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Chapter 12

COMPOUND BIOPOLYMERS AND BIOOLIGOMERS

In Chapters 7 - 11 of Volume B, separations of simple polymeric or oligomeric substances were treated, i.e., substances composed of repeated units of the same type, e.g., amino acids → peptides or proteins, nucleotides → oligonucleotides, polynucleotides or nucleic acids, simple sugars → oligosaccharides or polysaccharides. In this chapter the rapid chromatographic separation of natural oligomeric or polymeric compounds containing also important molecular moieties of a different type will be dealt with, such as nucleoprotein complexes, glycolipids, glycopeptides and glycoside oligomeric derivatives. In addition, separations of several natural complex substances that are not well known are discussed.

12.1 VIRUSES, BACTERIOPHAGES AND RIBOSOMES

Viruses, phages and ribosomes or their subunits are extremely high-molecular-weight, complex particles, differing substantially in their molecular size and shape, and therefore size exclusion chromatography (SEC) was the first chromatographic approach to separate them from each other and from various lower molecular weight substances such as proteins, peptides, oligonucleotides and sugars. In order to facilitate their rapid separation, highly macroporous and rigid support materials had to be found. Haller (1965) was the first to propose and develop controlled-pore glass (CPG) with pore diameters ranging from 100 to 1700 Å, suitable for this purpose. Fig. 1.1 in the introductory part of Volume A illustrated the success of this effort. The advantage of this support was not only that it separated compound biopolymers quickly, but also that it could be sterilized and cleaned with strong acids without adversely affecting their pore diameters. Unfortunately, the polar surface of CPG (and also of porous silica) was shown to sorb irreversibly and denature some sensitive biological substances. Therefore, methods were sought for protecting the active surface of CPG or silica either by covalent bonding (e.g., using a Glycophase layer, reviewed by Regnier et al., 1977), or by adsorbed protecting layers (described in Section 12.1.1).

However, during the further development of the rapid and selective isolation of viruses or their components, other separation modes [ion-exchange chromato-

graphy (IEC), reversed-phase chromatography (RPC), affinity chromatography etc.] were also tested and successfully used. Some of these methods will be reviewed in the following sections.

12.1.1 Size exclusion chromatography

12.1.1.1 Plant viruses and ribosomes

The first experiments were undertaken with plant viruses. An example of their rapid mutual separation using CPG and only hydrostatic pressure is illustrated by Fig. 12.1, taken from the pioneering work of Haller (1965). Almost complete separation was achieved in less then 10 min. It is interesting that TM-virus appeared at an elution volume of 18 ml, which is the dead-space of the column. As the virus consists mainly of rods 3000 Å long and 150 Å in diameter (cf., Laufer, 1944), this indicates that the length of the virus prevents it from entering the pores. The same phenomenon was observed by Steere (1964) on agar gel columns and was explained as being due to the rotation motion of the rod-shaped virus in the solution.

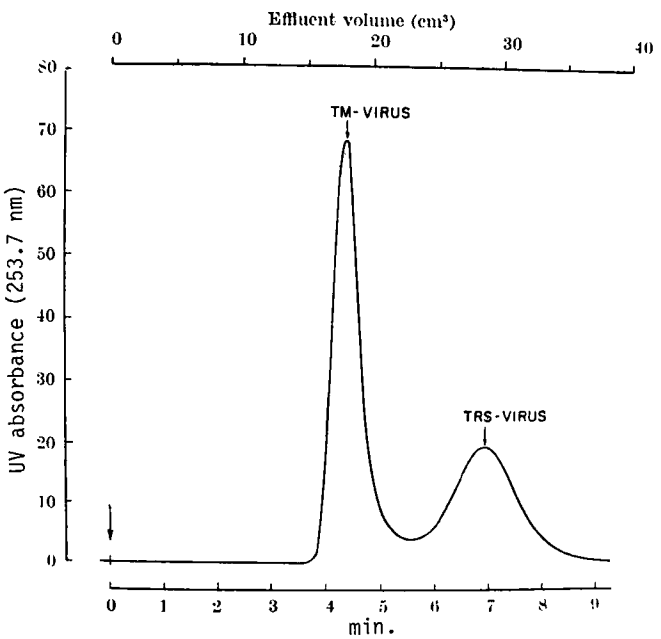

Fig. 12.1. Chromatographic separation of a mixture of tobacco mosaic virus (TM) and tobacco ring spot virus (TRS) on controlled-pore glass of average pore diameter 1700 Å. Bed dimensions: 50 cm x 1 cm. Sample: a mixture containing approximately 10^{11} particles of each virus in 0.06 ml of saline buffer. Eluent: 0.01 *M* phosphate buffer (pH 7.0) containing 0.85% NaCl. Flow by gravity was 5.2 ml/min cm^2. (Reprinted from Haller, 1965.)

TRS-virus, consisting of polyhedra of 260 Å diameter (Steere, 1956), readily entered the 1700 Å pores of glass; the elution peak was close to 30 ml, typical of admitted particles. Southern bean mosaic virus (SBMV) was also chromatographed (cf., Fig. 1.1 in Volume A); these particles had a diameter of 286 Å (Leonard et al., 1953), did not enter the 260 Å pores of the glass used and appeared at an elution volume of 18 ml, whereas the accompanying serum albumin of $M_r = 7 \cdot 10^4$ (Phelps and Putnam, 1960), which was smaller than the pores, was delayed and its position (slightly beyond 30 ml) was also influenced by the charge effect at the particular pH. In a subsequent paper on virus isolation with CPG, Haller (1967) described some other successful chromatographic experiments with plant viruses and bacteriophages (see the second half of this section).

However, Marcinka (1972), who also applied permeation chromatography on CPG in the purification of plant viruses, found that red clover mottle virus, tobacco mosaic virus, alfalfa mosaic virus and white clover mosaic virus were adsorbed under certain conditions. Čech et al. (1977) tried to separate tobacco mosaic virus on Spheron gel. This could be prepared in an extremely macroporous form, and is a fully organic copolymer having no silanol adsorbing groups, that could be used in high-performance liquid chromatography (HPLC).

Himmel and Squire (1981) described the HP-SEC of large biopolymers including viruses and ribosomes on a TSK Gel G 5000 PW preparative column. This support is also an organic polymer. It was used in the form of a 600 mm x 21.5 mm I.D. column, connected with a protective pre-column. The eluent was 10 m*M* phosphate buffer (pH 7), 100 m*M* in KCl, pumped at a flow-rate of 0.96 ml/min, and the effluent was monitored by UV by detection at 280 nm. The average sample load for calibration was 250 µl of a 3 mg/ml protein solution. In addition to a few proteins and dextrans, the following viruses were chromatographed: southern bean mosaic virus (SBMV, $M_r = 6.6 \cdot 10^6$; cf., Edsall, 1953), tomato bushy stunt virus (TBSV, $M_r = 8.9 \cdot 10^6$; cf., Edsall, 1953), tobacco mosaic virus (TMV, $M_r = 39.4 \cdot 10^6$; cf., Caspar, 1963), and turnip yellow mosaic virus (TYMV, $M_r = 5.4 \cdot 10^6$; cf., Matthews, 1970).

The GPC of ribosomes and ribosome subunits (30S and 50S from *Escherichia coli*, corresponding approximately to M_r $1.2 \cdot 10^6$ and $2.0 \cdot 10^6$, respectively) showed co-elution at the void volume, and seemed to indicate that their size exceeded that of the available pore openings. As the corresponding M_r values are not so high, in the opinion of the cited authors it seems likely that the cause of this phenomenon is the external size exclusion of these very large rods, whose length (0.3 µm) is comparable to the interstitial space between the spherical packing beads of radius ca. 9 µm. The cited authors have undertaken further detailed quantitative studies to test this hypothesis and the experiments are in progress. In spite of these problems, the TSK Gel column chromatography may be useful as a preparatory tool during the isolation and "clean-up" of ribosomes or their subunits.

Protein and virus samples were eluted as single symmetrical peaks with good resolution. GPC calibration constants (for dextrans) in this work were calculated both for molecular weights and for molecular radii and the data obtained are compared and discussed in relation to the other separated substances.

12.1.1.2 Animal viruses and bacteriophages

The first experiments on the rapid chromatography of bacteriophages and animal viruses were described by Haller (1967), who used CPG for the separation. MS_2 coliphage and Kilham rat virus were chromatographed. Purified preparations of φX-174, M13, M12, QB and T4 bacteriophages were obtained in large amounts by Gschwender et al. (1969), using a large-scale preparation on CPG. However, many investigators have found that some viruses adsorb so avidly to CPG that SEC cannot be applied. Hiatt et al. (1971) found some animal viruses (poliovirus, adenovirus, vesicular exanthema virus and viruses of vaccinia, yellow fever and rabies) that belonged to this adsorbed virus category. The adsorption of poliovirus was reduced (or eliminated) by pre-treatment of the glass with haemoglobin solutions followed by autoclaving (to denature haemoglobin in situ), but this technique was not found to be universal, e.g., it did not prevent the adsorption of rabies virus. The cited authors examined many techniques for preventing the adsorption of viruses and ultimately found that low concentrations of poly(ethylene oxide), a polyether of $M \approx 100\ 000$*, prevented the adsorption of rabies virus to porous glass of average diameter 1250 Å (CPG 10-1250, Corning Glass Works, Corning, NY, U.S.A.).

Application procedure. The polyether can either be added to the eluent at a concentration of 0.04% or used to pre-condition the column by passage of one void volume of 0.4% solution, followed by five or more void volumes of distilled water or buffered salt solution.

An elution pattern was published by Hiatt et al. (1971) of mouse-adapted rabies virus (fixed strain PV_{12}) after passage over a 450 mm x 11 mm I.D. column of powdered CPG (porosity 1250 Å) which was preconditioned with poly(ethylene oxide), as mentioned above. The charge was 0.6 ml of 20% mouse brain suspension. The eluent (isotonic phosphate-buffered saline solution, pH 7.3) was pumped at a flow-rate of 0.8 ml/min.

According to Hiatt et al. (1971), the effect of poly(ethylene oxide) can be explained as follows. Polyethers have a strong affinity for complex formation by hydrogen bonding (Smith et al., 1959; Rösch, 1963). It seems probable that

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*Polyox WSR IV-10, Union Carbide, New York, U.S.A. Poly(ethylene oxide) of M_r = 200 000 was also effective in preventing the adsorption. Polyethers of lower M_r were not tested; such compounds would be required for CPG of smaller pore size to ensure penetration.

multiple hydrogen bonds between the polyether and the electronegative oxygen atoms in the $-SiO_2-$ repeating structure of the glass account for the adsorption of poly(ethylene oxide). By competing for binding positions on the glass surface, poly(ethylene oxide) thus effectively blocks the adsorption of rabies virus.

According to Regnier et al. (1977), apparently polyethylene glycol (PEG) polymers hydrogen bond to surface silanol groups of CPG, decreasing the charge on the surface and preventing the adsorption of viruses and proteins. However, this is unfortunately a reversible process and PEG elutes from columns during continuous use. In spite of this, Darling et al. (1977) described the rapid purification of an RNA tumour virus and proteins by HP-SEC on bead columns made of CPG treated with PEG. Avian myeloblastosis virus (AMV) and hamster melanoma virus (HaMV) were purified from plasma proteins and tissue culture media components. The purified HaMV was still infectious, and the AMV-associated RNA-directed DNA polymerase ("reversed transcriptase") showed an 1100-fold purification of the virus after one column treatment. The time required for column purification was 5 min and electron microscopy of the purified virus showed intact particles.

The CPG (80-120 or 120-200 mesh) for this treatment was purchased from Analabs (North Haven, CT, U.S.A.) or Electro-Nucleonics (Fairfield, NJ, U.S.A.). The undesirable adsorption of CPG was greatly reduced by a modification of the method of Hawk et al. (1972), consisting in pre-treatment with PEG solution.

Pre-treatment procedure (Darling et al., 1977). A 100-g amount of CPG beads was added to 500 ml of degassed solution of 3% PEG 20M in distilled water. The suspension was maintained under vacuum to ensure that air held in the pores was removed to allow PEG to enter. The suspension was shaken occasionally to free any air bubbles adsorbed on the beads. After 30 min the suspension was connected to a vacuum line overnight. The excess of PEG was removed from the beads by repeated washing with distilled water until foam was no longer observed. The beads were air dried under vacuum (oven drying had to be avoided!).

Stainless-steel columns 1, 2 or 3 m long and 1/4 in. O.D. or 4.5 mm I.D. were used for chromatography. All columns were coiled, with a coil diameter of 30 cm. A simple dry-packing procedure and equilibration of the columns with eluent were applied. Separations were carried out at ambient temperature (for AMV) or at $0^{o}C$ (for HaMV). For example, for the separation of AMV and added alkaline phosphatase, a 2 m x 1/4 in. O.D. CPG-10-1250 pre-treated glass bead column was eluted with Tris buffer (0.01 *M*, pH 8.3) at a flow-rate 30 ml/h with a back-pressure of 100 p.s.i. Fractions of 0.5 ml were collected.

Eluting virions were detected (1) by assay for detergent-requiring polymerase activity versus protein concentration, (2) by a difference in UV absorption ($A_{254} - A_{280}$) and (3) by electron microscopy. Routinely, only the UV difference

spectrum was checked, as the position of the virus peak, once determined, did not vary by more than 0.1 ml, which was within experimental error. If it was desired to retain the infectivity of the viruses, the radiation damage caused by UV light had to be avoided in repeated experiments. The UV detector did not need to be employed, as the peaks positions were reproducible.

Bhown et al. (1981) studied the purification and characterization of the gag gene products of avian-type C retroviruses by HPLC. A rapid chromatographic separation of translational products of this gene using volatile solvents was described. The viral structural components p27 (M_r = 27 000), p19 (M_r = 19 000) and p15 (M_r = 15 000), which are the fragments (split by a proteinase) of the primary translational product, i.e., the precursor protein Pr 76 (M_r = 76 000), were purified in milligram amounts nearly to homogeneity and in high yields. For the purification of protein components of the disrupted virus a series of four ^{125}I gel permeation columns (Waters Assoc., 30 cm x 0.78 cm I.D.) were used, eluted with acetic acid-propanol-highly purified water (20:15:65) at a flow-rate of 0.2 ml/min. The load was 1 - 2 mg of the total protein dissolved in a volume not exceeding 100 µl. The effluent was monitored spectrophotometrically at 280 nm. The peaks were collected manually, lyophilized and further characterized.

12.1.2 Other chromatographic modes

In the last few years other methods have been applied to the chromatography of detergent-disrupted virus components, especially of proteins, in addition to SEC. For example, Henderson et al. (1981) have shown that RP-HPLC could be used to separate proteins in murine leukaemia virus. Kårsnäs et al. (1983) described the purification of surface proteins of bovine viral diarrhoea virus (BVDV) in detergent-containing buffers by fast protein liquid chromatography (FPLC). BVDV is an RNA virus of the genus *Pestivirus*, family Togaviridae. First, the virus was purified by *Crotalaria juncea* lectin chromatography and the glycoprotein peplomers were dissociated from the virion by treatment with 1% Berol 172 (a non-ionic detergent, purchased from Berol Scandinavia, Stenungsund, Sweden). In a second *Crotalaria* lectin affinity chromatographic step the peplomers were re-chromatographed and the glycoconjugates carrying terminal galactose were isolated. For further purification of these glycoproteins, FPLC was employed. In order to overcome the tendency of hydrophobic proteins to aggregate, and with the low UV transparency of most conventional detergents which were able to suppress the aggregation, Berol 172 was applied as a suitable eluent component.

For IEC in the FPLC system, a Mono-Q column (Pharmacia, Uppsala, Sweden) was used and eluted with 0.05 *M* Tris-HCl buffer containing 0.2% of Berol 172. After 20 ml of isocratic elution a linear gradient of 1 *M* NaCl was applied up to 60% (reached in ca. 40 ml) and then isocratic elution was continued at this NaCl concentration up to the final 50 ml. Virus-infected and uninfected materials were compared. Proteins of the infected material were eluted above 24-26% NaCl in one preparation line, and above 28% NaCl in the other. Re-chromatography of the pooled fractions using 0.02 *M* piperazine-HCl buffer (pH 6) + 0.2% of Berol 172 with a linear gradient of salt concentration was successfully applied and led to single peaks. The influence of the critical micellar concentration (CMC) of the detergent in the eluent on the chromatographic and detection processes was also investigated.

The experiments showed that FPLC on ion-exchange columns in detergent-containing buffers can be used effectively for the separation of protein components of virus surfaces.

Phelan and Cohen (1983) studied gradient optimization principles in RP-HPLC and the separation of influenza virus components. Major proteins of A/Bangkok 1/79 x 73 influenza virus were chromatographed. The purity of the isolated proteins, their yields and their reactivity with monoclonal antibodies were discussed. These proteins are important, because several studies of the influenza virus, its components and their roles in host infection and immune responses depended on the isolated viral components.

Theoretical considerations were discussed at the beginning, considering the dependence of the resolution, R_S, on various gradient parameters. The virus was purified by sucrose gradient centrifugation and dissociated in 8 *M* guanidine-HCl and 2 m*M* dithiothreitol (DTT) to a final protein concentration of 3.9 mg/ml for chromatography. A 25 cm x 0.46 cm I.D. Aquapore RP-300 column (CO3-10A; Rainin Instrument, Woburn, MA, U.S.A.) was used for the chromatography. For the separation of dissolved influenza X-73 virus, material containing 78 µg of total virus protein was chromatographed using a linear gradient of 0.05% trifluoro-acetic acid (TFA) in water to 0.05% TFA in acetonitrile over 33 min and a flow-rate of 1.0 ml/min. In another experiment, a gradient from 5% to 75% acetonitrile in 0.05% TFA was used. The influence of gradient time was examined in detailed experiments.

For detection, measurement of the absorbance at 220 nm was applied. In order to measure the protein concentration of virus and column eluates, the samples were hydrolysed in 5 *M* NaOH, neutralized with 5 *M* HCl, diluted with 1 *M* borate buffer of pH 10.5 (containing 0.1% of Triton X-100, 0.1% of 2-mercaptoethanol and 1% of methanol), mixed with an equal volume of borate buffer containing

0.08% (w/v) of *o*-phthalaldehyde (Pierce, Rockford, IL, U.S.A.) and the fluorescence excited at 340 nm was measured at 455 nm. Calculations were based on the measurement of standards. Column regeneration to improve its properties after prolonged use was also described. The experiments showed that dissolution of X-73 in 8 *M* guanidine hydrochloride and 2 m*M* DTT followed by RP-HPLC using a gradient of acetonitrile in 0.05% TFA separated the major proteins of this influenza virus. Although the total protein recovery was only ca. 20%, their purity was ≥88%.

A series of papers were published by Van der Zee, Welling and co-workers, describing various methods for the isolation and purification of proteins from detergent-extracted Sendai virus. In the first paper Van der Zee et al. (1983) described their purification by RPC.

Sendai virus is a paramyxovirus, ranging in size from 150 to 250 nm (Welling et al., 1983). In addition to the internal nucleoprotein (NP) and polymerase protein (P) in the nucleocapsis, there are three lipid-envelope-associated proteins (HN, F and M) in Sendai virus (Scheid and Choppin, 1974, 1977). According to Chopin et al. (1981), NH protein (M_r = 66 000) is responsible for haemagglutination and neuraminidase activities, and F (fusion) protein is involved in cell fusion, virus penetration and haemolysis. This last mentioned protein is present in the form of an inactive precursor F_0, from which it is generated by proteolytic cleavage. F protein consists of two disulphide-linked polypeptide chains, F_1 (M_r = 50 000 - 52 000) and F_2 (M_r = 13 500). Finally, M (matrix) protein of M_r = 38 000 is important for virus assembly and viral budding from the cell membrane. The envelope-associated proteins of Sendai virus and their separation were the main interest of the group concerned.

Van der Zee et al. (1983) disrupted the virions of Sendai virus by the addition of 2% (w/v) (final concentration) Triton X-100 for 30 min at room temperature. The detergent to viral protein ratio was 3.3 (w/v). The detergent was removed from the supernatant using Amberlite XAD-2, and at the same time the proteins were reduced by the addition of dithiothreitol (DTT) to a final concentration 20 m*M* (Amberlite XAD-2 was obtained from Serva, Heidelberg, F.R.G., and DTT from Sigma, St. Louis, MO, U.S.A.). Then the proteins were subjected to RP-HPLC. Two types of column were employed: (1) a 300 mm x 4.6 mm I.D. column was slurry packed with Nucleosil 10 C_{18} (Machery, Nagel & Co., Düren, F.R.G.), and (2) a 40 mm x 3.2 mm column containing Supelcosil LC-318 (Supelco, Bellefonte, PA, U.S.A.). The pore sizes were 10 and 30 nm, respectively.

Proteins containing extracts were chromatographed at a flow-rate of 1 ml/min with a linear gradient from 10 to 60% of solvent B in A for 24 min. Solvent A was 12 m*M* HCl in triply distilled water and B was 12 m*M* HCl in ethanol-1-butanol

(4:1, v/v). Both the organic solvents were obtained from E. Merck (Darmstadt, F.R.G.). The amount of proteins to be analysed was very small (10-50 µg). The purity of the isolated proteins was checked electrophoretically using SDS-PAGE. Protein F_2 was isolated in relatively large amounts (the yield was about 100%), whereas the recoveries of three other proteins (F_1, HN and M) were relatively low (from less than 5% to 50%). The amino acid analysis of F_2 was presented.

