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Biosynthesis and Release of Glycoproteins by Human Skin Fibroblasts in Culture

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1. Confluent human skin fibroblasts maintained in a chemically defined medium incorporate L-[1-^{3}H]fucose in a linear manner with time into non-diffusible macromolecules for up to 48h. Chromatographic analysis demonstrated that virtually all the macromolecule-associated ^{3}H was present as [^{3}H]fucose. 2. Equilibrium CsCl-density-gradient centrifugation established that [^{3}H]fucose-labelled macromolecules released into the medium were predominantly glycoproteins. Confirmation of this finding was provided by molecular-size analyses of the [^{3}H]fucose-labelled material before and after trypsin digestion. 3. The [^{3}H]fucose-labelled glycoproteins released into fibroblast culture medium were analysed by gel-filtration chromatography and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. These techniques demonstrated that the major fucosylated glycoprotein had an apparent mol.wt. of 230000–250000; several minor labelled species were also detected. 4. Dual-labelling experiments with [^{3}H]fucose and ^{14}C-labelled amino acids indicated that the major fucosylated glycoprotein was synthesized *de novo* by cultured fibroblasts. The non-collagenous nature of this glycoprotein was established by three independent methods. 5. Gel-filtration analysis before and after reduction with dithiothreitol showed that the major glycoprotein occurs as a disulphide-bonded dimer when analysed under denaturing conditions. Further experiments demonstrated that this glycoprotein was the predominant labelled species released into the medium when fibroblasts were incubated with [^{35}S]cysteine. 6. The relationship between the major fucosylated glycoprotein and a glycoprotein, or group of glycoproteins, variously known as fibronectin, LETS protein, cell-surface protein etc., is discussed.

It has been recognized for several years that connective tissues contain glycoproteins as well as collagen, elastin and the proteoglycan complexes. Some of these glycoproteins are readily extracted with dilute salt solutions and belong to the extravascular pool of plasma proteins (Anderson, 1976). Other glycoproteins, such as the link protein in proteoglycan aggregates from cartilage (Hascall & Sajdera, 1969) and the sialoprotein of bone (Herring, 1976), are more tightly bound to the insoluble matrix and are extracted only under severe chemical conditions or after enzymic removal of collagen. Another class of less well-defined glycoproteins derived from connective tissues has been designated as structural glycoproteins (Robert *et al.*, 1976), but the extraction methods used have often yielded heterogeneous preparations of denatured and partially degraded molecules (for review see Anderson, 1976).

Studies on the synthesis of glycoproteins by connective-tissue cells in culture offers an alternative approach to the isolation and characterization of glycoprotein constituents of the extracellular matrix. Human skin fibroblasts retain many of their specialized biosynthetic function *in vitro*, e.g. they synthesize the dermal collagens (Lichtenstein *et al.*, 1975) and complex polysaccharides (Matalon & Dorfman, 1969; Kleinman *et al.*, 1975), which can be recovered from the culture medium. However, there have been few systematic investigations of the non-collagenous glycoproteins released into fibroblast culture medium, although considerable interest has centred on glycoproteins present on the surface of fibroblast cell layers (for review see Hynes, 1976). In the present study human skin fibroblasts cultured under chemically defined conditions have been used to study the biosynthesis and release of glycoproteins labelled with [^{3}H]fucose. Some of the properties of the major fucosylated glycoprotein released into the medium are reported and the relationship of this component to cell-surface glycoproteins is discussed. Preliminary reports of part of this work have been presented (Sear *et al.*, 1975, 1976).

Experimental

Materials

Dulbecco's modification of Eagle's Minimum Essential Medium, the completely chemically defined medium MAB 87/3 (Gorham & Waymouth, 1965), Hanks balanced salts solution and foetal bovine serum were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland. L-[1-^{3}H]Fucose (3.3 Ci/mmol), L-[^{35}S]cysteine hydrochloride (60 mCi/mmol), U-^{14}C-labelled amino acid mixture (10 mCi/mmol) and ^3H_2O (5 Ci/ml) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Trypsin (grade TRL) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and highly purified bacterial collagenase was a generous gift from Dr. F. V. Lee-Own of this department. Acrylamide, *NN'*-methylenebisacrylamide, Bio-Gel P-30 and Bio-Gel A-5m were obtained from Bio-Rad Laboratories, Bromley, Kent, U.K., and Sepharose CL-2B and Blue Dextran 2000 were from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. The purified preparation of bovine tendon proteoglycan was a generous gift from Dr. J. C. Anderson of this department, and the human immunoglobulin preparation was kindly provided by Dr. P. Brenchley of the Department of Immunology, Medical School, University of Manchester, U.K. Myosin was prepared from rabbit skeletal muscle by the method of Weeds & Hartley (1968), and NCS Tissue Solubilizer (Amersham/Searle) was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. *N*-Ethylmaleimide, phenylmethanesulphonyl fluoride and dithiothreitol were purchased from Sigma (London) Chemical Co., London S.W.6, U.K., and SDS* (specially purified) from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K. All other chemicals were of AnalaR grade whenever available.

Cell culture

Fibroblasts were grown from human skin biopsy material by using the explant technique, and cultured in plastic flasks in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) foetal bovine serum (hereafter referred to as growth medium). Confluent cultures were subcultured at a split ratio of 1:4 by using 0.025% (w/v) trypsin in Hanks balanced salt solution buffered with 25 mM-Hepes at pH 7.7. The cultures grew in characteristic fashion, exhibiting a doubling time during exponential phase of approx. 24 h, and at confluency contained approx. 3×10^6 cells per flask of growth area 75 cm^2. The protein content of the cell layer at confluency was 0.43 ± 0.02 mg/10^6 cells (mean$\pm$S.E.M. of 13 determinations), as determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Fibroblast stocks were stored frozen in liquid N_2 and experiments were performed only on cells that were four to ten subcultures removed from the primary culture.

Unless otherwise stated, the experiments described in this study used cell line HF3, which was derived from a 33-year-old male. Chromosome analysis carried out in the Department of Medical Genetics, Chromosome Laboratory, St. Mary's Hospital, Manchester, U.K., indicated that this cell line was diploid, and the absence of mycoplasma contamination was confirmed by tests performed by Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.

Although serum-containing culture media are obligatory for the speedy attainment of confluency, their continued use for the maintenance of confluent fibroblasts presents considerable difficulties when newly synthesized extracellular glycoproteins are to be studied. The main problem is due to the presence of serum proteins in vast excess of any macromolecules released from the cells, thus making the isolation and chemical characterization of such molecules extremely difficult. There is also the possibility of 'non-enzymic tight binding' of radioactively labelled precursor molecules to serum components (Herrmann, 1974), thereby producing labelled macromolecules that have not been synthesized by the cells. Another problem concerns the enzyme content of serum. Degradative enzymes (presumably of lysosomal origin) have been detected in serum (Eriksson *et al.*, 1972) and might modify the structures of extracellular macromolecules. There is also some evidence that serum may induce or activate the cellular production and/or secretion of proteinases (Bankowski & Mitchell, 1973), although the presence of α_2-macroglobulin and other serum proteinase inhibitors might be expected to compensate for this.

