Separation of a Proteoglycan Fraction from Kurloff Cells Stimulating Protein Synthesis in Macrophages

By MICHAEL F. DEAN and HELEN MUIR

Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London W6 7DW, U.K.

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Proteoglycan from Kurloff cells, when present in the medium in low concentrations, increased the incorporation of [^3H]leucine into trichloroacetic acid-precipitable material by macrophages from peritoneal exudates, in addition to inhibiting their migration from capillary tubes, as observed previously. After treatment with 0.5M-NaOH, followed by dialysis or ultrafiltration, material with the distinctive u.v. and i.r. spectra of the whole proteoglycan appeared in the diffusate, and biological activity was lost from the proteoglycan which remained in the dialysis residue. The diffusible material absorbed near 260nm and had i.r. bands at 805cm^{-1} and 1260cm^{-1} , but did not display the i.r. bands characteristic of chondroitin 4-sulphate. It contained little sulphate, no hexosamine and less than 1% of the uronic acid present in the whole proteoglycan, and there were only trace amounts of amino acids, xylose and galactose. However, significant amounts of ribose and organic phosphate were present, each representing about 1% of the whole proteoglycan. After proteolysis and chondroitinase digestion of the proteoglycan, a fraction with absorbance at 260nm was eluted from Dowex 1 with water which stimulated the incorporation of [^3H]leucine by macrophages and inhibited their migration from capillary tubes.

Kurloff cells, which fill the spleens of guinea pigs after administration of oestrogen, contain a proteoglycan which has been well characterized (Dean & Muir, 1970). It resembles chondroitin 4-sulphate proteoglycans of cartilage in many ways, but is of smaller molecular size than most other proteoglycans as judged by agarose-gel chromatography. After proteolysis the resulting proteoglycan was eluted as single chains from Sephadex G-200, of rather higher molecular weight (Dean & Muir, 1970) than the average chondroitin 4-sulphate chains of cartilage (Tsiganos & Muir, 1969), but which were similarly degraded by testicular hyaluronidase (Muir & Marshall, 1961). From the molar ratios of serine to xylose and to galactose (Dean & Muir, 1970) the Kurloff-cell proteoglycan appears to possess the same linkage region as cartilage chondroitin sulphate (Rodén & Smith, 1966; Lindahl & Rodén, 1966) and hence undergoes an alkaline β -elimination reaction, with separation of carbohydrate from the protein part of the molecule and a concomitant loss of serine and threonine (Dean & Muir, 1970).

The Kurloff-cell proteoglycan, however, differs from the proteoglycans of cartilage in several respects. It has a u.v.-absorption spectrum with a maximum near 260nm and the i.r. spectrum has strong bands at 805cm^{-1} and 1260cm^{-1} (Dean *et al.*, 1971), in addition to bands characteristic of chondroitin 4-sulphate (Mathews, 1958) at 720cm^{-1} ,

850cm^{-1} and 928cm^{-1} . The Kurloff-cell proteoglycan and the corresponding glycosaminoglycan inhibited the migration of macrophages from capillary tubes *in vitro* at extremely low concentrations without effecting transformation of lymphocytes (Revell *et al.*, 1972), whereas proteoglycans and glycosaminoglycans isolated from pig tracheal cartilage by the same procedure had no such effect. Moreover, proteoglycans purified from sheep, pig, rat and human spleens, removed during or immediately after pregnancy, all inhibited macrophage migration and had u.v.- and i.r.-absorption spectra which resembled the spectra of the guinea-pig material (Dean *et al.*, 1971; Revell *et al.*, 1972).

In view of these properties, proteoglycan preparations from the spleens of oestrogen-treated guinea pigs have been investigated further in an attempt to determine which part of the molecule may be responsible for its biological activity.

