Biochem. J. (1980) **185**, 101–105
Printed in Great Britain

Quantitative Analysis of Hyaluronate in Nanogram Amounts

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(*Received* 23 *July* 1979)

A radiosorbent technique allowing the specific analysis of 10ng of hyaluronate has been developed.

Quantitative analysis of hyaluronate in the microgram range is usually carried out by colorimetry, for example by various modifications of the carbazole method for uronic acid (Bitter & Muir, 1962). Before such an analysis can be performed hyaluronate must be separated from other polysaccharides by ion-exchange chromatography (Hallén, 1972), by fractionation with quaternary ammonium compounds (Scott, 1960), enzymically (Linker *et al.*, 1969; Ohya & Kaucko, 1970) or by some other technique. A combination of enzymic degradation and isotope dilution has also been used (Laurent *et al.*, 1969). The discovery that proteins exist that specifically bind to hyaluronate (Hardingham & Muir, 1972; Hascall & Heinegård, 1974*a*) has given us a new tool for the analysis. It has already been utilized by Hardingham & Adams (1976), who bound cartilage proteoglycans to hyaluronate and analysed the complex isolated chromatographically. I have now prepared ^{125}I-labelled hyaluronate-binding cartilage proteins and measured their adsorption on a hyaluronate-substituted gel (Tengblad, 1979) in the presence of competing free hyaluronate. The binding to gel can be used as an assay for free hyaluronate.

Experimental

Materials

Hyaluronate from rooster comb (Healon) with a molecular weight of 3.5×10^6 was kindly supplied by AB Pharmacia, Uppsala, Sweden. Part of the sample was degraded enzymically (Tengblad, 1979) to a molecular weight of 85000. Chondroitin 4-sulphate (Wasteson, 1971), dermatan sulphate (Teien *et al.*, 1976) and heparin (Lindahl *et al.*, 1965) were kindly given by colleagues at this Institute. Heparan sulphate isolated from pig mucosa was a gift from Dr. L. Rodén, Birmingham, AL, U.S.A. Hyaluronidase from *Streptomyces hyalurolyticus* was purchased from Seikaku Kogyo Co., Tokyo, Japan, and bovine serum albumin from Sigma Chemical Co., St. Louis, MO, U.S.A.

Hyaluronate-substituted agarose gels have recently been described (Tengblad, 1979). A Sepharose 4B gel containing 1.2mg of hyaluronate/ml and a similarly treated control gel containing no hyaluronate were used. Hyaluronate-binding proteins [an equimolar mixture of the hyaluronate-binding globular part of cartilage proteoglycan (fraction BI) and link-protein (fraction CII)] were previously isolated by affinity chromatography (Tengblad, 1979). They were reaggregated with an excess of hyaluronate (mol.wt. 85000), dialysed against water and freeze-dried. An amount corresponding to 100μg of protein was dissolved in 50μl of 0.25M-sodium phosphate buffer, pH7.4, and iodinated with 0.5mCi of ^{125}I by the chloramine-T method essentially as described by Greenwood *et al.* (1963). The hyaluronate and any degraded proteins were then removed by affinity chromatography (Tengblad, 1979), and the intact iodinated proteins were eluted from the gel and kept at 0.7μg/ml in 4M-guanidinium chloride/0.5M-sodium acetate buffer, pH5.7, until further use. The specific radioactivity in various preparations was 0.8×10^6–1.4×10^6 c.p.m./μg of protein.

Methods

Hyaluronate and other polysaccharides were determined by the carbazole method (Bitter & Muir, 1962), and protein was measured by the method of Lowry *et al.* (1951), with glucuronate and bovine serum albumin as standards respectively. Radioactivity was measured in a Packard Autogamma model 5260 scintillation spectrometer. Digestion of 4mg of chondroitin sulphate with 2 units of *Streptomyces* hyaluronidase (Ohya & Kaucko, 1970) was performed in 1ml of water (pH4.7) for 20h at 20°C. The enzyme was inactivated at 100°C for 30min. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on reduced samples according to the procedure of Neville (1971), and the necessary reagents were from Eastman Kodak (Rochester, NY, U.S.A.). The protein bands were stained with Coomassie Brilliant Blue R-250 (Merck, Darmstadt, Germany) (Korn & Wright, 1973). The

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gels were sliced into 1 mm-thick slices and the radioactivity of each slice was measured. Bovine serum albumin and ovalbumin (from Sigma Chemical Co.) were used as reference proteins.