To avoid these difficulties some workers have used serum-depleted growth medium (Layman *et al.*, 1971; Goldberg *et al.*, 1972; Dell'Orco & Nash, 1973). However, when human dermal fibroblasts were maintained for up to 3 days in growth medium devoid of serum, or containing only 0.5% (v/v) serum, there was a marked decrease in cellular stability as evidenced by appreciable cell detachment from the substratum and loss of cellular protein (Sear *et al.*, 1975). Similar observations have been reported by Dell'Orco *et al.* (1973), but we have shown that it is possible to minimize these problems by maintaining the confluent cells in Waymouth's medium MAB 87/3 (Sear *et al.*, 1975). All the present experiments were carried out in this completely chemically defined medium, hereafter referred to as 'maintenance medium'.

Incubation of cells

Confluent cultures (75 cm^2) were washed free of growth medium with Hanks balanced salt solution

* Abbreviations: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

(3×5ml), which was then replaced with maintenance medium (10ml). After a preincubation period of 12h at 37°C the medium was again replaced with fresh maintenance medium (10ml), which was supplemented with L-[1-^{3}H]fucose (10μCi/ml) or, in some cases, with one of the other labelled compounds mentioned under 'Materials'. In experiments on the biosynthesis of collagenous molecules, the maintenance medium was also supplemented with L-ascorbic acid (50μg/ml) and β-aminopropionitrile fumarate (50μg/ml). Incubation periods lasted for up to 48h, after which the culture medium containing labelled macromolecules was decanted, centrifuged at 1200g for 10min to sediment any detached cells, and then dialysed against running tap water for 12–16h. During the characterization of the [^{3}H]fucose-labelled macromolecules released into the medium the need to add proteinase inhibitors was recognized, and in subsequent experiments *N*-ethylmaleimide (10mM), phenylmethanesulphonyl fluoride (2mM) and EDTA (tetrasodium salt; 25mM) were added as a routine to the centrifuged medium to the final concentrations indicated in parentheses.

Incorporation of [^{3}H]fucose into macromolecules

In experiments to investigate the nature of the macromolecule-associated ^{3}H after incubation of fibroblasts in the presence of L-[1-^{3}H]fucose for 24h, the culture medium was harvested, dialysed and freeze-dried. Samples were hydrolysed *in vacuo* in 0.3M-HCl at 110°C for 16h, freed of peptide material by passage through a column (1.1cm×6cm) of Dowex 50W (X8), deacidified on a column (1.1cm×6cm) of Amberlite IR-4B and analysed by paper chromatography in the solvent system ethyl acetate/pyridine/water (12:5:4, by vol.) or phenol/water (4:1, w/w). The overall recovery of ^{3}H during such analyses was 80%, of which 90–95% co-chromatographed with authentic L-fucose. Virtually identical results were obtained when the [^{3}H]fucose-labelled macromolecules from the cell layer were hydrolysed and chromatographed.

Equilibrium density-gradient ultracentrifugation

To confirm the glycoprotein nature of the [^{3}H]-fucose-labelled macromolecules released by cultured fibroblasts, samples of dialysed medium were analysed by CsCl-equilibrium-density-gradient centrifugation.

Experiments performed under denaturing conditions used aqueous mixtures (30ml) containing the radioactive sample (approx. 45000d.p.m.), foetal bovine serum (approx. 15mg) as glycoprotein/protein carrier, bovine tendon proteoglycan (2mg), guanidinium chloride to a final concentration of 4M and sufficient CsCl to give an initial density of approx. 1.4g/ml. The samples were centrifuged in an MSE Superspeed 65 preparative ultracentrifuge in angle rotors 59592 or 59594 for 65–88h at 20°C and 112000g ($r_{av.}$ 6.5cm). After centrifugation, the gradient was divided into eight or ten fractions, the densities of which were determined by weighing. The fractions were dialysed against running tap water for 12–16h and then analysed for protein content by measurements of A_{230}, and for hexuronic acid content by the method of Bitter & Muir (1962). The radioactivity in the fractions was determined by counting samples (1ml) in 10ml of scintillation fluid comprising 2vol. of toluene containing 2,5-diphenyloxazole (5g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/l), and 1vol. of Triton X-100.

Experiments under non-denaturing conditions were carried out as described above except that human immunoglobulin G (approx. 1mg) was used as glycoprotein carrier, the proteoglycan and guanidinium chloride were omitted, and the initial density was approx. 1.3g/ml.

Gel filtration of medium macromolecules on SDS/agarose

Gel-filtration chromatography was performed at room temperature (22°C) on columns (1.5cm×90cm) of 6% agarose (Bio-Gel A-5m) or cross-linked 2% agarose (Sepharose CL-2B). Descending elution was carried out at approx. 4ml/h by using 0.2M-Tris/HCl buffer, pH7.4, containing 0.1% SDS and 0.3% NaN_3. Fractions (approx. 2.4ml) were collected and samples taken for measurement of radioactivity in the toluene/Triton X-100 scintillation mixture described above.

Reduced and alkylated samples were prepared for chromatography in 50mM-Hepes buffer, pH7.4, containing 1% (w/v) SDS and 50mM-dithiothreitol, and were incubated at 100°C for 2min and then at 37°C for 2h. Alkylation was performed at room temperature for 30min with iodoacetamide (100mM) and the pH maintained at approx. 8 with phenolphthalein as internal indicator. After dialysis against a vast excess of column-elution buffer for 2h and removal of any insoluble material by centrifugation, the sample was applied to the column.

Unreduced samples were treated in a similar manner except that the dithiothreitol was omitted from the initial treatment with SDS, and iodoacetamide (100mM) was included to prevent the non-specific reduction of disulphide bonds that occurs with some samples of SDS (Parkhouse, 1971).

Columns were calibrated for molecular-weight determinations by preparing samples that included purified proteins (2–5mg) and/or isotopically labelled standards of known molecular weight, i.e. standardization was internal. Blue Dextran 2000 was used to determine the void volume (V_0) and ^3H_2O (approx. 0.05μCi) was used to determine the total column volume (V_t). The recovery of radioactivity from the SDS/agarose columns was consistently greater than 90%.

SDS/polyacrylamide-gel electrophoresis

Macromolecules released into fibroblast maintenance medium were prepared for electrophoretic analysis by precipitation with $(NH_4)_2SO_4$ at 75% saturation in the presence of proteinase inhibitors at the concentrations specified above. In some experiments where predominantly high-molecular-weight proteins were analysed, precipitation with $(NH_4)_2SO_4$ at 30% saturation was used.