Experimental

Chemicals and animals

General chemicals were purchased from British Drug Houses Ltd., Poole, Dorset, U.K., and were of analytical-reagent grade, except for cetyltrimethylammonium bromide and glucuronolactone. 9-Aminoacridine hydrochloride was supplied by Koch-Light Ltd., Colnbrook, Bucks., U.K. Deoxyribo-

nuclease and ribonuclease (from ox pancreas) were from Sigma (London) Chemical Co., Kingston, Surrey, U.K., and RR-grade acetone and ethanol from James Burroughs Ltd., London S.E.11, U.K. Sterile [^{35}S]sulphate for injection was from The Radiochemical Centre, Amersham, Bucks., U.K., and guinea pigs of the Dunkin Hartley strain came from a single supplier. Oestradiol monobenzoate was obtained from Evans Medical Ltd., Speke, Liverpool, U.K., and cell-culture media were from Flow Laboratories Ltd., Irvine, Ayrshire, U.K. NCS tissue solubilizer was supplied by Hopkin and Williams, Chadwell Heath, Essex, U.K.

Analytical methods

Hexuronic acid was determined by the method of Bitter & Muir (1962) with standards of glucuronolactone. Amino acid and hexosamine analyses were carried out on a Locarte analyser as described by Tsiganos & Muir (1969) after hydrolysis of samples for 24 h at 105°C in sealed tubes under N_2 . Neutral sugars were measured by g.l.c. after methanolysis in 2M-HCl, neutralization and conversion into their trimethylsilyl derivatives, by a modification (Ewins, 1972) of the method described by Clamp *et al.* (1967). Organic phosphate was determined by the procedure of Chen *et al.* (1956).

Measurement of radioactivity

Radioactive [^{35}S]sulphate was measured in a Packard model 3320 liquid-scintillation spectrometer. Samples (500 μl) were added to 10 ml of a solution containing 20 g of naphthalene, 1 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen (BBOT) and 130 ml of toluene, made up to 250 ml with 2-methoxy-ethanol (Hardingham & Phelps, 1968). Efficiency of counting was determined with an automatic external standard.

U.v. spectra

Samples were adjusted to an appropriate concentration in either 0.2M-sodium acetate, pH 6.8, or 0.5M-NaOH and spectra plotted by hand by using a Beckman DBG spectrophotometer.

I.r. spectra

Samples (about 1 mg) were dissolved in 10 ml of a 1.0% (w/v) solution of KCl, freeze-dried and pressed into a disc. The spectra were recorded on a Unicam SP. 200 spectrophotometer.

Preparation of Kurloff-cell proteoglycan

Batches of 30–40 female guinea pigs were injected intramuscularly with 500 μg doses of oestradiol monobenzoate at weekly intervals. Spleens were removed 30–35 days after the initial injection, homogenized in 0.9% (w/v) NaCl in a TenBroeck tube (two spleens and 10 ml of NaCl) and the homogenate

was heated for 60 min at 55°C to complete cell disruption and to denature the more labile proteins. Precipitated proteins were removed by centrifugation, the clear supernatant solution was set aside and the residue rehomogenized, heated and again centrifuged. Polyanions were precipitated from the combined supernatant solutions with 9-amino-acridine hydrochloride (Muir & Jacobs, 1967) and the proteoglycans isolated as described by Dean & Muir (1970).

Proteoglycans labelled with [^{35}S]sulphate were prepared by administering sterile radioactive sulphate during the period of oestrogen treatment. Four 500 μg doses of oestrogen were given, with an interval of 7 days between each dose, and 100 μCi of [^{35}S]sulphate was given intraperitoneally on the 5th, 7th, 9th and 11th days after the first injection of oestrogen, and doses of 500 μCi on days 20 and 22. The animals were killed on the 23rd day and the proteoglycans isolated as described above.

Alkaline treatment of proteoglycans

Samples containing 5–10 mg of proteoglycan were dissolved in 1–2 ml of 0.5M-NaOH in a stoppered tube and N_2 was bubbled through the solution for 10 min. After being left overnight at room temperature (about 21°C) samples were adjusted to pH 6.0 with 1.0M-HCl and either passively dialysed against 200 ml of water for 24 h at 4°C in heated dialysis tubing (Callanan *et al.*, 1957) or subjected to positive pressure dialysis in an ultrafiltration cell fitted with a UM-2 or UM-05 filter membrane (Amicon, High Wycombe, Bucks., U.K.). The solution was first passed through a UM-2 membrane and the filtrate then passed through a UM-05 membrane. The filtrate and residue from the second filtration were chromatographed on Sephadex G-10 and G-25 (see below, and Table 1). Where necessary diffusates and dialysis residues were freeze-dried for storage.

