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ETUDE DES SULFOGLYCOCONJUGUES DANS LA SECRETION BRONCHIQUE

Soutenue le 9 juillet 1990, devant la Commission d'Examen

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- **P. ROUSSEL**

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A la mémoire de mon père,

A ma mère,

A toute ma famille,

A mes amis,

En témoignage de mon affection.

Le travail exposé dans ce mémoire a été réalisé à l'Unité des Protéines (INSERM N° 16) à Lille, dirigée par Monsieur le Professeur Pierre-Marie DEGAND, sous la direction scientifique de Madame le Docteur Geneviève LAMBLIN et de Monsieur le Professeur Philippe Roussel, avec une participation des Laboratoires BAYER à Londres, sous la direction du Docteur Roderick L. Hall.

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ABREVIATIONS

Asn	Asparagine
CF	Cystic fibrosis (mucoviscidose)
CsBr	Cesium bromide
FAB	Fast Atom Bombardment
Fuc	Fucose
Gal	Galactose
GalNAc	N-acétylgalactosamine
Glc	Glucose
GlcNAc	N-acétylglucosamine
GalNAc-ol	N-acétylgalactosaminitol
GlcUA	Acide D-glucuronique
H A	Hyaluronic acid
^{1}H-NMR	Résonance Magnétique Nucléaire du proton
IduA	Acide L-iduronique
Man	Mannose
PAPS	3'-phosphoadénosine 5' phosphosulfate
SDS-PAGE	Sodium dodécyl sulfate-Polyacrylamide gel electrophoresis
Sia	Acide Sialique
Tris	Tris (hydroxymethyl)-aminomethane
Tyr	Tyrosine

INTRODUCTION

Les mucines représentent une vaste famille de glycoprotéines sécrétées par différentes muqueuses ou glandes muqueuses. Ces macromolécules, qui ont une conformation filamenteuse, ont une masse moleculaire généralement égale ou supérieure à 10^6 daltons, et résultent, pour près de 80% de leur composition, de phénomènes de glycosylation (1).

En pathologie humaine, les états d'hypersécrétion bronchique chronique sont fréquents. Ils s'accompagnent d'une augmentation de la sécrétion des mucines bronchiques qui constituent l'un des éléments majeurs du mucus bronchique. Les mécanismes qui régulent la sécrétion du mucus bronchique sont mal connus. Aussi les traitements therapeutiques appliqués aux hypersécrétions bronchiques chroniques sont-ils, malheureusement, souvent empiriques, avec une efficacité qui n'est pas toujours démontrée.

Il serait très important de pouvoir développer des modèles animaux permettant d'étudier la régulation de la sécrétion trachéo-bronchique et notamment celle des mucines. C'est le premier objectif du travail qui nous a été confié et qui a été réalisé en collaboration avec les Laboratoires Bayer.

Pour étudier cette sécrétion trachéo-bronchique, il nous a fallu choisir un marqueur peu coûteux et nous avons pensé au radiosulfate. Au cours des années 60, la notion de sulfatation des mucines, et des mucines bronchiques en particulier, s'est peu à peu imposée, au point que plusieurs laboratoires ont utilisé du radiosulfate pour étudier la sécrétion des mucines bronchiques (2).

Plus récemment, le développement des techniques de culture de cellules bronchiques est venu remettre en question cette notion, dans la mesure où une sécrétion de glycosaminoglycannes sulfatés a été mise en évidence par certains groupes (3).

Nous avons donc décidé de développer un modèle de culture organotypique de trachée chez l'animal et de caractériser les sulfoconjugués sécrétés.

Nous avons donc décidé de développer un modèle de culture organotypique de trachée chez l'animal et de caractériser les sulfoconjugués sécrétés.

A la suite de cette expérience, nous avons ensuite cherché à vérifier, dans la sécrétion bronchique humaine, les observations que nous avions pu faire chez l'animal.

Parallèlement à ces études, nous avons poursuivi un travail structural concernant la localisation du sulfate sur les chaînes glycanniques des mucines bronchiques humaines que nous avions entamé lors de notre DEA.

Avant de rapporter notre travail expérimental, nous présenterons un bref rappel concernant la diversité des sulfoconjugués et ce que l'on sait des sulfoconjugués dans l'arbre trachéo-bronchique.

* * *

L'ensemble des travaux que nous avons réalisés a fait l'objet des publications suivantes :

- 1 Sulfated O-glycoproteins secreted by guinea-pig trachea in organ culture.
Hassan Rahmoune, H. Paul Rounding, Wendy J. Mc Donald Gibson, Geneviève Lamblin, Roderick L. Hall, and Philippe Roussel.
Sous presse dans "**American Journal of Respiratory Cell and Molecular Biology**"

- 2 Chondroitin sulfate in infected sputum from patient with cystic fibrosis.
Hassan Rahmoune, Geneviève Lamblin, Jean-Jacques Laffite, Claude Galabert, Monique Filliat, and Philippe Roussel.
Sous presse dans "**American Journal of Respiratory Cell and Molecular Biology**"

- 3 Structure of two sulfated oligosaccharides from respiratory mucins of a patient suffering from cystic fibrosis. A Fast Atom Bombardment mass spectrometric and ^{1}H-NMR spectroscopic study.
Geneviève LAMBLIN, Hassan RAHMOUNE, Jean-Michel WIERUSEZSKI, Michel LHERMITTE, Gerard STRECKER and Philippe ROUSSEl.
Sous presse dans " **The Biochemical Journal** "

1ère Partie : RAPPELS BIBLIOGRAPHIQUES

I - RAPPEL SUR LES SULFOCONJUGUES.

Les fonctions des réactions de sulfatation sont beaucoup moins connues que celle des réactions de phosphorylation. La sulfatation est pourtant une réaction largement répandue qui intéresse de nombreuses classes de molécules, protéines, glycoconjugués ainsi que des molécules lipidiques ou stéroïdes ayant un noyau dérivé du stérane.

Ces réactions de sulfatation mettent en jeu des systèmes enzymatiques appelés sulfotransférases et un donneur de groupement sulfate, le 3'-phospho-adénosine 5'-phosphosulfate ou PAPS. Le substrat peut être sulfaté au niveau d'un résidu glucidique, d'un acide aminé ou d'un noyau de stérane.

1 - LES SULFOCONJUGUES.

Ils correspondent aux glycoprotéines, glycolipides ou protéoglycannes dont la partie glycannique peut être sulfatée. Il est parfois difficile d'établir une distinction entre glycoprotéines et protéoglycannes, dans la mesure où certaines molécules possèdent des éléments caractéristiques de ces deux types d'entités moléculaires ; aussi avons nous tenté d'établir une classification fondée sur la nature des chaînes glycanniques sulfatées.

a) Les glycosamino-glycurono-glycannes sulfatés

Les protéoglycannes sont des macromolécules comportant un axe peptidique ("core protein") hérissé d'un plus ou moins grand nombre de chaînes de glycosaminoglycannes qu'il est possible de distinguer en fonction de leur composition chimique (présence de N-acétylglucosamine et/ou de N-acétylgalactosamine; présence d'un acide uronique, D-glucuronique ou L-iduronique) et de leur sensibilité à certains enzymes spécifiques. La plupart de ces glycosaminoglycannes sont sulfatés (Tableau I) (4, 5).

On range habituellement, parmi les chaînes de glycosaminoglycannes, le kératane sulfate. Il sagit en fait de chaînes glycanniques qui ne comportent pas

TABLEAU I - PRICIPAUX GLYCOSAMINO-GLYCURONO-GLYCANNES SULFATES.

Variétés	Unité disaccharidique caractéristique	Sucre impliqué dans la liaison au sulfate	Position du sulfate sur le sucre	Enzymes spécifiques	Référ.
Chondroïtine sulfate A	$(GalNAc\ \beta\ 1,4\ GlcUA)_n$	GalNAc	4	Chondroïtinase ABC Chondroïtinase AC	(4, 5)
Chondroïtine sulfate C			6	Hyaluronidase tésticulaire	
Dermatane sulfate ou (Chondroïtine sulfate B)	$(GalNAc\ \beta\ 1,4\ IduA)_n$	GalNAc IduA	4 2	Chondroïtinase ABC	
Héparane sulfate	$(GlcNAc\ \alpha\ 1,4\ IduA)_n$	GlcNAc	6	Héparinase	
Héparine	$(GlcNAc\ \alpha\ 1,4\ IduA)_n$	GlcNAc IduA	2 et 6 6	Héparinase	(5)
Héparitine B	$(GlcNAc\ \alpha\ 1,4\ IduA)_n$	Glc NAc	2 et 6	Héparitinase	

d'acide uronique et qui ont des caractères communs avec d'autres glycannes. C'est la raison qui nous a incité à le traiter à part dans le paragraphe consacré aux polylactosaminoglycannes avec lesquels il a sans doute un grand nombre d'analogies structurales (6).

Outre la présence d'acide uronique, les chaînes de glycosamino-glycurono-glycannes ont une caractéristique commune, celle d'être liées à l'axe peptidique ("core protein") par des liaison alcali-labiles constituées d'un tétrasaccharide comprenant une molécule d'acide glucuronique, deux de galactose et une de xylose.

Nous avons fait figurer dans le Tableau I les principaux glycosamino-glycuronoglycannes qui entrent dans la constitution des protéoglycannes.(4-6). Ces protéoglycannes comportent une ou plusieurs molécules de glycosaminoglycurono-glycannes mais aussi, selon les cas, un nombre variable d'autres chaînes glycanniques, kératane sulfate, N-glycannes, O-glycannes (Figure 1) (7). Il s'agit d'un groupe de molécules que l'on trouve dans les tissus conjonctifs, le cartilage, à la surface des cellules, dans les membranes basales, dans les grains de sécrétion, dans le collagène, voire dans le plasma, sous des formes extrêmement diversifiées (8-12).

En fonction de leurs structures, les fonctions des protéoglycannes sont extrêmement diverses : structuration du tissu conjonctif et des membranes basales, adhésion cellulaire, contrôle de la prolifération cellulaire, coagulation, antiprotéase, activateur d'antiprotéase, récepteurs de la lipoprotéine lipase (10, 12).

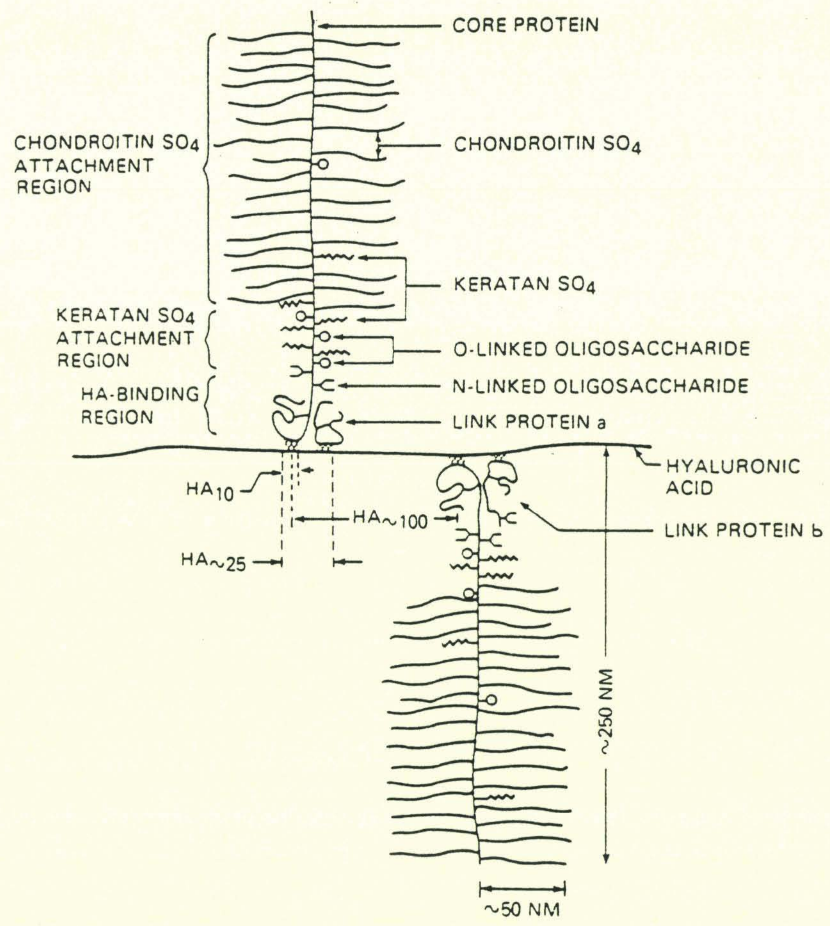


Figure 1 - Modèle de structure des agrégats de protéoglycannes de cartilage (7).

b) Les N-glycannes sulfatés

Les N-glycosylprotéines sont des glycoprotéines contenant une ou plusieurs chaînes glycanniques liées N-glycosidiquement à la partie peptidique. Cette liaison asparaginyl-N-acétylglucosamine est considérée comme alcali-stable (dans NaOH 0,1M à 45°C), mais peut être rompue dans des conditions plus drastiques (NaOH 1M à 80°C pendant 24 heures) (13).

Des résidus de sulfate ont été identifiés sur la partie glycannique de diverses N-glycosylprotéines (Tableau II) (14-20). Ce groupement sulfate pourrait intervenir comme :

- signal de reconnaissance pour la sécrétion folliculaire de la thyroglobuline (17),
- inhibiteur de la mannosidase II, stoppant ainsi le processing de la structure oligomannosidique (15),
- inhibiteur des exoglycosidases (19),
- facteur d'adsorption virale (18),
- élément favorisant l'embryogenèse (20).

c) Les O-glycannes sulfatés

Les mucines sont les principales glycoprotéines entrant dans la constitution des mucus bronchique, gastro-intestinal, cervical ou salivaire. Ces molécules sont caractérisées par leur masse moléculaire élevée et par la grande diversité des chaînes glycanniques (1) qui sont liés à l'axe peptidique par une liaison O-glycosidique (Figure 2), alcali-labile dans les conditions de Iyer *et al* (21).

Les mucines sont synthétisées dans des cellules particulières telles que les cellules caliciformes que l'on trouve dans de nombreux épithéliums ou les cellules muqueuses des glandes muqueuses ou séro-muqueuses.

De nombreuses mucines peuvent être sulfatées et quelques travaux ont été consacrés à la localisation du sulfate sur les mucines salivaires, bronchiques, gastriques (Tableau III) (22-29) ou coliques (30).

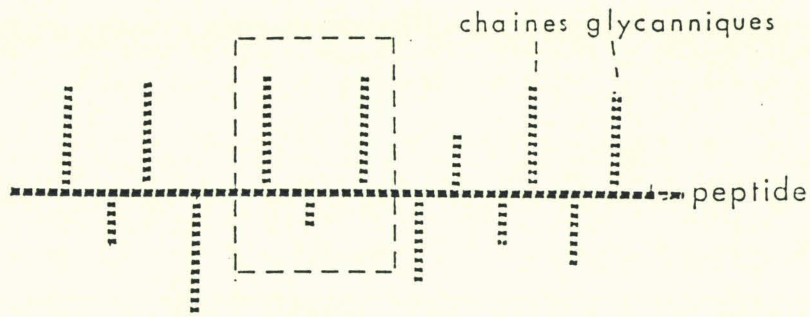
Il est à noter que le degré de sulfatation des mucines intervient dans leur hétérogénéité (31).

TABLEAU II - N-GLYCOSYLPROTEINES SULFATEES.

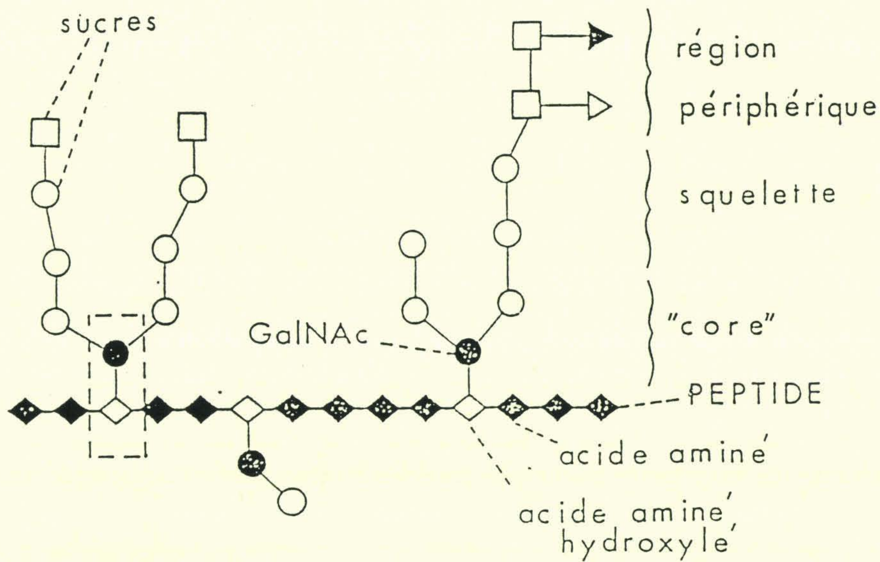
Varieté de glycoprotéine	Sucre impliqué dans la liaison au sulfate	Position du sulfate sur le sucre	Référ.
Glycoprotéine membranaire thyroidienne (Veau)	Man	6 et 4	(14)
Ovalbumine	Man	4	(15)
Hormone lutéïnisante (LH) (mouton)	GalNAc	4	(16)
Sous-unité B de la thyroglobuline .	Gal GlcNAc	3 6	(17)
Glycoprotéine du Paramyxovirus SV5	GlcNAc - Asn	6	(18)
Hormone lutéïnisante bovine	GalNAc GlcNAc	?	(19)

FIGURE 2

Schématisation des mucines



CHAINES GLYCANNIQUES



LIAISON

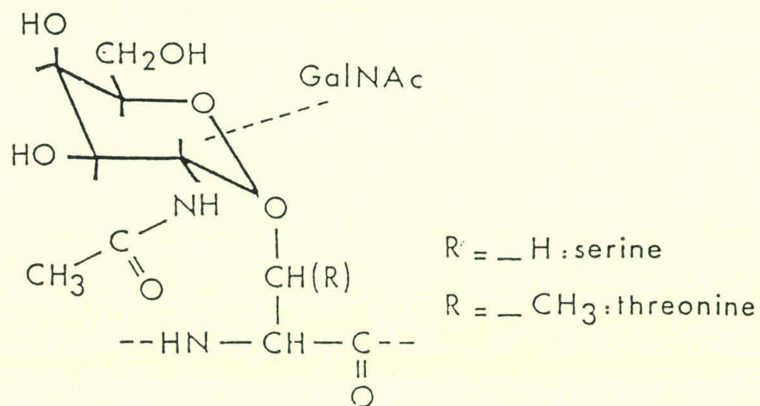


TABLEAU III - MUCINES SULFATEES.

Varieté de mucine sulfatée	Sucre impliqué dans la liaison au sulfate	Position du sulfate sur le sucre	Référ.
Ovomucine	GlcNAc	6	(22)
Mucines bronchiques	Gal	6	(23) (24)
Mucines salivaires	GlcNAc	3 ou 4	(25)
	GlcNAc	4 et 6	(26)
	GlcNAc	4	(27)
Mucines gastriques	GlcNAc	6	(28)
	N-Acétylhexosamine Gal	6 6	(29)

Les sulfomucines salivaires joueraient un rôle dans l'adhérence bactérienne (32), ou dans l'inhibition de l'action de la neuraminidase (33). Les sulfomucines gastriques inhiberaient l'action corrosive de la pepsine (34) et s'opposeraient à l'ulcération de l'estomac (35).

Au niveau des bronches, des études préliminaires ont montré que *Pseudomonas aeruginosa* adhérait aux glycopeptides neutres ou sialylés des mucines bronchiques humaines mais pas ou peu aux glycopeptides sulfatés (36).

Les travaux de Mian *et al* (37) ont aussi montré que le sulfate ainsi que l'acide sialique augmentaient la viscosité du mucus trachéal du *poulet.*

d) Le kératane sulfate et les polylactosaminoglycannes sulfatés

Le kératane sulfate est généralement étudié avec les protéoglycannes dont il peut constituer l'un des types de chaînes glycanniques (38, 39). On distingue le kératane sulfate de type I isolé de la cornée (40) et le kératane sulfate de type II isolé du cartilage (41). Les kératanes sulfate se distinguent essentiellement l'un de l'autre par le type de liaison entre l'axe peptidique ("core protein") et la partie glycannique (42). Cette liaison est alcali-stable dans le cas du kératane sulfate de type I et alcali-labile dans le cas du kératane sulfate de type II (Tableau IV). Les kératanes sulfate présentent des analogies avec les polylactosaminoglycannes qui, comme eux, sont formés d'enchaînements polylactosaminiques (6, 45).

Il est aussi important de signaler l'homologie structurale entre le kératane sulfate de type II et certaines chaînes glycanniques des sulfomucines isolées du colon (30) (Tableau IV). On y trouve :

- la même liaison O-glycosidique avec la partie peptidique,
- la même unité lactosaminique répétitive,
- la même position du sulfate sur le résidu de N-acetylglucosamine.

Les polylactosaminoglycannes sont en partie responsables de l'antigénicité de la surface cellulaire (6) : il en est ainsi des glycoconjugués qui portent les systèmes de groupe sanguin Ii et ABO (46). Ce caractère antigénique peut être masqué par les groupements sulfate dans le kératane sulfate (47).

TABLEAU IV - KERATANES SULFATES ET STRUCTURES APPARENTEES

Varieté	Unité disaccharidique caractéristique	Sucre impliqué dans la liaison au sulfate	Position du sulfate sur le sucre	Enzymes spécifiques	Référ.
Kératane sulfate I (N-glycosidique)		GlcNAc, Gal	6	Endo-ß-galactosidase	(42, 43)
Kératane sulfate II (O-glycosidique)		GlcNAc, Gal		Kératanase	
Lactosamino-glycannes sulfatés (mucines du colon)	$(Gal\ \beta 1,4\ GlcNAc)_n$	GlcNAc	6	Endo-ß-galactosidase	(30)
Sulfolactosamino-glycannes. Culture de cellules trachéales de chien		?	?	Endo-ß-galactosidase Kératanase	(44)

e) Les sulfoglycolipides

Les sulfoglycolipides se distinguent, selon l'alcool qui entre dans leur constitution, en *sphingo*-lipides et en *glycéro*-glucolipides (48-50) (Tableau V).

Les *sulfogalactoglycérolipides* sont encore appelés des séminolipides car ils constituent les sulfolipides majeurs au niveau des testicules ou du sperme de mammifères (51). Des formes mineures de diacyl- et alkyl-glycérol des sulfogalactosylglycérolipides ont été caractérisées dans le cerveau de *rat* (50).

Les *sulfatides* sont les composants majeurs des sulfolipides du système nerveux. Ces sulfatides pourraient intervenir dans :
- l'adhésion cellulaire (52),
- l'adhérence de germes pathogènes (53),
- la spermatogénèse (54),
- et dans la synaptogénèse (48).

2 - <u>LES PEPTIDES ET PROTEINES SULFATES.</u>

Certaines protéines comportent des résidus de tyrosine sulfate. La sulfatation de la tyrosine a été découverte dans le fibrinogène (55). Les liaisons sulfate-0-tyrosine sont alcali-stables (56). D'autres protéines sulfatées ont ensuite été décrites (Tableau VI) (57-61).

La sulfatation de la tyrosine jouerait un rôle dans la sécrétion des IgM, et dans leur stabilité après sécrétion (62). Dans le cas des IgG 2a, l'inhibition de la N-glycosylation par la tunicamycine s'accompagnerait d'une sulfatation de résidus de tyrosine qui compenserait l'absence de glycosylation (59). Freiderich *et al* (63) ont aussi montré qu'une inhibition de la sulfatation de la tyrosine diminuerait la vitesse de sécrétion des protéines.

TABLEAU V - SULFOGLYCOLIPIDES

Varieté	Sucre impliqué dans la liaison au sulfate	Position du sulfate sur le sucre	Référ.
Glycosphingolipides	Gal (t)	3	(49)
Gangliotriaosylceramide	Gal (i)	3	
	GalNAc (t) Gal (t)	3 3	
Lacto-triaosylceramide	GlcNAc (t)	6	
Lacto-neotriaosylceramide	GlcNAc (i)	6	
Glycolipides d'oursin	Sia (t) Sia (i)	8 8	
	GlcUA	3	
Triglucosylalkylacylglycerol	Glc	6	
Glycérolipides	Gal	3	(50)

(i) = position interne

(t) = position externe

TABLEAU VI - PEPTIDES ET PROTEINES SULFATES.

Varieté de peptide et de protéine	Type de macromolecule	Acide aminé impliqué	Liaison du sulfate sur l'aminoacide	Référ.
Gastrine II	peptide	Tyr	4	(57)
Enzymes des microvillosités intestinales	Protéines			(58)
IgG 2a	Immunoglobuline (Fc)			(59)
Chromogranine A	Glycoprotéine			(60)
Revue générale	peptide protéine glycoprotéine			(61)

3 - SULFATATION DES LIPIDES

La sulfatation du noyau stérane (Tableau VII) (64-67) est connue depuis longtemps: c'est un des mécanismes de conjugaison des hormones stéroïdiennes et de leurs dérivés (67). Cette sulfoconjugaison, comme la glucuronoconjugaison, facilite l'élimination urinaire des substances peu solubles dans l'eau en les rendant plus hydrophiles.

