Biochem. J. (1979) **179**, 465–471
Printed in Great Britain

The Isolation and Properties of a Second Glycoprotein (LGP-II) from the Articular Lubricating Fraction from Bovine Synovial Fluid

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(*Received* 3 *November* 1978)

A high-molecular-weight glycoprotein (LGP-I) was shown [Swann, Sotman, Dixon & Brooks (1977) *Biochem. J.* **161**, 473–485] to be the major constituent in the articular lubricating fraction from bovine synovial fluid. In addition to the LGP-I component, a second glycoprotein (LGP-II) was also present. After fractionation of bovine synovial fluid by sequential sedimentation in CsCl density gradients, the LGP-I and LGP-II components were separated by gel-permeation chromatography. The LGP-II component was then purified by chromatography on DEAE Bio-Gel A and Bio-Gel P-150. The molecular weight of the LGP-II component was 45800 calculated from sedimentation-equilibrium measurements. Amino acids represented 53% (w/w) and carbohydrate constituents 36% (w/w) of the molecule. Glutamic acid and lysine (144 and 100 residues/1000 residues) were the major amino acids. Glucosamine, mannose, galactose and *N*-acetylneuraminic acid [representing 8.0, 6.6, 9.5 and 11.9% (w/w) respectively] were the only carbohydrate constituents detected. Immunodiffusion analysis showed that LGP-II component did not form a detectable precipitin line with antiserum to bovine serum. It appears likely, therefore, that this glycoprotein is synthesized by the joint tissues and is not derived from serum.

A hyaluronic acid-free protein fraction of bovine synovial fluid possessing lubricating properties for articular cartilage *in vitro* (Radin *et al.*, 1970) was shown to contain two glycoprotein constituents (Swann & Radin, 1972). One of the glycoproteins, labelled LGP-I, was isolated and characterized as a 227500-dalton macromolecule consisting of a single polypeptide chain, 43% (w/w) amino acids and 44% (w/w) carbohydrate, with galactosamine, galactose and *N*-acetylneuraminic acid accounting for 98% of the total carbohydrate (Swann *et al.*, 1977).

The present report is concerned with the isolation and partial characterization of the second glycoprotein (LGP-II) also present in the lubricating fraction.

Materials and Methods

The glycoprotein constituents were isolated from bovine synovial fluid essentially as described by Swann *et al.* (1977), by using three consecutive CsCl-density-gradient procedures. The procedure used to pool the gradient fractions, however, was modified in that a lower density limit, 1.35g/ml instead of 1.40g/ml, was used for the collection of glycoprotein-I and -II fractions from the second and third gradients respectively.

Abbreviations used: SDS, sodium dodecyl sulphate; LGP, lubricating glycoprotein.

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Column chromatography

The glycoprotein-II fraction obtained by the third gradient (Fig. 1, Swann *et al.*, 1977) was concentrated by using an Amicon ultrafiltration cell with a Diaflo PM10 membrane (Amicon Corp., Lexington, MA 02173, U.S.A.) to a volume of 5ml, dialysed against 0.5M-guanidinium chloride/0.05M-Tris/HCl, pH7.6 (subsequently referred to as buffer A) and applied to a descending flow column (200cm × 2.5cm) packed with Bio-Gel A-5M (100–200 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) and equilibrated with buffer A. The column was operated at a flow rate of 30ml/h, and fractions (15ml) were collected.

The LGP-II-containing fraction (fraction A; Fig. 1), isolated by gel-permeation chromatography, was then concentrated to a volume of 10ml (Diaflo PM10 membrane) and dialysed exhaustively against deionized 4M-urea/0.005M-Tris/HCl, pH7.6 (buffer B). This sample was then fractionated on a column (30cm × 2.5cm) packed with DEAE Bio-Gel A (Bio-Rad Laboratories) that had been equilibrated against buffer B. After elution with buffer B, bound constituents were eluted with a linear NaCl gradient.

The starting solvent for the gradient was 500ml of buffer B and the limiting buffer was 500ml of buffer B containing 0.7M-NaCl. The column was operated at 20°C at a flow rate of 40ml/h, and 20ml fractions were collected.

The LGP-II-containing fraction obtained by DEAE ion-exchange chromatography (fraction B; Fig. 3) was then concentrated to 2.5ml, dialysed against buffer A and fractionated on a column (130cm×1.5cm) packed with Bio-Gel P-150 (100–200 mesh; Bio-Rad Laboratories) and equilibrated with buffer A. The column was operated at 20°C at a flow rate of 10ml/h, and fractions (2.5ml) were collected. The purified LGP-II component obtained was dialysed against distilled water/1M-NaCl (to obtain the Na^+ form), followed by dialysis against water to remove NaCl. The sample was then freeze-dried before analysis.