Determination of biological activity

(I) *Inhibition of macrophage migration.* The biological effects of Kurloff-cell proteoglycans and the subfractions derived from them were determined by using peritoneal-exudate cells induced with a single injection of 10 ml of paraffin oil. The exudates (85% macrophages) were collected 48 or 72 h after injection by washing out the peritoneal cavity with 60 ml of Hanks balanced salt solution cooled in ice and containing 5 units of heparin, 200 units of penicillin and 100 μg of streptomycin/ml.

The effect of proteoglycan fractions on the migration of peritoneal macrophages from capillary tubes *in vitro* was measured as described by Revell *et al.* (1972). Cells were suspended in TC 199 medium containing 200 units of penicillin and 100 μg of streptomycin/ml supplemented with 10% (v/v) bovine foetal

serum which had been heated at 57°C for 60 min to inactivate complement. Cell suspensions from single animals were used in each set of experiments and controls to avoid possible reactions between cells from different individuals. The migration of cells from groups of control and experimental capillaries (a minimum of 10 tubes was used for each group) was measured after 48 h (Revell *et al.*, 1972). The percentage inhibition of migration was calculated from the difference between the means of the control and experimental groups.

(II) *Incorporation of [³H]leucine.* To determine the effect of proteoglycan fractions on the incorporation of [³H]leucine, peritoneal-exudate cells obtained from a single animal were suspended in Eagle's minimum essential medium containing 20 000 units of penicillin 10 mg of streptomycin and 2 ml of 200 mM-glutamine per 100 ml, supplemented with 10% (v/v) bovine foetal serum. The total number of cells harvested was determined electronically by using a Coulter counter and the number adjusted to between 5.0×10^6 and 7.5×10^6 cells/ml of medium. Portions (1 ml) of this cell suspension and 8 ml of the supplemented Eagle's medium were incubated at 37°C in a shaking water bath together with 0.5 ml (5 or 10 μ Ci) of [³H]leucine and either 0.5 ml of test substance or 0.5 ml of Hanks balanced salt solution or cartilage proteoglycans as a control (total volume 10 ml). Duplicate samples (500 μ l) were removed from each cell suspension at intervals of 30 or 60 min, added to 5 ml portions of Hanks balanced salt solution in tapered centrifuge tubes and the cell pellets were spun down. Each pellet was resuspended in 5 ml of cold (4°C) 10% (w/v) trichloroacetic acid, recentrifuged in a bench centrifuge and the precipitated material dissolved in 1.0 ml of NCS tissue solubilizer for radioactivity counting. Samples (500 μ l) were taken for measurement of radioactivity.

Proteolysis of proteoglycans

Proteoglycans were dissolved in 0.2 M-sodium acetate containing 4 mM-EDTA and 20 mM-cysteine-HCl, pH 5.7, at a concentration of 2 mg/ml, and digested with 20 μ l (2 units) of a suspension of crystalline papain (EC 3.4.22.2). Digestion was continued overnight at 60°C under a layer of toluene, a further 10 μ l of enzyme added and incubation continued for another 6 h. The solution was then clarified by centrifugation. The glycosaminoglycans were precipitated with ethanol, washed in acetone and dried.

