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Lipid Modification of Proteins

A Practical Approach

Edited by

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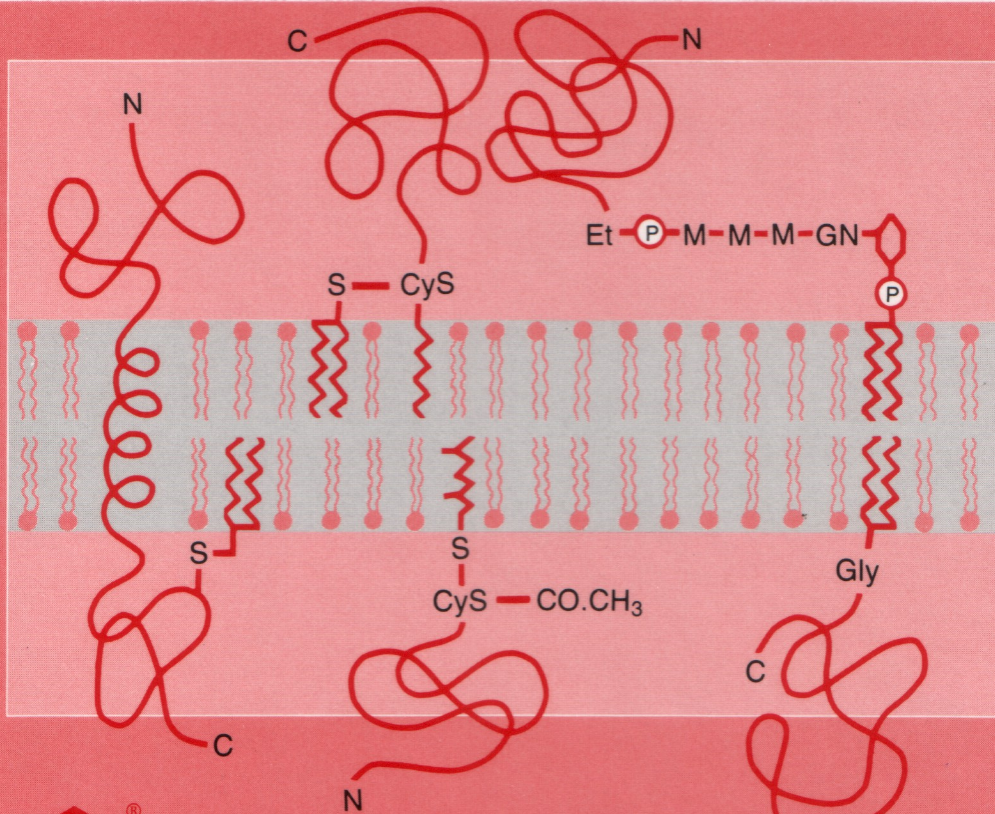


The Practical Approach Series

SERIES EDITORS: D. RICKWOOD and B. D. HAMES

7

Biosynthesis of glycosyl-phosphatidylinositol membrane protein anchors

MARK C. FIELD and ANANT K. MENON

1. Introduction

Different hydrophobic interactions are responsible for anchoring proteins to cell membranes. Classically, stretches of apolar amino acids in the protein itself are sufficiently hydrophobic to guarantee membrane association. In other cases membrane association is facilitated by fatty acids or complex lipids covalently linked to the protein. This chapter is concerned with the biosynthesis of a class of complex inositol-containing glycophospholipids (G-PIs) that serve as membrane anchors for a variety of eukaryotic cell surface proteins, including cell adhesion molecules, enzymes, complement regulatory proteins, lymphoid antigens, and protozoal coat proteins.

Much of the information on the structure and biosynthesis of glycolipid membrane anchors (*Figure 1–3*) has come from studies of the glycolipid-anchored variant surface glycoproteins (VSGs) of bloodstream forms of the parasitic protozoan *Trypanosoma brucei*. The parasites are easily obtained in large numbers (batches of 10^{10}–10^{11} cells) and have unique advantages as a model for the study of glycolipid anchor biosynthesis. Bloodstream trypanosomes devote a large fraction of their protein synthetic activity to producing VSG molecules (about 10^7 VSG molecules constitute the trypanosome's surface coat). Trypanosomes undergo profound developmental changes in the course of transmission from one mammal to another via an insect vector, the tsetse fly. The transformation from bloodstream trypanosomes to insect midgut stage cells (procyclic trypanosomes) can be achieved *in vitro*. Procyclics are easily cultured in the laboratory; they do not express VSG but, instead, express an equally abundant glycolipid-anchored cell surface protein termed procyclin or PARP (procyclic acidic repetitive protein) (1). Glycolipid precursors of the G-PI anchors of VSG and PARP have been isolated from both bloodstream and procyclic trypanosomes (*Figure 2*).

The purpose of this chapter is to describe techniques for G-PI labelling, extraction, and analysis that have been useful in studying glycolipid anchor

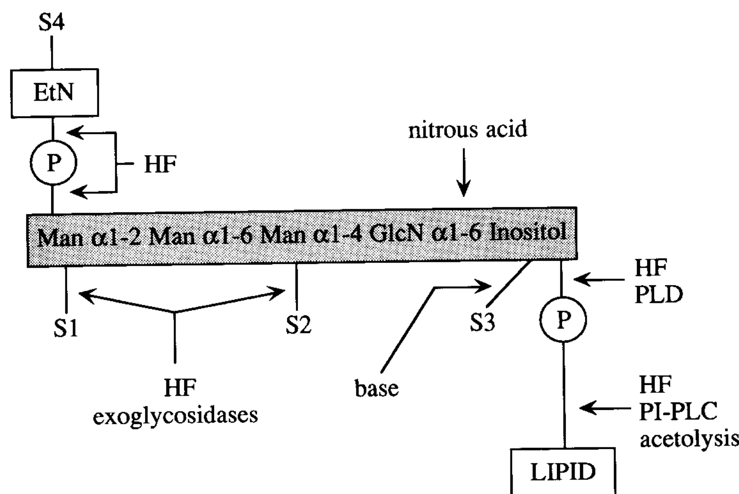


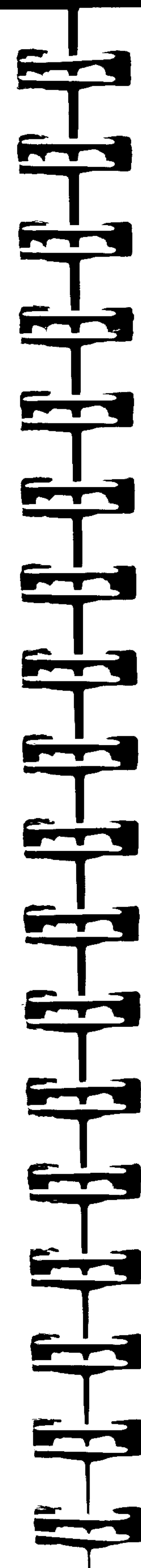
Figure 1. Glycosyl-phosphatidylinositol structure and cleavage sites.
The lipid moiety, phosphodiester-linked to inositol, can be dimyristoylglycerol [trypanosome VSG, (35)], 1-alkyl-2-acyl-glycerol [human erythrocyte acetylcholinesterase (E^{hu}AChE), (30), Leishmania PSP, (31)], or ceramide (32). S1, S2, and S3 represent substituents on the core glycan. Mannose is the only S1 substituent described thus far [Thy-1 anchor, (36)]. S2 substituents include ethanolamine-phosphate [Thy-1 (36), E^{hu}AChE (30)], *N*-acetyl-galactosamine [Thy-1 (36)], and galactose [trypanosome VSG 117 (16)]. The trypanosome G-PIs P2, P3, and PP1 (*Figure 2*) and the anchors of Leishmania PSP (31) and trypanosome VSG 118 (12) have no S1 or S2 substituents. S3 is an ester-linked fatty acid found in some G-PI structures: E^{hu}AChE (30) contains palmitic acid ester-linked to inositol, and the inositol residue of two trypanosome G-PIs (P3 and PP1, see *Figure 2*) can be labelled with [^{3}H]palmitic acid. S3 can be removed by base treatment; S1 and S2 may be removed by HF (in the case of S2 ethanolamine-phosphate) or exoglycosidases (depending on the substituent). In protein-linked G-PIs, the ethanolamine residue (EtN) is amide-linked to the carboxyl-terminal amino acid (S4) of the mature protein. The shaded box represents the glycan fragment released by HF dephosphorylation of the G-PI. Cleavages within the lipid moiety (for example, by phospholipase A_2 and base) are not shown.

biosynthesis in trypanosomes. The description focuses on free G-PIs (not attached to protein) isolated from biosynthetically labelled cells and from *in vitro* labelling reactions. With the exception of methods concerned with the growth and isolation of trypanosomes, it is expected that the labelling procedures and analytical techniques described in this chapter will be generally applicable to the study of G-PI biosynthesis in other cell types.

2. Metabolic labelling of trypanosomes

2.1 Growth and isolation of bloodstream trypanosomes

All strains of *T. brucei* must be considered as potential human pathogens; however, clones of *T. brucei brucei* are regarded as unlikely to be infective to



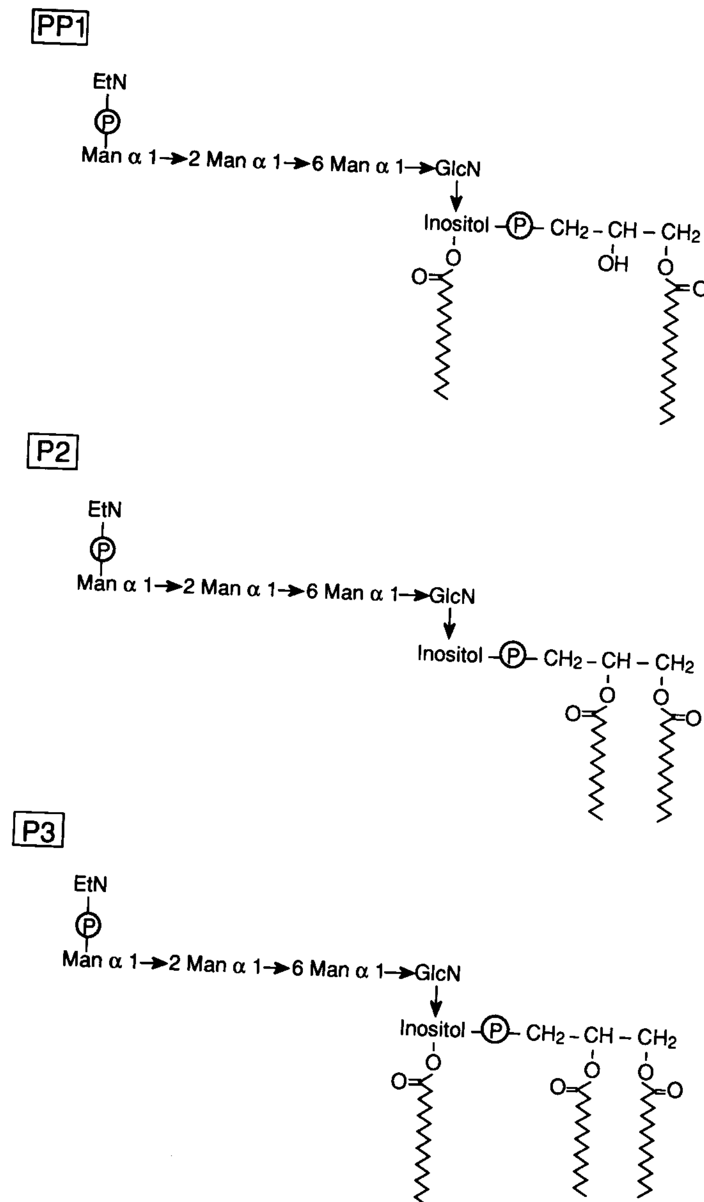


Figure 2. Structures of trypanosome glycosyl-phosphatidylinositols (PP1, P2, and P3). The structures are taken from Menon *et al.* (9), Mayor *et al.* (11, 12), and Field *et al.* (19). P2 and P3 are isolated from bloodstream trypanosomes; PP1 is the major G-PI lipid in procyclic trypanosomes. Both the glycerol-linked fatty acids in P2 and P3 are myristic acid. The glycerol-linked fatty acid in PP1 is stearic acid by radiolabelling. The inositol-linked fatty acid in both P3 and PP1 is palmitic acid by radiolabelling.

