Incorporation of *N*-Fluoroacetyl-D-glucosamine into Hyaluronate by Rabbit Tracheal Explants in Organ Culture

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1. Incubation of rabbit tracheal explants with *N*-[³H]acetyl-D-glucosamine and *N*-acetyl-D-[1-¹⁴C]glucosamine led to labelling of a number of soluble macromolecular products separable from the medium, after papain digestion, by ion-exchange chromatography. 2. With *N*-acetyl-D-[1-¹⁴C]glucosamine in the incubation medium, a neutral glycoprotein, two acidic glycoprotein fractions, hyaluronic acid and a glycosaminoglycan fraction were obtained and all were radioactively labelled. Similar labelling occurred with *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine or *N*-fluoro[³H]acetylglucosamine as precursor. 3. Maximal labelling was obtained at 96h after incubation of cultures. *N*-Fluoroacetylglucosamine under these conditions was incorporated into hyaluronate less efficiently than *N*-acetylglucosamine. 4. With *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine as precursor, a hyaluronate component was separated that on enzymic degradation by glycosidases (hyaluronidase, β -glucuronidase and *N*-acetyl- β -hexosaminidase) yielded a ¹⁴C-labelled oligosaccharide fraction together with *N*-acetyl-D-[1-¹⁴C]glucosamine and *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine, consistent with some exchange of *N*-acetyl groups having occurred. 5. The results on enzymic degradation of labelled macromolecules by glycosidases suggest that the presence of incorporated *N*-fluoroacetyl side chains may render the hyaluronate analogue more resistant to hyaluronidase.

The totality of potential biological information in a protein structure is not fully defined by the amino acid sequence alone and an additional hierarchy of information can be envisaged by post-polymerization structural modifications. Such modifications include phosphorylation, sulphation and in particular glycosylation, resulting in additional information, known to contribute crucially to features of biological recognition. It is therefore of interest to explore specific means by which intervention in the processes of protein glycosylation can be achieved with a view to modifying the final information content without disturbance of the general metabolism of the cells.

The deoxyfluoromonosaccharides (reviewed by Kent, 1972; Foster & Westwood, 1973) offer a way of exploring the hypothesis in mammalian cells. Existing evidence demonstrates that a variety of these sugars are readily transportable by mammalian cells (Barnett, 1972), where they can undergo phosphorylation and conversion into nucleotide sugar phosphates (Taylor, 1972; Schultz & Mora, 1975). In mammalian systems, so far, there is no evidence of metabolic breakdown of these fluorosugars into toxic

products as was at one time anticipated, though in some cases a limited oxidation and defluorination and in others reduction to hexitols has been observed (Kent & Wright, 1972).

A fluoro-analogue of GlcNAc, capable of occupying the same binding sites as GlcNAc in the active centres of lysozyme (Butchard *et al.*, 1972), was first reported to influence glycoprotein biosynthesis in fibroblast cell lines (Kent & Mora, 1973), after exploratory studies by Daniels (1972). More recent studies have, however, indicated that the fluoro-analogues interfere competitively with the incorporation of unsubstituted sugars. *N*-Trifluoroacetyl-D-glucosamine had been reported to compete with [¹⁴C]glucosamine incorporation by P288 mouse lymphoma cells, apparently decreasing the cellular incorporation of glucosamine to 31% of the control value within 5h, but having no effect on the incorporation of [³H]leucine by the cells (Bernacki *et al.*, 1977). Similarly, 2-fluoro-analogues of 2-deoxy-D-glucose and 2-deoxy-D-mannose had been found to behave as potent inhibitors in the synthesis of infectious Semliki forest and fowl plague viruses in chick-embryo cells and of pseudorabies virus in rabbit kidney cells (Schmidt *et al.*, 1976, 1978). The recently synthesized 2-deoxy-2-fluoro-L-fucose and its effect on glycoprotein-synthesizing cells is important in

Abbreviations used: GlcNAcF, *N*-fluoroacetylglucosamine; GlcNAc, *N*-acetylglucosamine.

general metabolic turnover (Winterbourne *et al.*, 1979).

In view of the structural complexity of glycoproteins and the difficulty in isolating them in a pure form, it was decided to investigate the incorporation of a fluoro-sugar into hyaluronate, which has a simple and well defined structure. With the availability of a biosynthetic system in the form of tracheal explants in organ culture (Gallagher & Kent, 1975), the present studies have been carried out to investigate the influence of fluoro-substitution in the side chain in GlcNAc on its incorporation into hyaluronate.

A preliminary communication of some of these results has been reported (Kent & Winterbourne, 1977).

Materials and Methods

Synthesis of radioactively labelled GlcNAcF

N-Fluoroacetyl-D-[1-¹⁴C]glucosamine. The method of Dwek *et al.* (1971) was adapted to prepare micro-quantities of the ring-labelled sugar.

Dicyclohexylcarbodi-imide (120mg) in pyridine (8ml) was added dropwise to a stirred solution of D-[1-¹⁴C]glucosamine hydrochloride (150 μ Ci, 0.25mmol) and an excess of sodium fluoroacetate (0.6mmol) in water (2ml). After being continuously stirred at room temperature (22°C) for 72h, the solution was diluted with 25ml of water, extracted with chloroform (25ml) and then with diethyl ether (2 $\times$ 25ml). After concentration to about 1ml, the aqueous solution was passed successively through two miniature ion-exchange columns (1.5cm $\times$ 6cm). The first contained Dowex 50W (X8; 200–400mesh, H⁺ form; Sigma Chemical Co., London SW6, U.K.) and the second Dowex 2 (X8; 200–400mesh, HCO₃⁻ form; Sigma). The eluate was concentrated and the labelled sugar was purified several times by preparative paper chromatography on Whatman 3MM with the upper phase of butanol/ethanol/water (4:1:5, by vol.) as solvent.

