THE PURIFICATION AND CYANOGEN BROMIDE CLEAVAGE OF THE 'LINK PROTEINS' FROM CARTILAGE PROTEOGLYCAN

John Baker and Bruce Caterson

Institute of Dental Research, School of Dentistry,
and Department of Medicine,
University of Alabama in Birmingham,
Birmingham, Alabama 35294

Received April 21, 1977

SUMMARY. The 'link proteins', which are an integral part of cartilage proteoglycan aggregates, have been previously partially purified by equilibrium density gradient procedures. In the present study, complete separation between 'link proteins' and proteoglycan has been achieved by chromatography on Ultrogel 34 in 1% SDS. Cyanogen bromide cleavage of purified 'link proteins' yielded one prominent peptide, which is not obtained from proteoglycan. These data suggest that the 'link proteins' and proteoglycan monomers of cartilage are structurally dissimilar.

INTRODUCTION. Proteoglycans have been shown to interact in vitro with hyaluronic acid to form aggregates[1], and it is believed that similar structures are the predominant form in which proteoglycans occur in cartilage[2]. Proteoglycan aggregates also possess a protein component, originally termed the 'glycoprotein link'[3], but now known as the 'link proteins'[4]. Some evidence indicates that they serve to stabilize the proteoglycan aggregates[5].

To isolate the 'link proteins', proteoglycan aggregates are initially prepared from cartilage extracts by equilibrium density gradient centrifugation under 'associative' conditions as described by Sajdera and Hascall[6]. Aggregates are subsequently dissociated in 4.0 M guanidine and the 'link proteins' are then free to occupy a position of low buoyant density (density < 1.45) in a second density gradient run. 'Link proteins' isolated in this manner were shown by acrylamide gel electrophoresis in SDS to contain two protein bands[4]. Their molecular weights have been estimated[7] to be

---

Abbreviation: SDS, sodium dodecyl sulfate. The abbreviations for proteoglycan aggregate (A1) and 'link protein' preparation (A1D5) follow the notation suggested by Heinegård[11].

ISSN 0006-291X

4-5 x 10^4. 'Link protein' preparations are contaminated by protein-rich proteoglycans, which cannot be entirely removed by refinement of the density gradient method. It has been claimed that chromatography on Sephadex G-200 in 4.0 M guanidine separates a voided peak of protein-rich proteoglycan from an included peak which contains both 'link proteins'[8], but analytical data on the separate fractions have not been reported.

Recent immunological evidence[9] has indicated that the 'link proteins' and the hyaluronic acid binding region may have antigenic sites in common. This result has naturally led to speculation that they may have a common origin (e.g. they could both be fragments of the proteoglycan's protein core).

In this paper, a novel method has been employed for the purification of the 'link proteins' (i.e. chromatography on Ultrogel 34 in 1% SDS). Both the 'link proteins' and the protein-rich proteoglycans have been treated with CNBr and their respective peptide profiles examined. The results indicate that 'link proteins' are not derived from proteoglycan.

METHODS. Fresh bovine nasal cartilage was sliced (0.5 mm thick) and extracted with 10 vol of 4.0 M guanidine, 0.1 M 6-aminohexanoic acid[10], 0.005 M benzamidine, 0.01 M EDTA and 0.05 M sodium acetate, pH 5.8, for 36 h at 4°. The extract was filtered, dialyzed to reduce the guanidine concentration to 0.4 M, brought to a density of 1.66 with cesium chloride and centrifuged in a Beckman Spinco L2-65B preparative ultracentrifuge (SW 27 rotor) at 23,500 rpm for 48 h at 10°. Tubes were sliced in two at a level where the density was approximately 1.61. The bottom fraction (A1) was made 4.0 M in guanidine at a density of 1.50 and recentrifuged at the same rate and temperature for 60 h. Tubes were sliced into five approximately equal segments, and the top fraction (A1D5) was dialyzed against 0.05 M sodium chloride, exhaustively against water and freeze dried.

