Stack, M. S., & Gray, R. D. (1989) *J. Biol. Chem. 264*, 4277–4281.
Steinbrink, D. R., Bond, M. D., & Van Wart, H. E. (1985) *J. Biol. Chem. 260*, 2771–2776.
Stöcker, W., Wolz, R. L., Zwilling, R., Strydom, D. A., & Auld, D. S. (1988) *Biochemistry 27*, 5026–5032.
Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., & Neurath, H. (1972) *Nature (London) New Biol. 238*, 35–37.
Titani, K., Torff, H.-J., Hormel, S., Kumar, S., Walsh, K. A., Rödl, J., Neurath, H., & Zwilling, R. (1987) *Biochemistry 26*, 222–226.
Torff, H.-J., Dörsam, H., & Zwilling, R. (1980) *Protides Biol. Fluids 28*, 107–110.
Vallee, B. L., & Auld, D. S. (1990a) *Biochemistry 29*, 5647–5659.
Vallee, B. L., & Auld, D. S. (1990b) in *Matrix Metalloproteinases and Inhibitors* (Birkedal-Hansen, H., Werb, Z., Welgus, H., & Van Wart, H. E., Eds.) Gustav Fischer Verlag, Stuttgart, West Germany (in press).
Vogt, G., Stöcker, W., Storch, V., & Zwilling, R. (1989) *Histochemistry 91*, 373–381.
Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., Kriz, M. J., Luxenberg, D. P., Sibley, B. S., & Wozney, J. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9484–9488.
Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H. J., Smith, B. J., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P., & Docherty, A. J. P. (1986) *Biochem. J. 240*, 913–916.
Wilhelm, S. M., Collier, I. E., Mormer, B. L., Eisen, A. Z., Grant, G. A., & Goldberg, G. I. (1989) *J. Biol. Chem. 264*, 17213–17221.
Wolz, R. L., & Zwilling, R. (1989) *J. Inorg. Biochem. 35*, 157–167.
Wolz, R. L., Stöcker, W., Auld, D. S., & Zwilling, R. (1987) 18th FEBS Meeting, June 28–July 3, Ljubljana, Yugoslavia.
Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., & Wang, E. A. (1988) *Science 242*, 1528–1534.
Zwilling, R., & Neurath, H. (1981) *Methods Enzymol. 80*, 633–664.
Zwilling, R., Dörsam, H., Torff, H.-J., & Rödl, J. (1981) *FEBS Lett. 127*, 75–78.

Characterization of an Intracellular Hyaluronic Acid Binding Site in Isolated Rat Hepatocytes†

Stephen J. Frost, Rampyari H. Raja,‡ and Paul H. Weigel*
The University of Texas Medical Branch, Department of Human Biological Chemistry and Genetics, Galveston, Texas 77550
Received May 4, 1990; Revised Manuscript Received August 14, 1990

ABSTRACT: ^{125}I-HA, prepared by chemical modification at the reducing sugar, specifically binds to rat hepatocytes in suspension or culture. Intact hepatocytes have relatively few surface ^{125}I-HA binding sites and show low specific binding. However, permeabilization of hepatocytes with the nonionic detergent digitonin results in increased specific ^{125}I-HA binding (45–65%) and a very large increase in the number of specific ^{125}I-HA binding sites. Scatchard analysis of equilibrium ^{125}I-HA binding to permeabilized hepatocytes in suspension at 4 °C indicates a $K_d = 1.8 \times 10^{-7}$ M and 1.3×10^6 molecules of HA ($M_r \sim 30\,000$) bound per cell at saturation. Hepatocytes in primary culture for 24 h show the same affinity but the total number of HA molecules bound per cell at saturation decreases to $\sim 6.2 \times 10^5$. Increasing the ionic strength above physiologic concentrations decreases ^{125}I-HA binding to permeable cells, whereas decreasing the ionic strength causes a ~4-fold increase. The divalent cation chelator EGTA does not prevent binding nor does it release ^{125}I-HA bound in the presence of 2 mM $CaCl_2$, although higher divalent cation concentrations stimulate ^{125}I-HA binding. Ten millimolar $CaCl_2$ or $MnCl_2$ increases HA binding 3–6-fold compared to EGTA-treated cells. Ten millimolar $MgCl_2$, $SrCl_2$, or $BaCl_2$ increased HA binding by 2-fold. The specific binding of ^{125}I-HA to digitonin-treated hepatocytes at 4 °C increased >10-fold at pH 5.0 as compared to pH 7. The kinetics of ^{125}I-HA binding to intact hepatocytes at 37 °C was rapid and similar to the kinetics of ^{125}I-HA binding at 4 °C ($t_{1/2} \sim 5$ min). Very little ^{125}I-HA was internalized after 4 h at 37 °C (460 molecules cell^{-1} h^{-1}). This rate is extremely slow (~1–3%) compared to the rate of receptor-mediated internalization of other ligands and indicates that HA uptake occurs by a noncoated pit pathway, probably reflecting general membrane pinocytosis. There is no evidence for recycling of the surface HA binding sites or use of the large intracellular reservoir for endocytosis.

