (analyzed as their alditol acetates). This showed that the glucosyl group is linked to O-2 of the galactose residue.

The disaccharide was cleaved by α-D-glucosidase (but not by β-D-glucosidase) into glucose and galactose when incubated for 40 h at 37° in acetate buffer, pH 5.0. This indicated the α-D configuration of the glucosyl group and thus the structure of the disaccharide is 2-*O*-α-D-glucopyranosylgalactose. Again, treatment of the galactosyloxylysine obtained by partial, acid hydrolysis of the *O*-glucosylgalactosyloxylysine with β-D-galactosidase for 96 h at 37° in phosphate buffer, pH 7.0, released galactose (α-D-galactosidase did not cleave the compound), indicating that the galactosyl residue has the β-D configuration when linked to the hydroxylysine. Thus, the structure of the carbohydrate–amino acid unit in the body-wall collagen of leech is *O*-α-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl-(1 → 5)-oxy-L-lysine. This unit has been found to be common to many of the vertebrate and invertebrate collagens. The frequency of occurrence of this unit in leech body-wall glycoprotein seems, however, to be relatively higher.

EXPERIMENTAL

General. — All evaporations of solutions of volatile solvents were conducted *in vacuo* at 30–40°. Aqueous solutions were lyophilized. Whatman No. 1 MM filter papers were used for partition paper-chromatography, and Whatman No. 3 MM papers for separating quantities of sugar mixtures up to 200 mg. The solvent mixtures (v/v) used for partition chromatography of sugars and their derivatives were: (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) upper layer of 4:1:5 1-butanol–acetic acid–water, (*C*) upper layer of 4:1:5 1-butanol–ethanol–water, (*D*) 20:60:20:3 1-propanol–ethanol–water–acetic acid, and (*E*) 10:1:2 1-butanol–ethanol–water. The spray reagents used were: (*a*) aniline oxalate, (*b*) alkaline silver nitrate, (*c*) benzidine periodate, and (*d*) ninhydrin. Silica Gel G (acc. to Stahl, Merck) was used for thin-layer chromatography; 40:10:1 (v/v) benzene–pyridine–acetic acid was used as the solvent, and concentrated sulfuric acid was the spray reagent. For gas–liquid chromatography, a Hewlett–Packard 5750 gas chromatograph with hydrogen-flame ionization detector was used. Chromatography of sugar derivatives was performed

on a glass column (1.83 m × 3.17 mm) containing (*a*) 3% of ECNSS-M on Gaschrom Q (100–120 mesh), at 190°, for alditol acetates of sugars, and (*b*) 5% of OV-225 on SIL RUB (80–100 mesh), at 190°, for alditol acetates of methylated sugars. U.v. and visible spectra were recorded with a Carl Zeiss VSU 2-P spectrophotometer. Infrared spectra were recorded with a Beckman Acculab-4 spectrophotometer. Total neutral hexoses were estimated by means of the anthrone reagent[12]. The mole fraction of each sugar was determined by the alkaline hypoiodite method[13]. The enzymes used were: (*1*) trypsin (vom Rind, Krist. lyophilisiert für biochemische Zwecke, Merck), (*2*) pronase E (für biochemische Zwecke, Merck), (*3*) α-D-glucosidase (aus Hefe, suspension, kühl lagern, Boehringer Mannheim, GmbH, W. Germany), (*4*) β-D-glucosidase (Süßmandeln, *Amygdalae dulces*, für analytische Zwecke, suspension, kühl lagern, Boehringer Mannheim, GmbH), (*5*) α-D-galactosidase (Semen *Coffeae arabicae*, für analytische Zwecke, suspension, kühl lagern, Boehringer Mannheim GmbH, W. Germany), and (*6*) β-D-galactosidase (für analytische Zwecke, Kristall-suspension, kühl lagern, Boehringer Mannheim GmbH, W. Germany).

Isolation of leech body-wall glycoprotein. — Body walls of a number of leeches were carefully scraped free of most of the inner layers. The walls were then washed with water, cut into very small pieces, and cleaned three times with 0.15M phosphate buffer, pH 8.0, at 4°, and dialyzed against distilled water for three days, with frequent changes of water. The residue (65 g, wet weight, in each lot) was mixed with water (100 mL), and the suspension was autoclaved at 15 lb. in.$^{-2}$ for 15 min. The mixture was cooled, and centrifuged at 6,000 r.p.m. for 30 min, and the supernatant liquor was collected. Water (80 mL) was added to the residue, and the mixture was similarly autoclaved. In this way, 8 extractions were made. The supernatant liquors were dialyzed against distilled water for 7 days at 4°, with frequent changes of distilled water, and then lyophilized. The yields of the successive extractions are given in Table I, which also shows the total hexose content in each fraction.