Acrylamide gel electrophoresis in SDS was performed according to the directions of Neville[12] on 11.1% gels.[13] Protein bands were located by prior labeling of samples with fluorescamine[13] or by staining gels with Coomassie Blue R 250. Densitometric scanning of Coomassie-stained bands was carried out in a Gilford 240 spectrophotometer fitted with a linear transporter. Peak areas, determined by densitometric scanning, were measured relative to a constant amount (25 µg) of ribonuclease-A added to each sample prior to electrophoresis.

Samples for amino acid analysis were hydrolyzed in 6 M HCl in vacuo at 105° for 20 h and before application to a Beckman 119 amino acid analyzer. Samples for hexosamine analyses were hydrolysed in 6 M HCl at 105° for 7 h. Hydrolysates were fractionated on a column (55 x 0.9 cm) of Aminex A-5 which was eluted with sodium citrate buffer, pH 5.28 (0.35 N in Na^+).

Gel chromatography was on a column (105 x 1.0 cm) of Ultrogel 34 developed in 1% SDS, 0.05 M sodium bicarbonate, pH 7.5, at room temperature.

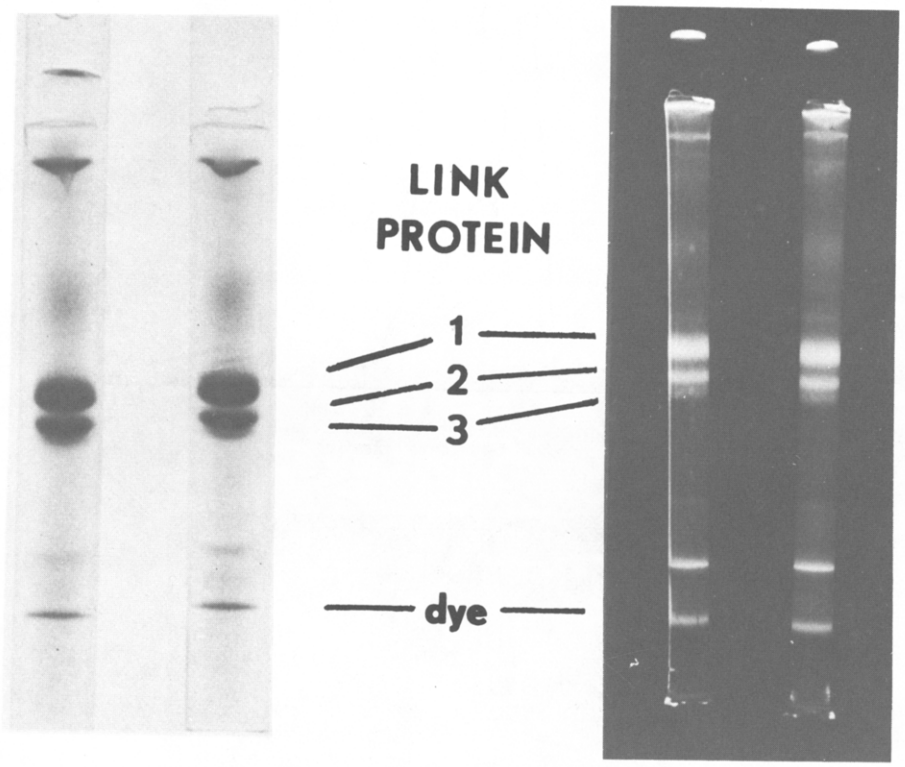

Figure 1. Acrylamide gel electrophoresis in SDS of A1D5.
a. Coomassie Blue-Stained.
b. Fluorescamine labeled prior to electrophoresis.

A flow rate of 3.0-3.5 ml/h was maintained, and fractions were collected every 30 min. Fractions were assayed by an automated Lowry protein procedure[14] and by electrophoresis.

