METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

Cancer Biology Research Laboratory
Stanford University Medical Center
Stanford, California

VOLUME 32

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METHODS OF BIOCHEMICAL ANALYSIS

Volume 32

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned not only with the results in the developing fields but also with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, applier, the teacher, and the student.

It is particularly important that review services of this nature should include the area of methods and techniques because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore, an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, and so on.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate, these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural

details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analysis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will be always welcome.

DAVID GLICK

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METHODS OF BIOCHEMICAL ANALYSIS

Volume 32

Activator Proteins for Lysosomal Glycolipid Hydrolysis

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I. INTRODUCTION

In 1964 Mehl and Jatzkewitz (1964) observed that after fractionation by free-flow electrophoresis a crude preparation of porcine arylsulfatase A had lost its ability to hydrolyze sulfatide (sulfogalactosylceramide) in the absence of detergents but remained fully active toward the water-

soluble artificial substrates. Addition of another fraction, which was enzymically inactive, to the assay restored the activity against the glycolipid. This effect could later be attributed to a small, acidic glycoprotein. A similar cofactor that promotes sulfatide catabolism was then shown to exist also in normal human liver as well as in tissues from patients with metachromatic leukodystrophy (Jatzkewitz and Stinshoff, 1973) and was designated as the "activator of cerebroside sulfatase." Although the name may be somewhat misleading because the cofactor does not "activate" the enzyme, such protein cofactors for the degradation of lipid substrates by lysosomal hydrolases are now generally referred to as "activator proteins."

The activator of cerebroside sulfatase (or "sulfatide activator") was purified by Fischer and Jatzkewitz (1975) from human liver and identified as a water-soluble glycoprotein with an isoelectric point at pH 4.3 and a molecular weight of approximately 22,000 Daltons. From kinetic and binding experiments and from the fact that this cofactor stimulated only the degradation of lipid substrates but not of artificial water-soluble ones, these authors concluded that the cofactor serves to solubilize the lipid by binding to it and extracting it from the membrane (or micelle). The resulting activator/lipid complex was assumed to be the true substrate of the enzymic reaction (Fischer and Jatzkewitz, 1977, 1978).

Li and coworkers (Li, Y.-T., et al., 1973; Li, S.-C., and Li, Y.-T., 1976) found that the enzymic hydrolysis of gangliosides G_{M1} and G_{M2} by β-galactosidase and β-hexosaminidase A, respectively, and of globotriaosylceramide by α-galactosidase A also requires the presence of low molecular weight protein cofactors. However, the protein cofactor they isolated was later shown to mediate the degradation of globotriaosylceramide and ganglioside G_{M1} but not of ganglioside G_{M2} and recently turned out to be identical with the sulfatide activator (Inui et al., 1983; Li, S.-C., et al., 1985).

The hydrolysis of ganglioside G_{M2} and related glycosphingolipids (e.g., glycolipid G_{A2}) by β-hexosaminidase A depends on the presence of another activator protein, the "G_{M2} activator" (Conzelmann and Sandhoff, 1978, 1979; Conzelmann et al., 1982), which has similar properties as the sulfatide activator but differs in glycolipid and enzyme specificity. The two proteins are encoded on different chromosomes (G_{M2} activator on chromosome 5 [Burg et al., 1985a], sulfatide activator on chromosome 10 [Inui et al., 1985]) and do not cross-react immunochemically (Li, S.-C., et al., 1979). The G_{M2} activator is also a glycoprotein, with a molecular weight of approximately 22,000 Daltons (Conzelmann et al., 1982; Burg et al., 1985b). On subcellular fractionation it codistributes with the lysosomal marker enzyme β-hexosaminidase

(Banerjee et al., 1984) and, like most other lysosomal proteins studied so far, is synthesized as a form with a slightly higher molecular weight (~ 24,000 Daltons [Burg et al., 1985b]), which is probably the precursor of the mature form.

Studies on the mechanism by which the G_{M2} activator promotes the enzymic degradation of lipid substrates (Conzelmann and Sandhoff, 1979; Conzelmann et al., 1982) demonstrated that this activator binds to ganglioside G_{M2} and solubilizes it as a water-soluble complex with a molar ratio of one glycolipid molecule per protein. Other related glycolipids are also bound but much less strongly. This complex formation is reversible, and hence the G_{M2} activator acts, at least in vitro, also as a glycolipid transfer protein (Conzelmann et al., 1982). Hexosaminidase A does not interact with the membrane-bound ganglioside. Instead, the G_{M2} activator protein binds to the ganglioside G_{M2} and extracts it from the membrane to form a water-soluble complex. This complex then recognizes a specific binding site on the alpha subunit of β-hexosaminidase A (Kytzia and Sandhoff, 1985) and binds in such a way that the terminal *N*-acetylgalactosamine moiety is correctly positioned for the enzymic hydrolysis of the glycosidic bond. The resulting ganglioside G_{M3}/activator complex dissociates then and the activator is set free for another round of catalysis.

The physiological significance of this kind of activator protein is demonstrated by two fatal lipid storage diseases that are caused by deficiencies of activator proteins rather than of the degrading enzymes: variant AB of infantile G_{M2} gangliosidosis results from a defect of the G_{M2} activator protein (Conzelmann and Sandhoff, 1978; Hechtman et al., 1982; Hirabayashi et al., 1983), whereas a deficiency of the sulfatide/G_{M1} activator leads to an atypical variant of juvenile metachromatic leukodystrophy (Stevens et al., 1981; Inui et al., 1983).

The specific interaction between β-hexosaminidase A and the G_{M2} activator has also been taken into account in the development of assay systems for ganglioside G_{M2} hydrolysis by extracts of cultured skin fibroblasts (Erzberger et al., 1980; Conzelmann et al., 1983). With such assays the residual activities in cells from patients with different variants of G_{M2} gangliosidosis could be determined very precisely (Conzelmann et al., 1983).