Welling et al. (1983) examined the anion-exchange chromatography of Sendai virus F protein in the presence of Triton X-100. Purified virions (46.5 mg/ml in 5 m*M* Tris-HCl, pH 7.23) were disrupted with Triton X-100 (purchased from BDH, Poole, U.K.) at a detergent to viral protein ratio of 0.9 (w/w) and dialysed against 0.02 *M* sodium phosphate buffer (pH 7.2). The retained material (containing mainly F and NH protein) was subjected to anion-exchange chromatography on a 50 mm x 5 mm I.D. Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) and eluted with a gradient from 0.15 to 1.5 *M* sodium chloride in 0.02 *M* sodium phosphate (pH 7.2) containing 0.1% (v/v) of Triton X-100; the flow-rate was 1 ml/min. For detection the absorbance at 260 nm was measured (owing to the presence of the aromatic ring in Triton, which did not allow the detection at 280 nm) and the peak fractions were collected manually in low-protein-adsorption (Minisorb) tubes (Nunc, Roskilde, Denmark). To remove most of Triton the tubes were covered with dialysis-membrane tubing and dialysed overnight at 4°C against water with Bio-Beads SM 2 (Bio-Rad Labs., Richmond, CA, U.S.A.; cf., Volsky and Loyter, 1978). The dialysed fractions were reduced and checked by SDS-PAGE.

The detergent extract contained F and HN proteins. HN was not retained by the column, and elution with a salt gradient resulted in several peaks, containing mainly or only F protein. As Brij 35 detergent did not absorb at 280 nm, the same chromatography was performed with 0.1% (v/v) Brij in the eluent. A similar separation resulted, although some tailing of peaks was observed. The ion-exchange procedure described was found to be a rapid method for the purification of viral proteins in the presence of non-ionic detergents.

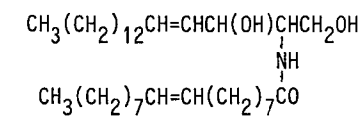
In continuation of these studies, Welling et al. (1984) described the isolation of detergent-extracted Sendai virus proteins by gel-filtration, ion-exchange and reversed-phase HPLC and the effect of immunological activity. Welling et al. (1985) combined size exclusion and reversed-phase HPLC of a detergent extract of Sendai virus, and Van der Zee and Welling (1985) studied the detection of Sendai virus proteins by RP-HPLC combined with immuno-chromatography.

Heukeshoven and Dernick (1985) described the characterization of a solvent system for the separation of water-insoluble poliovirus proteins by RP-HPLC, and Ricard and Sturman (1985) isolated the subunits of the coronavirus envelope glycoprotein E2 by hydroxyapatite HPLC.

12.2 GLYCOSPHINGOLIPIDS

12.2.1 Introduction

Glycosphingolipids are relatively complicated substances of great biological importance and therefore a concise chemical (biochemical) introduction will be given before a description of their chromatographic separations. It is known (Lehninger, 1978a) that cell coats of higher organisms consist of three main groups of compounds: (1) glycosphingolipids, (2) acid mucopolysaccharides and (3) glycoproteins. The first group are membrane components and belong to the complex lipids called sphingolipids. They contain long-chain aliphatic hydroxy-amino alcohols, saturated or unsaturated, or a related base as their backbone, e.g., sphingosine (4-sphingenine), $CH_3(CH_2)_{12}CH{=}CHCH(OH)CH(NH_2)CH_2OH$. This is the first building-block component. More than 30 different long-chain amino alcohols have been found in sphingolipids of various biological species. In sphingolipids, the amino group of sphingosine (or other amino alcohol) forms an amide bond with a long aliphatic fatty acid (saturated or unsaturated, containing 18 - 24 carbon atoms), e.g., oleic acid, $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$; the fatty acid is the second building-block component. Such amides are called ceramides and their structure can be illustrated by an example:

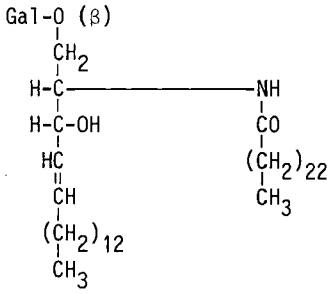


Ceramide

Ceramides create a molecular basis for the construction of sphingolipids. The third characteristic building-block component of sphingolipids is a polar head group, which may be very large and complex. In relatively simple sphingomyelins (which are not oligomers and will not be treated in this book) it contains phosphorylcholine; this component confers a zwitterionic nature on the resulting complex compound.

If sphingolipids contain one or more sugar residues in the position of their polar head, they are called neutral glycosphingolipids.

The simplest species of this group are cerebrosides, which contain a monosaccharide bound in a β-glycosidic linkage to the hydroxy group of ceramide. An example of galactocerebroside [containing galactose (Gal) and lignoceric acid (C_{24})] is illustrated by the formula



Galactocerebroside

If two galactose units are bound to create galactosylceramide, the structure of such a dihexoside may be symbolized as

$$\text{Gal1} \xrightarrow{\beta} \text{4Gal1} \xrightarrow{\beta} \text{Ceramide}$$

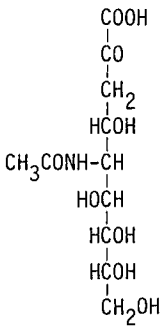
A compound containing one glucose (Glc) unit bound to ceramide is called glucocerebroside. Two or more galactose units containing glucosylceramides are symbolized as illustrated below by the example of tetrahexoside, containing terminal galactosamine (GalN) in the N-acetylated form (NAc):

$$\text{GalNAc1} \xrightarrow{\beta} \text{3Gal1} \xrightarrow{\beta} \text{4Gal1} \xrightarrow{\beta} \text{4Glc1} \xrightarrow{\beta} \text{Ceramide}$$

These are examples of so-called neutral glycosphingolipids. Di-, tri- and tetrahexosides belong to biooligomers and will be dealt with in the Section 12.2.

Sulphate esters of galactocerebrosides (at position 3 of Gal) are also found in nature as components of brain tissue; they are called sulphatides.

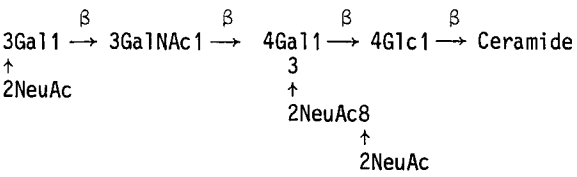
The most complex group of glycosphingolipids are gangliosides, containing 2-10 or more carbohydrates. In their polar oligosaccharide head they contain one or more residues of sialic acid, i.e., N-acetylneuraminic acid (NeuAc) (the older designation is NANA) with the formula



N-Acetylneuraminic acid (NeuAc) or sialic acid

[About 20 different sialic acids have been found in nature, but only a few have been detected in gangliosides (Ledeen and Yu, 1982). The above-mentioned NeuAc belong to the two major types.]

Most of the known gangliosides have Glc in the glycosidic linkage to ceramide. In the remaining part of the structure D-Gal or N-acetyl-D-GalN residues are often present. Nearly all vertebrate gangliosides are derived from lactosylceramide and hence possess the glucosylceramide structure; they can be classified into ganglio and neolacto series [Wiegandt (1979); Leeden and Yu (1983)]. Exceptionally, other types have also been found. If we consider variances in the sialic acid type, about 50 individual gangliosides have been described so far, differing in the number and relative positions of the hexose and sialic acid residues (Leeden and Yu, 1982), and this number is increasing as new discoveries are being reported. A complicated example of a trisialoganglioside is represented by



Even more complicated gangliosides have been found in tissues containing more sugar components, such as monosialomonofucooctahexaosylceramide.

Sphingolipids have been found in both plant and animal cells and are present in the largest amounts in brain and nerve tissues. The neutral glycosphingolipids play an important role in the cell surface (Lehninger, 1978a). Their non-polar tails penetrate into the lipid bilayer structure of cell membranes, whereas the polar heads protrude out from the surface and have various biological functions, e.g., they are responsible for blood-group specificity. In their positions on the cell surface the glycosphingolipids are concerned in organ and tissue specificity, in tissue immunity and cell-cell recognition processes (cancer cells have different glycosphingolipids from those in normal cells, which is important in cancer research and in clinical diagnostics). The accumulation of glycosphingolipids in cases of enzyme deficiency (genetic disorder in metabolism) leads to various lipid-storage diseases (glycosphingolipidoses; cf., Stanbury et al., 1978).

At present acidic glycosphingolipids (the gangliosides) are of great interest to biochemists and physicians as they are important membrane receptors (Svennerholm et al., 1980a; Hakomori, 1981; Fishman, 1982). They are components of the plasma membrane and contribute to the rich carbohydrate glycocylax, which determines the surface properties of cells (Ledeen and Yu, 1978, 1982; Wiegandt,

1982). In view of their localization in the plasma membrane outer leaflet, they participate in cell growth control and cell differentiation. Malignant transformation of cells triggers off major changes in their composition and therefore gangliosides can serve as tumour-associated markers (Hakomori and Kannagi, 1983). In addition, the gangliosides (abundant in nerve endings) have a function in the transmission of nerve impulses across synapses. Although originally discovered in the brain (especially the grey matter is very rich in gangliosides, where they represent 6% of the total lipids), these acidic glycosphingolipids have been found in other vertebrate tissues in smaller amounts. Several invertebrate gangliosides have also been identified.

In general, glycosphingolipids contain two long hydrophobic aliphatic hydrocarbon chains and are therefore soluble in organic solvents (e.g., chloroform or methanol), but they also contain a polar head. These are good properties for separations by means of partition chromatography in the normal-phase mode. Indeed, this technique has often been used for their fractionation. In some instance RPC has also been applied to their purification. Moreover, gangliosides contain ionogenic groups, allowing their simple isolation from neutral glycosphingolipids by anion-exchange chromatography and also their fractionation from each other. Both of these methods have often been used for the chromatographic separation of glycosphingolipids.

There are problems with the on-line detection of these substances if they are not modified with a suitable UV chromophore. Therefore, off-line detection has often been used for monitoring in column chromatography. A suitable and generally applicable method for the on-line detection of glycosphingolipids remains a problem.

12.2.2 Neutral glycosphingolipids

12.2.2.1 Unsubstituted natural products

HPLC has been applied for the analytical separation of glycosphingolipids (GSL) preferentially in the form of various derivatives, which will be discussed later in this section. However, the separation of GSL without derivatization has a great advantage, especially for preparations, because the separated lipids may be used directly in structural characterization and immunological analyses, and this is the main reason why it has been developed. Non-pressurized LC methods were often used in the beginning and various silica gel supports were applied as stationary phase for liquid-solid chromatography of GSL (Radin et al., 1956; Makita and Yamakawa, 1962; Gray, 1967; Vance and Sweeley, 1967; Siddiqui and McCluer, 1968; Puro, 1970; Hanahan et al., 1971; Ando and Yamakawa, 1973).

Pellicular supports have also been used for the separation of glycolipids, which allowed high-speed analyses (Stolyhwo and Privett, 1973; Sugita et al., 1974). Unfortunately, the low capacity of the last mentioned packing materials allowed only analytical applications.

Ando et al. (1976a) developed the high-performance preparative chromatography of lipids using totally porous silica (Iatrobeads), and applied it to the separation of molecular species of GSL. As the particle size was relatively large (60 μm), hydrostatic pressure was sufficient to achieve rapid separations. Ceramide dihexoside and ceramide trihexoside were both separated into two fractions of different molecular species, and two ceramide tetrasaccharides (globoside I and paragloboside) were also clearly separated. The separation of these molecular species was due to differences in their fatty acid and long-chain base compositions.

A sample of 400 mg of human erythrocyte glycolipid mixture was applied to a 120 cm x 1.7 cm I.D. column containing 140 g of Iatrobeads 6RS-8060 (Iatron Labs., Tokyo, Japan). The column was eluted with 1700 ml of a linear gradient of chloroform-methanol-water with the proportions changing from 83:16:0.5 to 55:42:3 (v/v) using two reservoirs. The column could be regenerated by washing with three column volumes of the starting solvent. In practice, an Iatrobeads column could be used for several chromatographic runs.

In another experiment, 100 mg of the fraction of human erythrocyte glycolipids (containing globoside I and paragloboside) were loaded on 85 g of Iatrobeads and the column was eluted with a convex gradient of chloroform-methanol-water with the proportions changing from 8:12:2 to 40:45:5 (v/v). The gradient was generated using three reservoirs in series containing the above eluent with proportions of 80:18:2, 55:42:3 and 40:55:5 (v/v).

The sugar content of each fraction was determined by the anthrone-sulphuric acid reaction, measuring the absorbance difference between 616 and 720 nm on a Hitachi Model 156 double-wavelength spectrometer. TLC was applied for the identification of the separated compounds. Also GLC (after methanolysis and trifluoroacetylation) and mass spectrometry were applied for detailed analysis of the fractions obtained. The physical properties of Iatrobeads were described in detail.

Watanabe and Arao (1981) described a new solvent system for the separation of neutral GSL. These glycolipids were extracted from erythrocyte membranes with hot ethanol and first purified by partitioning in chloroform-methanol (2:1). Acetylated glycolipids were further purified chromatographically on a Florisil column and neutral glycolipids were separated from gangliosides on an anion exchanger. Then the samples were applied to an HPLC column.

A stainless-steel column (500 mm x 4 mm I.D.) was slurry-packed with 10-μm Iatrobeads 6RS-8010 porous silica spheres (Iatron Labs.) using tetrabromoethane-tetrachloroethylene (60:40) at 400 kg/cm^2. The column was washed with isopropanol-hexane (55:45) and equilibrated with the starting solvent (e.g., isopropanol-hexane-water, 55:44:1). For elution a linear gradient of isopropanol-hexane-water was used (the concentrations of hexane and water were varied, depending on the glycolipid composition). The gradient was initiated immediately after injection at a flow-rate of 2.0 ml/min. Every 0.5 or 1 min one effluent fraction was collected in the fraction collector. After use, the column was regenerated by washing with isopropanol-hexane (55:45) at a flow-rate of 1 ml/min for 60 min and could be used again without any loss of efficiency.

No on-line sample detection was applied. The fractions were analysed by TLC [pre-coated silica gel G plates, developed with chloroform-methanol-water (60:35:8 or 65:25:4); detection by spraying with orcinol reagent]. The glycolipid content in each fraction was determined by the anthrone-sulphuric acid reaction and calculated as globotetraosylceramide (cf., Suzuki et al., 1976).

GSL with mono- to dodeca- or tetrakisdecasaccharides were separated highly reproducibly within 60 min. The method was applied to preparative separations of highly complex glycolipids with blood group activity.

12.2.2.2 Per-O,N-benzoyl derivatives

The lack of a suitable on-line detection method is a disadvantage of the chromatography of unsubstituted glycosphingolipids (GSL). A simple derivatization method was sought that would introduce a suitable UV chromophore into the glycolipid and allow the utilization of a sensitive UV detector. In addition, the modifying group should be easily split off after the chromatographic separation. This was believed to be a practical means of taking advantage of HPLC methods in glycolipid biochemistry.

Evans and McCluer (1972) reported a system for the separation of neutral glycolipids by HPLC of their benzoylated derivatives, prepared by reaction with benzoyl chloride, in a manner similar to that described by Acher and Kanfer (1972). They demonstrated that benzoylated cerebrosides could be readily separated into two fractions by HPLC on a silica gel column. It was originally believed that the derivatives prepared were only O-benzoyl derivatives and that the parent cerebrosides could be simply regenerated by catalytic deacylation with sodium methoxide in methanol. However, McCluer and Evans (1973) found that the reaction of cerebrosides containing non-hydroxy fatty acids with benzoyl chloride resulted in acylation of the amide in addition to the normal O-acylation (and mild alkali treatment of the N-diacyl derivative resulted in the formation of N-benzoylpsychosine). Cerebrosides containing hydroxy fatty acids did not

create the undesired N-benzoyl derivatives. Derivatization with benzoic anhydride avoided amide acylation even in the presence of non-hydroxy fatty acid components; however, this last reaction was not sufficiently fast.

McCluer and Evans (1973) described the benzoylation of cerebrosides and derivatives that contained hydroxy and non-hydroxy fatty acids were isolated by HPLC with UV detection.

Benzoylation procedure. Cerebroside samples (0.1-1.0 mg) were benzoylated with 0.6 ml of benzoyl chloride-pyridine (1:5, v/v) for 1 h at 60°C (similar amounts were benzoylated with 0.6 ml of 5% benzoic anhydride in pyridine for 18 h at 110°C). Two procedures were utilized to purify the benzoylated products, as follows. (A) The reaction mixture was dried under a stream of nitrogen and the residue was dissolved in 5 ml of hexane and successively washed with 3 ml each of 95% methanol saturated with Na_2CO_3, 0.6 *M* HCl in 95% methanol and 95% methanol. The hexane layer was dried under a stream of nitrogen. (B) The reaction product was refluxed under methanol and chloroform was used for extraction; after successive washing of the lower layer, the solution was evaporated and dissolved in hexane.

For HPLC a 50 cm x 2.1 mm I.D. stainless-steel column was dry-packed with Zipax pellicular packing (Instrument Products Division, DuPont, Wilmington, DE, U.S.A.) and eluted with 0.13% methanol in pentane at a flow-rate of 1.5 ml/min.

In addition to Zipax, a MicroPak NH_2 column (microparticulate silica gel with a bonded polar phase consisting of 3-aminopropylsilane groups) was used for the separation of perbenzoylated galactosyl- and glucosylceramides (McCluer and Evans, 1976). Suzuki et al. (1976) also described the separation of molecular species of glucosylceramide by HPLC of their benzoyl derivatives.

Jungalwala et al. (1977) reported the determination of less than 1 nmol of perbenzoylated cerebrosides by HPLC using gradient elution. Both hydroxy and non-hydroxy fatty-acid-containing cerebrosides were analysed with UV detection at 230 or 280 nm. The quantitative range of the method was 0.5-10 nmol of cerebrosides. The detection limit for injected samples was about 1 pmol and the analysis time was less than 5 min. No preliminary purification of the cerebrosides from other lipids in brain extracts was necessary. The moisture-free cerebrosides were first benzoylated with 50 µl of 10% benzoyl chloride in pyridine and then separated on a 50 cm x 2.1 mm I.D. column of 27 µm Zipax porous layer silica beads. If gradient elution with 2.8-5.5% dioxane in hexane was applied, monitoring at 230 nm (i.e., at the absorption maximum of benzoylated cerebrosides) was possible. The column was regenerated by reversing the gradient for 1 min and equilibrating the column with 2.8% dioxane in hexane for 3-4 min. This procedure was at least 10 times more sensitive than isocratic elution with detection at 280 nm (McCluer and Evans, 1976). Jungalwala et al. (1977) also de-

scribed a variant of gradient elution with detection at 280 nm, in which a linear gradient of 2-7% aqueous ethyl acetate in hexane was used for elution. The column was regenerated to its original polarity by reversing the gradient for 1 min and equilibrating the column with 2% aqueous ethyl acetate in hexane for 3-4 min. The methods described are applicable to biological materials containing minute amounts of cerebrosides.

Similar improved methods for the quantitative analysis or microanalysis of perbenzoylated neutral GSL were published by Ulman and McCluer (1977, 1978); the first paper describes HPLC with monitoring at 280 nm and the second at 230 nm. An example of separations reported in the first paper is illustrated in Fig. 12.2.

12.2.2.3 Per-O-benzoyl derivatives

The acylation of neutral glycosphingolipids (GSL) with benzoyl chloride was reviewed above. A disadvantage of this procedure is that N-benzoylation occurred in addition to the desired O-benzoylation, so that the recovery of the parent GSL after mild alkaline hydrolysis was not possible, owing to the formation of a mixture of products. Benzoylation with benzoic anhydride in pyridine did not lead to the formation of N-benzoylated products, but the anhydride reaction was sluggish, which is the reason why the benzoyl chloride method has been preferred.

Gross and McCluer (1980) described an HPLC analysis of neutral GSL per-O-benzoyl derivatives. The method was based on the observation of Gupta et al. (1977) that N,N-dimethyl-4-aminopyridine (DMAP) acted as a catalyst in the acylation of phospholipids by fatty acid anhydrides. Therefore, Gross and McCluer (1980) tried to acylate GSL with benzoic anhydride in the presence of DMAP. Single products were formed and the parent GSL could be regenerated by mild alkaline hydrolysis after chromatographic separation.

Per-O-benzoylation procedure (Gross and McCluer, 1980). Samples of 200 ng each of mono- to tetraglycosylceramides were dried under a stream of nitrogen in 100 x 13 mm screw-capped tubes and desiccated in vacuo over P_2O_5 for at least 2 h. A freshly prepared solution of 20% (w/v) benzoic anhydride and 5% (w/v) DMAP in pyridine (0.5 ml) was added. The tubes were briefly flushed with nitrogen, capped tightly and incubated at 37°C for 4 h; the pyridine was then removed under a stream of nitrogen. Hexane (3 ml) was added and the solution was washed four times with 1.8 ml of 80% methanol saturated with sodium carbonate and twice with 80% methanol, as described by Ulman and McCluer (1978). The solvent was removed under a stream of nitrogen and the benzoylated samples thus obtained were each dissolved in an appropriate amount of CCl_4 for injection on to the HPLC column.