Analytical procedures

The presence of protein constituents in column effluents was determined by measuring the absorbance at 280nm or 235nm by using a flow-through recording spectrophotometer (LKB Instruments, Rockwell, MD, U.S.A.). The neutral sugar content of column effluent was measured by a manual anthrone procedure (Roe, 1955). Uronic acid was determined by a manual carbazole method (Bitter & Muir, 1962), and sulphate by the procedure described by Antonopoulos (1962). Amino acids were determined after hydrolysis with 6M-HCl at 105°C for 24h in a laboratory-constructed semi-automated micro-modification of the two-column three-buffer system described by Moore *et al.* (1958). The acidic and neutral amino acids were eluted from a column (21cm×0.3cm) packed with Durrum DC-4A resin (Durrum Chemical Corp., Sunnyvale, CA, U.S.A.) with pH3.25 and pH4.25 buffers. Basic amino acids and hexosamines were eluted from a column (13cm×0.35cm) packed with the same DC-4A resin with the pH5.28 buffer. The analyser was constructed with parts obtained from the Durrum Chemical Corp., and used a ninhydrin reagent to detect and measure the presence and quantities of amino acids. Tryptophan was not determined. Glucosamine and galactosamine analyses were carried out in the amino acid analyser after hydrolysis with 6M-HCl at 105°C for 3h (Swann & Balazs, 1966). The fucose, mannose, galactose and *N*-acetylneuraminic acid contents of samples were determined by g.l.c. after methanolysis and preparation of the pertrimethylsilyl derivatives (Reinhold, 1972).

Double-diffusion analyses were performed using 1% agar prepared in phosphate-buffered saline (0.15M-NaCl/0.01M-sodium phosphate, pH7.4). Samples to be tested were prepared as solutions containing 0.1–5.0mg/ml of phosphate-buffered saline, and 40μl was applied to the well. Goat antiserum to bovine serum was purchased from ICN Pharmaceuticals, Cleveland, OH, U.S.A. Plates were kept at 4°C for 48h to allow the development of precipitin lines, and, after desiccation, were stained with Coomassie Blue.

The types of peptide constituents were determined by SDS/polyacrylamide-gel electrophoresis. Samples were dissolved in 0.1M-sodium phosphate buffer, pH7.2, containing 4M-urea and 0.5% (w/v) SDS. For reduction of samples, β-mercaptoethanol (10%, v/v) was added, followed by treatment at 37°C for 16h. Gels containing different concentrations of polyacrylamide were run in the presence of 0.1% SDS by using a continuous 0.1M-sodium phosphate, pH7.2, buffer system (Furthmayr & Timpl, 1971) at 6mA/gel for 6h. Electrophoresis in gradient gels in the presence of 0.1% SDS was performed by using a modification of a method described by Lambin *et al.* (1976). Samples were dissolved in 0.1M-sodium phosphate buffer containing 4M-urea and 0.5% SDS. Gradient gels with a slightly concave 4–26% acrylamide gradient (Ortec, Oak Ridge, TN, U.S.A.) were equilibrated with 0.1M-sodium phosphate buffer (pH7.2)/0.1% SDS by pre-electrophoresis in an Ortec slab gel apparatus using an Ortec 4100 pulsed constant power supply at 40mA, 100V and 70 pulses/s for 1h. After applying the samples, electrophoresis was carried out for 16h at 100 pulses/s. Gels were stained with Coomassie Blue to detect proteins and with periodic acid/Schiff reagent to detect glycoproteins.

Sedimentation analyses were carried out after dialysis of the LGP-II component against buffer A in a Beckman model-E analytical ultracentrifuge. For equilibrium measurements samples containing 0.1–0.3mg/ml were analysed by the short-column method described by Yphantis (1964). The sedimentation-equilibrium data were analysed by a computer procedure (Roark & Yphantis, 1969). Molecular weights were calculated by using the formula:

$$\text{Mol.wt.} = \frac{RT}{(1-\bar{v}\rho)\omega^2} \cdot \sigma c \rightarrow 0$$

where R is the gas constant, T the absolute temperature; ω the angular velocity, ρ the solution density and σ the number-average effective reduced molecular weight at the base of the cell. $\sigma c \rightarrow 0$ was obtained by plotting σ against the initial solute concentration and extrapolation to zero concentration. A value of 0.68 was used for the partial specific volume ($\bar{v}$) of component LGP-II. Velocity measurements were performed on samples containing 1.8–7.5mg of component LGP-II/ml of buffer A at 20°C, and the s^0_{20} values obtained by extrapolation to zero concentration.