Hyaluronic acid (HA)[1] is a ubiquitous component of the mammalian extracellular matrix (Fraser & Laurent, 1989). HA is an important mediator in many biological processes including cell adhesion (Underhill & Dorfman, 1978; Underhill, 1982), morphogenesis (Toole, 1981), wound healing (Toole, 1981; Weigel et al., 1986), tissue remodeling (Toole,

† This research was supported by National Institutes of Health Grant GM 35978.
* To whom correspondence should be addressed.
‡ Present address: Department of Medicine, Room 767, Diabetes Research Hospital, University of Alabama, Birmingham, AL 35294.

[1] Abbreviations: HA, hyaluronic acid; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hank's balanced salt solution; MES, 4-morpholineethanesulfonic acid.

0006-2960/90/0429-10425$02.50/0

1981), angiogenesis (West et al., 1985), and tumorigenesis (Toole et al., 1979; Knudson et al., 1984). The precise function of HA in these diverse physiological processes is not well understood. However, many studies have shown that HA interacts with other molecules on the cell surface and in the extracellular matrix. HA interacts with plasma components like fibrinogen (LeBoeuf et al., 1986), with cartilage proteoglycan and link protein in the extracellular matrix (Hascall, 1977), and with hyaluronectin on the surface of oligodendrial cells (Asou et al., 1983). Also, specific cell surface receptors or binding sites for HA have been reported on 3T3 and SV3T3 cells (Underhill & Toole, 1980), rat hepatocytes (Truppe et al., 1977), liver endothelial cells (Smedsrod et al., 1984; Raja et al., 1988), and alveolar macrophages (Love et al., 1979). The extent to which HA directly or indirectly interacts with cells and mediates subcellular events may be fundamental to understanding the actions of HA in a variety of biological processes.

Studies on HA interactions with isolated rat liver cells have recently received much attention because of the involvement of the liver in the clearance of HA from the circulation (Fraser et al., 1981) and the increasing therapeutic application of HA in the treatment of arthritis, tendon repair, and eye surgery (Gerke et al., 1985; St. Onge et al., 1980; Weiss et al., 1981). The earliest report on HA binding to liver cells concluded that cultured rat hepatocytes internalize HA by adsorptive endocytosis and then degrade it (Truppe et al., 1977). However, later studies showed that endothelial cells are the major sites of HA uptake and degradation in the liver (Fraser et al., 1981).

We addressed this apparent contradiction by studying HA binding to purified endothelial cells (Raja et al., 1988; McGary et al., 1989) and hepatocytes (Frost et al., 1988). In agreement with Laurent and co-workers (Smedsrod et al., 1984; Fraser et al., 1981) we conclude that liver endothelial cells actively endocytose HA via a recycling receptor system (McGary et al., 1989) that is specific for several types of glycosaminoglycans. We also reported the presence of an HA binding site in intact and permeabilized hepatocytes (Frost et al., 1988). In the present study, we have further characterized the ^{125}I-HA binding protein(s) in digitonin-treated hepatocytes.

Experimental Procedures

Materials. Digitonin was from Matheson, Coleman and Bell (Norwood, OH), Sigma Chemical Co. (St. Louis, MO), or Kodak Chemical Co. (Rochester, NY). Investigators should note that the purity of digitonin from Matheson, Coleman and Bell and Sigma has changed so that a 1.4% (w/v) stock solution in absolute ethanol can no longer be prepared. We find that Kodak Chemical Co. presently supplies the most suitable digitonin for these studies. 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (iodogen) was from Pierce Chemicals (Rockford, IL). Na^{125}I (10–20 mCi/μg iodine) was from Amersham Corp. ^{125}I-Asialoorosomucoid was prepared as described by Clark et al. (1987). BSA (CRG-7) was from Armour Biochemicals (Tarrytown, NY). Collagenase (type D) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Male Sprague-Dawley rats (200 g) were obtained from Harlan Breeding Laboratories, Houston, TX.

Preparation of the Hexylamine Derivative of HA and ^{125}I-HA. HA (human umbilical cord), obtained from Sigma Chemical Co., was further purified after complexing it with cetylpyridinium chloride by fractionation on Celite and ethanol precipitation as described by Scott (1960). Low molecular weight HA was obtained directly from Sigma Chemical Co (a lot of unusually low $M_r \sim 60000$) or native HA was sonicated under controlled conditions (Raja et al., 1988) to yield HA oligosaccharides with $M_r \sim 30000$. The HA was converted to a hexylamine derivative, which is chemically modified only at the terminal reducing sugar of the HA polymer and purified as described previously (Raja et al., 1984), with minor modifications (Frost et al., 1988). The Bolton-Hunter adduct of the hexylamine derivative of HA was prepared as described previously (Raja et al., 1984). The hydroxylphenyl propionyl derivative of HA thus obtained was then iodinated according to the procedure of Fraker and Speck (1978) with minor modifications (Raja et al., 1984). Specific activities are on the order of (2–4) $\times$ 10^6 dpm/μg HA. The ^{125}I-HA is stable for at least 20 days at 4 °C.