Digestion of glycosaminoglycans with chondroitinase

The glycosaminoglycans isolated after papain digestion of Kurloff-cell proteoglycans (Dean & Muir, 1970) were dissolved in 0.025 M-sodium acetate, pH 8.5, at a concentration of 1 mg/ml and 200 μ l of a solution of chondroitinase (chondroitin

sulphate ABC lyase, EC 4.2.2.4; a gift from Dr. E. Baxter) containing 1 unit (μ mol/min)/ml was added. After incubation for 4 h at 37°C 5 ml of the solution was layered on to a column (200 mm $\times$ 10 mm) packed with phosphocellulose that had been washed thoroughly in NaCl, then with water until free of chloride. The column was eluted with water, 5 ml fractions were collected and the position of the enzyme reaction products was followed by their absorbance at 232 nm. The chondroitinase remained bound to the column.

Ion-exchange chromatography

Depolymerized glycosaminoglycan fragments were removed from the phosphocellulose-column eluate by passing the latter through a column of Dowex 1 ($\times 2$; Cl⁻ form). Samples (15 ml) were applied to the column (200 mm $\times$ 10 mm, packed in water), 5 ml fractions were eluted with water and their uronic acid contents and absorbance at 257 nm measured. Fractions which had u.v. absorption were pooled and concentrated by positive pressure dialysis through a UM-05 membrane and stored at -20°C.

Gel chromatography

A column of Sephadex G-25 (10 mm $\times$ 1110 mm) and a column of Sephadex G-10 (9 mm $\times$ 650 mm) were packed in and eluted with 0.1 M-ammonium formate. Samples (1.0 ml) were applied to each column, 0.85 ml fractions were collected and their absorptions at 257 nm measured.

Results

When Kurloff-cell proteoglycan and [³H]leucine were together added to suspensions of peritoneal macrophages, the cells initially incorporated radioactivity into material insoluble in trichloroacetic acid at a rate similar to that for control cells that were incubated with equivalent amounts of cartilage proteoglycans. This initial phase lasted about 3 h, after which the rate of incorporation increased noticeably in the cell suspensions to which Kurloff-cell proteoglycan had been added (Fig. 1a), this increased rate being maintained in samples taken up to 20 h after the addition of [³H]leucine.

The increase in the incorporation of radioactivity varied between 25 and 70% when macrophages from a number of different animals were incubated for 4 h with a single preparation of Kurloff-cell proteoglycans at a concentration of 10 μ g/ml. Increased activity was still measurable at 0.5 μ g of proteoglycan/ml, the lowest concentration used so far.

The incorporation of radioactivity became negligible, however, in both the control and experimental cell suspensions when puromycin was added to the culture medium (Fig. 1a), which indicated that the observed increase in radioactivity was probably attributable to increased protein synthesis induced

Fig. 1. Incorporation of [³H]leucine into trichloroacetic acid-insoluble material by suspension cultures of peritoneal macrophages in the presence of proteoglycan from Kurloff cells or from cartilage

For details see the text. (a) 25 µCi of [³H]leucine was added at the same time to test and control cultures. ○, 20 µg of Kurloff-cell proteoglycan/ml; △, 20 µg of cartilage proteoglycan/ml; ●, 20 µg of Kurloff-cell proteoglycan/ml plus 50 µg of puromycin/ml; ▲, 20 µg of cartilage proteoglycan/ml plus 50 µg of puromycin/ml. (b) 5 µCi of [³H]leucine was added after preincubation for 2 h with 20 µg of Kurloff-cell (○) or cartilage (△) proteoglycan/ml. The final volume of all incubation mixtures was 10 ml. Each value plotted was the average of four samples. Zero values were not determined.

by the Kurloff-cell proteoglycan. The length of the initial lag period before the rate of synthesis increased could be shortened if cells were preincubated for several hours with Kurloff-cell proteoglycan before [³H]leucine was added (Fig. 1b).

The biological activity and u.v. absorption of the Kurloff-cell proteoglycan disappeared when it was treated with 0.5M-NaOH overnight, neutralized and either dialysed or filtered through a UM-2 membrane, which has a molecular-weight exclusion limit of about 1000. When the untreated proteoglycan was dialysed or ultrafiltered no u.v.-absorbing material

passed through the membrane, whereas after treatment, both the diffusate and the filtrate contained material whose u.v.-absorption spectrum was identical with that of the original proteoglycan, with a maximum at 265 nm at an alkaline pH (Fig. 2). Less than 1% of the uronic acid of the proteoglycan passed into the filtrate, however. Similarly, when ³⁵S-labelled Kurloff-cell proteoglycan (specific radioactivity 17000 d.p.m./µmol of uronic acid) was treated with alkali in the same way, less than 5% of the labelled sulphate passed through the UM-2 membrane.