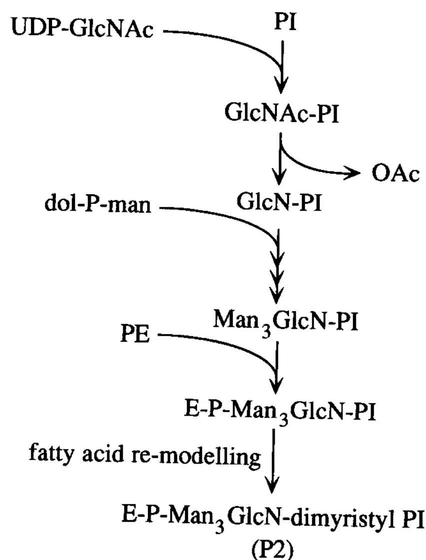


Figure 3. A model for glycosyl-phosphatidylinositol biosynthesis.
The figure represents the simplest model of G-PI biosynthesis (3, 10). The first committed step is the transfer of GlcNAc from UDP-GlcNAc to phosphatidylinositol (PI) to form GlcNAc-PI. GlcNAc-PI is then de-acetylated to give GlcN-PI (6). Three mannose residues are added sequentially from a lipid donor (dolichol-P-mannose) (37). The terminal ethanolamine residue is transferred from phosphatidylethanolamine (PE) (38). The completed G-PI structure then undergoes a series of fatty acid re-modelling reactions in which the glycerol-linked fatty acids are replaced by myristic acid (3, 7). Inositol acylation probably occurs early in the pathway [a G-PI similar to GlcN-acyl PI has been identified in yeast (39)]. The functional significance of inositol acylation is not clear, nor is the relation between inositol acylated G-PIs (such as P3 and PP1, *Figure 2*) and the G-PIs shown in the figure.

humans and have been widely used in laboratories without strict precautions. Nevertheless, it is prudent to observe elementary precautions (such as wearing gloves, no mouth pipetting, rinsing of used glassware before placing in regular glassware-washing containers, etc.) when working with these organisms. Bloodstream forms of *T. brucei brucei* can be stored essentially indefinitely in liquid nitrogen and can be grown to high yield (10^9 cells per millilitre of blood, roughly equivalent to the concentration of erythrocytes) in mice and rats. The trypanosomes can be easily isolated from the blood of infected rodents by centrifugation, followed, if necessary, by passage over a column of DEAE-cellulose (see *Protocols 1–4*).

Protocol 1. Cryopreservation of bloodstream trypanosomes

1. Collect blood from an infected mouse or rat (see *Protocol 2*). Transfer the

blood to a beaker on ice and add an equal volume of ice-cold dilution buffer[a] containing 20% (v/v) glycerol. Mix and keep on ice.

2. Take non-heparinized micro-haematocrit tubes (for example, Kimble #73811 disposable soda-lime glass micro-haematocrit tubes, approx. 7 cm long) and partly fill by dipping the tubes into the blood-dilution buffer mixture. Centre the blood in each tube leaving roughly 1.5 cm empty at each end, then seal the tube rapidly by introducing each end successively into a bunsen burner flame. Wait a few seconds to allow the tube to cool before dropping it into a beaker containing ice-cold water.
3. Wipe the tubes dry and put them in Nunc cryotubes. Include a slip of paper containing pertinent information in each cryotube.
4. Attach the cryotubes to a liquid nitrogen refrigerator plug (for example, Union Carbide BF-5 biological freezer) and cool slowly for 3 h in the vapour above the liquid nitrogen. Transfer the cryotubes to numbered canes and store immersed in liquid nitrogen.

[a] Dilution buffer: 5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4.7H_2O$, 20 mM Na_2HPO_4, 2 mM $NaH_2PO_4.2H_2O$, 20 mM glucose, pH 7.7.

Protocol 2. Infection of mice and rats

1. Withdraw a single haematocrit tube (*Protocol 1*) from liquid nitrogen storage (minimize warming of other stabilates), score, and snap off the ends, and expel the blood using a syringe pre-loaded with 1 ml dilution buffer (see *Protocol 1*) and fitted with a needle (23 or 25 gauge). It is important to wear a face-mask during this procedure since the haematocrit tubes occasionally explode.
2. Examine an aliquot of the sample (5–10 μl) in a light microscope to ensure that some trypanosomes are motile (the number of motile trypanosomes will increase as the sample warms up). Inject 200–300 μl into the peritoneal cavity of a mouse.
3. Parasitaemia may be monitored by cutting off the tip of the mouse's tail with a pair of scissors and milking a few drops of blood on to a glass slide; dilute the blood 1:200 in dilution buffer and count trypanosomes using a haemocytometer. The parasitaemia will reach $1–2 \times 10^9$ trypanosomes/ml of blood in about 3 days before the animal dies. For metabolic labelling purposes, parasites should be harvested at concentrations of 5×10^8/ml or less.
4. Anaesthetize the mouse by placing it in a covered glass beaker containing cotton soaked in ether or methoxyflurane (Metofane). Bleed the animal by cardiac puncture using a syringe pre-loaded with citrate-glucose

Protocol 2. *Continued*

anticoagulant (CGA),[a] or by opening the chest cavity and introducing 0.5 ml CGA before cutting the main dorsal vein and pipetting out blood as it fills the cavity. Transfer the blood to tubes (containing 1 ml CGA) on ice. Trypanosomes may be isolated by centrifugation and/or passage over DEAE-cellulose as described in *Protocol 4*. Approximately 10^9 trypanosomes may be obtained from an infected mouse.

5. If larger numbers of cells are required, the mouse blood should be used to infect one or more rats (after 3 days of infection, a rat will yield roughly 10^{10} trypanosomes). Count trypanosomes in the mouse blood and prepare a dilution in dilution buffer containing roughly 2.5×10^7 trypanosomes/ml. Inject a rat intra-peritoneally with 1 ml of the dilution and harvest trypanosomes after 3 days as described above.

[a] CGA: 0.1 M sodium citrate ($Na_3C_6H_5O_7.2H_2O$, 30 g/l), 0.04 M glucose (7.2 g/l), pH adjusted to 7.7. Freeze in 100-ml amounts. Store in a refrigerator during period of use and discard after two weeks.

Protocol 3. Preparation of DEAE-cellulose for trypanosome isolation

1. Take 1 kg Whatman DE52 in a beaker and add approx. 4 litres of 0.5 M HCl. Stir to disperse the cellulose and let stand for 30 min, stirring briefly every 10 min. Allow the cellulose to settle.
2. Remove most of the supernatant by aspiration and transfer the cellulose to a large plastic Buchner funnel lined with filter paper. Wash with distilled water until the effluent is about pH 4 (check with pH paper).
3. Return the cellulose to the beaker and proceed as for steps (1) and (2) using 0.5 M NaOH instead of HCl. Wash (using a Buchner funnel) until the effluent is about pH 8.
4. Transfer the cellulose to a beaker containing 1 litre separation buffer[a] (without glucose) and titrate to pH 8 with orthophosphoric acid (approx 9 ml required per 1 kg cellulose).
5. Wash (using a Buchner funnel) with separation buffer (without glucose) until the pH is identical to the original separation buffer (i.e. 8.0, checked accurately with a pH meter). Do not allow the cellulose to become dry. Transfer to a storage bottle with an equal volume of separation buffer (without glucose) plus 1 ml chloroform as a preservative. Store in a refrigerator or cold room.

[a] Separation buffer 44 mM NaCl, 57 mM Na_2HPO_4, 3 mM KH_2PO_4, 55 mM glucose. Check that the pH is 8.0 at 20 °C and adjust if necessary with NaOH.

Protocol 4. Isolation and purification of bloodstream trypanosomes

1. Bleed infected mice or rats as described in *Protocol 2*. Collect the blood in 16 × 125 mm disposable glass centrifuge tubes or 15-ml Corex tubes. Centrifuge the blood at 1500 *g* for 10 min at 4 °C: the trypanosomes will sediment as a dense white layer (the buffy coat) above the erythrocytes. Using a pipette, carefully remove the bulk of the supernatant plasma. Add up to 0.5 ml of separation buffer (*Protocol 3*) and resuspend the trypanosomes by stirring gently, taking care to avoid resuspending erythrocytes. Remove the trypanosomes and transfer to a fresh tube on ice. Buffy coat trypanosomes can be used directly (for metabolic labelling experiments) or after passage over a column of DEAE-cellulose as described below. If trypanosomes are being isolated from mouse blood, the centrifugation step can be omitted and whole blood can be passed directly over DEAE-cellulose.
2. Prepare a column of DEAE-cellulose (*Protocol 3*) using a fritted glass funnel or a syringe barrel plugged with cotton or glass wool; wash the column with at least 2 vol. of separation buffer to remove chloroform (it is best to pour and wash the column by gravity if there is time). Use a column of 10–15 ml to isolate trypanosomes in whole blood from one mouse; larger columns (30 ml) are required in order to run pooled buffy coats from 3–4 rats. Load the sample and elute with separation buffer. Blood cells are retained on the column while trypanosomes wash through as a visibly milky eluate. It is best to run the column in a cold room; if this is not possible, then care should be taken to collect the trypanosomes on ice.
3. The trypanosomes may be washed by centrifugation (4 °C, 1500 *g*, 10 min) and resuspension. It is important to keep them on ice, and to use them for metabolic labelling or membrane preparation as soon as possible (preferably within 1 h).