A basically different type of protein cofactor for lysosomal glycolipid hydrolases was described by Ho and O'Brien (1971) for acid β-glucosidase (glucosylceramidase). This factor accelerates the hydrolysis of glucosylceramide as well as of water-soluble artificial substrates by lysosomal β-glucosidase. It also seems to have an activating effect on acid sphingomyelinase and on galactosylceramide β-galactosidase (Christo-

manou, 1980; Wenger, et al., 1982). Unlike the activator proteins described earlier, this cofactor does not bind to the substrate (Berent and Radin, 1981a) but activates the enzyme itself. However, some controversy still persists as to the nature and the physiological relevance of this type of protein cofactor: the preparations isolated by various groups from different sources differ widely in specific activity (Peters et al., 1977a; Iyer et al., 1983), molecular weight (Ho and O'Brien, 1971; Peters et al., 1977a,b; Wenger and Roth, 1982), and extent and necessity of glycosylation (Ho and O'Brien, 1971; Peters et al., 1977a; Berent and Radin, 1981b).

Recently it was also found that when the natural substrate, glucosylceramide, is incorporated into phospholipid bilayers, together with a certain amount of acidic phospholipids such as phosphatidic acid, a high activity of glucosylceramidase is attained without the need for any activating protein cofactor (Sarmientos et al., 1986).

A new type of glucosylceramidase cofactor has recently been described by Vaccaro et al. (1985). This protein, which seems to be tightly associated with the enzyme, stimulates the hydrolysis of glucosylceramide but not that of the water-soluble substrate. Similarly two small proteins that stimulate specifically lysosomal sphingomyelinase were recently observed by Christomanou and Kleinschmidt (1985). In both cases it is, however, still too early to speculate on the role and significance of these proteins.

II. ASSAYS FOR ACTIVATOR PROTEINS

The quantification of activator proteins is generally based on their capability to stimulate in vitro the degradation of glycolipids by the corresponding hydrolases. The relation between concentration of activator in the assay mixture and resulting reaction rate is linear only within certain limits that depend not only on the reaction catalyzed and on the type of activator protein but also on the precise assay conditions.

Those activator proteins that promote the hydrolysis of glycolipid substrates by water-soluble hydrolases, namely the G_{M2} activator and the sulfatide activator, seem to act by similar mechanisms: they bind the glycolipid and extract it from the membrane to form a water-soluble complex that is the true substrate for the enzyme. Accordingly, the reaction rate depends on the concentration of the activator-lipid complex, with Michaelis-Menten-type saturation kinetics (Conzelmann and Sandhoff, 1979). Such a saturation curve is at no point exactly linear, but for practical purposes sufficient linearity can be assumed at activator concentrations below the K_M value of the enzyme for the respective activator-lipid complex as substrate.

It should also be considered that the formation of the complex between activator and lipid is an equilibrium reaction with a finite dissociation constant. Under the conditions used for the quantification of activators—that is, with pure glycolipid substrates at concentrations well above the K_D of the respective activator-lipid complex—the activator can be assumed to be saturated with the lipid, so that the activator concentration practically equals the concentration of the substrate of the reaction (the activator-lipid complex). However, the presence of other lipids such as phospholipids in the assay mixture may increase the experimental K_D by orders of magnitude since the mixed aggregates formed may be much more stable than the pure glycolipid micelles. (At a large excess of phospholipids as in the case of liposome-bound substrate, the K_D may depend linearly on the phospholipid concentration.) As a consequence the concentration of the activator-lipid complex may be far below the total activator concentration, and the enzymic reaction will accordingly be much slower than with pure glycolipid substrates.

Since, as in simple Michaelis-Menten kinetics, the reaction rate depends linearly on the enzyme concentration, the accuracy of activator determinations depends on the precision with which the enzyme concentration is known. Although it is not necessary to employ pure enzymes for the purpose of activator quantification, problems may arise if several isoenzymes exist of which only one is able to degrade the glycolipid substrate in the presence of the activator but all of them hydrolyze the artificial substrates employed for measuring the enzyme. This is, for example, the case for α-galactosidase, β-hexosaminidase, and arylsulfatase. In these cases separation of the isoenzymes, say by ion-exchange chromatography, is required.

It should also be noted that the Michaelis-Menten equation was derived under the assumption that the substrate concentration is always much higher than the enzyme concentration. This condition may not be met when the substrates involve macromolecules such as proteins. Thus at a low activator or high enzyme concentration some deviation from linearity may be encountered.

The mechanisms by which the cofactors for degradation of glucosylceramide and galactosylceramide stimulate the respective enzymes is not yet known. Quantification of these cofactors by their capacity to enhance enzymic glycolipid hydrolysis in vitro must therefore rely entirely on practical experiences. These will be discussed with the respective assay systems.

As noted before, it is usually not necessary to use highly purified enzymes. In most cases partially purified preparations will do, provided that they are sufficiently concentrated, essentially free of endogenous

activator, and contain only the one isoenzyme that does interact with the activator-lipid complex (or the isoenzyme composition is known, and the results can be corrected for it). For the water-soluble enzymes, satisfactory preparations can be obtained by affinity chromatography on Concanavalin A-Sepharose, followed by ion-exchange chromatography and, in some cases, gel filtration. A major exception is arylsulfatase A, which has to be quite pure since the degradation of sulfatide in the presence of the sulfatide activator is inhibited by many contaminating proteins (Fischer and Jatzkewitz, 1975). The membrane-associated enzymes glucosylceramide β-glucosidase and galactosylceramide β-galactosidase require solubilization with detergents prior to any chromatographic separation.

Activator proteins can also be quantified with immunochemical techniques if suitable antisera are available. Enzyme-linked immunosorbent assays (ELISA) have been developed for the sulfatide/G_{M1} activator (Gardas et al., 1984) and for the G_{M2} activator (Banerjee et al., 1984). The ELISA for the G_{M2} activator was more than 10 times as sensitive as enzymic assays and permitted the precise determination of G_{M2} activator in very dilute samples such as subcellular fractions of cultured skin fibroblasts (Banerjee et al., 1984).

1. Sulfatide/G_{M1} Activator

The sulfatide activator promotes, at least in vitro, several reactions. In principle, any one of them can be used to assay for this cofactor, but the degradation of ganglioside G_{M1} by β-galactosidase is most conveniently used since this reaction proceeds fast enough to permit sensitive measurements of activator content and is the least sensitive to disturbances by impurities. Globotriaosylceramide is much more tedious to prepare in pure form than ganglioside G_{M1}. Degradation of sulfatide by arylsulfatase A is comparatively slow and may be strongly inhibited by contaminating proteins and other compounds so that the assay requires the use of highly purified enzyme and activator. Reliable quantitation of the activator during purification is therefore not possible with this assay (Fischer and Jatzkewitz, 1975).