Samples (< 100 µg of protein) were treated with a 1% solution of CNBr in 70% formic acid (0.2 ml) for 24 h at 30°. Control samples were treated with 70% formic acid alone. Reaction was stopped by adding water (4.0 ml), and the mixture was freeze dried.

RESULTS AND DISCUSSION. A1D5 (50 µg) separated by electrophoresis and located by Coomassie blue or fluorescamine (Figures 1a and 1b, respectively), contained two major protein bands. When the same preparation was

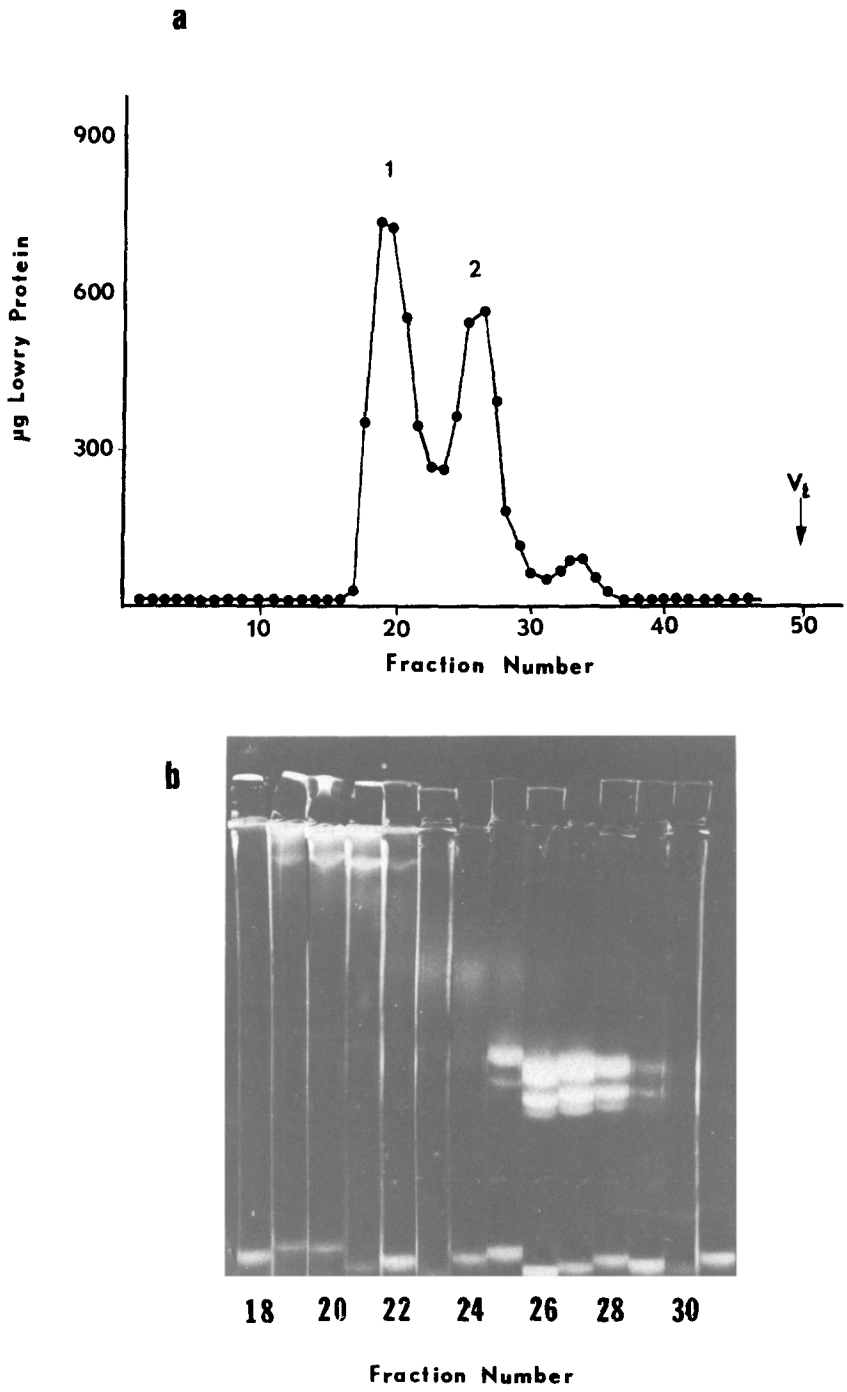

Figure 2. a. Fractionation of A1D5 on Ultrogel 34 in 1% SDS. 1 and 2 denote the two major protein peaks.
b. Electrophoresis of the Ultrogel 34 Fractions.

electrophoresed after reduction with mercaptoethanol, the two major bands migrated more slowly and had estimated molecular weights of 5.1×10^4 and 4.7×10^4. Clearly these two bands correspond to the two 'link proteins' reported by others[4,7]. The slower migrating band will be referred to as 'link protein' 1 and the faster one as 'link protein' 2. Another faint band, to be termed 'link protein' 3, migrated more rapidly than the others (Figure 1) This component, which was present in all A1D5 preparations examined, has not been previously reported. It appears that the high resolution which can be achieved using the discontinuous buffer systems of Neville[12] is responsible for the recognition of this additional band. Other bands, of protein-rich proteoglycans, are evident at the surface of stacker and running gels, and just within the running gels. Fluorescamine locates these bands with greater sensitivity than the Coomassie blue procedure as the fixation step employed in the latter method leads to the loss of protein-rich proteoglycan.