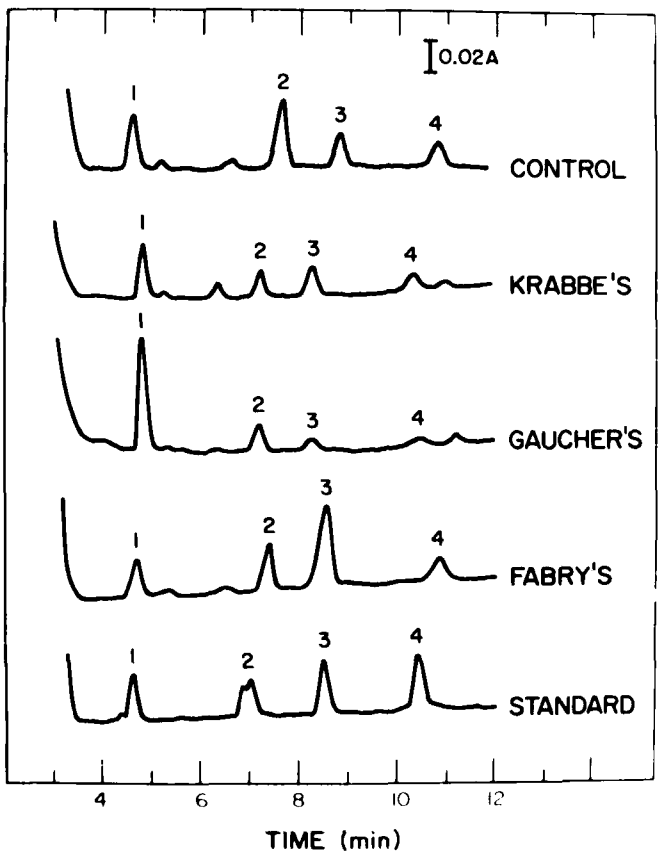


Fig. 12.2. HPLC analysis of plasma glycolipids from a healthy person and patients suffering from inborn errors of lipid metabolism. Glycolipid fractions from 1 ml of plasma were isolated, benzoylated and dissolved in 100 µl of CCl_4 and 40 µl were analysed on a pellicular silica gel (Zipax) column (50 cm x 2.1 mm I.D.) using a 10-min linear gradient of 2-17% ethyl acetate in hexane at a flow-rate of 2 ml/min. The absorbance at 280 nm was recorded. Peaks: 1 = Glc-Cer; 2 = Lac-Cer; 3 = Gal-Lac-Cer; 4 = globoside. The heights and the exact positions of the peaks are discussed in the original paper. On the right-hand side the diseases of the inborn errors of lipid metabolism are designated; for their characterization, see Table 14.4. (Reprinted from Ullman and McCluer, 1977.)

The HPLC column was a 50 cm x 2.1 mm I.D. stainless-steel tube packed with Zipax pellicular silica gel (DuPont, Wilmington, DE, U.S.A.). For elution a 13 min linear gradient from 2.5 to 25% dioxane in hexane was used, with a flow-rate of 2 ml/min. UV detection at 230 nm was applied.

Conversion of perbenzoylated products to the parent GSL (Gross and McCluer, 1980). The peaks obtained from HPLC were treated with 0.5 *M* methanolic sodium hydroxide solution for 1 h at 37°C. After partitioning in chloroform-methanol-water (8:4:3) the lipid products in the lower phase were examined by TLC.

Preparative and analytical HPLC of modified GSL using a similar method (per-O-benzoylation with benzoic anhydride catalysed by DMAP) was also described by McCluer and Ulman (1980).

12.2.2.4 O-Acetyl-N-p-nitrobenzoyl derivatives

Suzuki et al. (1977c) described the separation of molecular species of higher glycolipids by HPLC of their O-acetyl-N-*p*-nitrobenzoyl derivatives. The advantage of this modification of glycosphingolipids (GSL) for detection is the higher molar absorptivity at 254 nm compared with those of benzoyl derivatives. This labelling procedure can be easily performed on acetylated glycolipids. Suzuki et al. (1977c) separated O-acetyl-N-*p*-nitrobenzoyl derivatives of glucosylceramide, lactosylceramide, trihexosylceramide, globoside and haematoside (N-acetyl type) into their molecular species on the basis of fatty acid components by HPLC using a reversed-phase column (μBondapak C_{18}). Acetonitrile was used as the eluent.

Yamazaki et al. (1979) examined the consecutive analysis of GSL on the basis of sugar and ceramide moieties by HPLC. A quantitative method for the determination of GSL in biological materials was described and applied to erythrocyte glycolipids. A crude lipid extract was separated into neutral and acidic fractions on DEAE-Sephadex. GSL fractions were obtained by acetylation and Florisil column chromatography. Acetylated GSL samples were N-*p*-nitrobenzoylated and chromatographed on a Zorbax SIL silica gel column. For elution an isopropanol gradient in hexane-chloroform was used. Derivatives were separated on the basis of their sugar chains. The peaks obtained were further chromatographed on a μBondapak C_{18} reversed-phase column on the basis of their lipid portions.

An improved technique for the separation of neutral GSL by HPLC was described by Suzuki et al. (1980). O-Acetyl-N-*p*-nitrobenzoyl derivatives of six neutral GSL (glucosylceramide, lactosylceramide, globotriaosylceramide, lactotriaosylceramide, globotetraosylceramide and neolactotetraosylceramide) were separated. The recoveries of glucosylceramide and globotetraosylceramide for the derivatization procedure and chromatographic analysis were about 75%, and 1 nmol of glycolipid could be detected. The method was applied to the analysis of human erythrocyte neutral glycolipids and allowed the separation of certain glycolipids that contained equal numbers of sugar moieties. The derivatization technique was the same as that described by Yamazuki et al. (1979), including the purification of the derivatives on a Sephadex LH-20 column.

For the HPLC a 25 cm x 4.6 mm I.D. Zorbax SIL column was used and eluted at a flow-rate of 0.5 ml/min by programmed elution as follows: 5 min isocratic elution with 1% isopropanol in hexane-dichloroethane (2:1), 55 min with a linear gradient from 1 to 5% isopropanol in hexane-dichloroethane (2:1) and 10 min

isocratically with 5% isopropanol in hexane-dichloroethane (2:1). Before the next analysis, the column was reactivated with the initial solvent for 15 min.

12.2.3 Acidic glycosphingolipids - gangliosides

Gangliosides are glycolipids and the composition of their components determines their solubility in water and organic solvents. The original method for their isolation and purification (Folch et al., 1957) was based on partitioning from chloroform-methanol (into which they were extracted from natural sources) into the upper water-enriched phase, leaving behind the bulk of the other lipids. This procedure was later modified by various workers, e.g. by Suzuki (1965); a modification of this last-mentioned technique was described in detail in a review by Ledeen and Yu (1982), where other approaches were also discussed.

Gangliosides contain one or more residues of sialic acid, which opens up the possibility of ganglioside isolation using anion exchangers. The whole group of gangliosides can be separated in this way from neutral glycosphingolipids, but individual ganglioside subclasses (mono- to pentasialogangliosides) can be fractionated according to the number of sialic acid residues.

Because of the hybrid lipophilic-hydrophilic character of ganglioside fractions, LSC on silica gel has often been used sucessfully for their fractionation. In addition to column chromatography, TLC has also been applied for both analytical and semi-preparative separations.

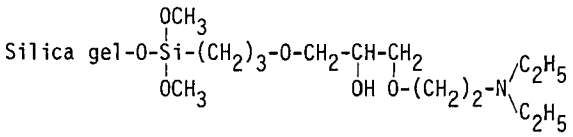
All the column chromatographic separation techniques for gangliosides will be reviewed in the following part of this Section. Most gangliosides have been chromatographed in the underivatized form, which will be dealt with first.

12.2.3.1 Ion-exchange chromatography

In 1971 Winterbourn published the first paper on the separation of brain gangliosides by column chromatography on DEAE-cellulose (Whatman DE-2), previously washed with ammonium acetate and methanol. Stepwise elution with gradually increasing concentrations of ammonium acetate in methanol (0.022, 0.04, 0.1 and 0.25 *M*) was applied and the separation of gangliosides according to the number of bound sialic acid residues was achieved. A group of gangliosides of human myelin with sialosylgalactosyceramide (G_7) as a major component was isolated from purified myelin using DEAE-Sephadex A-25 (Pharmacia) column chromatography by Ledeen et al. (1973); this was the first application of DEAE-Sephadex for the isolation of the total ganglioside group. Momoi et al. (1976) applied a concave gradient of ammonium acetate in methanol for the elution of gangliosides from a column of DEAE-Sephadex A-25, and these were further separated on silica gel. Ivamori and Nagai (1981a,b) combined a separation on DEAE-Sepharose with

Iatrobeads 6RS-8060 chromatography. A detailed description of the gradient elution of gangliosides from a DEAE-Sephadex A-25 column was described and discussed by Ledeen and Yu (1982). Comparing the separation of gangliosides on DEAE-Sephadex, QAE-Sephadex and DEAE-Sepharose, Ivamori and Nagai (1978a) found that the last material gave the best separation and yields. All these methods were based on the low-pressure chromatography.

The application of DEAE-silica gel as an ion exchanger for the isolation of glycolipids (Kundu and Roy, 1978) can be considered as the first step towards the development of the HPLC of gangliosides. A relatively rapid and quantitative method (but non-pressurized, with only gravitational elution) was presented for the isolation of gangliosides and neutral glycosphingolipids from animal tissues and cells. The advantages of this method in comparison with ion exchangers based on a carbohydrate matrix were: (1) faster flow-rate; (2) faster equilibration with the starting buffer; (3) easier regeneration; (4) greater economy; and (5) less susceptibility to microbial attack. Kundu and Roy (1978) described in detail the preparation of the DEAE derivatives of porous silica gel (pore diameter 200 nm; 200-400 mesh; surface area 150 m^2/g; pore volume 1.68 ml/g). γ-Glycidoxypropyltrimethoxysilane and N,N-diethylethanolamine were used for synthesis. The product had the formula



and was used in a 30 cm x 1.5 cm I.D. column, which was equilibrated with 0.2 *M* sodium acetate in methanol and thoroughly washed with methanol, and then washed with at least three bed volumes of chloroform-methanol-water(30:60:8, v/v) before use. A 10-ml volume of beef brain total lipid extract was diluted to 50 ml with chloroform-methanol-water (30:60:8, v/v) and allowed to flow through the column (10 g of DEAE-silica gel) under gravity. After 50 ml had been added, the column was eluted with 150 ml of the same solvent mixture. The combined eluates from this first fraction consisted of the uncharged and zwitterionic lipids. The second fraction, containing the acidic lipids, was eluted with 200 ml of chloroform-methanol-0.8 *M* sodium acetate (30:60:8, v/v). The total elution time was approximately 5 h.

Kundu et al. (1979) also developed the DEAE-silica gel and DEAE-controlled-pore glass (CPG) chromatography of gangliosides into a method that allowed the separation of gangliosides according to the number of sialic acid residues into mono-, di-, tri- and tetrasialogangliosides. The DEAE-silica gel was laboratory-prepared as described above. DEAE-CPG was obtained from Electro-Nucleonics

(Fairfield, NJ, U.S.A.). The sorbents were compared with DEAE-Sephadex and the best results were obtained with DEAE-silica gel. A 60 cm x 2 cm I.D. column containing 40 mg of ganglioside mixture was eluted with methanol, 0.2 *M* ammonium acetate in methanol and 0.5 *M* ammonium acetate in methanol. Volumes of 200 ml of each solvent were connected in series with each other through a gradient mixer. The flow-rate was 1.5 ml/min. Fractions of 15 ml of the effluent were collected and aliquots of 500 µl were used for sialic acid assay by the resorcinol method (Svennerholm, 1957; Miettiner et al., 1959; Suzuki, 1964). The gangliosides were isolated after the removal of ammonium acetate by dialysis against cold water. DEAE-silica gel and DEAE-CPG as ion exchangers for the isolation of glycolipids and their application to the separation of gangliosides were described in detail by Kundu (1981a). Kundu (1981b) also described in detail techniques for the TLC of neutral glycosphingolipids and gangliosides; these techniques have often been used for the evaluation of fractions obtained by IEC.

Fredman et al. (1980) tried to combine the good separation ability of DEAE-dextran for gangliosides with the rigidity of silica gel, suitable for HPLC, and developed a new type of anion-exchange support, Spherosil-DEAE-dextran, which consists of porous glass beads (Spherosil silica gel) covered with cross-linked DEAE-dextran. The invention of this combined ion exchanger was described earlier by Tayot et al. (1978). In principle, 1 kg of Spherosil was poured into 2.3 l of a 7.5% aqueous solution of DEAE-dextran at pH 11.5 and then dried for 15 h at 80^{o}C. DEAE-dextran was cross-linked with 1,4-butanediol diglycidyl ether (Aldrich, Milwaukee, WI, U.S.A.). The resin (200 ml) was freed from fines by decantation and then slurried into a glass column (3.0 cm I.D.) and converted into the acetate form by thorough washing with 2.0 *M* sodium acetate solution (five bed volumes). It was then rinsed with five bed volumes of distilled water and one bed volume of methanol. Before use one bed volume (200 ml) of chloroform-methanol-water (60:30:4.5, v/v) was passed through the column.

Dialysed brain gangliosides (corresponding to 1 mmol of neuraminic acid), dissolved in 200 ml of chloroform-methanol-water (66:30:4.5, v/v) were placed on the column and filtered at a flow-rate not exceeding 0.5 ml/min. The column was rinsed with 200 ml of the same chloroform-methanol-water mixture and 200 ml of methanol. The gangliosides were then eluted stepwise with (i) 1000 ml (five volumes) of 0.02 *M*, (ii) 2000 ml (ten volumes) of 0.10 *M*, (iii) 1600 ml (eight volumes) of 0.25 *M*, (iv) 1000 ml (five volumes) of 0.50 *M* and (v) 600 ml (three volumes) of 1.00 *M* potassium acetate in methanol. Mono-, di-, tri-, tetra- and pentasialoganglioside fractions were separated. The eluates were evaporated, dialysed, and their further purification was achieved by chromatography on silica gel or by TLC. This method has advantages over the previous preparative ion-exchange chromatography fractionation. The method was also described by Fredman (1980).

A very rapid method for the separation of gangliosides by anion-exchange chromatography on strongly basic Mono Q resin (9.8 μm, volume of the pre-packed bed = 1 ml) in combination with FPLC equipment (Pharmacia, Uppsala, Sweden) was presented by Månsson et al. (1985). The gangliosides were separated into mono-, di-, tri- and tetrasialoganglioside fractions by a discontinuous gradient of potassium acetate in methanol. The separation was completed in a volume of 50 ml (25 min). The Mono Q column was washed before use with 10 ml of methanol, 50 ml of 1 *M* potassium acetate in methanol and 10 ml of methanol at a flow-rate of 1 ml/min. A ganglioside sample dissolved in chloroform-methanol (1:2, v/v) was introduced on to the column at a flow-rate of 1 ml/min. The gangliosides were eluted with a stepwise gradient from 0 to 225 m*M* potassium acetate in methanol at a flow-rate of 2 ml/min. Fractions of 1 ml were collected and aliquots were assayed by HPTLC with 1-propanol-0.25% aqueous potassium chloride (3:1, v/v). The pooled fractions were dialysed against water and further assayed. After each analysis the column was washed with 5 ml of 1 *M* potassium acetate in methanol and 20 ml of methanol. Very good and rapid separations of gangliosides were achieved on Mono Q, but the application of this strongly basic anion exchanger had some disadvantages: part of the tetrasialoganglioside GQ1b was eluted among the monosialogangliosides, probably owing to lactonization of some of the sialic acid residues, and a certain non-specific adsorption effect also appeared.

Šmíd et al. (1986a) applied the medium-basic anion exchanger DEAE-Spheron to the separation of gangliosides. Linear gradient elution using ammonium acetate in methanol resulted in the complete separation of mono- to pentasialogangliosides in 35 min. The separation obtained, illustrated in Fig. 12.3, did not suffer from the undesirable catalytic activity of the anion exchanger on the sensitive gangliosides. Fractions were assayed by TLC and the technique of "ganglioside mapping" was employed (Ivamori and Nagai, 1978a) for their detailed identification. The rapid preparative separation of gangliosides on medium-basic anion exchangers was described by Šmíd et al. (1986b).

12.2.3.2 Chromatography on silica gel and controlled-pore glass

Ion-exchange chromatography permits only the group separation of gangliosides according to the number of sialic acid residues, and these groups usually contain more than one ganglioside species. Unfortunately, the present methods of IEC are not able to fractionate these groups into pure individual gangliosides and a subsequent separation is necessary. Some chromatographic methods allowing the direct fractionation of gangliosides (without any pre-fractionation by IEC) have been investigated. However, the combination of IEC with LSC (silica gel chromatography) was used most often and became almost customary.

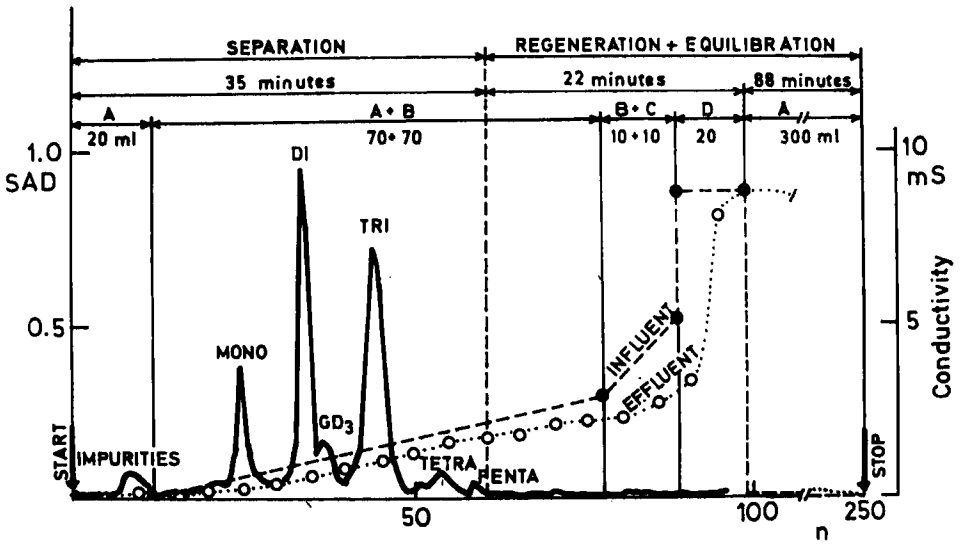


Fig. 12.3. Separation of gangliosides according to the number of sialic acid residues on the medium-basic anion exchanger Spheron 1000-DEAE (17 μm). The diagram illustrates the whole cycle of repeated accelerated IEC. The column (25 cm x 0.8 cm I.D.) was equilibrated with 2 *M* ammonium acetate in methanol and washed with methanol. The ganglioside sample (30 mg) was suspended in a mixture of 400 μl of water and 400 μl of methanol, cooled and applied using the underlayering method. Eluents: A = methanol; B = 0.2 *M* ammonium acetate in methanol; C = 0.5 *M* ammonium acetate in methanol; D = 2 *M* ammonium acetate in methanol. Fractions of 2 ml were taken at 35-s intervals. Temperature, 25°C; flow-rate, 3.4 ml/min (i.e., 6.8 ml/cm^2 min); pressure, 13 atm. After washing with methanol, the column was ready for the next sample. Mono to Penta refer to the number of sialic acid residues in ganglioside fractions. GD_3 is a disialoganglioside. SAD = relative colorimetric value (580 nm) of sialic acid detection (Miettinen and Takki-Luukkainen, 1959). The measurement of electrical conductivity (in millisiemens, mS) was found to be very useful for monitoring both the chromatography and the regeneration and equilibration. (Reprinted from Šmíd et al., 1986.)

Momoi et al. (1976) used DEAE-Sephadex for the pre-fractionation and the fractions obtained were further chromatographed on totally porous spheres, Iatrobeads 6RS-8060 (d_p 60 μm) (Iatron Labs., Tokyo, Japan). Using this system, brain gangliosides GM1, GD1a, GD1b and GT1 were obtained in high purity and in milligram ammounts. The principle of the procedure is as follows. Sluries of 130 g of Iatrobeads in mixtures of chloroform-methanol in various proportions (depending on the content of the various gangliosides in fractions I-V from IEC) were poured into 120 cm x 1.7 cm I.D. columns and left overnight. Then solutions of 100 mg of the lipid fractions in small amounts of the appropriate chloroform-methanol-water mixtures were applied to the column. Each column was eluted with a linear gradient of methanol and water in chloroform-methanol-water (the com-

position being specified for individual IEC fractions) at a flow-rate of 0.8-1.0 ml/min, and fractions of 20 ml were collected. The components of fraction II were not separated on Iatrobeads, but re-chromatographed on a DEAE-Sephadex column. Nagai and Ivamori (1980) also further separated the fractions of mono- to tetrasialogangliosides (originating from chromatography on DEAE-Sepharose) using an Iatrobeads column and obtained individual gangliosides.

Kundu and Scott (1982) described a rapid separation of gangliosides by HPLC. First they employed IEC on DEAE-silica gel (Kundu, 1981), in which the sample was fractionated into groups according to the number of sialic acid residues. Then individual ganglioside species were obtained by chromatography on a 25 cm x 4.6 mm I.D. column of porous silica gel (5-μm Zorbax Sil, obtained from DuPont, Wilmington, DE, U.S.A.), which was eluted by programmed gradient elution with a mixture with an increasing water content and a decreasing hexane content. The column was equilibrated with solvent A (see below) prior to injection of the sample. A mildly sonicated ganglioside sample (up to 500 μg) dissolved in 50 μl of isopropanol-hexane-water was injected. A linear gradient of isopropanol-hexane-water from 55:42:3 (solvent A) to 55:25:20 (solvent B) was used during a period of 2 h. The flow-rate was 0.5 ml/min. Fractions of 1 ml were obtained with a fraction collector and assayed by TLC. The column was regenerated after use with the initial solvent for 15 min. Major ganglioside components of human erythrocytes and beef brain were separated and the procedure was found to be highly reproducible.

Nakamura et al. (1983) studied gangliosides of hog skeletal muscle. Monosialoganglioside and disialoganglioside groups were isolated on a DEAE-Sephadex A-25 column , using a linear gradient of sodium acetate in methanol for elution, and purified to homogeneity of the individual fractions by Iatrobeads column chromatography. For the fractionation of about 3 g of the monosialoganglioside fraction, a 130 cm x 1.4 cm I.D. column containing 45 g of support was eluted with a linear gradient system prepared with 1.2 l each of chloroform-methanol-7.5 *M* ammonia solution (70:50:3 and 36:65:3, v/v). The gangliosides in the effluent were analysed by TLC. The monosialoganglioside fraction was separated into six portions and seven gangliosides were further purified by successive Iatrobeads column chromatography using various solvent systems to obtain homogeneous preparations. Approximately 500 mg of disialogangliosides were chromatographed on a 130 cm x 1.2 cm I.D. Iatrobeads column with a linear gradient of 1.5 l each of chloroform-methanol-7.5 *M* ammonia solution from 55:45:4 to 35:65:4 (v/v). TLC was used for the effluent analyses. Three pooled fractions were further purified by successive Iatrobeads column chromatography and five species were isolated. All the fractions obtained were examined using various chemical and biochemical modification reactions in order to determine their chemical composition and structure.