2.2 Metabolic labelling of bloodstream trypanosomes

Buffy coat or DEAE-purified bloodstream trypanosomes may be metabolically labelled with radioactive fatty acids, sugars, ethanolamine, inositol, or phosphate. Incubation for 1–2 h with precursors of high specific activity (^{3}H-labelled, 10–60 Ci/mmol) is sufficient to label G-PI species efficiently (see *Table 1*). The cells should be resuspended in the labelling medium (see *Protocol 5*) at a concentration no greater than 1×10^8/ml (usually 5×10^7/ml). Radiolabelled precursors (10–200 μCi/ml) should be added only after the cells have been pre-incubated for approx. 10 min at 37 °C and examined in a light microscope to assess viability (motility). Viability should also be checked periodically during the labelling incubation by withdrawing small aliquots

Table 1. Metabolic labelling of bloodstream and procyclic trypanosomes; incorporation of radioactivity into G-PI species

Radiolabel[a]	Concentration μCi/ml	P2[b]	P3[b] c.p.m./10^8 cells	PP1[c]
[^{3}H]myristic acid[d]	100–200	9×10^4	3×10^5	2×10^5
[^{3}H]palmitic acid[d]	100	4×10^3	3×10^5	ND[e]
[^{3}H]ethanolamine	25–50	5×10^3	3×10^4	9×10^4
[^{3}H]glucosamine	50	5×10^4	1×10^5	7×10^4
[^{3}H]mannose	25–50	8×10^3	2×10^4	1×10^5

[a] Specific activities of the radiolabelled compounds were in the range 20–55 Ci/mmol.
[b] GPI species in bloodstream trypanosomes (see *Figure 2*). Labelling was performed at 37 °C for 1–2 h with 0.5–1 × 10^8 cells/ml.
[c] GPI lipid in procyclic trypanosomes (see *Figure 2*). Labelling was performed at 27 °C for 4–16 h with 1–2 × 10^7 cells/ml.
[d] Trypanosomes elongate fatty acids; thus although both glycerol-linked fatty acids in [^{3}H]myristic acid labelled P2 and P3 are [^{3}H]myristic, the glycerol-linked fatty acid in [^{3}H]myristic acid labelled PP1 is [^{3}H]stearic acid and the inositol-linked fatty acid in [^{3}H]myristic acid labelled PP1 is [^{3}H]palmitic acid.
[e] Not determined.
(Data taken from refs 9, 12, and 19.)

(10 μl) of the cell suspension. To eliminate sugar labelling of dolichol-linked oligosaccharide species in the *N*-glycosylation pathway, labelling should be performed in the presence of tunicamycin (400 ng/ml, added from a 0.5 mg/ml stock solution in 95% ethanol) (note: the cells should be pre-incubated with tunicamycin for 30 min at 37 °C prior to addition of the radiolabel).

Protocol 5. Preparation of labelling media and metabolic labelling

1. Prepare the labelling medium: RPMI 1640 (Gibco or Specialty Media) supplemented with 5 μg/ml catalase (Boehringer Mannheim) and 7.1 mg/ml Hepes (buffered to pH 7.3). Except for metabolic labelling with radioactive fatty acids, the medium should also be supplemented with 3 mg/ml defatted bovine serum albumin (BSA) coupled to myristic acid at a molar ratio of 1:3.[a] For sugar labelling use glucose-free RPMI 1640 supplemented with 3 g/litre glycerol in addition to catalase, Hepes and BSA/myristic acid. For inositol labelling, use inositol-free RPMI 1640 supplemented as above.
2. Dry down the radiolabelled precursor (sugars, ethanolamine, or inositol) using a Speed-Vac evaporator (Savant Instruments), and resuspend in a small volume of labelling medium. Radiolabelled fatty acids should be coupled to defatted BSA at a molar ratio of 1:1 (see below) for addition to cells.[a] Labelling is typically carried out at 10–200 μCi/ml; the cost of the radiolabel usually limits the concentration range to 25–50 μCi/ml.

[a] To couple the fatty acids to defatted BSA dry the radiolabelled fatty acid or non-radioactive myristic acid in a Speed-Vac evaporator. Redissolve in a few drops of ethanol and add a solution of 1.8% defatted BSA: for radioactive fatty acids add an equivalent molar amount of BSA, for non-radioactive myristic acid add a threefold molar excess of BSA. Shake for 1 h at room temp. before use.

2.3 Culture and metabolic labelling of insect stage (procyclic) trypanosomes

Culture-adapted procyclic *T. brucei* can be grown at 27 °C in SDM-79 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco) and 7.5 μg/ml hemin (See *Protocol 6*) (2). Cells are usually maintained at a density of 5×10^5–1×10^7 parasites/ml and are best grown in polystyrene tissue culture flasks (for example, 25 cm^2, Corning), with the culture medium at a maximum depth of 0.5 cm. The caps should be tightly screwed on the flasks. Cells have a normal tendency to aggregate at low and high densities.

Protocol 6. Metabolic labelling of procyclic trypanosomes

1. Transfer the cultured cells to centrifuge tubes, pellet, wash three times with ice-cold phosphate-buffered saline (PBS) pH 7.5, and resuspend in labelling medium at 1–2 × 10^7 parasites/ml. Labelling is typically performed at 27 °C for 4–16 h using 10–200 μCi/ml of radiolabelled precursor (see *Table 1* for incorporation of radioactivity into PP1, the major G-PI in procyclic trypanosomes).
2. Labelling media. For sugar labelling use glucose-free RPMI 1640 supplemented with 7.1 mg/ml Hepes (pH 7.3), 0.6 mg/ml sodium citrate, 0.6 mg/ml L-proline, 7.5 μg/ml hemin and 1 mg/ml defatted BSA.[a] For ethanolamine labelling use SDM-79 supplemented with 10% FCS and 7.5 μg/ml hemin; for fatty-acid labelling use SDM-79 with 7.5 μg/ml hemin and 0.5 mg/ml bovine siderophillin; for inositol labelling use inositol-free RPMI 1640 supplemented as for sugar labelling. Prepare radiolabelled precursors as described in *Protocol 5*.

[a] If necessary, cells can be labelled in the presence of tunicamycin (1–2 μg/ml). Pre-incubate the cells with tunicamycin for 1 h at 27 °C prior to addition of radiolabel.

3. Cell-free synthesis of glycosyl-phosphatidylinositol lipids

Crude membrane preparations are capable of synthesizing G-PIs. The methods described in *Protocols 7* and *8* have been instrumental in outlining a

pathway for G-PI biosynthesis (*Figure 3*), and demonstrating the transfer of precursor glycolipids to protein acceptors (*Protocol 9*). Representative labelling data are summarized in *Table 2*. An essentially identical protocol has been successfully used to label G-PI *in vitro* in procyclic lysates (42).

Table 2. *In vitro* labelling of glycosyl-phosphatidylinositols (data from refs. 10 and 37 and unpublished results)

Radiolabel	Lipid species P2	P3	I	II	III	IIIC	IV	V	VI
					cpm × 10^{-3}				
Lysates									
GDP-[^{3}H]Man[a]	220	100	9.5	61.8	16	2.3	NL	NL	1.1
CDP-[^{3}H]EtN[b]	7.7	4.3	NL	NL	NL	NL	NL	NL	NL

NL-not labelled

Lipids are as follows: I, Man_3GlcN-PI; II, Man_2GlcN-PI; III, a mixture of Man_1GlcN-PI and Man_3GlcN-inositol acylated PI; IIIC, Man_2GlcN-inositol acylated PI; IV, GlcNAc-PI; V, GlcN-PI; VI, trypanosome dolichol-P-mannose.

[a] GDP-[^{3}H]Man (1.6×10^6 c.p.m.) was incubated with trypanosome lysates (8×10^7 cell equivalents, reaction volume 125 μl) in the presence of 1 mM non-radioactive UDP-GlcNAc for 40 min at 37 °C. See TLC profile of labelled lipids in *Figure 4*.

[b] CDP-[^{3}H]EtN (1.4×10^7 c.p.m.) was incubated with trypanosome lysates (1.2×10^8 cell equivalents, reaction volume 250 μl) for 30 min at 37 °C. Approx. 1.2×10^6 c.p.m. was recovered as phosphatidylethanolamine.

Protocol 7. Preparation of a trypanosome lysate

The preparation is essentially as described by Masterson *et al.* (3).

1. Isolate trypanosomes as described in *Protocol 4*.
2. Incubate the cells with tunicamycin (400 ng/ml) in RPMI 1640 medium for 30–60 min at 37 °C. Pellet the cells by centrifugation (wash once with ice-cold saline if necessary) and lyse by vortexing the pellet briefly with ice-cold water (containing 0.1 mM TLCK and 1 μg/ml leupeptin) at a concentration of 10^9 cells/ml.
3. Confirm the lysis by examining an aliquot of the preparation under a light microscope, then add an equal volume of ice-cold buffer (100 mM Na-Hepes (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM TLCK, 1 μg/ml leupeptin, 20% glycerol).
4. Snap-freeze 1 ml aliquots (5×10^8 cell equivalents) in Eppendorf tubes and store at −70 °C.

Protocol 8. *In vitro* labelling with trypanosome lysate

1. Rapidly thaw the frozen lysate immediately prior to use, and wash three times in ice-cold wash buffer[a] by centrifugation (Sorvall SS-34 rotor, 12 000 *g*, 10 min, 4 °C) and resuspension (10 ml wash buffer per millilitre of thawed lysate). Resuspend the membranes in incubation buffer (wash buffer + 5 mM $MnCl_2$) at 5×10^8 cell equivalents/ml.
2. Incubate the lysates ($0.5-1 \times 10^8$ cell equivalents) with GDP-[^{3}H]mannose, UDP-[^{3}H]GlcNAc, CDP-[^{3}H]ethanolamine (1–10 μCi, specific activity 15–30 Ci/mmol) or other radiolabelled precursors in the presence of 1 mM ATP, 1 mM CoA, 1 mM DTT, and 0.2 μg/ml tunicamycin (added as a solution of 95% ethanol, final concentration of ethanol $< 0.01\%$). Incubate for 15–90 min at 37 °C. The total volume of the reaction mixture is typically 100–250 μl.
3. The radiolabelled precursors are frequently supplied as ethanol:water mixtures and must be dried in the reaction tubes prior to addition of the other components. For UDP-[^{3}H]GlcNAc labelling, the reactions must be supplemented with non-radioactive GDP-mannose (1 mM) in order to synthesize labelled G-PIs other than GlcN-PI. Also, G-PI labelling via GDP-[^{3}H]mannose is considerably enhanced if labelling is performed in the presence of UDP-GlcNAc (1 mM).
4. Terminate labelling by adding ice-cold chloroform:methanol (1:1, v/v) to achieve a final composition of $CHCl_3$:CH_3OH:H_2O (10:10:3, v/v/v) (*Protocol 10*).

[a] Wash buffer; 50 mM Na–Hepes (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM TLCK, 1 μg/ml leupeptin

Protocol 9. Transfer of G-PI precursors to protein acceptors

The experimental procedure described here has been used to demonstrate the transfer of P2 and P3 (*Figure 2*) to endogenous VSG acceptors (4).

1. Incubate lysates for 15–90 min with 1–2 μCi GDP-[^{3}H]mannose or UDP-[^{3}H]GlcNAc as described in *Protocol 8* (include the appropriate nucleotide sugar (for example, 1 mM UDP-GlcNAc for GDP-[^{3}H]mannose labelling), 10 mM creatine phosphate and 8 U/ml creatine kinase in the reaction mixture).
2. At the end of the incubation, transfer an aliquot (50%, the remainder is taken for lipid extraction) of the reaction to an Eppendorf tube containing an equal volume of solubilization buffer (0.375 mM Tris–HCl, pH 6.8, 4% SDS) and heat at 100 °C for 5 min.

Protocol 9. *Continued*

3. Boil an aliquot of the solubilized reaction mixture with 0.2 vol. of 50% glycerol, 0.5 M DTT, 0.25% bromophenol blue, and analyse the sample by SDS-PAGE.
4. Labelled proteins (a single prominent VSG band approx 55 kd in the case of lysates prepared from bloodstream trypanosomes) can be located by fluorography. Labelled protein bands may also be excised from the gel and dissolved in 80% Solvable (Du Pont-New England Nuclear) at room temp. for 14 h, neutralized with glacial acetic acid, mixed with scintillation cocktail, and taken for liquid scintillation counting. For other analyses, the proteins may be electroeluted from the gel and treated as required (for example, preparation of a neutral glycan as described in Section 6).