The product (29 μ Ci) had a specific radioactivity of 300 μ Ci/mmol (yield 32% by wt.). Of the ¹⁴C radioactivity 99.9% resided in the finally purified *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine.

N-Fluoro[³H]acetyl-D-glucosamine. Twice-recrystallized sodium fluoroacetate was tritiated by catalytic exchange in an aqueous medium at The Radiochemical Centre, Amersham, Bucks., U.K. Radioactively labelled sodium fluoroacetate, as received, had a specific radioactivity of approx. 250mCi/mmol. Of this, 8mCi was added to twice-recrystallized sodium fluoroacetate (0.6mmol) and co-recrystallized in ethanol. The resulting purified material (2mCi, 0.34mmol) was added to an excess of glucosamine hydrochloride (0.5mmol) in 2ml of water and as before dicyclohexylcarbodi-imide (120mg) in pyridine (8ml) was added, while stirring continuously.

The reaction was allowed to proceed for six days at 20°C after which the solution was extracted and deionized as before. Purification was achieved by repeated preparative paper chromatography as described above. The solution of the tritiated products was passed through a Millipore filter (0.22 μ m) to remove traces of insoluble material. The solution was then freeze-dried giving a colourless solid (215 μ Ci, 33mg yield, 39% by wt.) having a specific radioactivity of 1.5mCi/mmol. Of the ³H radioactivity 99.9% resided in the finally purified *N*-fluoro-[³H]acetyl-D-glucosamine.

N-Acetyl[³H]glucosamine. *N*-Acetyl-D-[1-³H]-glucosamine (sp. radioactivity 3–10Ci/mmol) was obtained from The Radiochemical Centre.

Animals and materials

Medium 199 and NaHCO₃ were obtained from Wellcome Reagents, Beckenham, Kent, U.K. Gentamicin reagent solution was obtained from Essex Chemie A.-G., Lucerne, Switzerland. Fungizone was obtained from Squibb and Son, New York, NY, U.S.A. and penicillin and streptomycin, which were supplied in a single preparation called Crystamycin, were from Glaxo, Greenford, Middlesex, U.K. Rabbits were supplied by Hylyne Farms, Northwich, Cheshire, U.K.

Hyaluronidase (type VI, from bovine testis) and β -glucuronidase (type B-10, from bovine liver) were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. *N*-Acetyl- β -D-glucosaminidase (from bovine kidney) was obtained from Boehringer. One unit of hyaluronidase (EC 3.2.1.35) is defined as the activity contained in 100 μ g of the international standard preparation, and 1 unit of β -glucuronidase (EC 3.2.1.31) and *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) is defined as the enzyme activity capable of hydrolysing 1 μ mol of *p*-nitrophenyl β -D-glucuronide and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide/min at 37°C respectively.

Organ culture of rabbit trachea explants

This was as described by Gallagher & Kent (1975). Male rabbits (2.5kg) were killed by intravenous injection (60mg/kg body wt. via an ear vein) of sodium pentobarbitone (Sagatal; May and Baker, Dagenham, Essex, U.K.).

The trachea was exposed and dissected immediately and placed in dissection medium (Gallagher & Kent, 1975) maintained at 37°C. Under sterile conditions, each excised trachea was cleared of any extraneous material and washed several times in warm dissection medium. The trachea was cut longitudinally into two parts and then each part was cut laterally into five further pieces giving 10 tracheal segments, each approximately 0.5cm² in size. All the segments used in each experiment were pooled. Two segments with epithelial surface facing upwards were placed on the

incised surface of each of a series of plastic Petri dishes. Culture medium (5 ml) containing known concentrations of GlcNAc and/or GlcNAcF was prepared for each dish. The sugar solutions were sterilized before addition to the culture medium by filtration through Millipore filters. Usually five Petri dishes were incubated with medium containing GlcNAcF and five for GlcNAc. Explants were maintained in an atmosphere of O_2/CO_2 (19:1, v/v) in single-strength medium 199 containing the following additions: 0.22% $NaHCO_3$, 50 μg of gentamicin/ml, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 2.5 μg of fungizone/ml, for up to 10 days, at which point the ciliary activity began to diminish.

Single-label experiments

The tracheal segments were kept for 24 h at 37°C in culture medium during which time most serum components had passed into the solution. This was replaced by fresh media containing *N*-acetyl-D-[1- ^{14}C]glucosamine (0.5–0.1 $\mu Ci/ml$) or *N*-fluoroacetyl-D-[1- ^{14}C]glucosamine (0.5 $\mu Ci/ml$) or *N*-fluoro[3H]acetyl-D-glucosamine (0.2 $\mu Ci/ml$) in the appropriate dishes. The concentration of GlcNAc or GlcNAcF in the media was 0.1 mM throughout the incubation. The medium was changed after a further 2 day interval and towards the end of the experiment exchanged for a solution containing unlabelled precursor to 'chase' labelled secretions.

Spent medium was removed by the use of a sterile Pasteur pipette. Each segment was rinsed thoroughly with the corresponding medium to remove adhering mucus. The medium collected after different incubation periods was kept separately. The gelatinous mucus material present in the samples was separated by centrifugation at 4000g for 15 min and after several washings with medium was stored at -20°C. Finally at the end of each experiment, the tracheal segments were weighed and stored at -20°C for histological investigation.

The spent media containing soluble secreted fractions were exhaustively dialysed against water containing NaN_3 (0.02%, w/v), concentrated by rotary evaporation at 35°C for further fractionation and analysis.

Double-label experiments

In experiments with *N*-acetyl[3H]glucosamine and *N*-fluoroacetyl[1- ^{14}C]glucosamine simultaneously, cultures of rabbit tracheal explants were prepared as described previously. Tissue from five female Chinchilla rabbits were pooled and eight segments were made from each trachea. After 24 h incubation, the culture medium was replaced to include *N*-acetyl[3H]glucosamine and *N*-fluoroacetyl[1- ^{14}C]glucosamine (each 0.3 $\mu Ci/ml$).