Preparation of α_1-acid glycoprotein

An α_1-acid glycoprotein-containing fraction was prepared from bovine serum by DEAE-cellulose chromatography, as described by Shibata *et al.* (1975). A sample of Cohn fraction VI prepared from bovine synovial fluid was generously given by Dr. Karl Schmid Boston University Medical School, Boston, MA, U.S.A.

Results and Discussion

Isolation of the LGP-II component

Gel-permeation chromatography of the glycoprotein-II fraction, obtained by density-gradient fractionation of bovine synovial fluid, separated the LGP-I and LGP-II components into two discrete fractions (Fig. 1). Significantly greater quantities of the LGP-II component were obtained in these experiments compared with those reported previously (Swann *et al.*, 1977). The increased yield of this component was probably due to the fact that a lower density limit was used here when the gradient fractions were pooled to yield the glycoprotein fractions. Analysis by SDS/polyacrylamide-gel electrophoresis (Fig. 2) showed that fraction A obtained by gel-permeation chromatography contained component LGP-II and several other peptide constituents. Fractionation by chromatography on DEAE Bio-Gel A (Fig. 3) achieved a further purification of component LGP-II. This glycoprotein was eluted at a NaCl concentration of 0.15M, and gel electrophoresis (Fig. 2) showed that fraction B contained the

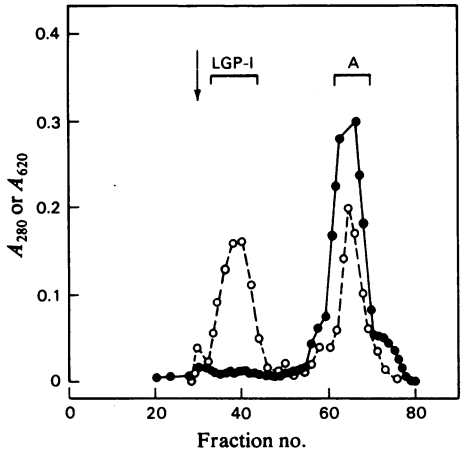

Fig. 1. *Fractionation of glycoprotein fraction II obtained by density-gradient sedimentation (Swann et al., 1977)*
Chromatography on a Bio-Gel A-5M column (200cm×2.5cm) eluted with buffer A. The LGP-I component was eluted near the column void volume (indicated by the arrow) and the LGP-II component was eluted in fraction A. The elution of protein was monitored by measuring the A_{280} (●) and carbohydrate by using the anthrone reaction (A_{620}, ○).

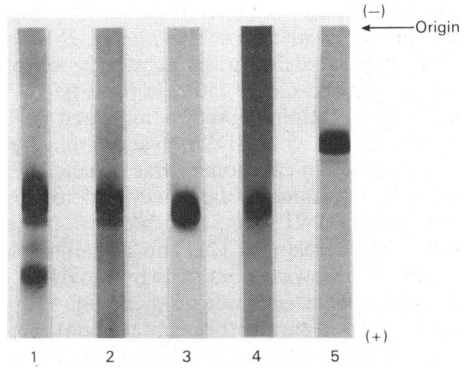

Fig. 2. *Electrophoresis of glycoprotein fractions and purified component LGP-II on polyacrylamide gels in the presence of SDS under non-reducing conditions*
Gel 1 shows fraction A, obtained by gel-permeation chromatography (Fig. 1). Gel 2 shows fraction B, obtained by refractionation of fraction A by chromatography on DEAE Bio-Gel A (Fig. 3). Gels 3, 4 and 5 show component LGP-II, obtained by chromatography on Bio-Gel P-150 (Fig. 4). Gels 1, 2, 3 and 4 were 5% polyacrylamide gels and gel 5 was 9%. Gels 1, 2, 3 and 5 were stained with Coomassie Blue and gel 4 with periodic acid/Schiff reagent.