A. ASSAY WITH GANGLIOSIDE G_{M1}/β-GALACTOSIDASE

1. Substrate. Ganglioside G_{M1} can be purified from brain lipid extracts by any one of several published methods (Svennerholm, 1972; Kundu, 1981; Ledeen and Yu, 1982). The yield of ganglioside G_{M1} can be increased several fold by converting higher gangliosides into ganglioside G_{M1} with sialidase: The crude ganglioside preparation (e.g., the

Folch upper phase [Folch et al., 1957]) is taken to dryness and dissolved in 50 m*M* acetate buffer, pH 5.5, 1 m*M* $CaCl_2$, and incubated overnight with sialidase from clostridium perfringens (10 units/liter) (G. Schwarzmann, personal communication).

For measuring its enzymic degradation, the substrate is most conveniently labeled in the terminal galactose moiety by the galactose oxidase/ [^{3}H]borohydride method of Radin (1972a), for example, as modified by Suzuki, K. (1977). (The reduction step of this procedure should be carried out at a pH value below 10. At a more alkaline pH some of the ganglioside G_{M1} is converted to a highly labeled product with the chromatographic mobility of ganglioside G_{M2}.)

Final purification of the labeled product is usually done by column chromatography according to Svennerholm (1972). If only small amounts of labeled ganglioside are needed, purification by preparative thin-layer chromatography is more convenient: the ganglioside solution in organic solvent is applied to a thin-layer plate (e.g., Merck Kieselgel G 60, 0.25 mm thickness) in a 16-cm wide streak. Analytical plates are preferable over preparative ones since they give a much better resolution. Up to 5 mg of lipid can be applied to one plate. The plate is developed in chloroform/methanol/15 m*M* aq $CaCl_2$ (55/45/10 by volume). Radioactive bands are located with a radioscanner, the plate is lightly sprayed with water, and the area containing the [^{3}H]ganglioside G_{M1} is scraped off. (If water is sprayed until the plate starts to become translucent, the lipid may be visible as a white band on a darker background.) The scrapings are suspended in 5 ml chloroform/methanol/water (55/45/10 by volume), packed into a suitable column (e.g., a large Pasteur pipette with glass wool plug) and eluted with 2×5 ml of the same solvent. The solvent is then evaporated under a stream of N_2, the dry residue is taken up in distilled water (~1 ml/mg lipid), dialyzed against distilled water and lyophilized.

The specific radioactivity of the product is best determined by measuring sialic acid content, for example, by the modification of Miettinen and Takki-Luukkainen (1959) of the method of Svennerholm (1957). Simply weighing the product may give wrong values due to the presence of some silicic acid.

2. β-Galactosidase. Lysosomal β-galactosidase can be purified from human liver or placenta with conventional methods (Meisler, 1972; Sloan, 1972; Miyatake and Suzuki, 1975) or with affinity chromatography on immobilized *p*-aminophenyl- or 6-aminohexyl-thio-β-D-galactoside (Miller et al., 1977; Lo et al., 1979). The most rapid and convenient procedure seems to be the two-step method of Miller et al. (1977), which is reported to give a more than 20,000-fold purification with 41% yield. The affinity gel is commercially available.

The activity of β-galactosidase is usually measured with the fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside, for example, 1 m*M* in 50 m*M* citrate/0.1 *M* phosphate buffer, pH 4.0, with 0.1 *M* NaCl (Suzuki, K., 1977). (The high ionic strength is needed to stabilize the enzyme.) One unit of β-galactosidase is generally defined as the amount of enzyme that splits 1 μmol of this substrate per minute under the above conditions, at 37°C.

3. Assay. The standard assay mixture for determination of the activator consists of 1 nmol ganglioside G_{M1}, [^{3}H]-labeled in the terminal galactose moiety (~100,000 cpm), 5 mU β-galactosidase and up to 25 μl of the suitably diluted activator sample, in a total volume of 50 μl of 50 m*M* citrate buffer, pH 4.5. The assays are incubated for 1 h at 37°C and then transferred to an ice-bath, and 1 ml of an ice-cold 1 m*M* galactose solution is added. The mixtures are loaded onto small (0.5–1 ml) columns of DEAE-cellulose (in Pasteur pipettes) that have been washed with distilled water. Liberated [^{3}H]galactose is washed out with 2 × 1 ml of 1 m*M* aqueous galactose solution, the combined effluents are collected in scintillation vials, and, after addition of 10 ml of scintillation fluid, their radioactivity is measured in a liquid scintillation counter. Blanks run with water instead of activator solution are subtracted.

The reaction depends almost linearly on the activator concentration up to about 5 μ*M* (~ 5 μg of activator/assay). The K_D of the activator/lipid complex is approximately 3 μ*M* (Vogel, 1985) so that the activator can be assumed to be saturated with the lipid under the preceding conditions.

B. ASSAY WITH SULFATIDE/ARYLSULFATASE A

1. Substrate. Sulfatides are commercially available, but they can also be prepared from crude brain lipid extracts by one of the conventional procedures (Rouser et al., 1969; Kwiterovich et al., 1970; Yu and Ledeen, 1972).

Three different strategies have been proposed for the radiolabeling of sulfatides:

a. Catalytic reduction of the sphingoid base by the method of Schwarzmann (1978), such as modified by Raghavan et al. (1981).

b. Preparation of lysosulfatide (sulfogalactosylsphingosine) by alkaline hydrolysis of sulfatide and reacylation of the sphingosine amino group with a radioactive fatty acid (Dubois et al., 1980).

c. Biosynthetic labeling of sulfatide by injecting [^{35}S]sulfate into the brains of still myelinating laboratory animals and isolation of the sulfatide formed after one or two days (Mehl and Jatzkewitz, 1964; Fluharty et al., 1974).

The product of the latter procedure, [^{35}S]sulfatide, permits a simple quantitation of enzymic sulfatide hydrolysis, by extracting the liberated sulfate into the aqueous phase of a two-phase system. However, the short half-life of ^{35}S (87 days) limits the usefulness of this method.

Of the other two methods, both of which employ long-lived isotopes (^{3}H or ^{14}C), the first one is less tedious and gives better yields.

2. Arylsulfatase A. As noted before, the arylsulfatase A preparations used for assays with sulfatide and the natural activator protein have to be rather pure since contaminating proteins may interfere with this reaction in vitro. A variety of methods have been published for purification of the enzyme from human liver (Draper et al., 1976; James and Austin, 1979), kidney (Stinshoff, 1972), placenta (Gniot-Szulzycka and Komoszynski, 1970), and urine (Breslow and Sloan, 1972; Stevens et al., 1975). In our laboratory the procedure of Stinshoff (1972) is usually followed.