4. Lipid extraction

G-PIs and other lipids may be extracted from metabolically labelled cells or *in vitro* labelling reactions by one of the following procedures. A TLC profile of chloroform:methanol:water-extracted [^{3}H]mannose-labelled G-PI species (*Protocol 10*) from an *in vitro* labelling reaction is shown in *Figure 4*.

Neutral lipids, phospholipids, some dolichol-linked sugars (for example, dolichol-P-mannose, dolichol-PP-GlcNAc$_{1-2}$) and non-ethanolamine-phosphate-containing G-PIs are soluble in chloroform:methanol (2:1, v/v). Extracing labelled cells or membranes with chloroform:methanol prior to chloroform:methanol:water extraction (*Protocol 11*) has the advantage of separating these lipids from mature ethanolamine-phosphate-containing G-PIs (such as P2, P3, PP1; see *Figure 2*) which are soluble in chloroform:methanol:water.

Protocol 10. One-step extraction of total lipid material

1. Centrifuge the labelled cells or membranes and resuspend the pellet in 1–5 ml ice-cold chlorofom:methanol:water (10:10:3, v/v/v). It is best to use a glass Pasteur pipette to disperse the pellet. Increase the volume of chloroform:methanol:water for larger samples.
2. Centrifugation prior to extraction has the advantage of removing unincorporated radiolabel, but in many cases (particularly for *in vitro* labelling reactions), it is simpler to add ice-cold chloroform:methanol (1:1, v/v) to give a final composition of chloroform:methanol:water (10:10:3) (1.7 ml chloroform:methanol (1:1) is required for a 250 µl aqueous reaction mixture).[a]

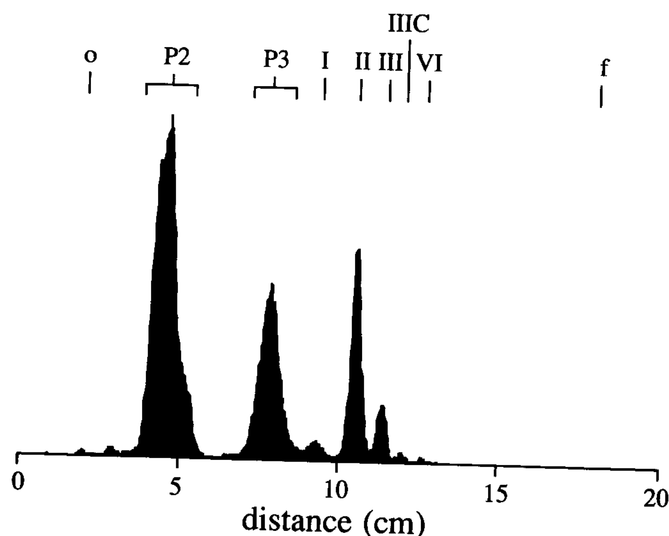


Figure 4. Thin-layer chromatogram of *in vitro* labelled glycosyl-phosphatidylinositols. See *Protocols 7* and *8* for detailed procedures. Trypanosome lysates (8×10^7 cell equivalents) were incubated with GDP-[^{3}H]mannose (1.6×10^6 c.p.m.) for 40 min at 37 °C. The total volume of the reaction was 125 µl. At the end of the incubation period, the reaction was stopped by adding 125 µl ice-cold incubation buffer and 1.7 ml ice-cold chloroform:methanol (1:1, v/v). The resulting chloroform:methanol:water, (10:10:3, v/v/v) extract was dried and partitioned between water and *n*-butanol. Lipids in the butanol phase were chromatographed on a silica 60 glass-backed thin-layer plate using solvent system B (chloroform:methanol:water 4:4:1, v/v/v, *Table 3*). The chromatogram shown in the figure was obtained using a TLC radioscanner and the vertical axis represents the scanner detector response. The broad regions marked P2 and P3 indicate the migration of fatty acid remodelling intermediates as well as the mature dimyristyl lipids. Other lipids are as follows: I, Man_3GlcN-PI; II, Man_2GlcN-PI; III, a mixture of Man_1GlcN-PI and Man_3GlcN-inositol acylated PI; IIIC, Man_2GlcN-inositol acylated PI; VI, trypanosome dolichol-P-mannose (trypanosome dolichols contain only 11 and 12 isoprene units; ref. 43). o = origin, f = solvent front.

3. For separation of lipids from non-lipid (water-soluble) material in the extract, dry the chloroform:methanol:water extract in a Speed-Vac evaporator. Add 750 µl each of water and *n*-butanol to the dried residue and mix thoroughly using a vortex mixer. Separate the phases by centrifugation (1000 *g*, 5–10 min),[b] transfer the butanol-rich upper phase to a fresh tube and re-extract the lower aqueous phase with water-saturated *n*-butanol. Pool the butanol phases and 'back-extract' with water. All G-PI species are quantitatively recovered in the butanol phase.

[a] Recovery of lipids is quite high (> 95%) and it is not necessary to repeat the extraction procedure for analytical samples.

[b] Let the sample stand at room temp. and centrifuge again if a sharp separation of phases is not achieved.

Protocol 11. Sequential extraction

1. Add 1.5 ml ice-cold chloroform:methanol (2:1 v/v) per 50 μl aqueous reaction mixture. Use enough chloroform:methanol to minimize the effect of water in cell pellets and in *in vitro* labelling reactions. Repeat the extraction with chloroform:methanol (less chloroform:methanol may be used for the second extract), and pool the extracts.
2. Aqueous contaminants in the chloroform:methanol extract may be removed by Folch washing. Add 4 mM $MgCl_2$ (chloroform:methanol extract: $MgCl_2$ = 5:1, v/v), vortex vigorously, and separate the phases by centrifugation. Remove the upper phase. Wash the lipid-containing lower phase with artificial upper phase (chloroform:methanol:water/1 M $MgCl_2$, 6:96:94:0.336, v/v/v/v) several times before lipid analysis.
3. Extract the partially delipidated residue with chloroform:methanol:water (10:10:3, v/v/v) and partition the extract between water and butanol as described in *Protocol 10*.

5. Extraction and analysis of water-soluble metabolites

Radiolabelled water-soluble metabolites can be recovered in perchloric acid extracts of labelled cells (*Protocol 12*), or in the aqueous phase obtained after drying and butanol/water partitioning of chloroform:methanol:water extracts (*Protocol 10*). The second procedure has the obvious advantage that both water-soluble metabolites and lipids can be obtained from a single extract.

Ethanolamine and serine metabolites can be easily resolved by stepwise elution from Dowex AG1X8 anion-exchange resin (formate form, Rio-Rad) (*Protocol 13*; method adapted from ref. 5). Perchloric acid extracts or chloroform:methanol:water-derived aqueous phases may also be analysed by TLC on cellulose sheets or by descending paper chromatography. Although TLC is quicker, large samples with significant non-radioactive impurities (salts, etc.) can only be conveniently analysed by paper. See *Table 3* for suitable TLC and paper chromatography systems.

Protocol 12. Perchloric acid extraction

1. Centrifuge the labelled cells (for example approx. 10^8 cells from a metabolic labelling experiment) and wash once with ice-cold fresh medium.
2. Resuspend the cell pellet in 1 ml ice-cold 0.9 M perchloric acid (use more perchloric acid or repeat the extraction several times if necessary).

3. On ice, add a few drops of phenol red and neutralize the extract using KOH; mix the solution frequently during the neutralization process (use a vortex mixer) and avoid over-titration. The dye will turn yellow before turning blue-red at the end of the titration. Check the pH with pH paper; let the solution stand for 30 min on ice and check the pH again if necessary.
4. Centrifuge to remove cell debris and store the supernatant extract at −20 °C until required for analysis.

Protocol 13. Dowex AG1 separation of ethanolamine and serine metabolites

1. Pack a 1 ml column of Dowex AG1X8 resin (formate form) in a syringe barrel plugged with glass wool. Load the sample in 20–100 μl.
2. Elute stepwise with water, 0.049 M formic acid, 0.147 M formic acid, and 0.49 M formic acid.

Serine and ethanolamine are eluted in water, ethanolamine phosphate with 0.049 M formic acid, and CDP-ethanolamine with 0.147 M formic acid. Analyse fractions by TLC (*Table 3*).

Table 3. Solvent systems for thin-layer chromatography

System	Solvent system (v/v)	Plate[a]	Comments[b]
A	$CHCl_3/CH_3OH$/30%NH_3/ 1M NH_4Ac/H_2O (180:140:9:8:23)	Si50000	Separation of polar glycolipids, for example, P2. Peaks are broad, and resolution is less than that for system B. Note that the lipophilic headgroup generated by G-PI-PLD treatment of P3 or PP1 has an R_f of ~0.45 in this system.
B	$CHCl_3/CH_3OH/H_2O$ (4:4:1) or (10:10:3)	Si60	Good resolution of all GPI species and phospholipids. The lipophilic head group generated by PLD treatment of P3 or PP1 does not migrate in this system.
C	$CHCl_3/CH_3OH/H_2O$ (65:25:4)	Si60	Separation of phospholipids (see also system K)
D	Petroleum ether/diethyl-ether/HAc (80:20:1) or (70:30:2)	Silica G	Analysis of di- and mono-acylglycerol, and di- and mono-acylglycerol acetates.
E	$CHCl_3/CH_3OH$/90% formic acid (50:30:7)	Si60	Analysis of phosphatidic acids and other phospholipids.
F	CH_3CN/HAc (1:1)	C18RP	Analysis of fatty acid methyl esters Radioactivity detection by scanner is poor.

Table 3. *Continued*

System	Solvent system (v/v)	Plate[a]	Comments[b]
G	$CHCl_3/CH_3OH$/30% NH_3 (65:25:5)	Si60	For assessing efficiency of methylation of fatty acids. Fatty acid methyl esters run at the front, fatty acids at R_f 0.45. Also for resolution of phospholipids (not as good as system K).
H	$CHCl_3/CH_3OH$ (9:1)	Si50000	Analysis of ceramides.
I	1-Butanol/HAc/H_2O (5:2:3)	Cellulose	Analysis of metabolites of ethanolamine and serine and resolution of ethanolamine from dansyl-ethanolamine which runs close to the front, ethanolamine has an R_f of ~0.65.
J	Benzene/acetone/H_2O/30% NH_3 (100:400:6:3)	Si60	For separation of O-methylated mannose derivatives.
K	$CHCl_3/CH_3OH$/HAc/H_2O (25:15:4:2)	Si60	Good resolution of phospholipids and non-ethanolamine-phosphate containing GPIs. Polar glycolipids (for example, P2, P3) remain at or close to origin.
L	$CHCl_3/CH_3OH$/1 M NH_4OH (10:10:3)	Si60	For resolving GPI-species, including GlcN-PI from GlcNAc-PI.
M	$CHCl_3/CH_3OH$/30% NH_4OH (65:35:5)	Si60	As system L.
N	$CHCl_3/CH_3OH$/1 M HCl (10:10:3)	Si60	As system L.
O	Ethanol/1 M NH_4Ac (pH 7.5) (5:2)	Paper[c]	Resolution of mannose, mannose phosphate, and nucleotide sugars
P	Ethanol/l M NH_4Ac (pH 3.5) (5:2)	Paper	Resolution of GlcN, GlcNAc, GlcNAc-P, and UDP-GlcNAc.
Q	*n*-Butanol/HAc/H_2O (5:2:3)	Cellulose	Ethanolamine and metabolites. Note that Serine and EthN-P co-migrate.