Plus récemment, on a observé la présence du cholestérol 3-sulfate (Tableau VII) au niveau de la membrane érythrocytaire (66) et de la membrane acrosomique, jouant un rôle de stabilisateur (65).

Rearick *et al* ont enfin montré que le cholestérol 3-sulfate était un marqueur de la différenciation cellulaire (64).

TABLEAU VII - STEROL-SULFATES.

Varieté	Entité impliquée dans la liaison au sulfate	Position du sulfate	Référ.
Cellules épithéliales de trachée de lapin	Cholestérol	3	(64)
Spermatozoïdes			(65)
Erythrocytes			(66)
Homme	Noyau stérol	3	(67)

II - RAPPEL SUR LES METHODES D'ETUDE DE LA SECRETION TRACHEO-BRONCHIQUE ET LES SULFOGLYCONJUGES

Pour étudier les glycoconjugués de la sécrétion bronchique, différentes approches ont jusqu'à maintenant été utilisées : l'étude directe des sécrétions trachéo-bronchiques produites *in vivo* et les sécrétions produites par des cultures organotypiques ou cellulaires.

1 - SECRETIONS TRACHEO-BRONCHIQUES

Les travaux concernant l'identification des sulfoglycoconjugés, notamment des glycosaminoglycannes, dans les lavages ou sécrétions bronchiques chez l'homme restent très controversés (Tableau VIII) (68-74).

Bhaskar *et al* (70) ont suggéré une sécrétion de glycosaminoglycannes chez le sujet sain et, dans les situations pathologiques, une diminution de cette sécrétion qui serait compensée par une hypersécrétion de mucines. Cette théorie est en contradiction avec les travaux de Lafitte *et al* (69) et de Thornton *et al* (75) qui n'ont pas observé de glycosaminoglycannes mais ont caractérisé des mucines respectivement dans le lavage et dans la sécrétion bronchique de sujets sains. Sahu et Lynn (68) ont, de leur côté, montré l'existence d'acide hyaluronique dans les lavages bonchoalvéolaires de sujets asthmatiques (Tableau VIII). Cet acide hyaluronique pourrait avoir une origine alvéolaire.

L'analyse de la sécrétion bronchique "normale" est, chez l'Homme, difficile pour plusieurs raisons :
- l'homme sain ne crache pas et, par fibroscopie, on ne peut recueillir que de faibles quantités de mucus ;
- l'utilisation des produits radiomarqués (^{35}S, ^{3}H) chez l'Homme pose des problémes éthiques.

Peu d'études ont été réalisées chez l'animal. Un modèle de poche trachéale du chien a cependant été décrit par Wardell *et al* (76) et utilisé pour caractériser les mucines sécrétées par cet animal (77).

TABLEAU VIII - SULFOGLYCOCONJUGUES PRESENTS DANS LES LAVAGES ET LES SECRETIONS TRACHEO-BRONCHIQUES HUMAINES.

Etat	Matériel	Type de macromolécules sécrétées	Référ.
Asthme	Lavage	Acide hyaluronique	(68)
Normal	Lavage	Mucines	(69)
Normal	Lavage	Protéoglycannes	(70)
Normal	Bronchoscopie	Mucines	(71)
Asthme ou bronchite chronique	Expectoration	Mucines	(72)
Mucoviscidose	Expectoration	Glycosaminoglycannes Mucines	(73)
Mucoviscidose	Expectoration	Mucines	(24) (74)

Parallèlement des techniques de cultures organotypiques et de cultures cellulaires ont été progressivement développées afin d'essayer d'analyser la sécrétion bronchique "normale".

2 - CULTURES ORGANOTYPIQUES

Des études histo-radiographiques réalisées sur des explants de muqueuse bronchique humaine, ont montré une incorporation intense de radiosulfate au niveau de l'épithélium de surface et dans les glandes séreuses de la sous-muqueuse ainsi qu'une incorporation moindre dans les chondrocytes (2).

D'autres travaux réalisés chez le *furet* ont confirmé ces résultats en montrant qu'il y avait une sulfatation rapide de l'épithélium de surface par rapport à la sous-muqueuse (78). Ces travaux montrent une diversité de répartition des sulfoglycoconjugués dans les différentes cellules trachéales.

Plusieurs modèles de culture organotypique ont été mis au point afin d'étudier la nature de la sécrétion trachéo-bronchique "normale" (Tableau IX) (79-90). Ces modèles permettent d'utiliser des marqueurs radioactifs mais ne permettent pas d'éliminer complètement la contamination du mucus sécrété au niveau de la surface épithéliale par des molécules synthétisées par le tissu conjonctif sous-jacent.

Gallagher *et al* ont suggéré que la composition des produits de sécrétion d'explants de trachée de *lapin* était fonction de l'origine cellulaire (82). Ainsi les cellules épithéliales sécréteraient des mucines tandis que le cartilage et le tissu conjonctif libéreraient du chondroïtine sulfate et de l'acide hyaluronique.

Dans des explants de trachée de *chien* débarrassé de tissu conjonctif, Stahl et Ellis (79, 80) ont montré :

- que la sécrétion globale était constituée de mucines,
- que la sous-muqueuse débarrassée de l'épithélium de surface produisait des mucines,

TABLEAU IX - CULTURES ORGANOTYPIQUES DE MUQUEUSE TRACHEO-BRONCHIQUE.

Origine	Matériel	Type de macromolécules sécrétées	Référ.
Chien	Explants	Mucines sulfatées	(79)
		Mucines	(80)
Chien et Homme	Explants	Mucines Chondroïtine sulfate Héparane sulfate Acide hyaluronique	(81)
Lapin	- Cellules épithéliales - Cartilage et tissu conjonctif	Mucines Chondroïtine sulfate Acide hyaluronique	(82)
Hamster	Explants	Mucines Protéoglycannes	(83)
	Explants	Chondroïtine sulfate Acide hyaluronique N- et O-glycoprotéines	(84)
Porc	Explants	Mucines	(85)
Furet	Explants	Glycosaminoglycannes Mucines	(86)
Poulet	Explants	Mucines	(87)
Homme	Explants	Mucines	(88)
Chat	Explants en chambre de Ussing	Mucines	(89)
Cobaye	Explants	Mucines Acide hyaluronique Chondroïtine sulfate	(90)

- et que l'épithélium de surface produisait aussi des mucines qui étaient plus sulfatées que celles produites par les glandes de la sous-muqueuse.

En étudiant la même espèce, Bhaskar *et al* ont rapporté que la sécrétion trachéo-bronchique était formée essentiellement de glycosaminoglycannes et ont suggéré que les cellules ciliées, qui incorporent fortement le sulfate, étaient à l'origine de cette sécrétion (81).

Chez le *hamster*, Breuer *et al* ont montré que l'action de l'élastase de polynucleaires neutrophiles sur la surface des cellules épithéliales permettait une libération de 85% à 90% de mucines et de 10 à 15% de protéoglycannes(83).

Les travaux de Daniel *et al* sur des explants de *hamster* dépourvus de tissu conjonctif, ont permis de mettre en évidence à la fois des protéoglycannes, des N-glycoprotéines et des O-glycoprotéines qui proviendraient respectivement du cartilage, des membranes plasmiques de l'épithélium et des cellules caliciformes (84).

Dans la plupart de ces travaux, il est difficile d'éviter la libération dans le milieu de molécules synthétisées dans le tissu conjonctif.

Les travaux de Bhaskar *et al* (81) et de Shelhamer *et al* (88) chez l'*homme*, sont en contradiction totale puisque les premiers affirment que la sécrétion bronchique "normale" est composée de protéoglycannes, tandis que les seconds montrent qu'elle est constituée de mucines. Il est important de signaler que Shelhamer *et al* (88) n'ont pas recherché la présence de protéoglycannes et que les arguments expérimentaux utilisés par Bhaskar *et al* (81) prêtent à discussion.

Chez le *furet*, Leigh *et al* (86) ont suggéré que la composition du mucus variait avec l'âge : la sécrétion du nouveau-né est constituée de mucines, alors que chez l'adulte elle serait composée majoritairement de glycosaminoglycannes.

En utilisant la chambre de Ussing qui évite les contaminations par les produits synthétisés par le tissu conjonctif, Phipps *et al* (89) ont montré, chez le *chat*, une sécrétion exclusive de mucines par les glandes de la sous-muqueuse (ils ont vérifié l'absence d'acide uronique dans cette sécrétion).

Les cultures organotypiques permettent l'étude de la sécrétion trachéo-bronchique "normale" en utilisant des marqueurs isotopiques (^{35}S, ^{3}H). Cependant il

est important de signaler que ces études *in vitro* souffrent d'un certain nombre de critiques par rapport à la situation *in vivo* :

- le tissu peut être endommagé ou altéré durant la dissection,
- les glandes de la sous-muqueuse ne sont plus soumises au contrôle du système nerveux,
- la perfusion de la surface épithéliale par un milieu nutritif ou le milieu baignant les explants peuvent influer sur la sécrétion,
- dans bien des systèmes, mis à part la chambre de Ussing, il est difficile de faire la part de (i) ce qui est sécrété par l'épithélium et par les glandes de la sous-muqueuse de (ii) ce qui est synthétisé par le tissu conjonctif sous-jacent.

Le modèle de culture organotypique que nous avons développé chez le *cobaye* permet de ne recueillir que la sécrétion produite dans la lumière trachéo-bronchique.

3 - <u>CULTURES CELLULAIRES</u>

La comparaison des différents types de macromolécules sécrétées par des cultures primaires de cellules trachéales, montre que la présence des mucines ou des glycosaminoglycannes varie en fonction du type de cellules mises en culture.

En effet, des cellules séreuses en culture (3) sécrètent en majorité des glycosaminoglycannes. Dans d'autres travaux, des cellules épithéliales ne sécrètent que des mucines (44, 91-93) (Tableau X).

Sur des cultures primaires de cellules épithéliales de *hamster*, Kim *et al* (94) ont montré que la sécrétion était constituée essentiellement de mucines et de 5% de glycosaminoglycannes et d'acide hyaluronique (Tableau X), la sécrétion de ces derniers pouvant être liée au degré de confluence et à la maturation des cellules.

Précédemment, cette même équipe avait montré que la sécrétion trachéale variait en fonction du support sur lequel les cellules étaient mises en culture : sur plastique, les cellules épithéliales de trachée de *lapin* sécrètaient de l'acide hyaluronique, et sur collagène, les mêmes cellules sécrètaient à la fois de l'acide hyaluronique et des mucines (95). Kim *et al* suggèrent donc que la nature du

TABLEAU X - CULTURES DE CELLULES TRACHEO-BRONCHIQUES.

Origine	Type de cellules en culture primaire	Type de macromolecules sécrétées	Référ.
Chien	Cellules épithéliales	Mucines Pas de protéoglycannes	(44)
	Cellules épithéliales	Mucines	(81)
Lapin	Cellules épithéliales	Mucines	(92)
Porc	Cellules épithéliales	Mucines	(93)
Hamster	Cellules épithéliales	Mucines Acide hyaluronique Chondroïtine sulfate Héparane sulfate N-glycoprotéines	(94)
Boeuf	Cellules séreuses	Acide hyaluronique Chondroïtine sulfate Héparane sulfate N- et O-glycoprotéines	(3)

support sur lequel les cellules sont cultivées joue un rôle important dans le maintien et la différenciation de ces cellules(96).

Enfin, Rearick *et al* ont confirmé les données précédentes et ont suggèré que les rétinoïdes, ajoutés au milieu extracellulaire, influaient sur la différenciation cellulaire et la sécrétion des mucines (92).

2ème Partie : TRAVAUX PERSONNELS

I- MISE AU POINT D'UN MODELE DE CULTURE ORGANOTYPIQUE DE TRACHEE DE COBAYE ET IDENTIFICATION DES SULFOGLYCOCONJUGUES SYNTHETISES

. La mise au point d'un système d'étude des sulfoglycoconjugués trachéo-bronchiques chez l'animal a représenté un travail long et difficile dont nous ne présenterons délibérement que les aspects positifs.

En effet, nous avions initialement imaginé que, pour étudier les sulfoglycoconjugués trachéo-bronchiques, nous pourrions utiliser l'animal entier.

Dans une première phase de nos recherches, nous nous sommes servis de la glucosamine et du sulfate radioactifs qui ont été injectés chez le *rat*. Nous avons alors essayé de caractériser les produits synthétisés par l'arbre trachéo-bronchique, en recueillant la sécrétion de rats normaux et d'animaux chez lesquels une exposition au SO_2 avait permis de provoquer un état d'hypersécrétion bronchique.

Ces expériences ont donné lieu à des résultats négatifs, à savoir qu'il a été d'abord pratiquement impossible de recueillir correctement la sécrétion des mucines "normales" chez l'animal vivant. Nous avons alors pensé recueillir la sécrétion par lavages de l'arbre trachéo-bronchique après sacrifice des animaux mais nous nous sommes aperçu que la radioactivité incorporée dans le matériel ainsi obtenu était très faible par rapport à celle qui était incorporée par la muqueuse digestive: ceci s'explique sans doute par la différence très importante qui existe entre la surface de la muqueuse trachéale et celle de la muqueuse digestive.

C'est la raison pour laquelle nous avons, avec les Laboratoires Bayer, développé un modèle de culture organotypique des voies aériennes chez le *cobaye*. Le choix de cet animal a été conditionné par le fait que sa trachée est un peu plus longue que celle du *rat*.

Le travail qui est exposé dans le premier manuscrit peut être résumé de la façon suivante.

Un modèle de culture organotypique de la trachée de *cobaye* a été développé en prélevant la trachée chez l'animal anesthésié, en ligaturant les extremités et en mettant cette trachée dans un milieu contenant du radiosulfate.

L'originalité de ce dispositif, par rapport à la plupart des modèles qui ont été décrits, réside dans le fait que la sécrétion recueillie dans la lumière trachéale après une survie de six heures ne peut contenir que le matériel sécrété par l'épithélium et

par les glandes de la sous-muqueuse. Aussi longtemps qu'il n'y a pas d'altération de l'épithélium, le matériel sécrété ne peut être contaminé par les molécules synthétisées par le tissu conjonctif sous-jacent. Cet écueil ne pouvait être évité dans la plupart des modèles utilisés jusqu'à présent, mise à part la chambre de Ussing.

Le radiosulfate, introduit dans le milieu où baigne la trachée, traverse la muqueuse et y est incorporé dans différents conjugués.

Dans le manuscrit qui suit, nous avons montré, que les macromolécules sulfatées qui ont été synthétisées et sécrétées dans la lumière trachéale étaient très diverses.

Une partie des macromolécules sulfatées synthétisées ont un comportement, en ultracentrifugation, en gel-filtration et après traitement alcalin , qui permet de les ranger dans la classe des mucines.

D'autres macromolécules sulfatées sont, comme les précédentes, sensibles au traitement alcalin mais résistantes aux enzymes capables de dégrader les glycosaminoglycannes. Elles sédimentent en gradient de densité de bromure de césium à des densités plus faibles que les mucines. La chromatographie de gel-filtration permet alors de les séparer en molécules dont le comportement chromatographique est le même que celui des mucines et d'autre part en molécules dont les masses sont nettement plus faibles (< 200 kDa) que celles qui sont couramment admises pour les mucines (> 1000 kDa).

On aboutit ainsi à la notion d'une population de glycoprotéines sulfatées à liaisons alcali-labiles. Si on exclue l'éventualité d'une dégradation protéolytique de certaines mucines dans la lumière trachéale, l'une des questions soulevées par ce travail est de savoir si des glycoprotéines sulfatées, à liaison alcali-labiles et de masse moléculaire relativement faibles, sont authentiquement des mucines.

A coté de ces O-glycoprotéines sulfatées, ce modèle de culture organotypique a permis de mettre en évidence une sécrétion de protéoglycannes à chondroitine sulfate dont l'existence avait jusqu'à présent été contestée dans d'autres espèces.

SULFATED O-GLYCOPROTEINS SECRETED BY GUINEA-PIG TRACHEA IN ORGAN CULTURE

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Running title : Sulfated O-glycoproteins secreted by guinea-pig trachea

ABSTRACT

Organ culture of guinea pig trachea was performed in the presence of [^{35}S]-sulfate in order to characterize the sulfated glycoproteins released from the respiratory epithelium and mucosa. The sulfated macromolecules that were synthesized during a 6 h incorporation were separated by CsBr density gradient centrifugation and gel-filtration chromatography successively.

Most of the sulfated secreted macromolecules corresponded to a population of glycoproteins sensitive to reductive β-elimination but resistant to both chondroitinase ABC and heparinase. These glycoproteins had different buoyant densities (ranging from 1.48 g/ml to 1.16 g/ml) and could be subfractionated according to molecular mass.

A major part of the radioactivity was incorporated into high molecular mass mucins which were exluded from a Sepharose CL-2B column and did not penetrate into polyacrylamide gel in PAGE. However a mixture of sulfated O-glycoproteins of much lower molecular mass was also characterized in addition to low amounts of chondroitin sulfate.

Epithelial goblet cells are the predominant mucin containing cells of the respiratory guinea pig trachea. Our results suggest that a wide range of sulfated O-glycoproteins are secreted by the guinea-pig tracheal mucosa.

Key Words : guinea-pig trachea, sulfated glycoproteins, chondroitin sulfate, mucins, organ culture.

Introduction

The respiratory tract is protected by mucus which is synthesized by goblet cells of the surface epithelium and, according to species, by submucosal glands. Respiratory mucus may contain several types of high molecular weight glycoconjugates, mucus glycoproteins or mucins, serum-type glycoproteins and proteoglycans (1). The principal macromolecules of respiratory mucus are mucins. These high molecular mass glycoproteins are heavily glycosylated and, at least in human, present a considerable diversity of carbohydrate chains. They are O-glycosidically attached to the peptide by alkali-labile linkages (2). Human respiratory mucins also show a wide diversity of contour length by electron microscopy (3,4).

Preliminary data have indicated the presence of glycosaminoglycans in the sputum of cystic fibrosis patients (5) or in bronchoalveolar lavage of volunteers (6). Hyaluronic acid has also been found in the bronchoalveolar lavage of asthmatics (7).

Sulfation is a post-translational modification which occurs on carbohydrates and which has been widely used to follow the biosynthesis or secretion of proteoglycans, mucins and other glycoconjugates such as N-glycoproteins (8,9). Tyrosine sulfation of proteins or peptides has also been described (10).

The sulfation of carbohydrate chains of respiratory mucins has been known for a long time (11,12), and this process may be increased in some pathological conditions (13-15). At the present time and to our knowledge, there are no reports concerning the direct sulfation of respiratory peptides or proteins.

Incorporation of radiolabelled sulfate into secreted glycoconjugates has often been used as a marker of mucin secretion by airway epithelium and epithelial cells in vitro (13,15-31). But many studies have not characterized the secreted products. This is important since there are examples where the major sulfated glycoconjugate products were not mucins but proteoglycans (25,18).

The apparent discrepancies between these results and the abundant literature concerning the identification of mucins as the main high molecular weight glycoconjugates in respiratory secretions may have several explanations. There are differences in the type of mucus synthesizing cells from species to species (32,33), and in the distribution of the cells at the different levels in the airway (34,35). In the case of explants, the preparation of a respiratory mucosa completely free of connective tisssue which has the ability to synthesize proteoglycans is not easy except when using Ussing chambers. For cells grown in cell culture, there is always the possibility that the secretion produced *in vitro* may differ from the material secreted by the respiratory mucosa *in vivo* (21). However, in a recent paper (36), Adler and associates used [^{3}H]-glucosamine as a marker and confirmed the synthesis and secretion of mucin-like glycoproteins by guinea pig tracheal epithelial cells in primary culture.

The purpose of the present study has been to label with [^{35}S]-sulfate and to identify the sulfated glycoconjugates secreted by trachea in an organ culture system where the collected material would not be in contact with the underlying connective tissue. This procedure has been applied to guinea pig trachea where the predominant mucin-synthesizing cells are goblet cells (32,33).

Under these conditions, most of the sulfated molecules which were secreted corresponded to a mixture of alkali-labile O-glycoproteins resistant to both chondroitinase ABC and heparinase. A minor fraction was represented by chondroitin sulfate - proteoglycans.

Materials and Methods

Radiochemicals, chemicals and solutions

Sodium [^{35}S]-sulfate (540 mCi/mmol) was from New England Nuclear Chemicals, Dreieich, Germany. All other chemicals and reagents used were of analytical grade: ethyl carbamate was from Sigma Chemical Company Ltd.

Tyrode's solution constituents were (mM) NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$; 2.1; NaH_2PO_4, 0.5; $NaHCO_3$, 11.9; D-glucose, 5.6: the solution was continuously gassed with 95% O_2, 5% CO_2. Urethane (ethyl carbamate) was used as a 25% wt / vol aqueous solution.

Cesium bromide was obtained from Merck; Sepharose CL-2B, Sephadex G-100 and the proteins standards for PAGE were from Pharmacia Fine Chemicals, Uppsala Sweden; guanidinium chloride was from Fluka A.G; the glycoprotein standard was serum α–1 glycoprotein from Sigma Chemical Co (St Louis, MO); Coomassie Brilliant blue R-250 was obtained from Serva; X-Omat S films were from Kodak; agarose was obtained from Litex, Denmark; chondroitinase ABC and endo-β-galactosidase from *E. freundii* were obtained from Miles; and heparinase and micrococcal nuclease were from Sigma. RNase A from bovine pancreas was from Boehringer. The scintillation cocktail (Aqualyte ™) was from J.T.Baker Reagent.

Animals

Dunkin Hartley male guinea pigs, 400-900 g, were supplied by Tuck and Harlan Olac, UK.

Organ culture of Guinea pig tracheas and collection of secretions

Guinea pigs were killed by intraperitoneal injection of urethane (7ml/kg). Each treatment group contained 30 animals.

Upper tracheal segments (20 to 30 mm long) were carefully dissected out from between the larynx and the top of the rib cage and immediately rinsed in ice-cold Tyrode to remove blood and secretions. Tracheal segments were trimmed of fat, blood vessels and connective tissue and pre-incubated at 37°C for 30 min in Tyrode. After the pre-incubation period the tracheal segments were tied tightly at both ends to form an enclosed sac of the luminal surface. The closed sacs were then placed in Tyrode containing sodium [^{35}S]-sulfate 100µCi / ml and incubated at 37°C for 6h.

Following the incubation period, the sacs were removed from the radiolabel solution. They were rinsed and cooled to 0°C in ice-cold Tyrode. To minimise contamination from cut mucosa sacs were held vertically and opened by cutting the top end. The lumenal secretions were carefully removed with a fine pasteur pipette, without touching the cut end. The sacs were further washed out with ice-cold Tyrode taking care that the wash solution did not contact the cut mucosa. Secretions from each group of 30 guinea pigs were pooled, centrifuged at 3,000 x *g* for 5 min to remove cellular debris, and the supernatants frozen prior to analysis.

After thawing, the secretions were dialyzed against distilled water at 4°C for 48h with several changes of water, freeze-dried and the weight of the total non-dialyzable labelled material was determined.

Histology

To determine an optimum incubation time, tracheas from 5 guinea pigs were incubated as above for 3, 7, 11, 16 or 21 h. The trachea from one guinea pig was taken without incubation as control. Following incubation each trachea was fixed in phosphate-buffered formalin (40% formaldehyde, 10% sodium phosphate, 0.9% NaCl) for 48 hours.They were then dehydrated and embedded in paraffin wax for sectioning. Sections of 5µm were cut from each trachea and stained either with Alcian blue/periodic acid Schiff at pH 2.6 for the visualisation of mucus cells, or with haemotoxylin and eosin for general histology.

CsBr density gradient centrifugation of secreted respiratory macromolecules

The non-dialyzable material (average 15 mg for 30 tracheas) was dissolved by stirring overnight at 4°C in 12 ml of 16.2 mM sodium phosphate buffer, pH 6.8, containing 33 mM NaCl, 0.02% of NaN_3 and 42% CsBr. This solution was transferred to a centrifuge tube (Beckman) and the sample was centrifuged for 72h at 10°C and at 43,500 rpm in a Beckman 70 Ti rotor (37).

After centrifugation, 13 fractions were collected from the tube and their density was measured by weighing 100 µl aliquots.

Radioactivity was determined after mixing a 20 µl sample from each fraction with 2 ml of scintillation cocktail (Aqualyte TM) by counting [^{35}S]-sulfate in a Beckman S 3801 instrument.

An aliquot of each fraction was diluted (1:5 with deionized water) and was assayed for hexose by an automated orcinol assay (38). The optical density at 278 nm was also measured.

Three fractions of decreasing density, 1, 2 and 3, were separated and dialysed at 4°C against distilled water.