Assay

The assay was performed in 3 ml plastic tubes that had been pretreated with bovine serum albumin dissolved in 0.5 M-sodium acetate buffer pH 5.7. Unless otherwise indicated, the concentration of bovine serum albumin was 0.1%. Hyaluronate-substituted gel suspended in buffer (1 M-NaCl/0.5 M-sodium acetate buffer, pH 5.7, containing 0.1% bovine serum albumin) was mixed with various amounts of free hyaluronate in a total volume of 500 μl. Then 50 μl of the solution of labelled protein (dissolved in 4 M-guanidinium chloride/0.5 M-sodium acetate buffer, pH 5.7) was added and the tubes were shaken at room temperature. The gel was pelleted by centrifugation at 900*g* for 10 min and the supernatant was removed. The gel was then washed and centrifuged three times with 3 ml of buffer before its radioactivity was measured.

In experiments designed to investigate the effect of pH on the binding the pH was varied by a change of buffer to a universal buffer containing diethylbarbituric acid, citric acid, potassium phosphate and boric acid (approx. 0.03 M each) and NaOH (0.02–0.1 M) (Britton & Welford, 1937). In other experiments the ionic strength was varied by the addition of NaCl.

The amounts of gel, free hyaluronate (or other polysaccharides) and protein as well as the incubation time were varied (see below). The standard incubation presently used in our laboratory contains 4 μl of wet gel, about 40 ng of protein with a total radioactivity of 40000 c.p.m. and 10–800 ng of free hyaluronate. Incubation time was 16 h.

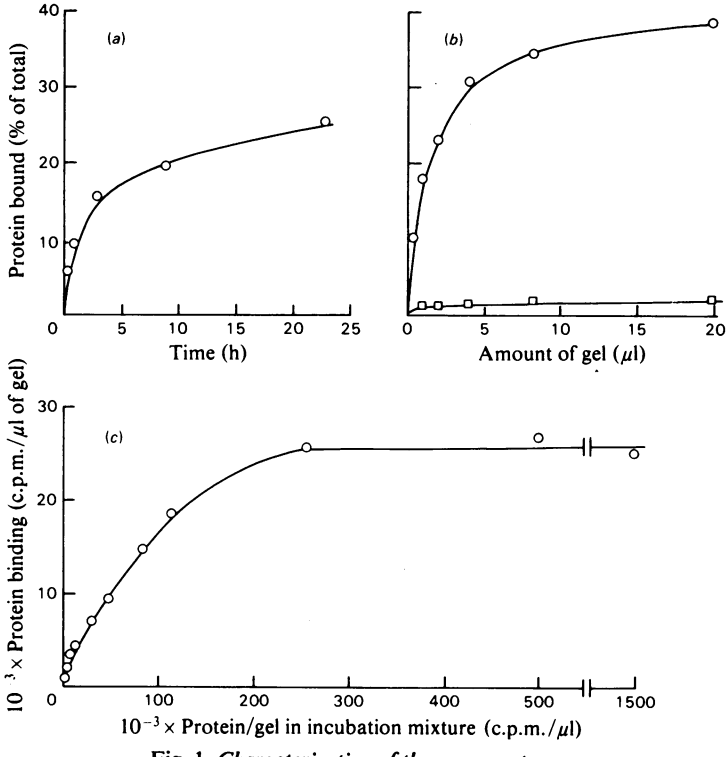

Fig. 1. *Characterization of the assay system*

^{125}I-labelled hyaluronate-binding protein and hyaluronate-Sepharose gel were mixed as described in the text and the binding of radioactivity to the gel was measured. (*a*) Time course of binding. A 4 μl portion of gel and about 35 ng of protein (35000 c.p.m.) were incubated for various time periods. (*b*) Binding capacity of the protein. Increasing amounts of hyaluronate–Sepharose gel (○) or control gel (□) were mixed with approx. 35 ng of protein and incubated for 16 h. Only about 40% of the protein seemed to have capacity for binding even at high gel concentrations. There is only a low unspecific adsorbtion on the control gel. (*c*) Binding capacity of the gel. Protein and gel were incubated for 16 h in different proportions. The gel content varied between 0.04 and 20 μl and the protein content between 15 and 52 ng (12000 and 50000 c.p.m.). Approx. 25000 c.p.m. (30 ng of protein) was bound/μl of gel at high protein/gel ratios.

Results and Discussion

In developing the present technique it was deemed necessary to use protein with the highest possible affinity and binding capacity for hyaluronate. I have therefore used a mixture of the globular part of the cartilage proteoglycan and the link protein, as it is known that these two components co-operate in binding to the polysaccharide (Hascall & Heinegård, 1974*b*). Iodination of the proteins was performed on the intact complex with hyaluronate to avoid substitution of the binding sites. The two proteins will presumably be labelled to different degrees, but for practical reasons I have assumed a uniform labelling in the calculations of protein content from radioactivity.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the material after elution from the hyaluronate–Sepharose showed two bands with mobilities corresponding to apparent mol.wts. of 45000 and 90000. This indicates that both the proteins (BI and CII) bound to the gel after the iodination.