Arylsulfatase activity is usually measured with the chromogenic substrate 4-nitrocatecholsulfate. (A fluorogenic substrate, 4-methylumbelliferylsulfate, is also available but is less frequently used because it is hydrolyzed only very slowly.) The assay system of Baum et al. (1959), which was developed to permit the determination of arylsulfatase A in the presence of the B isoenzyme, is still most widely used. The unusual time course of the hydrolysis of 4-nitrocatecholsulfate by arylsulfatase A (Roy, 1953; Baum et al., 1958; Stinshoff, 1972) may present a problem for standardization of the enzyme preparation and should be taken into account, such as by using short incubation times (15–30 min). The enzyme unit is usually defined as the amount of enzyme that hydrolyzes 1 μmol of this substrate per minute.

3. Assay. The standard assay mixture consists of 5 nmol sulfatide, either ^{3}H-labeled in the sphingoid base or ^{14}C-labeled in the fatty acid, 5 mU arylsulfatase A, and an aliquot of the activator sample to be assayed in a total volume of 100 μl of 0.1 *M* sodium acetate buffer, pH 5.0, with 0.1 *M* NaCl. After incubation for 3 h at 37°C, the reaction is stopped by addition of 400 μl of chloroform/methanol (2:1 by volume), and after thorough mixing, phases are separated by centrifugation. The (aqueous) upper phase is discarded, the organic phase is washed with 200 μl theoretical upper phase (chloroform/methanol/water, 3:48:47 by volume) and then loaded onto a 0.5 ml column of DEAE-cellulose (in a Pasteur pipette) equilibrated with methanol. Unbound lipids are eluted with 2 ml methanol, collected in a scintillation vial, and their radioactivity is quantified by liquid scintillation counting. Blanks that were run with water instead of activator solution are subtracted.

As noted earlier, the reaction is very sensitive to the presence of contaminating proteins and other compounds. Also arylsulfatase A is competitively inhibited by many anions such as phosphate (K_I = 0.6 m*M* [Gniot-Szulzycka, 1974]), sulfate, and others. Precise determination of activator content with this method is therefore very difficult.

C. ASSAY WITH GLOBOTRIAOSYLCERAMIDE/α-GALACTOSIDASE A

1. Substrate. Globotriaosylceramide can be isolated from the neutral lipid fraction of many tissues, including kidney, spleen, and liver. Porcine intestine (Dean and Sweeley, 1977) or erythrocytes (Taketomi and Kawamura, 1972) have been recommended as convenient sources. A particularly rich source is tissue of patients with Fabry's disease (Sweeley and Klionsky, 1963), especially liver and kidney. This disease is rare, however, and autopsy tissue is usually difficult to obtain.

Extraction and purification of globotriaosylceramide follows the usual methods described for neutral glycosphingolipids (Suzuki, C., et al., 1968; Esselman et al., 1972).

For enzymic studies, the glycolipid can be radiolabeled in the terminal galactose moiety by the galactose oxidase/[^{3}H]borohydride method of Radin (1972a), such as described by Suzuki, Y., and Suzuki, K. (1972) or Dean and Sweeley (1977).

2. α-Galactosidase A. The enzyme can be purified from human liver or placenta with conventional techniques (Beutler and Kuhl, 1972; Dean and Sweeley, 1979) or affinity chromatography (Bishop and Desnick, 1981). The enzyme is routinely assayed with the fluorogenic substrate 4-methylumbelliferyl-α-D-galactoside, most assays being modifications of the method of Desnick et al. (1973). Enzyme units (μmol/minute) are usually also defined with this substrate.

3. Assay. For the standard assay for activator protein, 2 nmol globotriaosylceramide, [^{3}H]-labeled in the terminal galactose residue (~22,000 cpm), are incubated with 1 mU α-galactosidase A and a suitable dilution of the sample to be assayed in a total volume of 50 μl of 50 m*M* sodium acetate buffer, pH 4.0, for 1–3 h at 37°C. (Assays with purified activator should contain some 5 μg of bovine serum albumin to prevent unspecific adsorption of protein on surfaces.) The reaction is terminated with 0.6 ml chloroform/methanol (2:1 by volume) and 0.1 ml of 1 m*M* galactose/1 *M* NaCl in water. After thorough mixing and centrifugation for 5 min in a benchtop centrifuge (Eppendorf), the lower phases are removed and discarded, the remaining upper phases are washed with 0.5 ml of theoretical lower phase (chloroform/methanol/water, 86:14:1 by volume) and then transferred to scintillation vials. Radioactivity is quanti-

fied by liquid scintillation counting, after addition of 5 ml scintillation fluid. Blanks run with water instead of activator solution are subtracted. The reaction rate increases linearly with activator concentration up to 2–3 μM (~2–3 μg/assay).

2. G_{M2} Activator

The G_{M2} activator is determined by its ability to promote the hydrolysis of ganglioside G_{M2} by β-hexosaminidase A.

1. Substrate. Ganglioside G_{M2} is found only in traces in most tissues, so purification from normal sources is extremely tedious. The ganglioside is usually isolated by the method of Svennerholm (1972) from brain tissue of patients who died from G_{M2} gangliosidosis. A more readily available source has recently been indicated by Li, Y.-T., et al. (1984) who found that ganglioside G_{M2} constitutes the main ganglioside of striped mullet (Mugil cephalus) roe. Care should however be exercised when using such preparations for kinetic studies since the ceramide moiety of the fish ganglioside differs somewhat from that of the human ganglioside.

The terminal *N*-acetylgalactosamine moiety can be radiolabeled with galactose oxidase/NaB^3H_4 (Suzuki, Y., and Suzuki, K., 1972) by essentially the same procedure as ganglioside G_{M1} (see the preceding section). However, the specific activity attainable is considerably lower than that of ganglioside G_{M1} or other glycolipids, due to an only limited degree of oxidation of the *N*-acetylgalactosamine residue. Unspecific labeling of other parts of the molecule may therefore contribute significantly to the specific activity of the labeled substrate, and it is recommended to reduce the starting material with unlabeled $NaBH_4$ prior to enzymic oxidation (Suzuki, Y., and Suzuki, K., 1972). As in the case of ganglioside G_{M1}, the amounts required for enzymic assays can be finally purified with preparative thin-layer chromatography and their specific radioactivity checked as described in the preceding section.