Several workers have tried to separate ganglioside mixtures in one chromatographic step. Tjaden et al. (1977) described the HPLC of glycosphingolipids (with special reference to gangliosides) within 40 min using 9 μm LiChrosorb Si 60 (E. Merck, Darmstadt, F.R.G.) as the stationary phase in a 25 cm x 2.8 mm I.D. column. Artificial mixtures of six gangliosides and four neutral glycolipids were analysed. For gangliosides the eluent was chloroform-methanol-aqueous HCl (60:35:4); the final HCl concentration was 0.01 *M*. For detection a moving vire was applied with a flame ionization detector. In addition to three unknown peaks, six known gangliosides were identified. In a separate chromatography of neutral glycosphingolipids, one unknown peak appeared in addition to four known species, when neutral chloroform-methanol (3:1) was used for elution. Also here the same universal detector was employed for monitoring. Tjaden et al. (1977) recommended the method for both analytical and micropreparative purposes.

Watanabe and Tomono (1984) developed a "one-step" fractionation of neutral and acidic glycosphingolipids by HPLC. They connected one column of DEAE-derivatized controlled-pore glass (CPG) serially with two columns of underivatized CPG. A mixture of gangliosides and neutral glycosphingolipids was loaded on the DEAE-CPG column and washed using gradient elution based on chloroform-methanol-water with increasing methanol and water contents, followed by a second gradient with an increasing amount of lithium acetate solution. In the first gradient elution neutral glycolipids (mono- to hexaglycosylceramides) were separated within 80 min, and in the second mono- to tetrasialogangliosides were separated within 60 min.

Controlled-pore glass (CPG-10) of pore diameter 75 Å, 200-400 mesh, and DEAE-CPG of pore diameter 170 Å, 200-400 mesh, were purchased from Electro-Nucleonics (Fairfield, NJ, U.S.A.). The first stainless-steel column (250 mm x 2.6 mm I.D.) was packed with DEAE-CPG. The ion exchanger was converted into the acetate from by washing with 0.1 *M* lithium acetate in solvent D (see below) at a flow-rate of 1 ml/min for 60 min, followed by washing with solvents C and B for 30 min each. The first column was connected with a second column (500 mm x 2.6 mm I.D.) packed with CPG-10, which was further connected to a third column identical with the second. The following mixtures were used as mobile phases: (A) chloroform-methanol (95:5, v/v); (B) chloroform-methanol-water (90:10:0.5, v/v); (C) chloroform-methanol-water (10:80:10, v/v); and (D) chloroform-methanol-1 *M* lithium acetate (10:80:10, v/v). Glycolipid mixtures were loaded on to the first column and eluted with a linear gradient of solvents B and C, followed by gradient elution from solvent C to D (for optimal proportions, see the original paper). The effluent was collected in a fraction collector and each fraction (or every second fraction) was examined by TLC or using resorcinol reagent (Suzuki, 1964).

A very good separation was obtained using this one-step fractionation method. This technique was applied to the determination of bovine brain gangliosides and neutral and acidic glycolipids isolated from rat kidney. The procedure was found to be highly reproducible.

12.2.3.3 Application of reversed-phase and normal-phase chromatography to the separation of gangliosides

In addition to IEC and LSC on silica gel, RPC and NPC have also been applied to separations of gangliosides. A great advantage of these methods is the possibility of a simple on-line detection of gangliosides in the effluent using the continuous measurement of the UV absorbance at low wavelengths. Kundu and Suzuki (1981) developed a simple micro-scale method for the isolation of gangliosides by RPC. A ganglioside fraction obtained by chromatography of the total lipid extract on DEAE-Sephadex (or DEAE-silica gel) was subjected to alkaline hydrolysis and salts and other non-lipid contaminants were removed by RPC on a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). The purified gangliosides were then obtained by chromatography on a small Iatrobeads (6RS-8060) or Unisil (200-325 mesh) column (obtained from Iatron Labs., Tokyo, Japan, and Clarkson Chemical, Williamsport, PA, U.S.A., respectively).

The residues of evaporated fractions after the IEC of gangliosides from human brain white matter and human erythrocytes were treated with 15 ml of 0.1 *M* sodium hydroxide in methanol and incubated at 37°C for 2 h to destroy the alkali-labile acidic phospholipids. After evaporation, the aqueous solution was neutralized with 0.5 *M* HCl and diluted to a concentration of 0.1 *M* salt and passed through a Sep-Pak C_{18} cartridge at a flow-rate of 1 ml/min. [The cartridge was pre-washed with 25 ml of chloroform-methanol (1:2), 25 ml of methanol and 50 ml of water before use.] The cartridge was washed with 25 ml of water and the gangliosides were then eluted with 5 ml of methanol followed by 25 ml of chloroform-methanol (1:2). The cartridge could be used again after washing with methanol and equilibration with water. Sulphatides and other coloured impurities were removed later by chromatography on Iatrobeads columns. In these experiments RPC on a C_{18} cartridge replaced a dialysis procedure in order to remove the salts and low-molecular-weight impurities. A quantitative recovery of gangliosides was obtained.

Sonnino et al. (1984) described the HPLC preparation of muscular species of GM1 and GD1a gangliosides with a homogeneous long-chain base composition (C_{18} or C_{20} sphingosine and C_{18} or C_{20} sphinganine, each in its natural *erythro* or unnatural *threo* form). An analytical or semi-preparative procedure was based on the application of HP-RPC under conditions suitable for resolving ganglioside species on the basis of (i) length, (ii) double bond occurrence and (iii) C-3 isomerism of the long-chain base.

The gangliosides were purified by Silica gel 100 (70-230 mesh, ASTM) column chromatography and structurally characterized as described by Ghidoni et al. (1980). After oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) and reduction with $NaBH_4$, the *erythro* form was partially converted into the *threo* form.

For the analytical separation, 1-10 µg of ganglioside, dissolved in 25 µl of redistilled water, was applied to an RP Spherisorb S5 OD S2 column (250 mm x 4.5 mm I.D.). The temperature was 18-20°C, the eluent was acetonitrile-5 m*M* sodium phosphate buffer (pH 7), in volume ratios of 7:3 for GM1 and 3:2 for GD1a, and the flow-rate was 1.0 ml/min. One complete analysis took about 40 min for GM1 and 20 min for GD1a. For semi-preparative experiments the same support was used in a 250 mm x 10 mm I.D. column (Phase Separations, Queensferry, U.K.) using the above conditions with the exception of the load (up to 5 mg of natural or DDQ/$NaBH_4$-treated gangliosides, dissolved in 25 µl of redistilled water) and flow-rate (7.5 ml/min). The elution was monitored by UV detection at 195 nm. The effluent was automatically collected on a computer programmed fraction collector on the basis of the UV signals. A complete cycle of analysis took 60 min for GM1 and 40 min for GD1. Natural GM1 and GD1a were separated by the HPLC preparative technique into four fractions, the composition of which was clarified using detailed chemical studies and further HPLC analyses.

In one working day four or five cycles as described above could be performed using a 30-min wash with the elution solvent between cycles, so that a total of 20-25 mg of gangliosides per day could be processed.

Gazzotti et al. (1984a) examined the analytical and preparative HPLC of gangliosides using an RP LiChrosorb RP-8 or µBondapak RP-18 column and a mixture of acetonitrile and phosphate buffer at fixed or varying volume ratios. The HPLC separation resolved all common gangliosides into four molecular species containing C_{18}-sphingosine, C_{18}-sphinganine, C_{20}-sphingosine and C_{20}-sphinganine. Both the sensitivity and the precision of the method were high (detection limit 0.1 nmol, relative standard deviation less than 10%). The semi-preparative method allowed the preparation of 100-mg amounts of gangliosides of each molecular species in 2-4 days in a fully automated process, starting from single gangliosides (such as GM1 and GD1a). In the preparative method acetonitrile-phosphate buffer-tetrahydrofuran was used as the eluent and the addition of the corresponding radioactive tracer was required; this procedure, applied to GM1, was devised for processing up to 50 mg of ganglioside per analysis.

LiChrosorb RP-8 (5 µm) was used in the analytical column (150 mm x 4.6 mm I.D.) (Merck, Darmstadt, F.R.G.). The chromatography of the total ganglioside mixture was carried out at 18-20°C with acetonitrile-5 m*M* sodium phosphate buffer (pH 7.0) in a volume ratio of 1:1 for 8 min, then the ratio was altered continuously to

3:2 during the following 6 min and finally kept at 3:2 until the end of the run. The flow-rate was 0.5 ml/min and UV detection at 195 nm (absorption maximum for gangliosides) was applied. A complete analysis took about 45 min. Analytical check of the purity of individual gangliosides were carried out under isocratic conditions with suitable mobile phase compositions.

In an automatic semi-preparative method a 10-μm μBondapak RP-18 column (250 mm x 10 mm I.D.) (Water Assoc., Milford, MA, U.S.A.) was used at 18-20°C. The eluent was acetonitrile-5 m*M* sodium phosphate buffer (pH 7.0) in a volume ratio of 7:3 for GM1 and 3:2 for GD1a. The flow-rate was 3.0 ml/min and UV detection at 195 nm was applied. The automation of the process was described. For fully preparative separations a 10-μm μBondapak column (250 mm x 10 mm I.D.) was used and eluted at 18-20°C with acetonitrile-5 m*M* sodium phosphate buffer (pH 7.0)-tetrahydrofuran (9:7:4, v/v) at a flow-rate of 7.5 ml/min. A 50 mg amount of GM1 was applied, mixed with 0.5 μCi of the tritiated compound, dissolved in 250 μl of redistilled water. The elution profile was monitored with a computer-assisted HPLC radiactivity monitor (Berthold, Model LB 503) equipped with a 120-μl solid scintillator cell.

In addition to RPC, NPC was also applied to the rapid fractionation of gangliosides. Gazzotti et al. (1985) described the analytical and semi-preparative NP-HPLC separation of underivatized brain ganglioside mixtures into individual components. Gangliosides were applied to a LiChrosorb-NH_2 column and eluted with acetonitrile-phosphate buffer mixtures. For on-line monitoring, flow-through detection at 215 nm was applied. Mono- and oligosialogangliosides were separated in one step in a total elution time of less than 90 min with high reproducibility (1-2% relative standard deviations of retention times).

A 0.1 - 20-nmol portion of pure gangliosides, or 1 - 50-nmol portions (as lipid-bound sialic acid) of calf-brain gangliosides were dissolved in 10 μl of redistilled water and applied to an analytical column (250 mm x 4 mm I.D.) containing 7-μm LiChrosorb-NH_2 (Merck, Darmstadt, F.R.G.), which was eluted at 20°C with the following solvent mixtures: (A) acetonitrile-5 m*M* phosphate buffer (pH 5.6) (83:17); (B) acetonitrile-20 m*M* phosphate buffer (pH 5.6) (1:1). A computer-controlled gradient programme was applied: 7 min elution with solvent A, 53 min with a linear gradient from A to B (66:34), 20 min with a linear gradient from A to B (66:34) to A to B (36:64). The flow-rate was 1 ml/min, and a complete analysis took 80 min.

For preparative HPLC, a 1-5 mg portion of ganglioside mixture (calculated as sialic acid) was dissolved in 100 μl of water and applied to a 250 mm x 25 mm I.D. column of LiChrosorb-NH_2 and eluted with the above-described gradient programme. The flow-rate was 39 ml/min. Fractions of 20 ml were collected au-

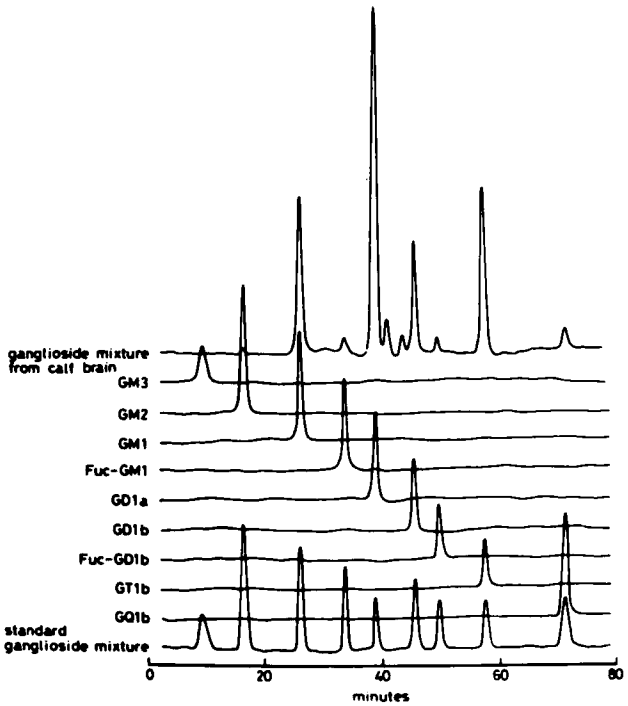


Fig. 12.4. Application of the NP-HPLC method to the separation of gangliosides present in a calf-brain ganglioside mixture. In the middle part of the figure analytical runs of the individual isolated gangliosides are illustrated. For comparison a profile of a standard mixture is given below. Chromatographic procedures are described in the text. (Reprinted from Gazzotti et al., 1985.)

tomatically, and 0.1-ml aliquots were dried and assayed by the HPTLC method described. Pooled fractions of purified gangliosides were submitted to the described analytical HPLC methods.

The following species were separated from a mixture of calf-brain gangliosides (in order of elution): GM3, GM2, GM1, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b and GQ1b.

Monitoring at 215 nm represents only 60% absorbance compared with the maximum absorption of gangliosides at 195 nm. However, the use of this longer wavelength allowed the baseline variations usual at 195 nm (if a gradient was used) due to the solvent absorption to be avoided. The purity of each separated ganglioside was at least 99%. Fractionation of gangliosides as described by Gazzotti et al. (1985) (cf., Fig. 12.4) seems to give the most complete separation of these underivatized acid glycosphingolipids described so far.

12.2.3.4 Separation of derivatized gangliosides

The derivatization of gangliosides with a UV chromophore opened up the way to the simple continuous monitoring of their separation even in LSC on silica gel. In experiments with neutral glycosphingolipids, perbenzoylation was found to be a suitable derivatization method (see Section 12.2.2). Such a modification was believed to allow the transformation of the old LSC into a modern HPLC technique, because on-line monitoring of the effluent was possible.

Bremer et al. (1979) described the quantitative analysis of monosialogangliosides by HPLC of their perbenzoyl derivatives. The method was very sensitive owing to the high molar absorptivity of the benzoyl derivatives at 230 nm. It was used to determine the GM3 content of human plasma.

As little as a 3-nmol sample of a monosialoganglioside fraction prepared from biological material by a preliminary fractionation (on a DEAE-Sephadex A-25 column, dialysis or using a Sep-Pak C_{18} cartridge, or on a Unisil silicic acid column) was dried and converted into the perbenzoyl derivative by reaction with 0.1 ml of 10% benzoyl chloride in pyridine at 60°C for 1 h. The product was purified by silicic acid chromatography and analysed by HPLC on a 50 cm x 2.1 mm I.D. LiChrospher Si 4000 column. The most satisfactory eluent was an 18-min linear gradient from 7 to 23% dioxane in hexane, or a 10-min gradient from 10 to 25% dioxane in hexane. The flow-rate was 2 ml/min and UV detection at 230 nm was applied; the detector response was found to be proportional to the amount of monosialoganglioside present. As little as 1 pmol of ganglioside could be conveniently estimated. After each gradient run was completed, a short reversed gradient (3 min) was used and a flow-through of the first solvent for 8 min returned the column to the original state, ready for further chromatography.

Lee et al. (1982) developed the HPLC of long-chain neutral glycosphingolipids and gangliosides. Perbenzoyl derivatives of both these types of glycolipids were chromatographed in a single HPLC system, consisting of Zipax stationary phase and a linear gradient of dioxane in hexane as the mobile phase. Twenty-four glycosphingolipids containing 1-10 sugars and 1 or 2 sialic acid residues were studied using this advantageous system. Gangliosides present in human leucocytes were evaluated.

A newer version of the quantitative analysis of more complex brain gangliosides by HPLC of their perbenzoyl derivatives was published by Ulman and McCluer (1985).

Traylor (1983) examined the HPLC resolution of *p*-nitrobenzyloxyamine derivatives of brain gangliosides, which were originally isolated from the natural material by the partitioning method of Folch et al. (1957) in the version of Suzuki (1965). The reversed-phase mode was used in these experiments with modified gangliosides. This derivatization involves ozonation and cleavage of

the ceramide double bond, followed by oxime formation to the nascent aldehyde. Ozonolysis at -70°C was performed with a Supelco Microozonizer at an oxygen flow-rate of 10 ml/min. Ozonation required about 2 min per milligram of ganglioside. Triphenylphosphine was added to cleave the ozonide selectively to the aldehyde (the fuchsin test for aldehydes was routinely positive within 1 min). A molar ratio of *p*-nitrobenzyloxyamine to the sum of ganglioside-aldehyde and long-chain fatty acid aldehyde of 1:3:1 was routinely used. The reaction mixture was incubated in methanol for 15 min at 40-45°C. Immediately following the derivatization, chloroform was added to the reaction mixture, the solvent composition was adjusted to methanol-chloroform (7:2) and the mixture was applied to a 25 cm x 2 cm I.D. DEAE-Sephadex A-25 column (Ledeen et al., 1973); the ion exchanger was first washed four times with chloroform-methanol-0.8 *M* sodium acetate (30:60:8) and three times with chloroform-methanol-water (30:60:9), and then poured in the form of a suspension in methanol-chloroform (7:2) into a glass column. The above-mentioned reaction mixture was applied to the column at a flow-rate of 1 ml/min and the column was eluted with 300 ml of methanol-chloroform (7:2). The labelled gangliosides were eluted with 200 ml of methanol-chloroform (7:2) that had been made 0.2 *M* in ammonium acetate; the effluent was monitored at 254 nm and the fractions were assayed by the resorcinol method of Svennerholm (1957). Dialyzed samples were lyophilized.

For HPLC a 30 cm x 2 mm I.D. reversed-phase 10-μm μBondapak C_{18} column (Waters Assoc.) was used and eluted at ambient temperature at a flow-rate of 1 ml/min. Solvent programming began with the initial solvent composition methanol-water (50:50), which was held constant for 1 min and then linearly adjusted during 15 min to methanol-water (70:30). Peak areas were quantitated by a Columbia Scientific Industries (Austin, TX, U.S.A.) Model CRS-208 integrator.

Individual gangliosides were collected and identified by TLC and GC of their monosaccharides. Quantitative results were obtained in this method, together with a marked increase in sensitivity in comparison with conventional analytical methods.

Nakabayashi et al. (1984) studied the analysis and quantitation of gangliosides as *p*-bromophenacyl derivatives by HPLC. This method was found to be highly sensitive, and involves a simple and specific derivatization of the carboxylic group. The molar absorptivity of the derivatives at maximum absorption (261 nm) was about 23 000. The acidic fractions from IEC pre-fractionation on DEAE-Sephadex could be directly derivatized and the reaction mixtures directly injected into the HPLC equipment without any preliminary purification. Both normal- and reversed-phase modes could be applied for the separation of ganglioside mixtures.

Derivatization of gangliosides (Nakabayashi et al., 1984). Ganglioside, containing 1-15 µg of sialic acid, was dried in vacuo in a PTFE-lined glass tube and reacted with 20 µl of *p*-bromophenacyl bromide (Gasukuro Kogeyo, Tokyo, Japan) in dimethylformamide (10 mg/ml) at 60°C for 1 h. When the reaction was completed, the solution was immediately checked by TLC and 5 µl of the reaction mixture was directly injected on to an HPLC column.

For HPLC in the NPC mode a 25 cm x 4.6 mm I.D. column packed with silica gel (5-6 µm, Zorbax SIL, DuPont) was used and eluted according to the following solvent programme: 1 min isocratic elution with solvent mixture A [isopropanol-*n*-hexane-water (50:49:1, v/v)], then from A to B [the same components (55:35:10, v/v)], at a rate of 2%/min. For HPLC in the RPC mode a 15 cm x 4.6 mm I.D. column packed with octasilylated silica gel (5-6 µm Zorbax C_8, DuPont) was used and eluted with acetonitrile-methanol (4:1, v/v). UV detection at 261 nm (the absorbance maximum of *p*-bromophenacyl derivatives) was applied and the peak areas were calculated with an integrator (Shimadzu C-R1A).

Monosialogangliosides GM4, GM3, GM2 and GM1 were well separated using the normal-phase mode. The calibration graph was linear up to 100 µg of ganglioside sialic acid; the detection limit was about 10 ng. The reversed-phase mode was also successfully applied to analyses of gangliosides.

12.2.3.5 Sulphatides

Sulphatides also belong to acid glycosphingolipids. From the chemical point of view they are sulphate esters of galactocerebrosides and create glycolipid components of brain and other tissues. Desulphated sulphatides are often treated together with cerebrosides. RPC of sulphatides was described by Yahara et al. (1980).

Shimomura and Kishimoto (1983) presented and improved a procedure for the quantitative determination and characterization of sulphatides in rat kidney and brain by RP-HPLC. A significant improvement was made in the desulphation step. A solution of trifluoroacetic acid in ethyl acetate was used for solvolysis. The revised method was used to determine the levels of cerebrosides and sulphatides in rat kidney.

12.2.4 Special separation techniques

In this section several miscellaneous techniques will be mentioned that are not based on liquid column chromatography, but which may be of interest for those investigators who are going to separate glycolipids by HPLC methods.

Young et al. (1979) described an improved method for the covalent attachment of glycosphingolipids to solid supports and macromolecules. The olefinic bond

of the sphingosine moiety was oxidized to a carboxyl group, which could be coupled to glass beads, agarose gel, proteins and polyacrylic hydrazide polymers. Solid supports and macromolecules prepared in this way may become useful for a variety of bioaffinity studies in cell biology and immunology.