Media and Buffers. Medium 1 contains modified Eagle's medium [Grand Island Biological Co. (GIBCO), Grand Island, NY] supplemented with 2.4 g/L HEPES, pH 7.4, and 0.22 g/L $NaHCO_3$. Medium 1/BSA is medium 1 with 0.1% (w/v) BSA. William's medium E obtained from Flow Laboratories Inc. (McLean, VA) was supplemented with 10 mM HEPES, pH 7.4, 2 mM glutamine, penicillin/streptomycin (100 units/mL), garamycin (0.02 mg/mL), insulin (50 munits/mL), dexamethasone (5 $\times$ 10^{-7} M), and 5% (v/v) fetal calf serum obtained from Flow Laboratories. Buffer 1 contains 143 mM NaCl, 6.8 mM KCl, and 10 mM HEPES (pH 7.4). Buffer 1/BSA is buffer 1 with 1.5% (w/v) BSA. Hank's balanced salt solution (HBSS) was formulated as described in the GIBCO catalogue.

Cell Preparations. Rat hepatocytes were prepared by collagenase perfusion of the liver according to the procedure of Seglen (1973) with minor modifications (Clarke et al., 1987). Final cell pellets were resuspended at 4 °C in medium 1/BSA and were routinely >85% viable as judged by trypan blue exclusion. For studies with hepatocytes in suspension, the cells were incubated for 1 h at 2 $\times$ 10^6 cells/mL in medium 1/BSA in a gyratory shaker at 100 rpm to allow them to recover from the collagenase perfusion. The hepatocytes were then further purified by fractionation over discontinuous Percoll gradients to separate nonparenchymal cells and dead hepatocytes from viable hepatocytes (Dalet et al., 1982). The viable hepatocytes pellet to the bottom of the gradient. The parenchymal cells (99% purity and 98% viability) were resuspended in either medium 1/BSA or buffer 1/BSA. Hepatocytes were also cultured and maintained as described previously (Oka & Weigel, 1987) in complete William's media E on uncoated 35-mm tissue culture dishes (Falcon) for 24 h prior to use.

^{125}I-HA Binding to Permeabilized Hepatocytes. Freshly purified rat hepatocytes [(2–5) $\times$ 10^6 cells/mL] were incubated with ^{125}I-HA in the absence (total binding) or presence (nonspecific binding) of ≥100-fold excess of nonradiolabeled HA in 12 mm $\times$ 75 mm plastic tubes at 4 °C for 60–90 min with gentle mixing by hand every 5 min. After the incubation, aliquots of the cell suspension (500–550 μL) were layered over 900 μL of an oil mixture consisting of dibutyl phthalate and dioctyl phthalate (2:1). The cells were pelleted by centrifugation in a microfuge at 12000 rpm for 30 s (intact hepatocytes) or 3 min (permeabilized hepatocytes). The tips of the microfuge tubes containing the cell pellets were cut and placed in 12 mm $\times$ 75 mm γ tubes. This procedure was fast and had minimal loss of hepatocytes. When DNA was used to estimate the number of cells after ^{125}I-HA binding, permeabilized hepatocytes were washed three times by centrifugation at 1200 rpm for 2 min at 4 °C in a Beckman tabletop centrifuge (Model TJ-6) with the buffer used for ^{125}I-HA binding. After the cells were washed, the bound ^{125}I-HA was determined and the specific binding calculated by subtracting the nonspecific

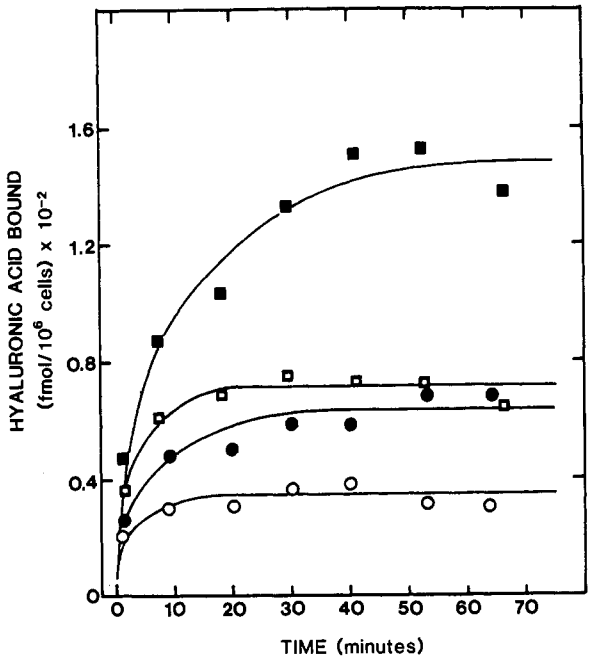

FIGURE 1: Kinetics of ^{125}I-HA binding to intact or permeabilized hepatocytes in suspension. Purified hepatocytes (2×10^6 cells/mL) were incubated with 3×10^{-8} M ^{125}I-HA (M_r ~30000) at 4 °C either in the presence (□, ■) or the absence (○, ●) of 0.055% digitonin and in the absence (●, ■) or presence (□, ○) of 2×10^{-5} M unlabeled HA. At the indicated times, 500-μL aliquots of the cell suspension were withdrawn and washed by centrifugation through oil, and bound ^{125}I-HA was determined as described under Experimental Procedures.