Moreover, after the alkali treatment of the proteoglycan, the material in the diffusate had an i.r. spectrum with bands at 805 cm⁻¹ and 1260 cm⁻¹ (Fig. 3), but no bands at 720 cm⁻¹, 850 cm⁻¹ and 928 cm⁻¹ (Mathews, 1958), which are characteristic of chondroitin 4-sulphate. Thus the appearance of material in the diffusates with the peculiar spectral characteristics of the Kurloff-cell proteoglycan coincided with loss of biological activity from the proteoglycan.

The material which had passed through the UM-2

Fig. 2. U.v.-absorption spectra of Kurloff-cell proteoglycan and of the fractions obtained after treatment with NaOH

For details see the text. ○, 80 µg of whole proteoglycan/ml in 0.5M-NaOH. △, Dialysis residue from 1.4 mg of proteoglycan/ml in 10 ml of water. ●, Diffusate from 1 mg of proteoglycan in 10 ml of water.

Fig. 3. I.r.-absorption spectra of whole Kurloff-cell proteoglycan and of the fractions separated from it after treatment with NaOH

For details see the text. In each case the material was pressed into a disc with 100mg of KCl. (A) 1.0mg of proteoglycan. (B) Dialysis residue from 1.0mg of proteoglycan. (C) Diffusate from 1.0mg of proteoglycan.

membrane was further fractionated by filtration through a UM-05 membrane with a molecular-weight exclusion limit of about 500. About 75% of the u.v.-absorbing material passed through the membrane, but neither the material in the filtrate nor the residue was homogeneous on gel chromatography. Thus the material in the residue separated into three u.v.-absorbing fractions on Sephadex G-25, the largest of which (fraction I) was eluted at a $V_{max.}/V_0$ value of 1.2. The middle component (fraction II) showed a distinct shoulder and was eluted at a $V_{max.}/V_0$ value of 1.6 and fraction III appeared at V_r . The material that had passed through the UM-05 membrane was separated into two fractions on

Sephadex G-10, with $V_{max.}/V_0$ values of 1.1 and 1.5 respectively

Equal volumes from each of these five fractions were analysed for their amino acid contents. Trace amounts (nm quantities) of several amino acids were detected in each (Table 1), with glutamic acid, glycine and serine predominating, but the overall composition of each fraction was very similar to that of the whole Kurloff-cell proteoglycan (Dean & Muir, 1970). However, the total amino acids present in all five fractions together amounted to less than 1% of that of the whole proteoglycan before alkali treatment. In addition, no hexosamines were detected in any of the fractions analysed.

Equal volumes of the fraction with the highest absorbance at 257 nm from Sephadex G-25 and G-10 chromatography and the same volume of buffer eluate as a control were dried separately *in vacuo* over P_2O_5 and the neutral sugars were determined after methanolysis by g.l.c. A small amount of ribose was detected in the fraction from Sephadex G-25 and, xylose, galactose and ribose were found in the relative proportions by wt. of 1:3:18 respectively in the fraction from Sephadex G-10. The ribose in this fraction accounted for approx. 1% by wt. of the original proteoglycan. Traces of glucose were found in all three samples, including the control, which presumably came from traces of Sephadex in the eluates.

Further analysis showed that the whole UM-05 diffusate contained 1% of organic phosphate but no inorganic phosphate and this, together with the

Table 1. Amino acid content of fractions separated on Sephadex G-10 and G-25 from Kurloff-cell proteoglycan after treatment with 0.5M-NaOH for 18 h at 20°C

For details see the text. After alkali treatment, the solution was neutralized and filtered through a UM-2 membrane and the filtrate passed through a UM-05 membrane. The second filtrate was applied to a column of Sephadex G-10 and the residue to a column of Sephadex G-25.