After a further 48 h incubation, the medium was replaced by fresh medium with the same isotopically

labelled metabolites and incubation continued for a further 48 h. At the termination of the experiment tissue specimens still exhibited vigorous ciliary motion. The spent media collected after 48 h and 96 h were separately freed from gelatinous mucus by centrifugation at 4000g for 15 min and washed several times with the medium. The supernatants containing soluble mucus were exhaustively dialysed against water containing NaN_3 (0.02%, w/v), and concentrated by rotary evaporation at 35°C.

Papain digestion

Freeze-dried samples of tracheal secretions (1–5 mg/ml) were suspended in 0.1 M-sodium citrate buffer (pH 5.6) containing 1 M-NaCl, 5 mM-EDTA, 1 mM-cysteine and crystalline papain (0.5 mg/ml; Sigma). Incubation was carried out at 60°C for 4 h, when further papain (0.2 mg/ml) was added and kept at 60°C (20 h). After cooling, protein was precipitated by the addition of trichloroacetic acid (90%, w/v) to a final concentration of 10% (w/v) and left at 4°C for 4 h. The precipitate was removed by centrifugation, washed with 10% trichloroacetic acid, re-centrifuged and the supernatant was pooled, dialysed against water and concentrated by rotary evaporation.

Ion-exchange chromatography

In previous experiments papain-digested samples of double-labelled tracheal secretions were fractionated on a DEAE-cellulose column (5 cm $\times$ 1.2 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 8.0. The column was eluted with an NaCl linear gradient (0 to 0.75 M) in the same buffer and 3 ml fractions were collected and ^{14}C and 3H radioactivity were counted. Separated peaks were concentrated and dialysed against water and freeze-dried.

Papain-digested samples of tracheal secretions from single-label experiments were initially dialysed against 20 mM- Na_2HPO_4/NaH_2PO_4 buffer, pH 7.2, containing 10 mM-NaCl and 6 M-urea and fractionated on a DEAE-cellulose column (5 cm $\times$ 1.2 cm) equilibrated with the same buffer. The column was first eluted with 15 ml of the above buffer and then with a linear gradient of 0.01 M- to 0.4 M-NaCl in a total volume of 100 ml made up in the same buffer. Fractions of 3 ml in volume were collected at a flow rate of 15 ml/h. Portions were taken from each fraction and the radioactivity was measured in 5 ml of NE260 scintillation fluid (Nuclear Enterprises, Edinburgh, Scotland, U.K.). Separated peaks were concentrated by rotary evaporation at 30°C and dialysed against 20 mM- Na_2HPO_4/NaH_2PO_4 buffer, pH 7.2, containing 10 mM-NaCl and 6 M-urea. The dialysed samples were fractionated again on a DEAE-cellulose column under the same conditions but using a stepwise elution. Finally, separated compo-

nents were exhaustively dialysed against water and concentrated either by rotary evaporation or by freeze-drying.

Cellulose acetate electrophoresis

Column fractions and chromatographically separated components of tracheal secretions were analysed by zonal electrophoresis on cellulose acetate strips in 0.8M-sodium barbitone/barbituric acid buffer (pH 8.6) at 250 V for 30 min. The strips were fixed in 1% (w/v) acetic acid and/or stained with periodate/Schiff reagent. Unstained strips were cut into 0.5 cm segments and counted in 5 ml of NE260 scintillation fluid.

G.l.c.

The carbohydrate composition of freeze-dried samples was analysed in this laboratory by Dr. Maria C. Dillon after methanolysis, by the method of Clamp *et al.* (1971).

Digestion of hyaluronate fractions by a mixture of glycosidases

Fractions of radioactively labelled hyaluronate purified by ion-exchange chromatography were subjected to degradation by a mixture of hyaluronidase (100 units), β -glucuronidase (200 units) and *N*-acetyl- β -D-glucosaminidase (0.2 unit) in 0.1 M-sodium acetate/acetic acid buffer, pH 5.0, containing 0.4 M- $(\text{NH}_4)_2\text{SO}_4$ and 0.15 M-NaCl at 37°C in a total volume of 0.2 ml. The amount of each glycosidase was doubled and trebled after 5 and 24 h respectively and portions of the digest were taken at each stage for chromatographic analysis. The digestion was carried out for a maximum period of 12 h. Digested samples were applied to Whatman no. 1 chromatography paper and run in pyridine/ethyl acetate/water, (2:8:1, by vol.) as solvent for 20 h at 22°C. In this solvent, GlcNAcF (R_F 0.27) and GlcNAc (R_F 0.15) were clearly separated. Strips (1 cm) were cut and counted in 5 ml of NE260 scintillation fluid.

Presentation of the incorporation data

The incorporation of a radioactive precursor into hyaluronate is expressed as a percentage of the initial total amount of radioactive precursor used or of its specific radioactivity as stated for individual experiments.