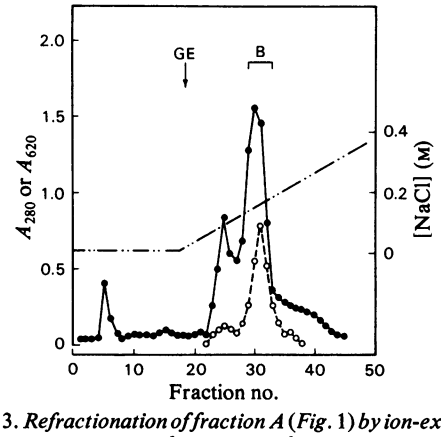

Fig. 3. *Refractionation of fraction A (Fig. 1) by ion-exchange chromatography*
A DEAE Bio-Gel A column (30cm×2.5cm) equilibrated with buffer A and a linear NaCl gradient in the same buffer were used (for details, see the text). The fractions were analysed for protein (A_{280}; ●), and for carbohydrate (A_{620}; ○) by the anthrone reaction. The elution position of NaCl used in the gradient is indicated by the arrow labelled GE. The NaCl concentration was determined by conductivity measurements. The fractions indicated (B) were pooled for rechromatography on Bio-Gel P-105 (Fig. 4). —·—, NaCl concentration.

LGP-II component and a second peptide component with a slightly greater mobility. The LGP-II component was separated from this peptide by chromatography on the Bio-Gel P-150 column (Fig. 4). When the LGP-II-containing fraction indicated in Fig. 4 was analysed on 5% SDS/polyacrylamide gels, a single glycoprotein component was detected (Fig. 2). A trace of contaminant was, however, detected when component LGP-II was analysed by using 9% polyacrylamide gels (Fig. 2), and a similar electrophoretic profile was obtained by analysis on a 4–26% SDS/polyacrylamide gradient gel.

Attempts to separate this contaminant were not successful, and, since the electrophoresis results indicated that it was present in only trace amounts, samples prepared by the above fractionation scheme have been used for the characterization of the LGP-II glycoprotein.

Chemical composition

The amino acid composition of component LGP-II is shown in Table 1. It had a high content of glutamic acid, leucine and lysine, and the amino acids determined accounted for 53% of the total composition, and the only constituents detected by g.l.c. and hexosamine analyses were glucosamine, mannose, galactose and *N*-acetylneuraminic acid, which represented 8, 6.6, 9.5 and 11.9% (w/w) of freeze-dried sample respectively. A manual carbazole assay for uronic acid showed an A_{530} corresponding to a uronic acid content of 1% (w/w). The absorption spectrum of coloured products, however, was different from that of the glucuronolactone standard. Fetuin, a glycoprotein with a similar carbohydrate content, but which does not contain uronic acid (Spiro & Bhoyroo, 1974), reacted in a similar manner. It was concluded, therefore, that the LGP-II component does not contain uronic acid. Sulphate was not detected.

Table 1. *Chemical composition of component LGP-II*

	Amino acid content (residues/1000 residues)
Aspartic acid	83
Threonine	63
Serine	56
Glutamic acid	144
Proline	40
Glycine	54
Alanine	77
Half-cystine	29
Alanine	52
Methionine	13
Isoleucine	42
Leucine	94
Tyrosine	41
Phenylalanine	40
Lysine	100
Histidine	25
Arginine	49
Total amino acids (%, w/w)	53
	Carbohydrate content (%, w/w)
Glucosamine	8.0
Galactosamine	0
Fucose	0
Mannose	6.6
Galactose	9.5
N-Acetylneuraminic acid	11.9
Total	36.0

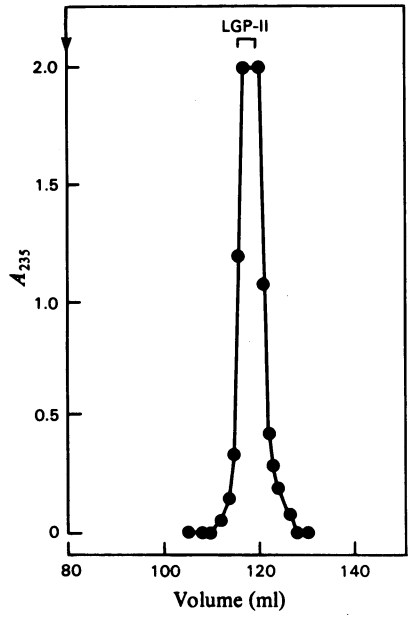

Fig. 4. *Refractionation of fraction B (Fig. 3)*
Chromatography was on a Bio-Gel P-150 column (130cm × 1.5cm) eluted with buffer A (see the text). Glycoprotein components were detected by measuring the A_{235}, and the fractions indicated were pooled, dialysed and freeze-dried to yield purified component LGP-II. The elution position of Blue Dextran 2000 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.), which indicates the void volume, is shown by the arrow.