Amino acid	Content (nmol)				
	Sephadex G-25 fractions in order of elution			Sephadex G-10 fractions in order of elution	
	I	II	III	I	II
Asp	3.23	1.37	0.66	1.50	0.64
Thr	1.31	0.85	0.54	0.74	0.40
Ser	2.73	2.92	1.17	5.94	1.50
Glu	6.57	3.02	1.35	2.72	1.70
Gly	4.86	4.17	13.0	9.12	2.04
Ala	3.38	2.06	0.66	2.55	1.13
Val	2.62	1.53	0.44	—	0.49
Leu	3.64	1.49	0.74	—	8.61

presence of ribose and the strong u.v. absorption near 260nm, suggested that a ribonucleotide-like material might be present. When a control sample of 50 μ g of yeast RNA/ml with an absorbance of 1.0 at 260nm (a gift from Dr. C. P. Tsiganos) was subjected to positive pressure dialysis through a UM-2 filter membrane all of the u.v.-absorbing material remained behind in the dialysis residue. However, after an equivalent sample was left overnight in 0.5M-NaOH all of the u.v.-absorbing material was rendered small enough in size to pass through a UM-2 filter membrane, and 38% of the UM-2 filtrate was small enough to pass through a UM-05 membrane.

Because of the possibility that the ribonucleotide-like material which separated from the Kurloff-cell proteoglycan after treatment with NaOH may have represented fragments of a larger molecule susceptible to alkaline degradation, other methods for separating the u.v.-absorbing moiety from the remainder of the proteoglycan were examined. Previous work (Revell *et al.*, 1972) had established that glycosaminoglycans isolated from Kurloff-cell proteoglycan after proteolysis remained biologically active. A sample of Kurloff-cell proteoglycan was therefore digested with papain, the glycosaminoglycans were reisolated and then digested with chondroitinase. The resulting digest was exhaustively dialysed through a UM-2 membrane until the filtrate no longer absorbed at 232nm (i.e. was free of unsaturated disaccharides). The dialysis residue contained only 2.5% of the original uronic acid but still absorbed strongly at 257nm in sodium acetate at pH 6.8. Samples of this dialysis residue containing 1 μ g of uronic acid/ml, with an absorbance of 0.1 at 257nm, and samples of the filtrate containing 10 μ g of uronic acid/ml but with no u.v. absorbance, were examined for their ability to inhibit the migration of peritoneal macrophages. The dialysis residue inhibited the migration by 53% with a significance of difference from the controls greater than 0.001 measured after 24h, whereas the filtrate showed only 20% inhibition with a significance of difference of only 0.2.

Further samples of Kurloff-cell proteoglycan were subjected to proteolysis, and the isolated glycosaminoglycan was digested with chondroitinase. The reaction mixture was finally passed through a column of phosphocellulose to remove the chondroitinase. Samples of this enzyme-free mixture, which consisted of unsaturated disaccharides plus the u.v.-absorbing material, were then eluted with water from a column of Dowex 1 ($\times 2$; Cl^- form). No uronic acid was detected in the eluate, which contained 22% of the u.v.-absorbing material and was free of protein. It was highly active in stimulating [^3H]leucine incorporation by peritoneal macrophages (Fig. 4). Thus a sample having an absorbance at 257nm of only 0.0025

Fig. 4. Stimulation of [^3H]leucine incorporation into trichloroacetic acid-insoluble material by peritoneal macrophages in the presence of a fraction with absorption maximum at 260nm obtained from Kurloff-cell proteoglycan

For details see the text. [^3H]Leucine (5 μCi) was added at the same time to test and control cultures: O, 0.5ml of eluate from Dowex 1 (Cl^- form); Δ , 0.5ml of Hanks balanced salt solution. The final volume of incubation mixtures was 10ml. Each value plotted was the average of four samples and zero-time values were subtracted throughout.