Results

Incorporation of *N*-acetylglucosamine and its fluoro-analogues into tracheal secretion

N-Acetyl-D-[1- ^{14}C]glucosamine, used as precursor, was incorporated into all four principal protein-polysaccharide products isolated from the culture fluid (Fig. 1). The use of *N*-fluoroacetyl[1- ^{14}C]glucosamine and *N*-fluoro[^3H]acetyl-D-glucosamine

Fig. 1. Ion-exchange chromatography of soluble secreted products from rabbit tracheal explants incubated for 144 h with *N*-acetyl-D-[1- ^{14}C]glucosamine

Papain-digested samples of tracheal secretion were dialysed against 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer pH 7.2, containing 10 mM-NaCl and 6 M-urea and were then fractionated on a DEAE-cellulose column (5 cm $\times$ 1.2 cm) equilibrated with the same buffer. The column was eluted first with 15 ml of the above buffer followed by a linear gradient of 0.01 M- to 0.4 M-NaCl in a total volume of 100 ml in the same buffer. The fractions (3 ml) were collected at a flow rate of 15 ml/h. Neutral glycoprotein was fractions 1-11 inclusive, acidic glycoproteins were fractions 16-29 inclusive and hyaluronate was fractions 30-34 inclusive. The sulphated glycoaminoglycans were in fractions 40-45. Symbol: $\bullet$, [NaCl].

as precursors showed no qualitative differences in the incorporation profiles when compared with that of *N*-acetyl-D-[1- ^{14}C]glucosamine. A comparison of the amounts of all three precursors present in papain-digested secreted products is shown in Table 1. It was observed that GlcNAcF-labelled products consistently required a slightly higher molar concentration of NaCl in the gradient for their displacement in DEAE-cellulose chromatography. The hyaluronate component, though quantitatively minor was nevertheless substantially labelled, 16.8% incorporation from the *N*-fluoroacetyl-D-[1- ^{14}C]glucosamine precursor compared with 10.7% from the *N*-fluoro[^3H]acetyl-D-glucosamine precursor. Double labelling experiments with *N*-fluoroacetyl-D-[1- ^{14}C]glucosamine and *N*-acetyl-D-[^3H]glucosamine in 96 h incubations showed an apparent enrichment of hyaluronate fraction in ^3H compared with ^{14}C ($^{14}\text{C}/^3\text{H}$ ratio, 0.088:1) since the $^{14}\text{C}/^3\text{H}$ ratio originally present in the culture medium was 0.117:1 (Table 2). The isotopic yields of the hyaluronate were 2.3% with respect to ^{14}C (*N*-fluoroacetylglucosamine) and 5.7% with respect to the ^3H -labelled fluorine-free compound (GlcNAc). In these experiments, both

Table 1. *Distribution of ^{14}C and ^3H radioactivity in papain-digested soluble secreted products of rabbit tracheal explants*
Pooled tissue from three animals was incubated for 240 h in medium (100 ml) containing labelled amino-sugar (single label). Tracheal secretions were exhaustively dialysed against water and freeze-dried and their papain-digested products were separated on a DEAE-cellulose column (5 cm $\times$ 1.2 cm) equilibrated with 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, containing 6 M-urea. The column was eluted with a linear gradient of 0 to 0.4 M-NaCl in the same buffer. Separated products were identified as described in the text. No measurements were made for the glycosaminoglycan fraction eluted after the NaCl gradient concentration reached 0.3 M.

Secreted products	NaCl concentration of gradient (M)		Radioactivity in chromatographically isolated products (%)		
			^{14}C -labelled GlcNAc (0.1 $\mu\text{Ci/ml}\dagger$)	^{14}C -labelled GlcNAcF (0.05 $\mu\text{Ci/ml}\dagger$)	^3H -labelled GlcNAcF (0.25 $\mu\text{Ci/ml}\dagger$)
	GlcNAc	GlcNAcF*			
Neutral glycoprotein	0–0.01	0–0.01	31.4	32.1	32.3
Acidic glycoprotein I	0.05	0.055	57.0	51.1	57.0
Acidic glycoprotein II	0.085	0.10			
Hyaluronic acid	0.19	0.22	11.6	16.8	10.7

* ^{14}C - or ^3H -labelled sugar.

$\dagger$ Initial value in medium.

Table 2. *Ratio of $^{14}\text{C}/^3\text{H}$ labelling of papain-digested secretions of rabbit tracheal explants incubated simultaneously with N -fluoroacetyl-D-[1- ^{14}C]glucosamine and N -acetyl-D-[^3H]glucosamine*

Incubations were carried out for 96 h with pooled tissues; products were papain-digested and separated on DEAE-cellulose columns as described in Table 1.

Secreted products	Effluent volume (ml)	NaCl concentration of eluting gradient (M)	$^{14}\text{C}/^3\text{H}$ in isolated product*
Neutral glycoprotein	4–17	0	0.037
Acidic glycoprotein I	23–27	0.08	0.042
Acidic glycoprotein II	28–34	0.10	0.047
Hyaluronic acid	36, 37	0.23	0.088
Glycosaminoglycan I	41–45	0.45	0.07
Glycosaminoglycan II	47–52	0.50	0.094

* The ratio $^{14}\text{C}/^3\text{H}$ in the initial culture fluid was 0.117:1. The value was 0.044:1 for the papain-digested mixed products, before DEAE-cellulose fractionation.

isotopic precursors were present in all the secreted components separated chromatographically.

In view of the proximity of fractions eluted by ion-exchange chromatography, acidic glycoprotein fractions I and II (Fig. 1, fractions 11–29 inclusive) were refractionated on DEAE-cellulose column with an NaCl gradient, but using stepwise elution. The labelled glycoproteins were quantitatively recovered and the amount of hyaluronate as a contaminant was negligible (Fig. 2a). When the hyaluronate fraction (Fig. 1, fractions 30–34) was refractionated under

similar chromatographic conditions, 95% of the label was recovered as hyaluronate (Fig. 2b). Zonal electrophoresis of this material on cellulose acetate showed a single component staining with Alcian Blue and having the same mobility as standard hyaluronate (BDH Chemicals, Poole, Dorset, U.K.). The hyaluronate fraction was clearly separated from added chondroitin sulphate on electrophoresis on cellulose acetate strips.

Extent of incorporation of GlcNAc and its fluoro-analogues

In studies on the amount of incorporation of GlcNAc and GlcNAcF into total macromolecular secreted products, it was found that 3.19% of ^{14}C radioactivity originating from N -acetyl-D-[1- ^{14}C]glucosamine was present after the first 24 h period and 2.55% after the second such period of incubation.

