Journal of Cellular Biochemistry 38:77–86 (1988)

Oligosaccharide Heterogeneity of Glycoproteins Sulfated During the Vegetative Growth of *Dictyostelium discoideum*

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Macromolecules are sulfated during the vegetative growth of *Dictyostelium discoideum*. A characterisation of the structures of sulfated oligosaccharides associated with these macromolecules indicates that the oligosaccharides are heterogeneous. Endoglycosidase and pronase digestion were used with gel-filtration chromatography to obtain two different oligosaccharide fractions and a glycopeptide fraction; these were further characterised by ion-exchange and lectin-affinity chromatography and by acid hydrolysis. The data indicate that up to 43% of the sulfate is associated with typical *N*-linked oligosaccharides, that up to 5% is associated with *N*-linked oligosaccharides that are either very large or extremely highly charged, and that the remaining sulfate is associated with oligosaccharides non-*N*-linked to protein. Each fraction was also shown to be heterogeneous at most other structural levels. Electrophoretic analyses following the endoglycosidase and pronase treatments indicated that all of the macromolecules are glycoproteins and suggested further that at least two of the oligosaccharide fractions are located on different groups of glycoproteins.

Key words: macromolecule sulfation, oligosaccharide structures, life cycle

Dictyostelium discoideum is uniquely suited to the biochemical analysis of cell growth and development. The earliest developmental event in the organism's life cycle is the transition from the vegetative growth phase to the developmental phase.

The role of macromolecule sulfation in the life cycle of *D. discoideum* has been the primary subject of research in this laboratory. In early experiments an inhibitor of sulfation, selenate, was shown to arrest the vegetative growth of *D. discoideum* amoebae [1]. In contrast, the development of amoebae is largely unaffected by selenate, although the possibility that the amoebae become impermeable to the inhibitor has not been formally excluded. [^{35}S]Sulfate ($^{35}SO_4$)-incorporation experiments indicated that during vegetative growth at least ten macromolecules become highly sulfated [2]. These ma-

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Received July 28, 1987; accepted February 8, 1988.

cromolecules are sulfated on carbohydrate residues rather than on tyrosine and have relatively low subunit molecular weights and isoelectric points [2]. Although these macromolecules share some of the properties of the lysosomal enzymes, the majority of these macromolecules do not react with monoclonal antibody to the sulfated common antigenic determinant present on these enzymes [3,4]. Among the most interesting properties of the macromolecules is the common decline in their sulfation and their subsequent loss en masse to the extracellular medium without major degradation during early development [2]. Together these studies suggest 1) that sulfation of one or more of the macromolecules is necessary for the vegetative growth of amoebae, 2) that the macromolecules might be functionally and/or temporally related via sulfation, and 3) that the status of the macromolecules changes upon the transition to the developmental phase of the life cycle.

In this report the structures of the sulfated oligosaccharides associated with the macromolecules have been partially characterised as part of an effort to gain insights into the function(s) of the macromolecules and the role, if any, of sulfation in mediating the function(s). Groups of macromolecules with similar functions have been shown previously to share essential oligosaccharide determinants. The best-understood example is that of the lysosomal enzymes of mammalian cells, which share *N*-linked oligosaccharides with phosphorylated mannose residues essential for the targeting of the enzymes [5,6]. The mannose 6-phosphate residues mediate the interaction of the lysosomal enzymes with two alternative specific receptors, which direct the deposition of the enzymes in the lysosome [5,6].

Standard procedures used elsewhere to characterise the oligosaccharides of the lysosomal enzymes of *D. discoideum* [7] were employed here to show that instead of sharing uniquely modified oligosaccharides of a single class the sulfated macromolecules are glycoproteins that carry extremely heterogeneous oligosaccharides. These data may be useful in efforts to determine the function of sulfated oligosaccharides in the life cycle of *D. discoideum*.

MATERIALS AND METHODS

Materials

Media components were obtained from Difco Pty. Ltd. Pronase and concanavalin A-Sepharose were purchased from Pharmacia Fine Chemicals. QAE-Sephadex, Sephadex G-50, and Sephacryl S-200 were purchased from the Sigma Chemical Company. Peptide:*N*-glycosidase F (PNGase F), purified by the method of Tarentino et al. [8], was generously supplied by Drs. L. Roux and G. Sunblad of the University of California Cancer Center, San Diego. All other materials used were also of the highest quality available.