Fractionation of glycoprotein on a column of DEAE-cellulose. — Glycoprotein (115 mg) was mixed with a small amount of DEAE-cellulose, and the mixture was applied to the top of a column (3 × 23 cm) of DEAE-cellulose that had been washed with water. The column was successively eluted with water, 0.05M sodium chloride, and 0.5M sodium chloride. The fractions containing glycoproteins (tested for protein at 280 nm in a spectrophotometer, and for carbohydrate, by the anthrone reagent) were collected separately, dialyzed against distilled water, and lyophilized. In this way, three fractions were obtained.

Proteolytic degradation of glycoprotein. — Glycoprotein (1 g) was taken up in water (60 mL), the pH of the solution was adjusted to 8.4 with 0.1M sodium hydroxide, and trypsin (15 mg) was added. A few drops of toluene were added (to inhibit bacterial growth), and the mixture was incubated for 20 h at 37°. The solution was then boiled for 10 min, and lyophilized. The resulting material was dissolved in water (10 mL), and treated with a 2% solution of cetylpyridinium chloride (15 mL). The precipitate formed was centrifuged off, and the material in the supernatant liquor was precipitated

with five volumes of ethanol. The resulting precipitate was centrifuged off, washed three times with ethanol, dissolved in water, and the solution lyophilized.

To a solution of the lyophilized product in 0.1M Tris acetate buffer, pH 7.8 (9 mL) containing 5mM calcium chloride, was added pronase (2.8 mg), and the mixture was incubated at 37° in the presence of toluene. Further additions of pronase (~1 mg) were made at the 18th and 47th hours. Degradation was complete in 72 h (as shown by the ninhydrin test). The suspension was heated for 15 min at ~65° to deactivate the enzyme, the suspension was centrifuged, and the supernatant liquor was lyophilized.

Separation of glycopeptides from peptides and amino acids. — Pronase-treated material was dissolved in solvent *D* (5 mL) and applied to the top of a column (3.8 × 52 cm) of cellulose pre-equilibrated with the same solvent. The amino acids and the carbohydrate-free peptides were eluted with solvent *D* (2 L). The glycopeptides were then eluted with water, and the eluate lyophilized (yield 0.1341 g).

Fractionation of the glycopeptide on a column of O-*(carboxymethyl)cellulose.* — A solution of glycopeptide was fractionated on a column (1.8 cm × 32 cm) of *O*-(carboxymethyl)cellulose that had been extensively prewashed and equilibrated with 10mM citrate buffer, pH 3.2, containing 5mM sodium chloride. The column was eluted with a linear gradient consisting of 250 mL of the starting buffer and 250 mL of 10mM citrate buffer, pH 3.2, containing 50mM sodium chloride. Aliquots of the eluate were analyzed for hexose and peptides.

Alkaline degradation of the glycopeptide into O-*glucosylgalactosyloxylysine.* — Glycopeptide (100 mg) was heated with 2M sodium hydroxide (12 mL) for 24 h at 105° in a stoppered poly(propylene) tube, the air having been previously removed by bubbling nitrogen into it. The hydrolyzate was made neutral, the pH adjusted to 2.0 with hydrochloric acid, and applied to a column of Dowex 50 W-X8 (2.8 × 38.5 cm). The column was washed with water, and when the water eluate was free from chloride ions, the column was eluted with 1.5M ammonium hydroxide. Ammonia was removed from the eluate by lyophilization, and *O*-glucosylgalactosyloxylysine was obtained by preparative paper-chromatography after resolving the mixture on thick papers in solvent *B* for 110 h and eluting the zone corresponding to glucosylgalactosyloxylysine (R_{HOLys} 0.70). A very faint spot, corresponding to galactosyloxylysine, was also detected (R_{HOLys} 0.75).