Gel-filtration chromatography

Fractions 1 and 2 from density gradient centrifugation were subjected to chromatography on a Sepharose CL-2B column (30 x 1 cm) equilibrated in 6 M guanidinium chloride. Fractions (0.9 or 1ml) were collected and analyzed by counting [^{35}S]-sulfate as already indicated. Subfractions 1a (high mol. wt.) and 1b (lower mol. wt.) were obtained from fraction 1, and 2a and 2b from fraction 2. They were dialyzed and freeze-dried.

Fraction 1a and 2a were submitted to various treatments and rerun on the same column.

Fraction 2b was subfractionated on a Sephadex G-100 column (30 x 1 cm) in 0.1 M Tris-HCl, pH 8, containing 0.25 M NaCl and of 0.02% NaN_3 and was

analyzed by counting [^{35}S]-sulfate. After dialysis and lyophilization, fraction 2b was submitted to different treatments and then rerun on the same column.

Alkali treatment

The labeled fractions 1a, 2a and 2b were treated with 2 M $NaBH_4$ in 50 mM NaOH for 16h at 45°C (2). The mixtures were then adjusted to pH 5.4 with 4 M acetic acid and studied by gel filtration chromatography. The alkali treatment applied to labeled fractions 2b1 and 2b2 before PAGE was carried out without $NaBH_4$.

Enzymatic digestion

The lyophilized [^{35}S]-labelled products 1a, 1b, 2a and 2b were dissolved in physiological saline (300 µl). Aliquots (100 µl) were digested with chondroitinase ABC, heparinase or endo-β-galactosidase under the following conditions:

- chondroitinase ABC was added (0.5 unit in 20 µl of 0.1M Tris-acetate, pH 7.3) and incubated at 37°C for 18 h (17);
- heparinase was added (0.5 unit in 10 µl of 0.1M potassium phosphate, pH 7.0) and incubated at 37°C for 18 h (39);
- endo-β-galactosidase (0.1 unit) was used in 0.1 M Tris-acetate, pH 5.8, and the mixture was incubated at 37°C for 24 h (40).

For treatment by nucleases, fraction 1b was incubated with micrococcal nuclease (0.5 U) and RNAse A (20 µg) in 0.5 ml of 5 mM sodium borate, pH 8.8, containing 0.1% of bovine serum albumin for 18 h at 37°C (41).

After each enzymatic digestion, the mixture was subjected to chromatography or studied by agarose gel electrophoresis and PAGE.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed in veronal buffer at pH 8.2 (ionic strength 0.1) as previously described (42). The slides were studied by toluidine blue

(43). After staining, they were exposed to Kodak X-Omat S film at -80°C in the dark for 2 wk and developed.

Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel electophoresis of labelled products (1a, 1b, 2a, 2b, $2b_1$ and $2b_2$) was performed in polyacrylamide gel slabs (5%-15%) using the buffer system of Laemmli (44). After electrophoresis, the slabs were stained for protein with Coomassie Brilliant Blue R-250 or for carbohydrate with Schiff / periodate (45). The dried gels were exposed to Kodak X-Omat S film at - 80°C in the dark for 3 wk and developed.

RESULTS

Histological control of guinea pig trachea in organ culture and collection of the respiratory mucus

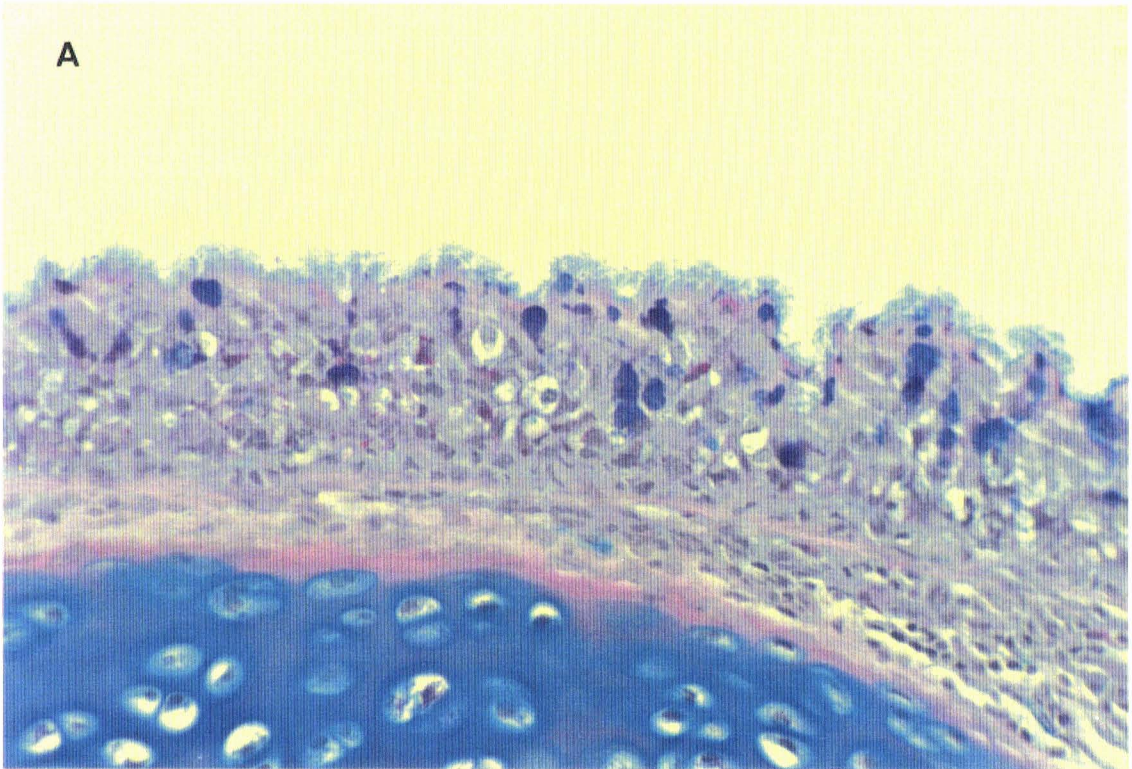
At 0 h incubation the epithelium of the guinea pig trachea was pseudostratified, ciliated and contained numerous mucous cells, mostly stained purple (Figure 1a). Sub-mucosal glands containing cells which stained blue, red / blue and red with Alcian blue/periodic acid Schiff could also be seen but only 2-3 glands were seen in each longitudinal tracheal section. After 3 and 7 h (Figures 1b &1c) incubation there was some loss of Alcian blue/periodic acid Schiff-stained cells from the epithelium, although not from the glands. Up to 7 h the epithelium remained intact (Figures 1b &1c). After 16 and 21 h incubation Alcian blue/periodic acid Schiff stained cells could be seen but there was progressive sloughing of epithelial cells. At 21 h there were large areas of exposed basement membrane. On the basis of these results we chose an incubation time of 6 h, a time at which no epithelial sloughing occured. After culture, 20 to 50 μl of secreted material were collected from each trachea. Secretions from 30 animals were pooled and fractionated (Figure 2).

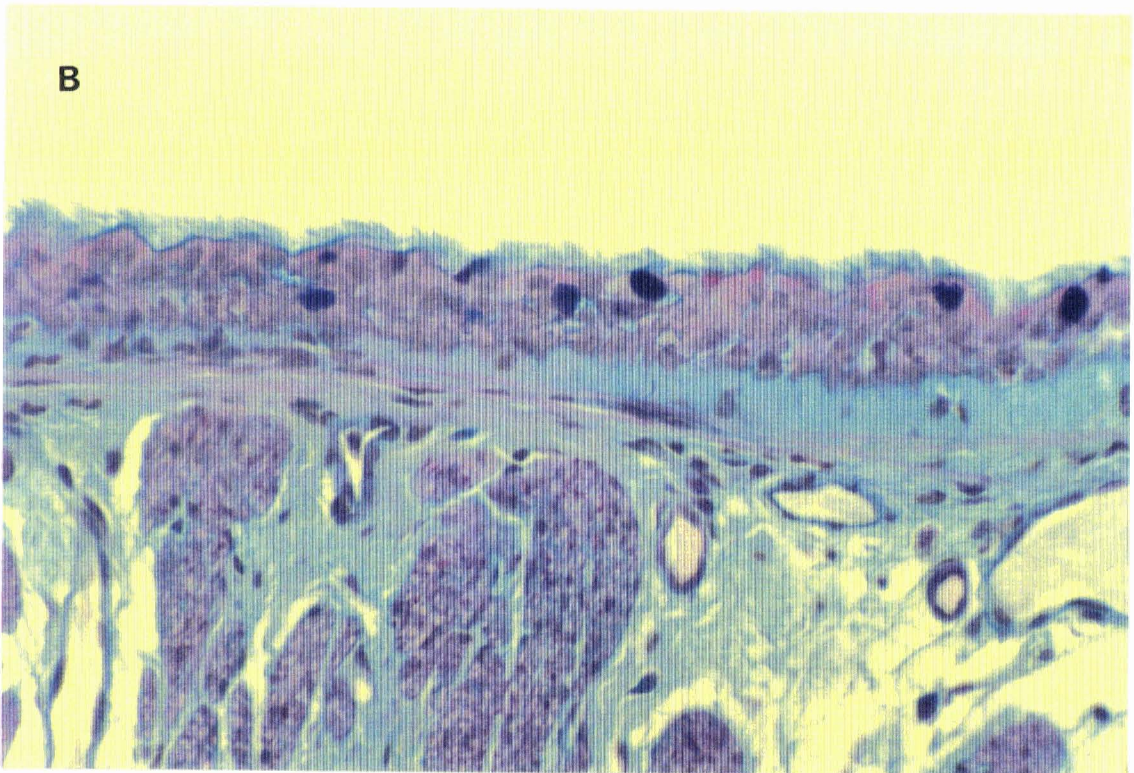
Fractionation of sulfated glycoconjugates secreted by guinea pig mucosa

The collected secretions (an average 15 mg) were separated by CsBr density gradient centifugation (Figure 3). Three fractions were obtained, 1, 2 and 3 : after dialysis and lyophilisation, the average recovered radioactivity for the three fractions was 40, 40 and 20% respectively.

Fraction 1 had the highest radioactive sulfate and carbohydrate content as visualized in Figure 3 and had a density ranging from 1.48 to 1.43 g / ml (n = 4 experiments). Fraction 2 had a density ranging from 1.38 to 1.23g / ml (n = 4 experiments), had a lower radioactive sulfate and carbohydrate content and had a high absorbance at 278 nm (Figure 3). Fraction 3 which had a much lower density ranging from 1.21 to 1.16 g / ml (n = 4 experiments) was turbid before collection

Figure 1. Micrographs of guinea pig trachea stained with Alcian blue/periodic acid Schiff : (A) 0 h ; (B) after 3 h incubation in Tyrode ; (C) after 7 h incubation in Tyrode.


A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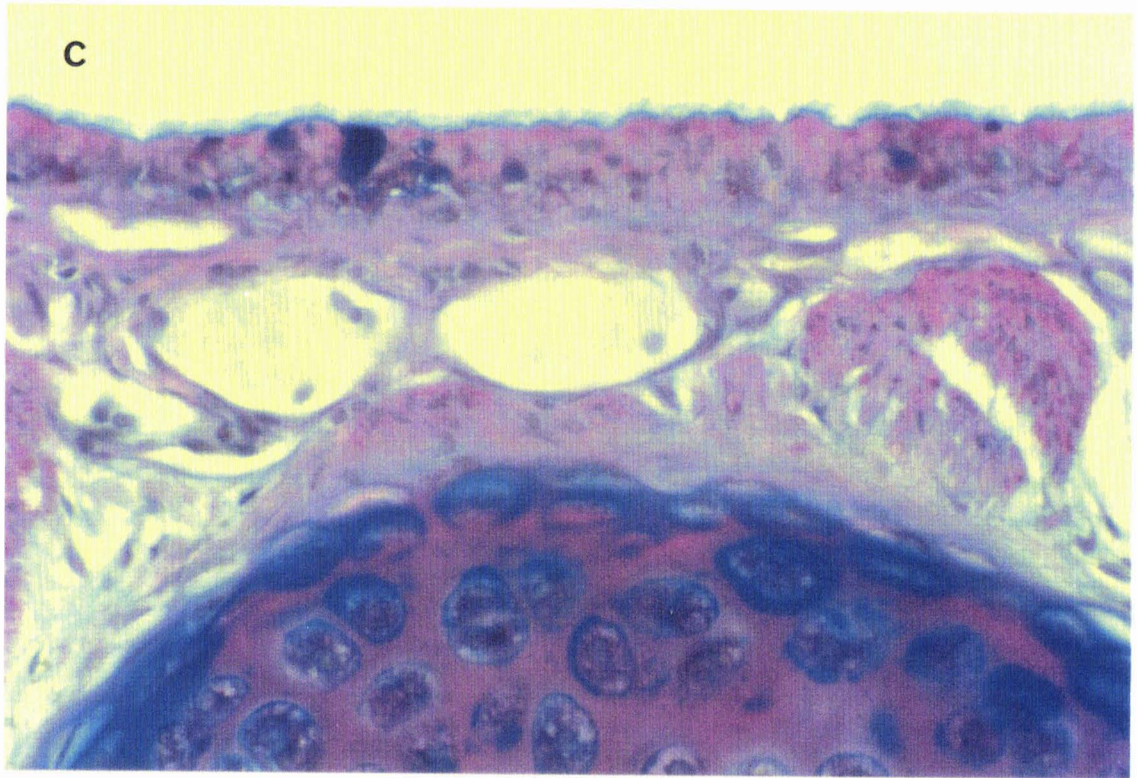
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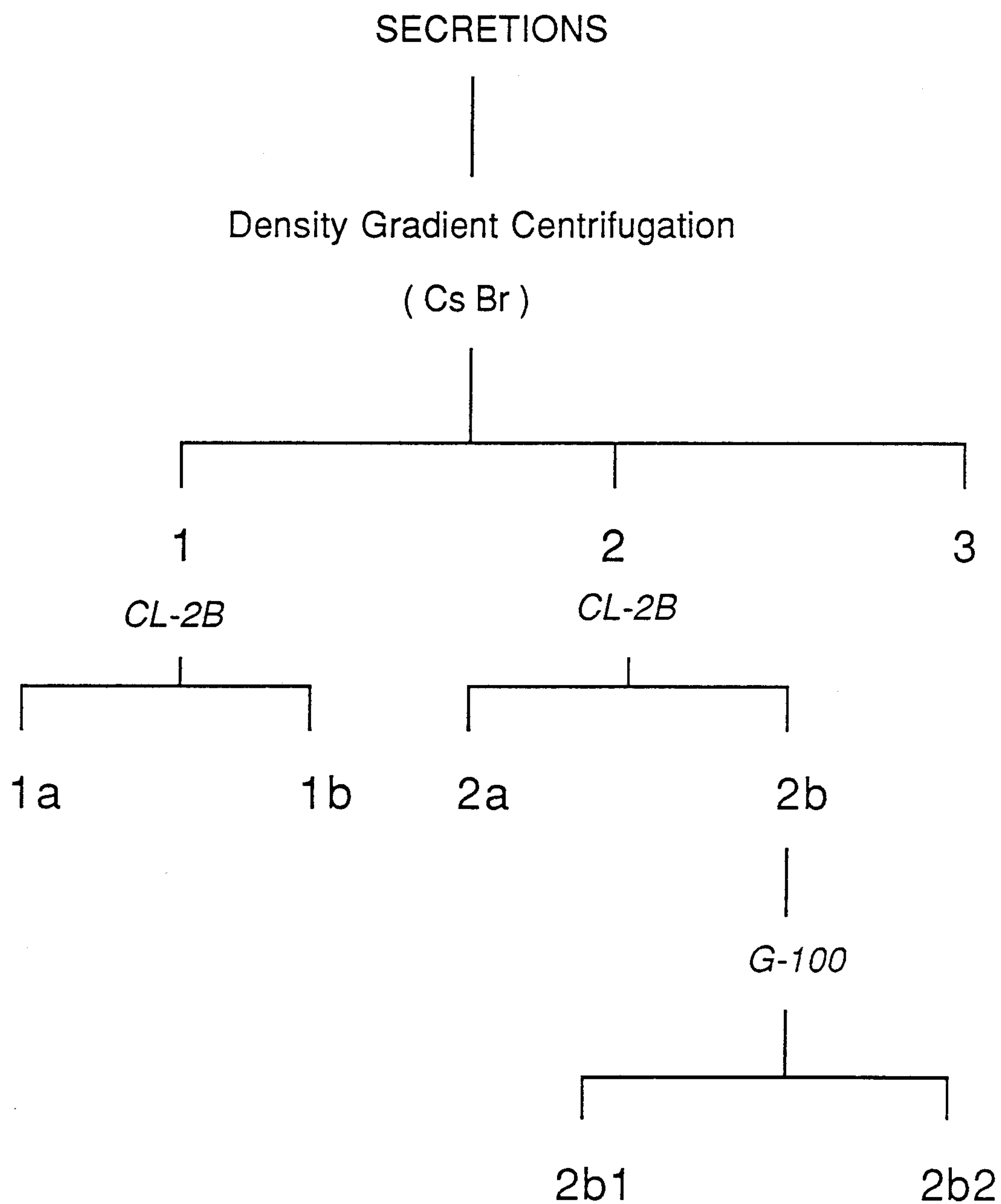

Figure 2.- Fractionation scheme of secreted guinea-pig secretions.

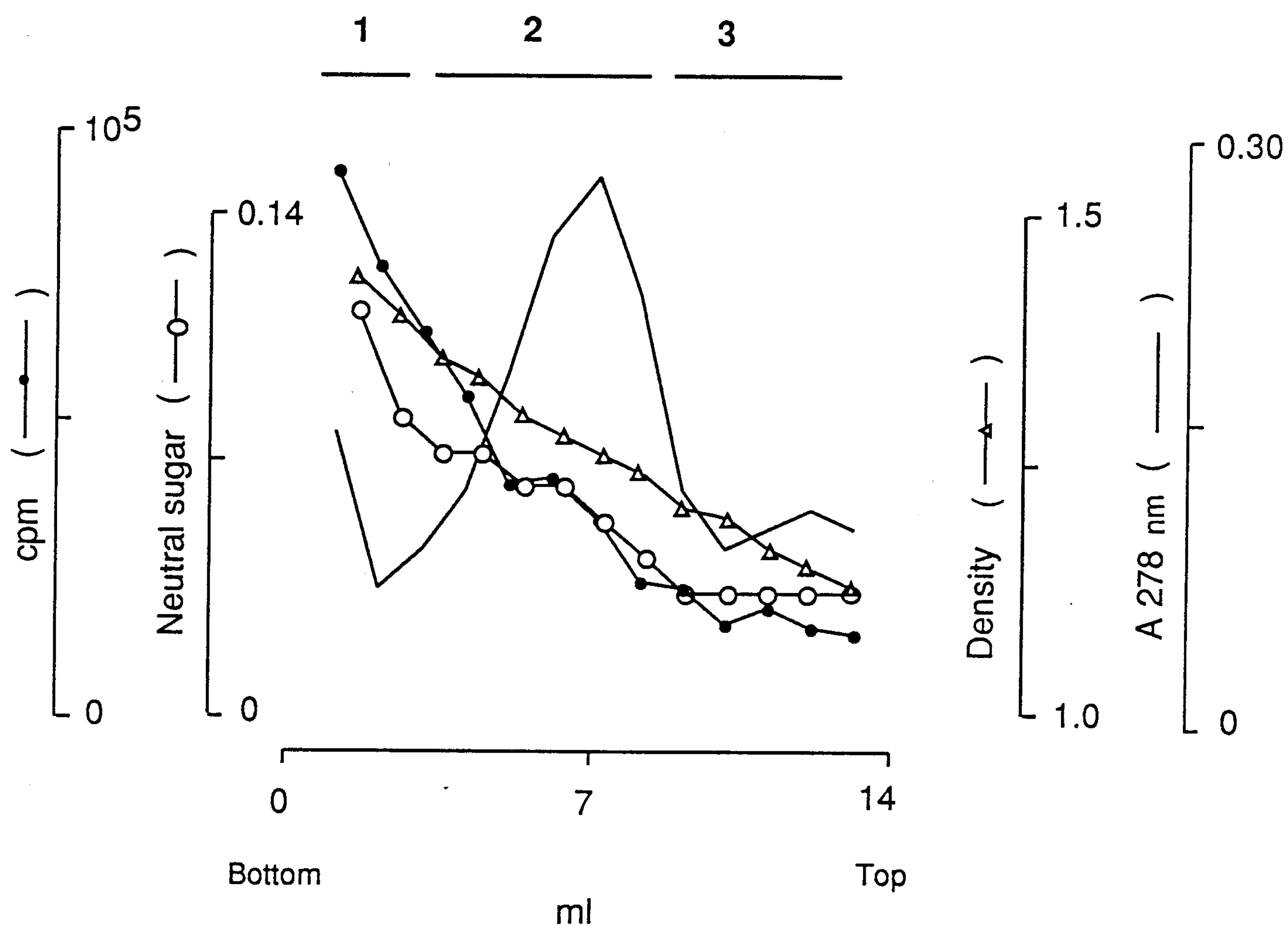

Figure 3.- CsBr-density gradient centrifugation of guinea-pig respiratory mucus. Bronchial mucus (15 mg) was centrifuged for 72 h at 43 500 rpm. Fractions were collected and analyzed for absorbance at 278 nm (closed line), for neutral sugar (open circle) and for [^{35}S]-sulfate (closed circle). Density was measured by weighing (open triangles). Fractions 1,2 and 3 indicated by bars were dialyzed and lyophilyzed.

and had low radiosulfate and carbohydrate contents. It was therefore not studied further.

Fractions 1 and 2 were subfractionated by gel chromatography on Sepharose CL-2B to give Fractions 1a, 1b, 2a and 2b which corresponded to 60% and 40%, 30% and 70% of the radioactivity recovered after CL-2B chromatography respectively (Figure 4).

Identification of the radiolabeled components of fractions 1a and 2a

The excluded peaks 1a and 2a were treated either by chondroitinase ABC or by heparinase and rerun on the same Sepharose CL-2B column. The elution pattern of the radioactive sulfate was not modified by these treatments indicating that these fractions did not contain glycosaminoglycan chains (Figure 5). In one experiment peaks 1a and 2a were treated by endo-β-galactosidase and rerun on the same Sepharose CL-2B column. The elution pattern of the radioactive sulfate was not modified (data not shown).

Fractions 1a and 2a were also submitted to reductive β-elimination and then applied to the Sepharose CL-2B column. In these conditions, the elution pattern of radioactive sulfate was totally included (Figure 5).

Fractions 1a and 2a were then studied by agarose gel electrophoresis followed by autoradiography; it showed that most of the radioactivity hardly penetrated into the agarose gel and was unaffected by chondroitinase ABC and heparinase (Figure 6).

Fractions 1a and 2a were finally analyzed by SDS-PAGE and staining by Coomassie blue or Schiff / periodate before being studied by autoradiography (Figure 7). Autoradiograms of fractions 1a and 2a showed that most of the radiolabelled components stayed in the stacking gel, in areas stained by Schiff reagent. The intensity of staining and labelling was higher in fraction 1a than in fraction 2a. The electrophoretic patterns of these two fractions were identical with those of human respiratory mucins (3).

