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A METHOD FOR THE DETERMINATION OF THE CHEMICAL STRUCTURE OF GLYCOLIPIDS BIOSYNTHESIZED *in vitro* AND THE SPECIFICITY AND ACTIVITY OF GLYCOSYLTRANSFERASES*

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ABSTRACT

A very sensitive method for the qualitative and quantitative investigation of the biosynthesis of the oligosaccharide chains of glycolipids in such tissues as cultured cells, biopsies, or cell organelles, is described. It permits the determination, on an ultramicro-scale, of the specificity of glycosyltransferases in terms of the exact chemical structure of the products that are synthesized from known labeled glycolipid precursors, and defines without ambiguity the precursor-to-product relationship in biosynthetic incubations *in vitro*.

INTRODUCTION

In the course of the past 15 years or so, accumulated evidence has indicated that glycolipids and glycoproteins, components of the plasma membrane of cells, are involved through their carbohydrate moieties in the control of cell social behavior, recognition, differentiation, and malignant transformation. Glycolipids have also been shown to act as specific receptors for toxins, viruses, and hormones. The mechanisms of these biological phenomena, as well as the mechanism by which oncogenic agents interfere with the metabolism of glycolipids and glycoconjugates in general, in transformed and cancer cells, are still largely unknown[2–5].

Since small changes in the chemical structure of glycolipids result in marked changes in their biological properties, particularly immunological properties and susceptibility to enzymes, it is of primordial importance to determine their precise chemical structure, *i.e.*, the nature and the sequence of the carbohydrate components, and the position and anomeric configuration of the linkages between these components in order to gain an understanding of the role and function of glycoconjugates in terms of

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precise molecular mechanisms. It is also necessary to establish the specificity of the glycosyltransferases that catalyze the sequential steps of addition of monosaccharide units to form the oligosaccharide chains in glycolipids, and to detect any changes, not only in the activity but also in the specificity of these enzymes, that may correspond to differentiation or transformation.

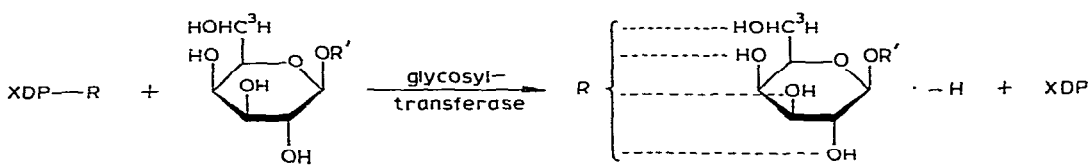
Concurrent with the increasing interest in studies of the role and function of glycolipids, and of glycoconjugates in general, extensive progress has been made toward the development of new techniques for the determination of the chemical structure of these substances. However, in studies of the biosynthetic capability of tissues that are available in limited quantity, such as cultured cells, the amounts of products formed in incubations *in vitro* are extremely small. Thus, existing methods are not sensitive enough to determine the chemical structure of these products, and consequently to establish the specificity of the glycosyltransferases that catalyze the biosynthesis, taking into consideration the structure of the linkages formed. The methods here described have been developed in order to fulfill this need.

DISCUSSION

Determination of the chemical structure of glycolipids biosynthesized in vitro. — The method consists of three essential steps:

(*a*) *Incubation.* Homogenates or subcellular fractions of the tissue under study are used as enzyme source in incubations with precursor glycolipids of known structure and labeled with radioactive elements specifically in the glycosyl unit that acts as the receptor (**2**) of the nonlabeled glycosyl residue from the donor sugar nucleotide (**1**). This is in contrast with previous studies in which unlabeled acceptor glycolipid and labeled sugar nucleotides were used.