[a] Si50000, Si60 and C18 reverse-phase glass-backed plates can be obtained from Merck. The normal phase plates should be activated at 120 °C for at least two hours prior to use. High performance RPHPTLC C18 plates provide better resolution than the standard plates, but the R_f is decreased so that C18:0 chromatographs with an R_f of only 0.2. Plastic-backed cellulose plates can be obtained from Eastman Kodak (Kodak Chromagram sheet 13254 or 13256).
[b] Suggested applications and points to note. R_f values for some lipids and fragments from *T. brucei* on some of the systems are given in *Table 4*.
[c] Suitable paper for chromatography is Whatman 3MM. Strips are developed as descending paper chromatograms, with a minimum length of 40 cm to provide sufficient resolution.

5.1 Analysis of sugar metabolites by anion-exchange chromatography

Mannose and glucosamine metabolites can be analysed by TLC or paper chromatography as described above, or by anion-exchange chromatography

on a Dionex HPLC system using program III or IV (see *Table 7* for details). In addition, anion exchange on Mono-Q using the conditions described by Doering *et al.* can be used for partial separation of glucosamine metabolites (6).

6. Methods for structural analysis of glycosyl-phosphatidylinositol lipids

6.1 Identification of glycosyl-phosphatidylinositol

Several criteria can be used to identify species containing the glycosyl-phosphatidylinositol core structure. The analytical approach presented in this chapter involves *in vivo* and *in vitro* labelling with G-PI components (for example, mannose, ethanolamine; see *Tables 1* and *2*), and specific cleavage reactions, especially those involving phospholipases (such as phosphatidyl-inositol-specific phospholipase C) and nitrous acid.

Identification by incorporation of a radiolabel clearly relies on the ability to separate the components of interest from other labelled species. The reader is directed to *Table 3* for a list of useful TLC systems, and should also consult original papers (3, 7–12) for examples of actual separations (see also *Figure 4*).

Metabolic labelling with [^{3}H]ethanolamine is probably the most informative single labelling experiment to perform, as few components other than phosphatidylethanolamine and G-PIs are labelled; phosphatidylethanolamine can be separated from polar G-PI lipids by a differential extraction protocol (*Protocol 11*) and by TLC (*Table 4*). The interpretation of sugar labelling experiments can be more complex. Identification of G-PIs labelled with mannose and glucosamine can be confused by the presence of dolichol-linked oligosaccharide species (dolichol-PP-GlcNAc$_{1-2}$Man$_{1-9}$). These glycolipids can be discriminated from authentic G-PI species by sensitivity of the labelling to tunicamycin (3, 11), or by release of the radiolabelled glycan as an aqueous soluble fragment with mild acid (*Table 5*). Labelling with [^{3}H]inositol, while diagnostic for G-PI structures, is not always successful. In Leishmania, toxoplasma, and yeast (13–15), [^{3}H]inositol incorporation into G-PI is highly efficient, but in *T. brucei* only phosphatidylinositol is labelled, and more polar species are not seen. However, the presence of inositol in the candidate G-PI can be probed with enzymes specific for inositol or inositol-containing glycans (for example, PI-PLC or G-PI-PLD; see Chapters 5 and 6).

Incorporation of [^{3}H] fatty acids is less informative for initial identification purposes, but is very important for full structural analysis. As G-PI species are comparatively minor membrane components, even in *T. brucei*, extracts labelled with radioactive fatty acids mostly contain labelled phospholipids. The two-step extraction protocol is very useful in this instance, since the input radioactivity and labelled phospholipids are removed in the first step.

However, a number of polar species, for example CDP-diglyceride and fatty acyl-CoA are included in the second extract, and these may confuse the labelling profile. Fatty acyl–CoA can be hydrolysed by treatment with 100 mM DTT (60 °C, 1 h).

Table 4. Thin-layer chromatography: R_f values[a]

System	Compound (R_f)
A	P2(0.42), P3(0.55), PP1(0.43), PLD-released lipophilic P3 headgroup (0.43).
B (10:10:3)	P2(0.34), P3(0.51), lysoP2(0.25), P2′(0.44), P3′(0.53), M_3NPI(0.56), M_2NPI(0.59), M_1NPI(0.62), M_2N(acyl)PI (0.65), PP1(0.43), PI(0.63), lipophilic P3 headgroup (PLD released) (0.05), Dol_t-P-M(0.73).
B (4:4:1)	P2(0.16), PP1(0.27), P3(0.36), P2′(0.19), PI(0.84), lysoPA (0.56), PA(0.72), P3-headgroup (PLD released) (0.03), M_3NPI(0.45), M_2NPI(0.52), M_3N(acyl)-PI(0.58), M_1NPI (0.58), Dol_t-P-M(0.66), M_2N(Acyl)PI(0.62).
K	M_3NPI(0.08), M_2NPI(0.15), M_3N(Acyl)PI(0.23), M_1NPI (0.23), GlcNAcPI(0.47), NPI(0.53), Dol_t-P-M(0.66), PP1/P2/ P3 (origin), PC(0.24), PI(0.55), PS(0.55), PE(0.68), PA(0.83)
J	2,3,4,6-tetra-O-methyl-mannose (terminal) (0.57), 3,4,6-tri-O-methyl-mannose(2-linked) (0.22), 2,3,4-tri-O-methyl-mannose (6-linked) (0.30).

R_f values are intended as a guide only, and should be used only in conjunction with standard compounds. Migration positions can alter by > 10% with different equilibration times of TLC tanks prior to chromatography.

Abbreviations: M = mannose, N = GlcN, PI = phosphatidylinositol, PS = phosphatidylserine, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PA = phosphatidic acid, Dol_t-P = trypanosome dolichol phosphate.

[a] Data are given for representative TLC separations performed in our laboratory using the systems described in *Table 3*.

Table 5. Conditions for commonly used chemical modifications in G-PI structure analysis

This table is provided as a condensed reference for many of the protocols utilized in G-PI structural studies. For fuller discussions see ref. 9–12, 19, and 37.

Protocol for Table 5:

1. Dry the sample in an Eppendorf microcentrifuge tube in a Speed-Vac. Include *relevant* positive and negative controls along with the sample being analysed.
2. Add the specified volume of the reagent (**Reagent**) given below to the tube for the desired reaction (**Reaction**), and incubate under the conditions described (**Conditions**).
3. After this time, the sample is freed from reagents and worked up by one of the procedures A–D (**Work up**) as specified in the table.
4. Additional information is given in the footnotes and in the text of Section 6.

Table 5. *Continued*

Reaction	**Reagent** Composition/volume	**Conditions** Temp (°C)/time (h)	**Work up**
Nitrous acid[a]	200 mM sodium acetate pH 3.7 + 250 mM sodium nitrate + 0.1% Nonidet P–40/400 μl	RT/12.0[b]	A
Base; mild	30% NH_4OH/CH_3OH (1:1, v/v)/200 μl	37/4.0	B
Base; strong	50 mM KOH in 90% CH_3OH (aq)/200 μl	RT/0.5	B
Hydrofluoric acid[c]	50% HF(aq)/50 μl	0/60.0	C
Acid; mild[d]	500 mM HCl + *n*-propanol/500 + 500 μl	55/0.25	B
Acid; strong	4M TFA/200 μl	80/6.0	B
Acetolysis	acetic acid + acetic anhydride (3:2, v/v)/200 μl	105/6.0	B
N-Acetylation	saturated sodium bicarbonate/ 100 μl+ 2 × acetic anhydride/5μl[e]	RT/1.0	D

Work up conditions

A: Terminate reaction by addition of 5 μl 6M HCl. For water-soluble starting material, desalt by passing the products through a column containing 200 μl AG3(OH^-) and eluting with three column volumes of water. For lipid samples, add *n*-butanol (500 μl), vortex to mix, and separate the phases by centrifugation; analyse the butanol and aqueous phases separately.
B: Evaporate the reaction mixture to dryness in a Speed-Vac, and partition products between water and butanol; fatty acids (released by base treatment of fatty-acid-labelled GPIs) can also be extracted with hexane or toluene.
C: Neutralize reaction with saturated LiOH (see *Protocol 17* for full experimental details).
D: Pass reaction mixture through a column of 500 μl AG50(H^+) and elute with three column volumes of water. If a lipid is being *N*-acetylated, recover the product by butanol/water phase separation as described for work up 'A'.

[a] The reaction is very sensitive to pH, and therefore it is recommended that the pH of a 1 μl aliquot of the reaction mixture is checked with pH paper before the incubation. Adjust with acetic acid or dilute NaOH as necessary. Detergent in the incubation is only necessary for deamination of lipids and should be left out when water-soluble materials (for example, dephosphorylated glycans) are to be deaminated.
[b] The deamination stains the plastic a brown colour during the incubation. This should happen, and is a good indication that nitrous acid has been generated during the incubation.
[c] The HF reagent is highly reactive, and must not be allowed to warm. Complete hydrolysis of all glycosidic linkages will result if the sample is allowed to warm whilst in the presence of the HF reagent. See *Protocol 17* for details.
[d] Under these conditions dol-P linked glycans are released with > 85% efficiency, whilst little hydrolysis (< 25%) of G-PIs is seen.
[e] Add the first aliquot of acetic anhydride at the start of the reaction, and the second after 10 min. Detergent (for example, 0.1% Nonidet P-40) may be included in the reaction mixture if a lipid sample is to be N-acetylated.
RT = room temp.

It is important to correlate labelling information with data on purity of a given peak as visualized by TLC. This is especially important if data obtained with more than one radioactive compound are to be combined. Co-chromatography on at least two different TLC systems (ideally three) is an absolute minimum when assigning two radiolabelled components to the same molecule.

A lipid species may be tentatively classified as a G-PI based on incorporation of several radiolabelled compounds. However, further evidence is required to confirm the assignment. First-order structural data may be obtained by PI-PLC or G-PI-PLD cleavage (see *Table 6* for reaction conditions). PI-PLC will cleave phosphatidylinositol-containing lipids, and will release the headgroup as a water-soluble species. Substitution of the inositol by fatty acid prevents the action of PI-PLC, but the G-PI-PLD from rabbit serum will still act to cleave the molecule (12). In this case the headgroup is lipophilic, and cleavage can be assessed by chromatography on system B (*Tables 3* and *4*).

Table 6. Conditions for enzyme digestions used in G-PI structure analysis

Protocol for Table 6:

1. Dry the sample in microcentrifuge tube using a Speed-Vac.
2. Add the relevant buffer (A–F) and an aliquot of the enzyme stock to give the final concentration specified in the table. If samples are aqueous the detergent can be omitted.
3. Incubate for 12 h at 37 °C. Terminate the lipase digestions by the addition of 10 µl glacial acetic acid, or, for the glycosidases, boil for 2 min. Note that the glycosidase conditions are given for free glycans and may need adjustment for lipid- or protein-linked substrates.
4. Partition the digestion products with butanol (*Protocol 14*) if required.