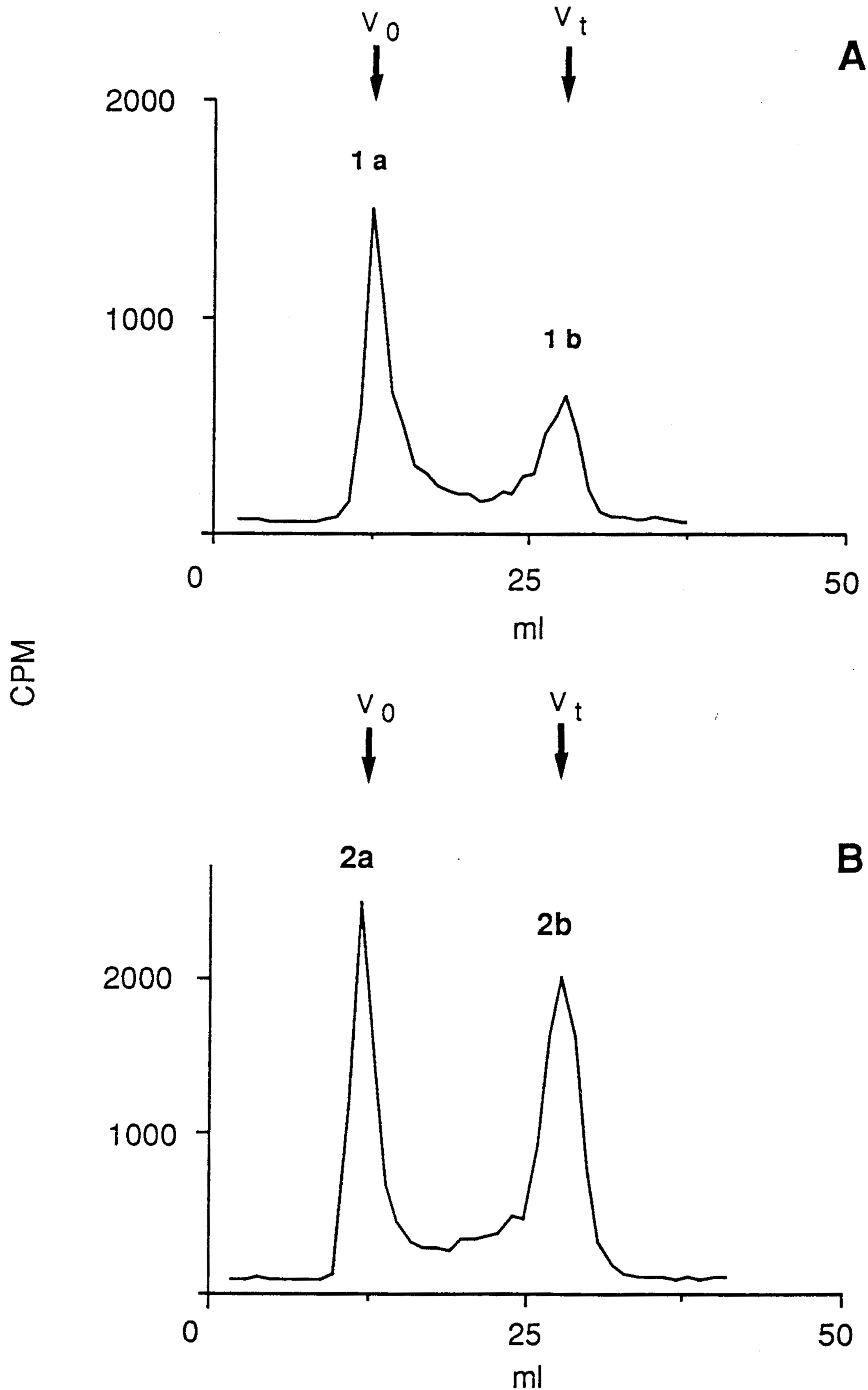

Figure 4.- Sepharose CL-2B (1x30 cm) chromatography of fraction 1 (A) and fraction 2 (B) from density gradient centrifugation. Fractions (1 ml) were collected and 20 µl aliquots were tested for their [^{35}S]-sulfate (solid line) activity.

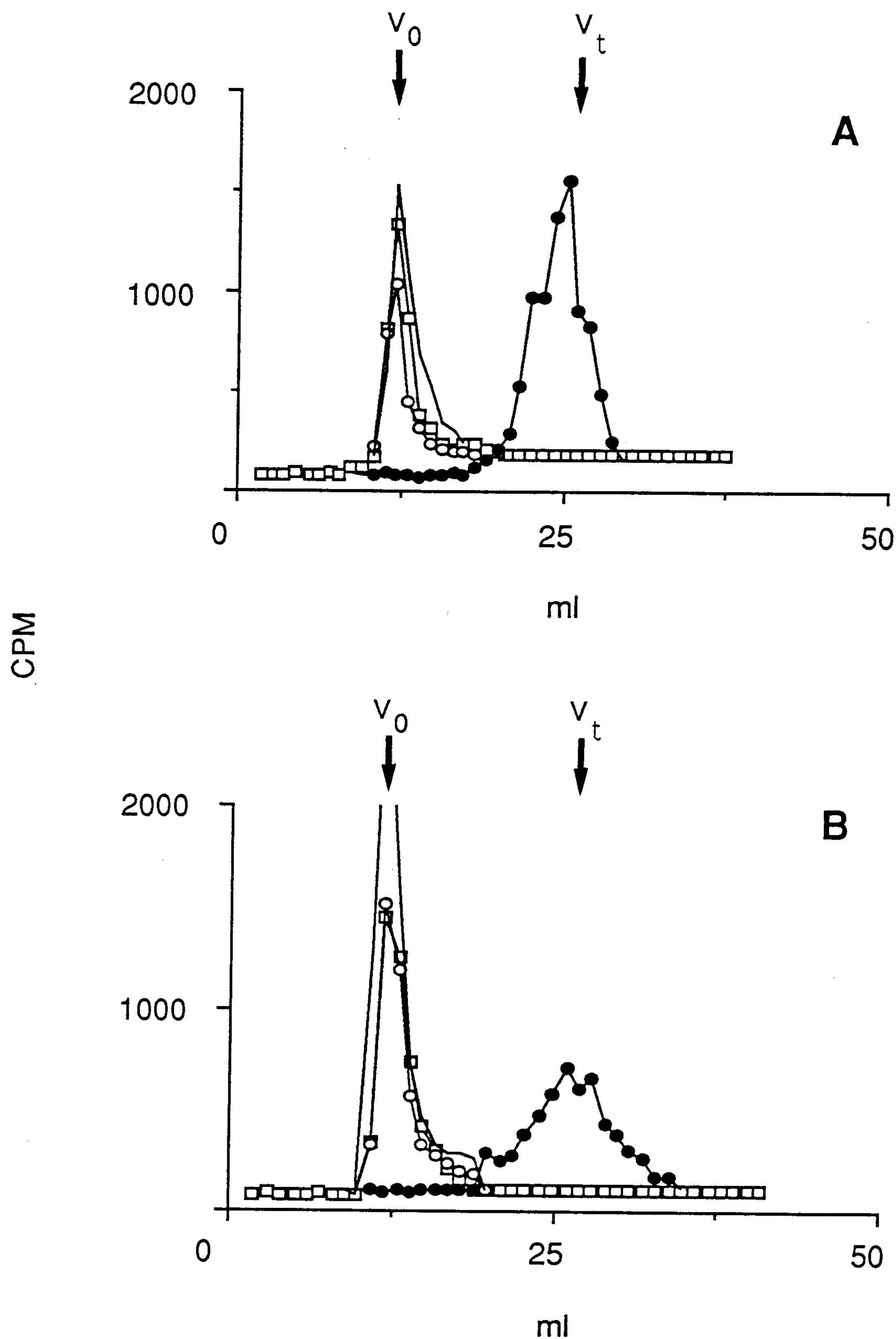

Figure 5.- Rechromatography of the excluded peak 1a (A), and 2a (B) on Sepharose CL-2B column (1x30 cm). Aliquots of peaks 1a and 2a were used as control (solid line), treated separately with heparinase (open squares), chondroitinase ABC (open circles) and were subjeted to alkali-borohydride treatment (closed circles). The eluted fractions were analyzed as described in figure 2.

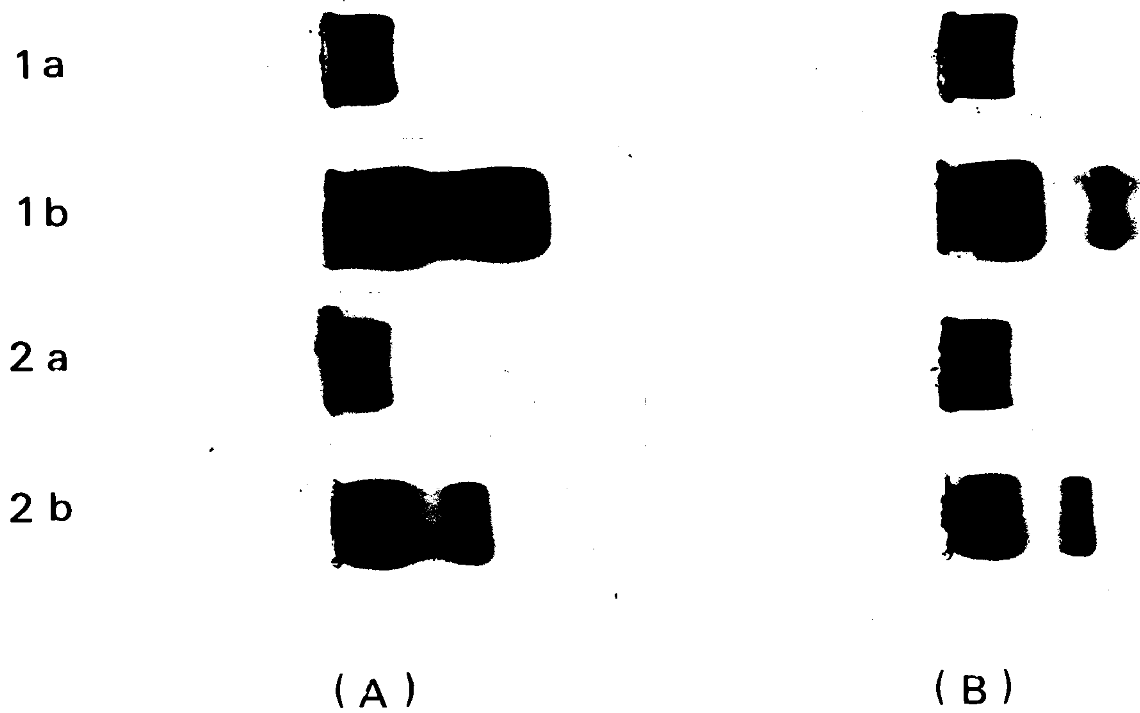

Figure 6 - Autoradiography of agarose gel electrophoresis of the different glycoconjugate fractions obtained from guinea pig tracheal secretion before (A) and after (B) chondroitinase ABC and heparinase.

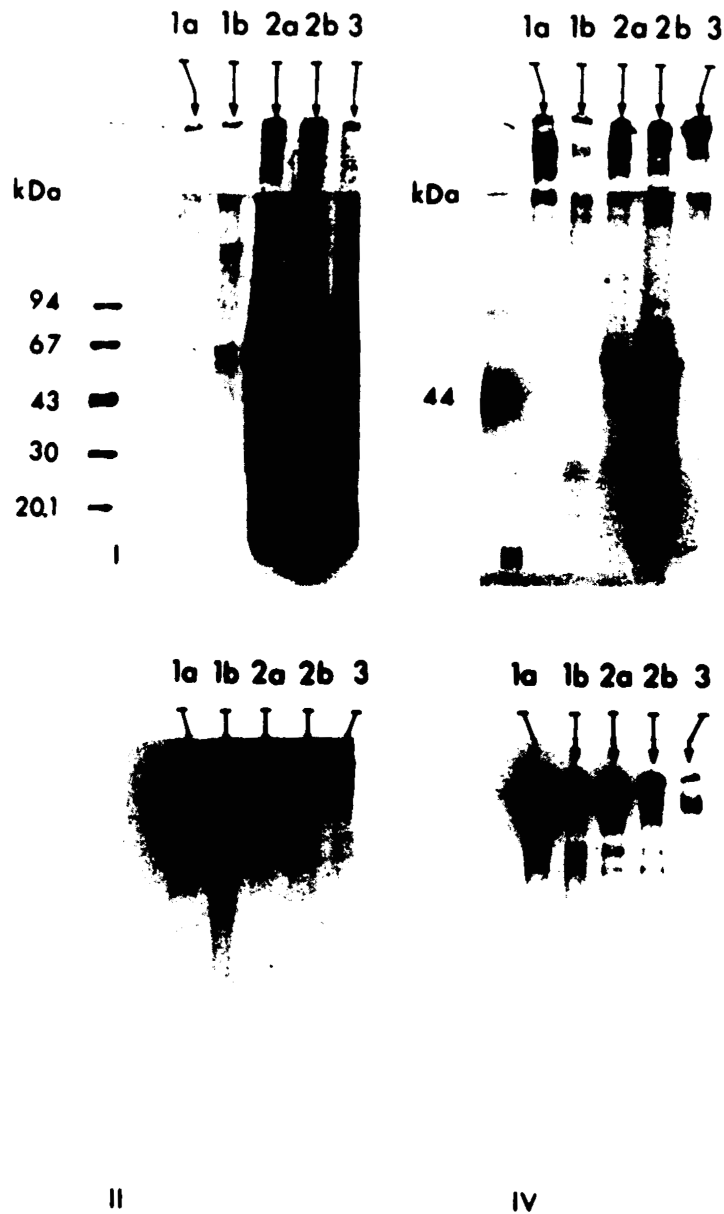

Figure 7 - SDS-PAGE of the different glycoconjugate fractions obtained from guinea pig tracheas. Gels were stained by coomassie blue (I), and periodate / Schiff (III) before being studied by autoradiography (II and IV).

Therefore fractions 1a and 2a behave like high molecular weight and sulfated glycoproteins of different buoyant density.

Identification of radiolabelled components of fraction 1b

The included peak 1b represented 40% of the radioactivity contained in fraction 1. It was studied by agarose gel electrophoresis followed by autoradiography. Two main radioactive components were observed (Figure 6).

The fastest fraction corresponded to an area stained by toluidine blue. Both radioactivity and staining were decreased after treatment of fraction 1b by chondroitinase ABC alone or by a mixture of heparinase and chondroitinase ABC (Figure 6). The staining by toluidine blue was partially eliminated after prior treatment with nucleases (data not shown). Therefore this anodic fraction, which may be estimated to about 50% of the radioactivity of fraction 1b, mainly corresponds to chondroitin sulfate and, to a lesser extent, to radioactive material resistant to chondroitinase (Figure 6). It was also mixed with a small amount of nucleic acids.

The radiolabelled fraction with the lowest mobility was, in two experiments, slightly stained by Schiff / periodate and toluidine blue and was resistant to both chondroitinase ABC and heparinase (data not shown). In a third experiment no staining could be observed although the autoradioactivity of the band was intense.

Fraction 1b was also studied by PAGE followed by autoradiography (Figure 7). The radioactivity was visualized in the stacking gel and as a smear in the resolving gel. Both areas were slightly stained by Schiff / periodate (Figure 7). The radioactivity of the smear was slightly decreased after chondroitinase ABC and heparinase treatment (data not shown).

Therefore the radioactive components of fraction 1b can be considered as a mixture of chondroitin sulfate containing proteoglycans and sulfated glycoproteins.

Identification of sulfated glycoconjugates contained in Fraction 2b

The included peak 2b represented 70% of the radioactivity contained in Fraction 2.It was studied by agarose gel electrophoresis followed by autoradiography. Two radioactive components were observed (Figure 6). Fraction 2b was studied by gel filtration chromatography on Sephadex G-100 and was separated into two fractions containing radiosulfate : 2b1 and 2b2 (Figure 8). The elution profile of Fraction 2b was not modified after prior treatment by chondroitinase ABC and heparinase. However the pattern of radiosulfate was completely changed after reductive β-elimination (Figure 8).

In PAGE, fraction 2b contained a mixture of radiolabelled glycoconjugates stained by Schiff / periodate and presenting a wide range of mobility from the stacking gel to an area corresponding to an approximate Mr 10 to 15 kDa (Figure 7).

Fractions 2b1 and 2b2 were analyzed by PAGE (Figure 9). In addition to some radioactive components remaining in the stacking gel, fraction 2b1 contained a diffuse radioactive zone corresponding to Mr greater than 60 kDa which was slightly stained by Schiff / periodate. Fraction 2b2 contained (i) radioactive components remaining in the stacking gel (probably aggregates), (ii) a discrete radioactive component which slightly penetrated into the polyacrylamide gel and had an Mr above 200 kDa and (iii) components with Mr less than 30 kDa that were also stained by Schiff / periodate. Most of these components disappeared after non reductive β-elimination (Figure 9, lanes 2 and 6).

Therefore fraction 2b mostly corresponded to a mixture of low molecular weight sulfated glycoproteins.

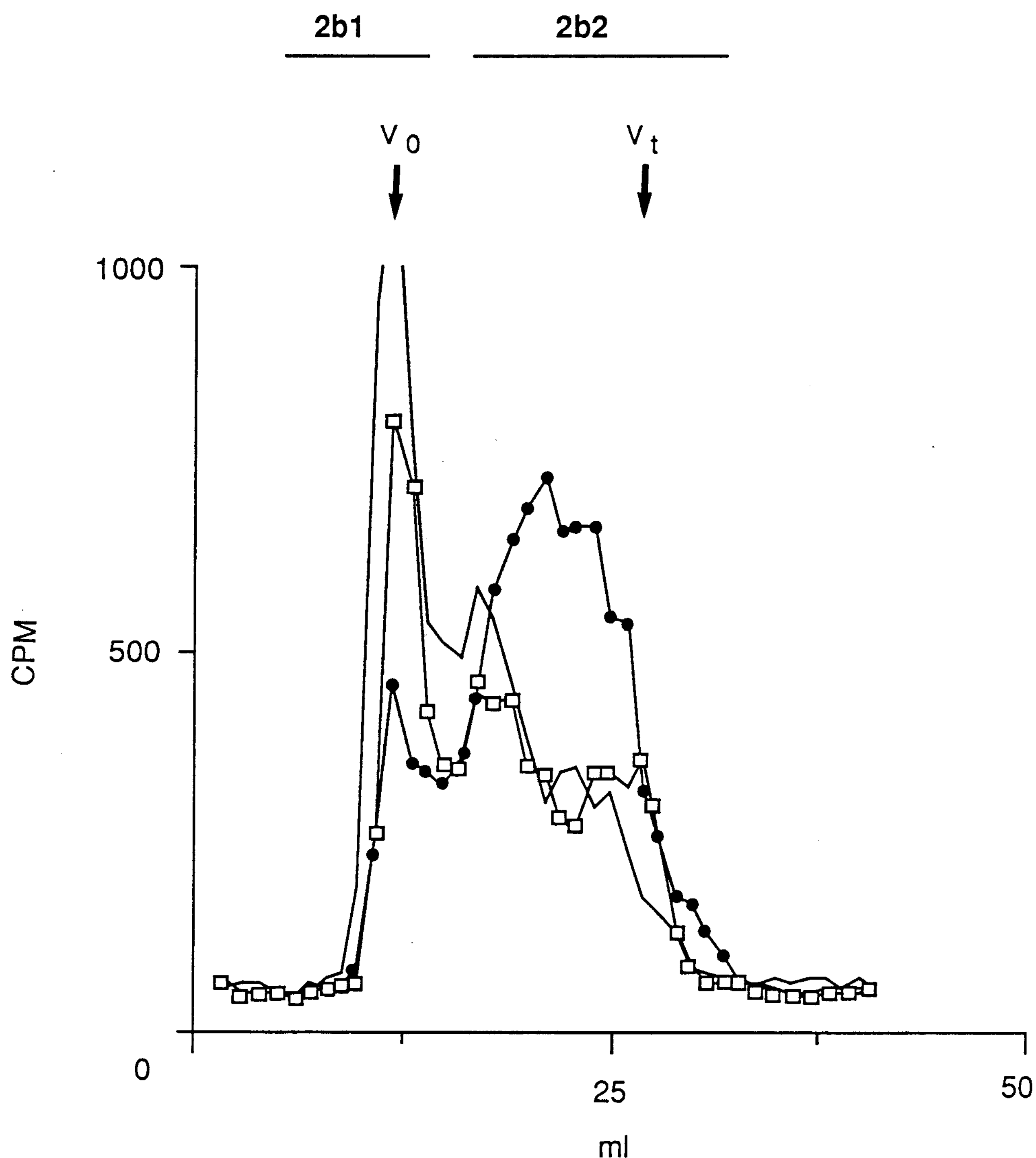

Figure 8 - Subfractionation of fraction 2b by chromatography on a Sephadex G-100 column (1x30 cm) eluted with 0.1 M Tris-HCl buffer, pH 8.O, contaning 0.25 M NaCl and 0.02% of NaN_3. Aliquots of peak 2b were used as control (solid line), or treated by heparinase and chondroitinase ABC (open squares), or were subjected to alkali-borohydride treatment (closed circles). The eluted fractions were analyzed as described in figure 2.

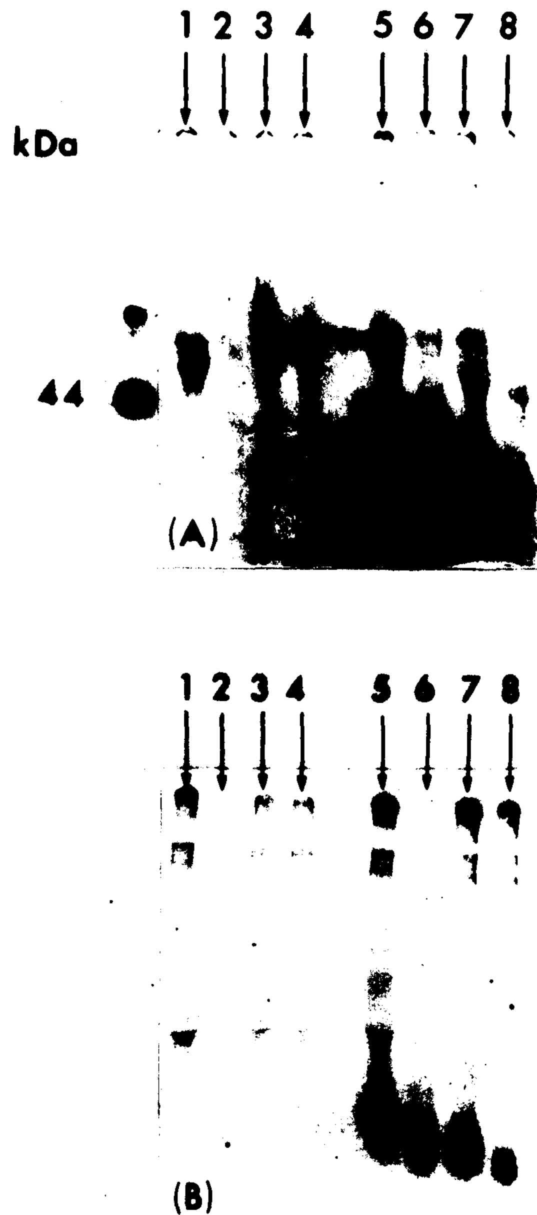

Figure 9 - SDS- PAGE of fractions 2b1 and 2b2 eluted from Sephadex G-100 column. Gel was stained by periodate / Schiff (A), before being studied by autoradiography (B). Lanes: 1 through 4, fraction 2b1; lanes 5 through 8, fraction 2b2. Lanes 1 and 5 correspond to the controls. These fractions have been treated by non reductive β elimination (lanes 2 and 6), heparinase and chondroitinase ABC (lanes 3 and 7) and endo-β-galactosidase (lanes 4 and 8) respectively.

DISCUSSION

Organ culture of guinea pig trachea maintained as a closed sac for 6 h is a suitable procedure to study the biosynthesis and secretion of respiratory glycoconjugates. Radiosulfate added externally to the medium bathing the tracheas was used by the tracheal tissue for the synthesis of sulfated glycoconjugates secreted in the tracheal lumen. An advantage of this system is that it eliminates the possibility of proteoglycans coming from the peripheral airways. And although we can never be completely certain that some secretions were not produced by cut mucosa, we believe that the rigorous precautions of the collection method kept this possibility to a minimum.

Macromolecules secreted by human bronchus have already been labelled by [^{35}S]-sulfate using Ussing chamber (31): they were excluded in CL-2B Sepharose chromatography.

In the present study the radiolabelled molecules were characterized using different criteria: elution pattern in gel-filtration, agarose and polyacrylamide gel electrophoreses, sensitivity to glycosaminoglycan-degrading enzymes and to β-elimination.

Iyer & Carlson (46) have described a reductive β-elimination which releases O-linked carbohydrate chains as oligosaccharide-alditols. When applied to mucins, this procedure allows their complete breakdown (16). However at least two degradative processes can and do occur simultaneously. Removal of O-linked carbohydrate chains, and breakdown of the polypeptide core with O-linked carbohydrate chains remaining attached to short peptides to form small glycopeptides (12). The action of reductive β-elimination on N-linked glycoproteins which were previously supposed to be resistant to alkali has also been investigated by several laboratories (47-51). The picture which emerges from these studies is that reductive β-elimination has a minor action on the peptide-

carbohydrate linkages of N-linked carbohydrate chains but does cleave the peptide of these N-glycoproteins. Non-reductive β-elimination has no action on the peptide of these glycoproteins (51).

The present study clearly showed that the radioactive sulfate was incorporated in a wide range of molecules secreted by guinea pig trachea (fractions 1a, 1b, 2a and 2b). The radiosulfate incorporated in fractions 1a, 2a and 2b corresponded to more than 80% of the total radioactivity recovered in fractions 1 and 2 after ultracentrifugation. These sulfated molecules were sensitive to reductive β-elimination and resistant to both chondroitinase ABC and heparinase. Moreover, they were stained by Schiff / periodate and therefore could be defined as sulfated glycoproteins.

The sulfated glycoproteins of fraction 1a represented 30% of the material recovered in fractions 1 and 2 after ultracentrifugation. Like mucins, they were high molecular mass, had a high density in cesium bromide, did not penetrate into polyacrylamide gel electrophoresis and hardly in agarose electrophoresis. Like hamster respiratory mucins (16), they were completely degraded by reductive β-elimination. Therefore, they could be identified as high molecular weight mucin-like glycoproteins.
In contrast to fraction 1a, the identification of the sulfated glycoproteins contained in fraction 2a and 2b was a little more difficult.