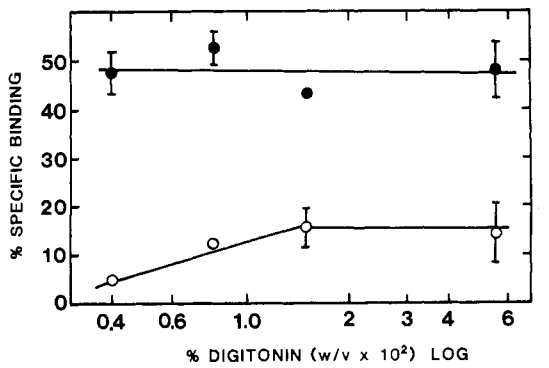

FIGURE 2: Effect of digitonin concentration on the competition for ^{125}I-HA binding by high and low molecular weight HA. Purified rat hepatocytes (4×10^6 cells/mL) were permeabilized for 20 min at 4 °C with the indicated concentrations of digitonin in buffer 1 plus 1.5% BSA and 133 μM PMSF. The cells were washed three times with buffer 1, resuspended in medium 1/BSA at 1×10^7 cells/mL, and 100 μL was added to 100 μL of 2×10^{-7} M ^{125}I-HA in the presence or absence of a 100-fold weight excess of nonradiolabeled HA of M_r ~60K (●) or >400K (○). The cells were incubated for 1 h at 4 °C and washed by centrifugation, and radioactivity and DNA content were determined as described under Experimental Procedures. Each point represents the average of triplicate determinations. The slopes of the lines derived from the least-squares linear regression analysis were analyzed by a Student's t test to determine whether they were significantly greater than zero; t = slope/(the sample standard deviation of the slope). Using the Student's t test, the slope of the line with larger HA (○) was statistically greater than zero ($p < 0.01$) between 0.004% and 0.015% digitonin. The slope of the line with low MW HA was not significantly greater than 0.

binding from the total binding. The percent specific binding (SB%) was calculated by dividing each individual nonspecific binding point (NS) by the average of the total binding (T) and subtracting this from 1. That is, SB% = [1 - (NS/T)] × 100.

^{125}I-HA Binding to Cultured Hepatocytes. Cells in culture were washed twice with HBSS just before the experiment. Cultured hepatocytes (1×10^6 cells/plate) were incubated with medium 1/BSA containing ^{125}I-HA in the absence (total binding) or the presence (nonspecific binding) of ≥100-fold excess of nonradiolabeled HA at 4 °C for 1 h. After the incubation, the plates were quickly washed at 4 °C once with 2 mL of medium 1/BSA and three times with 2 mL of HBSS. Under these conditions, the ^{125}I-HA binding to the dish itself was negligible. The cells were then solubilized in 0.3 N NaOH and placed into 12 × 75 mm γ tubes and the bound ^{125}I-HA was determined. Specific binding was calculated as above.

General. Protein was measured by using the method of Bradford (1976) with BSA as a standard. ^{125}I radioactivity was determined by using a Packard Multiprias 2 γ spectrometer. DNA was determined by the procedure of Labarca and Paigen (1980) with calf thymus DNA as a standard. One million hepatocytes have ~18 μg of DNA. In experiments with duplicate or triplicate samples, the error bars represent the sample standard deviation. Error bars not shown are within the symbols.

RESULTS

Kinetics of ^{125}I-HA Binding to Intact and Permeabilized Hepatocytes. The distribution of membrane-bound receptors or enzymes between the cell surface and the cell interior can be determined in the presence of the nonionic detergent digitonin without gross disruption of cell organelles (Weigel et al., 1983). Digitonin at 0.055% (w/v) permeabilizes hepatocytes and releases soluble cytoplasmic proteins (~50% of the total protein) with subunit molecular weights of at least 200000.

We used intact or digitonin-treated cells to examine whether hepatocytes had surface and/or intracellular binding sites for ^{125}I-HA. The kinetics of specific ^{125}I-HA binding to intact or permeabilized hepatocytes at 4 °C was rapid and was 50% complete in ~10 min (Figure 1). Specific ^{125}I-HA binding was maximal within 60 min for either intact or permeable cells. In most experiments, the percent specific binding of ^{125}I-HA ranged from 15% to 40% for intact hepatocytes and from 45% to 65% for permeabilized hepatocytes. Moreover, at low nonsaturating concentrations, the amount of ^{125}I-HA (M_r ~30000) specifically bound to permeabilized hepatocytes was ~3–4 times greater than that to intact cells (Figure 1). Thus, between 67% and 75% of the total cellular HA binding sites may be intracellular. Using a larger ^{125}I-HA (M_r ~60000) in another study, we found ~90% of the total cellular HA binding sites to be intracellular (Frost et al., 1988).

Intracellular Location of HA Binding Sites Exposed by Digitonin. Because of its polymeric nature HA can exhibit a considerable range of size (e.g., 10000 < M_r > 1000000). Since increasing concentrations of digitonin cause increasingly greater permeabilization of the plasma membrane and intracellular membranes (Weigel et al., 1983), we could use this property of HA to confirm the intracellular localization of these HA binding sites in rat hepatocytes. The rationale is that large HA (but not small HA) would have difficulty penetrating the plasma membrane of rat hepatocytes only mildly permeabilized with low concentrations of digitonin. Therefore, large HA would not be able to compete as well as small HA for the binding of the smaller ^{125}I-HA to intracellular binding sites. Higher concentrations of digitonin, which cause larger holes in the membranes, would eliminate this effect and would allow larger HA to compete for ^{125}I-HA binding (Raja et al., 1988).