A1D5 (10 mg) fractionated on a column of Ultrogel 34 in 1% SDS gave the profile illustrated in Figure 2a. Two major protein peaks are evident: one at the void volume (peak 1) and the other well-included (K_{av}= 0.25-0.28, peak 2). Samples of each fraction (50 µl) were assayed by electrophoresis to determine the location of the 'link proteins' and protein-rich proteoglycans. The pattern of fluorescamine-labeled bands obtained is shown in Figure 2b. Fractions from the void volume peak (18-21) contained much fluorescent material at or near the surfaces of the stacker and running gels, which are the protein-rich proteoglycans. No 'link protein' bands are discernible in these fractions. Fractions through peak 2 (26-28, Figure 2a) contain the 'link protein' bands, but no bands of lesser mobility. There is no evidence that 'link proteins' 1,2 and 3 have been fractionated from each other through the fractions of peak 2. This fractionation is reminiscent of that obtained on Sephadex G-200 in 4.0 M guanidine[10], but Ultrogel has the advantage of greater stability toward denaturants and permits higher flow rates. Further-

Amino acid and amino sugar analyses of peaks 1 and 2 from Ultrogel 34.

Table 1

AMINO ACID	PEAK 1 Fractions 19-21	PEAK 2 Fractions 26-28
	Residues per 1000 residues of amino acid	
S-Carboxymethylcysteine	19	26
Aspartic Acid	97	130
Threonine	49	44
Serine	75	62
Glutamic Acid	124	88
Proline	90	51
Glycine	106	95
Alanine	81	75
Valine	57	57
Methionine	12	6
Isoleucine	36	31
Leucine	81	82
Tyrosine	25	58
Phenylalanine	41	50
Histidine	16	25
Lysine	39	53
Arginine	52	67
Glucosamine	80	24
Galactosamine	132	11
	Molar ratio	
Galactosamine:Glucosamine	1.65	0.46

more, the location of protein bands and subsequent electrophoresis analysis is greatly facilitated by replacing guanidine with SDS.