Enzyme	Reaction Volume (µl)	Enzyme Concentration (U/ml)	Buffer	Source[a]
Lipases				
B. thuringiensis PI-PLC	100	1	A	B
Rabbit serum GPI-PLD	100	1 µl[b]	A[c]	Serum[d]
C. adamanteus PLA_2	100	250	A[c]	S
Sphingomyelinase (human placental or *S. aureus*)	400[e]	2.5	F	B
Glycohydrolases				
S. plicatus Endoglycosidase H	50	0.01	B	B, OG
C. ensiformis α-mannosidase	30	50	C	S, OG

Table 6. *Continued*

Coffee bean α-galactosidase	15	5	D	B, OG
A. phoenicus α-mannosidase	20	2	D	K, OG
S. pneumoniae ß-hexosaminidase[g]	20	0.3	B	B
Bovine epididymal ß-galactosidase	25	2	E	B

Buffer systems

A: 100 mM Tris–HCl, pH 7.4, 0.1% sodium deoxycholate
B: 100 mM citrate-phosphate buffer, pH 6.0
C: 100 mM citrate-phosphate buffer, pH 4.5
D: 100 mM sodium acetate, pH 6.0
E: 100 mM citrate-phosphate buffer, pH 3.5
F: 20 µl $CHCl_3$, 10 µg Triton-100, 300 µl diethylether, 20 µl $MgCl_2$ and 40 µl buffer (1 M sodium acetate pH 5.2 [human], 1 M Tris–acetate pH 7.6 [*S. aureus*]).

[a] Recommended commercial sources of the enzymes at a suitable purity are given: B = Boehringer Mannheim, S = Sigma, K = Seikagaku Kogyo Co., OG = Oxford Glycosystems.
[b] Actual activity not assayed. Add the volume of rabbit serum to the sample in buffer. Note that the titre of this enzyme is variable between different individuals and therefore each batch should be assayed before use.
[c] Supplement buffer A with 2.5 mM $CaCl_2$.
[d] G-PI-PLD is an unstable enzyme. It is recommended that serum is stored as small aliquots at −80 °C, and thawed once for use. After thawing the serum can be stored at +4 °C for up to one week.
[e] Digest should be stirred during the incubation, and the ceramide extracted with diethylether after the reaction.
[f] Activity specified in mg/ml.
[g] Under these conditions the enzyme shows no sensitivity towards the 'bisect' GlcNAc.

Nitrous acid treatment converts the nonacetylated glucosamine in the core glycan to 2,5-anhydromannose (16), and cleaves the glucosamine-inositol bond to release a water-soluble headgroup. Conditions for this reaction are given in *Table 5*. The reaction does not always go to completion, and a 80% release of the headgroup of a purified lipid over the control is a respectable result. The efficiency of the reaction can be increased by a second addition of 250 mM sodium nitrite after 12 h to the reaction vessel.

Treating whole extracts with PI-PLC, G-PI-PLD and nitrous acid is an efficient way to screen labelled material; TLC analysis of the extract following treatment will identify which species are of interest for further study. Detergent from the enzyme digestion buffer can cause problems with the TLC analysis; chromatograph as small an aliquot of the reaction as possible in order to minimize this problem.

In most cases, the cleavage reactions can be processed by butanol partitioning (*Protocol 14*). This technique is extremely useful for analysis of radiolabelled extracts and quantification of cleavage reactions, as well as for preparation of fragments for subsequent analysis (see also Chapter 6).

Protocol 14. Butanol partitioning for analysis of G-PI cleavage

Reactions are conveniently performed in Eppendorf microcentrifuge tubes.

1. Following the reaction (deamination, PI-PLC digestion, etc.), acidify the mixture if necessary by the addition of 5 μl acetic acid, and adjust the volume to 500 μl with distilled water.
2. Add 500 μl of water-saturated *n*-butanol, vortex the tube for 10 sec and centrifuge to resolve the phases.
3. Transfer the upper phase to a clean vessel and repeat step 2.
4. Pool both butanol (upper) extracts. Assess cleavage by taking aliquots of each phase (butanol and aqueous) for scintillation counting.

6.2 Purification of glycosyl-phosphatidylinositol lipids

6.2.1 Thin-layer chromatography

Standard TLC methodology is both rapid and easy to perform. Typically, chromatograms require 1–2 h for development. Solvent systems A, B, C, and K (*Table 3*) are recommended for purification of G-PIs. It is best to use solvent B initially as the peaks are close together in this system (*Figure 4*). At least two different systems must be used to ensure purity. System K can be useful for removal of phospholipids, as polar G-PI lipids remain close to the origin. TLC is useful for the purification of small amounts of material (10–100 μg), for larger samples preparative TLC plates are available (Merck), with the same coatings as their analytical counterparts (note: R_f values may be slightly altered on the thicker preparative plates).

i. Visualization of lipids on thin layer plates

By far the most sensitive and rapid way to locate radiolabelled lipids in a thin-layer chromatogram is to use a linear radioscanner (for example, Berthold model LB 2842). Scanners also generate data than can be integrated using the software provided with the instrument. A second method is fluorography. With ^{3}H-labelled compounds the TLC plate must be sprayed with En^{3}Hance reagent (Dupont) prior to exposure to the film (Kodak X-OMat is suitable for this purpose). Quantification of the data then relies either on densitometry of the fluorogram (accurate within the linear range of the film) or by scraping off the bands and liquid scintillation counting. Lipids that have been exposed to En^{3}Hance are not then suitable for fatty acid or amine analysis. Standard detection techniques (for example, iodine, ninhydrin) for larger samples can be used in conjunction with radioactive visualization.

ii. Extraction of lipids from thin-layer plates.

Low yields are a major drawback of TLC as a preparative method. A return

of 50–60% from a plate following exhaustive extraction is about the most that can be expected. Yields are maximized by preventing extensive drying of the plate before extraction. The extraction procedure is summarized in *Protocol 15*.

Protocol 15. Extraction of lipids from TLC plates

1. Scrape the region of the plate containing the lipid of interest and transfer the powder to a glass tube.
2. Add 1 ml of solvent (usually chloroform:methanol:water 10:10:3, v/v/v; for lipophilic headgroups generated by G-PI-PLC treatment use solvent system A (*Table 3*)), and sonicate for 15 min. Centrifuge the sample and transfer the supernatant solvent to a fresh tube.
3. Repeat the extraction and pool the extracts. Filter the extract if necessary [using a Centrex 0.45 μm nylon centrifugal filter (Schleicher & Schuell)] and dry in a Speed-Vac.[a]
4. Partition the residue between water and *n*-butanol and recover the butanol phase.

[a] Do not dry the sample completely if there are traces of silica in the extract, as the yield will be compromised.

6.2.2 Liquid chromatography

i. Iatrobeads

The most applicable liquid chromatography method for G-PI isolation involves Iatrobeads (see *Protocol 16*) (large porous silica spheres of about 60 μ diameter, Iatron Laboratories, Tokyo, Japan; ref. 17). The yield from this method can be 90% or more, and the same protocol can be used with milligram amounts of material. Iatrobead chromatography provides a good first step for the fractionation of lipids from a large cell pellet where TLC would be unsuitable. See ref. 44 for an example of Iatrobead chromatography of G-PI biosynthetic intermediates.

Protocol 16. Iatrobead chromatography

1. Standard liquid chromatography equipment is required, with a system that is resistant to chlorinated solvents; for example, the FPLC system from Pharmacia-LKB. The bed volume depends on capacity required, 1 × 25 cm is suitable for most applications.
2. Pack the column with the beads in a slurry (1:1 solvent and resin). Programme the chromatography system to deliver four column volumes of chloroform:methanol (11:9 v/v) followed by four column volumes of

Protocol 16. *Continued*

chloroform:methanol:water (11:9:2 v/v/v) (3). As with any liquid chromatography system, the flow rate and fraction size must be optimized, but at least ten fractions per column volume of solvent should be collected.

3. Run a dummy gradient to ensure that pressure and volume alterations will not be problematic. Load the sample and elute the column.
4. Monitor the elution by scintillation counting, and identify peaks by TLC.

ii. Octyl-sepharose

Glycophospholipids can be chromatographed on octyl-Sepharose (Pharmacia or Sigma) using a linear gradient of 1-propanol (5–70%, v/v). (13, 18).

iii. Anion-exchange

DEAE–cellulose (Whatman DE-52, acetate form, equilibrated with 99% methanol) or Mono Q (HR 5/5, Pharmacia) can be used to partially resolve phospholipids and glycophospholipids. Both columns are eluted with 0 to 400 mM ammonium acetate in 99% methanol.

6.3 Generation of a G-PI glycan fragment

A G-PI glycan fragment can be prepared by cleaving the phosphodiester bond linking the glycan to the lipid moiety or by cleaving the glucosamine-inositol linkage. The latter cleavage can be achieved by nitrous acid treatment and several phospholipases (PI-PLC, G-PI-PLC, G-PI-PLD) can be used to cleave the glycan–lipid phosphodiester linkage (*Figure 1*). However, the most useful way to produce a G-PI glycan for analysis is first to dephosphorylate the G-PI with aqueous hydrofluoric acid, and then convert the released glycan to a neutral species by *N*-acetylation or nitrous acid deamination and reduction. All methods require sugar-labelled G-PI, although analysis of the nitrous acid released headgroup (NAhg) can be done with ethanolamine-labelled material as well. Methodology for the structural elucidation of G-PI by direct chemical means can be found in Chapter 8.

6.3.1 Hydrofluoric acid dephosphorylation and preparation of a neutral glycan

The phosphodiester bridge between ethanolamine and the terminal mannose residue (*Figures 1* and *2*) is cleaved within 24 h of HF treatment (*Protocol 17*). However, complete cleavage of the phosphodiester bond linking inositol to the lipid moiety takes approx 60 h (11). The inositol acyl group in P3 (and other inositol acylated G-PIs) appears to be quite resistant to HF (< 20% hydrolysis in 60 h) under the conditions used, and butanol/water phase separation (Section 6) of the HF reaction mixture results in the recovery of the inositol-acylated glycan in the butanol phase, along with the released lipid

moiety. Mixtures of inositol- and acyl-inositol-containing G-PIs may be identified in this way (10).

Protocol 17. Dephosphorylation with hydrofluoric acid.

1. Dry the sample in a microcentrifuge tube. Cool the tube by placing it on an ice–water mixture in a Dewar flask, then add 50 μl ice-cold 50% aqueous HF (EM Science). Leave the flask in a cold room during the incubation. Include six blank tubes (containing 50 μl HF each) with the sample.
2. Quench the reaction after approximately 60 h by transferring the reaction mixture to a frozen saturated solution of LiOH (about 280 μl, pre-frozen on dry ice). To ensure that the pH does not rise above 8.0 during the neutralization process, estimate the amount of saturated LiOH required for the neutralization of 50 μl HF by titrating the blank samples and measuring the end-point with pH paper (E. Merck, 0–6 pH range). Place 10 μl less than the estimated volume of LiOH in a microcentrifuge tube and freeze the solution in a dry-ice–ethanol mixture. Transfer the HF reaction mixture to the frozen LiOH and let the sample thaw on ice. Add 10 μl aliquots of saturated $NaHCO_3$ until the reaction ceases to effervesce. If the reaction fails to effervesce add drops of glacial acetic acid to the mixture until effervescence is observed. This method of neutralization prevents removal of the inositol acyl groups on the released dephosphorylated glycan moiety, and provides a useful way of obtaining a butanol-soluble glycan from PI-PLC resistant (i.e. inositol acylated) G-PI species.
3. Centrifuge the neutralized sample in a microcentrifuge, and return the supernatant and a 100 μl water wash of the LiF precipitate to the original reaction tube. Re-adjust the pH of the solution with saturated $NaHCO_3$ to approximately pH 4.0. If the sample has not been base-treated and may have an acyl-inositol, butanol partition the products at this point. Count an aliquot of each phase. For P3, > 80% of the sugar label will be recovered in the butanol phase.
4. Desalt the aqueous phase by passage over 0.4 ml AG3X4A(OH^-) resin. Wash the column with 3 × 0.4 ml water. Pool the eluate and washings, and proceed with deamination and reduction (step 6), *or* *N*-acetylation (step 5). Glycans with acylated inositol will be recovered in the butanol phase. These must be deacylated with mild base (*Table 5*) prior to further manipulation.
5. To neutralize the glycan the NH_2 group of the glucosamine residue may be derivatized by acetylation (*Table 5*). Neutral glycans prepared by this route contain inositol and cannot be reduced by borohydride treatment. The inositol group reduces the retention time of the neutral glycan on

Protocol 17. *Continued*

Dionex compared to the deaminated glycan (refs. 10 and 11, and see *Table 7*). Analysis prior to, and post, reduction provides one means of distinguishing neutral G-PI glycans from other contaminating glycans that possess a reducing terminus.