$^{35}SO_4$ Labeling

Vegetative *D. discoideum* amoebae, maintained as described previously [1,2], were cultured to a density of 2.5×10^6 cells/ml with *K. aerogenes* (25 mg/ml wet weight) in KK2 (2.6 g KH_2PO_4, 0.66 g K_2HPO_4, and 0.41 g $MgCl_2$ per liter, pH 6.2) containing 0.4 mM L-cystine. These cells were then labeled in 10-ml cultures for 6 h after the addition of $^{35}SO_4$ to a final concentration of 50 μCi/ml.

After labeling, the amoebae were washed three times by centrifugation (200g, 5 min) in KK2 containing 1 mM Na_2SO_4 prior to being lysed at 100°C in 10 mM Tris-

HCl, 1% w/v sodium dodecyl sulfate (SDS), and 1 M mercaptoethanol pH 7.5. The cell lysates were frozen at −20° prior to use.

Preparation of Oligosaccharide and Glycopeptide Fractions

The cell lysates were chromatographed on a 0.7 cm × 50-cm column of Sephadex G-50 equilibrated with 10 mM Tris-HCl, 0.1% w/v SDS, pH 7.5. The macromolecules eluting in the void region (pool a, Fig. 1A) were pooled and precipitated with 9 volumes of acetone at −20°C. The precipitated macromolecules were collected by centrifugation (2,000g, 20 min) and redissolved in 20 mM sodium phosphate buffer, pH 6.8, containing 0.2% v/v mercaptoethanol, 0.5% v/v NP40, and 0.1% w/v SDS. Five milliunits (mU) of PNGase F was added to the solution, which was incubated at 37°C. After 12 h incubation an additional 5 mU was added. After a further 12 h at 37°C, the solution was boiled for 2 min. Precipitated protein was removed by centrifugation (10,000g, 5 min), and the supernatant was rechromatographed on Sephadex G-50. The fractions containing the released oligosaccharides that were retained by the column (fraction I, Fig. 1B) were pooled and frozen at −20°C. The fractions containing the excluded macromolecules (pool b, Fig. 1B) were pooled, concentrated to 0.5 ml by lyophilization, and chromatographed on a 0.7 cm × 50-cm column of Sephacryl S-200 equilibrated with 10 mM Tris-HCl, 0.1% w/v SDS, pH 7.5. The fractions containing released oligosaccharides retained by S-200 (fraction II, Fig. 1C) were pooled and frozen at −20°C. The fractions containing excluded macromolecules (pool c, Fig. 1C) were precipitated with 9 volumes of acetone at −20°C, collected by centrifugation (2,000g, 20 min), and dissolved in 200 mM Tris-HCl, pH 8.5, containing 2 mg/ml pronase. After 5 h at 55°C the solution was boiled for 2 min and rechromatographed on Sephacryl S-200. Fractions containing glycopeptides retained by column (fraction III, Fig. 1D) were pooled and frozen at −20°C.