N-*Acetylation of the glycopeptide.* — A solution of the glycopeptide (100 mg) in 4.5M sodium acetate (15 mL) was treated with acetic anhydride (25 mL) in five equal portions during 1 h. The reaction was terminated by diluting the mixture with water (20 vol.) and heating on a boiling-water bath for 10 min. The sodium ions were removed by passage through a column of Dowex 50W-X8 (H^+), and the solution was lyophilized (yield 98 mg). Several batches of this material were prepared.

Preparation of the disaccharide. — The *N*-acetylated glycopeptide (200 mg) was hydrolyzed with 0.1M hydrochloric acid (7 mL) in a sealed tube at 100°. The hydrolyzate was made neutral with Dowex-1 X-8 (HCO_3^-), and the solution was lyophilized. The residue was dissolved in water, and then resolved on paper (in solvent *E*), glucose,

galactose, and a disaccharide ($R_{lactose}$ 1.28) being detected. The disaccharide was isolated by resolving the mixture on thick papers in solvent *E* (yield 18 mg).

The disaccharide (~2 mg) was hydrolyzed with M hydrochloric acid in a sealed tube. Another batch of the disaccharide (~2 mg) in water (1 mL) was reduced with sodium borohydride (2 mg) for 5 h at room temperature, and the borate was removed as methyl borate. This reduced disaccharide was hydrolyzed with M hydrochloric acid, as for the original disaccharide. After removal of ions, the product was analyzed by paper chromatography.

Smith degradation[14] *of the disaccharide.* — A solution of the disaccharide (5 mg) in 0.25M sodium metaperiodate (1 mL) was kept in the dark for 24 h at 4°. The iodate and periodate ions were removed as their barium salts, and the solution was passed through a column of Dowex 50W-X8 (H^+). The eluate was concentrated to ~1 mL, and the product reduced with a 4% solution of sodium borohydride (1 mL) for 4 h. The product was acidified with acetic acid, and the boric acid was removed as methyl borate. The reduced material was then hydrolyzed with M hydrochloric acid for 2 h in a boiling-water bath. The acid was removed from the hydrolyzate, and the product was converted into the alditol acetate, which was analyzed by g.l.c. (column *a*). A peak corresponding to glycerol triacetate was obtained, but no tetritol tetraacetate or any higher polyol acetates were found.

Methylation of the disaccharide. — To a solution of the disaccharide (~5 mg) in *N,N*-dimethylformamide (1.2 mL) were added silver oxide (0.25 g) and methyl iodide (1 mL). The mixture was shaken in the dark for 2 days, and filtered, and the insoluble salts were washed with *N,N*-dimethylformamide. The filtrate was concentrated to a small volume *in vacuo*. Chloroform was added, and the resulting precipitate was removed by filtration. The filtrate was washed with water (4 times), dried (sodium sulfate), and evaporated to dryness. The product was remethylated by the Purdie method, to yield a fully methylated derivative showing no OH absorption band in its infrared spectrum; yield (5.4 mg).

Methylated product (~2 mg) was hydrolyzed with M sulfuric acid for 3 h, and the solution was made neutral with barium carbonate, the suspension filtered, and the filtrate concentrated. Methylated sugars were identified by paper chromatography (solvent *C*), and by g.l.c. on column *b* (as their alditol acetates).

N-*(Dinitrophenyl)ation of* O-*glucosylgalactosyloxylysine.* — A solution of *O*-glucosylgalactosyloxylysine (~2 mg) in water (0.8 mL) was added, with stirring, to triethylamine (0.2 mL), and then a 5% solution of 1-fluoro-2,4-dinitrobenzene in ethanol (3.2 mL) was added. The mixture was kept in the dark for 2 h at room temperature, acidified, diluted with water, and washed several times with ether. The aqueous phase was evaporated to dryness, and the residue hydrolyzed with M sulfuric acid (~1 mL) in a sealed tube at 100°. The hydrolyzate was diluted with water, extracted with ether, and the ether and aqueous phases separately evaporated to dryness. The products were resolved by t.l.c. on silica gel, and compared with 2,6-bis-*N*-(2,4-dinitrophenyl)-5-hydroxy-L-lysine prepared according to the procedure of Sanger and Thompson[9].