(*b*) *Permethylation.* Because of the sensitivity of the methods of detection with radioactive elements, it is possible not only to identify the radioactive product(s) (**3**), but also to determine the position of attachment of the new residue (R) to the radioactively labeled receptor glycosyl unit of the acceptor glycolipid. Therefore, the product glycolipid, purified by t.l.c. and detected by autoradiography, is permethylated and hydrolyzed. The only radioactive compound in the hydrolyzate is the derivative of the receptor sugar that is methylated in all positions, except the one substituted by the glycosyl residue (R) from the sugar nucleotide. This methylated radioactive compound (in the example given, tri-*O*-methyl-D-galactose) is then identified by comparison on chromatograms with authentic methylated derivatives of the acceptor sugar.



1 2 3

(*c*) *Degradation of the product with a specific glycosidase.* The degree of certainty of the results obtained by this method is further increased by a reversal of the procedure. Part of the product glycolipid is hydrolyzed with the specific glycosidase that is capable of splitting off the newly attached glycosyl unit originating from the donor sugar nucleotide. This establishes the anomeric configuration of the new linkage, and, moreover, the resulting radioactive glycolipid fragment is compared by t.l.c. with the original precursor in order to demonstrate that no change has occurred in the precursor moiety during the incubation. The "regenerated" precursor glycolipid is permethylated and, after hydrolysis, the permethylated derivative of the terminal sugar residue (in the example given, 2,3,4,6-tetra-*O*-methyl-D-galactose) is characterized by comparison with standards. As a result of these experiments, it is possible to demonstrate unequivocally that the radioactive material that is obtained by permethylation of the product glycolipid and that migrates like a trimethyl derivative of the terminal sugar residue of the precursor is indeed this derivative. Consequently, the structure of the product glycolipid may be clearly established.

EXPERIMENTAL

Procedure. — The procedure involves the following steps:

(*a*) *Preparation of radioactive precursors.* Glycolipids with terminal D-galactopyranosyl or 2-acetamido-2-deoxy-D-galactopyranosyl residues were labeled specifically in these residues by oxidation with D-galactose oxidase and subsequent reduction with sodium borotritide of high specific activity[6,7]. The success of these operations, which were carried out in buffered tetrahydrofuran (oxolane) solution, required the use of tetrahydrofuran freshly distilled over calcium hydride. In our experience, tetrahydrofuran forms compounds (probably peroxides) that interfere with these reactions (especially the reduction), even when the distilled solvent is kept over calcium hydride. These compounds were detected as black spots on thin-layer chromatograms sprayed with sulfuric acid and heated.

Purification of the radioactive glycolipid was accomplished by repeated preparative t.l.c. in different solvent systems. A convenient streaking sample-applicator has been described[8]. After each run, the material that was detected by autoradiography and migrated like a standard of the expected product, *i.e.*, the original glycolipid, was recovered from the t.l.c. plate. The silica gel was collected on a fritted-glass filter, and the glycolipid was eluted with a large volume of a suitable solvent, usually chloroform–methanol–water.

When the oxidation was carried out on 10-mg samples of glycolipid with ~2,000 units of D-galactose oxidase (A. B. Kabi, Stockholm, Sweden) in two additions, and the oxidized material was subsequently reduced with sodium borotritide of high specific activity (0.1 Ci), the purified radioactive product had a specific activity of ~0.2–0.3 Ci/mmol. Proof of the selective labeling of the terminal D-galactose (or 2-amino-2-deoxy-D-galactose) residue was obtained in the following two steps: (i) Autoradiography of chromatograms of a total hydrolyzate revealed only radio-

actively labeled D-galactose or 2-amino-2-deoxy-D-galactose respectively. (ii) Another portion of the radioactive glycolipid was permethylated and then hydrolyzed. In this case, only 2,3,4,6-tetra-*O*-methyl-D-galactose (or 2-amino-2-deoxy-3,4,6-tri-*O*-methyl-D-galactose) was detected by autoradiography of the chromatograms. In our experiments, when this labeling procedure was applied to glycolipids containing internal D-galactopyranosyl residues in addition to the terminal one, no radioactive tri-O-methyl-D-galactose was detected, thus indicating that the internal residues were not affected by D-galactose oxidase.