Polyacrylamide-gel electrophoresis was carried out under reducing conditions in the presence of 0.1% SDS as described by Laemmli (1970), but with the addition of urea (2M) in the sample-preparation buffer. Residual $(NH_4)_2SO_4$ was removed from the samples by dialysis against sample-preparation buffer. Polyacrylamide gels were prepared in glass tubes of internal dimensions 0.6cm×12cm, and consisted of a separating gel (10cm) containing 8% or 5% (w/v) polyacrylamide and a stacking gel (1cm) containing 3% (w/v) polyacrylamide. Electrophoresis was carried out at room temperature and at 1.5mA/gel. Gels were stained with Coomassie Brilliant Blue R or the periodic acid/Schiff reagents by the methods of Fairbanks *et al.* (1971). Molecular-weight estimations were made by the method of Weber & Osborn (1969). The localization of radioactive glycoproteins was determined by slicing the frozen gel with a parallel array of razor blades (providing slices 1.15mm thick), followed by digestion of each slice in a closed scintillation vial containing 0.5ml of NCS/water (9:1, by vol.) for 16h at room temperature. Scintillation fluid [2,5-diphenyloxazole (5g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/l) dissolved in toluene] (10ml) was then added to each vial and radioactivity measured after a further 4-day incubation at room temperature.

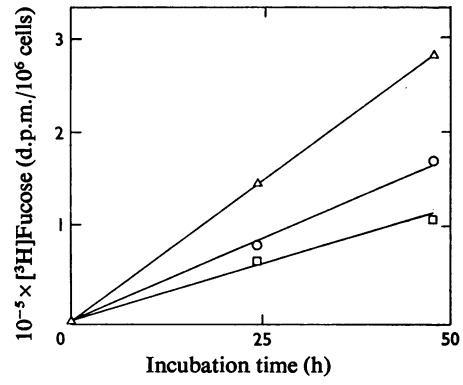

Fig. 1. *Incorporation of* L-[1-^{3}*H*]*fucose into macromolecules synthesized by cultured human skin fibroblasts*

Replicate confluent cultures containing approx. 10^6 cells per flask (25cm^2) were incubated for up to 48h with L-[1-^{3}H]fucose (10μCi) in maintenance medium (3ml). At daily intervals duplicate cultures were harvested and the medium was dialysed against a vast excess of water at 4°C. The non-diffusible [^{3}H]fucose-labelled macromolecules in the medium were measured by liquid-scintillation spectrometry. Cells were enumerated after trypsin treatment, sedimented and the pellet was dialysed as above. The [^{3}H]fucose-labelled glycopeptides present in the trypsin digest were recovered by ion-exchange chromatography [Dowex 50W (X8; H^+ form)], combined with the dialysed cells and freeze-dried. The radioactivity in this cell-layer fraction was determined after digestion in NCS/water (9:1, v/v). Each point represents the mean of two determinations. △, Total [^{3}H]fucose-labelled macromolecules; ○, [^{3}H]fucose-labelled macromolecules present in the medium; □, [^{3}H]-fucose-labelled macromolecules associated with the cell layer.

Results

Incorporation of [^{3}*H*]*fucose into glycoproteins*

When confluent human skin fibroblasts were maintained in medium MAB 87/3 the incorporation of [^{3}H]fucose into non-diffusible macromolecules was linear with time over a 48h period (Fig. 1). Almost identical results were obtained with six different cell lines, and of the total [^{3}H]fucose incorporated 40–48% was in cell-layer material and 52–60% in macromolecules released into the maintenance medium (Table 1). The mean total incorporation of [^{3}H]fucose was 14×10^4d.p.m./24h per 10^6 cells, which represents approx. 0.6% of the exogenously supplied fucose. Chromatographic analysis of acid hydrolysates of the newly fucosylated macromolecules indicated that virtually all the ^{3}H recovered was present as [^{3}H]fucose.

The nature of the [^{3}H]fucose-labelled macromolecules released into the maintenance medium was investigated by CsCl-equilibrium-density-gradient centrifugation. Under denaturing conditions, which permitted the separation of proteins from proteoglycans (Fig. 2*a*), approx. 90% of the radioactivity was located with protein carrier in the uppermost fractions of density below 1.34g/ml. These results suggested that the [^{3}H]fucose had been incorporated predominantly into proteinaceous macromolecules, i.e. glycoproteins rather than proteoglycans. In further experiments to determine whether the released material also contained [^{3}H]fucose-labelled glycolipids or lipoproteins, the macromolecules in the medium were analysed under conditions designed to separate lipid-containing species (of buoyant density <1.20g/ml) from proteins. Fig. 2(*b*) demonstrates that the [^{3}H]fucose-labelled sample sedimented as a single peak of buoyant density 1.32g/ml, which is

within the range of densities (1.25–1.35 g/ml) exhibited by proteins in general (Anderson, 1976). A negligible amount of radioactivity was located in the uppermost fractions of density less than 1.25 g/ml. The carrier glycoprotein, human immunoglobulin G, sedimented at a buoyant density of 1.29–1.30 g/ml.

Further evidence for the glycoprotein nature of the fucosylated macromolecules was obtained by studying their susceptibility to trypsin. Predominantly proteinaceous molecules are degraded by trypsin to relatively small peptides and glycopeptides, whereas complex polysaccharides are either not digested (e.g. hyaluronic acid) or are decreased in size to relatively high-molecular-weight peptidoglycans (Mathews, 1971; Heinegård & Hascall, 1974). When undigested [^{3}H]fucose-labelled material was chromatographed on Bio-Gel P-30 in the presence of 0.1% SDS, 85–90% of the radioactivity was eluted in a symmetrical peak at the void volume of the column. After trypsin digestion, only 4–5% of the ^{3}H was eluted at the void volume, and the remaining radioactivity was recovered in fractions corresponding to an apparent mol.wt. of 20000 or less (Fig. 3).

Table 1. *Incorporation of [^{3}H]fucose into macromolecules synthesized by six human skin-fibroblast cell lines*

Replicate confluent cultures containing approx. 10^6 cells per flask (25 cm^2) were incubated for up to 48 h with [^{3}H]fucose (10 μCi) in maintenance medium (3 ml). At daily intervals duplicate cultures were harvested and the [^{3}H]fucose-labelled macromolecules released into the medium, and those associated with the cell layer, were determined as described in the legend to Fig. 1. Cell lines established in this laboratory were derived from skin biopsy specimens of a female aged 60 years (HF1), a male aged 33 years (HF3), two males each aged 2 years (HF11 and HF12), and a male aged 4½ years (HF16). Cell line F7000 was obtained from Flow Laboratories.