The sulfated glycoproteins contained in fraction 2a had a lower buoyant density than ordinary mucins which would have sedimented with fraction 1a. But, like the components of fraction 1a, they were completely degraded by reductive β-elimination. They had the same electrophoretic behaviour and did not migrate in polyacrylamide or agarose electrophoresis. The occurence of mucins with a lower buoyant density corresponding to non-covalent association with lipid has already been reported (52). Leigh and coworkers (28) have also characterized changes of

buoyant density of reduced mucins during development and suggested that they could be accounted for by differences in glycosylation. Recently Van Beurden-Lamers and colleagues (53) have attributed heterogeneity of gastric mucins in buoyant density to a different degree of sulfation. One should also notice that the separation in density gradient ultracentrifugation between fraction 1 and fraction 2 is not clear cut. This may due to high molecular weight mucin-like glycoproteins secreted by guinea pig trachea being a very diverse population like human mucins (3,4). Since sulfated polylactosaminoglycans can be released by trypsin treatment of dog tracheal cells in culture (54), the high molecular weight mucin-like glycoproteins from guinea-pig were submitted to endo-β-galactosidase. No modification of the elution profile of these glycoproteins could be observed. They were therefore not polylactosaminoglycans.

The identification of the lower molecular mass sulfated glycoproteins secreted by the guinea-pig trachea was even more difficult. These sulfated glycoconjugates did not correspond to chondroitin sulfate or heparan sulfate but were sensitive to both mild alkali treatment (Figure 8) and to mild alkaline borohydride treatment (Figure 7) and therefore most probably correspond to low-molecular mass sulfated O-glycoproteins. There were at least three components with approximate molecular mass of 30, 60 and above 200 kDa. They represented 35 % of the material recovered in fractions 1 and 2 after ultracentrifugation. It is possible that these components could have been produced by some proteolytic degradation occuring in the tracheal lumen. To our knowledge, no similar components have ever been described in the respiratory mucus, except in chicken trachea exposed to calcium stimulation where glycoconjugates with Mr of 325 and 46.2 kDa have been identified (26). The concentration of Ca^{2+} used in our study (2.4 mM) was unlikely to have produced such an effect. Hamster tracheal surface epithelial cells in primary culture (17) have also yielded significant amounts of unidentified sulfated

glycoconjugates that were resistant to chondroitinase and included in Sepharose CL-4B chromatography.

Fraction 1b which only represents 18 % of the material recovered in fractions 1 and 2 after ultracentrifugation clearly contained material susceptible to chondroitinase and can be identified as chondroitin sulfate proteoglycan (26).

Chondroitin sulfate proteoglycans also represented a small percentage of the macromolecules secreted by primary hamster tracheal surface epithelial cells in culture (17). These data contrast with that of Bhaskar and associates (25) for human and canine airway organ explant cultures. Here (25) proteoglycans were the major secreted components with little mucin of typical buoyant density. This was possibly a consequence of the organ culture method where proteoglycans may have been produced by the cut sub-mucosa. Mucins of typical buoyant density in CsBr have been reported as the major components from in vivo collection in the dog tracheal pouch (55) and from "normal" human tracheobronchial secretion (56).

Both epithelial goblet cells and submucosal glands were present in the guinea pig tracheal epithelium and both probably contributed to the secretions studied. But there is evidence that suggests that, in bovine and canine mucosae, serous and possibly ciliated cells also contribute to airway secretions (18, 54). Further studies are needed to characterize the source of the different components identified in secretions from guinea pig trachea.

In conclusion this model of organ culture might be valuable to study the effects of different stimuli applied either on the luminal or on the submucosal side of the trachea. The present results demonstrate that in this system [^{35}S] sulfate is incorporated into both "classical" respiratory mucins, a class of lower molecular mass O-glycoproteins and into a low amount of chondroitin sulfate containing proteoglycans. Moreover, they need to be confirmed in other mucosae, especially in human, and should stimulate a re-investigation of the sulfated molecules found in human bronchial secretion.

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II- RECHERCHE DES GLYCOSAMINOGLYCANNES DANS LA SECRETION BRONCHIQUE HUMAINE

Dans le chapitre précédent, nous avons montré que la trachée de cobaye en culture organotypique sécrétait un peu de protéoglycannes à chondroïtine sulfate. Ceci nous a amené à réexaminer une question à laquelle les réponses apportées jusqu'à présent sont contradictoires: y a-t-il des glycosaminoglycannes dans la sécrétion bronchique humaine?

On peut rappeler à cet égard que Bhaskar *et al* ont publié un travail tendant à démontrer qu'à l'état physiologique, la muqueuse bronchique humaine ne sécrète pas de mucines mais des protéoglycannes (70). Cette notion est en contradiction totale avec les travaux de Lafitte *et al* (69) et de Thornton *et al* (75) qui ont parfaitement démontré la présence de mucines dans la lumière trachéo-bronchique humaine mais qui n'ont pas trouvé (Thornton) ou signalé (Lafitte) de protéoglycannes. Toutefois des résultats préliminaires de Le Treut semblaient indiquer que, dans certaines situations pathologiques, il était parfois possible d'observer des glycosamino-glycurono-glycannes (73).

Nous avons donc repris cette étude et dans un premier temps, nous avons mis au point un protocole d'étude des glycosamino-glycurono-glycannes libérés par protéolyse du mucus bronchique. Ce protocole est fondé sur l'électrophorèse en agarose et sur l'utilisation de divers d'enzymes : nucléases, chondroïtinases ABC et AC, héparinase, hyaluronidase de *Streptomyces* et endo-β-galactosidase, visant à mettre en évidence les différents constituants acides du mucus bronchique préalablement protéolysé: acides nucléiques, acide hyaluronique, chondroïtines, héparine, kératane et glycopeptides de mucines.

Ce protocole a été appliqué à l'analyse de deux séries d'expectoration, les unes provenant d'une part de 11 malades souffrant de bronchite chronique et les autres provenant d'une série de 13 sujets atteints de mucoviscidose. Dans chacun des deux groupes, certaines expectorations étaient très infectées et très riches en acides nucléiques alors que d'autres l'étaient peu ou pas du tout, ne comportant que peu ou pas d'acides nucléiques.

Ces expériences ont permis de montrer que:

- (i) dans tous les cas de mucoviscidose très infectés, on pouvait mettre en évidence du chondroïtine sulfate,
- alors que (ii) dans tous les cas de bronchite chronique, sauf un, on ne pouvait déceler de chondroïtine sulfate.

Ces résultats posent le problème de l'origine de ce chondroïtine sulfate. Les cellules séreuses des glandes de la sous-muqueuse bronchique humaine incorporent fortement le sulfate (2). Des cultures de cellules séreuses trachéales bovines sécrètent des protéoglycannes à chondroïtine sulfate (3). Est-il possible que de tels protéoglycannes soient effectivement sécrétés et secondairement dégradés dans les canaux des glandes de la sous-muqueuse ou à la surface de l'épithélium? Il faudra à tout le moins étudier la sécrétion trachéo-bronchique et rechercher si, à l'état normal ou dans des situations d'hypersécrétion bronchique, elle ne contient pas une chondroïtinase capable de dégrader les protéoglycannes à chondroïtine sulfate (97).

CHONDROITIN SULFATE IN INFECTED SPUTUM FROM PATIENTS WITH CYSTIC FIBROSIS

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Running title: Chondroitin sulfate in sputum

ABSTRACT

In order to ascertain whether or not the presence of glycosaminoglycans in sputum of patients suffering from chronic bronchial disorders was related to tracheobronchial infection, an electrophoretic procedure was set up. The different acidic macromolecular components of sputum, namely nucleic acids, glycosaminoglycans and bronchial glycopeptides could be identified in proteolyzed sputum using agarose electrophoresis before and after the action of different enzymes: nucleases, chondroitinases, hyaluronidase and heparinase. This procedure was used to anlayse 13 sputa from patients suffering from cystic fibrosis and 12 sputa from patients suffering from chronic bronchitis. Chondroitin sulfate was identified in 11 infected sputa from patients with cystic fibrosis and also in the non infected sputum from a patient with chronic bronchitis. These data suggest a relationship between the presence of chondroitin sulfate proteoglycans in sputum and severe tracheobronchial infection in cystic fibrosis.

Key Words : Sputum, Cystic Fibrosis, Chondroitin sulfate, nucleic acids.

Abbreviations: CF, Cystic Fibrosis

INTRODUCTION

Sputum consists of a complex mixture of macromolecules including proteins, serum-type glycoproteins, mucins, lipids and possibly nucleic acids (1) proteoglycans or glycosaminoglycans (2,3).

Bhaskar and associates (2) have described material with features of proteoglycans in bronchial mucus aspirates from healthy volunteers (smokers and non-smokers) and, in a preliminary report, Le Treut and coworkers (3) have shown some evidence for the presence of proteoglycans in the sputum of patients suffering from severe chronic bronchial hypersecretion, mostly cystic fibrosis. Hyaluronic acid has also been characterized in the bronchoalveolar lavage of asthmatic patients (4).

The fact that proteoglycans have been described in sputum raises the question as to whether they are part of the normal bronchial secretions or are related to infection or to an underlying disease especially since there are reports indicating the secretion of glycosaminoglycans by organ cultures of airway mucosa (5, 6) or by respiratory cells grown in culture (7,8). There are also reports of an increased sulfation of glycoconjugates including glycosaminoglycans by cultured nasal epithelial cells from patients with cystic fibrosis (9) raising the question as to whether they may be normally present in CF secretions.

The aim of the present work was therefore to look for the presence of glycosaminoglycans in the sputum of patients suffering from chronic bronchial disorders, and to find out whether or not it was related to tracheobronchial infection. For this purpose, an electrophoretic procedure was set up to identify the different acidic macromolecular components of sputum, namely nucleic acids, glycosaminoglycans and bronchial glycopeptides in proteolyzed sputum. Since alginate is an acidic polysaccharide which might be present in *Pseudomonas* infected sputum, its behaviour in that procedure was also analyzed. This procedure was used to investigate infected and non-infected sputa from patients suffering from cystic fibrosis or from chronic bronchitis.

The study suggests that chondroitin sulfate was mainly associated with infection of cystic fibrosis sputa.

MATERIALS and METHODS

Materials

Pronase was purchased from Calbiochem (La Jolla,CA,USA); agarose was obtained from Litex (FMC-Bioproducts, Denmark); chondroitin sulfate, endo-β-galactosidase (from *E. freundii*), chondroitinases AC and ABC were obtained from Miles (Lisle, IL), hyaluronic acid (bovine trachea), hyaluronidase from *Streptomyces hyalurolyticus*, heparinase and micrococcal nuclease were from Sigma (St louis, MO); RNAse A from bovine pancreas was from Boehringer (Boehringer Mannheim, W. Germany). Alginate was a generous gift from Dr Gerald Pier (Harvard Medical School, Boston, USA) and acidic bronchial glycopeptides (fraction F2b) were prepared as already described (10).

Collection and dilution of mucus

Sputum (200 ml) was collected from 11 patients suffering from chronic bronchitis and 13 patients suffering from cystic fibrosis (sputum was collected twice from patient n°11 with a 5 yr interval). Samples 11b (CB) and 12-22 (CF) were purulent. Every sample was culured and the presence or the absence of *Pseudomonas aeruginosa* was noted (Table I). The collected sputum was kept frozen until used. It was thawed at 4°C, diluted 1 to 12 with deionised water containing 0.02% NaN_3, stirred overnight at 4°C and centrifuged at 3000 x g for 30 min. The supernatant and the pellet containing cellular debris were dialyzed and lyophilized (11).

CASES n°	AGE yr	DISEASES*	PSEUDOMONAS	BRONCHIAL GLYCOPEPTIDES	NUCLEIC ACID	GAG*	OTHER BAND
1	57	B	-	+	-	-	-
2	50	B	-	+	-	-	-
3	58	B	-	+	-	-	-
4	61	B	-	+	-	-	-
5	49	B	-	+	-	-	-
6	66	B	-	+	-	-	-
7	55	B	-	+	-	-	+
8	58	B	-	+	-	-	+
9	52	B	-	+	-	-	+
10	62	B	-	+	-	+	-
11a	54	B	-	+	-	-	+
11b	59	B	+	+	+	-	-
12	22	CF	+	+	+	+	-
13	10	CF	+	+	+	+	-
14	9	CF	+	+	+	+	-
15	19	CF	+	+	+	+	-
16	19	CF	+	+	+	tr	-
17	15	CF	+	+	+	+	-
18	13	CF	+	+	+	+	-
19	27	CF	+	+	+	+	tr
20	6	CF	+	+	+	+	tr
21	8	CF	+	+	+	+	tr
22	27	CF	+	+	+	+	tr
23	13	CF	+	+	tr	-	+
24	10	CF	-	+	tr	-	-

*B = Chronic Bronchitis; CF = Cystic Fibrosis; GAG = glycosaminoglycans; + = presence; 0 = absence; tr = traces.

Pronase digestion of lyophilized mucus supernatant

The lyophilized supernatant (80 mg of each) was digested with pronase in 0.01 M calcium acetate buffer at pH 7.0 for 48 h at 37°C using an enzyme / substrate ratio of 1 / 40 and a fresh addition of enzyme at 24 h. After 48 h the pronase treated material was boiled at 100 °C for 3 min and centrifuged ; the supernatant containing bronchial glycopeptides, and possibly nucleic acids and / or glycosaminoglycans was dialyzed and lyophilized.

Digestion of the macromolecule mixture with nucleases, glycosaminoglycans degrading enzymes and endo-β-galactosidase

The lyophilized mixture was treated as follows.

(i) Treatment by nucleases : two milligrams were dissolved in 0.5 ml of 5 mM sodium borate, pH 8.8, containing 0.1 % of bovine serum albumin and incubated with micrococcal nuclease (0.5 unit in 10 µl of distilled water) and RNAse A (20 µg in 20 µl of distilled water) for 18 h at 37°C (12); after incubation, the mixture was lyophilized and dissolved in 100 µl of deionized water.

(ii) Treatment by chondroitinase ABC and / or heparinase : two milligrams were dissolved in 100 µl of physiological saline and treated for 18 h at 37 °C with 0.5 unit of chondroitinase ABC in 20 µl of 0.1M Tris-acetate, pH 7.3 (8) and / or with 0.5 unit of heparinase in 10 µl of 0.1M potassium phosphate, pH 7.0 (13). After digestion, the mixture was lyophilized and dissolved in 100 µl of deionized water.

(iii) Simultaneous treatments by nucleases, heparinase and chondroitinase ABC : two milligrams were dissolved in 0.5 ml of 5 mM sodium borate, pH 8.8, containing 0.1 % of bovine serum albumin and the different enzymes were added at the same time (0.5 unit of micrococcal nuclease ; 20 µg of RNAse A; 0.5 unit of both chondroitinase ABC and heparinase). After digestion for 18 h at 37 °C, the mixture was lyophilized and dissolved in 100 µl of deionized water.

(iv) Treatment by chondroitinase AC: as for chondroitinase ABC, except for the buffer which was 0.1 M sodium acetate pH 6.0.

(v) In the samples where material sensitive to chondroitinase ABC was detected, the action of hyaluronidase from *Streptomyces hyalurolyticus* was also checked: 1 unit of hyaluronidase in 10 µl of 0.1 M sodium acetate, pH 6.0, was incubated with a solution of the mixture dissolved in 100 µl of physiological saline at 37°C for 24 h (15).

(vi) In some instances, the macromolecule mixture was dissolved in 100 µl of physiological saline and treated for 24 h at 37 °C with 0.01 unit of endo-β-galactosidase in 0.1 M Tris acetate, pH 5.8 (15).

Agarose gel electrophoresis

After each enzymatic digestion, a volume corresponding to 400 µg of digested products was subjected to agarose gel electrophoresis. An untreated control (400 µg in 20 µl of physiological saline) was run simultaneously.

Agarose gel electrophoresis was performed as described (16) using 1% agarose in veronal buffer at pH 8.2 (ionic strengh 0.1) on 26 X 75 mm microscope slides. A potential of 2.6 V per cm width was applied for 1 h at room temperature. Slides were stained with toludine blue (17).

In order to evaluate the sensitivity of the method to detect the presence of theses acidic compounds, decreasing amounts of pure chondroitin sulfate and alginate were studied by this method.

RESULTS

Electrophoretic examination of acidic compounds from proteolyzed sputum

In theory the pronase digested sputum should yield a mixture of peptides, glycopeptides and, if any, nucleic acids and glycosaminoglycans. The peptides were eliminated by dialysis and the acidic components of the remaining mixture, mostly glycopeptides, were analysed using agarose gel electrophoresis followed by

staining with toluidine blue which reveals acidic components. The migration patterns of the possible components are shown in Figure 1 (C), using the purified compounds Under these conditions, nucleic acids, hyaluronic acid and chondroitin sulfate have a fast anodic mobility whereas purified bronchial glycopeptides usually have a less anodic mobility. Moreover, since some sputa were collected from patients infected by *Pseudomonas aeruginosa* which may secrete alginate, the electrophoretic behaviour of pure alginate is also shown (Fig. 1).

Figure 1 (EZ) demonstrates that the different compounds were sensitive to the appropriate enzymes. The sensitivity of the method was also evaluated: the lowest amounts of chondroitin sulfate and alginate detected by this method were respectively 10 µg and 2 µg (Fig. 1).

Electrophoretic study of sputum digests from patients with CF or chronic bronchitis.

Mucin glycopeptides - A fraction stained with toluidine, moving just ahead of the slot (e.g. fraction "G" of Fig. 1; see also Figures 2-5) and corresponding to the acidic bronchial mucin glycopeptides was observed in each sample (Table I).

Nucleic acids - A fast moving band containing nucleic acids was observed in samples n°11b to 24 (Table I): it was totally (Figure 3 and samples n°11b and 24 in Table I) or partially (samples n° 12-23) sensitive to nucleases. Nucleic acids were present in all heavily infected CF sputum, in traces amount for two CF samples (n° 23 and 24) and in one infected sputum from a patient with chronic bronchitis (sample n° 11b). Nucleic acids were absent in 11 out of 12 samples from chronic bronchitis which were not infected (Table I: samples n°1 to 11a).

Glycosaminoglycans - Chondroitin sulfate was observed in one sputum sample from a patient with non-infected chronic bronchitis (sample n° 10 in Table I): it migrated as a fast-moving band not sensitive to nucleases, heparinase or

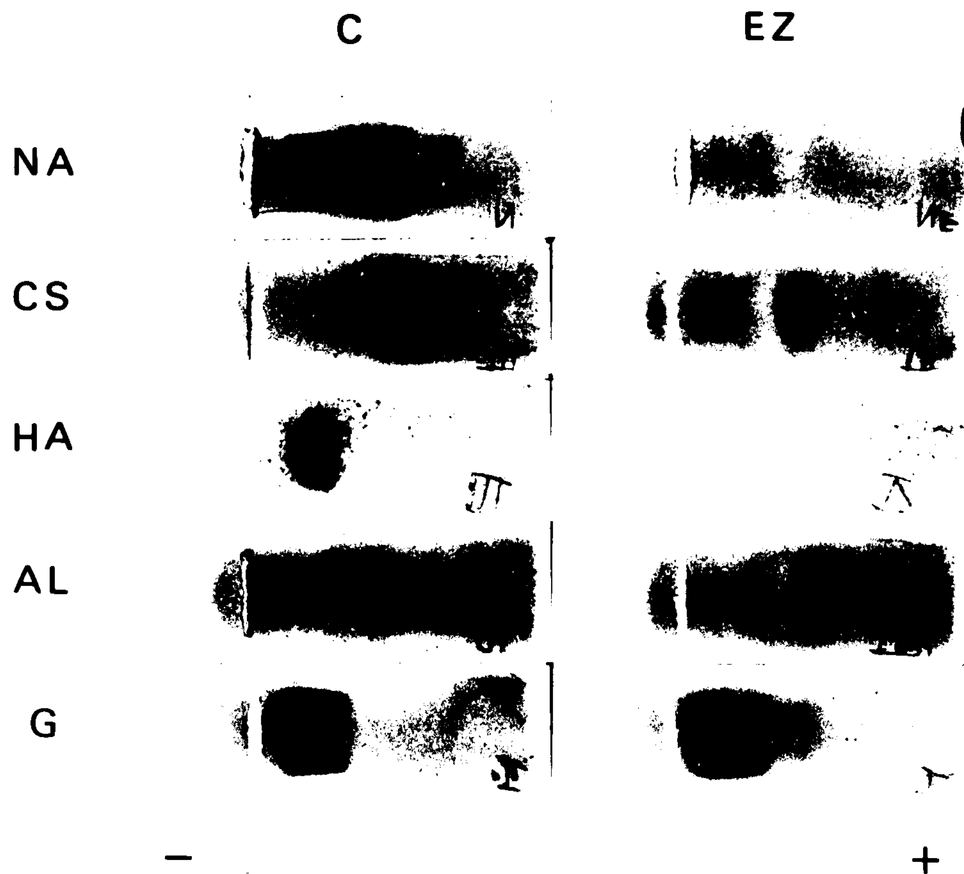

Figure 1 : Agarose gel electrophoresis of 50 µg of purified nucleic acids (NA), of 10 µg chondroitin sulfate (CS), of 2 µg alginate (AL), of 20 µg hyaluronic acid (HA) and of 400 µg acidic tracheobronchial glycopeptides (G). Each preparation was analysed before (C) and after treatment with a mixture of nucleases, chondroitinase ABC, heparinase (EZ). The slides were stained with toluidine blue.

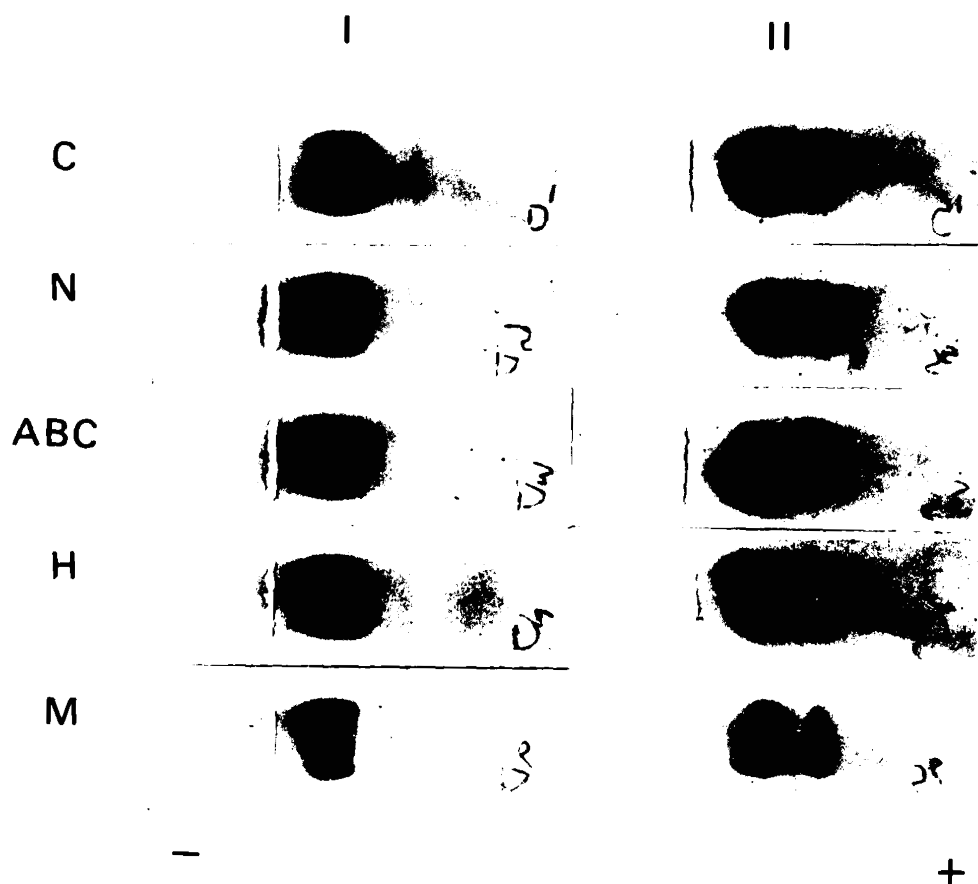

Figure 2 : Agarose gel electrophoresis of digested sputum from two non-infected patients suffering from chronic bronchitis (case n°.6 (I) and 8 (II) of table I). The digests were analysed before (C) and after treatment with nucleases (N) , chondroitinase ABC (ABC) , heparinase (H), a mixture of these three enzymes (M) The slides were stained with toluidine blue.