The validity of the incorporation was further demonstrated by subsequent sequential 'chase' experiments with non-radioactive precursor present, with a 10-fold decrease in the amount of ^{14}C radioactivity (originated from N -acetyl-D-[1- ^{14}C]glucosamine) present in the total secretions (Table 3). In simultaneous experiments, labelling with N -fluoroacetyl-D-[1- ^{14}C]glucosamine was 1.11% (1.20%, duplicate value) and 1.14% (1.53%, duplicate) for two sequential periods of incubation each 24 h. The corresponding labelling yield was 0.86% (1.24%, duplicate) for N -fluoro[^3H]acetyl-D-glucosamine. Similarly in the case of fluoroacetyl analogues the amount of ^{14}C - or ^3H -labelled material present in the secreted products was substantially diminished in 'chase' conditions, the decrease being less compared with that observed in the presence of unsubstituted GlcNAc (Table 3).

Fig. 2. Refractionation of acidic glycoprotein fractions I and II and hyaluronate from rabbit tracheal secretion on ion-exchange chromatography

Acidic glycoprotein fractions I and II and hyaluronate fractions collected from the first DEAE-cellulose column were concentrated. Acidic glycoprotein fractions were dialysed against 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, containing 6 M-urea, and hyaluronate fractions were dialysed against 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, containing 0.17 M-NaCl and 6 M-urea. (a) Acidic glycoprotein fractions I and II were fractionated on a DEAE-cellulose column (5 cm $\times$ 1.2 cm) equilibrated with 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, containing 6 M-urea. The column was eluted with a linear gradient of 0 to 0.17 M-NaCl in the same buffer and fractions (4 ml) were collected. (b) Hyaluronate was fractionated on a DEAE-cellulose column (5 cm $\times$ 1.2 cm) equilibrated with 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, containing 0.17 M-NaCl and 6 M-urea. The column was eluted with a linear gradient of 0.17 M- to 0.4 M-NaCl in the same buffer and fractions (4 ml) were collected. Symbols: $\bullet$, [NaCl]; $\times$, radioactivity.

Table 3. Labelling of total native soluble secretions of rabbit tracheal explants labelled by incubation with *N*-acetyl-[1- ^{14}C]glucosamine, or *N*-fluoroacetyl-D-[1- ^{14}C]glucosamine or *N*-fluoro[^3H]acetyl-D-glucosamine (single label experiments)

Incubations were carried out for 96 h with 40 ml of culture fluid, in the presence of various labelled precursors. The medium was then exchanged with a solution of identical molar concentration of unlabelled precursor and incubation was continued for a further 48 h. The spent media were dialysed exhaustively at 4°C before counting for radioactivity. Values are expressed as total radioactivity (nCi) and as a percentage recovery of initial radioisotope present in the media.

Incubation period (h)	Radioactive label in medium									
	Expt. 1				Expt. 2					
	^{14}C -labelled GlcNAc (825 nCi; 0.1 mM)		^{14}C -labelled GlcNAcF (972 nCi; 0.1 mM)		^{14}C -labelled GlcNAc (2.24 mCi; 0.1 mM)		^{14}C -labelled GlcNAcF (989 nCi; 0.1 mM)		^3H -labelled GlcNAcF (3.345 mCi; 0.1 mM)	
	(nCi)	(%)	(nCi)	(%)	(nCi)	(%)	(nCi)	(%)	(nCi)	(%)
	(nCi)	(%)	(nCi)	(%)	(nCi)	(%)	(nCi)	(%)	(nCi)	(%)
24-48	27.4	3.19	10.81	1.11	73.6	3.29	11.97	1.20	28.8	0.86
48-96	21.9	2.55	11.1	1.14	69.6	3.11	15.1	1.53	41.4	1.24
96-120	2.9		4.1		22.6					
120-140	2.6		3.9		9.3	4.5		10.2		

Table 4. Time course of distribution of label into native soluble secretions of rabbit tracheal explants incubated with *N*-acetyl-D-[1-¹⁴C]glucosamine or *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine

Incubations were carried out sequentially for three periods of 48h with respective labelled precursors for the first two periods as in Table 2 (Expt. 2). In the third incubation period, unlabelled medium contained identical molar concentration of unlabelled precursors. Values are percentages of total radioactivity eluted from DEAE-cellulose fractionation of the mixed components.

Incubation period (h)	Components ... Labelled precursor ...	Radioactivity (% of total radioactivity eluted)					
		Neutral glycoprotein		Acidic glycoproteins		Hyaluronate	
		¹⁴ C-labelled GlcNAc	¹⁴ C-labelled GlcNAcF	¹⁴ C-labelled GlcNAc	¹⁴ C-labelled GlcNAcF	¹⁴ C-labelled GlcNAc	¹⁴ C-labelled GlcNAcF
0-48		25.9	20.0	52.6	57.5	21.4	22.5
48-96		30.5	23.6	44.5	47.0	25.0	29.4
96-144		19.6	29.4	48.1	40.9	32.3	29.3

Table 5. ¹⁴C or ³H radioactivity in the refractionated hyaluronate component, after papain digestion, from rabbit tracheal explants incubated in the presence of *N*-acetyl-D-[¹⁴C]glucosamine, *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine or *N*-fluoro[³H]acetyl-D-glucosamine

Incubations (48h) were carried out as described in Table 4. Values are expressed as % of total radioactivity present originally in culture media.