Fibroblast cell line	10^{-4} × Non-diffusible [^{3}H]fucose-labelled macromolecules (d.p.m./24 h per 10^6 cells)		
	Cell layer	Medium	Total
HF1	6.7	10.0	16.7
HF3	6.3	8.2	14.5
HF11	5.6	8.2	13.8
HF12	5.6	6.1	11.7
HF16	5.7	8.2	13.9
F7000	6.3	6.8	13.1

Molecular-size analysis of [^{3}H]fucose-labelled glycoproteins

During preliminary experiments in which [^{3}H]-fucose-labelled medium macromolecules were denatured in SDS, reduced, alkylated and chromatography on SDS/agarose, the elution profile shown in Fig. 4(*a*) was frequently obtained. However, the occasional appearance of a high-molecular-weight peak of radioactivity suggested that some degradation of fucosylated macromolecules might be occurring. When proteinase inhibitors were incorporated in the processing of the maintenance medium, as described in the Experimental section, the elution profile shown in Fig. 4(*b*) was obtained. The most prominent peak (R1), representing approx. 30–50% of the recovered radioactivity, was eluted with an apparent mol.wt. of 250000. Five other peaks of radioactivity (R2–R6), having apparent mol.wts. of approx.

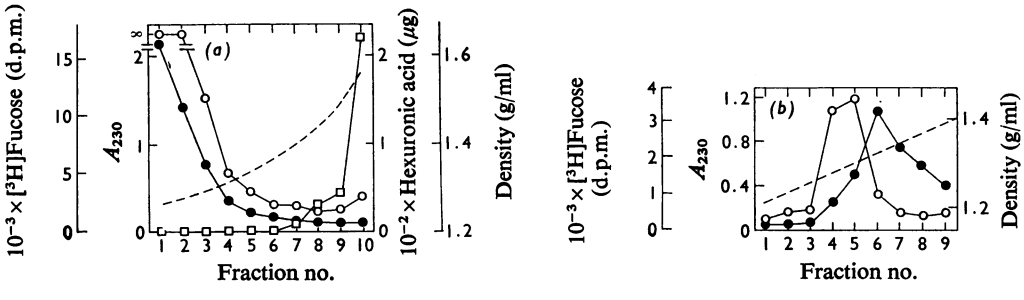

Fig. 2. *Equilibrium CsCl-density-gradient centrifugation of extracellular [^{3}H]fucose-labelled macromolecules in (a) the presence and (b) the absence of* 4M-*guanidinium chloride*

Non-diffusible [^{3}H]fucose-labelled macromolecules released into the medium by confluent human skin fibroblasts during a 24 h incubation period were prepared and analysed in the ultracentrifuge as described in the text. When analysed under denaturing conditions the sample had an initial density of 1.39 g/ml and included serum proteins and tendon proteoglycans as carrier macromolecules. In the absence of guanidinium chloride the sample had an initial density of 1.30 g/ml, and human immunoglobulin G was included as a glycoprotein carrier. After centrifugation the fractions were analysed for radioactivity (●), protein content as determined by A_{230} (○), hexuronic acid content (□) and density (----).

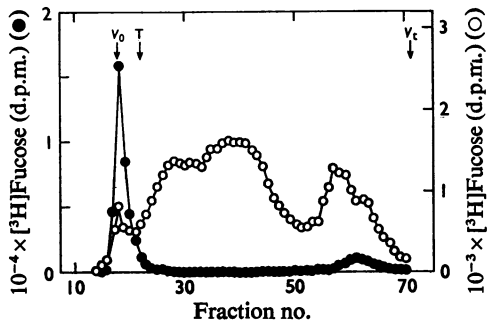

Fig. 3. *Gel-filtration chromatography of extracellular [^{3}H]fucose-labelled macromolecules before and after digestion with trypsin*

[^{3}H]Fucose-labelled macromolecules released into fibroblast maintenance medium were digested with trypsin for 24h at 37°C in 0.05M-Tris/HCl buffer, pH8.0 (enzyme/substrate ratio approx. 1:40, w/w). After incubation digests were made 1% (w/v) in SDS and 5% (v/v) in 2-mercaptoethanol and incubated at 100°C for 2min. Samples were then chromatographed on a column (1.6cm×60cm) of Bio-Gel P-30 equilibrated with 0.02M-Tris/HCl buffer, pH7.4, containing 0.1% SDS and 0.02% NaN_3. Fractions (1.6ml) were collected and portions taken for the measurement of radioactivity. An undigested sample of [^{3}H]fucose-labelled macromolecules was also chromatographed. The elution positions of Blue Dextran 2000, trypsin and ^3H_2O are indicated by V_0, T and V_t respectively. ●, Undigested sample; ○, trypsin-digested sample.

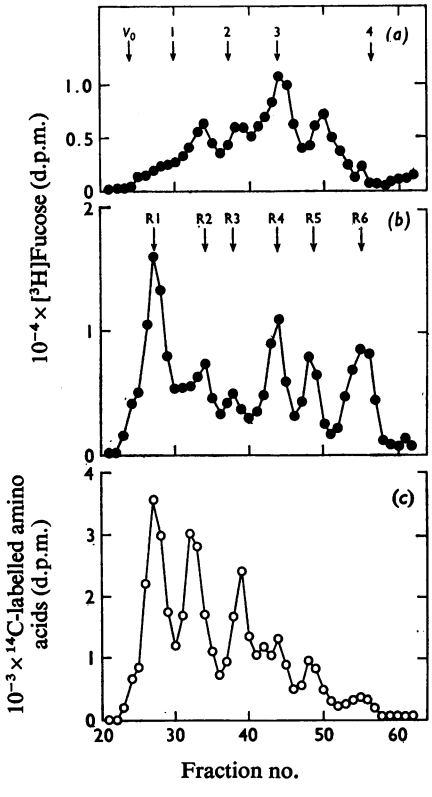

Fig. 4. *Gel filtration on SDS/agarose (Bio-Gel A-5m) of newly synthesized glycoproteins released into fibroblast maintenance medium*

Confluent fibroblast cultures (75cm^2) were labelled for 24h with [^{3}H]fucose (10μCi/ml) or ^{14}C-labelled amino acid mixture (0.5μCi/ml). In (*a*) the medium was dialysed exhaustively against water at 4°C and freeze-dried, whereas in (*b*) and (*c*) proteinase inhibitors (see the text) were added to the medium before precipitation of glycoproteins at 4°C with $(NH_4)_2SO_4$ (75% satn). Samples were denatured in SDS, reduced with dithiothreitol, alkylated with iodoacetamide and dialysed against 0.02M-Tris/HCl buffer (pH7.4) containing 0.1% SDS and 0.03% NaN_3 before chromatography. The elution positions of standards are indicated as follows: 1, rat tail tendon collagen β-chains; 2, collagen α-chains; 3, ovalbumin; 4, ribonuclease A; V_0, Blue Dextran 2000. For reference purposes the peaks of radioactivity in (*b*) have been designated R1–R6 as in Sear *et al.* (1976). ●, [^{3}H]Fucose; ○, ^{14}C-labelled amino acids.

130000, 85000, 45000, 30000 and 15000 respectively, were also observed. The area under peak R6 varied from experiment to experiment. To investigate whether proteins of similar size were being synthesized *de novo*, medium from cultures maintained for 24h in the presence of a ^{14}C-labelled amino acid mixture was analysed by gel filtration (Fig. 4*c*). The profiles shown in Figs. 4(*b*) and 4(*c*) exhibit a high degree of coincidence, suggesting that the fucosylated macromolecules were newly synthesized glycoproteins and that peak R1 probably represented the major protein released by cultured fibroblasts when maintained in medium MAB 87/3.