2. β-Hexosaminidase A. The enzyme can be purified from normal human liver (Sandhoff et al., 1977), placenta (Lee and Yoshida, 1976; Geiger and Arnon, 1978), kidney (Wiktorowicz et al., 1977), brain (Aruna and Basu, 1976), or urine (Marinkovic and Marinkovic, 1978). The enzyme preparation used does not have to be pure but it is essential to remove β-hexosaminidase B (or at least to know the proportions of the two enzymes) since only the A isoenzyme interacts with the activator/lipid complex. A preparation suitable for activator determination can be obtained from liver or placenta extract (20% in water) with chromatography on Concanavalin A-Sepharose 4B as described by Sandhoff et al. (1977),

followed by ion-exchange chromatography on DEAE-cellulose in 10 m*M* phosphate buffer, pH 6.0. (Column vol. ~1 ml per 5 mg protein to be applied.) Hexosaminidase B is first washed out with 2 column volumes of buffer, then hexosaminidase A is eluted with 0.5 *M* NaCl in the same buffer. The preparation must be dialyzed against distilled water before use because the enzymic reaction rate is inversely proportional to the ionic strength (Conzelmann et al., 1982).

The enzyme is most conveniently assayed with the fluorogenic substrate 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide (Leaback and Walker, 1961; Dance et al., 1969; Sandhoff et al., 1977) (1 m*M* in 50 m*M* citrate buffer, pH 4.5). One enzyme unit is usually defined as the amount of enzyme that splits 1 μmol of this substrate per minute under standard conditions.

3. Assay. The standard assay for quantification of the G_{M2} activator contains 5 nmol of [^{3}H]ganglioside G_{M2} (specific radioactivity at least 5–10 Ci/mol), 2.5 μg bovine serum albumin, 100 mU β-hexosaminidase A, and up to 25 μl of the suitably diluted activator sample in a total volume of 40 μl m*M* citrate buffer, pH 4.0, and is incubated for 1–4 h at 37°C. Samples are then transferred to an ice bath and loaded onto 1 ml columns of DEAE-cellulose (in Pasteur pipettes) that have been washed with distilled water. Liberated [^{3}H]*N*-acetylgalactosamine is eluted with 2 × 1 ml of a 1 m*M* aq *N*-acetylgalactosamine solution. The combined effluents are collected in scintillation vials, and after addition of 10 ml scintillation fluid, their radioactivity is measured. Blanks run with water instead of activator solution are subtracted.

The reaction rate is very low without activator and increases practically linearly up to 1 μg of G_{M2} activator/assay, corresponding to a degradation rate of approximately 50 nmol/(h × U hex A). The absolute reaction rate decreases strongly with increasing ionic strength (Conzelmann et al., 1982). Samples should therefore be dialyzed against water when the activator content is to be quantified precisely.

3. Cofactors for Glucosylceramide β-Glucosidase and Galactosylceramide β-Galactosidase

A. ASSAY WITH β-GLUCOSIDASE

1. Substrates. In most normal tissues, glucosylceramide is only a trace component. Tissues of patients with Gaucher's disease (particularly, spleen and liver) contain large amounts of this glycolipid, due to the deficiency of the catabolizing enzyme, and Gaucher spleen is the most frequent source of the glucosylceramide used in enzymic and other studies.

Extraction and purification can be done as described by Radin (1976) or by Peters et al. (1977c).

Glucosylceramide can also be synthesized chemically (Brady et al., 1965; Stoffyn et al., 1971). It has the advantage of yielding homogeneous products with respect to fatty acid and sphingoid base but is, of course, more laborious.

Radioactive labeling can most easily be achieved by catalytic reduction of the sphingosine double bond (Schwarzmann, 1978). Another possibility is amidation of a radioactive fatty acid with glucosylsphingosine prepared from glucosylceramide by alkaline hydrolysis (Erickson and Radin, 1973). However, the most useful substrate for enzymic assays is the one labeled in the glucose moiety. This can be obtained either by chemical synthesis with radioactive glucose or by labeling intact glucosylceramide by the method of McMaster and Radin (1977).

2. Acid β-glucosidase. Like other lysosomal enzymes, glucosylceramide β-glucosidase (acid β-glucosidase) can be isolated from almost any human tissue. Placenta has proved to be a good source that is readily available. The enzyme can be obtained in water-soluble form after extraction of crude preparations with *n*-butanol or other organic solvents (Blonder et al., 1976; Dale et al., 1976). High purity with good yield was reported with conventional techniques (Dale and Beutler, 1976; Furbish et al., 1978) as well as with affinity chromatography (Strasberg et al., 1982; Grabowski and Dagan, 1984).

Acid β-glucosidase is routinely assayed with the fluorogenic 4-methylumbelliferyl-β-D-glucoside. Standardization of enzyme preparations is, however, problematic since the enzyme is stimulated by acidic phospholipids (Ho and Light, 1973; Dale et al., 1976; Peters et al., 1977a) or detergents (Ho, 1973; Dale et al., 1976; Peters et al., 1976) so that the absolute activity depends strongly on the amount and nature of such additions.

3. Assay. Acid β-glucosidase hydrolyzes both water-soluble and lipid substrates. The activity, and hence the activation of this enzyme by cofactors, can therefore be determined with either kind of substrate. Due to the rapidity and convenience of assays with fluorogenic substrates, most workers prefer to use the commercially available 4-methylumbelliferyl-β-D-glucoside. In some cases it may, however, be desirable, or even necessary, to confirm the results with the natural substrate, glucosylceramide. Both types of assays will therefore be described here.

A central problem in both cases is the definition of "optimal" conditions for the quantification of a stimulating cofactor. Generally, conditions must be sought that lead to maximal stimulation of an otherwise

small basal activity. As outlined earlier, β-glucosidase activity depends strongly on the nature and amount of additions to the assay (acidic phospholipids, anionic detergents). It is therefore difficult to define the activator activity in absolute terms such as an increase in the amount of substrate hydrolyzed per enzyme unit. Most workers express the activation therefore as multiples of basal activity ("-fold activation").

The interaction between acid β-glucosidase and activating protein cofactors depends on the presence of acidic lipids (Ho and Light, 1973), so a certain amount of such lipids has to be added to the assay system. (The partially purified enzyme preparations used contain frequently some lipids, including acidic ones.) On the other hand, higher concentrations of acidic lipids may stimulate the enzyme to almost maximal activity (Berent and Radin, 1981a) and may render protein cofactors obsolete.