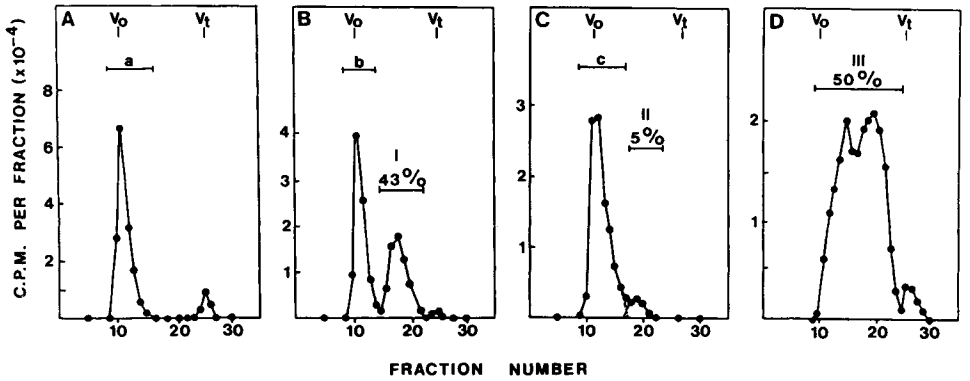

Fig. 1. Preparation of oligosaccharide and glycopeptide fractions from the major sulfated macromolecules. Vegetative *D. discoideum* strain NP73 amoebae were incubated with [^{35}S] sulfate and SDS/mercaptoethanol extracts chromatographed on Sephadex G-50 (**A**). After acetone precipitation, the macromolecules (pool a) were digested with 2 × 5 mU PNGase F and rechromatographed on Sephadex G-50 (**B**). Material remaining in the void fractions (pool b) was concentrated and chromatographed on Sephacryl S-200 (**C**). The final macromolecular component (pool c) was digested with Pronase and rechromatographed on Sephadex S-200 (**D**). The oligosaccharide fractions (I and II) and glycopeptide fraction (III) were desalted on Sephadex G-25. The small peak eluting after fraction III (D), which contained 2% of the total ^{35}S counts, was not characterised. The proportion of the total ^{35}S counts (●) eluting as fractions I, II, or III is indicated as a percentage above the horizontal bars.

Fractions I, II, and III were finally desalted on 0.7 cm × 50-cm Sephadex G-25 columns equilibrated with distilled water. In all cases 0.6-ml fractions were collected, and the elution profiles were determined by scintillation counting 1% of each fraction. Each elution profile, along with the lectin-affinity and QAE-Sephadex elution profiles described below, were reproduced at least once.

Gel Electrophoresis

$^{35}SO_4$-labeled macromolecules were incubated with 2 × 5 mU of PNGase F, and an aliquot was subjected to an additional incubation with pronase under the conditions described above. The digested macromolecules (20,000 cpm) were analysed by SDS gel electrophoresis along with untreated macromolecules and fluorographed as described by Laskey and Mills [9].

QAE-Sephadex Chromatography

The desalted fractions I, II, or III in 500–1,000 cpm aliquots were analysed on 2-ml QAE-Sephadex columns constructed in Pasteur pipettes and equilibrated with 2 mM Tris base according to the method of Freeze and Wolgast [7]. Fractions with zero to six negative charges were eluted with three 1.5-ml washes of 0, 20, 70, 125, 200, 285, or 400 mM NaCl in 2 mM Tris base, respectively. The columns were finally eluted with three 1.5-ml washes of 1 M NaCl in 2 mM Tris base. The radioactivity in each fraction was determined by scintillation counting. The radioactivity remaining bound to the column was determined by subtracting the salt-eluted radioactivity from the total radioactivity applied to the column.

Lectin-Affinity Chromatography

Concanavalin A-Sepharose chromatography was also performed on 2-cm columns constructed in Pasteur pipettes according to the method of Freeze and Wolgast [7]. After application of the desalted fractions I, II, or III in 500–1,000 cpm aliquots the columns were washed three times with 1.5 ml 10 mM phosphate buffered saline, pH 6.5. The lectin-binding fractions were eluted with three 1.5-ml washes of 10 mM phosphate-buffered saline containing 10 mM alpha-methyl glucoside at 25°C followed by three 1.5-ml washes with 10 mM phosphase-buffered saline containing 100 mM alpha-methyl mannoside at 55°C. Radioactivity in each fraction was determined by scintillation counting.

Kinetics of Acid Hydrolysis

Aliquots of fractions I, II, or III containing 1,000 cpm of $^{35}SO_4$ were hydrolysed in 0.25 N HCl at 100°C as described by Freeze and Wolgast [7]. The free sulfate liberated from fraction I oligosaccharides was determined by barium precipitation. Because it was found that barium precipitated undigested fractions II and III, chromatography on Sephadex G-25 columns equilibrated with 150 mM NaCl was used to determine the extent of hydrolysis of these fractions.