Pick et al. (1984a,b) used a method for overpressured TLC (Tyihak et al., 1979), designated OPTLC, and used it for the class fractionation of neutral and acidic glycosphingolipids.

Otsuka and Yamakawa (1981) and Otsuka et al. (1983) described the application of droplet counter-current chromatography (DCC) for the separation of acidic glycosphingolipids from brain and neutral glycosphingolipids from mammalian erythrocytes (blood group substances).

12.2.5 Applications of HPLC of glycosphingolipids

12.2.5.1 Discoveries of novel glycosphingolipids

The investigation of glycosphingolipids is a very systematic, thorough and progressive process: nearly every year brings knew knowledge in this field and new species are discovered and structurally characterized; many of them are gangliosides.

Ando et al. (1976b) described the existence and structure of glucosamyl-lactosylceramide [lacto-N-triose(II)ceramide, amino CTH-I] in human erythrocyte membranes as a possible precursor of paragloboside and group-active glycolipids; high-speed Iatrobeads chromatography was used for its purification. Ando and Yu (1977) reported the isolation and structural characterization of a trisialoganglioside, GT1a, from human brain; it represented 0.6% of the total gangliosides. Also here Iatrobeads chromatography was applied as a final fractionation and purification step of the trisialoganglioside fraction obtained by IEC. Ivamori and Nagai (1978b) presented results of the isolation and characterization of a ganglioside (monosialosylpentahexaosylceramide) from human brain. Using a combination of IEC and Iatrobeads chromatography, approximately 2.1 μmol of an unknown ganglioside were obtained from 1 kg of human brain (this amount comprised 0.09% of the total lipid-bound sialic acid in the brain). The characterization procedures were described.

Ivamori and Nagai (1981c) studied monosialogangliosides of rabbit skeletal muscle and characterized a novel N-acetylneuraminosyl lacto-N-noroctaosyl ceramide. After IEC (DEAE-Sephadex, DEAE-Sepharose) the monosialoganglioside fraction was further fractionated by LCS on Silica gel 60 and Iatrobeads columns. As in the above-mentioned papers, also here the purity of the obtained fractions was examined by TLC. The newly isolated ganglioside represented 5.1% of the monosialoganglioside fraction. Chien and Hogan (1983) described a novel pentasia-

lohexaosyl ganglioside of the globo series purified from chicken muscle. This monosialosyl ganglioside was isolated from a tetrahydrofuran-aqueous KCl extract of chicken pectoral muscle by a combination of IEC (DEAE-Sephadex) and silicic acid (Bio-Sil A) chromatography. The fractions were checked by TLC.

12.2.5.2 Glycosphingolipids in research on and diagnostics of cancer

Glycosphingolipids participate in cell recognition processes and their changes are closely connected with the process of tumour growth. This is the reason why they are intensively studied also from the medical point of view and high-speed chromatographic methods for glycosphingolipids can not only contribute to research on cancer, but they can be also used as rapid diagnostic methods in clinical biochemistry.

Hakomori et al. (1982) described the common structure of fucosyllactosaminolipids accumulating in human adenocarcinomas and their possible absence in normal tissue. Two major glycolipids accumulating in human primary liver carcinoma (absent in normal liver) were characterized as lacto-N-fucopentaosyl(III)ceramide and difucosyllacto-N-norhexaosylceramide. The tissue extract [in isopropanol-hexane-water (55:25:20)] was partitioned four times (according to Folch et al., 1951, as modified by Svennerholm, 1963) and then separated by successive chromatography on DEAE-Sephadex (Yu and Ledeen, 1972). Neutral glycolipids were purified by three stages of HPLC: (i) low-pressure chromatography on an Iatrobeads 6RS-8060 column, (ii) re-chromatography of the selected fractions on an Iatrobeads 6RS-8060 column and (iii) repeated HPLC purification; for details see the original paper. Chemical characterization of the isolated species was described and its comparison with other related glycolipids was discussed in connection with oncogenesis.

Holmes and Hakomori (1982) isolated and characterized a new fucoganglioside accumulated in precancerous rat liver and in hepatoma induced by N-2-acetylaminofluorene. This novel ganglioside was found to be accumulated in the liver of rats fed with N-2-acetylaminofluorene before the development of hepatoma. This new fucoganglioside was absent in normal rat liver and in livers of rats fed with non-hepatitic carcinogens.

The animals were labelled in vivo with [^{3}H]fucose. The crude gangliosides obtained after tissue extraction, Folch partitioning (for citations see above in the preceding paper) and IEC on DEAE-Sephadex A-25 were further chromatographed on an Iatrobeads (6RS-8010) column (50 cm x 1 cm I.D.), eluting with a gradient system of isopropanol-hexane-water from 55:40:45 to 55:30:15 over 150 min at a flow-rate of 1 ml/min. The eluted gangliosides were detected both by liquid scintillation counting and by TLC. Fractions containing [^{3}H]fucosyl gangliosides were pooled and purified by HPLC on an analytical Iatrobeads

6RS-8010 column (80 cm x 4 mm I.D.) using the gradient described above and a flow-rate of 0.5 ml/min. The biochemical and chemical degradation of the novel ganglioside in order to establish the structure was described.

In addition to the mentioned species, also another (second) fucoganglioside was detected in a smaller amount in the precancerous liver and in hepatoma in vivo, but not in control tissue. The results indicated that the synthesis of new fucolipids was induced at an early stage during the process of carcinogenesis; this could be a unique marker for the diagnosis of hepatoma and its premalignancy.

Magnani et al. (1982) proved that monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose(II). Monoclonal antibodies were produced by hybridomas obtained from a mouse immunized with colorectal carcinoma cells. These antibodies bind specifically to human gastrointestinal cancer cells. The antigen corresponsing to this antibody was isolated and chemically determined. It appeared to be a monosialoganglioside and its structure was proposed. The ganglioside antigen was isolated from human adenocarcinoma cell line SW 1116. The extract was first fractionated by IEC on DEAE-Sepharose CL-6B and the monosialoganglioside fraction was further separated on a Bio-Sil HA (-325 mesh) column (100 cm x 0.9 cm I.D.) using concave gradient elution from chloroform-methanol (4:1, v/v) to methanol-0.2% calcium chloride solution (50:3, v/v). Fractions containing antigen and exhibiting only one band in TLC (migrating between G_{M1} and G_{D1}) gave 300 µg of the monosialoganglioside, which was chemically characterized and examined by immunoassays.

Westrick et al. (1983a) studied gangliosides of human acute leukaemia cells (from cells of eight patients: four lymphoblastic and four non-lymphoblastic) and found great differences in the absolute amounts of gangliosides (they were lower than in normal cells), a simplified ganglioside pattern and reduced amounts of N-acetylneuraminosyllactotriaosylceramide. In one instance (a patient with acute non-lymphoblastic leukaemia) a disialylated ganglioside (GD_3) was proved, which was not found in normal leukocytes. TLC and HPLC methods in combination with glycosidase treatments were used for the identification of gangliosides. Westric et al. (1983b) described the isolation and characterization of gangliosides from chronic myelogenous leukaemia cells. Similarly to the above, they found differences in the patterns (lower total content of gangliosides). The major gangliosides were G_{M3}, a sialosylparagloboside, and another ganglioside of the reported structure (previously designated as an "i" active compound). No other gangliosides containing more than one sialic acid residue were detected in chronic myelogenous leukaemia glycosphingolipids. Folch partitioning (mentioned at the beginning of this section), DEAE-Sephadex chromatography and Bio-Sil A

or Florisil chromatography were used for the isolation, and TLC, HPLC and biochemical and chemical methods were used for the degradation and identification of these glycosphingolipids. Differences between chronic myelogenous leukaemia and acute leukaemia were discussed.

Blaszczyk et al. (1984) studied a foetal glycolipid expressed on adenocarcinomas of the colon. Using IEC, HPLC and TLC they reported the detection and characterization of tumour-associated glycolipids from colorectal carcinoma and meconium, which resemble the gastrointestinal cancer antigen described by Magnani et al. (1981). These glycolipids contain the known X-determinant or stage-specific embryonic antigen-1, modified by a single sialo residue. Although the X-determinant was present in the normal colon, the sialylated-X glycolipids were absent and found only in adenocarcinomas. These monosialo-X glycolipid antigen might serve as potential tumour markers. Gonwa et al. (1984) reported the inhibition of mitogen- and antigen-induced lymphocyte activation by human leukaemia cell gangliosides. The number of viable cells was not reduced and the inhibition was due to blast formation. Three (in vitro) inhibiting gangliosides were isolated, which were not unique to leukaemia cells, but their concentration was increased in patients with cancer. Siddiqui et al. (1984) studied the differential expression of ganglioside G_{D3} by human leukocytes and leukaemia cells (both acute and chronic). Among myeloid cells acute leukaemia cells were positive for G_{D3}, whereas chronic leukaemia cells and normal neutrophils did not have detectable G_{D3}. All lymphocytic leukaemia cells (chronic and acute) contained G_{D3}, which were not detected in normal lymphocytes. The procedure was as follows. The upper phase from Folch partitioning was chromatographed on an Iatrobeads 6RS-8010) column using a linear gradient of 2-propanol-hexane-water from 55:35:10 to 55:30:15 at 2 ml/min for 150 min. The fractions were analysed on HPTLC plates, using detection with resorcinol reagent or by immunostaining.

12.2.5.3 Miscellaneous biomedical applications

The analytical chromatographic separation of glycosphingolipids has been used for the diagnostics of some inherited anomalies (enzyme deficiency diseases). Ullman et al. (1980) described the application of HPLC to the study of sphingolipidoses. Perbenzoylated sphingolipids, prepared from plasma from 32 Gaucher (β-glucosidase deficiency) and 6 Fabry (α-galactosidase deficiency) patients by solvent partition, were chromatographically separated on silicic acid or reversed-phase columns. Chromatographic analysis of sphingolipids provided useful supportive information for diagnoses because affected individuals were shown to possess increased circulating concentrations of pathognomic sphingolipid. Strasberg et al. (1983) also studied the HPLC analysis of perbenzoylated neutral glycolipids as an aid in the diagnosis of lysozomal storage

disease. Concentrations of four glycolipids (GL1a, GL2a, GL3a and GL4a) were determined in normal plasma and compared with the concentrations in plasma from patients with Gaucher, Krabbe, Fabry, Sandhoff and Tay-Sachs diseases and also with hypocholesterolaemia. An Ultrasphere silica column (5 μm) eluted with a gradient of isopropanol in hexane proved useful for the analyses (see also Fig. 12.2).

Nagai and Ivamori (1980b) examined brain and thymus gangliosides, their molecular diversity, their biological implications and a dynamic annular model for their function in cell surface membranes. Iatrobeads column chromatography and ganglioside mapping were used. Gravotta and Maccioni (1985) studied gangliosides and sialoglycoproteins in coated vesicles from bovine brain.

Uemura et al. (1978) described the characterization of major glycolipids in bovine erythrocyte membrane; a combination of IEC (DEAE-Sephadex A-25) and chromatography on a mixed silicic acid-Hyflo Super Cell (3:1) column were used for the fractionation of neutral and acidic glycosphingolipids. Yamakawa et al. (1980) reported the analysis of red blood cell glycolipids by HPLC of acetylated and perbenzoylated derivatives; the derivatives of erythrocyte glycolipids were separated on a silica gel column on the basis of their sugar chain and the fractions were analysed by RPC on the basis of their lipid portions.

Suzuki et al. (1977a) used perbenzoylated derivatives of neutral glycosphingolipids for the micro-scale determination of seminolipids by HPLC and studied their application to the determination of the seminolipid content of boar spermatozoa. The decrease in the seminolipid content of testes of rats with vitamin A deficiency was also determined by HPLC using similar methods (Suzuki et al., 1977b).

Chien Jaw-Long and Hogan (1980) studied glycosphingolipids of skeletal muscle. Kosai et al. (1982) described a convenient method for the large-scale preparation of sialo-G_{M1} ganglioside from brain. Gazzotti et al. (1984b) reported the preparation of tritiated molecular forms of gangliosides with a homogeneous long-chain base composition; gangliosides were labelled at C-6 of the terminal galactose or N-acetylgalactosamine or at C-3 of the long-chain base, and using an HPLC procedure eight different molecular species were prepared from each labelled ganglioside, which were further characterized. The reversed-phase mode according to Sonnino et al. (1984) was applied, employing the experimental conditions described in Section 12.2.3 (NPC and RPC). For radioactivity counting a 120-μl solid scintillator cell (Berthold LB 503 radioactivity monitor) was used). A computer-assisted automatic fraction collector (Gilson 201) was programmed to separate peaks on the basis of UV or radioactivity signals.

12.2.6 Comments on literature

In addition to the references given in the introductory Section 12.2.1 the following citations are important. Glycolipids were reviewed by McKibbin (1970). Weigandt (1980) wrote introductory remarks on the chemical structure of gangliosides, including the classification of glycosphingolipids. The proposed pathways for the ganglioside biosynthesis were discussed by Yu and Ando (1980). Svennerholm et al. (1980b) edited symposium proceedings in which much useful information on gangliosides can be found. Macher and Sweeley (1978) dealt with the structure, biological sources and properties of glycosphingolipids. Ledeen and Yu (1982) have written a methodological review on the structure, isolation and analysis of gangliosides (including glycolipid classification). Kanfer and Hakomori (1983) have written a book on sphingolipid biochemistry. Eberendu et al. (1985) presented a review of chromatographic techniques for the analysis of glycolipids and phospholipids, based on 125 references. McCluer et al. (1986) have written a modern review on the HPLC of glycosphingolipids and phospholipids. Nagai et al. (1986) described the procedure of gene transfer as a novel approach to the gene-controlled mechanism of the cellular expression of glycosphingolipids.

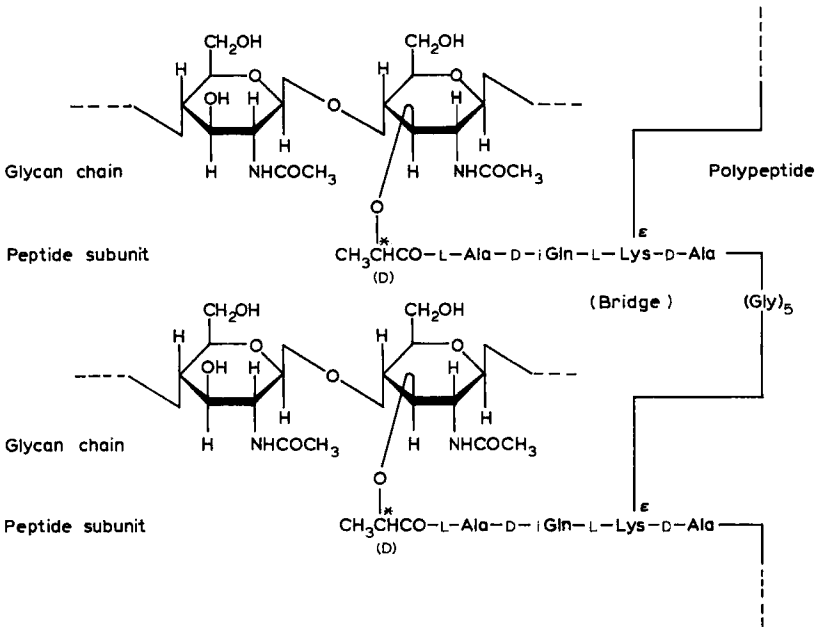
The nomenclature of glycolipids is sometimes complicated and several recommendations have been made by individual authors, and proposals accepted by the official nomenclature commissions have also been published. A practical shortened ganglioside nomenclature was proposed by Svennerholm (1963, 1964), and the IUPAC-IUB Commissions (1977, 1978) recommended a unified nomenclature for lipids.

12.3 GLYCOPEPTIDES AND PEPTIDES OF BACTERIAL CELL WALLS, IMMUNOMODULATORS AND SYNTHETIC VACCINES

12.3.1 Introduction

The polysaccharides, peptidoglycans and proteins on the surface of bacterial cell walls give the main impulse for the immune response of macroorganisms attacked by microbes. The detailed mechanism of the immune response process is very complicated, but two general types of compounds can be classified on the bacterial cell walls, which are the most important to start with: (i) antigens, against which antibodies are synthesized by the macroorganism, and (ii) immunomodulators, modifying the immune response and the activity of antigens. In Sections 12.3.1-12.3.3 the second type of compounds will be dealt with, and the first type will be discussed in the Section 12.3.4. In research on both types of compounds chromatographic methods have played an important role.

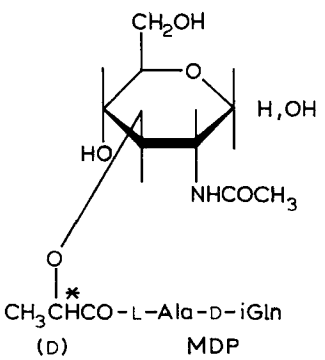
Gram-positive and Gram-negative bacterial cell walls have a common feature, viz., the rigid structural framework consisting of parallel polysaccharide chains covalently cross-linked by peptide chains, and this framework constitutes 10-50% or even more of the weight of the cell wall. We shall not comment here on the accessory components, which are different in the two types of bacterial cell, but will focus our attention on the main macromolecular framework, a heteropolymer, which is called peptidoglycan or murein (murus means wall in Latin). The peptidoglycan forms a completely continuous covalent structure around the cell. For example, Gram-positive bacteria are encased in up to 20 layers of cross-linked peptidoglycan (Lehninger, 1978b). The basic recurring unit in peptidoglycans (creating long polysaccharide chains) is a disaccharide of N-acetyl-D-glucosamine and N-acetylmuramic acid in a β(1→4) linkage, to the carboxyl group of which a peptide is attached by means of the N-terminus. The peptidoglycan cross-linking in the *Staphylococcus aureus* cell wall is given below as an example (according to Strominger and Ghuysen, 1967; see also peptidoglycan biosynthesis, dealt with by Strominger et al. 1967).



A tetrapeptide L-Ala-D-isoGln-L-Lys-D-Ala is joined by a pentaglycine bridge bound with its N-terminus to the carboxyl group of D-Ala in one chain and by its C-terminus to the ε-amino group of Lys in the other chain. In the cell walls of other bacteria similar but not identical peptide cross-linking patterns have been

found [for surveys, see Rogers and Perkins (1968), Schleifer and Kandler (1972), Leive (1973) and Ježek (1986)].

It was found that for the immunization of the macroorganism (i.e., for the prevention of infection) the presence of intact bacterial cells is not necessary; some parts of cells were sufficient, especially bacterial cell walls and their oligomeric components. Peptidoglycan and their fragments showed in both in vitro and in vivo tests about 40 types of biological activity (Kotani et al., 1983; Adam and Lederer, 1984; Lederer, 1986; Ježek, 1986), such as modulation (potentiation or inhibition) of the immune response, pyrogenicity, stimulation of the natural non-specific resistence against microbial, viral and parasite infections and growth of tumours and modulation of cells participating in the process of the natural or induced immunity. Some of these biological activities did not seem to have any direct connection with the immune response process, e.g., the influence on the increase in the duration of slow-wave sleep (somnogenic activity). Many papers have been published describing the biological properties of various peptidoglycan fragments and their analogues. The turning-point in research on these compounds was the period 1974-75, when Adam et al. (1974), Ellouz et al. (1974) and independently Kotani et al. (1975) proved that the minimum size fragment showing high immunoadjuvant activity is N-acetylmuramyl-L-alanyl-D-isoglutamin, often designated muramyl dipeptide (MDP) or MDP-Pasteur in the literature, owing to its discovery in the Pasteur Institute in Paris.



Adjuvant activity is the stimulation of the production of antibodies against an antigen injected simultaneously with the adjuvant glycopeptide. Many experiments have been undertaken to substitute chemically or modify this basic active unit (MDP) in order to prepare analogues with various biological activities; for reviews see, e.g., Lederer (1986) and Ježek (1986).

Synthetic glycopeptides represent a very important contribution to the endeavour of discovering the best immunomodulators. The first peptide fragments of bacterial cell walls (the lysine pentapeptides) were synthesized by Garg et al. (1962), the first lysine glycopeptides (containing N-acetylmuramic acid) were prepared by Lanzilotti et al. (1964) and the first glycopeptides containing diaminopimelic acid were prepared by Arendt et al. (1974). The synthetic approach required the working out of some auxiliary synthetic methods, for instance for isoglutamine or isoasparagine derivatives (e.g., Straka and Zaoral, 1977) or for removal of protecting groups (e.g., Zaoral et al., 1978). Immunoadjuvant activity and structure specificity of some synthetic glycopeptides were studied (e.g., Mašek et al., 1978, 1979; Adam and Lederer, 1984; Lederer, 1986). Ovchinnikov et al. (1979) prepared the longest oligosaccharide glycopeptide (the tetrasaccharide with two bound dipeptides L-Ala-D-iGln) and the longest peptide bound to disaccharide (i.e., the pentapeptide) using semi-synthetic methods. The largest lysine glycopeptides [bis(N-Ac-Mur)tridecapeptides with a pentaglycine or penta-L-alanine bridge moiety] were synthesized by Zaoral et al. (1980) and tris(N-Ac-Mur)octadecapeptide by Ježek et al. (1987). Up to now about 1000 synthetic glycopeptides have been reported, and most of them have been patented (cf., e.g., Lefrancier and Lederer, 1981; Straka 1983; Ježek, 1986).