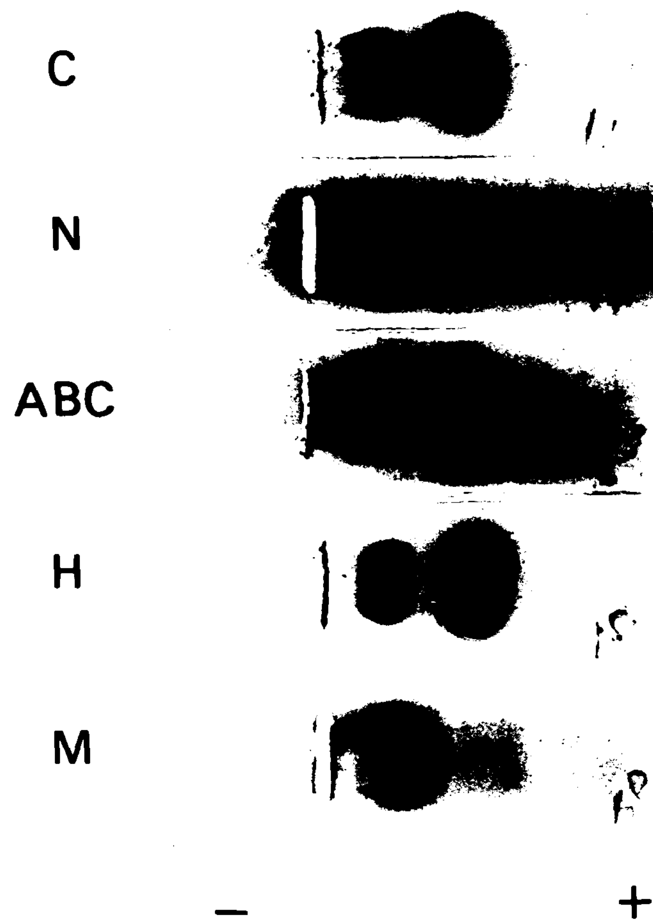

Figure 3 : Agarose gel electrophoresis of digested sputum from an infected patient suffering from chronic bronchitis (case N° 11b). The digest was analysed before (C) and after treatment with nucleases (N) , chondroitinase ABC (ABC) , heparinase (H) or the mixture of the three enzymes (M). The slides were stained with toluidine blue.

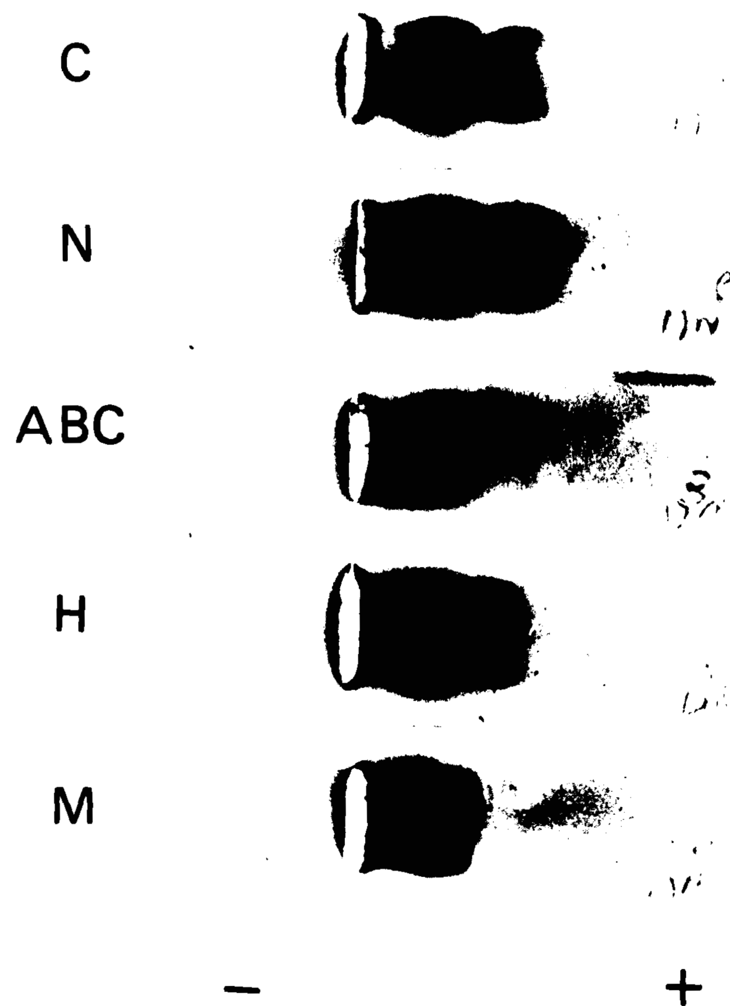

Figure 4 : Agarose gel electrophoresis of digested sputum from an non infected patient suffering from chronic bronchitis (case N° 10). The digest was analysed before (C) and after treatment with nucleases (N) , chondroitinase ABC (ABC) , heparinase (H) or the mixture of the three enzymes (M). The slides were stained with toluidine blue.

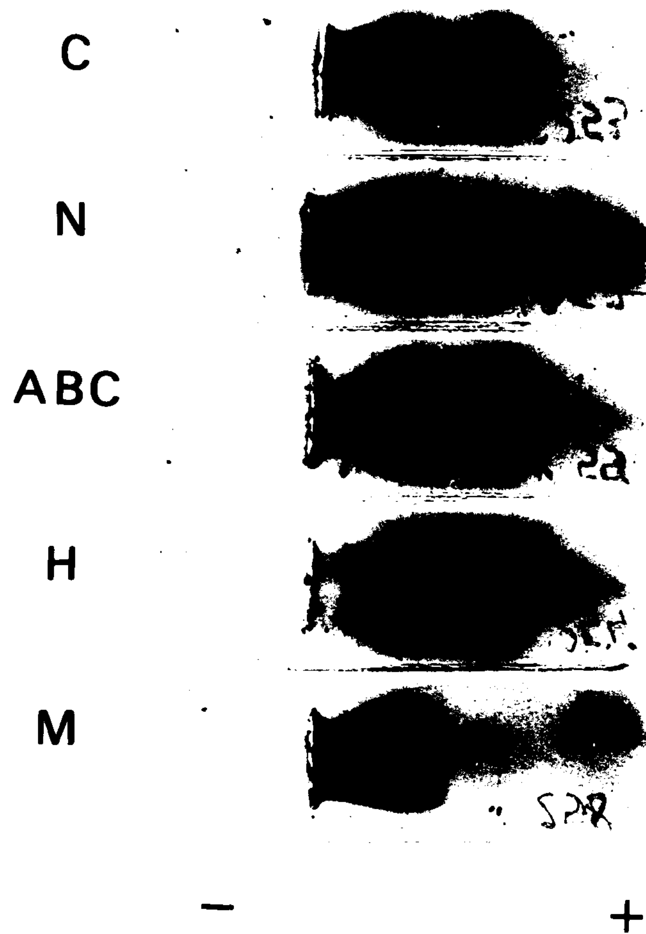

Figure 5 : Agarose gel electrophoresis of digested sputum from an infected patient suffering from cystic fibrosis (case N° 15). The digest was analysed before (C) and after treatment with nucleases (N) , chondroitinase ABC (ABC) , heparinase (H) or the mixture of the three enzymes (Mx). The slides were stained with toluidine blue.

hyaluronidase from *Streptomyces hyalurolyticus* , but completely degraded by chondroitinase ABC (Figure n°4) or AC. 11 samples from patients with cystic fibrosis contained chondroitin sulfate in addition to nucleic acids (samples n° 12-22 in Table I) since in these samples the fast moving band was resistant to heparinase but disappeared completely (Figure n°5) or partially after action of chondroitinase ABC or AC and nucleases.

Other acidic compounds - Components with a fast electrophoretic mobility and stained by toluidine blue remained in 5 CF samples (n°19-23) after treatment by all the mentioned enzymes. In these CF samples infected by *Pseudomonas aeruginosa* this band might correspond to alginate or to a more acidic fraction of mucin glycopeptides. However components with similar mobility , staining and resistance to enzymes were observed in 4 samples from non infected chronic bronchitis (Figure 2 -II and samples n° 7-9 and 11a in Table I). The sputum of patient n°11 was analyzed twice, first when the sputum was not infected and five years later, when it was infected by *Pseudomonas aeruginosa*: When it was sudied for the first time, it was not infected and had the same electrophoretic pattern as cases n°7-9 (i.e. a fast moving band resistant to all the enzymes). Five years later, this latter band was replaced by a band completely sensitive to nucleases alone.

DISCUSSION

Agarose electrophoresis followed by staining with toluidine blue is a sensitive and convenient method to separate acidic bronchial mucin glycopeptides from other acidic components such as nucleic acids, chondroitin sulfate, keratan sulfate and alginate which are more acidic and have a close electrophoretic mobility. However, since infected sputum contains nucleic acids, the precise identification of the different acidic glycoconjugates needs a comparison of agarose electrophoretic profiles before and after treatment with nucleases, chondroitinase ABC, heparinase, hyaluronidase from *Streptomyces hyalurolyticus* and endo-β-galactosidase.

There was no detectable nucleic acid in the sputum from 10 patients suffering from non-infected chronic bronchitis. However, four of them had an aditionnal acidic fraction completely resistant to all the enzymes that were used (Fig. 2). Such a fraction has never been reported so far in respiratory secretions, except in the material secreted in organ culture (18). It will be necessary to find out if this fraction originates from some variety of respiratory mucins or from another secreted or shed glycoconjugate.

All the infected sputa from patients suffering from cystic fibrosis contained nucleic acids. The amount of nuclease sensitive material seemed to be roughly correlated with the degree of purulence. Therefore the present work confirms previous data which clearly indicate that the presence of nucleic acids in infected sputum reflects the extent of respiratory infection (19). Lethem and coworkers have recently shown that most of the nucleic acids in infected sputum come from human DNA (20).

Chondroitin sulfate was present in 11 out of 13 patients with cystic fibrosis. This finding may be related to the disease itself since CF respiratory cells in culture

have been reported to produce an increased secretion of chondroitin sulfate (9). However the present study shows (i) that these 11 patients were heavily infected by *Pseudomonas aeruginosa*, (ii) that chondroitin sulfate is not specific for infected CF sputum since it was also observed in a non infected sputum from a patient with chronic bronchitis (case n°10) and (iii) that chondroitin sulfate was not detected in the sputum from non infected CF patients. However chondroitin sulfate was also not detectable in the sputum from a patient with chronic bronchitis who was infected by *Pseudomonas aeruginosa* (case n°11b).

Among the sputa infected by *Pseudomonas aeruginosa*, there were four cases (cases n° 19-22) where a faint band was still visible after the action of the different enzymes, corresponding either to another variety of bronchial mucin glycopeptides (as in cases n° 7 -10, non infected chronic bronchitis) or to alginate. Given the sensitivity of the method and the amount of digested sputum that was analyzed (400 µg), if this band was alginate, then in all the other infected CF sputa from (cases n° 12 to 18), the alginate content was less than 0.5%, if any.

Serous cells of human bronchial mucosa have been shown to strongly incorporate radiosulfate (21) and proteoglycans have also been identified in the secretion from cultures of serous cells (7). However, the present results are somehow at variance with the data of Bhaskar and colleagues (2) who found material with some features of proteoglycan but no respiratory mucin in the bronchial aspirates of six healthy volunteers. In the present work, chondroitin sulfate was observed almost entirely in infected CF sputum.

The reasons for these discrepancies are unclear. The present data are in agreement with the findings of Thornton and colleagues (22) who studied aspirates from normal bronchial mucosa and found mucins but no proteoglycans. On the other hand, they confirm a preliminary report suggesting the presence of proteoglycans in infected CF sputum (3).

The presence of chondroitin sulfate in the sputum of severely infected patients is puzzling: it may be due to the action of proteases, such as

Pseudomonas aeruginosa elastase, leading to degradation of the epithelium surface, the basement membrane or the connective tissue.

Chace and colleagues (23) have observed a correlation between the concentration of highly sulfated mucins in cystic fibrosis sputum and the severity of the disease but did not look for the presence of glycosaminoglycans.

One may therefore wonder whether proteoglycans, if they were normally secreted by serous glands, would be degraded in the gland ducts or in the respiratory lumen. It has been reported that proteoglycan-degrading enzymes could be secreted by macrophages (24) and, considering the present data, it will be interesting to look for the presence of this enzyme in different sputa and also to compare infected and non-infected sputa.

Regardless of the mechanisms involved in the release of chondroitin sulfate containing proteoglycans, the present work suggests that the simultaneous presence of nucleic acids and chondroitin sulfate in sputum may be evidence for severe bronchial infection in cystic fibrosis.

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Acknowledgments: AFLM

III ETUDE D'OLIGOSACCHARIDES SULFATES DE MUCINES BRONCHIQUES HUMAINES.

Dans la première partie de notre travail nous avons mis au point un modèle d'etude des glycoconjugués sulfatés sécrétés par la trachée de *cobaye* en culture organotypique. Ce travail a abouti à la caractérisation de mucines sulfatées qui présentent des propriétés (en ultracentrifugation, chromatographie de gel filtration...) qui sont tout a fait comparables à celles des mucines bronchiques humaine isolées à partir d'expectoration ou de lavages bronchiques. Par contre il a aussi débouché sur deux observations plus inattendues d'une part celle de l'existence de protéoglycannes à chondroïtine sulfate et d'autre part celle d'une population de glycoprotéines O-sulfatées n'ayant pas toutes les propriétés chimiques des mucines bronchiques humaines.

Dans la seconde partie de notre travail, nous avons cherché à vérifier dans la sécrétion bronchique humaine la présence de protéoglycannes qui sont des protéoglycannes à chondroïtine sulfate.

La logique aurait alors voulu que nous essayons de vérifier dans la sécrétion bronchique humaine la présence d'une population de glycoprotéines se distinguant des mucines. Nous avons hésité à nous lancer dans cette direction pour au moins deux raisons (i) l'absence de méthodes non isotopiques utilisant de faible échantillons permettant de détecter de faibles quantités de sulfate dans un éluat de colonne ou fractionnement par ultracentrifugation (ii) une contrainte de temps : il semblait plus difficile de pouvoir mener ce travail à bien dans la dernière année de thèse qui nous était impartie.

Laissant cette entreprise à d'autres, nous avons cherché à terminer le travail entamé lors de notre DEA sur l'identification de chaînes glycanniques sulfatées dans des mucus bronchiques.

Ce travail est nécessaire pour toute une série de raisons :

- il semble y avoir de nombreuses population de chaînes glycannique sulfatées et les travaux réalisés dans ce domaine (cF tableau III de l'introduction) sont peu nombreux; on ne sait pas du tout si les liaisons sucre-sulfate sont aussi diverses que les liaisons α–neuraminyl ou -fucosyl qui se situent à la périphérie des glycannes;
- si l'existence d'une population de glycoprotéines sulfatées differentes des mucines se confirme, il faudra aussi comparer les systèmes de sulfatation de ces glycoprotéines avec ceux des mucines bronchiques;

Nous avons donc développé une stratégie de fractionnement des oligosaccharides de mucines bronchiques sulfatés en nous fondant sur les techniques de gel filtration sur Biogel. A l'évidence ces chaînes glycanniques sont très diverses. Nous avons eu la chance de disposer des techniques de spectroscopie ^{1}HNMR et de spectrométrie de masse (FAB) et de pouvoir identifier deux oligosaccharides inconnus. Ces oligosaccharides contrairement à tous les oligosaccharides décrits jusqu' ici présentent une liaison sulfate-O 3-galactose.

STRUCTURE OF TWO SULFATED OLIGOSACCHARIDES FROM RESPIRATORY MUCINS OF A PATIENT SUFFERING FROM CYSTIC FIBROSIS : A FAST ATOM BOMBARDMENT MASS SPECTROMETRIC AND ^{1}H-N.M.R. SPECTROSCOPIC STUDY

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Section: Carbohydrate

SUMMARY

Human respiratory mucin glycopeptides were isolated from the sputum of a patient suffering from cystic fibrosis. They were treated by alkaline-borohydride treatment. Application of ion-exchange chromatography afforded carbohydrate fractions containing sulfate. One of these fractions containing a mixture of sulfated oligosaccharides was subsequently submitted to gel-filtration and high performance liquid chromatography. Two sulfated oligosaccharide-alditols, A and B, were prepared; their structure was determined using 400 MHz ^{1}H-n.m.r. and FAB mass spectrometry. They had a core type 2 and the sulfate was 3-linked to a terminal galactose residue:

SO_3H - 3 Gal (β1-4) GlcNAc (β1-6)
GalNAc-ol   A
Fuc (α1-2) Gal (β1-3)

SO_3H - 3 Gal (β1-4) GlcNAc (β1-6)
GalNAc-ol   B
Gal (β1-3)

Abbreviations: FAB m.s., Fast atom bombardment mass spectrometry; CF, Cystic Fibrosis; CFTR, cystic fibrosis transmembrane regulator;

INTRODUCTION

Cystic fibrosis is the most common fatal autosomal recessive disease in Caucasian population with an incidence of 1 on 2,000 birth. It is characterized by abnormal electrolytes transport in the apical membrane of several epithelia (Quinton *et al*, 1989). In the large airways epithelium, failure to secrete Cl^- and excessive reabsorption of Na^+ (Knowles *et al*, 1986) lead to mucus dehydratation, abnormal mucociliary clearance and mucus obstruction.

But this abnormal electrolytes transport alone cannot explain the persistent infection of CF patients by selective microbial pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* , bacteria that destroy the lung tissue (Hoiby, 1988). Chronic infections by these microorganisms do not occur so rapidly in patients suffering from other genetic diseases with failure of mucociliary clearance such as the dismotile cilia syndrom (Eliason *et al*, 1977).

Identification of the gene defect that causes CF (Riordan *et al*,1989) suggests that the protein responsible for CF, or cystic fibrosis transmembrane regulator, belongs to the superfamily of membrane-associated transport proteins, P-glycoproteins (Juranka *et al*, 1989). The cellular localization and the precise function of this CFTR protein are still unknown.

Previous studies by our group and others (Roussel *et al*, 1975; Lamblin *et al*, 1977 a; Boat *et al*, 1977) have shown that respiratory mucins secreted by CF patients were highly sulfated. Recently, it has been reported that "mucins" synthesized by cultured CF nasal epithelial cells were more sulfated than "mucins" produced by normal cells (Cheng *et al*, 1989). Sulfate uptake has also been shown to increase in skin fibroblasts from CF patients (Elgavish & Meezan, 1988).

In order to further investigate the sulfation of respiratory mucins in CF, we have started a structural comparison of sulfated oligosaccharides from respiratory mucins secreted by CF patients and by patients suffering from other chronic bronchial diseases. In the present work, we describe the isolation, purification and structure of two sulfated oligosaccharides isolated from respiratory mucins of a patient suffering from CF.

MATERIAL AND METHODS.

Respiratory mucus sampling

Human sputum from a patient (Lab...) with blood-group 0 sufffering from cystic fibrosis was collected every day and kept frozen until used. The bronchial secretions were then thawed at 4°C, diluted 1 to 12 with deionized water and stirred overnight at 4°C (Slayter *et al*, 1984). The diluted mucus was then centrifuged at 3,000 g for 30 min dialyzed and lyophilized.

Preparation of respiratory mucin glycopeptides

The lyophilized supernatant was digested at 37°C by pronase (pronase R proteinase, Calbiochem, San Diego CA) in 0.01M calcium acetate buffer (pH 7.0) using an enzyme/substrate ratio of 1 : 40 and a fresh addition of enzyme at 24 h. After 48 h, the mixture was centrifuged and the supernatant was dialyzed and lyophilized.

Aliquots (200mg) of freeze-dried pronase digested supernatant were submitted to gel filtration on a Sepharose CL-4B (Pharmacia) column (2,5 x 48 cm) equilibrated and eluted with 0.2 M NaCl, 0.1 M Tris-HCl, pH 8.0. Fractions (10 ml) were collected and analyzed for absorbance at 278 nm and for hexose by an automated orcinol assay (Demaille *et al*, 1965). Three fractions F_1, F_2, F_3 were separated, dialyzed and lyophilized.

Isolation of oligosaccharides alditols

O-linked oligosaccharides were released from fraction F_2 by alkaline borohydride treatment in 2 M sodium borohydride, 0.05 M sodium hydroxide at 45° C for 16h (Roussel *et al*, 1975). The solution was applied to a column (2.5 x 54 cm) of AG 50W X 8 (Bio-Rad, Richmond CA, U.S.A.) in order to remove salts and then the mixture was fractionated by ion exchange chromatography (AG 1 X 2) and gel-filtration (Bio-Gel P4) according to acidity and molecular size respectively as previously described (Lamblin *et al*, 1977 b). By this procedure, four pools of oligosaccharide-alditols were obtained : Ic (neutral), IIc (sialytated) and IIIc and IVc (sulfated).

The sulfated oligosaccharide fraction IIIc was subsequently applied to a column (1 x 150 cm) of Bio-Gel P2 (200 - 400 mesh, Bio-Rad Richmond CA, U.S.A.) using 0.1 M ammonium acetate as eluent, resulting in the separation of 6 oligosaccharide fractions (IIIc1 - IIIc6).

Fractionation of acidic-oligosaccharide alditols of fraction IIIc6 was carried out by reverse phase chromatography on a Ultra-sphere IP column (Beckman) (250 x 4.6 mm, particle size 5 µm) run isocratically with water as eluent at a flow

rate of 0.5 ml/min at room temperature. Oligosaccharide-alditols were detected by measuring absorption at 206 nm.

Analytical methods

Amino acid analyses were performed as already described (Lamblin *et al*, 1977 b).

Sugar analysis was carried out by glc of trimethylsilyl derivatives of methylglycosides formed by methanolysis in 1.5 M HCl in methanol at 80°C for 24 h (Lamblin *et al*, 1984 a). N-acetyl neuraminic acid and sulfate were measured as already described (Roussel *et al*, 1975).

Fast atom bombardment-Mass spectometry

FAB - MS of native oligosaccharide alditols was performed using a Kratos MS - 50 mass spectrometer. The samples (1 µg) were applied to the target in aqueous solution, glycerol was used as matrix. The target was bombarded with xenon atoms having a kinetic energy equivalent to 9 Kev. The spectra were recorded in negative ion mode at 7 kv acceleration voltage, in a mass-controlled linear scan at a resolution of 300 ppm.

400 MHz ^{1}H-NMR spectroscopy

Oligosaccharide-alditols were repeatedly exchanged in $^{2}H_{2}0$ (99.96 atom % ^{2}H, Aldrich, Milwaukee, WI, U.S.A.) with intermediate lyophilization and analyzed with a Bruker AM-400 WB spectrometer operating at 400 MHz in the Fourier transform mode at a probe temperature of 300°K. Chemical shifts are given relative to sodium - 4,4, dimethyl-4-silapentane-sulfonate but were actually measured indirectly to acetone in $^{2}H_{2}0$ ($\gamma = 2.225$ ppm).

The 2D-homonuclear Cosy 45 experiments were performed by use of the standard Bruker pulse program Cosy.

RESULTS

The carbohydrate composition of bronchial mucin glycopeptides from patient Lab... suffering from cystic-fibrosis (Fraction 2 in Figure 1) is given in Table I.

890 mg of glycopeptides F_2 were obtained from 650 ml of sputum. Fraction F_2 was submitted to alkaline borohydride degradation and fractionated by ion-exchange chromatography in four fraction (Lamblin *et al*, 1977 b). Fraction III, eluted from the Dowex AG1 X 2 with 0.025 M H_2SO_4, was subfractionated into IIIa, IIIb and IIIc by chromatography on Biogel P4 (Figure 2) (Lamblin *et al*, 1977 b). Fraction IIIc (18 mg) corresponded to a mixture of sulfated and sialylated oligosaccharide-alditols as shown by its carbohydrate composition (Table II).

In order to separate the different oligosaccharide-alditols present in fraction IIIc, this fraction was subsequently applied to a Biogel P2 column eluted with 0.1M ammonium acetate (Figure 3). Five main fractions were obtained and pooled as indicated : $IIIc_2$ (1 mg), $IIIc_3$ (1.5 mg), $IIIc_4$ (2.9 mg), $IIIc_5$ (2.2 mg), $IIIc_6$ (1.5 mg) (Figure 3). The carbohydrate compositions of the subfractions from pool IIIc are compiled in Table III. All fractions contain GalNAc-ol, GlcNAc, Gal, Fuc. Fractions $IIIc_2$ and $IIIc_3$ contain both sialic acid and sulfate, whereas $IIIc_4$, $IIIc_5$ and $IIIc_6$ contain only sulfate. Based on the assumption of one residue of GalNAc-ol per oligosaccharide-alditol molecule, the components obtained have an average carbohydrate chain-length from 4.8 sugars ($IIIc_6$) to 10 sugars ($IIIc_2$).

The molecular weights of oligosaccharide-alditols were measured by FAB-mass spectrometry in the negative ion mode. Only the spectrum of Fraction $IIIc_6$ was interpretable, the others being too complexe and corresponding to mixtures of a large numbers of oligosaccharide-alditols. The FAB spectrum of $IIIc_6$ (Figure 4) shows the presence of two major pseudomolecular ions $(M-H)^-$ at m/e 975 and 829, which correspond to the pentasaccharide sulfate, Fuc, Gal_2, GlcNac, GalNac-ol and the tetra-saccharide sulfate, Gal_2, GlcNac, GalNac-ol. The fraction $IIIc_6$ is contamined by hexasaccharides sulfate, with m/e at 1178 (Fuc, $GlcNac_2$, Gal_2, GalNac-ol) and 1121 (sulfate, $GlcNac_2$, Gal_2, GalNac-ol), which are present in low amount.