RESULTS

The oligosaccharides of macromolecules sulfated during the vegetative growth of *D. discoideum* were first characterized by gel filtration chromatography after incubating the amoebae with $^{35}SO_4$ and digesting the solubilised, labeled macromolecules with endoglycosidase and pronase (Fig. 1). These chromatographic analyses were highly

reproducible: variation in the relative proportions of $^{35}SO_4$ label associated with each of the oligosaccharide and glycopeptide fractions was of the order of 10–15%. The macromolecular fraction (pool a, Fig. 1A) obtained by chromatography of the solubilised, labeled macromolecules on Sephadex G-50 was acetone precipitated and digested with PNGase F prior to rechromatography on Sephadex G-50 (Fig. 1B). PNGase F released up to 43% of the $^{35}SO_4$ from the macromolecules (fraction I, Fig. 1B); a second incubation of the macromolecules with additional PNGase F failed to release more $^{35}SO_4$ (data not shown). Chromatography of the macromolecules eluting with the void volume (pool b, Fig. 1B) from Sephacryl S-200 revealed a smaller peak of PNGase F-released $^{35}SO_4$-labeled oligosaccharides (fraction II, Fig. 1C) constituting up to 5% of the total. The remaining macromolecules (pool c, Fig. 1C) were acetone precipitated, digested with pronase, and the glycopeptides were rechromatographed on Sephacryl S-200 (fraction III, Fig. 1D).

These data suggest that at least three different classes of oligosaccharides are sulfated in *D. discoideum*. Since PNGase F cleaves the asparagine-*N*-acetyl-glucosamine bond of the chitobiosyl core of *N*-linked oligosaccharides [8], up to 43% of the sulfate incorporated by vegetative amoebae is associated with typical *N*-linked oligosaccharides (fraction I), and up to 5% is linked to *N*-linked oligosaccharides (fraction II) that are either very large or are so negatively charge that they are retarded by Sephadex and Sephacryl. The remaining sulfate (approximately 50%) is bound to macromolecules bearing non-*N*-linked oligosaccharides (fraction III). This is supported by the observation that essentially none of the fraction III glycopeptides bound to the *N*-linked oligosaccharide-binding lectin concanavalin A (data not shown).

These observations were extended by an electrophoretic analysis of the PNGase F- and pronase-treated sulfated macromolecules. Macromolecules with PNGase F-sensitive oligosaccharides (marked with arrows, lane 1, Fig. 2), which can be identified by the loss of the $^{35}SO_4$ label upon PNGase F treatment, have a wide range of molecular weights, but all appear as discrete bands after electrophoresis. The majority of the PNGase F-insensitive sulfated macromolecules, however, which have similarly low molecular weights, appear as a very diffuse band after elctrophoresis (lane 2, Fig. 2). This suggests that the PNGase F-sensitive and PNGase F-insensitive oligosaccharides are associated with different types of macromolecules. However, the possibility that the two oligosaccharide fractions are both present on macromolecules which will therefore retain label after PNGase F treatment, is not formally excluded by such an analysis. The pronase sensitivity of the sulfated macromolecules (lane 3, Fig. 2) suggests that there is no polysaccharide present and therefore that all of the macromolecules are glycoproteins. These oligosaccharide and glycopeptide fractions were further characterised by ion-exchange and lectin-affinity chromatography and acid hydrolysis.

QAE-Sephadex ion-exchange chromatography was used to characterise the variability in the degree of substitution of fraction I, II, and III oligosaccharides and glycopeptides. Species with zero to six charges were eluted with a stepwise salt gradient after application of the sample to a column of QAE-Sephadex. The oligosaccharides of fraction I carried a range of negative charges between one and in excess of six and were completely eluted by 1 M NaCl (Fig. 3A). This suggests that there is substantial heterogeneity in the degree of substitution of these oligosaccharides.

Fraction II and III oligosaccharides and glycopeptides had similar polyanionic properties, which differed markedly from those of fraction I. Fraction II oligosaccharides had either three or at least six negative charges (Fig. 3B). Similarly, the number of