Labelled precursor ...	Radioactivity in medium					
	¹⁴ C-labelled GlcNAc		¹⁴ C-labelled GlcNAcF		³ H-labelled GlcNAcF	
	(nCi)	(%)	(nCi)	(%)	(nCi)	(%)
Exp. 1.	2.50	0.15	3.09	0.16	—	—
Exp. 2.	5.68	0.13	3.14	0.16	5.36	0.08

Time course of distribution of labelled *N*-acetylglucosamine and its fluoro-analogues into native soluble secretions of rabbit tracheal explants

Over a period of 144h, the labelling of the neutral glycoprotein fraction (Fig. 1, fractions 1-11 inclusive) with *N*-acetyl-D-[1-¹⁴C]glucosamine decreased from 25.9% (first 48h) to 19.6% 96 to 144h period of incubation). In contrast, the uptake of *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine as precursor into this fraction increased from 20.0% to 29.4% in the corresponding periods. Among the acidic glycoprotein fractions, the rate of labelling of the major radioactive product with either *N*-acetyl-D-[1-¹⁴C]glucosamine or *N*-fluoroacetyl-D-[1-¹⁴C]glucosamine decreased in both cases over 144h (Table 4). In the same periods, the labelling of the hyaluronate increased from 21.4% (first 48h) to 32.3% (96-144h) with the ¹⁴C-labelled GlcNAc compared with an increase from 22.5% to 29.3% in the case of the ¹⁴C-labelled GlcNAcF. Further details of the labelled refractionated hyaluronate are given in Table 5.

Identification of radioactively labelled sugars in hyaluronate fractions

The purified hyaluronate (fractions 30-34, Fig. 2) containing ¹⁴C-labelled GlcNAc, ¹⁴C-labelled GlcNAcF or ³H-labelled GlcNAcF was digested by using a mixture of hyaluronidase, β -glucuronidase and *N*-acetyl- β -D-glucosaminidase. Digestion was carried out for 48, 72 and 120h and after each time interval the products were separated by paper chromatography.

Paper-chromatographic analysis of the products released by enzymic digestion (120h) of hyaluronate labelled with ¹⁴C-labelled GlcNAc showed that 45.9% of the label was released as an oligosaccharide/disaccharide fraction (R_F 0), whereas 54.1% was in the form of ¹⁴C-labelled GlcNAc (R_F 0.15). Glucuronic acid was also readily detected colorimetrically, though this was of course unlabelled. Similar analysis of hyaluronate labelled with ¹⁴C-labelled GlcNAcF indicated that 74.6% of the label was released as an oligosaccharide/disaccharide fraction, 19.5% as ¹⁴C-labelled GlcNAc (R_F 0.15) and 5.9% as ¹⁴C-labelled GlcNAcF (R_F 0.27). In similar experiments where ³H-labelled GlcNAcF had been used as a precursor, 95.4% of the label was recovered in the oligosaccharide/disaccharide fraction (R_F 0) and 4.6% as ³H-labelled GlcNAcF (R_F 0.27). Unlabelled GlcNAc was also present, which was detected colorimetrically on the chromatograms (Table 6). In further experiments more extensive chromatographic separation of the enzymically digested products indicated the presence of a disaccharide component (*N*-acetylhyalobiuronic acid) containing 3.1% as ¹⁴C-labelled GlcNAc and 2.3% as ¹⁴C-labelled GlcNAc and of a distinctive disaccharide component (believed to be *N*-fluoroacetylhyalobiuronic acid) only where GlcNAcF had been used as a precursor, and this represented 3.1% of the ¹⁴C radioactivity incorporated into hyaluro-

Table 6. Distribution of ^{14}C and ^3H in products of mixed-glycosidase digestion of purified hyaluronate fraction obtained by incubation of rabbit tracheal explants with ^{14}C -labelled GlcNAc or GlcNAcF (^{14}C - or ^3H -labelled)

Refractionated hyaluronate component, separated as described in the Materials and Methods section and in Tables 3 and 4, was incubated with a mixture of hyaluronidase, β -glucuronidase and *N*-acetyl- β -D-hexosaminidase for 48, 72 and 120 h respectively (for conditions see the Materials and Methods section.) Hydrolysis products were separated chromatographically on paper, located and counted for radioactivity. Values given for chromatographic components are radioactivity counts detected as a percentage of counts applied to the paper chromatogram.

Chromatographic component	Incubation time (h)	...	Radioactivity (% of radioactivity applied to chromatogram)							
			Labelled precursor used in incubation			...			Radioactivity (% of radioactivity applied to chromatogram)	
			^{14}C -labelled GlcNAc			^{14}C -labelled GlcNAcF			^3H -labelled GlcNAcF	
			48	72	120	48	72	120	72	120
Oligosaccharide ($R_F 0$)			50.2	38.9	45.9	63.0	66.9	74.6	88.8	95.4
GlcNAc ($R_F 0.15$)			49.8	61.1	54.1	25.1	26.5	19.5	0	0
GlcNAcF ($R_F 0.27$)			0	0	0	11.9	6.5	5.9	11.2	4.6

Table 7. Extended chromatographic separation of products of mixed glycosidase degradation of hyaluronate isotopically labelled from ^{14}C -labelled GlcNAcF or ^{14}C -labelled GlcNAc

The enzymic hydrolysis was conducted for 48 h, and the analysis was carried out as described in the Materials and Methods section. Hydrolysis products were separated chromatographically on paper by using pyridine/ethyl acetate/water (2:8:1, by vol.) as solvent. Values are expressed as a percentage of the counts applied to paper.

Products of hydrolysis	Precursor ...	Radioactivity (%)	
		^{14}C -labelled GlcNAcF	^{14}C -labelled GlcNAc
Oligosaccharide ($R_F 0$)		59.3	46.0
Acetylhyaluronate		10.3	2.3
Fluoroacetylhyaluronate		3.1	—
GlcNAc		21.2	51.4
GlcNAcF		5.9	—

nate from ^{14}C -labelled GlcNAcF. The complete analysis is given in Table 7.