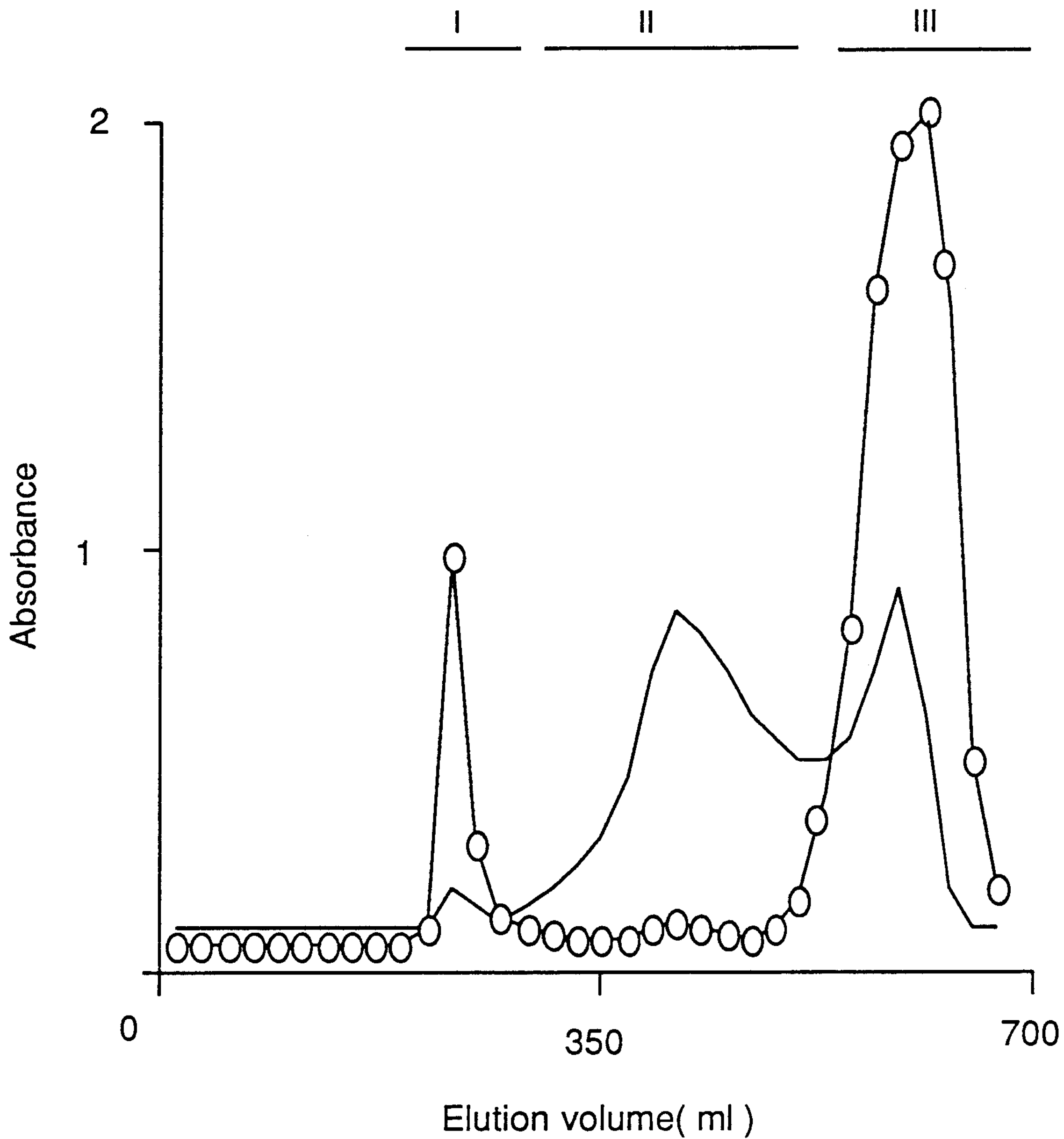


Fig. 1- Sepharose CL-4B (4 X50 cm) chromatography of mucus (Lab...) supernatant after pronase digestion (210 mg). The column was eluted with O.2 M NaCl 0.1 M Tris-HCl pH 8.0. 10 ml fractions were collected and analyzed for absorbance at 278 nm (open circles) and for hexose (solide line). The fractions indicated by bars were dialysed and lyophilized.

Table I. Chemical composition of mucus glycopeptides (F2) from CF sputum after pronase and chromatography on Sepharose 4B column

Components	Amount	Total
	mmol / g	% (by mass)
Aspartic acid	26	
Threonine	330	
Serine	140	
Glutamic acid	39	
Proline	119	
Glycine	63	
Alanine	97	
Valine	39	
Methionine	6	
Isoleucine	22	
Leucine	43	
Tyrosine	2	
Phenylalanine	10	
Histidine	21	
Lysine	19	
Arginine	23	
Total amino acids	999	9.8
N-acetylneuraminic acid	240	
Fucose	1210	
Galactose	1180	
N-acetylglucosamine	1030	
N-acetylgalactosamine	510	
Total carbohydrate	4170	82.7
Sulfate	235	

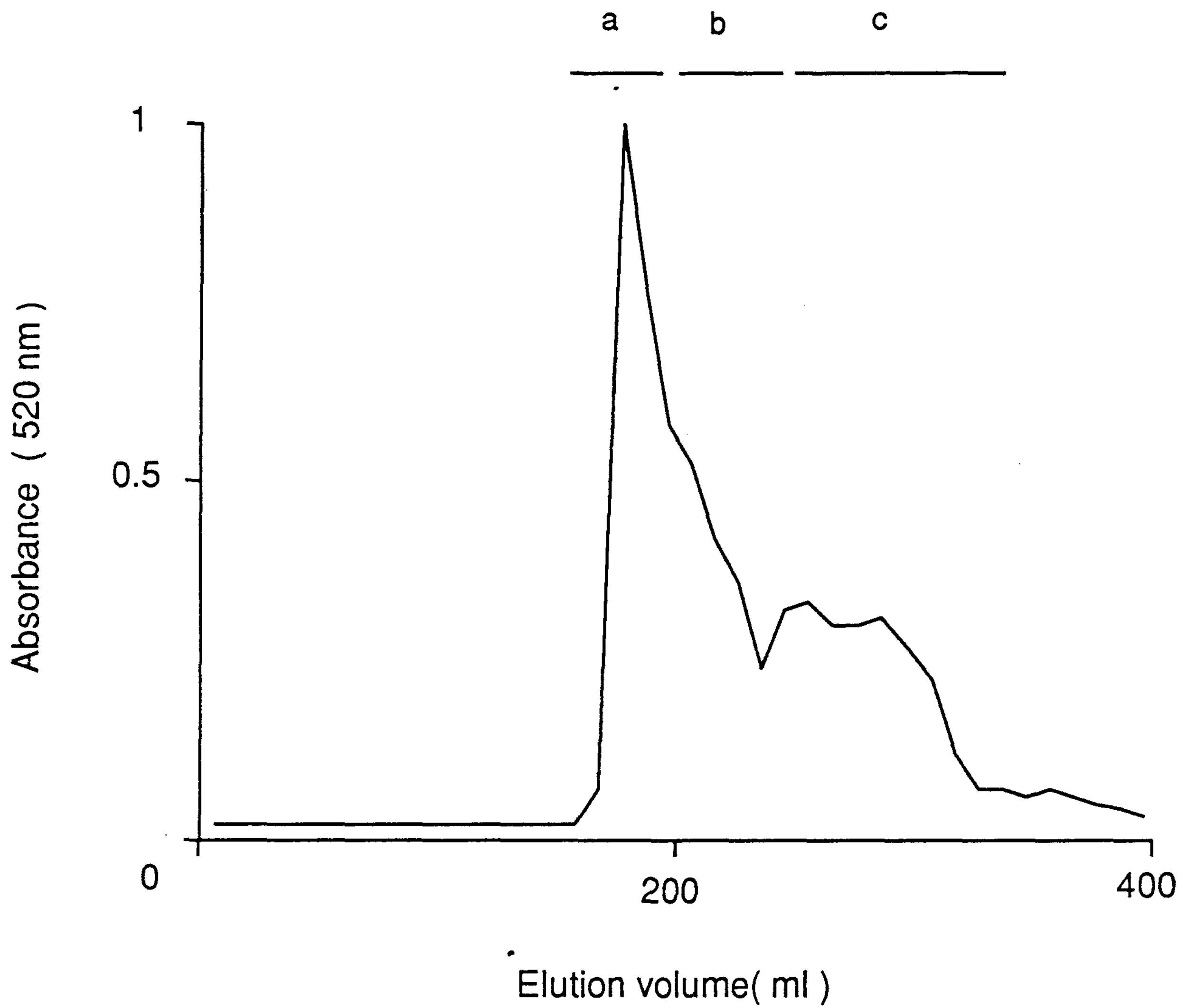


Fig. 2 - Fractionation of fraction III obtained after AG1 X2 column by chromatography on Bio-Gel P4 column (2 X 98 cm) eluted with 0.1 M ammonium acetate. 10 ml fractions were collected and analyzed for hexose (solide line). Fractions a, b, c indicated by bars were lyophilized.

Table II : Carbohydrate and sulfate composition of fractions IIIa, IIIb and IIIc.

	III a		III b		III c	
	μmole/g	molar ratio [a]	μmole/g	molar ratio	μmole/g	molar ratio
Carbohydrates						
N-acetylneuraminic acid	480	1.2	400	0.7	810	1
Fucose	855	2.2	725	1.4	585	0.7
Galactose	1170	3	1150	2.1	1070	1.3
N-acetylglucosamine	760	1.9	805	1.5	680	0.8
N-acetylgalactosamine	295		245		180	
N-acetylgalactosaminitol	95	1	290	1	660	1
Sulfate	185	0.5	295	0.6	720	0.9
Carbohydrate chain-length [b]	9.3		6.7		4.8	

[a] - The molar ratio of the oligosaccharide-alditols was calculated on the basis of one residue of GalNAc-ol (or GalNAc-ol + GalNAc) per molecule.

[b] - Ratio of total carbohydrate residues to the sum of N-acetylgalactosamine and N-acetylgalactosaminitol.

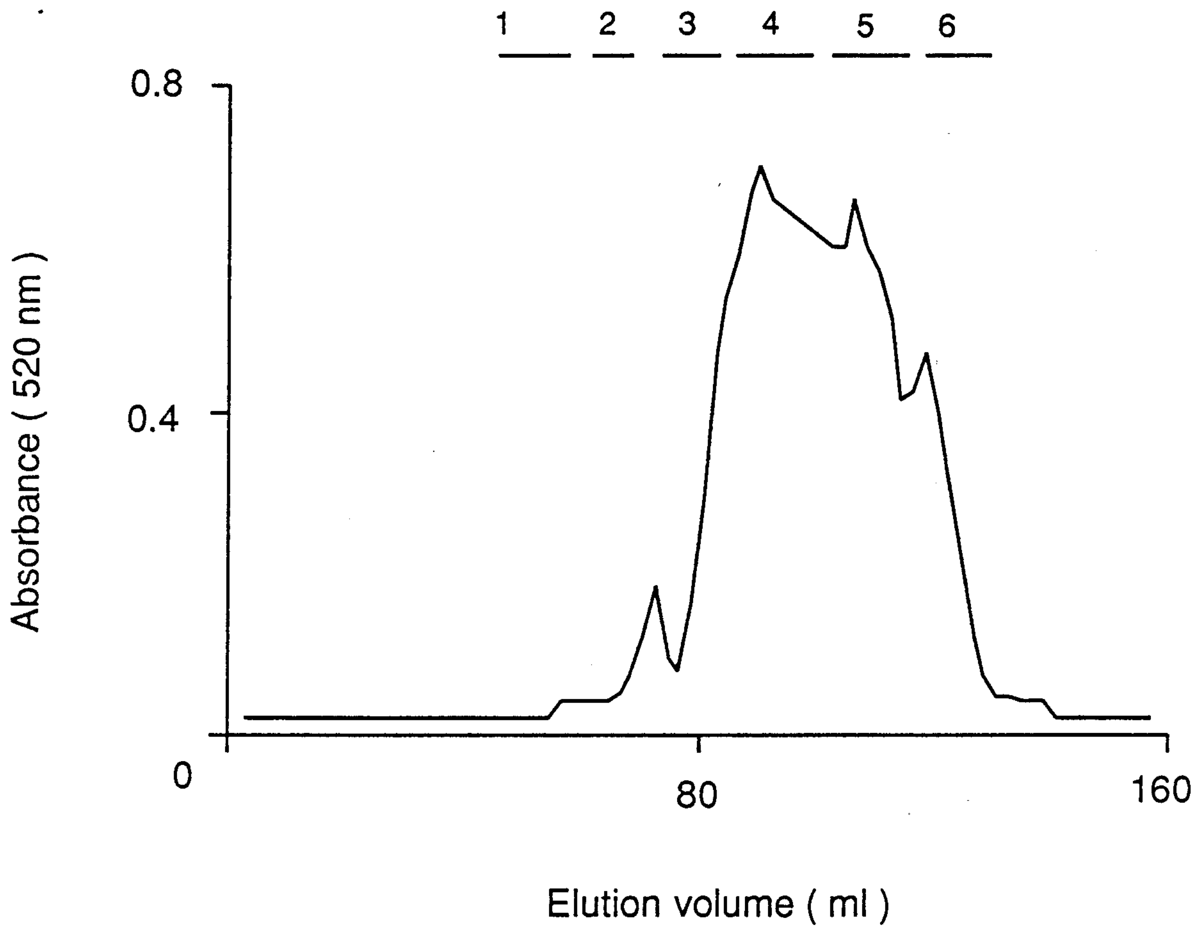


Fig. 3 - Fractionation of IIIc by chromatography on Bio-Gel P2 column (1 X 150 cm) eluted with 0.1 M ammonium acetate. 2 ml fractions were collected and analyzed for hexose (solide line). Fractions indicated by bars were lyophilized.

Table III : Carbohydrate and sulfate composition of fractions eluted from Bio-Gel P2 column (IIIc2, III c3, III c4, III c5 and IIIc6).

	III c2		III c3		III c4		III c5		III c6	
	µmole/g	molar ratio [a]	µmole/g	molar ratio	µmole/g	molar ratio	µmole/g	molar ratio	µmole/g	molar ratio
Carbohydrates										
N-acetylneuraminic acid	150	0.4	440	0.8	0		0		0	
Fucose	1790	5.0	1640	2.9	1150	1.9	840	1.6	420	0.7
Galactose	920	2.6	1300	2.4	1155	1.9	1150	2.0	1270	2.1
N-acetylglucosamine	355	1	390	0.7	590	1.0	615	0.9	590	1.0
N-acetylgalactosamine	270		120		0			0		0
N-acetylgalactosaminitol	90	1	430	1	605	1	705	1	610	1
Sulfate	925	2.5	370	0.7	495	0.8	850	1.2	690	1.1
Carbohydrate chain-length [b]	10		7.8		5.8		5.5		4.8	

[a] - The molar ratio of the oligosaccharide-alditols was calculated on the basis of one residue of GalNac-ol (or GalNAc-ol + GalNac) per molecule.

[b] - Ratio of total carbohydrate residues to the sum of N-acetylgalactosamine and N-acetylgalactosaminitol.

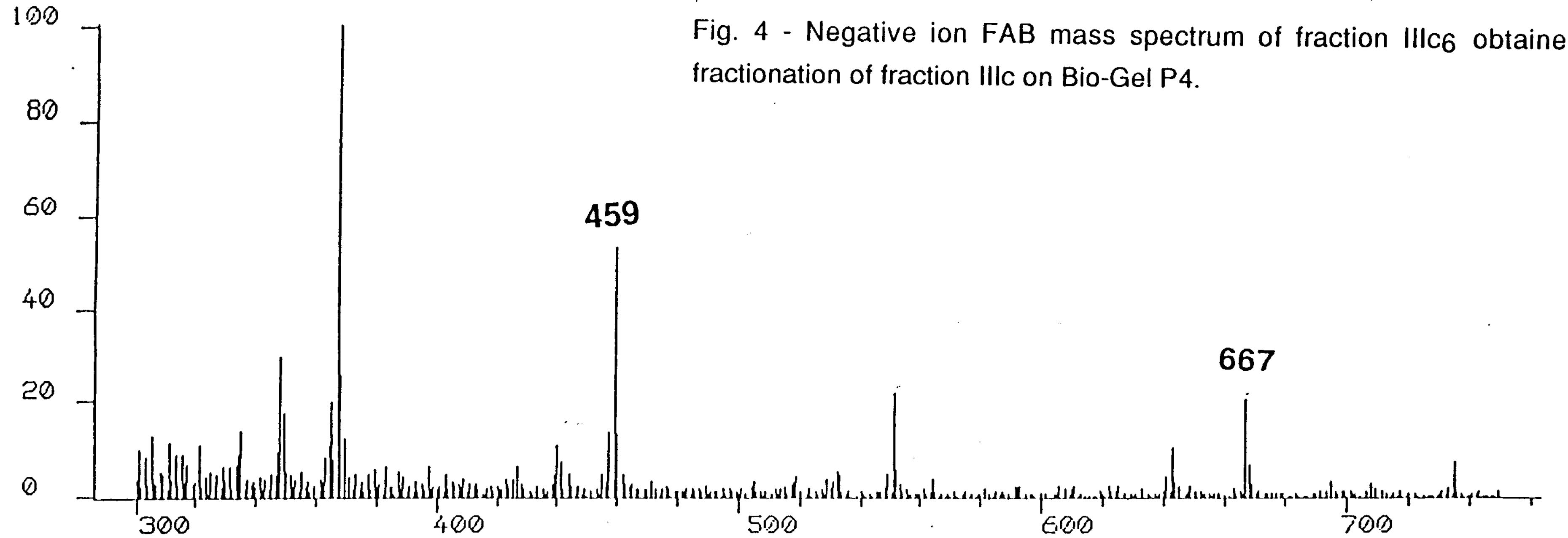
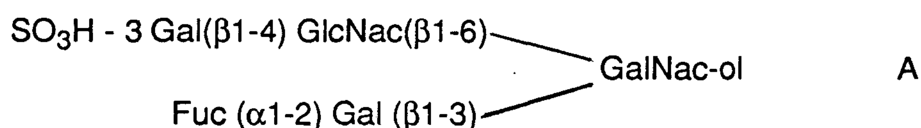


Fig. 4 - Negative ion FAB mass spectrum of fraction IIIc6 obtained after fractionation of fraction IIIc on Bio-Gel P4.

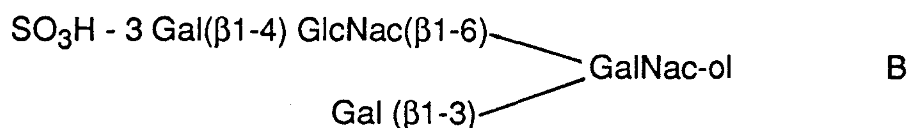
The 1D-^{1}H-NMR spectrum of III c_6 is depicted in Figure 5. The chemical shifts of the H-2 and H-5 resonances of GalNAc-ol (at δ = 4.40 and 4.27 ppm, respectively) are characteristic of the core Gal(β1-3) [GlcNAc (β1-6)] GalNAc-ol (Vliegenthart *et al*, 1981). The H-1 resonance observed at δ = 4.587 ppm is connected, on the COSY spectrum (Figure 6), to the H-2 (δ = 3.670 ppm), H-3 (δ = 4.337 ppm) and H-4 (δ = 4.292 ppm) resonances of the sugar unit. Particularly, the low coupling constant $J_{4,5}$ indicates the sugar to be a galactose residue. Its downfield-shift H-3 resonance is attributable to the C-3 substitution by a sulfate residue.

The fucose residue is α–1,2 linked to Gal^3, as shown by the typical H-1, H-5 and H-6 resonances of Fuc and the H-1 resonance of Gal^3, observed at 4.572 ppm, as in reference compounds (Klein *et al*, 1988). The H-1 resonance of $GlcNAc^6$ (at δ = 4.572 ppm) of the Gal (β1-4) GlcNAc unit is characteristic of the β-1,4-substitution $GlcNAc^6$ residue (Klein *et al*, 1988).

On the basis of these observations, the following structure was attributed to the major oligosaccharide-alditol of the fraction $IIIc_6$:

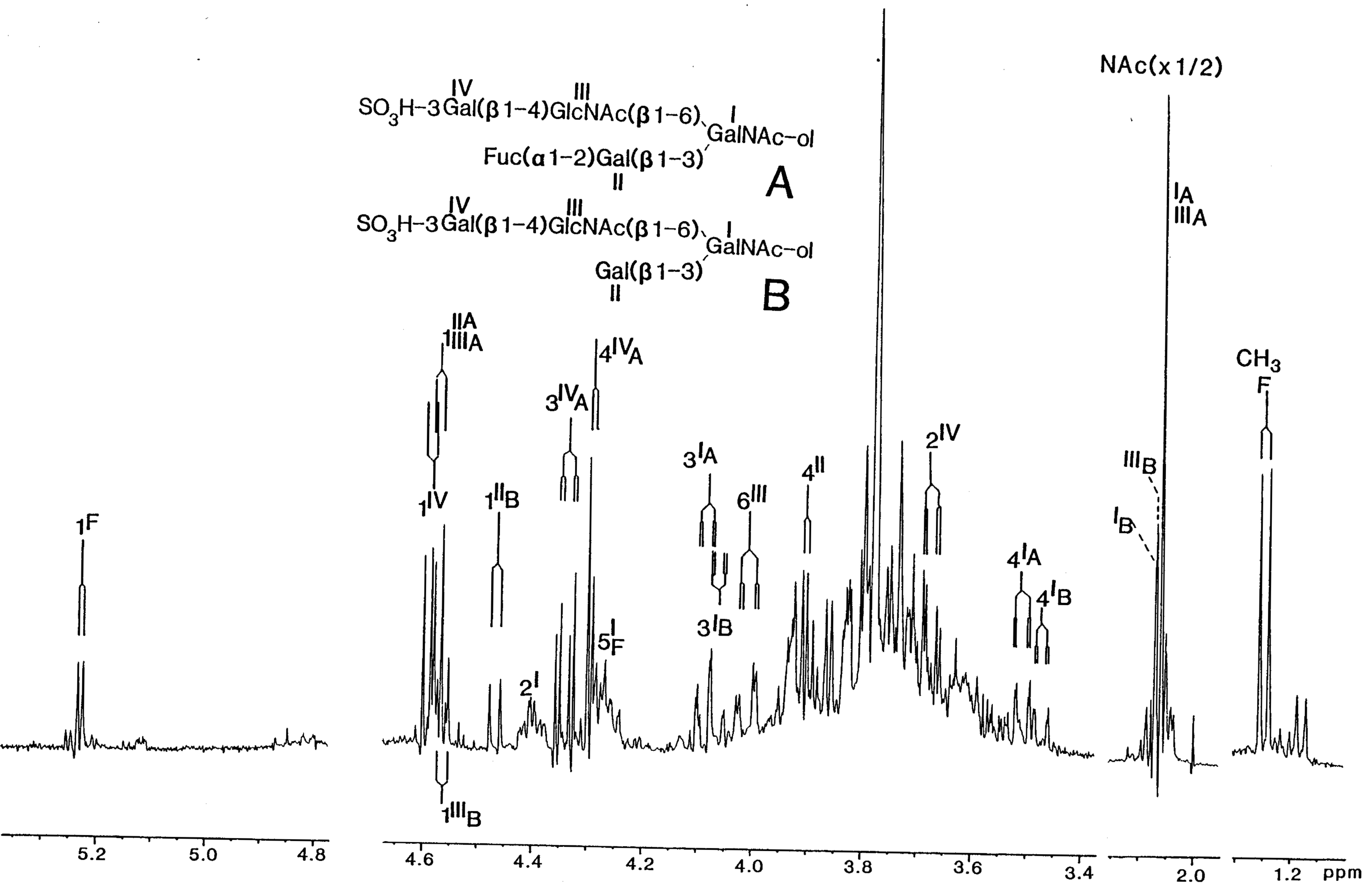


The second compound, devoid of fucose, possess an H-1 Gal resonance at 4.464 ppm, characteristic of the terminal, non reducing, Gal^3 residue (Klein *et al*, 1988) and, consequently, was established to be :



The two oligosaccharides A and B are divided up into a ratio of 70 and 30 percent. They have been separated further by HPLC (as described in material and methods) and the RMN spectra obtained from the two major peaks 4 and 13 (Figure 7) confirmed that they correspond to the structures determined in $IIIc_6$ (Figure. 8) : Peak 4 is the oligosaccharide B and peak 13 is the oligosaccharide A.

Fig. 5 - 400 MHz ^{1}H-NMR spectrum of fraction IIIc6.