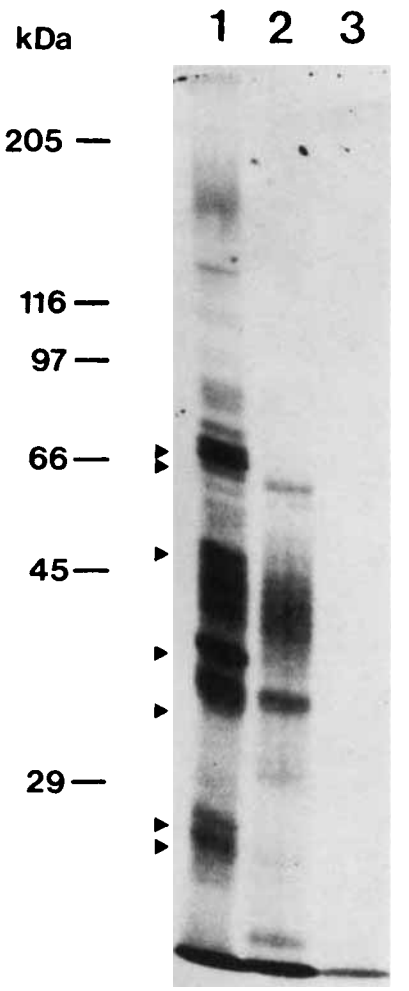

Fig. 2. Electrophoretic analysis of the effects of PNGase F and pronase on the [^{35}S] sulfate-labeled macromolecules. [^{35}S] sulfate-labeled macromolecules from vegetative *D. discoideum* strain NP73 amoebae were treated with PNGase F and pronase as described in "Materials and Methods." Aliquots containing approximately 20,000 cpm of the original (**lane 1**), PNGase F-(**lane 2**), and pronase-(**lane 3**) treated preparations were analysed by SDS polyacrylamide gel electrophoresis. A fluorograph of the gel is shown. PNGase F-sensitive macromolecules are indicated (arrowheads).

negative charges on fraction III glycopeptides varied from two to in excess of six charges (Fig. 3C). A significant proportion of each fraction (approximately 54% and 38%, respectively) remained bound to the QAE-Sephadex after washes with 1 M NaCl, which indicates that these oligosaccharides include variants with extremely high charge densities. The possibility that the peptides contribute to the charge properties of fraction III glycopeptides cannot be ruled out, however.

The lectin concanavalin A has different affinities for the various subclasses of *N*-linked oligosaccharides [10]. Biantennary complex-type oligosaccharides bind concan-

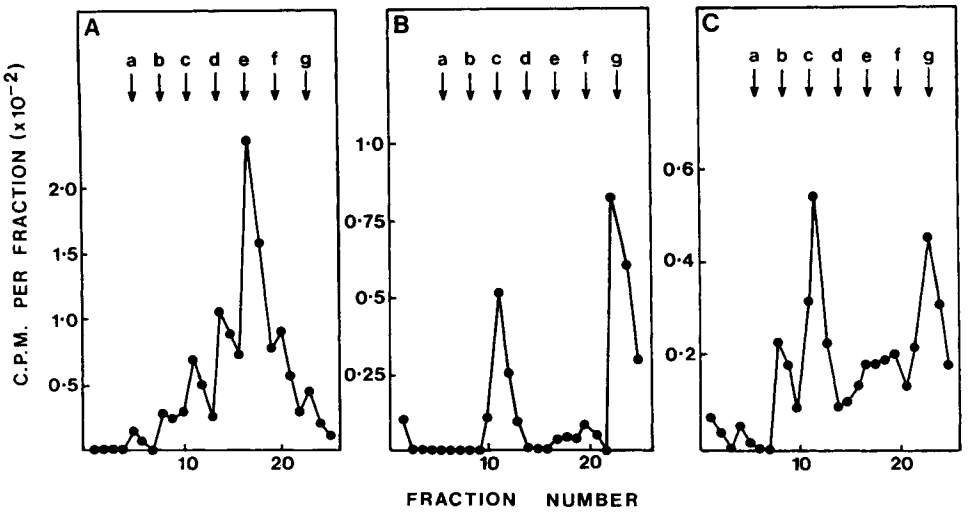

Fig. 3. QAE-Sephadex ion-exchange chromatography of the fraction I **(A)**, II **(B)**, III **(C)** oligosaccharides and glycopeptides. Aliquots of the desalted fractions (500–1,000 cpm) were applied to QAE-Sephadex columns constructed in Pasteur pipettes. As indicated (arrows) charged species were eluted with a stepwise salt gradient consisting of 20 mM **(a)**, 70 mM **(b)**, 125 mM **(c)**, 200 mM **(d)**, 285 mM **(e)**, 400 mM **(f)**, and 1 M **(g)** NaCl in 2 mM Tris base. The ^{35}S counts eluting from the column are indicated (●).