Exploratory experiments are described in Fig. 1. Several hours of incubation were required before a reasonable equilibrium in protein binding to the gel was attained (Fig. 1*a*). A standard incubation time of 16h was therefore chosen in subsequent work. The experiment described in Fig. 1(*b*) shows that, in spite of an increase in gel concentration, only about 40% of the total labelled protein was adsorbed on the gel. In similar experiments with other batches of proteins different percentages were adsorbed. The difference might be due to a decrease in the capacity of the protein batch to bind with increased storage time. At 2 weeks after the elution from the hyaluronate–Sepharose 42% of the protein was bound under the assay conditions and 28 days later only 30% was bound. Some of the protein may also have lost its binding ability as a result of the desorption with 4M-guanidinium chloride from the hyaluronate–Sepharose affinity column. Furthermore, some of the proteins might not be able to bind to the hyaluronate–Sepharose owing to protein aggregation, which occurs at 0.4M-guanidinium chloride, which is the final concentration of guanidinium chloride in the binding assay. I have in subsequent experiments expressed the binding of protein as a percentage of maximal binding in the system used rather than as a percentage of total added protein. A series of experiments in which the ratio of protein to gel in the incubation mixture was varied over a large range is described in Fig. 1(*c*). At a large excess of protein (saturation conditions) there was approx. 30ng of protein bound/μl of gel. This number, as well as the whole binding profile shown in Fig. 1(*c*), should be slightly higher at higher incubation times, since the binding was not complete at 16h (Fig. 1*a*).

The influences of pH, temperature, ionic strength and albumin concentration are summarized in Table 1. The variations were small in the region of the incubation conditions chosen for the standard assay. The increase in binding at zero salt concentration was due to unspecific binding to the gel and the tubes. The albumin decreased the unspecific binding to the tubes from more than 5% to 0%.

The inhibiting effect of free hyaluronate on the protein binding to the gel is shown in Fig. 2. At the higher gel concentrations there is a linear relationship between protein bound and the logarithm of added free hyaluronate (in the range 5–500ng). The experiments have a high reproducibility, and the curves in Fig. 2 can be used as standard curves in determinations of unknown amounts of hyaluronate. The linearity disappears at the lower gel concentrations, and the binding becomes sensitive to lower concen-

Table 1. *Influence of pH, salt (NaCl), albumin concentration and temperature on the binding of ^{125}I-labelled protein to hyaluronate–Sepharose gel*

Relative binding is the ratio of the amount of bound protein at the specified condition to that bound at standard assay conditions. At a given condition specified in the Table all the other conditions were those of the standard assay conditions.

pH	Relative binding	Concn. of NaCl (M)	Relative binding	Concn. of albumin (g/l)	Relative binding	Temperature (°C)	Relative binding
2.5	0.43	0	1.22	0	0.84	3	0.53
3.4	1.00	0.4	0.94	0.7	1.05	6	0.68
4.4	1.13	0.8	0.96	1.7	0.97	16	0.91
5.6	1.16	1.2	1.00	3.5	1.00	26	0.89
6.4	1.03	1.6	1.00	7.0	0.71	31	1.04
7.0	1.19	2.0	0.83				
8.0	1.05	2.3	0.75				
8.6	0.99	2.7	0.32				
10.3	0.62	3.1	0.35				
11.2	0.25						
11.9	0.10						