Fractions from peaks 1 (18-20) and 2 (26-28) were dialyzed exhaustively against water and freeze dried. Duplicate samples (150-200 μg of protein)

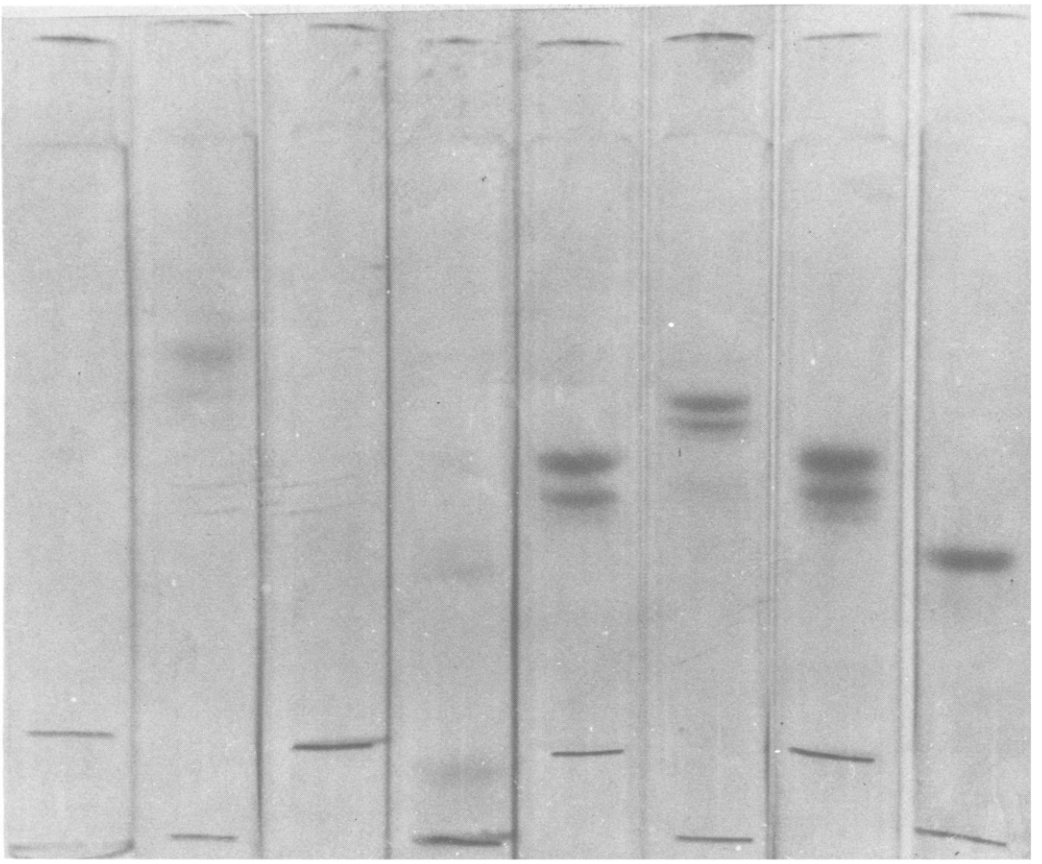

Figure 3. Electrophoresis of peak 1 and 2 samples (Fig. 2a) after cleavage with CNBr. Subscripts 1 and 2, denote that samples were from peaks 1 and 2, respectively. Before electrophoresis, samples were treated as follows:

Gel	Cyanogen Bromide	Mercaptoethanol
a	-	-
b	-	+
c	+	-
d	+	+

from both peaks were hydrolyzed and analyzed for amino acid and hexosamine content (Table 1). The most striking feature of these analyses is the low hexosamine content of the purified 'link protein' preparation (i.e. 35 residues of hexosamine/1000 residues of amino acid), which contrasts with 212 residues

of hexosamine/1000 residues of amino acid in the protein-rich proteoglycan fraction. Compared to peak 1 fractions, the 'link proteins' have a relatively higher aspartic acid, cystine, tyrosine, lysine and arginine content, but glutamic acid and proline are lower. A similar trend has been noted in the compositions of proteoglycans fractionated in a density gradient[15,16] (i.e. with decreasing buoyant density, proteoglycans tend to be smaller and more like the 'link proteins' in composition).