Discussion

The identification of the hyaluronate fraction, obtained from the tracheal explants in the organ culture by its chemical analysis and its susceptibility to the action of hyaluronidase has been previously described (Gallagher & Kent, 1975). These studies indicated that the hyaluronate fraction was readily labelled with D-[1- ^{14}C]glucosamine as precursor. The present work demonstrated that although

N-acetyl-D-[1- ^{14}C]glucosamine acted as a precursor in the biosynthesis of hyaluronate, the extent of its incorporation was less compared with that of D-[1- ^{14}C]glucosamine (Gallagher & Kent, 1975). The hyaluronate fraction, purified from tracheal secretion after incubation of the explants with *N*-acetyl-D-[1- ^{14}C]glucosamine as precursor, moved as a single zone on cellulose acetate electrophoresis, and acted as a substrate for hyaluronidase. The refractionated material on DEAE-cellulose chromatography had a specific radioactivity of 90 $\mu\text{Ci}/\text{mmol}$ of uronic acid when labelled with *N*-acetyl-D-[1- ^{14}C]glucosamine (8.28 μCi being originally present in 100 ml of culture medium). Incubation with a mixture of glycosidases (hyaluronidase, β -glucuronidase and *N*-acetyl- β -hexosaminidase) released 54 % of the labelled GlcNAc as free sugar, whereas the rest of the radioactivity remained in the oligomeric products. Similar observations on the hydrolysis of hyaluronate by these glycosidases had been made by Hayashi (1977). Despite extensive refractionation, monosaccharide analysis on g.l.c. showed that other sugars, notably galactose and mannose, were present in the tracheal hyaluronate preparation either arising from some impurity or as a part of some more complex structure with which hyaluronate is associated.

Labelling of the hyaluronate fraction with D-[1- ^{14}C]galactose has been reported previously (Gallagher & Kent, 1975). Some recent exploratory double-labelling experiments (performed by Ms. Caroline Anderson in this laboratory), however, showed that the hyaluronate component could be labelled with both D-[1- ^{14}C]glucosamine and L-[U- ^3H]leucine, but not with L-[^3H]fucose and $^{35}\text{SO}_4^{2-}$, when both the last two compounds were present simultaneously. The possibility exists therefore that the hyaluronate so obtained may indeed exist in some complex form with protein and the matter remains to be investigated. The results are similar to those of Stoolmiller & Dorfman (1970) in which hyaluronate from culture

fluids of Group A streptococci (papain-digested, chromatographically separated and purified by precipitation with cetylpyridinium chloride) contained about 2 μ mol of amino acids, including leucine, per 1000 μ mol of glucosamine. Unlike chondroitin sulphate, the biosynthesis of hyaluronate is not apparently sensitive to puromycin or chloramphenicol and thus is not thought to require essential peptide chains (Stoolmiller & Dorfman, 1970).

Detailed comparison of the effect of fluorine present as the fluoroacetyl side group attached to glucosamine was made possible by the synthesis (Dwek *et al.*, 1971) of *N*-fluoroacetyl-D-[1- 14 C]-glucosamine and *N*-fluoro[3 H]acetyl-D-glucosamine in highly purified form. Both fluoro-analogues provided metabolic sources of labelled GlcNAc for all the soluble secreted protein-polysaccharide com-

plexes, and hyaluronate, synthesized by rabbit tracheal explants. The possible metabolic pathway involved in the incorporation of the fluoro-analogues is summarized in Scheme 1. This Scheme, at present, is hypothetical and requires further data on the comparative kinetics of incorporation of GlcNAc and GlcNAcF into hyaluronate and on the effect of GlcNAcF on the incorporation of GlcNAc into hyaluronate, and vice versa, to clearly understand the competition between the two sugars during their incorporation into hyaluronate. The data on incorporation indicate that either by transport or by enzymic specificity, GlcNAc was utilized to a greater extent than the fluoro-analogues (Tables 2 and 3) and it was considered that identical initial enzymes were involved (Scheme 1, steps 1, 2 and 3). In attempts to increase the extent of labelling by prolonged incubation of tissue with these metabolites, it was found that enhanced labelling of hyaluronate resulted with both the GlcNAc and its fluoro-analogues up to 144 h of incubation with a concurrent decrease in the incorporation of the labelled material into the accompanying neutral and acidic secreted-glycoprotein fractions. The striking differences in the hyaluronate labelling arising from 14 C- and 3 H-labelled GlcNAcF (Table 5) indicated strongly the possibility of partial loss or replacement of the side-chain acyl group (Tables 2 and 5) at any of the sugar phosphate intermediary states (Scheme 1, step 4). Further evidence on this point was obtained by study of the products of hydrolysis of the hyaluronates labelled with the two labelled fluoro-sugars followed by the action of the mixture of glycosidases. With 14 C-labelled GlcNAcF as a metabolite, the action of mixed glycosidases (for 48 h) as before resulted in recovery of 11.9% of the radioactivity in the form of free fluoro-sugar separated and identified chromatographically. This value decreased to 5.9% on prolonged glycosidase action with a corresponding increase in the labelling of the oligomeric component, thus clearly indicating the occurrence of some transglycosidation reaction. In addition, however, 25.1% of the 14 C radioactivity was present as GlcNAc, showing that some side-chain displacement must have occurred. The remaining radioactive isotope (63.0%) was present in the oligomeric material, substantially greater than that found (50%) for fluorine-free hyaluronate. Similarly with 3 H-labelled metabolite, 11.2% of the radioactivity could be recovered after mixed-glycosidase degradation (for 24 h) and chromatographically identified as the GlcNAcF (Table 6). The major part of the 3 H radioactivity remained in oligomeric products, and somewhat more extensive chromatography (Table 7) enabled comparison to be made of the glycosidase-degradation products.