6. Dephosphorylated glycans can be deaminated to convert them to neutral species. Dry the sample in a Speed-Vac, and deaminate as described (*Table 5*). For reduction, terminate the reaction by adding 300 μl 400 mM H_3BO_3, followed by 130 μl 1 M NaOH and 100 μl 2 M $NaBH_4$ in 0.3 M NaOH. Check the pH (adjust to 11 if necessary). Incubate the tube at room temp. for 12 h and terminate the reaction by adding 20 μl glacial acetic acid. Desalt the sample on a 1-ml column of AG50WX12(H^+). Wash the column with 3 × 1 ml water. Pool the column eluate and washings and dry in a Speed-Vac, then flash evaporate the dried residue, first with 5% acetic acid in methanol (2 × 200 μl), then with toluene (3 × 20 μl), and resuspend in water.

6.3.2 Glycan released by nitrous acid cleavage

Nitrous acid cleaves the glucosamine-inositol bond in G-PIs, resulting in a water-soluble headgroup (NAhg) and an inositol phospholipid; the NAhg can be analysed by Dionex HPLC, Bio-Gel P4, and Mono-Q. The NAhg will still contain any ethanolamine-phosphate moieties originally linked to the glycan; treatment of the NAhg with α-mannosidase before and after HF treatment has been used to locate the position of the ethanolamine-phosphate group in *T. brucei* G-PIs (11, 19). Deaminate the lipid as described (*Table 5*) and partition between water and butanol. Recover the glycan in the aqueous phase.

Note that the glycan is still negatively charged as the phosphate groups are still present, so that the NAhg cannot be desalted with AG50. The deamination reaction is less than 100% efficient. Reaction efficiency can be improved considerably by increasing the solubility of the G-PI in the reaction mixture; this is done by including detergent in the buffer (*Table 5*), or deacylating the G-PI with mild base beforehand. Note that the deamination reaction is not completely understood and that alternative deamination products (not involving cleavage of the glucosamine–inositol bond) may be recovered in the butanol phase, in addition to the starting material (see ref. 12 for a discussion). In this context it should also be noted that the ethanolamine residue in ethanolamine-phosphate-containing G-PIs can be deaminated (i.e. the NAhg contains deaminated ethanolamine, not ethanolamine).

6.4 Analysis of glycan fragments

A few very specific methods are detailed below. The reader should consult

the relevant volumes of *Methods in enzymology* for a fuller treatment of glycan sequence analysis, and Chapter 8 for a description of mass spectrometric analyses of both glycans and lipids.

6.4.1 Liquid chromatography

The most powerful liquid chromatography methods for G-PI glycan analysis are Dionex anion-exchange HPLC (*Protocol 18*) and Bio-Gel P4 gel filtration chromatography (*Protocol 19*). Ion exchange chromatography on Mono Q is a useful supplementary method for analysis of NAhg that have not been subjected to HF dephosphorylation (*Protocol 20*). Discussions of pulsed amperometric detection and the use of anion exchange chromatography to separate monosaccharides and oligosaccharides may be found in refs. 20–22.

Protocol 18. Dionex anion-exchange chromatography

For most applications this is the method of choice. One drawback is that the recovery from the column is low (35–50% when working with small amounts of metabolically labelled material), and therefore sequential analyses are not advised.

1. Analysis is performed using a Dionex Basic Chromatography System (Dionex Corporation, Sunnyvale, CA, USA), with an HPIC AS6 separation and guard column. Separation is accomplished by gradient elution (flow rate 1 ml/min) using one of the programs listed in *Table 7*. The sample should be prepared as for chromatography on Bio-Gel P4 (*Protocol 19*). It is especially important to filter the samples [using, for example, 0.45 μm Acrodise syringe filters (Gelman Sciences)] before injection to maximize the life of the column.
2. The column eluant is neutralized with an anion micromembrane suppressor (Dionex Corporation, 0.1 M H_2SO_4 counter-flow regenerant). Collect fractions, typically 0.25 or 0.4 min-fraction, and determine the radioactivity in each fraction by liquid scintillation counting. Failure to neutralize the eluant may result in abnormally high background scintillation with some liquid scintillation cocktails which may be reduced by adding acetic acid (to pH 5.0) to the fractions. As little as 200 c.p.m. can be reliably detected if all the radioactivity chromatographs as a single peak.
3. Dextran oligomers (1–2 μg) should be included in each run and detected using pulsed amperometric detection. Retention times for glucose homopolymers and G-PI glycans and glycan fragments derived from *T. brucei* lipids are listed in *Table 7*.
4. It is important to de-gas the water before adding the salts, and to keep the buffers under argon when made up. For operation procedures and maintenance, refer to instructions supplied by Dionex Corporation.

Table 7. Chromatographic separations using the Dionex anion-exchange HPLC system[a]

Program[b]	Retention time (min)
I	*Glucose oligomers*: Glc1 (6.0), Glc2 (10.4), Glc3 (14.8) *GPI fragments*: AHM (5.0), M_1AHM (6.0), M_2AHM (10.5), M_3AHM (13.5) *Other*: GlcNAc-ol (2.6), GlcNAcα1-6inositol (*N*-acetylated GPI fragment) (2.6), GlcNAcGlcNAc-ol (reduced chitobiose) (3.5), GlcNAc (6.0)
II	*Glucose oligomers (non-reduced)*: Glc1 (6.0), Glc2 (10.9), Glc3 (19.2), Glc 4 (24.8), Glc5 (29.4) *Glucose oligomers (reduced)*: Glc1 (2.1), Glc2 (2.9), Glc3 (4.1), Glc4 (7.3) *GPI fragments*: AHM (5.0), M_3GlcNAc-inositol (6.0), M_3AHM (15.2) *Other*: mannitol (3.9), GlcNAc (5.8), mannose (5.8), chitobiose (7.6), M_5GlcNAcGlcNAc-ol (19.6)[c]
III	M (3.9), M-1-P (14.6), M-6-P (16.5), UDP-GlcNAc (26.5), GDP-M (31.5)
IV	UDP-GlcN (32.3), UDP-GlcNAc (33.8)

[a] Retention times are not absolute and are intended as a guide to devising chromatographic separations. *Abbreviations*: AHM, 2,5-anhydromannitol, Glc, glucose, GlcN, glucosamine, GlcNAc, *N*-acetylglucosamine, GDP, guanosine 5′-diphospho-, M, mannose, UDP, uridine 5′-diphospho-, P, phosphate.

[b] All separations performed on a Dionex Basic Chromatography System using a HPICAS6 column preceded by a HPICAG6 guard column. *Program I*: Solution A (100 mM NaOH), Solution B (0.5 M sodium acetate, 100 mM NaOH). Elution gradient: 100% solution A up to 3 min after sample injection, then a linear gradient to 55% A, 45% B at 33 min. Wash: continue to 100% B over 5 min, maintain 100% B for an additional 5 min before returning to 100% A. *Program II*: Solutions A and B as for Program I. Elution gradient: 100% A for 6 min after sample injection, then a linear gradient to 85% A, 15% B at 36 min. Wash: similar to *Program III*: A (200 mM NaOH), B (200 mM NaOH, 1 M sodium acetate). Elution gradient: 100% A for 5 min after sample injection, then a linear gradient to 100% B at 25 min. *Program IV*: Solutions A and B as for Program III. Elution gradient: 100% A for 5 min after sample injection, then a linear gradient to 100% B at 35 min.

[c] Defined isomer of M_5GlcNAcGlcNAc-ol (40).

Protocol 19. Bio-Gel P4 gel filtration

Sodium acetate buffer is used for most applications; water is less easy to use for technical reasons, including the inability to chromatograph charged compounds.

1. Swell the P4 gel (Bio-Rad Laboratories) in an excess of 50 mM sodium acetate (pH 5.5) for 4 h. Remove fines in the gel by letting it stand for 30 min and decanting the supernatant (do this at least twice). Resuspend the gel as a 1:1 slurry in the buffer and pour into a 1.5 × 100 cm glass column equipped with a plunger. Allow the gel to pack, and pump the column at 6 ml/h with acetate buffer for 24 h to allow the bed to settle. A P500 FPLC pump (Pharmacia-LKB) or an HPLC pump equipped with a back-pressure device is ideal for this application.
2. Calibrate the column with [^{3}H]-reduced isomaltose oligomers (23).

Dextran may be easily hydrolysed to produce a series of oligomers by dialysing T200 dextran (Sigma) against water, followed by partial hydrolysis of the retentate with 100 mM HCl at 100 °C for 6 h. The dextran hydrolysate may be reduced with sodium borotritide (see Chapter 8) in order to generate radiolabelled standards. A void volume marker (such as bovine serum albumin) should be included in each run.

3. Prepare neutral glycans by passage through a layered column containing 100 µl each of the following (top to bottom); Chelex 100(Na^+), AG50(H^+), AG3(OH^-), QAE-Sephadex. For charged glycans omit the AG3 and QAE. All samples should be filtered through a 0.45 µm PTFE filter before injection. Load the glycan or phosphoglycan in 200 µl water and collect fractions (typically 0.5–1.0 ml). Locate the glycan by scintillation counting of aliquots of the fractions. Recovery of radioactive material is 90–95% from these columns, and runs are highly reproducible.
4. Calculate the hydrodynamic volume (in glucose units, Gu) of the glycan by reference to the elution of the dextran standards. Note that glucitol elutes as 1.5 Gu, hexoses as 0.9–1.0 Gu, HexNAc as 2.0, and anhydromannitol (AHM) as 1.7 Gu. Neutral G-PI glycans elute as follows: Man_3AHM (4.2 Gu), Man_2AHM (3.2 Gu), Man_1AHM (2.4 Gu). The glycan generated by nitrous acid deamination and borohydride reduction of PP1, P2, and P3 (*Figure 2*) chromatographs as approx 7 Gu (approx 6.2 Gu in 100 mM sodium acetate).

Protocol 20. Mono Q anion exchange chromatography

Ethanolamine-phosphate groups in the G-PI will remain on the glycan fragment generated by nitrous acid treatment, and the charges on the phosphate esters can be assayed by ion-exchange. Use a gradient FPLC system (or equivalent) and an HR5/5 Mono-Q column (Pharmacia-LKB).

1. Prepare the following buffers: A, deionized water; B, 200 mM ammonium acetate pH 5.2. Filter the buffers through a 0.45 µm PTFE membrane.
2. Program the pumps for the following gradient, flow rate 1 ml/min; 0 to 7 min isocratic 100% buffer A, 7 to 30 min linear gradient to 25% buffer B, 30 to 40 min linear increase to 100% B, and then re-equilibrate.
3. Inject the sample in 100 µl deionized water, and collect 1 ml fractions. Take aliquots of the fractions for scintillation counting.