avalin A weakly and are displaced by alpha-methyl glucoside. Hybrid and high-mannose type oligosaccharides bind more strongly to the lectin and are only displaced by high concentrations of alpha-methyl mannoside. Triantennary- and tetra-antennary-complex-type oligosaccharides fail to bind to concanavalin A [10]. This lectin can therefore be used to characterise the heterogeneity in the chain organisation of *N*-linked oligosaccharides. Since fraction II and III oligosaccharides and glycopeptides failed to bind to concanavalin A-affinity columns (data not shown), this technique was only used to characterise fraction I oligosaccharides. This fraction consists of nonbinding, alpha-methyl glucoside-eluted and alpha-methyl mannoside-eluted components (Fig. 4). It is likely that the presence of sulfate esters inhibits the binding of some of these oligosaccharides as has been demonstrated for the oligosaccharides of the lysosomal enzymes [7]. Thus, it is not possible to predict the structural subclasses to which these oligosaccharides belong. Nevertheless, these data indicate that considerable heterogeneity exists in the organisation of the oligosaccharide chains of fraction I *N*-linked oligosaccharides. Moreover, these oligosaccharides appear to be more complex and variable than the sulfated oligosaccharides of the lysosomal enzymes of *D. discoideum*, the majority of which bind to concanavalin A [7].

The sensitivity of sulfate esters to acid-catalysed hydrolysis is of the order equatorial > axial > primary [11,12]. The half-lives of each of these esters in 0.25 N HCl at 100°C are 6–25 min, 60–84 min, and 90–120 min, respectively. The hydrolysis under these conditions of fractions I, II, and III was examined to characterise the nature of the sulfate esters associated with these glycopeptides and oligosaccharides. The half-lives for hydrolysis of fractions I, II and III sulfate esters were 60 min, 26 min, and 15

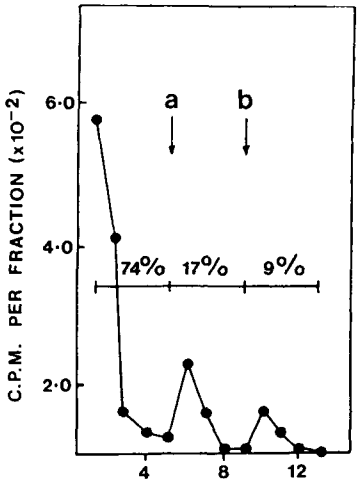

Fig. 4. Lectin-affinity chromatography of fraction III oligosaccharides. An aliquot of the desalted fraction (500–1,000 cpm) was applied to a concanavalin A-Sepharose column constructed in a Pasteur pipette. After eluting nonbinding material with 10 mM phosphate-buffered saline, bound species were eluted with 10 mM alpha-methyl glucoside (**a**) and 100 mM alpha-methyl mannoside (**b**) in 10 mM phosphate-buffered saline. The proportions of the total ^{35}S counts eluting from the column (●) after each wash are indicated as a percentage above the horizontal bar.

min, respectively (Fig. 5). These data suggest that sulfate is linked axially in fraction I oligosaccharides and equatorially in fractions II and III oligosaccharides and glycopeptides.

DISCUSSION

The analyses described in this report indicate that macromolecules sulfated during the vegetative growth of *D. discoideum* are glycoproteins and that these glycoproteins carry classes of oligosaccharides with properties that differ from those previously described in *D. discoideum*. The endoglycosidase-resistant oligosaccharides, which are polyanionic and sulfated equatorially (constituting fraction III glycopeptides) and which are associated with glycoproteins with relatively low molecular weights, might be similar to the glycosaminoglycans bound to the proteoglycans of higher animals, which constitute the connective tissue and extracellular matrices of higher animals [10]. Fraction II oligosaccharides share several of the properties of fraction III glycopeptides but are unusual in being *N*-linked to protein. It should be noted, however, that these analyses are not comprehensive and that additional work is necessary to elucidate the detailed structures of these glycoproteins.