Assay with 4-methylumbelliferyl-β-D-glucoside: The activator sample to be assayed is incubated with 2 m*M* substrate in 0.2 *M* acetate buffer, pH 5.5 (Peters et al., 1977a,b), or 50 m*M* acetate, pH 4.5 (Berent and Radin, 1981a; Wenger and Roth, 1982), with an appropriate amount of partially purified enzyme and, if necessary, with 5 μg phosphatidylserine, in a total volume of 100 μl. After incubation (1 h, 37°C), the reaction is terminated by the addition of 0.5 ml 0.2 *M* glycine/0.2 *M* sodium carbonate, and the liberated 4-methylumbelliferone is determined fluorimetrically (excitation 365 nm, emission 440 nm).

Assay with glucosylceramide: Assay conditions are essentially the same as with the fluorogenic substrate except that [glucose-6-^{3}H]glucosylceramide is used as substrate, at a concentration of 0.3–0.4 m*M* (Berent and Radin, 1981b; Wenger and Roth, 1982). After incubation, 5 volumes of chloroform/methanol (2:1 by volume) and 0.5 volumes of an aqueous solution of 0.2 *M* glucose/0.2 *M* KCl are added. The mixture is thoroughly vortexed and then centrifuged and the liberated [^{3}H]glucose is measured in an aliquot of the upper phase. (The sensitivity of the method can be improved, if necessary, by washing the upper phase with 2 volumes of theoretical lower phase {chloroform/methanol/water, 64:9:1, by volume}.)

B. ASSAY WITH GALACTOSYLCERAMIDE β-GALACTOSIDASE

1. Substrate. Galactosylceramide can be purified from the neutral fractions of brain lipid extracts (Radin, 1972a). The galactose moiety can easily be tritium-labeled by the galactose oxidase/sodium borohydride method (Radin, 1972a).

2. Enzyme preparation. Wenger et al. (1982) studied the stimulating effect of protein cofactors on galactosylceramide hydrolysis with crude tissue homogenates or extracts as enzyme sources. Purification of

galactosylceramide β-galactosidase, which seems to be somewhat difficult (Suzuki, K., 1977), may therefore not be necessary.

If a more purified preparation is desired, the enzyme, like acid β-glucosidase, can be converted into a water-soluble form by extraction with organic solvents (Tanaka and Suzuki, 1976, 1977). However, the purification protocols published so far (Radin, 1972b; Wenger et al., 1975) rely mainly on extraction with detergents.

3. Assay. The assay method described is that used by Wenger et al. (1982). [^{3}H]Galactosylceramide (10 nmol; specific activity ~1 mCi/mmol) and 25 μg pure phosphatidylserine are dispersed in 0.1 ml 0.1 *M* citrate/ 0.2 *M* phosphate buffer, pH 4.6, by sonication. Enzyme, activator sample, and distilled water are added to a final volume of 0.2 ml. After incubation at 37°C for 1 h, 1 ml of chloroform/methanol (2:1 by volume) is added, and the liberated [^{3}H]galactose, which partitions into the upper phase, is determined by liquid scintillation counting.

The reaction rate is reported to increase linearly with the amount of cofactor added up to 5–10-fold stimulation (Wenger et al., 1982).

III. PURIFICATION OF ACTIVATOR PROTEINS

1. G_{M2} Activator

The richest source of human G_{M2} activator is kidney tissue (Conzelmann and Sandhoff, 1978, 1979), which contains some 800 ng activator/mg extract protein, as compared to 100 ng/mg for brain and 50 ng/mg for liver (Banerjee et al., 1984). Another, more convenient, source is urine (Li, Y.-T., et al., 1983), with a G_{M2} activator content between 200 and 1400 ng/mg protein (Banerjee et al., 1984).

The following purification scheme from human kidney is a simplified version of the one originally developed in our laboratory (Conzelmann and Sandhoff, 1979). Human urine, concentrated 10-fold by ultrafiltration (exclusion limit ~10,000 Da) may be substituted for the crude kidney extract.

All steps are performed at 4°C unless otherwise stated. Postmortem human kidney (1 kg) obtained within less than 24 h after death and stored frozen (−20°C) is homogenized in 4 volumes of distilled water, with an Ultra-Turrax or a Polytron homogenizer and centrifuged at 13,000 × g (9000 rpm in a Sorvall GSA or GS-3 rotor) for 30 min. The pellet is reextracted with 3 volumes of water.

The combined supernatants are heated to 60°C in a water-bath for 2 h, and precipitated material is removed by centrifugation (as before). The extract is adjusted to pH 3.0 by slow addition of 10% trichloroacetic acid,

under rapid stirring, and then stirred for another 2 h (or overnight). Precipitated protein is spun off, the clear supernatant is dialyzed against 10 m*M* phosphate buffer, pH 6.0. After filtration, the solution is loaded onto a column of DEAE-cellulose (5 × 30 cm) equilibrated with the same phosphate buffer. The column is washed with 1 l phosphate buffer; then the activator is eluted with a linear gradient of NaCl (0–0.5 *M* in 1 l phosphate buffer). Fractions of 20 ml are collected and monitored for G_{M2} activator. Since this step, ion-exchange chromatography, separates the G_{M2} activator from the sulfatide/G_{M1} activator, the latter may also be recovered and further purified as described shortly, if desired.

Fractions containing the G_{M2} activator (usually those with more than 20% of maximal activity) are pooled, dialyzed against distilled water, and lyophilized. At this point several preparations may be combined and processed together if larger quantities of the activator protein are to be prepared.

The dry residue is dissolved in 10–15 ml of 50 m*M* citrate buffer, pH 4.2, and, after removal of insoluble material by centrifugation, is passed over a gel-filtration column (Sephadex G-100; 2.6 × 90 cm) in the same buffer, at a flow rate of approximately 20 ml/h.

Activator-containing fractions are combined and loaded onto an octyl-Sepharose column (10 ml) packed in water. Unbound protein is washed out with 3 volumes 10 m*M* phosphate buffer, pH 6.0; then the activator is eluted with a linear gradient of 0–1% sodium cholate (analytical grade) in 50 ml of the same buffer. Fractions of 1 ml are collected and analyzed for G_{M2} activator. The active fractions are pooled and dialyzed exhaustively against several changes of distilled water and then lyophilized.