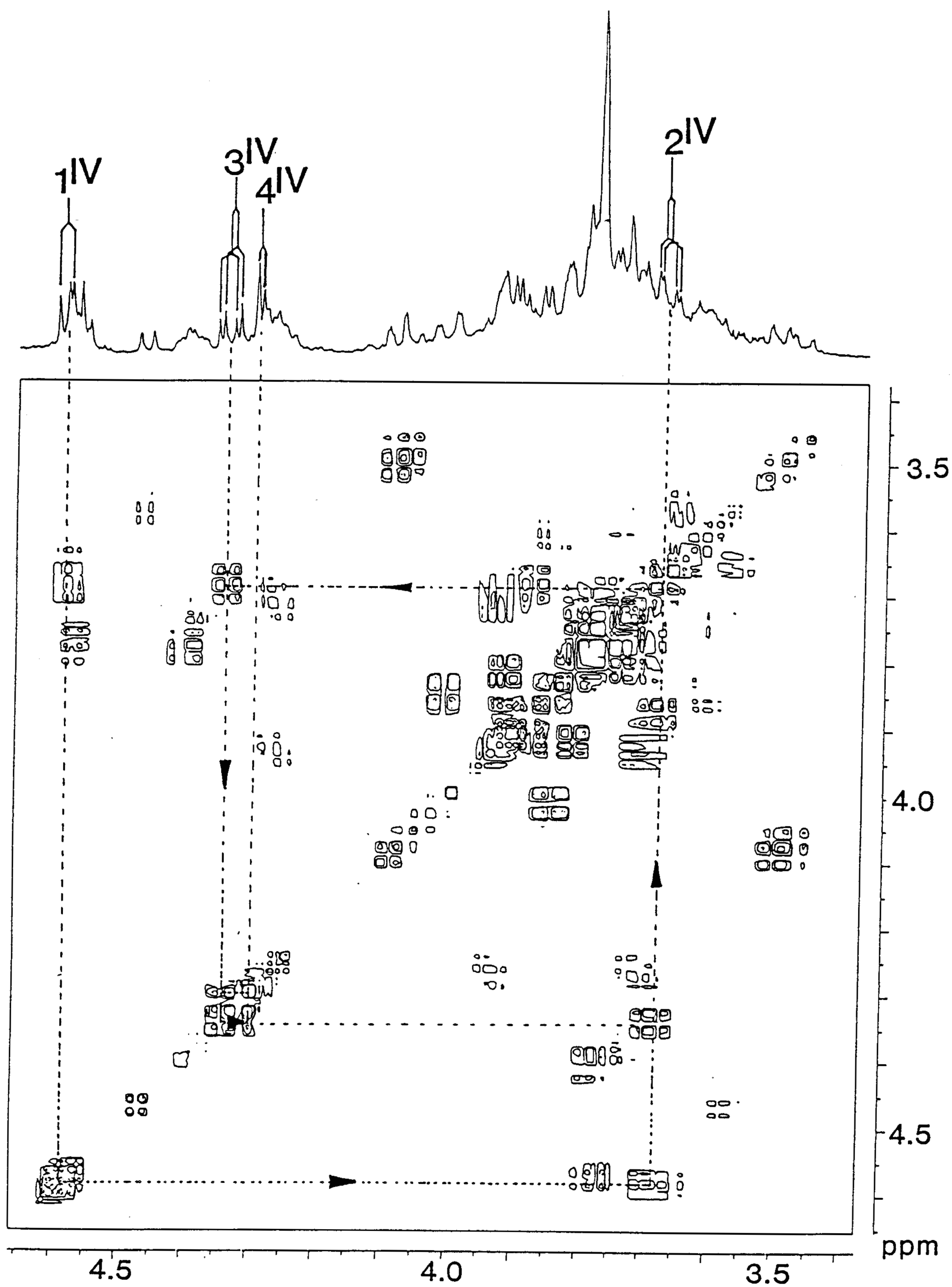


Fig. 6 - 2D- Homonuclear COSY spectrum of fraction IIIc$_6$.

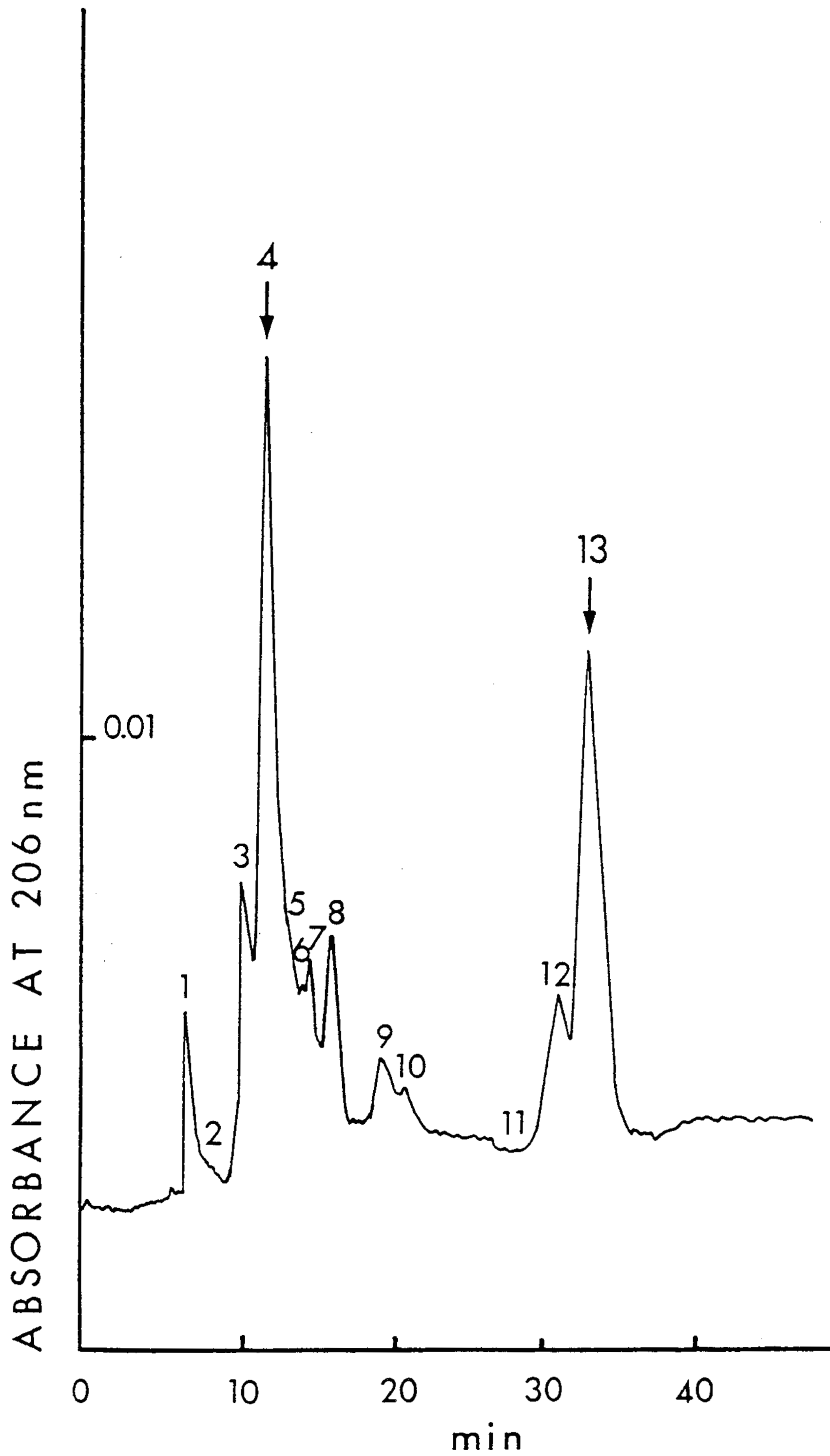


Fig. 7 - Fractionation of IIIc$_6$ by HPLC. Elution was performed isocratically with water on a 5 μm Ultrasphere C18 IP. Oligosaccharides peaks were detected by absorbance at 206 nm. Peaks 4 and 13 were collected and lyophilized.

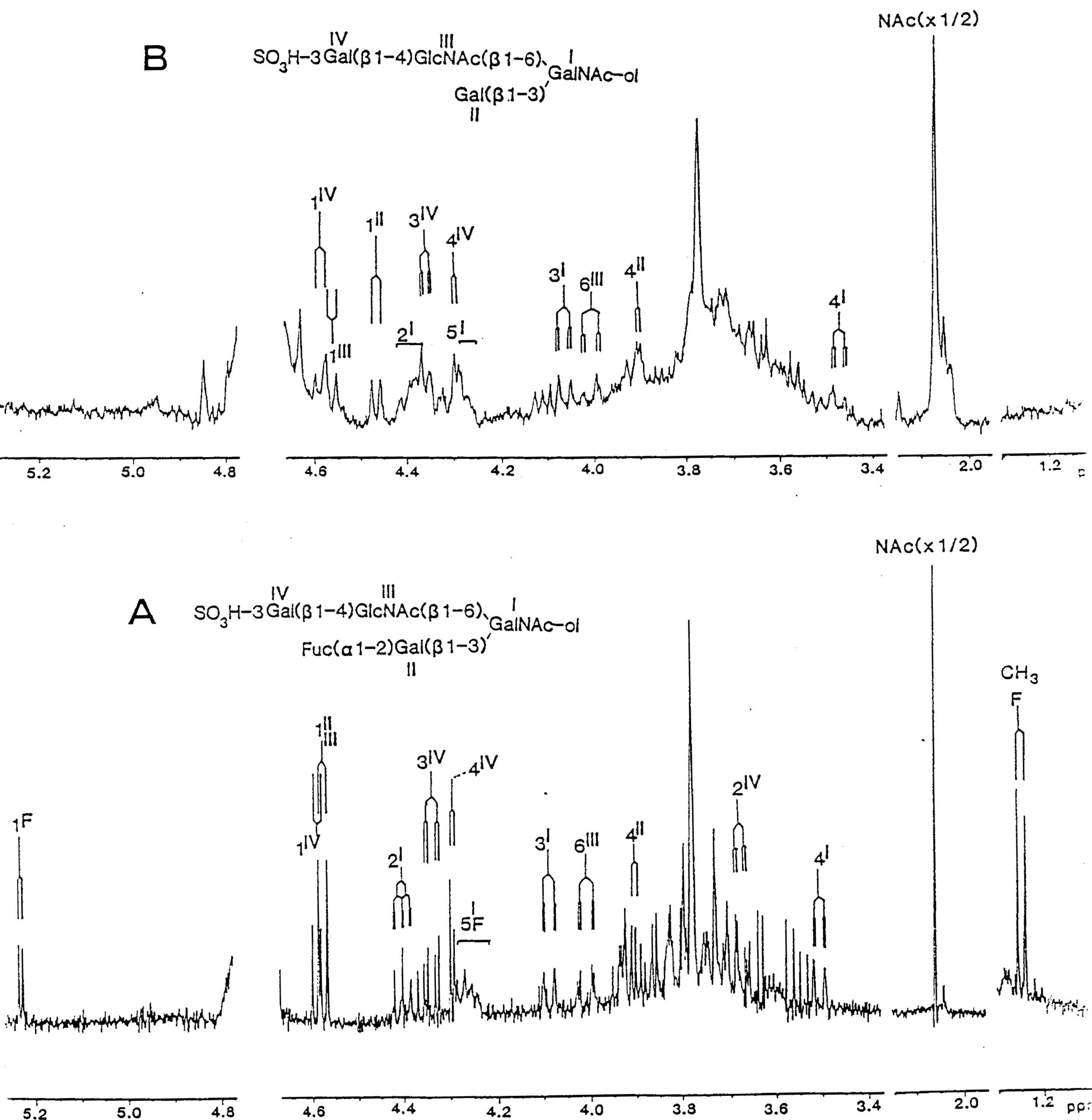


Fig. 8 - 400 MHz ^{1}H-NMR spectrum of compounds A and B obtained after fractionation of IIIc6 by HPLC.

DISCUSSION

Human respiratory mucins have been shown to be very heterogeneous with regard to acidity, molecular size and structure of their carbohydrate chains (Roussel *et al*, 1975; Lamblin *et al*, 1979; Lamblin *et al*, 1984 a & b; Breg *et al*, 1987,1988; Klein *et al*, 1988; Van Halbeek *et al*, 1988). Sulfated chains are abundant, especially in the case of mucins from patients with cystic fibrosis. However, our knowledge of the structures of sulfated chains has been so far limited, due to the predominance of sulfate ester on long chain oligosaccharides (Roussel *et al*, 1975; Lamblin *et al*, 1979), very hard to isolate in a pure state necessary for further structure determinations.

In the present work, ^{1}H-n.m.r. spectroscopy in combination with fast ion bombardment-mass spectroscopy in negative ion mode were used for investigation of two new structures of sulfated oligosaccharides with core 2 and sulfate 3-linked to galactose.

The n.m.r. data presented in Table IV illustrate the possibility of determining the exact position of sulfate in carbohydrate chains. The H-3 resonance of Gal^4 residue was observed at $\delta = 4.337$ ppm, which is identical to the value observed in a sulfated oligosaccharide-alditol found in human meconium (Capon *et al*, 1989). By comparison with compound 11 of Table IV for which the complete assignement of n.m.r. parameters has been recently described (Strecker *et al*, 1989), the H-3 resonance of the SO_3H 3-Gal^4 residue was found to be downfield shifted of + 0.668 p.p.m. Similarly, by comparison with pure methyl α–D-galactopyranoside 3-sulfate for which the complete assignement of n.m.r. parameters has been reported (Contreras *et al*, 1988), the H-4 resonance of the SO_3H 3-Gal^4 residue was also found to be downfield shifted of + 0.369 p.p.m.

A 3-O-sulfation of terminal β-D-galactosyl residues has already been observed in N-linked carbohydrate units of calf and human thyroglobulin (Spiro & Bhoyroo, 1988) and in an oligosaccharide-alditol found in human meconium (Capon *et al*, 1989). A PAPS : galactosyl 3-O-sulfotransferase has been localized in the Golgi compartment of thyroid (Kato & Spiro, 1989) and, in the future, it will be interesting to find out if this enzyme is also expressed in the Golgi compartment of respiratory mucins synthesizing cells.

Other sulfated sugars have been identified in different mucins. The presence of galactose-6-sulfate has been already suggested in human respiratory (Roussel *et al*, 1975; Mawhinney *et al*, 1987) and rat gastric mucins

TABLE IV. Chemical shifts of structural reporter-group protons of the constituent monosaccharides for sulfated oligosaccharide-alditols derived from respiratory mucins of a CF patient and for reference compounds 11 and 12 (Lamblin *et al*, 1984)
◇ = GalNAc-ol, ■ = Gal, ● = GlcNAc, □ = Fuc.

Residue	Reporter Group	Chemical shift in compound			
		(11)	B	(12)	A
			SO_3H		SO_3H
GalNAc-ol	H-2	4.399	4.391	4.405	4.400
	H-3	4.061	4.060	4.085	4.085
	H-4	3.460	3.468	3.499	3.502
	H-5	4.285	4.271	4.269	4.265
	NAc	2.067	2.067	2.055	2.056
Gal[3]	H-1	4.463	4.464	4.573	4.572
	H-4	3.898	3.901	3.923	3.923
GlcNAc[6]	H-1	4.557	4.560	4.570	4.572
	H-6	3.996	4.006	3.998	4.006
	NAc	2.066	2.064	2.055	2.056
Gal[4]	H-1	4.469	4.587	4.469	4.587
	H-2	-	3.670	-	3.670
	H-3	-	4.337	-	4.337
	H-4	3.923	4.292	3.923	4.292
Fuc[2]	H-1	-	-	5.221	5.225
	H-5	-	-	4.278	4.288
	CH3	-	-	1.244	1.245

(Liau *et al*, 1982) whereas N-acetylglucosamine 6-sulfate has been identified in ovomucin (Strecker *et al*, 1989), rat salivary (Green *et al*, 1987) and gastric mucins (Carter *et al*, 1988). N-acetylglucosamine 4-sulfate has been found in dog (Lombart *et al*, 1974), rat (Liau *et al*, 1982) and monkey (Tabak *et al*, 1981) salivary mucins.

In the present work, FAB spectrometry of the four pools of oligosaccharides contained in the other fractions eluted from the Bio-Gel P2 column (IIIc$_2$, IIIc$_3$, IIIc$_4$ and IIIc$_5$) has shown that they corresponded to very complex mixtures of sulfated and sulfated/sialylated oligosaccharides (data not shown). Using a different fractionation procedure, Mawhinney *et al*, (1987) have already described small linear sulfated oligosaccharides from respiratory mucins with core 1 or 3 and sulfate 6-linked to galactose in human respiratory from CF patients.

Therefore, in CF respiratory mucins, sulfate may be located, at least, at the C-6 (Mawhinney *et al*, 1987) or at the C-3 of terminal galactose residues and it is highly probable that several sulfotransferases are responsible for the sulfation of those respiratory mucins.

In the future, it will be interesting to set up methods allowing the isolation of the different sulfated oligosaccharides, to compare their distribution (6-sulfated *vs* 3-sulfated) in mucins from patients suffering from CF and from other bronchial disorders and to define their role in the selection of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the airways of CF patients.

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DISCUSSION GENERALE
ET
CONCLUSIONS

Au cours de ce travail, un premier objectif consistait à développer un modèle de culture organotypique de muqueuse trachéale de cobaye permettant l'étude de la sécrétion bronchique et de facteurs susceptibles de l'influencer. Cet objectif a été atteint et un marqueur, le radiosulfate, ajouté dans le milieu où baigne la trachée permet de suivre la sécrétion des sulfoglycoconjugués qui ont été synthétisés et sécrétés dans la lumière trachéale.

Ce modèle de culture organotypique est intéressant. On pourra, grâce à lui, explorer les effets, sur la sécrétion des sulfoglycoconjugués de cobaye, de certains agents ajoutés soit à la périphérie de la trachée (le milieu dans lequel elle baigne), soit directement dans la lumière trachéale.

Il n'est cependant pas sûr qu'en utilisant le radio-sulfate, on explore bien toutes les mucines synthétisées par la muqueuse : il est en effet probable que certaines mucines ne soient pas sulfatées et qu'elles échappent à ce type d'exploration. Il faudrait donc utiliser d'autres marqueurs, comme la glucosamine, en espérant que leur diffusion au travers de la muqueuse soit aussi bonne que celle du sulfate. Pour des raisons de coût, nous n'avons pas pu explorer cette possibilité.

L'utilisation du radiosulfate a permis de définir les sulfoglycoconjugués sécrétés dans la lumière trachéale. Trois variétés de molécules ont été identifiés.

- Les premières possèdent les caractéristiques des mucines: sédimentation dans les solutions de sels de césium de haute densité, haute masse moléculaire (> 10^6 Da) et sensibilité à la β-élimination.

- Les secondes ne possèdent pas toutes les propriétés des mucines: elles sédimentent dans des solutions de densité moins élevée que les mucines classiques, leur masse moléculaire peut être plus faible mais elles sont sensibles à la β-élimination. Cette population de O-glycoprotéines sulfatées pose un problème, celui de son appartenance à la classe des mucines. On dispose aujourd'hui de toute une série d'arguments expérimentaux (microscopie électronique, étude de biosynthèse) montrant que les mucines sont assez polydispersées (1); il se pourrait que cette polydispersion soit encore plus importante qu'on ne le pense généralement et que la classe des mucines comprenne aussi des molécules de faible masse moléculaire, ce qui pourrait amener à revoir leur définition.

- La trachée de cobaye sécrète enfin une faible proportion de protéoglycannes à chondroïtine sulfate. Leur origine est incertaine, desquamation des cellules de la surface épithéliale, produit de synthèse des cellules ayant un caractère glandulaire? En tout état de cause, il ne s'agit probablement pas de protéoglycannes provenant de la sous-muqueuse sous-jacente car nous n'avons pas trouvé pas de kératane-sulfate qui est un des constituants glycanniques des protéoglycannes du tissu conjonctif.

En fonction de ces résultats, à côté des mucines qui en constituent les principales macromolécules de la sécrétion bronchique humaine, nous avons cherché à vérifier la présence de protéoglycannes à chondroïtine sulfate. Il s'agit là encore d'un domaine très controversé.

Nous avons travaillé sur des expectorations recueillies chez 12 malades souffrant de bronchite chronique, la plupart non surinfectées, et sur celles de 13 malades atteints de mucoviscidose, la plupart infectées par *Pseudomonas aeruginosa*, le germe de surinfection le plus couramment rencontré dans la mucoviscidose.

Nous avons mis en évidence du chondroïtine sulfate dans la plupart des expectorations surinfectées de malades atteints de mucoviscidose. Toutefois cette présence n'est ni spécifique à la mucoviscidose, ni spécifique à la surinfection par *Pseudomonas aeruginosa.* puisqu'une expectoration non surinfectée d'un malade atteint de bronchite chronique contenait un peu de chondroïtine sulfate.

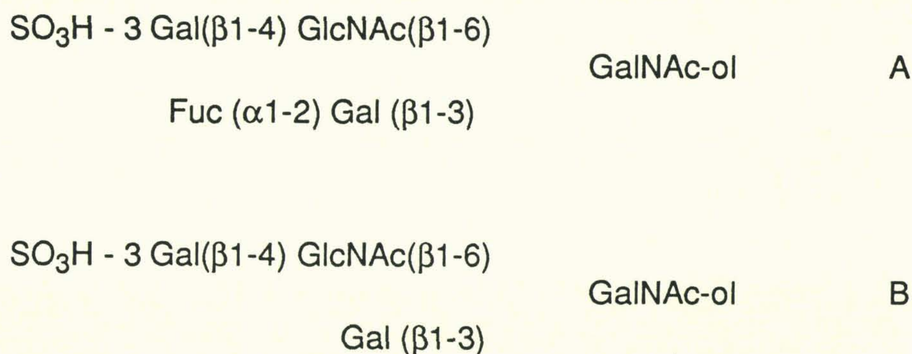
Il faudra là encore essayer de déterminer l'origine de ce chondroïtine sulfate: desquamation du glycocalyx des cellules de la surface ? sécrétion du contenu des cellules séreuses qui, en culture organotypique (2), incorporent fortement le radio-sulfate et qui, en culture cellulaire (3), sécrètent du chondroïtine sulfate ?

Nous n'avons pas vérifié si les leucocytes, très nombreux dans les expectorations surinfectées ou très inflammatoires, étaient particulièrement riches en chondroïtine sulfate.

Dans la dernière partie de notre travail, nous nous sommes intéressé à la localisation du sulfate dans certaines chaînes glycanniques des mucines bronchiques humaines.

Les chaînes glycanniques sulfatées sont très diverses (24; 98). On connait peu de choses sur la localisation du sulfate sur ces oligosaccharides si ce n'est

qu'un certain nombre de chaînes glycanniques contiennent du sulfate localisé sur le C-6 de résidus de galactose (23; 24). Nous avons repris une étude ancienne du Laboratoire sur les chaînes glycanniques sulfatées de mucines sécrétées au cours de la mucoviscidose et pu isoler deux oligosaccharides sulfatés comptant du sulfate lié au C-3 de résidus de galactose terminaux:



Il y a donc dans les mucines de mucoviscidose des groupements sulfate liés en C-3 ou en C-6 à des résidus de galactose.

On a décrit il y a longtemps une hypersulfatation des mucines de sujets souffrant de mucoviscidose (98; 99) et on a trouvé plus récemment des anomalies de la sulfatation dans les cellules épithéliales de mucoviscidose (100) et des anomalies du transport de sulfate dans les fibroblastes de sujets atteints de mucoviscidose (101).

Cette étude nous a permis d'entrevoir également l'extrême hétérogénéité qui existe dans les oligosaccharides sulfatés des mucines bronchiques d'enfants atteints de mucoviscidose. A partir de cette constatation et de la mise en évidence de différents types de liaisons ester-sulfate dans ces oligosaccharides, il serait sans doute intéressant de pouvoir mesurer l'activité des différentes sulfo-transferases de la muqueuse bronchique humaine dans des situations pathologiques. Des anomalies de ces réactions pourraient s'observer de façon non spécifique dans certaines hypersécrétions bronchiques chroniques ou peut-être de façon spécifique dans la mucoviscidose.

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RESUME

Au cours de ce travail sur les sulfoglycoconjugués trachéo-bronchiques, (i) un système de culture organo-typique de la muqueuse trachéo-bronchique de cobaye a été mis au point: il a permis de définir les sulfoglycoconjugués normalement sécrétes par cette muqueuse, O-glycoprotéines sulfatées et protéoglycannes à chondroïtine sulfate;

(ii) en fonction de ces résultats, la présence de chodroitïne-sulfate dans la sécrétion bronchique humaine a été recherchée: certaines sécrétion bronchiques très infectées, notamment au cours de la mucoviscidose, contiennent du chondroïtine-sulfate;

(iii) les caractéristiques de la sulfatation des mucines bronchiques humaines ont été étudiées et des liaisons ester-sulfate sur le C-3 de résidus de galactose terminaux, liaisons qui étaient jusqu'a présent inconnues dans les chaînes glycanniques des mucines, ont été mises en évidence.