The experiments described in this report also indicate that the oligosaccharides of glycoproteins for which sulfation declines after the vegetative growth of *D. discoideum* amoebae are extremely heterogeneous. For each of the three classes of oligosaccharides, heterogeneity exists at all of the structural levels examined: in the nature of the link to the protein, in the size and organisation of the oligosaccharide chains and the degree of

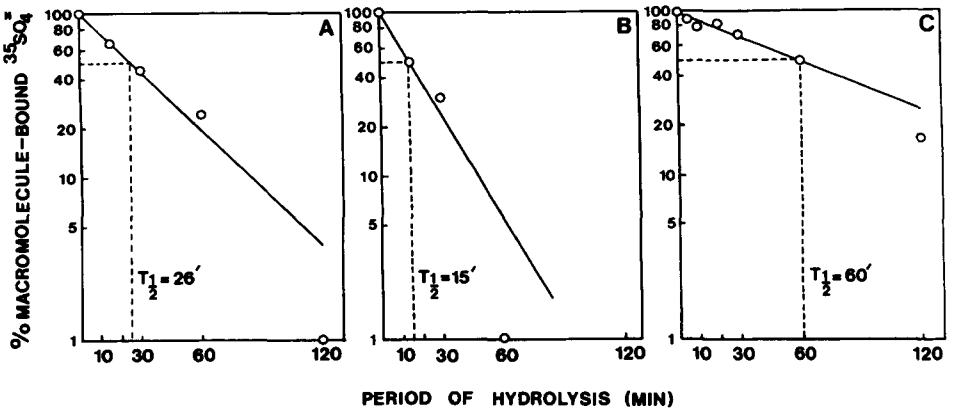

Fig. 5. Kinetics of acid hydrolysis of the sulfate esters linked to fraction I, II, and III oligosaccharides and glycopeptides. Aliquots of the desalted oligosaccharide fractions I (**A**), II (**B**), and III (**C**) were treated with 0.25 N HCl for various times, and the released sulfate was separated by gel filtration or barium precipitation as described in "Materials and Methods." Data are presented as the means of duplicate determinations.

their substitution, and in the nature of the sulfate-ester link to the oligosaccharides. Moreover, there is suggestive evidence (Fig. 2) that at least two of these oligosaccharide classes are associated with different types of proteins. It is most unlikely, therefore, that these glycoproteins share a functional relationship that is dependent on the presence of a unique class of oligosaccharides modified with sulfate in the way the lysosomal enzymes of mammalian cells are related by the phosphomannosyl residues of their *N*-linked oligosaccharides [5,6].

The functions of the sulfated glycoproteins remain to be determined. Analyses of the subcellular distribution of the glycoproteins and the three classes of oligosaccharides are likely to be of considerable value in reducing the complexity of this problem.

ACKNOWLEDGMENTS

This work was made possible by a Flinders University Overseas Traveling Fellowship and an Amy Forwood Travel Award awarded to the author. The author is grateful for the assistance rendered by Dr. Hudson H. Freeze and the members of his laboratory.

REFERENCES

1. Davis SJ, Wheldrake JF: FEMS Micro Lett 30:353, 1985.
2. Davis SJ, Wheldrake JF: Eur J Biochem 158:179, 1986.
3. Davis SJ, Freeze HH, Wheldrake JF: J Cell Biochem (in press).
4. Knecht DA, Dimond RK, Wheeler S, Loomis WF: J Biol Chem 259:10633, 1984.
5. Kornfeld R, Kornfeld S: Annu Rev Biochem 54:631, 1985.
6. von Figura K, Hasilik A: Annu Rev Biochem 55:167, 1986.
7. Freeze HH, Wolgast D: J Biol Chem 261:127, 1986.

8. Tarentino AL, Gomez CM, Plummer TH: Biochemistry 25:4665, 1985.
9. Laskey RA, Mills AD: FEBS Lett 82:314, 1977.
10. Beeley JG: "Laboratory Techniques in Biochemistry and Molecular Biology." Amsterdam: Elsevier Publ. Co., 1985.
11. Percival E: In Schweiger RG (ed): "Carbohydrate Sulfates," ACS Symposium Series 77. Washington: American Chemical Society, 1978, p 213.
12. Rees DA: Biochem J 88:343, 1963.