Duplicate samples of peaks 1 and 2 (31 and 15 μg of Lowry protein, respectively) were treated with CNBr. Prior to electrophoresis, one sample from each pair was reduced with mercaptoethanol, and the other remained unreduced. The controls detailed in the legend to Figure 3 were also examined. CNBr treatment of the 'link proteins' followed by reduction (Figure 3, gel d_2) caused loss of the 'link proteins' and the appearance of a new peptide band (peptide 1, mol. wt. approx. 2×10^4). Presumably other small peptides were also formed but were not stained with Coomassie blue. A sample which had not been CNBR treated, but was reduced, still showed the characteristic 'link protein' pattern of three bands (gel b_2). CNBr treatment without reduction (gel c_2) gave a 'link protein' profile which was identical to that of the unreduced control (gel a_2). Thus, reduction with mercaptoethanol after CNBr treatment is necessary for the disintegration of the 'link proteins' into smaller peptides. Apparently, the CNBr sensitive sites of the 'link proteins' are bridged by disulfide bonds. CNBr treatment of the protein-rich proteoglycan sample (peak 1) followed by reduction liberated a small amount of peptide 1 (gel d_1), but it was also noted, with some surprise, that reduction of peak 1 caused the liberation of some 'link protein' (gel b_1) which was not seen in an unreduced sample (gel a_1). It is likely that this small amount of 'link protein' is firmly associated with protein-rich proteoglycan by disulfide linkage, as heating in 1% SDS failed to dissociate it.

To determine whether the amount of peptide 1 formed from protein-rich proteoglycan (peak 1) corresponded to the small amount of 'link protein'

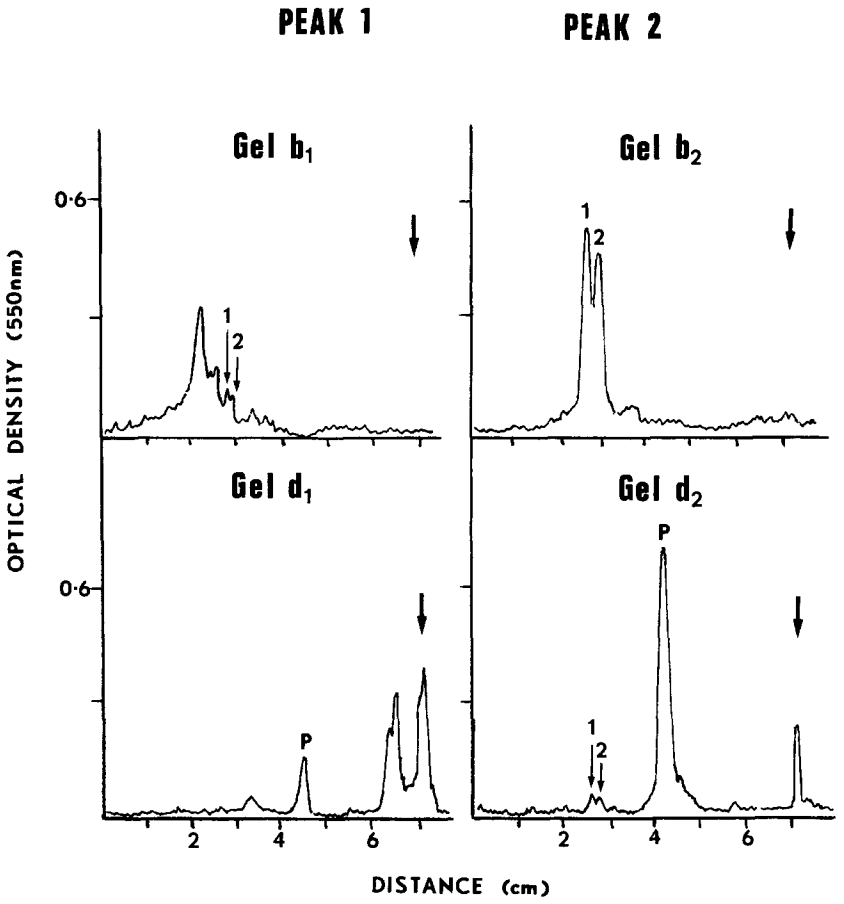

Figure 4. Densitometric scans of the Coomassie blue-stained gels. Labeling of gels (i.e. b_1, b_2, d_1 and d_2) is as given in the legend to Figure 3. Bands 1, 2 and P correspond to 'link proteins' 1 and 2 and cyanogen bromide peptide 1, respectively.