At this stage the preparation is approximately 80% pure as judged by analytical HPLC gel filtration. Recovery is approximately 30–50%. If an even purer preparation is desired, final purification can be achieved with HPLC gel filtration on a TSK G 2000 SW column (150 μl per run, on a 13 ml column, in 0.1 *M* phosphate buffer, pH 6.0, flow rate 0.5 ml/min) or HPLC anion-exchange chromatography on a TSK 545 DEAE column (both columns are from LKB, Sweden). In the latter procedure 10 mg of activator (dissolved in 10 m*M* phosphate buffer, pH 6.0) can be applied to a 6-ml column which is then eluted with a linear gradient of 0–0.3 *M* NaCl in 60 ml of the same buffer.

The purity of G_{M2} activator is difficult to assess with electrophoretic methods since the material isolated from tissue or urine appears to have a very heterogeneous carbohydrate portion and runs on SDS electrophoresis as a broad, diffuse band, with an apparent M_R of approximately 22,000 Da.

2. Sulfatide/G_{M1} Activator

Purification of the human sulfatide activator to a preparation that yielded one band in nondenaturing electophoresis was reported by Fischer and Jatzkewitz (1975). Li and Li (1976) purified the G_{M1} activator, which later turned out to be the same protein. Both procedures exploit the unusual thermal stability of this protein which can be heated to 95°C for several minutes without loss of activity. A gentler procedure that allows the simultaneous preparation of both, the sulfatide/G_{M1} activator and the more sensitive G_{M2} activator, was also suggested by Li and Li (1982). Unfortunately neither publication contains information on yield and purity of the final preparations. Also the steps involving chromatography on DEAE-Sephadex A-50 proved to be quite cumbersome in our hands. The method of Fischer and Jatzkewitz appears to give comparatively pure products, with reasonable yields (5.5 mg/kg tissue), but employing acetone precipitation as the first purification step leads to very large volumes that have to be centrifuged.

We use the following procedures. All steps are performed at 4°C unless otherwise stated. The human autopsy tissues used as sources should be obtained within 24 h after death and should be stored at or below −20°C until used.

A. PURIFICATION FROM HUMAN LIVER

Human autopsy liver (500 g) is homogenized in 4 volumes of 10 m*M* phosphate buffer, pH 6.5, with an Ultra-Turrax or Polytron homogenizer for 5 min. The homogenate is centrifuged at 100,000 × g for 30 min. The pellet is reextracted with 4 volumes of the same buffer, and the combined supernatants are filtered through glass wool.

Solid ammonium sulfate is slowly (within 1 h) added with stirring, up to 60% saturation, and stirring is continued overnight. Precipitated material is spun down (13,000 × g, 1 h), redissolved in approximately 100 ml 10 m*M* phosphate buffer, pH 6.5, and dialyzed against two changes of 10 l each of the same buffer. This solution is then loaded onto a DEAE-cellulose column (2.6 × 40 cm) equilibrated with the same phosphate buffer. After washing with 2 column volumes of buffer, the column is eluted with a linear gradient of 0−0.3 *M* NaCl in 1 l phosphate buffer (flow rate 40 ml/h). Fractions of 15 ml are collected and analyzed for activator content. The fractions with an activator concentration of more than 20% of maximum are pooled, dialyzed against distilled water, lyophilized, and then dissolved in 20 ml 10 m*M* phosphate buffer, pH 6.5, with 100 m*M* NaCl. The solution is passed over a Sephadex G-75 sf column

(2.6 × 86 cm) in the same buffer, at a flow rate of 10 ml/h. Fractions of 10 ml are collected.

The activator-containing fractions are loaded onto a 15 ml octyl-Sepharose column packed in 10 m*M* phosphate buffer, pH 6.5. The column is washed with 2 volumes of 50% ethylene glycol in water and eluted with 1% cholic acid (analytical grade) in 10 m*M* phosphate buffer, pH 6.5. The protein-containing fractions are pooled, dialyzed first against 2 × 2 l 10 m*M* phosphate buffer, pH 6.5, then against 2 × 2 l distilled water and finally lyophilized.

The preparation is finally purified with ion-exchange HPLC: the sample is dissolved in 0.5–1 ml of 10 m*M* phosphate buffer, pH 6.0, and after removal of any insoluble material by centrifugation (Eppendorf benchtop centrifuge), up to 10 mg protein per run are loaded onto a 6 ml TSK 545 DEAE column (LKB, Sweden). The column is eluted at room temperature with a linear gradient of 0–0.3 *M* NaCl in the same phosphate buffer (flow rate 0.5 ml/min). Fractions of 0.5 ml are collected and analyzed for activator protein.

The overall yield of this procedure is approximately 15% (30% before HPLC). The final product yields a single symmetrical peak in analytical HPLC gel filtration, whereas on ion-exchange HPLC several peaks can be resolved, all of which have the same specific activity. The existence of these different forms is probably due to heterogeneity of the carbohydrate portion. The electrophoretic purity is difficult to assess since the activator, which according to gel-filtration experiments seems to have a molecular weight of approximately 22,000 Da, consistently yields several bands in the range between 6,000 and 10,000 Da on SDS electrophoresis.

B. PURIFICATION FROM HUMAN KIDNEY AND URINE

1. Kidney. Human autopsy kidney tissue (500 g) is homogenized with an Ultra-Turrax or Polytron homogenizer in 3 volumes of 10 m*M* phosphate buffer, pH 6.0, and then centrifuged at 13,000 × g for 30 min. The supernatant is heated to 60°C for 30 min in a water bath. Precipitated material is removed by centrifugation (13,000 × g, 30 min). The extract is adjusted to pH 4.0 with 1 *M* citric acid and stirred overnight. After removal of precipitated protein by centrifugation, the solution is dialyzed against 10 m*M* phosphate buffer, pH 6.0, concentrated at room temperature with a rotary evaporator to a volume of 40 ml and centrifuged again. The supernatant is applied to a Sephadex G-75 column (2.6 × 60 cm) in portions of 20 ml and chromatographed in 10 m*M* phosphate buffer, pH 6.0. Fractions with activator content of more than 20% of maximum are pooled and loaded onto a DEAE-cellulose column (2.6 × 25 cm) equili-

brated with 10 m*M* phosphate buffer, pH 6.0. After washing with 2 volumes of this buffer, the column is eluted with a linear gradient of 0–0.5 *M* NaCl in 1 l of the same buffer. The activator-containing fractions are pooled and dialyzed overnight against 1% glycine solution.