SDS/polyacrylamide-gel electrophoresis of the macromolecules released into fibroblast culture medium revealed several prominent Coomassie Blue-staining bands migrating with apparent mol.wts. ranging from approx. 260000 to 75000, three of which also stained strongly with the periodic acid/Schiff reagent (Fig. 5). The major species revealed by both staining techniques migrated with an apparent mol.wt. of 230000.

Newly released glycoproteins dual-labelled with ^{14}C-labelled amino acids and [^{3}H]fucose were subjected to electrophoretic analysis, and the profiles obtained when gels were sliced and analysed for radioactivity are shown in Fig. 5. A close correlation was observed between the ^{14}C profile and the protein bands revealed by Coomassie Blue staining, indicating that these components had been

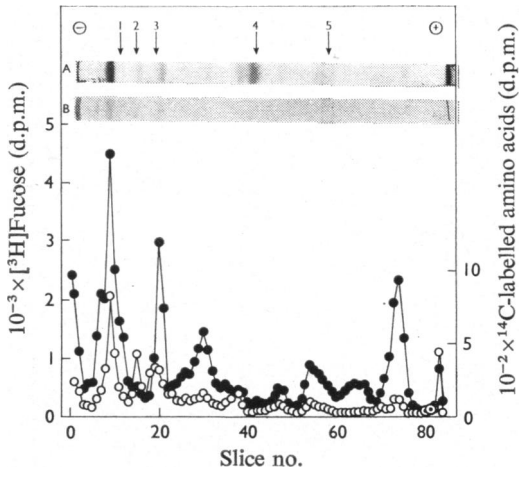

Fig. 5. *SDS/polyacrylamide-gel electrophoresis of macromolecules released into fibroblast maintenance medium*
A confluent fibroblast culture (75cm^2) was dual-labelled for 24h with [^{3}H]fucose (10μCi/ml) and ^{14}C-labelled amino acid mixture (2μCi/ml). Macromolecules from the medium were prepared by $(NH_4)_2SO_4$ precipitation in the presence of proteinase inhibitors and processed as described in the text for electrophoresis on gels containing 8% (w/v) acrylamide. Various portions of the reduced and denatured macromolecules from the medium were electrophoresed in parallel and stained with Coomassie Blue (gel A, containing approx. one-eighth of the sample) or periodic acid/Schiff reagent (gel B, containing approx. three-eighths of the sample). Another gel, containing approx. one-eighth of the sample, was sliced and assayed for radioactivity. The mobilities of the following standards are indicated: 1, rabbit skeletal-muscle myosin; 2, chick tendon procollagen pro-α1 chains; 3, chick tendon procollagen pro-α2 chains; 4, bovine serum albumin; 5, ovalbumin. ●, [^{3}H]Fucose; ○, ^{14}C-labelled amino acids.

newly synthesized by the cells. A correspondence was also observed between the [^{3}H]fucose profile and the periodic acid/Schiff-stained bands. The most prominent species labelled with both ^{14}C-labelled amino acids and [^{3}H]fucose co-migrated with the major glycoprotein of apparent mol.wt. 230000. The second most prominent [^{3}H]fucose-labelled species co-migrated with the Coomassie Blue and periodic acid/Schiff-positive species of apparent mol.wt. 140000. It is noteworthy that in these electrophoretic analyses the yields of this latter glycoprotein were generally higher than the yields of material of similar molecular weight recovered from the gel-filtration column (peak R2 in Fig. 4*b*). As proteinase inhibitors were not present in the agarose-column elution buffer, this finding may reflect the susceptibility of this glycoprotein to enzymic degradation.

Distributions of radioactivity almost identical with that shown in Fig. 5 were obtained after incubation of fibroblasts for 5, 12 or 24h with [^{3}H]fucose, demonstrating that fucosylated glycoproteins were being continuously released into the culture medium in approximately the same relative proportions.

The above electrophoretic analyses thus demonstrate the presence in human fibroblast culture medium of a major fucosylated glycoprotein (apparent mol.wt. 230000). Further experiments established that this molecule was identical with the major [^{3}H]fucose-containing component detected by gel-filtration analysis (peak R1 in Fig. 4*b*). This major fucosylated glycoprotein, designated MFGP in a preliminary report (Sear *et al.*, 1976), was selected for further study.

Confirmation of the non-collagenous nature of the major fucosylated glycoprotein

Church & Tanzer (1975) have described in human fibroblast culture medium an unusually large glycoprotein species having some properties characteristic of collagenous molecules. The possibility that the major glycoprotein described above, or another of the fucosylated glycoproteins synthesized and released by cultured fibroblasts, might represent such a collagen–glycoprotein hybrid was therefore investigated.

Since the stimulatory effect of ascorbate on collagen synthesis and secretion is well documented (Barnes, 1975; Levene & Bates, 1975), a comparison of the influence of ascorbate on [^{3}H]fucose incorporation into non-diffusible macromolecules and on hydroxy[^{14}C]proline synthesis was investigated. The synthesis and release of [^{3}H]fucose-labelled macromolecules were only slightly elevated under ascorbate-supplemented conditions when compared with the ascorbate-deficient conditions (Figs. 6*a* and 6*b*). However, in the ascorbate-supplemented cultures there was a 3–4-fold stimulation in the synthesis of hydroxy[^{14}C]proline-containing macromolecules, which gave rise to a 5–6-fold increase in collagenous molecules released into the medium (Figs. 6*c* and 6*d*). These observations demonstrated that the synthesis and release of newly fucosylated macromolecules was independent of the ascorbate-mediated stimulation of collagen biosynthesis. Further, the profile of the newly released [^{3}H]fucose-labelled glycoproteins obtained by SDS/agarose chromatography was unaffected by the ascorbate content of the maintenance medium. These results therefore suggested that the [^{3}H]fucose-labelled glycoproteins were unrelated to precursors of collagen.