These predictions were validated when we assessed the differential effect of low molecular weight HA (M_r ~60000)

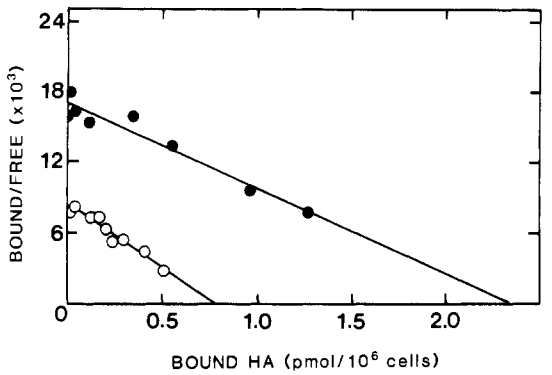

FIGURE 3: Scatchard analysis of equilibrium binding of ^{125}I-HA to permeabilized hepatocytes in suspension or culture. Isolated rat hepatocytes in suspension (2×10^6 cells/mL; ●) or culture (1×10^6 cells/plate; ○) were incubated with increasing concentrations of ^{125}I-HA (M_r ~30000) in the presence of 0.055% digitonin with and without 3×10^{-5} M unlabeled HA at 4 °C for 90 min. At the end of the incubation, cell-free supernatant was removed to determine the free HA concentration. The suspension cells were washed by centrifugation through oil, and the hepatocyte cultures were rinsed three times in HBSS. The bound ^{125}I-HA was determined as described under Experimental Procedures and the data were analyzed by the procedure of Scatchard (1949). Each point represents the average of duplicate determinations. The lines were calculated by least-squares linear regression analysis (●, $r = -0.996$; ○, $r = -0.973$).

versus higher molecular weight HA (M_r >400000) to compete for the binding of ^{125}I-HA (M_r ~60000) to permeabilized hepatocytes (Figure 2). The percent specific binding (specific binding/total binding) with low MW HA as competitor did not increase with increasing digitonin concentration. This means that both ^{125}I-HA and the low molecular weight HA had equal access to the HA binding sites. However, the percent specific binding with higher MW HA as competitor did increase as the digitonin concentration increased from 0.004% to 0.015%. There is a possibility that the large HA may be sterically inaccessible to HA binding sites, since the competition of high MW HA did not reach the same competition as the low MW HA. To assess this a dot-blot assay was performed on detergent extracts immobilized onto nitrocellulose with ^{125}I-HA in the absence or presence of small or large MW HA. In this situation steric hindrance should be minimal. The high MW HA was still only about 50% as effective as the low MW HA in competing ^{125}I-HA binding in this assay. This is virtually the same result as seen in Figure 2. The lower competition of ^{125}I-HA with high MW HA may reflect the lower molar concentration (and excess) of high MW HA (>15-fold) as compared to low MW HA (100-fold). This result suggests that large HA can compete for ^{125}I-HA binding only when the degree of permeabilization has passed the size-exclusion limit for this HA. Thus we conclude that the HA binding sites exposed by digitonin are localized intracellularly and not on the cell surface; they are not just a byproduct of the digitonin treatment.

Saturation of ^{125}I-HA Binding by Permeabilized Hepatocytes at 4 °C. Isolated intact hepatocytes have at least 9000 specific surface HA binding sites/cell at 1×10^{-7} M ^{125}I-HA (Frost et al., 1988). However, this concentration of HA is subsaturating and is an underestimate of the actual number of surface binding sites. Also high nonspecific binding of HA (~80%) made equilibrium binding studies difficult to perform and these studies did not consistently demonstrate saturability. Therefore, Scatchard analysis was not applicable for determining the affinity or the number of HA binding sites on intact hepatocytes. However, when equilibrium binding studies were performed by using permeabilized hepatocytes either in suspension or in culture, saturation of ^{125}I-HA (M_r ~30000) binding was observed at ~5×10^{-7} M HA (Figure 3). Scatchard analyses from this and other experiments are summarized in Table I. The dissociation constant, K_d, for ^{125}I-HA binding to cells in suspension or in culture was $(1.8 \pm 0.8) \times 10^{-7}$ M ($n = 3$) and $(1.6 \pm 0.9) \times 10^{-7}$ M ($n = 3$), respectively. The number of binding sites/cell for the cells in suspension was $(1.3 \pm 0.1) \times 10^6$, whereas cells in culture exhibited fewer sites per cell, $(0.62 \pm 0.13) \times 10^6$.

Table I: Scatchard Analysis of ^{125}I-HA Binding to Permeabilized Hepatocytes in Suspension and in Culture[a]

experiment	K_d ($\times 10^7$ M)[b]	B_{max} ($\times 10^{-6}$)[c]
Hepatocytes in Suspension		
1	0.8	1.3
2	2.1	1.2
3	2.4	1.4
mean	1.8 ± 0.8	1.3 ± 0.1
Hepatocytes in Culture		
1	1.0	0.47
2	2.7	0.70
3	1.2	0.68
mean	1.6 ± 0.9	0.62 ± 0.13

[a] Equilibrium binding experiments were performed at 4 °C and analyzed as described in Figures 3 and 4. [b] Dissociation constant. [c] Molecules of HA bound per cell.