For isolelectric focusing the sample is mixed into a linear sucrose gradient from 35 to 0% (w/v), containing 5% (by volume) of carrier ampholyte solution, pH 4–7, (equivalent to an ampholyte concentration of 2%, w/v) in a 110-ml electrofocusing column (LKB, Sweden). The cathode space is filled with 1.5% (w/v) of ethylene diamine in a 50% (w/v) sucrose solution; the sample space is topped off with 0.1% aqueous H_2SO_4 as anode solution. Focusing is performed for 72 h at 500 V; then the contents are collected in 3-ml fractions.

Activator-containing fractions are combined and loaded onto a small (1-ml) column of octyl-Sepharose. Ampholytes are washed out with 5 ml of 10 m*M* phosphate buffer, pH 7; then the activator is eluted with 1% cholic acid (analytical grade) in the same buffer. After addition of 0.1 mg cytochrome c/ml (as protection against adsorption of the small amounts of protein onto dialysis tubing and other surfaces) the sample is dialyzed extensively against distilled water and then lyophilized.

The preparation is finally purified by HPLC as described earlier for the liver protein. Yield and purity of the final preparation are comparable to those of the first method.

2. Urine. Freshly voided human urine is filtered through ordinary filter paper in a Büchner funnel, concentrated 10-fold by ultrafiltration (exclusion limit of the membrane 10,000 Da), dialyzed against distilled water, lyophilized, and taken up in 40 ml of distilled water.

Further processing of the sample is as described earlier for the kidney extract, starting with the Sephadex G-75 gel filtration. Yield and quality of the final product are comparable to those of the other methods described earlier.

During this procedure the G_{M2} activator is separated from the sulfatide activator by the ion-exchange chromatography step and can be further purified as described earlier.

3. Co-glucosidase

Since the initial report by Ho and O'Brien (1971) that lysosomal β-glucosidase activity toward water-soluble substrates as well as glucosylceramide (Ho et al., 1973) is greatly stimulated by small nonenzymic protein cofactors that are particularly abundant in Gaucher spleen, such factors have been purified by several groups from human spleen (Ho and O'Brien, 1971; Peters et al., 1977a, b; Chiao et al., 1978; Iyer et

al., 1983) and brain (Wenger and Roth, 1982), and from bovine spleen (Berent and Radin, 1981a, b). Some of these preparations were also tested with other enzymes and were found to stimulate also the hydrolysis of galactosylceramide by the corresponding β-galactosidase (Wenger et al., 1982).

It is, however, very difficult to assess the various purification procedures suggested. A comparison of the products obtained from various sources with different methods (or even with the same method) indicates a tremendous heterogeneity with respect to molecular weight (Ho and O'Brien, 1971; Peters et al., 1977a, b; Wenger and Roth, 1982), specific activity (Peters et al., 1977a; Iyer et al., 1983), and extent and necessity of glycosylation (Ho and O'Brien, 1971; Peters et al., 1977a; Berent and Radin 1981b). Recent evidence (Sarmientos et al., 1986) also suggests that acid β-glucosidase may be able to degrade its membrane-bound glycolipid substrate without the aid of any protein cofactor. (The role of the intrinsic cofactor for glucosylceramide β-glucosidase recently described by Vaccaro et al. [1985] may be different but cannot yet be assigned.)

A convenient method that yields essentially pure products, from human tissue, is that of Peters et al. (1977a).

However, as long as it is not known which of the stimulating factors for glucosylceramide and galactosylceramide degradation, if any, is required in vivo, we feel that it is impossible to decide on which one of the purification methods currently used to recommend.

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Isolation and Analysis of Cell Walls from Plant Material

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I. INTRODUCTION: SOME GENERAL CONSIDERATIONS

Understanding of the role of plant cell wall carbohydrate polymers was restricted before 1965 by the following factors: (1) Most of the detailed

chemical work on cell wall polysaccharides was carried out by chemists, at a time when the significance of the localization of these polymers in the walls of various tissue types was only just being appreciated. The main emphasis was on the elucidation of the major structural features of a great variety of polysaccharides present in a range of plants. (2) The methods developed essentially for the isolation of cell wall polysaccharides from wood were applied to other tissues, indiscriminately, and little consideration was given to material lost in the extraction fluids (e.g., delignification liquors) and in the supernatants after precipitation with aqueous alcohol. (3) Mainly chemical fractionation techniques were used to resolve the polysaccharides; although these methods can give quantitative recovery of the polysaccharides, the resolutions obtained by such methods alone are inadequate. (4) Relatively large amounts of polysaccharides (>100 mg) were required for detailed structural work. (5) Enzymes capable of cleaving specific glycosidic linkages were not available commercially, and there were relatively few good methods for isolating the necessary enzymes. (6) Very little encouragement was given to work on the cell walls of edible plants, because cell walls and products derived from them were regarded as nutritionally unimportant.

The whole scene has, however, changed over the past 15–20 years, probably for the following reasons: (1) Increasing numbers of plant biochemists and plant physiologists have been studying cell walls, often in collaboration with cell wall and carbohydrate chemists. (2) There has been greater interest in the efficient utilization of cell walls from forages, grasses, and so forth, by ruminants. (3) Dietary fiber in food is considered to be more important. (4) There have been improved methods for the isolation and analysis of cell wall material (CWM) from starch- and protein-rich foods, and from suspension cultured tissues. (5) There are now better methods for resolving cell wall polymers by ion-exchange, cellulose and affinity chromatography, gel filtration, electrophoresis, and sedimentation analysis by ultracentrifugation. (6) It has become practicable to use highly purified enzymes to cleave specific glycosidic linkages in cell wall polymers. (7) There have been important recent developments in various techniques for identifying carbohydrate derivatives, for example, the use of improved methods of methylation analysis, coupled with gas liquid chromatography-mass spectrometry (GLC-MS) of the derived products, permits the scale of experiments to be reduced drastically so that only a few mg of material are necessary. (8) Partial acid hydrolysis, acetolysis, and purified enzymes have been used to degrade the polysaccharides (or their derivatives) into fragments that can be identified, after suitable derivatization, by GLC-MS or high performance liquid chromatography-mass spectrometry (HPLC-MS). This makes sequenc-