Glucosylceramide labeled in the glucose unit may be prepared according to McMaster and Radin[9]. Glycolipids that are not amenable to the D-galactose oxidase–sodium borotritide treatment, or which would be too difficult to obtain by chemical synthesis, may be prepared by biosynthesis *in vitro* from the appropriate precursor and a sugar nucleotide labeled with carbon-14. This applies, for example, to lactotriaosylceramide which has a 2-acetamido-2-deoxy-D-glucose terminal residue. Hematoside labeled in the D-galactose residue may be prepared by biosynthesis, in a yield of ~15%, from lactosylceramide labeled with tritium in the D-galactose residue and nonradioactive CMPNeuNAc by use of young rat brain as a source of sialosyltransferase.

(*b*) *Incubations.* The conditions for incubations of tissue homogenates or subcellular fractions with radioactive glycolipid acceptor and unlabeled sugar nucleotide are similar to those described earlier by several authors. An amount of 10–100 mg of wet cells is generally sufficient to produce enough product for the subsequent establishment of its structure.

(*c*) *Purification of the glycolipid product.* After the incubation, the reaction was stopped by addition of 2:1 (v/v) chloroform–methanol in an amount sufficient to obtain a mixture containing 5% of water. This solution was passed through a column containing Sephadex G-25 Superfine pre-equilibrated with the same solvent mixture[10]. Short columns (10–30 mm diameter) containing 5–20 g of Sephadex were found effective in removing the nucleotides. Elution of the radioactive glycolipid was complete with ~40–120 mL of solvent. The eluate was evaporated to dryness, and the residue, dissolved in a small amount of 2:1 (v/v) chloroform–methanol, was applied at the starting line of preparative thin-layer chromatograms. The unchanged precursor and radioactive products were separated and purified by t.l.c. in suitable solvent systems. Standards of the precursor and of expected product(s) were used for preliminary identification. The radioactive bands located by autoradiography were scraped from the plate, and the lipids were eluted with chloroform–methanol–water from the silica collected on fritted glass. The purity of the eluted products was determined by rechromatography of an aliquot. In these chromatographic comparisons, it was necessary to apply the radioactive unknown compound in admixture with the nonradioactive standard. Indeed, comparison by running the unknown and the standard compounds side by side is often misleading, because the unknown compounds may still contain detergent or impurities that alter their migration rate. Because the radioactive unknown compounds are detected by autoradiography and

the standards by spraying with a color-developing reagent, *e.g.*, anthrone–sulfuric acid, both can be detected separately when cochromatographed. In this way, the standard and the unknown compounds were chromatographed under exactly identical conditions, and very small differences in migration were detectable, even when the radioactive spot partially overlapped that of the standard compound.

(*d*) *Permethylation.* Methylation was carried out on a very small scale by the method of Hakomori[11]. To the radioactive glycolipid recovered from the thin-layer chromatogram and dried in a short test tube, dimethyl sulfoxide (0.1–0.5 mL) was added, and then, a solution of dimethylsulfinyl carbanion (0.1–0.5 mL), prepared by dissolving sodium hydride in oil (250 mg) in dimethyl sulfoxide (10 mL) for 30 min at 70° under nitrogen and with magnetic stirring. (It is not necessary to eliminate the oil.) After 15 min at room temperature, methyl iodide (0.05–0.1 mL) was added, and the mixture was kept for another 15 min. Then, the content of the tube, held in a slanted position, was frozen and the dimethyl sulfoxide was lyophilized (it may melt, but nevertheless it evaporates in a few hours under vacuum). The dry residue was taken up in water (5 ml), and the lipid was extracted with chloroform (six 10-mL portions). The combined extract was diluted with methanol (0.5 vol.) and passed through a column containing Sephadex G-25 Superfine (5 g) previously equilibrated in 40:20:3 (v/v) chloroform–methanol–water. Elution was continued until no more radioactivity was found in the effluent. The eluate was evaporated at low temperature under vacuum, and a small amount of ethanol was added toward the end in order to facilitate evaporation of the water. The residue was transferred with small portions of 2:1 (v/v) chloroform–methanol to a borosilicate glass tube (7 mm diam. × 20 cm) sealed at one end. Standard methylated sugars, which serve also as carrier, were added (~30 μg each), and the solvent was evaporated under a stream of nitrogen, and then under vacuum. Evaporation under a stream of nitrogen had to be carried out at 25–30° maximum and the flow of gas stopped as soon as the solvent had evaporated, as methylated sugars are appreciably volatile under these conditions. Final drying under vacuum did not result in significant losses.