(equivalent to 2 μ g of the original proteoglycan/ml) increased the incorporation by more than 100% after 4h of incubation, and inhibited the migration of macrophages from capillary tubes by 27% with a significance of difference from the controls of 0.01.

Discussion

Previously published data (Dean & Muir, 1970; Revell *et al.*, 1972) have shown that although proteoglycans and glycosaminoglycans isolated from Kurloff cells of the guinea pig were similar in composition to other chondroitin sulphate proteoglycans they differed in two important respects. First, they absorbed strongly near 260nm and their i.r. spectrum had two prominent bands at 805 cm^{-1} and 1260 cm^{-1} , in addition to the 'fingerprint' bands characteristic of chondroitin 4-sulphate. Secondly, they inhibited the migration of macrophages from capillary tubes *in vitro* at very low concentrations. These properties, which persisted through exhaustive purification procedures, including incubation with deoxyribonuclease, ribonuclease and proteolysis, were not possessed by the chondroitin sulphate proteoglycans of pig cartilage prepared by identical procedures.

The results presented here show that another action of the Kurloff-cell proteoglycan on macrophages was

the stimulation of protein synthesis as judged by the incorporation of [^3H]leucine. It is not yet certain whether protein synthesis was specifically stimulated by Kurloff-cell proteoglycans. However, peritoneal macrophages do not divide, since they incorporate less than 1% of externally administered tritiated thymidine (Vernon-Roberts, 1972) but when stimulated they do synthesize and secrete a variety of lysosomal and other enzymes (Cardella *et al.*, 1974; Davies *et al.*, 1974; Gordon *et al.*, 1974). Hence, the observed increase in protein synthesis may have been channelled in this direction. Preliminary results after cell fractionation by density-gradient centrifugation suggest that not all cell populations are stimulated to the same extent by Kurloff-cell proteoglycan.

The distinctive spectra of the Kurloff-cell proteoglycan seemed to be related to its biological activity, since after treatment with alkali followed by dialysis or ultrafiltration, material with these spectral characteristics appeared in the diffusate, whereas biological activity was lost from the proteoglycan which remained in the dialysis residue. This did not happen, however, after dialysis or ultrafiltration alone. The moiety in the diffusate, to which the distinctive spectral features belonged, had none of the characteristics of a proteoglycan, since it did not contain uronic acid, hexosamine or sulphate and the i.r. bands characteristic of chondroitin 4-sulphate were absent. On the other hand, it had some of the characteristics of a ribonucleotide, since it contained organic phosphate and ribose and the absorption spectrum had a maximum near 260 nm.

The u.v.-absorbing material in the diffusate was, however, of low molecular weight, since it would pass through a UM-2 membrane, which has a molecular-weight exclusion limit of 1000, and much of the material would also pass through a UM-05 membrane which has an exclusion limit of 500. On gel chromatography it was quite polydisperse. Thus it appears that the biological activity was attributable to a ribonucleotide which, until degraded by alkali, was associated in some way with the proteoglycan. Alternatively, RNA sufficiently similar in physical properties to the proteoglycan may remain closely associated with it during the purification procedure, which nevertheless succeeded in removing all yeast RNA from cartilage proteoglycan in an artificial mixture with an equivalent absorbance at 260 nm to the Kurloff-cell proteoglycan.

The proteoglycan does not itself appear to influence the biological action either on protein synthesis by macrophages, or on their migration from capillary tubes, because an active fraction was recovered from Dowex 1 chromatography after the protein core of the proteoglycan had been digested with papain and

the chondroitin sulphate chains with chondroitinase. Moreover, cartilage proteoglycans had no action even when applied to the cells at higher concentrations than were used with the Kurloff-cell proteoglycan.

The role of Kurloff-cell proteoglycan *in vivo*, however, is not known, although it has been suggested that it may interfere with cell-mediated immunity (Marshall *et al.*, 1971). The bulk of the molecule, although it does not affect biological activity *in vitro*, may have some function in storage, or transport to the site of action, or perhaps have some protective role.

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