Partial, acid hydrolysis of O-*glucosylgalactosyloxylysine.* — The *O*-glucosylgalactosyloxylysine was separately subjected to hydrolysis with 0.1M and 1.0M hydrochloric acid at 100°. At regular periods, an aliquot of the hydrolyzate was removed, made neutral, centrifuged, and the supernatant liquor examined by paper chromatography. With 0.1M hydrochloric acid, most of the glucose was removed in 8 h (in the hydrolyzate, glucose:galactose = 10:1). The hydrolyzate after 10 h was resolved on thick filter-papers with solvent *B*, as for *O*-glucosylgalactosyloxylysine. The galactosyloxylysine was isolated by elution of that fraction from the paper; however, this fraction contained a trace of *O*-glucosylgalactosyloxylysine, but it could be used for the enzyme treatment.

Digestion with α-D-*glucosidase.* — To a solution (~1 mg) of the disaccharide in 0.2M sodium acetate buffer (pH 5.0, 2 mL) were added α-D-glucosidase (1 mL) and toluene (to prevent bacterial growth). The solutions were incubated for 40 h at 37°, the enzymic digestion being monitored by controls. At the end of the incubation, the enzyme was deactivated by heating for 10 min at 60°. The resulting mixtures were passed through small columns of charcoal–Celite, and washed with water (4 vol.). The solutions were desalted by successive passage through columns of Dowex 50 W-X8 (H^+) and Dowex 2 X-8 (OH^-), and the sugars were identified by paper chromatography.

Digestion with β-D-*glucosidase.* — The disaccharide (~1 mg) was treated with β-D-glucosidase (1 mL) under the same conditions as for α-D-glucosidase.

Digestion with α-D-*galactosidase and with* β-D-*galactosidase.* — A solution of galactosyloxylysine (~1 mg), obtained as described earlier, in 0.2M sodium acetate buffer (2 mL; pH 5.0) was incubated with α-D-galactosidase (1 mL) for 96 h at 37°. Simultaneously, a solution of galactosyloxylysine (~1 mg) in 0.05M potassium phosphate (2 mL), at pH 7.0, in the presence of 0.01M magnesium sulfate was incubated with β-D-galactosidase for 96 h at 37°. Both of the enzymic digestions were accompanied by controls, and toluene was added to prevent bacterial growth.

At the end of the incubations, the enzymes were deactivated, and the released monosaccharides were separated from the enzyme and the rest of the substrate by passage through small columns of charcoal–Celite, and the solutions were desalted by subsequent passage through columns of Dowex 50 W-X8 (H^+) and Dowex 2 X-8 (OH^-). The released sugars were detected by paper chromatography.

ACKNOWLEDGMENTS

The authors thank Mr. S. Ganguli, Head of the Department of Biology, Vivekananda College, Calcutta, for suggesting methods of obtaining the leech (*H. medicinalis*) body-wall free from other parts of the body, and Dr. N. Roy of this Department for supplying some of the partially methylated sugars as standard compounds.

REFERENCES

1 W. T. Butler and L. W. Cunningham, *J. Biol. Chem.*, 241 (1966) 3882–3888.
2 R. G. Spiro, *J. Biol. Chem.*, 242 (1967) 4813–4823.
3 Y. C. Lee and D. Lang, *J. Biol. Chem.*, 243 (1968) 677–680.
4 R. G. Spiro, *J. Biol. Chem.*, 244 (1969) 602–612.
5 R. G. Spiro and S. Fukushi, *J. Biol. Chem.*, 244 (1969) 2049–2058.
6 L. Muir and Y. C. Lee, *J. Biol. Chem.*, 244 (1969) 2343–2349.
7 R. L. Katzman and R. W. Jeanloz, *Chem. Mol. Biol. Intercell. Matrix Adv. Study Inst.*, 1 (1969) 217–227.
8 R. L. Katzman, A. K. Bhattacharya, and R. W. Jeanloz, *Biochim. Biophys. Acta*, 184 (1969) 523–528.
9 F. Sanger and E. O. P. Thompson, *Biochem. J.*, 53 (1953) 353–366.
10 H. G. Walker, Jr., M. Gee, and R. M. McCready, *J. Org. Chem.*, 27 (1962) 2100–2102.
11 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 85 (1904) 1049–1070.
12 J. H. Roe, *J. Biol. Chem.*, 212 (1955) 335–343.
13 E. L. Hirst, L. Hough, and J. K. N. Jones, *J. Chem. Soc.*, (1949) 928–933.
14 H. Abdel-Akher, J. K. Hamilton, R. Montgomery, and F. Smith, *J. Am. Chem. Soc.*, 74 (1952) 4970–4971.