A 15% solution of dry hydrogen chloride in methanol (0.5 ml) was added, and then overlayered with pure methanol (1 mL). The tube, cooled in Dry Ice, was sealed, and methanolysis was carried out for 24 h at 100°. The tube, cooled again in Dry Ice, was opened, and the solvent was evaporated under a stream of nitrogen, at first without warming in order to avoid boiling over due to the presence of low-boiling methyl chloride, then at 30°, and finally under vacuum. The residue was hydrolyzed in the same tube with M sulfuric acid (0.5 ml) for 24 h at 100°. The solution was de-ionized by passing through a column of Dowex 50 (H^+, 0.5 ml, 100–200 mesh) surmounted with Dowex 1 (AcO^-, 2 ml) ion-exchange resins. The methylated sugars were eluted with 1:4 (v/v) methanol–water (10 mL). The solvent was evaporated under vacuum in a rotary evaporator at 30° maximum, and the residue, dissolved in a small amount of methanol, was applied at the start of a t.l.c. plate (Silica gel G). The chromatogram was developed with an appropriate solvent. In the case of *O*-methyl-D-galactoses, 500:9 (v/v) acetone–5M ammonia was suitable. Autoradiography was carried out

on X-ray film. If the radioactive label was tritium*, intensification with PPO was needed[12]. The plate was sprayed with aniline phthalate or anthrone–sulfuric acid in order to detect the internal and separate standards.

Determination of the activity of glycosyltransferases. — When radioactive-labeled glycolipid precursors and nonradioactive sugar nucleotides were used in biosynthetic incubations, instead of the opposite conventional way, it became clear that the radioactive-labeled products formed were derived from the known exogenous precursor, and not from endogenous acceptors that may be present in the enzyme preparation. By measuring the yield of conversion of the precursor to the product(s), this straightforward precursor-to-product relationship allowed a precise estimation of the activity of the glycosyltransferase acting on the known exogenous precursor. Thus, direct conversion of the precursor glycolipid into the product was observed and measured, instead of stimulation of incorporation resulting from addition of a nonradioactive precursor.

Control experiments were performed in the following ways:

(*a*) Incubations were carried out with the same amount of enzyme preparation, without adding an exogenous glycolipid precursor, but adding a ^{14}C-labeled sugar nucleotide. As a result, ^{14}C-labeled products derived only from endogenous acceptors were detected by the autoradiography of chromatograms.

(*b*) Incubations were carried out with labeled, exogenous glycolipid precursors but without the addition of a sugar nucleotide. In this case, the possible biosynthesis of products by linking the sugar component from endogenous sugar nucleotides to the exogenous glycolipid could be detected.

(*c*) When a glycolipid, obtained as the product of a previous biosynthetic experiment, and thus labeled in the penultimate sugar residue, was reincubated with the enzyme preparation that was used for its biosynthesis, it was possible to detect the products resulting from the action of glycosidases. Thus, the extent of the degradation may be taken into account as a correction factor in the estimation of the activity of glycosyltransferases.