In addition to glycopeptides and peptides from bacterial cell walls, a number of other natural peptides (and their synthetic analogues) also show important activities in the immune response process [cf., e.g., the review (460 references) by Leclers and Vogel (1986)]. Similar activity was found, e.g., in the natural tetrapeptide tuftsin (Thr-Lys-Pro-Arg), which is a phagocytosis-stimulating factor isolated from the γ-globulin fraction of human blood serum and activates all functions of phagocytic cells (Najar and Nishioka, 1970; Najar et al., 1981). Tuftsin binds to specific receptors on phagocytic cells (Wleklik et al., 1986). In addition to tuftsin and its analogues, the natural pentapeptide proctolin (Arg-Tyr-Leu-Pro-Thr), a neuropeptide from the hindgut of the cockroach, *Periplaneta americana* L., also shows a positive activity on restoration of human PMN leukocytes defected by acute lymphoblastic leukaemia (Konopinska et al., 1986). In contrast, Orefici et al. (1986) found that peptides are not necessary in the modulation of all the immune responses: polysaccharides secreted by streptococci (streptococcal exopolysaccharides alone) could induce a variety of effector cells in the peritoneum of normal experimental mice, including natural killer lymphocytes showing cytotoxic activity against some tumour cell targets.

12.3.2 Chromatography of glycopeptide immunomodulators

The conditions for the chromatography of glycopeptides are limited to the pH range of stability and also by the solubility of these substances. N-Acetylmuramyl peptides are readily soluble in water and in lower alcohols. They are very sensitive to alkaline solutions, e.g., 0.05 *M* ammonia solution (at 37°C) can split off lactyl or lactopeptide in 1 h, and aqueous ammonia (pH 12) will destroy the muramyl peptide immediately (for citations, see Ježek, 1986). In a mildly acidic solution (pH 2-4) muramyl peptides are relatively stable, whereas in strongly acid media muramic acid is decomposed. For monitoring in column liquid chromatography, measurement of the UV absorbance at 200-210 nm may be used. In preparative separations TLC is often applied for monitoring the purity of fractions, in addition to HPLC.

In the study of natural glycopeptides, IEC has been used for the separation of peptidoglycan fragments. Bacteriolytic peptidases are capable of breaking some peptides that interlink the peptidoglycan strands of microbial cell walls and the wall material is thus transferred into solution. Ion exchangers were used to fractionate such a partial hydrolysate (cf., review by Mikeš and Šebesta, 1979). Tipper et al. (1967) split the peptide cross-bridges that interlink the peptidoglycan strands of *Staphylococcus aureus* cell walls by means of exopeptidases produced by *Myxobacter*, and the digest was chromatographed on a 23 cm x 3 cm I.D. ECTEOLA-cellulose (Bio-Rad Cellex E) column using brief isocratic elution with water and then a linear gradient of LiCl concentration. The elution rate was 1 ml/min and the fraction volume was 15 ml. The desalted fractions were further fractionated on Sephadex and CM-cellulose. On the basis of the study of isolated polysaccharides and peptides, the structure of peptidoglycan was proposed.

Lefrancier et al. (1977) also used IEC (AG1 X-2 resin from Bio-Rad Labs., Richmond, CA, U.S.A. and Amberlite IRA-68 resin from Serva, Heidelberg, F.R.G.) for the fractionation of synthetic glycopeptides [N-acetylmuramyl-L-Ala-D-iGln, i.e., muramyl dipeptide (MDP) and analogues] in addition to preparative silica gel column chromatography. Zaoral et al. (1978), in synthetic experiments on the glycopeptide series, purified 270 mg of the crude intermediate N-acetyl-D-glucosamine by chromatography on a silica gel (60-120 μm) column (60 cm x 2.5 cm I.D.) eluted with 1-butanol-acetic acid-water (4:1:1) at a flow-rate of 50 ml/h and with a fraction volume of 10 ml. The pure product was eluted in fractions 40-56 and, after evaporation at reduced pressure, a yield of 203 mg (73%) was obtained. The same chromatographic method was also used for the purification of other intermediates. In an additional contribution to the glycopeptide series

(Zaoral et al., 1980), some intermediates were purified by GPC on a 100 cm x 1 cm I.D. column of Sephadex in 1 *M* acetic acid at a flow-rate of 6 ml/h, monitored by TLC. No apparent loss due to decomposition of the products was observed in these experiments.

Lefrancier and Lederer (1981) found HPLC of MDP to be a promising method for various analytical purposes (to check its chemical or even stereochemical homogeneity, to study its stability or to quantify its dosage forms). Spherisorb ODS and LiChrosorb-NH_2 columns eluted with ammonium acetate-acetonitrile mixtures were found to be very effective even for the separation of isomers (see later). Phillips et al. (1984) studied the synthesis and fast atom bombardment-mass spectrometry of MDP and for the HPLC of glycopeptide samples used a Varian (Palo Alto, CA, U.S.A.) MicroPak AX-10 column (30 cm x 4 mm I.D.), eluted at 50°C with acetonitrile-triethylammonium trifluoroacetate (pH 3.1) (2:3) at 1 ml/min; the pressure was 9.6 MPa and UV detection at 206 nm was applied.

If the results of glycopeptide HPLC are to be interpreted, it is necessary to realize that the free muramyl peptides contain a half-acetal hydroxy group and are therefore subject to mutarotation. Because the half-time of the mutarotation is one order of magnitude longer than the chromatographic process, it is possible to employ this technique for the separation of individual anomers in the pyranoid or furanoid forms (e.g., see Ježek, 1981, 1986).

Halls et al. (1980) studied the HPLC of MDP and some derivatives on a Spherisororb ODS (5 μm) column (25 cm x 3 cm I.D.) using 5 m*M* ammonium acetate (pH 2.5)-acetonitrile (199:1, v/v) as eluent and found that this approach allowed the separation of α- and β-anomers in 6 min. This time was short enough to allow (with fairly good accuracy) the study of the ratio of the two anomers and its variation with time as a result of mutarotation (which proceeds in a time measurable in hours). Ježek (1981) and Buděšínský et al. (1982) examined in detail the cyclization and mutarotation processes, which influence the separation of individual components of glycopeptides: double, triple or quadruple peaks of the same component were often found and the isolated individual split again into the original forms if there was enough time to reach equilibrium in the solution.

Ježek (1981) studied the synthesis of glycopeptides of bacterial cell walls. Synthetically prepared MDP contained four substances, which were preparatively separated on a 25 cm x 0.4 cm I.D. column of Separon Si C_{18} (10 μm) (Laboratory Instruments Works, Prague, Czechoslovakia), eluted with 0.01 *M* phosphate buffer (pH 5.0) containing 5% methanol at a flow-rate of 1.5 ml/min and with UV detection at 210 nm. Two pairs of anomers were obtained; the first two isomers were the main products and the other two were impurities. The fractions were identified as follows: 1 (k' = 4.7) = β-pyranoid isomer; 2 (k' = 8.5) = α-pyranoid;

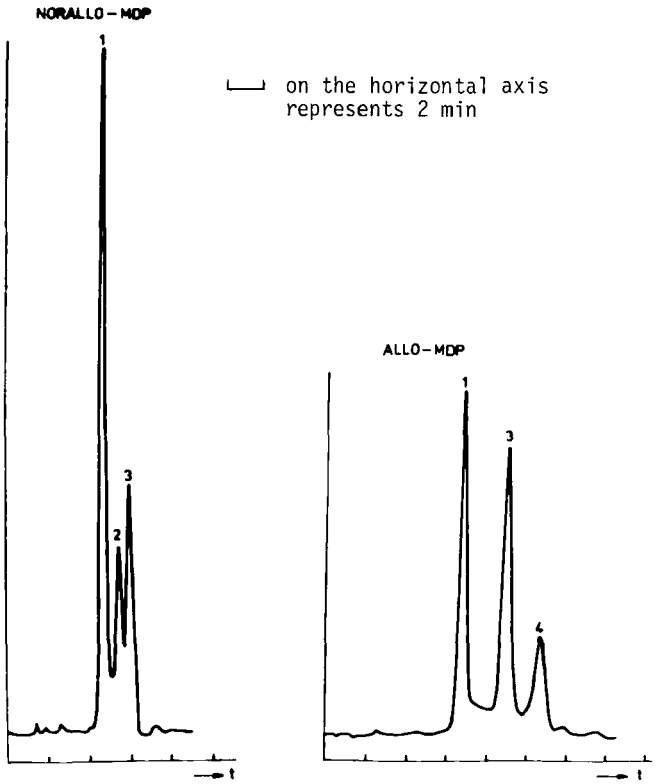


Fig. 12.5. Chromatography of synthetic glycopeptides by means of HP-RPC (for conditions, see text). Left: separation of nor-*allo*-MDP (i.e., N-Ac-Nor-*allo*-Mur-Ala-iGln); 1 = β-pyranoid isomer; 2 = α-furanoid form; 3 = α-pyranoid; 4 = β-furanoid (present in only a small amount; not numbered). Right: separation of *allo*-MDP (i.e., N-Ac-*allo*-Mur-Ala-D-iGln); 1 = β-pyranoid isomer; 2 = β-furanoid form (present in only a small amount in some experiments owing to rapid isomerization; not-numbered); 3 = α-pyranoid; 4 = α-furanoid. (Reprinted from Ježek, 1981; see also Budějínský et al., 1982.)

3 (k' = 9.8) = β-pyranoid, isomuramic isomer; and 4 (k' = 15.8) = α-pyranoid, isomuramic isomer. Owing to the mutarotation process, the freshly prepared solution of *allo*-MDP contained four peaks before equilibrium and three peaks after equilibrium had been achieved.

Similar experiments with other synthetic glycopeptides (N-Ac-galacto-Mur-L-Ala-D-iGln, N-Ac-nor-*allo*-Mur-L-Ala-D-iGln and N-Ac-*allo*-Mur-L-Ala-D-iGln) provided a mixture of at least three forms in each instance (cf., Fig. 12.5). All the isolated individual components 1, 2, 3 or 1, 3, 4 after standing at an appropriate pH yielded a mixture of components of the original composition, i.e., containing α- and β-anomers in the pyranoid and furanoid form.

Lefrancier and Lederer (1981) used HPLC of MDP on a Spherisorb ODS (5 μm) column eluted with $5 \cdot 10^{-3}$ *M* ammonium acetate (pH 2.5)-acetonitrile (995:5) and separated α- and β-anomers; this method was able to follow their mutarotation. Using a LiChrosorb-NH_2 column eluted with $5 \cdot 10^{-3}$ *M* ammonium acetate (pH 3)-acetonitrile (10:90) the two anomers of MDP could be separated from the two peaks of its diastereoisomer containing a D-Ala residue. Lebl and Gut (1983) used the data from HPLC of muramyldipeptide anomers [MDP was prepared by Zaoral et al. (1978)] for the calculation of the rate constant of the reversible anomerization reaction. They employed a Zorbax ODS column (DuPont, Wilmington, DE, U.S.A.) eluted with 0.1% trifluoroacetic acid-1% methanol at 34°C. For the determination of the rate constant with an isolated anomer, 0.5 mg of MDP was separated at a flow-rate of 1.5 ml/min. The time of elution of the anomer peak that was collected was taken as zero. The anomer solution was kept at 34°C and samples were taken at several time intervals and analysed at a flow-rate of 6.8 ml/min. The equilibrium constant of the reaction was determined at the same flow-rate with a sample of MDP dissolved in the mobile phase equilibrated at 34°C for 2 days. The rate constant for β- to α-anomer conversion was found to be $k_2 = 3.25 \cdot 10^{-2}$ min^{-1} or $3.45 \cdot 10^{-2}$ min^{-1} at 34°C. An advantage of the method is the need for only two chromatographic experiments for the determination and no strict requirements with regard to the purity of the studied compounds.

12.3.3 Examples of prepared immunomodulators

A series of papers were published on studies of the biological activity of synthetic subunits of *Streptococcus* peptidoglycan. Rotta et al. (1979) studied the pyrogenic and thrombocytolytic activity of several analogues and found that synthetic glycopeptides displayed both activities comparable to those of natural peptidoglycans. Rotta et al. (1983) examined the relationship between peptidoglycan subunits and series of analogues and the fever effect and induction of tolerance to pyrogenicity. The homogeneity of the tested peptides was checked by TLC, NMR, HPLC and amino acid analyses. Structure-function relationships were discussed. Pekárek et al. (1985) described a relationship between peptidoglycan subunit and analogue structures and adjuvant activity in cell-mediated immunity. Eight compounds (homogeneous according to TLC, paper electrophoresis and HPLC) were tested; the examined compounds were three desmuramyl peptides, MDP-polylysine, *allo*- and nor-*allo*-MDP, [Abu]-nor-MDP and MDP. Their structure-function relationships were discussed. Rotta et al. (1986) studied the pyrogenicity and hypersensitivity inducing activities of streptococcal peptidoglycan and its synthetic analogues. Ovalbumin and M25 *Streptococcus* extract were

used as antigens, and six glycopeptides (from glycodipeptide up to glycotridecapeptide) as immunoadjuvants. Rýc et al. (1986) described the effect of *Streptococcus* peptidoglycan and its synthetic analogues on rabbit blood cells. Four linear and one cross-linked desmuramylpeptide, four linear and one cross-linked muramylpeptide and twelve other analogues were investigated. Morphological and ultrastructural changes in platelets were followed. Pekárek et al. (1986) examined experimental allergic encephalomyelities (EAE) and the production of lymphokins in animals stimulated by *Streptococcus* peptidoglycan and synthetic analogues. Thirty peptides (both desmuramyl and muramyl derivatives, some of them cross-linked) were tested; ten showed immunoadjuvant activity and twenty were inactive.

Ivanov et al. (1981) studied the synthesis, structure and biological properties of glycopeptides containing the N-Ac-Glc-(β1→4)-N-Ac-Mur-disaccharide unit. The antitumour activity of eleven synthetic peptides and the adjuvant activity of eight synthetic peptides were tested. The energetically preferred conformation of the disaccharide dipeptide N-Ac-Glc-N-Ac-Mur-L-Ala-D-iGln (GMDP) was discussed. Ivanov (1986) described studies on bacterial glycopeptides (analogues of di- and tetrasaccharide muramylpeptides found in blastolysin, the antitumour preparation isolated from *Lactobacillus bulgaricus* cells). Nine synthetic glycopeptides were prepared and their immunomodulating properties were examined, both in antitumour activity and experimental bacterial infections.

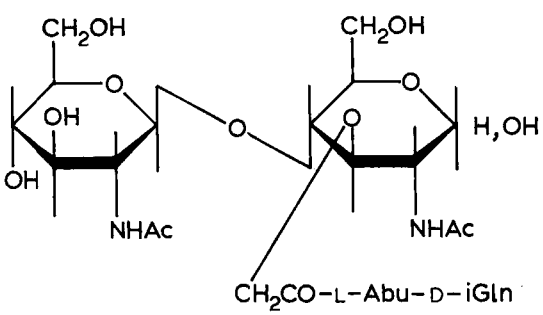
Zaoral et al. (1982) described the preparation and some biological properties of three MDP analogues and Krchňák et al. (1983) those of nor-Mur-N-Ac-α-aminobutyryl-D-iGln-Lys-Lys-Lys, which was prepared by solid-phase synthesis. Preparative HPLC was performed on a 25 cm x 2 cm I.D. column of Separon R (Laboratory Instrument Works, Prague, Czechoslovakia) using methanol-water-trifluoroacetic acid (5:95:0.2) for elution and UV detection at 210 nm. The compound forms a mixture of α- and β-anomers. Mašek and Flegl (1983) prepared an unusual type of hydrophobic desmuramyldipeptide, L-Ala-D-iGln-adamantylamide, which showed increased delayed hypersensitivity.

During the last 10 years, Kotani and co-workers have examined a series of immunoadjuvant glycopeptides and their application forms and activities [see, e.g., the paper by Kotani et al. (1977) in which the effect of administration of glycopeptides with liposomes was reported]. Kotani et al. (1986) also studied synthetic immunomodulators mimicking bacterial cell surface components. A description of the acyl derivatives of muramylpeptides and low toxic lipid A analogues as possible adjuvants for vaccines was presented. 6-O-(2-Tetradecylhexadecanoyl)-Mur-N-Ac-L-Ala-D-iGln (the so-called B 30 MDP), N^{α}-(Mur-N-Ac)-N^{α}-methyl-L-Ala-D-iGln-N^{ε}-stearoyl-L-Lys and Mur-N-Ac-L-Ala-D-Gln α-*n*-butyl ester

(Murabutide; cf., Lefrancier et al., 1982) were prepared and tested in various forms, and also in the form of virosome influenza vaccine. Masihi and Lange (1986) reported the stimulation of non-specific resistance against respiratory infections by immunomodulators. Eleven MDP analogues were found to be effective (when combined with 6,6'-trehalose dimycolate) against influenza virus or *Mycobacterium tuberculosis* infection.

Ježek et al. (1986, 1987) continued the synthesis of peptides and glycopeptides of bacterial cell walls. By means of solid-phase synthesis and synthesis in solution, 21 tetra- to octapeptides and 4 glycopeptides [N-acetylmuramylhexapeptide to tris(N-acetylmuramyl)octadecapeptide] were prepared and tested for immunoadjuvant activity and pyrogenicity. For the preparative separation of glycopeptides (30-50 mg) a 250 mm x 9 mm I.D. column of 10-μm Partisil ODS-2 (Whatman, Clifton, NJ, U.S.A.) was used, eluted with a gradient system of 0.1% trifluoroacetic acid solution (or 0.05 *M* NaH_2PO_4-0.05 *M* H_3PO_4) and methanol, the methanol concentration being increased at 1%/min. For analytical checking of purity, a Spherisorb ODS (5 μm) column (250 mm x 4.6 mm I.D.) (Phase Separations, Queensferry, U.K.) was applied, using the same mobile phases. Two of the prepared peptides were pyrogenic. Tris(N-acetylmuramyl)octapeptide showed immunoadjuvant activity comparable to MDP and was not pyrogenic.

Farkaš et al. (1986, 1987) described the synthesis of a new disaccharide analogue of MDP, O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-N-acetyl-normuramoyl-L-aminobutyryl-D-isoglutamine:



Some intermediates were purified by preparative column chromatography on silica gel (30-60 μm) using acetone-chloroform (1:9, v/v), chloroform-ethyl acetate (2:1, v/v), ethyl acetate-toluene (2:1, v/v) or chloroform-methanol (50:1 or 25:1, v/v), and checked by RPC on C_{18} silica gel (10-15 μm) in methanol-water (3:1 or 97:3, v/v) or 60% methanol. The final product was chromatographed

by RP-HPLC on a 25 cm x 0.4 cm I.D. column of C_{18} silica (Laboratory Instrument Works, Prague, Czechoslovakia) using water-methanol (97:3, v/v) for elution. The immunoadjuvant activity of the final compound was determined in guinea pigs by the delayed-type hypersensitivity assay using ovalbumin as antigen. The disaccharide, when applied with Freund's incomplete adjuvant, produced a 3.4-fold higher immunoadjuvant activity than MDP in control experiments. The undesirable pyrogenic effect of the product, unlike that in MDP, was very low (cf., Rotta et al., 1986).

12.3.4 Synthetic vaccines

12.3.4.1 Introduction

Not only peptidoglycan macromolecules on the bacterial cell surface but also protein macromolecules do not need to be in the intact macromolecular form to be able to elicit the immune response in the attacked macroorganism. Fragments of proteins are sufficient to act as antigens. Antigenic determinants (so-called protective epitopes) are relatively low-molecular-weight segments of antigens and therefore the possibility of preparing synthetic vaccines is opened up if the antigenic determinants of the natural antigens are reliably determined (Shinnic et al., 1983; Arnon, 1984). Many studies on the identification of the epitopes have been published. The antigenic determinants are believed to be present in the accessible hydrophilic areas on the surface of protein antigens, which can be found by theoretical prediction.

Hopp and Woods (1981) published a method for the prediction of antigenic determinants from amino acid sequences, assigning a hydrophilicity value for each amino acid, repetitively averaging these values along the chain (a table of hydrophilicity values of amino acids was published). Kyte and Doolitle (1982) presented a simple method for displaying the hydropathic character of proteins; a computer program was written that evaluated the hydrophilicity and hydrophobicity of proteins along the chain. However, the choice of peptides to be synthesized is not always straightforward (Van Regenmortel, 1986), as many antigenic determinants of proteins seem to be conformational (depending on the three-dimensional folding of the native molecule) rather than sequentional (conditioned only by the amino acid sequence of the isolated peptide segment, not influenced by the total structure of the protein). Different strategies have been developed for searching for the appropriate antigenic sites (Atasi, 1984; Weijer et al., 1986). Also the atomic mobility sites in the three-dimensional X-ray structure of proteins (Tainer et al., 1984) and their segmental mobility (Westhoff et al., 1984) were correlated with the location of antigenic determinants and their reactivity.

In addition to theoretical methods, experimental techniques have also been proposed for searching for determinants. Jemmerson and Paterson (1986b) presented a method for mapping epitopes on a protein antigen by partial proteolysis of the antigen-antibody complexes, in which chromatography played an important role. An antibody bound to a protein antigen showed a steric hindrance effect and decreased the rate of proteolytic cleavage of the antigen in regions involved in the antibody-antigen contact. RP-HPLC of the partial digest has made it possible to identify the contact area according to the relative amount (peak heights) and composition of the isolated peptides of the antigen, provided that the primary structure of the antigen was known and that a protease digest of the single antibody and the single antigen were chromatographed in preliminary experiments in order to localize their peptide peaks in the chromatogram. Cytochrome *c* and monoclonal antibody against it were used in these experiments. A 30-min tryptic digestion was found to be optimal. For HPLC a 250 mm x 4.6 mm I.D. column of C_{18} Spherisorb ODS-1 (Custom LC, Houston, TX, U.S.A.) was eluted with a linear gradient from 0 to 70% acetonitrile (0.1% TFA) during 90 min at a flow-rate of 1 ml/min. UV detection at 214 nm was applied. Collected peaks of the antigen were hydrolysed and analysed in an amino acid analyser. The applicability of this method was discussed in detail. Another, but very expensive and time-consuming, experimental method for determining the contact area between the antibody and antigen is X-ray crystallography of the antibody-antigen complex (Amit et al., 1985), from which the epitopes could be derived. This method is very precise but it cannot be generally used.