In further experiments the susceptibility of the major glycoprotein to highly purified bacterial

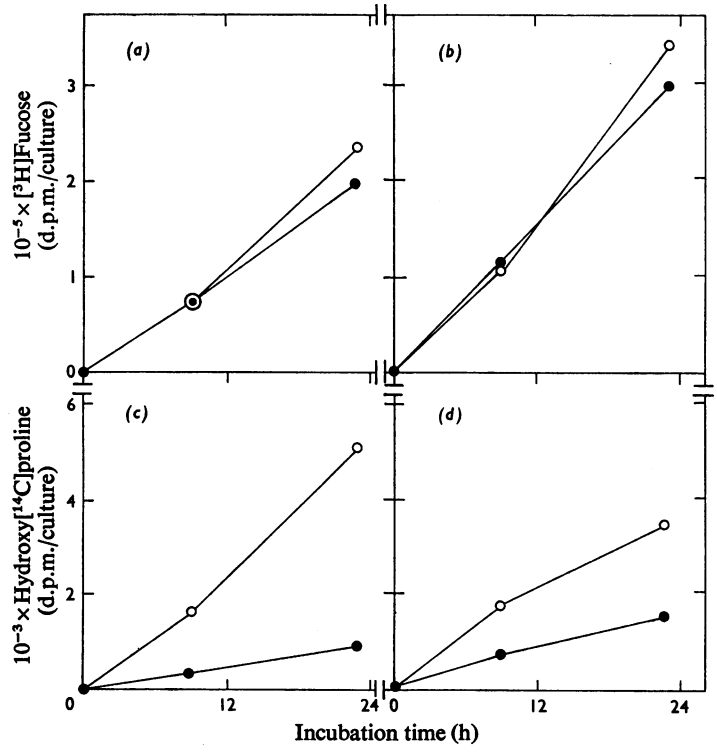

Fig. 6. *Effect of ascorbate supplementation of the medium on the biosynthesis of [^{3}H]fucose-labelled glycoproteins and hydroxy[^{14}C]proline-containing macromolecules by cultured fibroblasts*

Replicate confluent cultures (25 cm^2) were incubated in maintenance medium (3 ml) containing [^{3}H]fucose (10 μCi/ml) and [U-^{14}C]proline (1 μCi/ml) with or without ascorbic acid (50 μg/ml). β-Aminopropionitrile (50 μg/ml) was included in all the culture media to inhibit the formation of lysine-derived collagen cross-links. At various intervals cultures were harvested and proteinase inhibitors were added to the media before exhaustive dialysis against water. Non-diffusible [^{3}H]fucose-labelled macromolecules were measured by liquid-scintillation spectrometry of a portion of the dialysed medium and the remainder was hydrolysed in 6M-HCl for 18h at 110°C and assayed for hydroxy[^{14}C]-proline by the method of Juva & Prockop (1966). The cell layers were scraped from the flasks into 0.1 M-Tris/HCl buffer (pH 8.0) containing 2% SDS, 0.1% Triton X-100 and proteinase inhibitors. Complete dissolution of the cell-layer fractions was achieved by incubation at 100°C for 2 min followed by a further 2h at room temperature. The samples were dialysed, freeze-dried and either analysed for ^{3}H after digestion in NCS/water (9:1, v/v) or hydrolysed and assayed for hydroxy[^{14}C]proline as above. Incorporation of [^{3}H]fucose into macromolecules (*a*) released into the medium and (*b*) present in the cell layer is compared with the synthesis of hydroxy[^{14}C]proline-containing macromolecules (*c*) released into the medium, and (*d*) present in the cell layer. Each point represents the mean of two to four determinations. ●, Ascorbate-deficient conditions; ○, ascorbate-supplemented conditions.

collagenase was investigated. High-molecular-weight proteins released into fibroblast-maintenance medium were concentrated by precipitation with 30%-satd. $(NH_4)_2SO_4$ and analysed by SDS/polyacrylamide-gel electrophoresis after incubation in the presence or absence of collagenase (Fig. 7). The control sample consisted predominantly of the major glycoprotein and two other prominent bands. These latter two bands correspond to the two Coomassie Blue-positive species (apparent mol.wt. 145000–160000) which were not stained with the periodic acid/Schiff reagent (Fig. 5) and had the same mobilities as pro-α1(I) and pro-α2 chains of chick tendon procollagen (result not shown). These presumptive procollagen species were absent from the digested sample, but the intensity of the band corresponding to the major glycoprotein remained virtually unchanged (Fig. 7). When the distribution of radioactivity in the gels was determined the predominant peak had the same mobility as the Coomassie Blue-stained band representing the major glycoprotein and there was no significant difference between the profiles of the control and the collagenase-treated sample (Fig. 7).

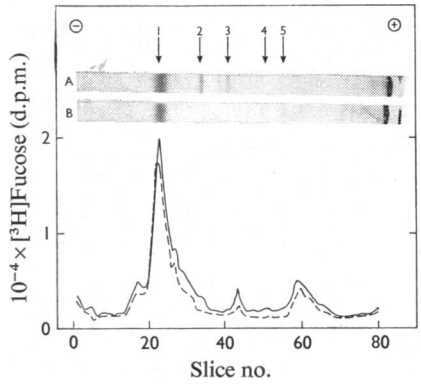

Fig. 7. *Effect of bacterial collagenase on the high-molecular-weight proteins released into the medium by cultured fibroblasts*

A confluent fibroblast culture (175 cm^2) was incubated in maintenance medium (20ml) for 24h with [^{3}H]-fucose (2.5μCi/ml) and the high-molecular-weight proteins released into the medium were precipitated with 30%-satd. $(NH_4)_2SO_4$ in the presence of proteinase inhibitors as described in the text. The precipitate was dispersed at 4°C in 0.05M-Tris/HCl buffer (pH7.4) containing 5mM-$CaCl_2$, 1mM-phenylmethanesulphonyl fluoride and 2mM-*N*-ethylmaleimide. The suspension was dialysed at 4°C against 3×100ml of this buffered solution. Half of the sample was incubated with highly purified bacterial collagenase (approx. enzyme/substrate ratio 1:100, w/w) at 37°C for 2h, and the other half was incubated without enzyme. Equal amounts of each sample were then subjected to SDS/polyacrylamide-gel electrophoresis in duplicate. The gels contained 5% (w/v) acrylamide and were either stained with Coomassie Blue or sliced and assayed for radioactivity. Gel A contained the control sample and gel B the digested sample. The mobilities of the following are indicated: 1, major fucosylated glycoprotein; 2, procollagen pro-α1 chains; 3, procollagen pro-α2 chains; 4 and 5 are the major polypeptides in the collagenase preparation. ——, Control sample; ----, digested sample.

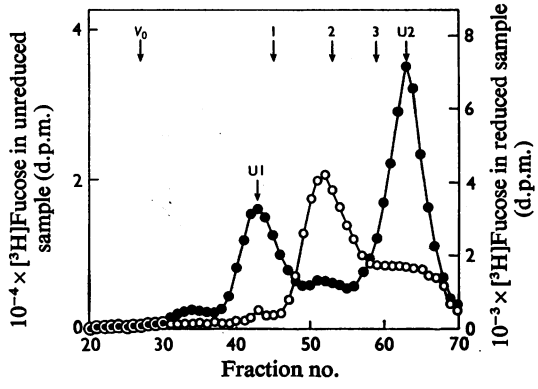

Fig. 8. *Gel filtration on SDS/agarose (Sepharose CL-2B) of [^{3}H]fucose-labelled glycoproteins released into fibroblast maintenance medium*

A confluent fibroblast culture (75 cm^2) was incubated for 24h with [^{3}H]fucose (15μCi/ml), after which the medium was decanted and proteinase inhibitors added before dialysis overnight against water. A portion of the dialysed medium was freeze-dried, and then denatured with SDS in the presence of iodoacetamide as described in the text. This unreduced sample was co-chromatographed with [^{14}C]proline-labelled chick tendon procollagen (approx. 30000d.p.m.) and 5mg of rat tail-tendon collagen, which were added to the sample before solubilization. The elution positions of standards are indicated as follows: 1, procollagen pro-γ-chains; 2, collagen β-chains; 3, collagen α-chains; V_0, Blue Dextran 2000. Two major peaks of [^{3}H]fucose-labelled glycoproteins are designated U1 and U2. Peak U1, comprising fractions 40–46, was concentrated, reduced, alkylated and a portion rechromatographed. ●, Unreduced sample; ○, reduced and alkylated material from peak U1.