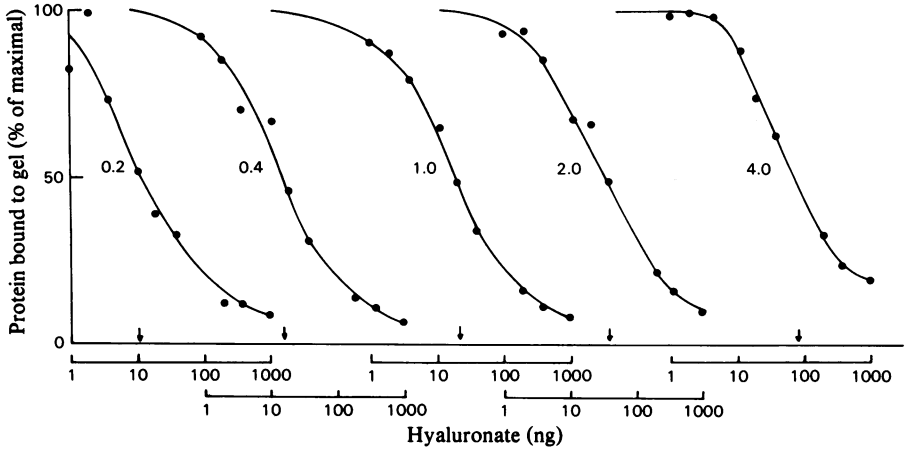

Fig. 2. *Inhibition of protein binding to gel by free hyaluronate*
Hyaluronate-binding protein (approx. 35ng), hyaluronate–Sepharose gel (0.2, 0.4, 1, 2 and 4 μl in five separate series) and free hyaluronate (0–1000ng; mol.wt. 85000) were incubated for 16h as described in the text. The amount of protein bound is given on the ordinate as a percentage of the binding in the absence of free hyaluronate. The arrows at 11, 15, 20, 36 and 79ng of hyaluronate indicate the amounts of free polysaccharide that are needed for 50% inhibition of the protein binding to the different quantities of gel.

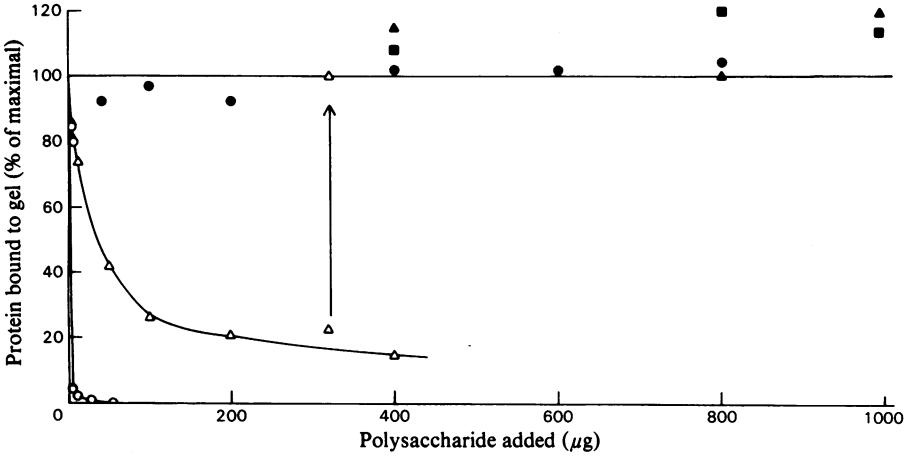

Fig. 3. *Effect of different polysaccharides on the binding of iodinated proteins to hyaluronate–Sepharose gel*
The capacities of the following polysaccharides for inhibiting protein binding to the hyaluronate–Sepharose gel were tested: hyaluronate (○), chondroitin sulphate (△), dermatan sulphate (▲), heparan sulphate (●) and heparin (■). The incubation system contained 4 μl of gel and about 30ng of iodinated protein. Only hyaluronate and chondroitin sulphate inhibited the binding. The effect of the latter polysaccharide was due to contamination with hyaluronate, because it disappeared after digestion with a specific hyaluronate-degrading enzyme (demonstrated by an arrow in the Figure) between an undigested and a digested sample.

trations of free hyaluronate. It is possible to detect, although with low precision, as little as 2ng of free hyaluronate. The experiments presented in Fig. 2 were performed with hyaluronate of mol.wt. 85000. Identical results were obtained with the sample of mol.wt. 3.5×10^6.

I have chosen to work with assay systems containing 4 μl of gel. Ten samples containing 10ng and ten samples containing 100ng of hyaluronate were analysed in this system. The resulting mean values ± S.D. were 10.3 ± 1.4 and 100 ± 12 respectively. The error in the analysis is thus about 15%.

The specificity in the analysis was tested by adding other glycosaminoglycans than hyaluronate to the incubations. As demonstrated in Fig. 3, even 1000-fold larger amounts of dermatan sulphate, heparan sulphate or heparin did not inhibit the protein binding to the gel. The polysaccharides actually caused a small increase in binding, which could be explained by a polymer exclusion effect (Comper & Laurent, 1978). The chondroitin sulphate preparation did prevent protein binding to the gel in concentrations about 100-fold higher than that of hyaluronate. This turned out to be due to a contamination by hyaluronate, which could be removed by digestion with *Streptomyces* hyaluronidase, an enzyme specific for hyaluronate.

The technique seems to have a great potential in biological experiments. It is simple to perform and the number of analyses that can be carried out simultaneously is large. Its sensitivity and specificity make it useful for analysis of tissue fluids, although possible interactions from other tissue components have not yet been analysed.

I thank Professor T. C. Laurent for his continued advice and interest in this project. This work has been supported by grants from the Swedish Medical Research Council (Project no. 13x-4), Svenska Sällskapet för Medicinsk Forskning and the University of Uppsala.

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