The theoretically proposed or practically found antigen determinants are the starting point for the peptide synthesis (Shinnick et al., 1983; Walter, 1986). However, so far the purified peptides corresponding to protective epitopes often possess poor immunogenicity, "at least partly because of the removal of components carrying adjuvant activity of the original vaccines (Kotani et al., 1986); hence the development of new types of vaccines requires studies of chemically well defined immunoadjuvants, which effectively potentiate the immunogenicity of protective epitopes". Also, the effect of additivity or synergism caused by a large number of determinants on the same natural macromolecular antigen contributes to the stronger antigenicity of natural antigens in comparison with single synthetic peptides.

The chromatography of synthetically prepared peptides for vaccination does not differ from the separation techniques for any other simple peptides, which were described in detail in Chapter 11, and therefore in the following part of this section only a few examples of synthetic vaccines will be presented.

12.3.4.2 Examples of efforts to prepare synthetic vaccines

First, synthetic vaccines against bacterial infections will be considered. Synthetic streptococcal M-protein vaccines were chosen because of their great medical importance. The usual vaccination against streptococcal infections with killed streptococci (or with a crude extract) is not possible owing to serious complications with rheumatic fever (Massel et al., 1969). The only known factor of the virulence and antigenicity of *Streptococcus pyogenes* is the M-protein, emanating as fibrils from the surface of streptococcal cells (Fox, 1974). The single-chain protein molecule is present as a dimer and has the form of an α-helical coiled-coil structure (Phillips et al., 1981). The isolated M-protein is an unsuitable antigen for vaccination owing to immune cross-reactions leading to acute rheumatic fever (Dale and Beachey, 1985). It has become apparent that the whole M-protein molecule is not required to evoke protective immunity and that various extracts containing only polypeptide fragments of M-protein retain type-specific protective immunogenicity [for references see Beachey et al. (1986) and Kühnemund et al. (1986)]. A solution to the vaccination problem might be seen in the preparation of synthetic peptide vaccines (as short as possible) that could retain antigenic determinants for the production of protective antibodies, but which would not contain segments responsible for the toxic cross-reactions (Beachey and Stollerman, 1971). Therefore, experiments with further cleavage of the fragments continued and yielded peptides containing as few as twelve amino acid residues that retained protective epitopes (Beachey et al., 1981).

These problems were discussed in detail by Beachey et al. (1986), who carried out experiments on the immune responses in rabbits immunized with synthetic streptococcal M-protein peptides (M5 and M6, containing 20 amino acid residues from the N-terminus of M-protein, and M24, containing two 35-residue peptides) conjugated to polylysine or tetanus toxoid. The location and synthesis of protective epitopes of type 5 streptococcal M-protein were studied by Seyer et al. (1986). Six synthetic peptides were prepared from M5 region 1-35 and tested. The segment SM5 (1-35) was immunogenic in rabbits and evoked opsonic antibodies against type 5 streptococci, that did not negatively cross-react with human tissues. All of the protective epitopes resided between amino acid residues 14 and 26. Kühnemund et al. (1986) studied types 1 and 12 of group A streptococci. The M1 and M12 proteins were prepared using phage-associated lysin (PAL) extraction and affinity chromatography. The maps of tryptic digests of samples of the two proteins were compared by two-dimensional TLC peptide mapping and nine common spots were found. A mild pepsin extraction to isolate M1 protein led to a protein fragment of M_r = 20 000, which (following affinity chromatography on immobilized fibrinogen) was identified as the N-terminal part of M-protein. Morávek et al.

(1986) further studied this purified fragment of M1 protein, which represents the exposed area of the molecule on the surface fibrils of streptococcal cells, which seems to be very important for differentiation of the individual serological types. The sequence of 39 amino acid residues was determined and the homology with the N-terminus of M5, M6 and M24 proteins was discussed, and also with the internal homology in repeated areas of M24 protein. All the mentioned results provide a great impulse to efforts for the preparation of synthetic vaccines.

In the second part of this section, synthetic antiviral vaccines (Brown, 1984) will be mentioned and the examples will be focused on influenza viruses. Synthetic vaccines against influenza viruses, which are of great public-health importance, are a great challenge for organic chemists synthesizing peptides. Their efforts are directed to the preparation of segments mimicking viral surface proteins which elicit the production of antibodies in the attacked macroorganism. There are two surface glycoproteins in influenza virus, haemagglutinin and neuramidinase. The first protein is the major viral surface antigen and will be dealt with later (cf., Müller et al., 1982; Shapira et al., 1984; Wabuke-Bunoti, 1984a,b; Hamšíková et al., 1986, 1987). It is strain-specific and can be seen by electron microscopy to form spikes protruding from the spherical lipid envelope in the form of a trimer; the monomer consists of two polypeptide chains denoted HA-1 and HA-2. A concise chronological review of viral antigen examinations will illustrate the research efforts that have been made and reasons for the selection of particular peptide sequences for experiments.

As early as in 1964 Laver reported structural studies on the protein subunits from three strains of influenza virus. Brand and Skehel (1972) described crystalline antigen from the influenza virus envelope. Jackson et al. (1979) studied the antigenic determinant of influenza virus in detail and described the immunogenicity of fragments isolated from haemagglutinin of A/Memphis/72 virus. The cyanogen bromide cleavage fragment (designated CN-1), prepared from the heavy-chain subunit HA-1, was responsible for immunogenic activity, but it was too large for synthesis because it contained 170 amino acid residues. Wilson et al. (1981) presented the three-dimensional structure of the haemagglutinin membrane glycoprotein determined by X-ray crystallography at 3 Å resolution. Wiley et al. (1981) identified the structures of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in the antigenic variations. Ward (1981) reviewed the structure of the influenza virus haemagglutinin; it was apparent that the fragment Ser 91 to Leu 108 of CN-1 (from HA-1) contained two Pro and three Tyr residues (important for antigenicity) and a folder corner in the X-ray structure; this was suggesting because there was a chance that this

area might be hopeful for synthetic efforts. Müller et al. (1982) reported an anti-influenza response achieved by immunization with a synthetic conjugate containing the mentioned amino acid sequence. Atassi and Kurisaki (1984) published a novel approach to the location of the continuous protein antigenic sites by comprehensive synthetic surface scanning: antibody and T-cell activity to several influenza haemagglutinin synthetic sites. Arnon and Shapira (1984) dealt with anti-influenza synthetic vaccines and reported that at least twelve H3N2 influenza virus strains contained the above-mentioned sequence Ser 91 to Leu 108, so that it is very common in influenza viruses. Synthetic peptides corresponding to the antigenic site of haemagglutinin of various length were described by Shapira et al. (1984), and Wabuke-Bunoti et al. (1984a,b) studied cytolytic T-lymphocytes in relation to antibody responses to synthetic peptides of influenza virus haemagglutinin. Shapira et al. (1985) described a synthetic vaccine against influenza with built-in adjuvant (HA fragment 91-108 was conjugated to tetanus toxoid and then to MDP-Lys) and reported protective activity in mice immunized with such a system.

Bláha et al. (1986) synthesized by a solid-phase method haemagglutinin HA-2 fragments Ser 91 to Leu 108 (corresponding to H3N2-type haemagglutinin) in which Cys 97 was replaced with Ala 97 (peptide I) or Met 97 (peptide II), and HA-2 (1-13) (peptide III), and HA-1 (185-200) (peptide IV). Peptide I was identical with the Müller et al.'s (1982) peptide. For the purification of I, II and IV a column of Partisil ODS-2 or Separon Si C_{18} was used, eluted with a gradient 30-80% of methanol-water (+0.1% trifluoroacetic acid). All peptides were checked by elemental and amino acid analysis, Edman degradation and peptide mapping and behaved as homogeneous in TLC and analytical HPLC. With the exception of III the peptides were conjugated to tetanus toxoid and mixed with Freund's adjuvant. Hamšíková et al. (1986, 1987) studied the immunogenic properties of the above-mentioned synthetic peptides I and II. The induced antibodies reacted with synthetic peptides (coupled to bovine serum albumin) and with the haemagglutinins of the homotypic H3 viruses, but not with the haemagglutinins of the heterotypic influenza A viruses (when using purified virions as antigens, marked cross-reactivity was observed). The petide II conjugates appeared to be a more potent immunogen then I (probably owing to the presence of Met). None of the antibodies raised against the peptide conjugates displayed any activity in the haemagglutinin-inhibition neutralization or complement-fixation tests. In addition, in mice immunized with the same preparation, intranasally administrated H3N2 virus replicated to the same extent as in the control animals; these findings did not agree with the observation of Shapira et al. (1985). The data obtained suggest that the region corresponding to Ser 91-Leu 108 is not immunogenic under conditions of natural infection or in comparison with immunization with more complex

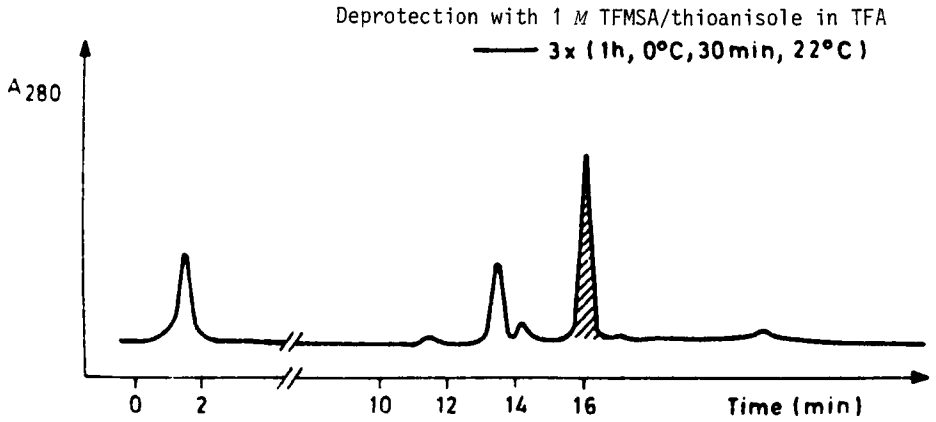


Fig. 12.6. HPLC of deprotected [Tyr]^{161}-VP$_1$(141-161) peptide preparation. For chromatographic conditions, see text. TSMFA = trifluoromethanesulphonic acid. (Reprinted from Dölling et al., 1986.)

virus material. It can be seen from this short history that the preparation of an effective synthetic vaccine will not be easy.

Dölling et al. (1986) described the synthesis of peptide sequences belonging to foot-and-mouth disease virus (FMDV) using a biphasic solvent system. The major immunogenic region of FMDV is located on the viral capsid protein VP$_1$ in the amino acid sequence 141-160 (Pfaff et al., 1982) with the accent on the Leu residues 148 and 151 of the immunogenic epitope 146-152 (Geysen et al., 1984). So far several attempts to prepare peptides from this region have been published. For references, see Dölling et al. (1986), who used a special solution synthesis for the preparation of VP$_1$ fragment 141-160, C-terminally extended by a tyrosine residue Tyr^{161} in order to allow specific coupling to proteins. The detailed strategy was discussed. For analyses of deprotected peptides a 5-µm Vydac C$_4$ (300 Å) column was used (cf., Fig. 12.6), eluted with a gradient of 2%/min of B in 0-40 min at a flow-rate of 1 ml/min; solutions: A = 0.08% TFA in water - *n*-propanol (9:1); B = 0.05% TFA in water - *n*-propanol (1:1).

The Tyr^{161} peptide was linked with human serum albumin using bis-diazotized benzidine. In preliminary experiments, the peptide showed good reactivity with an antiserum directed toward the complete virus, but immunization with the albumin-conjugate (peptide to protein ratio = 8:1) gave no virus neutralizing antibodies.

Krchňák and Malý (1986) used modern technology to prepare and test synthetic antigen fragments in which the isolation of the real intact-protein antigen was not necessary. The principle of this approach can be illustrated by the following steps: (1) determination of the DNA sequence expressing the antigen; (2) selection of the corresponding protein fragment(s) based on theoretical translation;

(3) synthetsis of the selected peptide(s); (4) preparation of hapten-carrier conjugate; (5) immunization of experimental animals; (6) titration of the antibody; (7) isolation of the peptide-specific antibody (may be omitted); and (8) study of the interaction of the antibody with the protein. Krchňák and Malý (1986) used this method to study the antigenic properties of oncoprotein of avian myeloblastosis virus (AMV). AMV is a member of the avian retroviruses, which are deffective for replication and cause leukaemia. The AMV oncogens are expressed in the transformed cell in the form of subgenomic spliced mRNA (which is also present in the virion) and the product of its translation is a protein of M_r = 48 000 (Boyle et al., 1983; Klempnauer et al., 1983; Malý and Krchňák, 1984).

Krchňák and Malý (1986) started from the published nucleotide sequence of the transforming gene (Rushlow et al., 1982) and selected the fragment 92-110, expected to be the most hopeful. The nonadecapeptide was synthesized by the Merrifield solid-phase method. The product was purified on Bio-Gel P-4 and by HPLC. The homogeneous preparation was coupled to keyhole limpet haemocyanin as the carrier, emulsified in complete Freund's adjuvant and administered intracutaneously to rabbits. High titres were obtained. The detailed immunological assays were discussed.

Mach et al. (1986) studied antibodies against a synthetic decapeptide precipitating phosphokinase (measured by enzymic activity) expressed by src gene, which had been originally detected in Rous sarcoma virus. Oncogenes (i.e., genes responsible for the induction and maintenance of the transformed state of a cell) are intensively studied, because they give rise to tumours not only in model systems, but even in humans. The detection of protein products expressed by pathological genes permits their easier investigation and specific antibodies can be simply used for this purpose.

Owing to difficulties in the preparation of a potent specific antibody against the products of src gene, new ways were sought and fragments of phosphokinase (M_r = 60 000) were believed to be useful for the synthesis of antigens. Because the amino acid sequence of $pp60^{src}$ was known, Mach et al. selected a surface hydrophilic domain, which could be characterized by a decapeptide containing a Tyr residue. This decapeptide was prepared by solid-phase synthesis and purified to 99% purity by HPLC. The peptide was coupled to serum albumin as a carrier and used for immunization of rabbits using Freund's complete adjuvant. All the immunized rabbits produced antibodies against the synthetic peptide in titres from 1:5000 to 1:10 000. The antibody against the synthetic peptide reacted even with native protein $pp60^{src}$. The amino acid sequence of the decapeptide used was compared with sequences of four other published heptapeptides (corresponding to different regions of the protein) eliciting antibodies which reacted with native $pp60^{src}$.

12.3.5 Comments on literature

First the literature relating to glycopeptide adjuvants will be summarized. Kotani et al. (1982) dealt with the non-specific and antigen-specific stimulation of host defence mechanism by liophilic derivatives of muramyl dipeptides, Leclerc et al. (1983) reviewed the potential use of synthetic muramyl peptides as immunoregulating molecules, Straka (1983) the preparation of fragments of bacterial cell wall peptidoglycan and analogues (in Czech; 280 references), Adam and Lederer (1984) muramyl peptides, immunomodulators, sleep factors and vitamins and Takada and Kotani (1985) immunopharmaceutical activities of synthetic muramyl peptides. Ježek (1986) (in Czech, 234 references) dealt with glycopeptides of bacterial cell walls from the synthetic point of view. Lederer (1986) presented an opening lecture, "Muramyl Peptides and Their Use in Vaccines", at a Symposium and its expanded version contained 133 references. Books on this theme have also been published by Friedman et al. (1981) on immunomodulation by bacteria and by Adam (1985) on synthetic adjuvants, and Zaoral et al. (1986) edited Symposium Proceedings on synthetic immunomodulators and vaccines.

Secondly, a survey of the literature relating to the problem of the prediction of peptide antigenic determinants will be given, because of its importance with regard to synthetic approaches. Hopp and Woods (1981) described the prediction of protein antigenic determinants from amino acid sequences, and Kyte and Doolitle (1982) a simple method for displaying the hydropathic character of proteins. Sutcliffe et al. (1983) dealt with antibodies that reacted with predetermined sites on proteins, and Berzofsky (1985) intrinsic and extrinsic factors in protein antigenic structure. Tainer et al. (1985) reviewed the atomic mobility component of protein antigenicity, and Jemmerson and Paterson (1986a) the mapping of antigenic sites on proteins with implications for the design of synthetic vaccines.

Thirdly, several reviews on synthetic vaccines will be summarized. Shinnick et al. (1983) dealt with synthetic peptide immunogens as vaccines, Benjamin et al. (1984) have written a reappraisal of the antigenic structure of proteins, Arnon (1984) reviewed synthetic vaccines, Brown (1984) presented a specialized review on synthetic antiviral vaccines and Walter (1986) commented the production and use of antibodies against synthetic peptides. Rowland (1986) reviewed the synthetic antigen approach to the production of vaccines and a new generation of antiviral vaccines was discussed by Bittle (1986).

12.4 MISCELLANEOUS GLYCOPEPTIDES

12.4.1 Viral glycopeptides

Hollaway et al. (1979) described a procedure for the rapid separation of Rauscher murine leukaemia virus type I and II glycopeptides, which consisted of one to three amino acid residues and a large oligosaccharide chain. The desalted glycosylated components obtained from radiolabelled virions or from gp70 viral glycoprotein were dissolved in solvent A, i.e., in 0.1% acetic acid (pH 3.2) or 0.1% phosphoric acid (pH 2.85) and subjected to IP-RPC using a μBondapak C_{18} column (30 cm x 4 mm I.D.) and brief isocratic elution, followed by a linear gradient up to 30% acetonitrile (solvent B) at ambient temperature and a flow-rate of 2 ml/min. The [^{3}H]glucosamine or [^{3}H]mannose employed for labelling were detected by measurement of their decompositions per minute. The isolated components could be re-chromatographed using a similar procedure. The utilization of an ion-pair technique eliminated the necessity for derivatization of the primary amino group and hence the subsequent analysis of the sample was simple.

Basak and Compans (1981a) dealt with variations of glycosylation sites in H_1N_1 strains of influenza virus. The glycosylated sites of haemagglutinin glycoprotein of series of H_1N_1 strains (after pronase digestion) were compared by GPC of glycopeptides on a 115 cm x 1 cm I.D. Bio-Gel P-6 column, and also (after tryptic digestion) by mapping using RP-HPLC on a Waters μBondapak C_{18} column, eluted with a 0.1% phosphoric acid-*n*-propanol (70:30) gradient. HPLC was found to be effective for the detection of changes in glycosylation sites. Basak and Compans (1981b) published an improved separation of glycosylated tryptic peptides of haemagglutinin (HA) glycoprotein of influenza virus. For the RPC a Waters μBondapak C_{18} column (30 cm x 3.9 mm I.D.) was eluted using a gradient from 0.1% phosphoric acid (solvent A) to either acetonitrile (containing 0.1% of phosphoric acid)-0.1% phosphoric acid (40:60) or *n*-propanol (containing 0.1% of phosphoric acid)-0.1% phosphoric acid (30:70) at room temperature and a flow-rate of 2 ml/min; the gradient time was 150 min. Radiolabelled fractions were mixed with Scintiverse (Fisher Scientific) and counted in a Beckman scintillation counter. Excellent separation was achieved with a recovery of 90-95%. When reduction and alkylation were carried out before tryptic digestion of HA glycoprotein, eight tryptic peptide classes originating from HA glycoprotein of the A/USSS-R/90/77 virus strain were perfectly resolved.

Kemp et al. (1981) described the IP-RPC of viral tryptic glycoproteins from influenza A/WSN(H_0N_1) and mink cell focus (MCF)-inducing (MCF-247) murine leukaemia virus. The chromatography was developed for mapping purposes. Hydrophilic ion pairing was accomplished using 0.1% phosphoric acid on μBondapak and

LiChrosorb RP-18 ODS columns. Solvent A was 0.1% phosphoric acid (pH 2.85) and solvent B was 60% acetonitrile in solvent A. A brief isocratic elution (10 min) was followed by a linear gradient starting from 0% acetonitrile to 40% acetonitrile at a flow-rate of 2 ml/min, or starting from 12.5% acetonitrile to the final concentration of 35% at a flow-rate of 1 ml/min. The glycopeptides originating from [^{3}H]mannose-labelled cells and the radioactivity of 1-min fractions were determined by liquid scintillation countings. The chromatogram of the total HA tryptic glycopeptides was compared with those of HA_1 and HA_2 tryptic glycopeptides, prepared from haemagglutinin previously separated into HA_1 and HA_2 glycoproteins, which differed in electrophoresis. The recovery of glycopeptides was in the range 70-80%.

12.4.2 Glycopeptides indicating lysosomal storage disorders

Calatroni and Tira (1976) reported the isolation of acidic glycopeptides by means of an anion-exchange resin (Dowex 1) and the application of this method to some cases of glycosphingolipidosis or mucolipidosis.

Ng Ying-Kin and Wolfe (1980) described the HPLC of oligosaccharides and glycopeptides accumulating in lysosomal storage disorders. These types of compounds are excreted in the urine of a number of lysosomal storage disorders. The diseases studied were mannosidosis, fucosidosis, G_{M1}-gangliosidosis, G_{M2}-gangliosidosis variant O, sialidosis, and aspartylglucosaminuria. A table was published giving the structures of oligosaccharides and glycopeptides accumulating in tissues and excreted in the urine of patients suffering from the above diseases. A 30 cm x 4 mm I.D. μBondapak/carbohydrate column was isocratically eluted with acetonitrile-1% acetic acid (60:40, v/v) for sialic acid-free glycopeptides and acetonitrile-0.1 *M* sodium acetate-acetic acid buffer (pH 5.6) (54:45, v/v) for sialyl oligosaccharides and glycopeptides. Refractive index measurement was used for monitoring. A large detector response in the first 3 min represented the low-molecular-weight material present in the urine. The chromatographic separation could be completed in 20 min.

Lou et al. (1980) reported a split-stream cation-exchange chromatographic method for isolating glycopeptides from biological fluids, using Aminex A-7 in a carbohydrate analyser and citrate buffers for elution. Both ninhydrin and orcinol reaction patterns could be obtained.