Physicochemical characterization

Sedimentation-velocity measurements performed with different concentrations of component LGP-II in buffer A gave a value of 2.97S for s_{20} at zero concentration (Fig.5). The $s_{20,w}$ value at zero concentration, calculated by using values of 1.028 and 1.05 for the relative viscosity and density of buffer A at 20°C

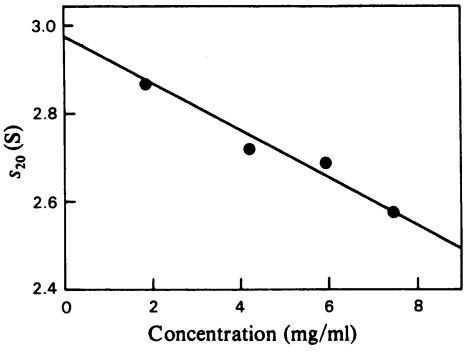

Fig. 5. *Concentration-dependence of the sedimentation coefficient of component LGP-II*
Samples prepared by dissolving component LGP-II in buffer A (see the text), followed by dialysis against the same buffer, were analysed by sedimentation at 42040rev./min at 20°C in the analytical ultracentrifuge with the use of schlieren optics and double-sector cells, one of which was fitted with a 1° window.

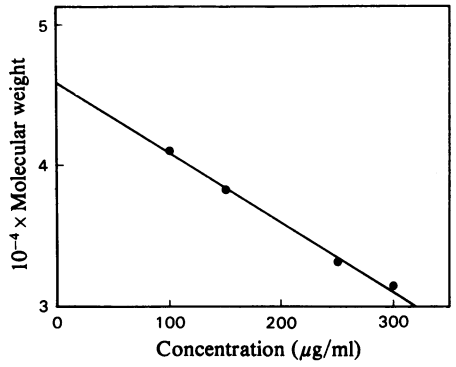

Fig. 6. *Concentration-dependence of the molecular weight of component LGP-II determined by sedimentation-equilibrium analysis*
Samples dissolved in and dialysed against buffer A (see the text) were analysed by sedimentation at 24615rev./min at 4°C in the analytical ultracentrifuge using interference optics and a cell containing the six-chamber centrepiece. Photographs were taken after sedimentation for 64h. Molecular weights were calculated as described in the text and the values obtained plotted against the initial concentration of component LGP-II in the sample.

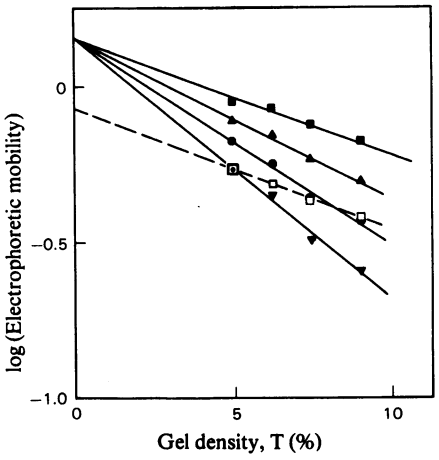

Fig. 7. *Relationship between the electrophoretic mobility of component LGP-II and standard proteins on gels containing different concentrations of polyacrylamide*
The mobilities of ribonuclease (■), chymotrypsin (▲), ovalbumin (●) and bovine serum albumin (▼) were compared with those of component LGP-II (□). In each case a linear relationship was observed, but the data for component LGP-II were extrapolated to a different intercept, indicating anomalous behaviour for this glycoprotein.

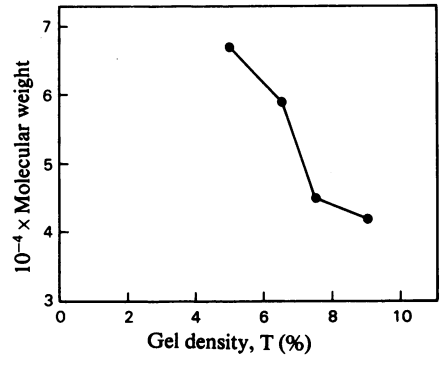

Fig. 8. *Molecular weight of component LGP-II determined by SDS/polyacrylamide-gel-electrophoresis measurements on gels containing different concentrations of polyacrylamide*
The apparent molecular weights of component LGP-II were calculated from the relative mobility of component LGP-II and proteins of known molecular weight (Fig. 7).

respectively, is 3.42S. Sedimentation-equilibrium measurements also showed a concentration-dependence (Fig. 6), and the weight-average molecular weight at zero concentration was calculated to be 45800.