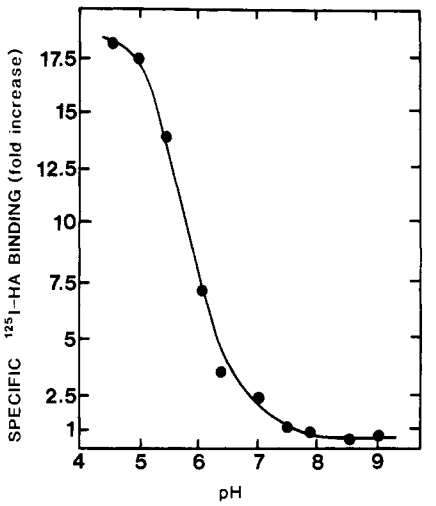

FIGURE 4: Effect of pH on ^{125}I-HA binding to permeabilized hepatocytes in suspension. Purified hepatocytes (2×10^6 cells/mL) were incubated with 1×10^{-7} M ^{125}I-HA (M_r ~30000) and 0.055% digitonin in the presence or absence of 8.33×10^{-6} M nonradioactive HA in 0.02 M MES or 0.02 M HEPES buffers at the indicated pH values for 1 h at 4 °C. The cells were then washed by centrifugation through oil, and the bound ^{125}I-HA was determined as described under Experimental Procedures. The points represent the average of duplicates or triplicates, and the sample standard deviation was not greater than ±12% of the mean for each point.

Effect of pH on ^{125}I-HA Binding to Permeabilized Hepatocytes. Since permeabilized hepatocytes have a large number of intracellular HA binding sites, they may have an important cellular function. Therefore, we wanted to characterize further the HA binding site(s) in permeabilized hepatocytes. We first examined the effect of pH on ^{125}I-HA binding to these cells (Figure 4). Unlike many receptor–ligand complexes that dissociate at low pH (Yamashiro & Maxfield, 1984), specific ^{125}I-HA binding by hepatocytes treated with digitonin demonstrated a ≥10-fold increase in specific ^{125}I-HA binding at pH 5.0 compared to physiological pH. This increased HA binding capacity of hepatocytes at low pH was reversible. Permeabilized cells, which were first allowed to bind ^{125}I-HA at pH 5 for 1 h, were then washed with HBSS at pH 7.4. These cells specifically bound lower amounts of ^{125}I-HA

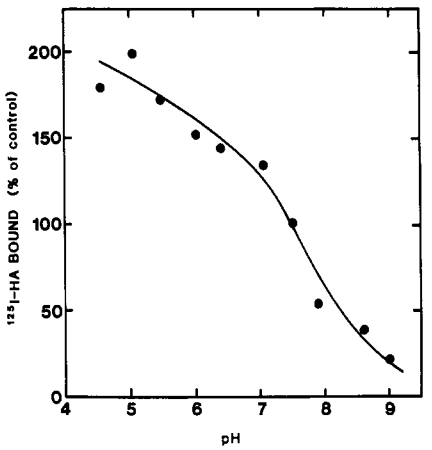

FIGURE 5: Effect of pH on dissociation of prebound ^{125}I-HA from permeabilized hepatocytes. Purified hepatocytes (5×10^6 cells/mL) in medium 1/BSA were incubated for 1 h at 4 °C with 1×10^{-7} M ^{125}I-HA (M_r ~30000) and 0.055% digitonin in the presence or absence of a 100-fold excess of nonradiolabeled HA. The cells were then washed once by centrifugation with medium 1/BSA and resuspended in 10 mL of medium 1/BSA, and 1-mL aliquots were transferred to 13 × 100 mm tubes and centrifuged at 200g for 2 min. The supernatant fluid was removed by aspiration and the cell pellets were resuspended in 2 mL of buffer at the indicated pH. Buffers contained 143 mM NaCl, 6.8 mM KCl, 2 mM $CaCl_2$, 0.1% BSA, and either 20 mM HEPES for the pH range of 6.5–9.0 or 20 mM MES for the pH range of 4.5–6.0. The cells were incubated for 30 min at 4 °C and centrifuged, and the supernatant fluid was removed. The cell pellet was dissolved in 0.3 N NaOH, and protein and bound ^{125}I-HA were determined as described under Experimental Procedures. Each point is an individual determination.

(~40%) as compared with permeabilized cells that were incubated with ^{125}I-HA at pH 7.4 and washed at pH 7.4.

Since ^{125}I-HA binding by hepatocytes was greatly reduced above pH 8 (Figure 4), the effect of pH on the dissociation of ^{125}I-HA prebound at pH 7.4 was examined by washing cells at 4 °C at different pHs. Dissociation of prebound ^{125}I-HA is greatly enhanced at pH > 8 (Figure 5). Conversely, incubation of hepatocytes at pH < 7 decreases the rate of dissociation of the prebound ^{125}I-HA.

Effect of Ionic Strength on ^{125}I-HA Binding to Permeabilized Hepatocytes. Since HA is negatively charged at pH 7 and this charge may be important in HA binding to permeabilized hepatocytes, the ionic strength may affect this interaction. When ^{125}I-HA binding to permeable cells was performed in the presence of increasing NaCl concentration, specific ^{125}I-HA binding decreased (Figure 6). Binding was maximal at ≤50 mM NaCl and then decreased as the NaCl concentration increased to 200 mM. Binding remained low at concentrations from 250 mM to 1 M NaCl. Despite this sensitivity to salt concentration, however, significant ^{125}I-HA binding occurs at physiologic ionic strength. The addition of 0.5 M NaCl to the binding mix decreased the amount of specific ^{125}I-HA binding by hepatocytes at both pH 5 and 7.4 by 75% (not shown).