These experiments, together with data demonstrating the virtual absence of hydroxyproline in the major glycoprotein (Sear *et al.*, 1976), clearly establish that the latter is a non-collagenous glycoprotein.

Disulphide-bonded nature of the major glycoprotein newly released by cultured fibroblasts

In preliminary experiments in which [^{3}H]fucose-labelled glycoproteins released into fibroblast maintenance medium were chromatographed on an SDS/agarose (Bio-Gel A-5m) column under non-reducing conditions, a major peak of radioactivity was eluted in the void volume (Sear *et al.*, 1976). Reduction and alkylation of this high-molecular-weight material followed by rechromatography on the SDS/agarose column yielded a single peak corresponding to peak R1 in Fig. 4(*b*), and electrophoretic analysis established that this reduced species had the same mobility as the major glycoprotein (result not shown). In further studies to determine the molecular size of the unreduced glycoprotein aggregates, non-diffusible [^{3}H]fucose-labelled glycoproteins were chromatographed under non-reducing conditions on an SDS/Sepharose CL-2B column and two prominent peaks U1 and U2 were obtained (Fig. 8). The major peak, U2, was eluted after an internal standard of tropocollagen α-chains and therefore represented material of mol.wts. less than 100000. The other peak, U1, was eluted just ahead of unreduced tendon procollagen molecules (pro-γ-chains, mol.wt. approx. 450000), and extrapolation of a molecular-weight calibration curve for this column indicated that U1 had an apparent mol.wt. in the range 480000–570000. Reduction, alkylation

and rechromatography of peak-U1 fractions gave a single peak (apparent mol.wt. 240000) corresponding to the major glycoprotein (Fig. 8). These results suggest that the major glycoprotein, newly released from cultured fibroblasts, occurs in an aggregated form, and that these aggregates, when analysed under denaturing conditions in the presence of SDS, contain disulphide-linked glycoprotein dimers.

The presence of intermolecular disulphide bonds is obviously dependent on the presence of peptidyl-cysteine, and therefore a study was made of the incorporation of [^{35}S]cysteine into molecules newly released into fibroblast maintenance medium. Cells were dual-labelled with [^{35}S]cysteine and [^{3}H]fucose for 24h and high-molecular-weight macromolecules in the medium were precipitated with 30%-satd. $(NH_4)_2SO_4$. Gel-filtration analysis of this fraction after reduction and alkylation demonstrated that the predominant [^{35}S]cysteine-labelled species co-eluted with the [^{3}H]fucose-labelled major glycoprotein (Fig. 9*a*). Similar analyses of the supernatant obtained after $(NH_4)_2SO_4$ treatment revealed only minor [^{35}S]cysteine-labelled components (Fig. 9*b*). The major [^{35}S]cysteine-containing polypeptide could not be separated from the [^{3}H]fucose-labelled major glycoprotein during several fractionation procedures, including SDS/polyacrylamide-gel electrophoresis and ion-exchange chromatography. These experiments thus suggest that the major fucosylated glycoprotein is also the major cysteine-containing component released by cultured human skin fibroblasts into the extracellular space.

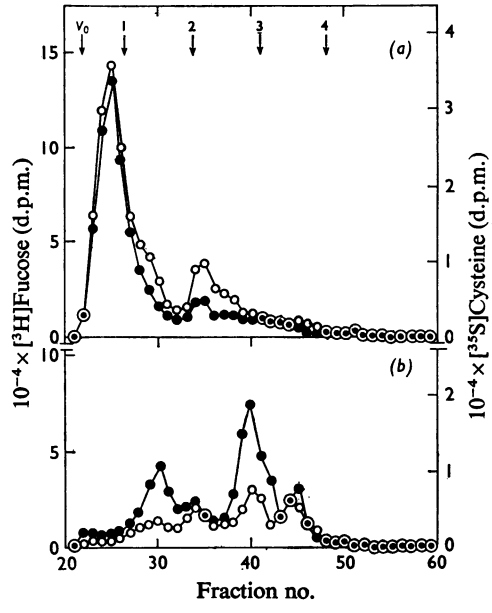

Fig. 9. *Gel filtration on SDS/agarose (Bio-Gel A-5m) of macromolecules released into the medium by fibroblasts maintained in the presence of [^{3}H]fucose and [^{35}S]cysteine*

A confluent fibroblast culture (175cm^2) was incubated for 24h in maintenance medium (20ml) containing [^{3}H]fucose (10μCi/ml) and [^{35}S]cysteine (2μCi/ml). High-molecular-weight proteins released into the medium were precipitated with 30%-satd. $(NH_4)_2SO_4$ in the presence of proteinase inhibitors. (*a*) The precipitated protein fraction was denatured in SDS, reduced with dithiothreitol, alkylated with iodoacetamide and dialysed against 0.02M-Tris/HCl buffer (pH7.4) containing 0.1% SDS and 0.03% NaN_3 before chromatography. (*b*) The supernatant fraction was concentrated and freed of $(NH_4)_2SO_4$ by ultrafiltration, denatured in SDS, reduced, alkylated and rechromatographed. The elution positions of standards are indicated as follows: 1, collagen β-chains; 2, collagen α-chains; 3, ovalbumin; 4, ribonuclease A; V_0, Blue Dextran 2000. ●, [^{3}H]Fucose; ○, [^{35}S]cysteine.

Discussion

Isotopically labelled L-fucose has found wide use as a glycoprotein-specific marker in metabolic and radioautographic studies with a variety of tissues (Coffey *et al.*, 1964; Bekesi & Winzler, 1967; Bennet & Leblond, 1970; Sturgess *et al.*, 1973; Atkinson, 1975). However, these techniques do not appear to have been applied to the study of extracellular glycoproteins synthesized by diploid human skin fibroblasts. Initial studies were therefore concerned with the fate of [^{3}H]fucose when added to cultures of human skin fibroblasts. In these experiments only 0.6% of the exogenously supplied sugar was utilized in the synthesis of macromolecules during a 24h incubation period. This observation is consistent with the fact that L-fucose is a minor component of glycoproteins, occupying a terminal position on the oligosaccharide side chains (Winzler, 1973; Spiro, 1973). The demonstration that virtually all of the macromolecule-associated ^{3}H was present as [^{3}H]fucose agrees with previous studies indicating that mammalian cells do not convert exogenous L-fucose into other saccharide moieties found in macromolecules (see, e.g., Kaufman & Ginsburg, 1968; Della Corte & Parkhouse, 1973). These preliminary experiments showed that the ^{3}H associated with L-[1-^{3}H]fucose was a faithful means of identifying fucosyl moieties covalently attached to macromolecules newly synthesized by cultured human skin fibroblasts.