The apparent molecular weight calculated from the relative mobility of component LGP-II on SDS/polyacrylamide gels was dependent upon the concentration of acrylamide employed (Figs. 7 and 8). Similar anomalous behaviour has been observed with other glycoproteins (Banker & Cotman, 1972; Segrest *et al.*, 1971). The most likely explanation for this phenomenon is the altered binding of SDS to the

peptide core of the glycoprotein compared with the standard proteins (Grefrath & Reynolds, 1974; Segrest *et al.*, 1971). It is not known to what extent this is related to the composition and or the conformation of the carbohydrate side chains. In addition the altered mobility of the LGP-II component before and after reduction (see Plate 1 of Swann *et al.*, 1977) indicated that the conformation of the peptide core may also influence the mobility in SDS/polyacrylamide gels. The apparent molecular weights calculated from mobility results (Fig. 7), however, conform to the previously observed pattern (Segrest *et al.*, 1971; Rodbard, 1976; Marguardt *et al.*, 1977) in that they decrease with increasing acrylamide concentration (Fig. 8). Segrest *et al.* (1971) considered that molecular-weight determinations were more accurate at high gel concentrations ($T > 10$, where gel density T is the percentage concentration of acrylamide+*NN'*-methylenebisacrylamide), because of the molecular-sieving properties of the gel. The apparent molecular weight of component LGP-II obtained by using 9% gels was 42000 (Fig. 8) and that obtained by using the 4–26% gradient gels was calculated to be 37000. According to the data shown in Fig. 8, a molecular weight that was the same as that obtained by sedimentation-equilibrium analysis (45800) could be obtained with an acrylamide concentration of 7.4%. These results illustrate the problem of using SDS/polyacrylamide-gel electrophoresis to determine the molecular weight of constituents that exhibit anomalous electrophoretic behaviour and indicate that the mobility probably depends upon the particular structure of the constituent.

Immunological identity of component LGP-II

Although the chemical composition of component LGP-II differs from that reported previously for α_1-acid glycoprotein isolated from bovine serum (Bezkorovainy & Doherty, 1962; Endresen & Christensen, 1972) and other species (Schmid, 1975), these two glycoproteins have a similar overall composition and molecular weight. For this reason the ability of component LGP-II to cross-react with an antiserum to bovine serum was compared with that of samples of α_1-acid glycoproteins prepared from bovine serum and bovine synovial fluid. Cross-reacting precipitin lines were observed with the Cohn fraction VI, the α_1-acid glycoprotein sample prepared from bovine serum and the fraction A sample (Fig. 1) from which component LGP-II was purified by ion-exchange (Fig. 3) and gel-permeation (Fig. 4) chromatography. Component LGP-II, however, failed to form a precipitin line with anti-bovine serum at concentrations of up to 5 mg of LGP-II/ml, and appears to be a distinct immunological entity.

General discussion

Our interest in the LGP-II component stems from the fact that this hitherto undescribed glycoprotein was first noted as a component present in the articular lubricating fraction of bovine synovial fluid (Swann & Radin, 1972; Swann *et al.*, 1977). Although lubrication tests have indicated that the boundary lubricating properties of synovial fluid are primarily associated with the LGP-1 component (Swann, 1978; D. A. Swann & E. L. Radin, unpublished work), a consistent finding in our studies has been that the purification of this component results in a loss of boundary lubricating activity compared with that of intact synovial fluid. This raises the possibility that boundary lubrication of articular cartilage is a co-operative phenomenon involving more than one synovial-fluid constituent (Swann, 1978). It is not known at present whether the LGP-II component plays a role in joint lubrication, but the observation that this glycoprotein does not cross-react with an antiserum to bovine serum indicates that it is not a serum component. Its presence in synovial fluid indicates that it is synthesized by the joint tissues and that it has some role in joint function.

This work was supported by research funds from the Shriners Burns Institute, Boston Unit, and Grant no. AM 19835 from the National Institutes of Health. We thank Stuart Sotman, Susan Powell, Heather Hermann and Paegan Deering for skilled technical assistance and Dr. K. Schmid for helpful discussions and the donation of samples.

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