(*d*) "Double-label" incubations were performed with, for example, a glycolipid precursor labeled with tritium, and a sugar nucleotide labeled with carbon-14 in the sugar component. Autoradiography of thin-layer chromatograms of the lipid extract of such incubations revealed: (i) The unchanged precursor labeled only with tritium; (ii) the products of incorporation of the ^{14}C-labeled sugar into endogenous acceptors, these products being labeled with carbon-14 only; and (iii) the doubly labeled products resulting from the combination of the exogenous precursor with the sugar nucleotide.

The isotope or isotopes in each radioactive band observed on the autoradiogram were identified by differential counting of the radioactivity of the silica scraped from the thin-layer chromatogram and suspended in a gelling scintillation fluid. A

**Note added in proof.* — Increased sensitivity in autoradiography of tritium-labelled compounds is obtained with LKB Ultrofilm (LKB Produkter AB, Sweden). No PPO is necessary, and the radioactive products can be recovered.

quantitative determination of the various products was thereby possible. As a result of these experiments, a precise overall-view of the course of the enzymic reactions involving exogenous as well as endogenous substrates was obtained. The double-label experiments were also useful for the identification of products of incubations that give more than one product from the glycolipid precursor. Indeed, comparison of the ratio of carbon-14 (from the sugar nucleotide) to tritium (from the glycolipid receptor) in the different, doubly labeled products indicates the relative number of sugar residues that have been linked to one molecule of the glycolipid acceptor.

CONCLUSIONS

The method described presents the following advantages:

(a) It detects specifically the position of the newly formed linkage between the *known* glycolipid acceptor and the glycosyl residue from the *known* donor sugar nucleotide. A very clear interpretation of the result is thereby possible.

(*b*) It unequivocally demonstrates the structure of glycolipids formed in amounts of the order of 20 pmol.

(*c*) When applied in a sequential fashion (*i.e.*, by use of a series of precursor glycolipids, from the simplest monohexosylceramides to the more complex polyhexosylceramides), it constitutes an analysis of the enzymic potential of the tissue under study, and establishes the pathway of biosynthesis of glycolipids in the particular biological material used as enzyme source.

(*d*) It establishes the specificity of the glycosyltransferases that catalyze each step in terms of the structures of the compounds formed as well as substrates, because the precise structure of the compounds formed is established at each step of the biosynthesis.

(*e*) It is based on radioactive products that are derived from known exogenous radioactive precursors and, in control experiments, it shows the participation of endogenous glycolipids as acceptors or the possible degradation by glycosidases.

(*f*) By use of glycolipids labeled with radioactive elements, it allows competitive inhibition experiments between different possible glycolipid acceptors of a particular sugar nucleotide in a way that clearly indicates the precursor-to-product relationship. Therefore, incubations are carried out in which one glycolipid acceptor is radioactively labeled, whereas neither the competitor glycolipid, added in various amounts, nor the sugar nucleotide present in saturating amounts is labeled. For confirmation, the experiment is repeated, but in reversing the labeling between the competing glycolipid acceptors. In this way, the radioactive product that is formed is marked as a direct derivative of the known radioactive exogenous acceptor, and the effect of varying amounts of the nonradioactive competitor on the yield of a defined product can be measured. The participation of endogenous glycolipids present in the enzyme preparation, and which may compete with the exogenous acceptor, can be made apparent and estimated in control experiments carried out as just described.

Altogether, the methodology described here provides a precise approach that

adds a new dimension to the study of the biosynthetic capability of tissues, *i.e.*, the unambiguous determination of the chemical structure of the products formed *in vitro*, and thereby the establishing of the activity and specificity of glycosyltransferases, not only with respect to their substrate, but also in terms of the structure of the product formed. The fundamental importance of the precise determination of the chemical structure of products biosynthesized *in vitro* becomes apparent when, in three cases that were previously investigated[13–16], the products formed *in vitro* were found to be chemically different from those expected on the basis of previous structural studies on similar natural products.

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