Because L-fucose is a constituent of keratan sulphate (Roden, 1970) and some glycolipids (Steiner *et al.*, 1973), as well as glycoproteins, it was necessary to determine the nature of the newly synthesized [^{3}H]fucose-labelled macromolecules released into fibroblast culture medium. At least 90%

of these fucosylated macromolecules sedimented as glycoproteins when analysed on CsCl density gradients (Figs. 2*a* and 2*b*), and this observation was supported by the susceptibility of the labelled species to trypsin (Fig. 3). The absence of [^{3}H]fucose-labelled glycolipids in the culture medium suggests that these plasma-membrane components are not shed from the cell layer under the conditions of culture used. Further, the failure to detect fucosylated proteoglycan components suggests that these cells do not synthesize and secrete keratan sulphate. This finding agrees with a previous study on the glycosaminoglycans synthesized by cultured human skin fibroblasts (Matalon & Dorfman, 1969).

Preliminary characterization of the [^{3}H]fucose-labelled glycoproteins released from the cultured fibroblasts was performed by gel-filtration analysis under denaturing conditions. A relatively simple elution profile was obtained as a routine (Fig. 4*b*), provided that proteinase inhibitors were present during the processing of the samples for analysis. This requirement suggested that the [^{3}H]fucose-labelled species, and in particular the larger glycoproteins, were susceptible to digestion by proteinases present in the culture medium. The production of such enzymes may be an integral part of the fibroblasts' response to cultivation *in vitro* and may not be representative of the normal situation *in vivo*. On the other hand, enzymic modifications are of considerable importance in the extracellular processing of at least two connective-tissue constituents (collagen and elastin) and may also play an essential role in the temporal and spatial organization of newly synthesized glycoproteins destined for the extracellular matrix.

Molecular-size analyses of reduced [^{3}H]fucose-labelled polypeptides by SDS/agarose gel-filtration chromatography and SDS/polyacrylamide-gel electrophoresis indicated the presence of at least six distinct glycoprotein species (Figs. 4 and 5). The major fucosylated glycoprotein had an apparent mol.wt. of 250000 as judged by gel-filtration analysis and 230000 as determined by gel electrophoresis. Our previous electrophoretic studies indicated that this glycoprotein had an apparent mol.wt. of 200000 (Sear *et al.*, 1976), but the use of standards of higher purity, and in particular the fact that the glycoprotein migrates more slowly than skeletal-muscle myosin (Fig. 5), suggest that the higher value is more reliable. However, since SDS/glycoprotein complexes are known to behave anomalously during molecular-permeation analyses (Weber & Osborn, 1976), these molecular-weight estimates must be regarded as approximations.

It is noteworthy that, under the ascorbate-deficient conditions prevailing when fibroblasts are maintained in medium MAB 87/3, procollagen polypeptides did not represent major constituents released into the medium. However, supplementation with ascorbate promoted a significant increase in hydroxyproline synthesis and secretion but had little effect on the synthesis and release of fucosylated glycoproteins (Fig. 6). In further studies it was shown that the major fucosylated glycoprotein was not susceptible to digestion by highly purified bacterial collagenase (Fig. 7), nor did it contain hydroxyproline (Sear *et al.*, 1976). The major glycoprotein released by human skin fibroblasts was thus shown to be non-collagenous and unrelated to the high-molecular-weight glycoprotein–collagen complex detected under similar conditions by Church & Tanzer (1975).

Muir *et al.* (1976) have described a high-molecular-weight glycoprotein that is synthesized by monkey arterial smooth-muscle cells in culture. This glycoprotein is similar in many respects to the major fucosylated glycoprotein identified in the present study, e.g. the two glycoproteins have similar molecular sizes in the presence of SDS, and both are the predominant labelled species released into the medium when cells are incubated with [^{35}S]-cysteine (Fig. 9) or [^{3}H]cysteine (Muir *et al.*, 1976). These latter authors suggested that this glycoprotein may be a subunit of the microfibrillar protein found in elastic tissues, but current investigations in our laboratory show a closer relationship between the glycoprotein of mol.wt. 140000 (peak R2 of Fig. 4*b*; Fig. 5) and microfibrillar protein (Sear *et al.*, 1977).

However, the glycoprotein described by Muir *et al.* (1976) and the major fucosylated glycoprotein released by human skin fibroblasts do appear to share several characteristics with a glycoprotein that has been localized on the surface of fibroblast cell layers. For example, this latter species, variously termed LETS (large, external, transformation-sensitive) glycoprotein (Hynes, 1973), CSP (cell-surface protein) (Yamada & Weston, 1974) or fibronectin (Vaheri *et al.*, 1976), is also a non-collagenous molecule of mol.wt. 210000–270000, depending on the analytical system used, and is extremely sensitive to proteinase digestion (for review see Hynes, 1976). The demonstration that the major fucosylated glycoprotein released by human skin fibroblasts occurs predominantly as a disulphide-linked dimer when analysed under non-reducing conditions (Fig. 8) represents a further point of similarity, since Keski-Oja *et al.* (1977) have proposed that fibronectin from fibroblast cell layers can also exist as a disulphide-bonded dimer. In preliminary studies (C. H. J. Sear, M. E. Grant & D. S. Jackson, unpublished work) we have also shown that this fucosylated glycoprotein is localized on the surface of human skin fibroblast-cell layers as assessed by its availability for lactoperoxidase-mediated iodination and extraction by dilute solutions of urea (Yamada *et al.*, 1975). These properties provide further evidence for a relationship between the major fucosylated glycoprotein detected in the

present study and fibronectin, although formal proof of their identity remains to be established.

As yet the biological function of fibronectin is unknown, but various roles have been proposed, e.g., as a determinant of cell adhesion and morphology (Yamada *et al.*, 1976) and as a structural glycoprotein (Linder *et al.*, 1975; Graham *et al.*, 1975) that may mediate the adhesion of fibroblasts to collagen (Pearlstein, 1976). Our finding that a fibronectin-like species is the major fucosylated glycoprotein released into the maintenance medium by confluent human skin fibroblasts also suggests an extracellular role for this macromolecule. A somewhat analogous situation pertains for collagen, which occurs (as procollagen) in fibroblast culture medium and is also laid down in the cell layer (Schafer *et al.*, 1967; Taubman & Goldberg, 1976). It is tempting to speculate therefore that collagen and this fucosylated glycoprotein may be associated *in vivo* in a pericellular matrix that constitutes the immediate environment of connective-tissue cells and that may play an important part in the development of tissue morphology.

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