Effect of Divalent Cations on ^{125}I-HA Binding to Permeabilized Hepatocytes. When ^{125}I-HA was bound to hepatocytes in the presence of medium 1/BSA, which contains 1.8 mM $CaCl_2$, the specifically bound HA could not be released by incubation with EGTA (not shown). This suggests that divalent cations are not required for binding. Yet surprisingly, when higher concentrations of Ca^{2+} (up to 10 mM) were present, the specific ^{125}I-HA binding increased linearly (80 fmol of ^{125}I-HA bound/mM Ca^{2+} at 10^{-7} M ^{125}I-HA; r = 0.996). Both the nonspecific binding and the total binding increased. Other divalent cations also increased the specific

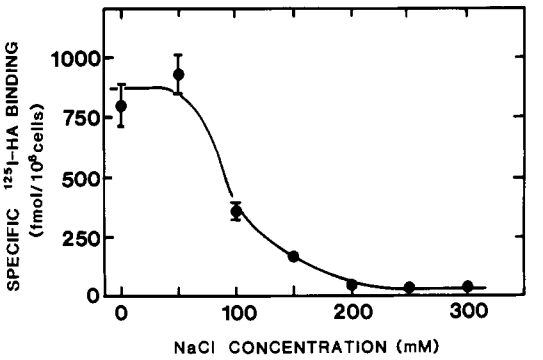

FIGURE 6: Effect of ionic strength on ^{125}I-HA binding to permeabilized hepatocytes in suspension. Purified hepatocytes were permeabilized with 0.055% digitonin for 20 min in buffer 1/BSA in the presence of 133 μM PMSF. The permeabilized cells were washed three times and resuspended at 10^7 cells/mL in 0.02 M HEPES, pH 7.4. One hundred microliters of cells was added to 100 μL of solutions containing 2×10^{-7} M ^{125}I-HA (M_r ~60000) in the presence and absence of 2×10^{-5} M unlabeled HA and increasing NaCl concentrations. The cells were incubated for 1 h at 4 °C and washed by centrifugation, and bound ^{125}I-HA and DNA content were determined as described under Experimental Procedures. Each point represents the average of triplicates.

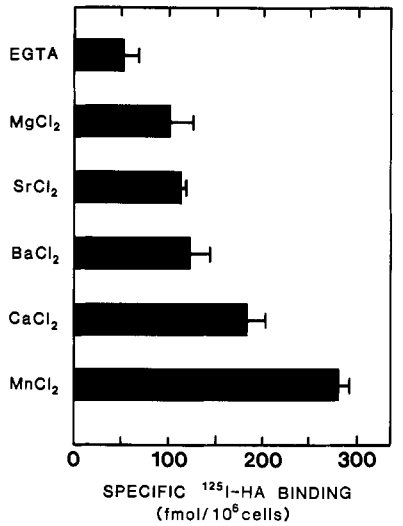

FIGURE 7: Effect of divalent cations on ^{125}I-HA binding to permeabilized hepatocytes in suspension. Purified hepatocytes were permeabilized as described in Figure 6. After permeabilization, the cells were washed three times with buffer 1 and were resuspended at 10^7 cells/mL in 20 mM solutions of the indicated salts or 4 mM of EGTA in 2× buffer 1 with 0.02% BSA. One hundred microliters of cells was immediately diluted with 100 μL of 2×10^{-7} M ^{125}I-HA (M_r ~60000) in the presence or absence of 2×10^{-5} M nonradiolabeled HA. At the end of the incubation, the cells were washed by centrifugation, and bound ^{125}I-HA and DNA content was determined as described under Experimental Procedures. Each point represents the mean of triplicates.

^{125}I-HA binding to permeabilized hepatocytes (Figure 7). Ten millimolar $CaCl_2$ and $MnCl_2$ increased ^{125}I-HA binding 3–6-fold compared to binding in the presence of EGTA. Other divalent cations also increased HA binding but to a lesser extent. Only a 2-fold increase in specific ^{125}I-HA binding was observed with 10 mM $MgCl_2$, $SrCl_2$, and $BaCl_2$.

The Uptake of ^{125}I-HA by Hepatocytes in Suspension. The kinetics of ^{125}I-HA uptake by hepatocytes at 37 °C was studied to determine whether hepatocytes can internalize HA by an adsorptive or a fluid-phase endocytic process. The rate of ^{125}I-HA association with intact hepatocytes was initially fast and was comparable to the kinetics of HA binding at 4 °C (see Figure 1). A rapid increase in cell-associated ^{125}I-HA was observed within 10 min and thereafter the increase was