The presence of fluoroacetylglucosaminyl residues in hyaluronate, which the evidence established, also appeared to lead to greater stability towards hyalur-

Scheme 1. Competitive metabolism of GlcNAc and GlcNAcF in hyaluronate biosynthesis

Abbreviations: GlcNAc 6P, *N*-acetylglucosamine 6-phosphate; GlcNAc 1P, *N*-acetylglucosamine 1-phosphate; UDP-GlcNAc, uridine 5'-diphosphate *N*-acetylglucosamine; UDP-GlcA, uridine 5'-diphosphate glucuronic acid. GlcNAcF 6P, GlcNAcF 1P and UDP-GlcNAcF are the corresponding fluoro-analogues of GlcNAc 6P, GlcNAc 1P and UDP-GlcNAc respectively.

onidase degradation, compared with fluorine-free hyaluronate. This was further supported by the considerably greater yield under given conditions of enzymic hydrolysis of GlcNAc from normal hyaluronate than from that in which fluoroacetylglucosaminyl residues were present. Yamamoto (1973) showed that *p*-nitrophenyl 2-deoxy-2-fluoroacetamido- β -D-glycopyranoside was hydrolysed by purified *N*-acetyl- β -hexosaminidase at 65% of the rate found for the unsubstituted derivative, further supporting the constraining effect exerted by fluorine in this structural position on the enzymic hydrolysis of the substituted glycoside.

It is tempting to suggest that the accumulation of labelled GlcNAcF residues in the glycosidase-resistant oligosaccharide arises from enhanced resistance to hydrolysis of bonds between fluoroacetylhyalobiuronyl sequences in the polymer, compared with more susceptible fluorine-free regions. In a review, Muir & Hardingham (1975) draw attention to the powerful structural effect that the acetamido groups of hyaluronate possess and it is reasonable to suppose that introductions of fluorine atoms at such points will markedly enhance the bonding capacity of sites.

It is concluded that GlcNAcF is utilized by tracheal cells in a manner closely similar to GlcNAc, that it is converted into the corresponding UDP-nucleotide precursor (Schultz & Mora, 1975) and thence is incorporated into hyaluronate from which it can be recovered by enzymic degradation. The latter step is considered to be essential to the argument, especially in view of the metabolic transformations that the fluoro-analogue may undergo. In the course of these reactions, some enzymic de-*N*-acylation must be inferred, fluoroacetyl substituents being replaced by acetyl groups.

Since the primary aim of the present study was to see whether or not GlcNAcF was incorporated into hyaluronate, the effects of these fluoro-sugars on the general metabolism of the tracheal epithelial cells were not investigated. Although information on the protein-synthetic activity of the tracheal epithelial cells in the presence and absence of fluoro-sugars would have been of advantage in the analysis of the incorporation of the two sugars, it did not fall within the scope of the present investigation. However, the present findings that GlcNAcF was incorporated into hyaluronate would indicate that these fluoro-sugars did not have a deleterious effect on at least some of the biosynthetic pathways of the cells. Secondly the light-microscopic observations that the epithelium of the tracheal explants incubated with GlcNAcF for 96h still exhibited vigorous ciliary

motion would suggest that the presence of fluoro-sugars did not affect the general physiological behaviour of the cells.

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References

- Barnett, J. E. G. (1972) *Carbon-Fluorine Compounds; CIBA Found. Symp.* pp. 95–110, Elsevier/Excerpta Medica, Amsterdam
- Bernacki, R. J., Sharma, M., Porter, N. K., Rustum, Y., Paul, B. & Korytnyk, W. (1977) *J. Supramol. Struct.* **7**, 235–250
- Butchard, C. G., Dwek, R. A., Kent, P. W., Williams, R. J. P. & Xavier A. V. (1972) *Eur. J. Biochem.* **27**, 548–553
- Clamp, J. R., Bhattie, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–234
- Daniels, P. F. (1972) Ph.D. thesis, Oxford University
- Dwek, R. A., Kent, P. W. & Xavier, A. V. (1971) *Eur. J. Biochem.* **23**, 343–348
- Foster, A. B. & Westwood, J. H. (1973) *Pure Appl. Chem.* **35**, 147–168
- Gallagher, J. T. & Kent, P. W. (1975) *Biochem. J.* **148**, 187–196
- Hayashi (1977) *J. Biochem. (Tokyo)* **82**, 1287–1295
- Kent, P. W. (1972) *Carbon-Fluorine Compounds; CIBA Found. Symp.* pp. 169–213, Elsevier/Excerpta Medica, Amsterdam
- Kent, P. W. & Mora, P. T. (1973) in *Membrane Mediated Information* (Kent, P. W., ed.), vol. 1. pp. 129–137, Medical & Technical Publishers, Lancaster
- Kent, P. W. & Winterbourne, D. J. (1977) *Biochem. Soc. Trans.* **5**, 439–440
- Kent, P. W. & Wright, J. R. (1972) *Carbohydr. Res.* **22**, 193–200
- Muir, H. M. & Hardingham T. E. (1975) in *Biochemistry of Carbohydrates* (Whelan, W. J., ed.), pp. 153–222, Butterworths, London
- Schmidt, M. F. G., Schwarz, R. T. & Ludwig, H. (1976) *J. Virol.* **18**, 819–823
- Schmidt, M. F. G., Biely, P., Kraty, Z. & Schwarz, R. T. (1978) *Eur. J. Biochem.* **87**, 55–68
- Schultz, A. M. & Mora, P. T. (1975) *Carbohydr. Res.* **40**, 119–127
- Stoolmiller, A. C. & Dorfman, A. (1970) *Exposés Annuels de Biochimie Medicale* ser **30**, 65–78.
- Taylor, N. F. (1972) *Carbon-Fluorine Compounds; CIBA Found. Symp.* pp. 215–235, Elsevier/Excerpta Medica, Amsterdam
- Winterbourne, D. J., Butchard, G. C. & Kent, P. W. (1979) *Biochem. Biophys. Res. Commun.* **87**, 989–992
- Yamamoto, K. (1973) *J. Biochem. (Tokyo)* **73**, 749–753