[^{3}H]-reduced glucitol (or any other neutral sugar) and glucitol-6-phosphate are useful standards. The gradient system will resolve glycans with 0–3 negative charges on them. The percentage of B can be increased if all the material is not eluted by this procedure.

6.4.2 Fragmentation reactions and other analyses

i. Methylation analysis of [^{3}H]mannose-labelled glycans

This method (*Protocol 21*) provides data on linkages between mannose residues in the G-PI glycan and is comparable to the commonly used GC-MS methylation analysis (24).

ii. Acetolysis

Acetolysis can be used for the selective cleavage of α1–6 bonds between monosaccharides [such as the Manα1–6Man linkage found in the G-PI core glycan (*Figures 1* and *2*)] (*Protocol 22* and ref. 26).

iii. Dansylation

Dansylation is used to identify underivatized ethanolamine-phosphate residues in [^{3}H]ethanolamine-labelled G-PIs (*Protocol 23*).

iv. Exoglycosidase digestion

Conditions for the commonly used exoglycosidases in G-PI analysis are given in *Table 6*. The *Aspergillus phoenicis* α-mannosidase is Manα1–2 specific. Endoglycosidase H is useful for the removal of oligomannose N-linked glycans. Reaction conditions for several additional exoglycosidases can be found in 'Biochemica Information', available from Boehringer Mannheim.

Protocol 21. Methylation linkage analysis

1. Dry the glycan in a 1 ml Reacti-Vial (Pierce Chemical Co.). Add 50 μl dimethyl-sulfoxide (DMSO) and stir continuously with a magnetic stirrer (Pierce Chemical Co.) designed to fit the conical interior of the vial. The sample must be stirred throughout the permethylation procedure. Finely powder NaOH pellets in a mortar and pestle, and immediately make a 120 mg/ml suspension in DMSO. Add 50 μl of the suspension to the glycan sample and continue stirring. Make three additions each of 10 μl iodomethane (CH_3I, Aldrich Chemical Company) after 5, 15, and 20 min.
2. Terminate the reaction 10 min after the final addition by adding 200 μl sodium thiosulphate (100 mg/ml) and transfer the contents to a 5-ml glass tube. Rinse the Reacti-Vial successively with 600 μl sodium thiosulphate and 3 × 200 μl chloroform. Transfer the two-phase reaction mixture and rinses to the glass tube. The permethylated glycans will be recovered in the lower chloroform phase, which can be separated by centrifugation. Transfer the chloroform phase to a fresh tube and wash with water (5 × 1 ml, vortex the mixture and centrifuge to separate the two phases each time) and finally transfer the washed chloroform phase to a Reacti-Vial. An aliquot of the chloroform may be taken for scintillation counting to assess recovery of material (typically 50–65%).

3. Dry the sample in a Speed-Vac evaporator and hydrolyse the residue for 4 h at 120 °C in 200 µl 2 M trifluoroacetic acid (Pierce Chemical Co.). After hydrolysis, evaporate the trifluoroacetic acid to dryness, and flash-evaporate the residual acid with 3 × 100 µlCH_3OH. Resuspend the samples in 50% aqueous CH_3OH and analyse by TLC system J (*Table 3*).
4. Mixtures of partially O-methylated methylmannosides may be prepared according to the procedure of Hull and Turco (25), and hydrolysed as described above to generate appropriate standards. Alternatively, completely characterized [^{3}H]mannose-labelled glycans derived from known G-PI anchors, dolichol-linked oligosaccharides, or N-linked glycans may be analysed alongside the sample of interest to provide a spectrum of partially O-methylated mannose markers. Approx R_f values of the partially methylated mannoses recovered from the G-PI core glycan are given in *Table 4*.

Protocol 22. Acetolysis

1. Dry the sample in a glass Reacti-Vial.
2. Per-acetylate the glycan with 40 µl pyridine:acetic anhydride (1:1) for 16 h at room temp., and dry. Add 30 µl of acetic anhydride:acetic acid:sulfuric acid (10:10:3) and incubate the mixture at 37 °C for 6 h.
3. Stop the reaction by adding 10 µl pyridine and partition between 400 µl water and 200 µl $CHCl_3$. Recover the products in the organic phase, and wash with 400 µl water. De-acetylate the glycan by treatment with mild base (*Table 5*) for 16 h at 37 °C.
4. Analyse the sample by Bio-Gel P4 gel filtration chromatography (*Protocol 19*).

Protocol 23. Dansylation

1. Dry the [^{3}H]ethanolamine-labelled G-PI sample in a 100 µl Reacti-Vial and dansylate either prior to hydrolysis or after hydrolysis. Dansylation prior to hydrolysis will permit the identification of N-unsubstituted ethanolamine residues in the intact G-PI.
2. Dansylate the sample by resuspending the residue in 10 µl of 0.2 M $NaHCO_3$, pH 9, and 10 µl 2.5 mg/ml 5-dimethylaminonapthalene-1-sulfonyl chloride (dansyl chloride; Pierce Chemical Co,) in acetone. Incubate the sample at 37 °C for 30 min and evaporate the reaction mixture to dryness.

Protocol 23. *Continued*

3. Hydrolyse the samples in 10 µl 6 M HCl for 8–10 h at 110 °C. Evaporate the hydrolysis mixture in a Speed-Vac prior to dansylation or thin layer chromatography analysis using system I (*Table 3*).

Quantification of free and derivatized ethanolamine content can be obtained by radiomethylation with [^{14}C]HCHO (27–29).

6.5 Analysis of the lipid moieties of glycosyl-phosphatidylinositols

The methods described in this section are included in *Table 6* and require G-PIs biosynthetically labelled with fatty acid. Only methods that have been useful in the analysis of *T. brucei* G-PIs will be discussed (complete analysis of alkylglycerol and ceramide lipid fragments is not dealt with here; for information see refs. 30–32). For details of GC-MS and fast atom bombardment techniques see Chapter 8.

6.5.1 Base hydrolysis

Strong base hydrolysis (*Table 5*) will quantitatively release ester-linked fatty acids. Analysis of material labelled with headgroup components (such as [^{3}H]mannose, [^{3}H]glucosamine) should also be performed to determine the fate of the remainder of the molecule.

6.5.2 Arrangement of fatty acid substituents

i. PI-PLC

PI-PLC releases a glyceride moiety from glycerophospholipid-based G-PIs (for example PI-PLC releases dimyristoylglycerol from P2 (*Figure 2*)). Analyse the reaction products by TLC using system D (70:30:2, *Table 3*). Di- and mono-acylglycerols of known fatty acid composition (available from Sigma) should be included as standards. Standards can be visualized by exposing the plate to iodine vapour, or by spraying the plate with 10% sulfuric acid and heating at 110 °C to char.

ii. G-PI-PLD

The activity (found in mammalian serum) releases a phosphatidic acid moiety from glycerophospholipid-based G-PI (see Chapters 5 and 6); G-PIs that are resistant to PI-PLC may still be sensitive to G-PI-PLD. Chromatograph the enzyme digestion products on TLC system B or E along with phosphatidic acid standards (Sigma; lysophosphatidic acid can be made by phospholipase A_2 digestion of phosphatidic acid). Standards can be visualized with molybdic acid spray reagent. In order to demonstrate that non-specific lipolysis is not

taking place, the serum may be treated with 1,10-*o*-phenanthroline (0.5 mM, pre-incubated with the serum for 30 min at 0 °C) to inhibit the PLD (33, 34). The inositol residue in PI-PLC resistant G-PIs from *T. brucei* (P3 and PP1, *Figure 2*) is also labelled with [^{3}H]fatty acid. Thus G-PI-PLD treatment of P3 and PP1 produces [^{3}H]fatty-acid-labelled phosphatidic acid as well as a [^{3}H]fatty-acid-labelled glycan. This lipophilic glycan migrates close to the origin of the TLC plate (system B). This provides a convenient means for the separation of the inositol-linked fatty acids from the glycerol-linked fatty acids, therefore allowing separate analysis (such as described in Section 6.5.3 and in ref. 19).

iii. Nitrous acid

This reagent will release phosphatidylinositol from G-PIs. Comparison of the mobility of the released fragment with diacyl phosphatidylinositol (Sigma) can provide information about the number of fatty acids present. Lipids with two fatty acyl chains co-chromatograph with diacylphosphatidylinositol, those with three (for example, that obtained from P3; ref 12) run ahead of it. Nitrous acid cleavage is diagnostic for the N-unsubstituted glucosamine residue and is especially useful when analysing G-PI linked to protein, where enzyme cleavage may be inefficient.

iv. Phospholipase A_2

This enzyme selectively removes the fatty acid from the 2-position on the glycerol. Digest the lipid as described (*Table 6*), and analyse the product by TLC on system B. Suitable controls should be included; it may be necessary to mix a control substrate with the unknown to ensure that the enzyme is active. Free fatty acid released by this method can either be removed by toluene extraction of the reaction mixture, or resolved from the other products by TLC (system B). It is important that the correct phospholipase A_2 is used. We have found that the *Crotalus adamanteus* enzyme (Sigma) is effective on G-PI substrates, whereas the bee venom enzyme (Sigma) is not.

v. Acetolysis

Glyceride acetates are released by acetolysis of glycerophospholipid-based G-PIs (for example, acetolysis of P2 (*Figure 2*) gives 1,2-dimyristyl-3-acetyl-glycerol). The reaction can be used to release a glyceride if both PI-PLC and G-PI-PLD fail. Conditions are given in *Table 5*. Analyse the products by TLC (system D, 80:20:1, *Table 3*).

6.5.3 Identification of [^{3}H]fatty acids biosynthetically incorporated into G-PI species

This is performed by release and conversion to fatty acid methyl esters, followed by analysis on TLC (*Protocol 24*).

Protocol 24. Analysis of biosynthetically-labelled fatty acids

1. Release the labelled fatty acids by hydrolysis in 160 μl 0.5 M NaOH in methanol (2 min, 100 °C) for ester-linked fatty acids or 1 M KOH in 80% methanol (18 h, 70 °C) for amide-linked fatty acids such as those found in ceramides.
2. Esterify the released fatty acids by heating with 200 μl BF_3/methanol (12% w/w, Supelco Inc.) at 100 °C for 2 min (41). Add 200 μl 5 M NaCl in 0.5 M HAc and extract the fatty acid methyl esters into toluene.
3. Analyse the fatty acid methyl esters, along with standards, on RP-18 F254 S thin-layer plates (20 × 20 cm, Merck) using system F (*Table 3*). Detect radioactivity by scanning or fluorography.
4. To establish that the released fatty acids are quantitatively converted to the corresponding methyl esters, chromatograph aliquots of the toluene phase on silica 60 plates using system G (*Table 3*). Radioactivity should chromatograph quantitatively with fatty acid methyl ester standards (R_f 0.97) and not with free fatty acids (R_f 0.45).
5. Detect non-radioactive lipid standards by spraying the plates with 10% phosphomolybdic acid in ethanol (Sigma Chemical Co.) and heating at 120 °C for 20 min.

Detection of radioactivity using the TLC scanner is less efficient for the RP plates than for silica 60 TLC plates. At least a few thousand counts per minute of radioactivity in each peak are necessary to detect the radiolabelled methyl esters. Alternatively, the matrix can be scraped from the plates in 0.25-cm-wide strips, transferred into scintillation vials and counted.

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