present in that sample, the ratio of peptide 1 in gel d_1 to 'link proteins' in gel b_1 was compared with the ratio of peptide 1 in gel d_2 to 'link proteins in gel b_2. The ratios (0.59 and 0.82, respectively) were sufficiently similar to show that any peptide 1 derived from peak 1 was a product of the small amount of associated 'link protein'. The lower ratio for the peak 1 sample probably results from an overestimation of the small amount of 'link proteins' present (Figure 4, gel b_1). If peptide 1 were derived from protein-rich proteoglycan in peak 1, much more peptide 1 would have been seen in gel d_1.

From the amount of peptide 1 (gel d_2, Figure 4) formed from 'link protein' (gel b_2), it appears likely that both 'link proteins' 1 and 2 are cleaved by CNBr to yield this peptide. That 'link proteins' 1 and 2 are probably structurally similar also follows from this result. The separation and structural relationship of 'link proteins' 1 and 2 are the topics of a future communication[13].

On the basis of immunological data[9], it has been suggested that the 'link proteins' are small fragments of proteoglycan which still retain the hyaluronic acid binding region. The CNBr cleavage data presented in this paper are incompatible with this notion. The 'link proteins' are apparently structurally distinct from the protein core of proteoglycan monomers.

ACKNOWLEDGEMENTS. This work was supported by NIH Grants DE 02670 and HL 11310. The expert technical assistance of Ms. J.M. Johnson and Mr. J.T. Ford is gratefully acknowledged.

REFERENCES

1. Hardingham, T.E. and Muir, H. (1972) Biochim. Biophys. Acta 279, 401-405.
2. Muir, H. and Hardingham, T.E. (1975) in Biochemistry of Carbohydrates (Ed. Whelan, W.J.) pp. 153-222 University Park Press.
3. Hascall, V.C. and Sajdera, S.W. (1969) J. Biol. Chem. 244, 2384-2396.
4. Keiser, H.D., Shulman, H.J. and Sandson, J.I. (1972) Biochem. J. 126, 163-169.
5. Gregory, J.D. (1973) Biochem. J. 133, 383-386.
6. Sajdera, S.W. and Hascall, V.C. (1969) J. Biol. Chem. 244, 77-87.
7. Hascall, V.C. and Heinegård, D. (1974) J. Biol. Chem. 249, 4232-4241.
8. Gregory, J.D. (1974) in Protides of the Biological Fluids (Ed. Peeters, H.), pp. 171-176 Pergamon Press.
9. Keiser, H.D. (1975) Biochemistry, 14, 5304-5307.
10. Oegema, T.P., Hascall, V.C. and Dziewiatkowski, D.D. (1975) J. Biol. Chem. 250, 6151-6159.
11. Heinegård, D. (1972) Biochim. Biophys. Acta 285, 181-192.
12. Neville, D.M. (1971) J. Biol. Chem. 245, 6328-6334.
13. Baker, J.R. and Caterson, B. (manuscript in preparation).
14. Heinegård, D. (1973) Chem. Scr. 4, 199-201.
15. Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S. and Strider, W. (1976) J. Biol. Chem. 251, 6439-6444.
16. Hardingham, T.E., Ewins, R.J.F. and Muir, H. (1976) Biochem. J. 157, 127-143.
17. Heinegård, D. and Hascall, V.C. (1974) J. Biol Chem. 249, 4251-4256.