12.5 MISCELLANEOUS POLYMERIC AND OLIGOMERIC SUBSTANCES

12.5.1 Complex carbohydrates

The chromatography of polysaccharide-protein complexes was reviewed by Juřicová and Deyl (1975); glycosaminoglycans (mucopolysaccharides), glycoproteins and glycopeptides were dealt with. The separation of dolichylpyrophosphoryl oligosaccharides by LC was studied by Wells et al. (1981). Fourteen compounds of this type (precursors of the asparagine-linked oligosaccharides in proteins) were separated on base-treated (Smith and Lester, 1974) silicic acid Porasil A-60 (37-75 μm) (Waters Assoc., Milford, MA, U.S.A.), packed into six 100 cm x 0.32 cm I.D. columns linked in series. In some instances a 3.5 cm x 0.32 cm I.D. pre-column was used. Up to 500 μl of sample dissolved in chloroform-methanol-water (10:10:3) or in chloroform-methanol-concentrated ammonia solution-water (16:16:1:4) was applied to the column equilibrated with the initial solvent, which was then pumped for 5 min prior to the gradient. The flow-rate was 2.0 ml/min and pressures of 1000 and 2000 p.s.i. were applied.

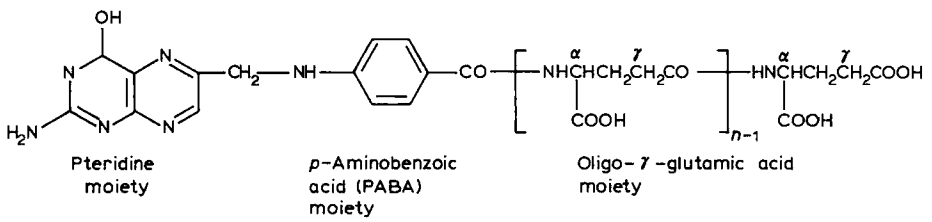
For the separation of dolichylpyrophosphoryl-N,N'-diacetylchitobiose, a programmed non-linear gradient was used, formed by mixing solvent A (chloroform-methanol-concentrated ammonia solution, 65:29:6) and solvent B (chloroform-methanol-concentrated ammonia solution-water, 43:43:6:8). The total gradient time was 60 min, at which the solvent composition was 65% A - 35% B, and this mobile phase was pumped isocratically for a further 30 min. The gradient defined the composition at t min representing a percentage of solvent B of $35(t/60)^{1/3}$. Separation of more complicated dolichylpyrophosphoryl oligosaccharides was carried out with a programmed non-linear gradient formed with solvent A and solvent C (chloroform-methanol-concentrated ammonia solution-water, 40:42:6:12). The gradient was run for 120 min from 0 to 100% solvent C, followed by pumping solvent C for 75 min isocratically. In this instance the solvent composition during the gradient at t min was represented by a percentage of solvent C of $100(t/120)^{1/2}$. The complex oligosaccharides thus resolved retained their function as substrates in enzyme-catalysed reactions.

The chromatographic procedures described for the first time made available many of these single intermediates for further study. Wells et al. (1982) reviewed the resolution of dolichylpyrophosphoryl oligosaccharides by HPLC.

Roughley and Mort (1985) described the resolution of cartilage proteoglycan and its proteolytic degradation products by HPLC.

12.5.2 Pteroyl oligoglutamates

Pteroyl oligo-γ-L-glutamates ($PtGlu_n$) are folic acid (FA) derivatives and have the general formula



(for the parent FA n= 1 and for Pt-oligoglutamates n= 2-9), and have important biological functions as coenzymes essential in the synthesis of proteins and nucleic acids (Baugh and Drumendieck, 1971) and other functions. Because the FA derivatives are acids or oligoacids, IEC on microparticulate bonded phases was used for their rapid separation first (Reed and Archer, 1976; Stout et al., 1976). Naturally, the retention of $PtGlu_n$ in anion-exchange chromatography depends on the number of carboxyl groups, being highest with species containing most Glu residues.

Bush et al. (1979) studied the retention behaviour of $PtGlu_n$ in RPC on an ODS-silica column and found this method to be very suitable for good resolution and rapid analysis. When the carboxylic groups are largely undissociated (at pH 2), the retention of $PtGlu_n$ increases with the number of Glu residues and the elution order parallels that in IEC. At sufficiently high pH (e.g., at pH 4.5) the carboxilic groups are dissociated and the elution order is reversed (cf., Fig. 12.7). The logarithm of the capacity factor is linearly dependent (with the exception of FA) on the number of Glu residues over a wide range of eluent pH (cf., Fig. 4 in the original paper). As can be seen from Fig. 12.8, at pH 6 (at which the carboxyl groups are almost completely dissociated) the retention decreases with increasing number of Glu residues, so that both methods (IEC and RPC) complement each other. In general, the efficiency of IEC with bonded phases appears to be higher than that of RPC at low pH on ODS with the same particle size and column dimensions. IEC is recommended when the elution

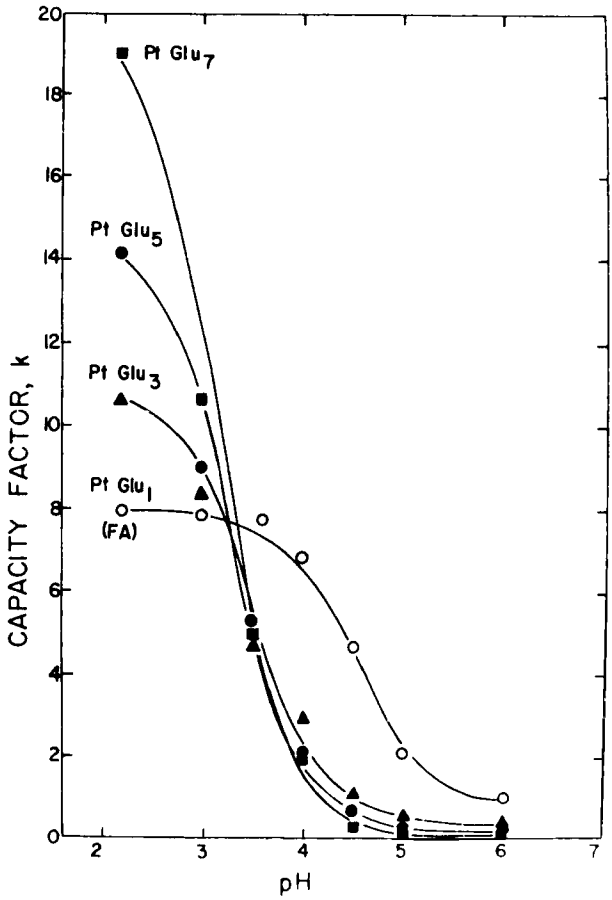


Fig. 12.7. Capacity factors of pteroyl-γ-glutamates as a function of the eluent pH in RPC. Column, 5-μm Partisil ODS-2; eluent, 0.1 *M* phosphate buffer containing 6% (v/v) acetonitrile; temperature, 45°C. (Reprinted from Bush et al., 1979.)

order of increasing numbers of Glu residues is desired. RPC is eminently suitable for separations with a reversed elution order, as at high eluent pH adequate resolution can be obtained by isocratic or (with more complex mixtures) gradient elution.

Cashmore et al. (1980) reviewed the separation of $PtGlu_n$ by HPLC. Both RPC and IEC methods were described in detail and compared. The conditions for the IEC of $PtGlu_n$ and PABA-oligo-γ-L-glutamates were as follows: a 25 cm x 4.6 mm I.D. column of 10-μm Partisil 10 SAX (Whatman, Clifton, NJ, U.S.A.), eluted with a linear gradient from 0.01 to 1.0 *M* sodium phosphate (pH 3.3); temperature, 50°C; inlet pressure at the start, 300 lb/in^2; flow-rate, 0.8 ml/min; sample, 3-10 μg of each component in 0.1 *M* phosphate buffer (pH 3.0); detection, UV (280 nm); and time required, 50 min.

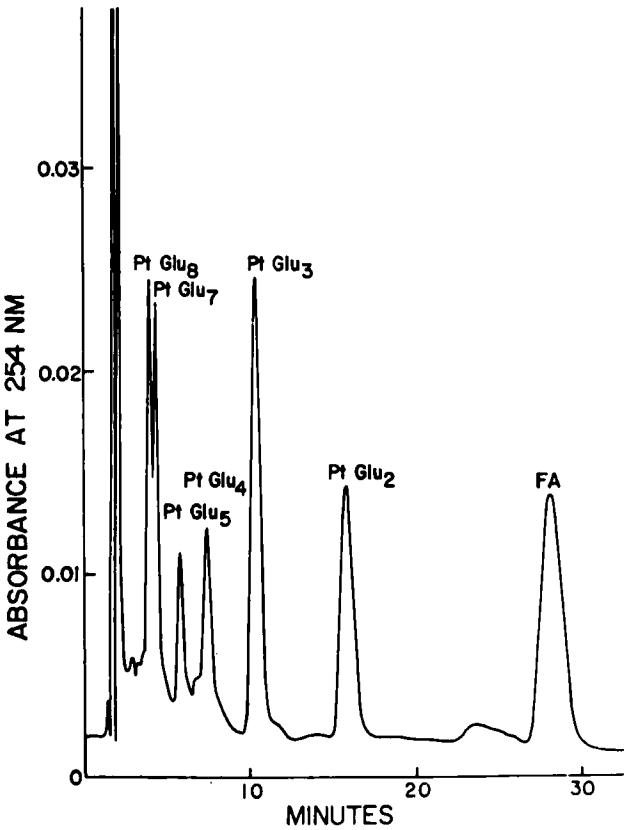


Fig. 12.8. RPC of pteroyl-γ-glutamates ($PtGlu_n$) obtained by isocratic elution at pH 6. Column, Spherisorb ODS (250 mm x 4.6 mm I.D.); eluent, 0.1 *M* phosphate buffer (pH 6) containing 1% (v/v) acetonitrile; flow-rate, 1.5 ml/min; inlet pressure, 11.42 MN/m^2; temperature, 45°C; sample size, ca. 10 µg of each component. FA = Folic acid. (Reprinted from Bush et al., 1979.)

Eto and Krumdieck (1982) studied the determination of three different pools of reduced one-carbon substituted folates and described the RP-HPLC of the azo dye derivatives of *p*-aminobenzoyl poly-γ-glutamates ($AzoGlu_n$) and its application to the study of unlabelled endogenous $PtGlu_n$ of rat liver. Picomole amounts of the $AzoGlu_n$ could be separated and quantitated. Azo dye derivatives were prepared from the corresponding PABA-γ-glutamates (containing 1-7 Glu residues) by a Braton-Marshall procedure slightly modified by Eto and Krumdieck (1980) and were purified by Bio-Gel P-2 polyacrylamide gel chromatography.

All the analyses were performed on a 25 cm x 0.46 cm I.D. column packed with 5-µm Spherisorb S5 ODS (carbon load approximately 7%) (Applied Science Division, Milton Roy, Philadelphia, PA, U.S.A.). Before sample application the column was

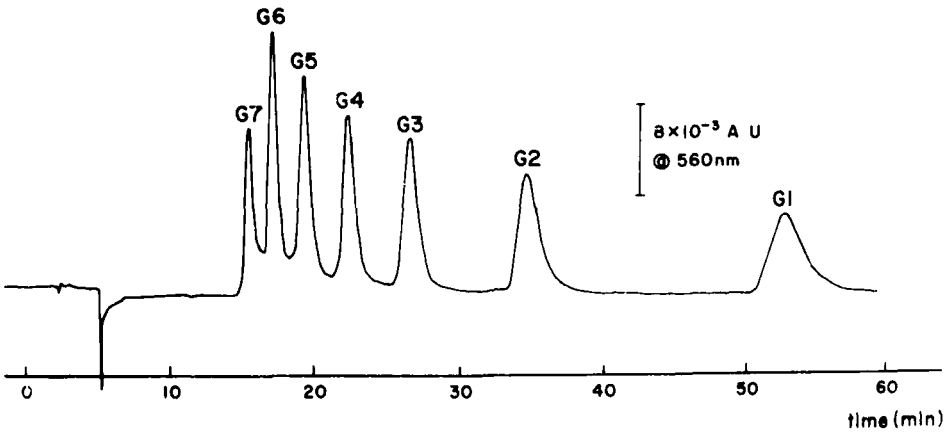


Fig. 12.9. Separation of synthetic azo-*p*-aminobenzoyl poly-γ-glutamates ($AzoGlu_n$) by RP-HPLC. For conditions, see text. G1-G7 indicate the number of glutamyl residues. (Reprinted from Eto and Krumdieck, 1982.)

routinely washed with 30 ml of solvent B (1-propanol-acetic acid-water, 10:7.5:82.5) followed by 30 ml of solvent A (acetic acid-water, 7.5:92.5). The lyophilized $AzoGlu_n$ were dissolved in 1.0 ml of solvent A and 20-70 µl were injected. Immediately thereafter the concentration of 1-propanol in the mobile phase was increased to 10% (v/v) within 3 min, switching from solvent A to B employing a steep convex gradient. Isocratic elution followed at a flow-rate of 1 ml/min. UV detection at 560 nm was applied. The resolution was completed within 1 h. In the case of pulse labelling with radioactive FA (carried out by injecting rats intraperitoneally with 1.0 ml of a solution of 0.24 µg of [^{3}H]FA (25 µCi) in physiologic saline), the radioactivity of the isolated products was monitored with a Beckman LS-250 liquid scintillation spectrometer using Scinti-Verse (Fisher Scientific) scintillation fluid. An example of the HPLC elution pattern is illustrated in Fig. 12.9.

12.5.3 Oligomers containing nucleobases

Fiedler (1981a) described the quantitation of nikkomycins in biological fluids by IP-RPC. These antibiotics are produced by *Streptomyces tendae* and are interesting from an agricultural point of view because they possess high and selective antifungal and insecticidal activity (they interfere in the biosynthesis of chitin cell walls). Nikkomycins are nucleoside-peptide antibiotics, similar to polyoxins. Using a 120 mm x 4.6 mm I.D. column packed with 5-µm LiChrosorb RP-8, equipped with a 40 mm x 4.6 mm I.D. pre-column (Knauer, West

Berlin, F.R.G.), Fiedler was able to determine five nikkomycin components in the filtrate of the fermentation broth in which they were cultured.

Both types of elution were used for effective separation. For isocratic elution the mobile phase employed contained 80 m*M* ammonium formate buffer (pH 4.7) and 1 m*M* heptanesulphonic acid. The flow-rate was 1 ml/min and the pressure was 100 bar. For gradient elution, solvent A was 30 m*M* ammonium formate (pH 3.75) containing 1 m*M* heptanesulphonic acid and solvent B was methanol containing 30 m*M* ammonium formate (pH 3.75) and 1 m*M* heptanesulphonic acid. The flow-rate was 1 ml/min. UV detection at 290 nm was applied. A 10-µl volume of clear supernatant of the fermentation broth was injected. When using gradient elution, only a short equilibration time under initial conditions was necessary to achieve reproducible results. In isocratic elution the column had to be equilibrated for 3 days after the regeneration with methanol in order to obtain reproducible results; about 300 injections could be performed before regeneration of the column and when using a pre-column about 3000 injections could be effected (however, it was necessary to change the pre-column after 500 injections). The advantages of HPLC of nikkomycins were obvious.

Van Haastert (1981) studied the HPLC of nucleobases, nucleosides and nucleotides, and in this cited second paper of the series the mobile phase composition for the separation of charged solutes by IEC was described. He examined the application of a cation exchanger (Partisil 10 SCX) and an anion exchanger (Partisil 10 SAX) and concluded that these ion exchangers also had reversed-phase and normal-phase properties; their occurrence was determined by the polarity of the mobile phase. In addition to various tested compounds, S-adenoxyl-L-methionine (SAM) was chromatographed on a 250 mm x 4.6 mm I.D. column of Partisil 10 SCX, using as the mobile phase 1.1 *M* ammonium acetate (pH 5.6) and a flow-rate of 2 ml/min. SAM was separated into two peaks (stereoisomers at the sulphonium S^+ atom), SAM I = (-)-S-adenysyl-L-methionine and SAM II = (+)-S-adenosyl-L-methionine. The two peaks were interconvertible by heating. The twin peaks appeared only if reversed-phase and cation-exchange properties of the support were present simultaneously, i.e., in the mixed-mode chromatography process. An increase in buffer concentration or the addition of propanol shifted the peak of SAM in the opposite direction.

Yip and Albarella (1985) described the use of HPLC in the preparation of flavin adenine dinucleotide conjugates, and Ehrat et al. (1985), in the context of research on neonucleoproteins, described the preparation and HPLC characterization of succinyl-lysozyme-diaminooctylpolycytidylic acid and realted polycytidylic acid conjugates.

12.5.4 Miscellaneous substances

Species of complex glycosides, reviewed by Courtois and Percheron (1970), also belong to biooligomers, and examples of their separation and detection will be mentioned in this section. Erni and Frei (1977) described a comparison of reversed-phase and partition or adsorption HPLC of some digitalis glycosides. Digitalis glycosides of the cardenolide groups are important drugs for the treatment of heart diseases. The authors attempted to find a simple isocratic system for a good separation with sufficient reproducibility. RPC could offer some advantages with regard to sample preparation of pharmaceutical formulations. Digitalis glycosides of the C series contain (on the hydroxy group in position 3 of the steroid skeleton) chains of repeated digitose or acetyldigitose residues, sometimes bound to glucose. A chromatogram of this group of compounds is illustrated in Fig. 12.10. The detection limits for digitalis glycosides in the above-mentioned paper varied between 10 and 100 ng per injection and permitted the analysis of by-products even in low-dosage pharmaceutical formulations. The reproducibility for repetitive chromatograms was about 1% (relative standard deviation); significantly better results were obtained with automatic injections.

In Nibbering's (1982) review on ionization methods with emphasis on liquid chromatography-mass spectrometry, an analysis of oligomeric purpureaglycoside A (M_r = 926) was mentioned in addition to other glycosides, according to papers by Bruins (1980a,b).

Saito and Hayano (1979) examined the application of HP-GPC to humic substances in marine sediments. Humic and fulvic acids were extracted with 0.1 *M* $Na_4P_2O_7$-0.1 *M* NaOH solution from the marine sediment obtained from Sagami Bay (Japan) at a depth of 88 m and 11 km from the shore. The humic acids were preliminarily purified and injected (in volumes of 0.025-0.1 ml) into a TSK Gel G 3000 SW column (60 cm x 0.75 cm I.D.). Pure water and 0.1 *M* NaCl were used for elution at flow-rates of 0.5 and 1.0 ml/min. The pressure for a 1.0 ml/min flow-rate was 30 atm. UV detection at 254 nm was applied. The void volume (V_0) and the total effective column volume ($V_0 + V_i$) were determined using Blue Dextran 2000 and acetone, respectively. In water the coulombic repulsion forces shifted the chromatographed compounds to the void volume, but in 0.1 *M* NaCl a normal chromatogram was obtained containing the eluted compounds between V_0 and $V_0 + V_i$. Part of the humic acid was of the higher molecular weight type and required a more porous support.

Melander et al. (1979) studied mobile phase effects in RPC and described changes in the conformation and retention of oligo(ethylene glycol) derivatives with temperature and eluent composition. Zorbax ODS and LiChrosorb RP-8 columns

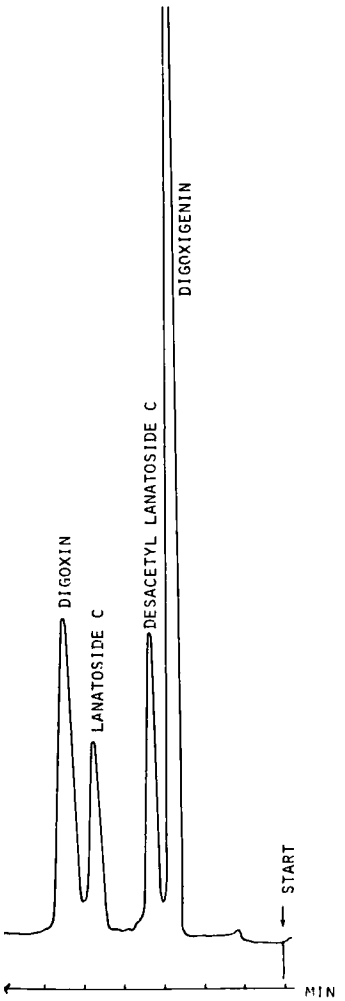


Fig. 12.10. Separation of the C series of digitalis glycosides by RPC. A 30 cm x 3.5 mm I.D. column of 10-µm Nucleosil C_{18} (Machery, Nagel & Co., DÜren, F.R.G.) was eluted with a 40% solution of acetonitrile-dioxan (1:1) in water; flow-rate, 1.3 ml/min; pressure, 140 bar; injection, 25-µl loop; detection, 220 nm. (Reprinted from Erni and Frei, 1977.)

were used. The results contributed to the verification of various theoretical approaches to chromatography and could serve as a model for studying the chromatographic behaviour of biological substances such as peptides and oligonucleotides.

Fiedler (1981b) described the preparative-scale HPLC of ferricrocin, a microbial product. The cyclic hexapeptide (containing three acetylated N^{δ}-hydroxy-

ornithine residues, capable of binding one Fe^{3+} ion in a complex bond) is a metabolic product of *Aspergillus viridi-mutans* and was isolated from the fermentation broth by means of XAD-2 adsorption. It is a member of a broad and interesting family of natural hydroxamates (growth factors and antibiotics) (cf., review by Mikeš and Turková, 1964) and close to the structure of the antibiotic albomycin (cf., Turková et al. 1964, 1965) or grisein (Turková et al., 1966), which contains a pyrimidine moiety in addition to cyclic hexapeptide with three hydroxamate residues and one iron atom in a complex bond.

Ferricrocin could be purified to a high degree of homogeneity by means of RPC on a C_8 support. Columns 250 mm x 16 or 4.6 mm I.D. packed with 7-µm LiChrosorb RP-8 eluted with water-acetonitrile (9:1) at a linear flow-rate of 12 cm/min and ambient temperature were used for separation. UV detection at 270 nm was applied. The capacity of the separation was reached with a sample weight of 20 mg per gram of stationary phase.

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