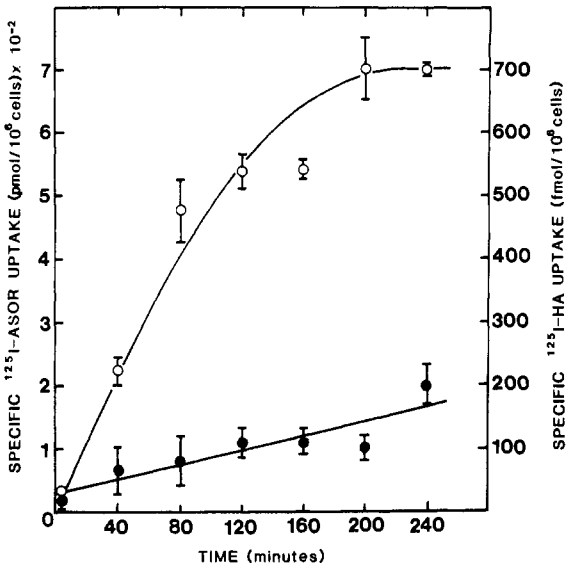

FIGURE 8: Internalization of ^{125}I-asialoorosomucoid and ^{125}I-HA by hepatocytes in suspension at 37 °C. Purified hepatocytes (2 mL, 2 × 10^6 cells/mL) were incubated in 25-mL flasks in a gyratory shaking water bath with either 1 × 10^{-7} M ^{125}I-HA (M_r ~60 000) at 37 °C in the absence or presence of 1 × 10^{-5} M unlabeled HA or 1.5 μg/mL of ^{125}I-asialoorosomucoid in the absence or presence of 75 μg/mL unlabeled asialoorosomucoid. At the indicated times, three aliquots (600 μL) were sampled from each flask and placed either in 2 mL of HBSS with 7.5 mM EGTA for asialoorosomucoid samples or in 2 mL of 0.625 M NaCl with 0.05 mM HEPES, pH 9.0, for HA samples. The cells were incubated for 30 min at 4 °C and pelleted by centrifugation. The cell pellets were resuspended in 200 μL of the same buffer and the cell suspension was layered onto 800 μL of 40% Percoll. Viable hepatocytes were pelleted by centrifugation at 1200 rpm in a Beckman tabletop centrifuge (Model TJ-6) for 5 min at 4 °C. The cells were resuspended in HBSS and quickly washed by centrifugation. These washing procedures remove the surface-bound ligands. The specific internalized ^{125}I-HA (●), ^{125}I-asialoorosomucoid (○), and protein content were determined as described under Experimental Procedures. Each point represents the mean of triplicate determinations.

very slow. The latter kinetics of HA accumulation at 37 °C is consistent with either an adsorptive or fluid-phase pinocytic process but not receptor-mediated endocytosis via a coated-pit pathway. The steady-state rate of accumulation after 10 min was only ~100 HA molecules cell^{-1} min^{-1}. An endocytic receptor system operating via a coated-pit pathway would internalize ~40 000 molecules cell^{-1} min^{-1} (Weigel & Oka, 1982; Stahl et al., 1984). This result indicates a rapid ^{125}I-HA binding at 37 °C to relatively few surface binding sites and the possibility that only a single round of endocytosis of this bound HA could occur.

To further examine the question of whether hepatocytes can internalize ^{125}I-HA at 37 °C, conditions were chosen that would remove surface-bound ^{125}I-HA at 4 °C. Earlier experiments indicated that high pH and high ionic strength decreased ^{125}I-HA binding (Figures 4 and 6). Two washes with 0.5 M NaCl and 20 mM HEPES, pH 9.0, removed 80% of the ^{125}I-HA specifically prebound to digitonin-treated hepatocytes at 4 °C. Therefore, this treatment was employed to strip surface-bound ^{125}I-HA from intact hepatocytes in order to assess intracellular ^{125}I-HA.

Hepatocytes in suspension were incubated with ^{125}I-HA at 37 °C for various times or at 4 °C for 1 h (Figure 8). The cells were then washed at 4 °C with the high pH/high salt buffer to remove surface-bound ^{125}I-HA. After these treatments, >80% of the cells are viable by trypan blue exclusion. Though less than 20% of the hepatocytes are dead, the exposed intracellular HA binding sites may have some residual bound ^{125}I-HA after the stripping procedure. This would lead to an overestimate of the amount of ^{125}I-HA internalized. Therefore, viable hepatocytes were isolated over Percoll gradients. After 4 h at 37 °C, a significant uptake of ^{125}I-HA was observed, but, again, the rate of internalization was very low (460 HA molecules cell^{-1} min^{-1}). As a positive control, the internalization of asialoorosomucoid mediated by the asialoglycoprotein receptor was also determined. The internalization rate of this ligand was 36 000 molecules cell^{-1} min^{-1}. This hepatocyte asialoglycoprotein receptor, which endocytoses asialoorosomucoid by a coated-pit pathway, internalized its cell surface equivalent of receptor–ligand complexes in 6 min, while the HA binding protein took 25 min to internalize its cell surface equivalent of bound HA.

These results suggest that HA may be continually internalized but at a very slow rate and not via a coated-pit pathway. This was tested further by treating hepatocytes with hyperosmolar sucrose, which completely inhibits the coated-pit pathway without affecting fluid-phase pinocytosis (Oka et al., 1989) in hepatocytes. No significant difference in the uptake of ^{125}I-HA by hepatocytes was observed in the presence or absence of 0.2 M sucrose (not shown). In a parallel experiment the internalization of asialoorosomucoid by the asialoglycoprotein receptor was inhibited >85% by 0.2 M sucrose. This result verifies the conclusion that adsorptive uptake of HA is not via a coated-pit pathway.

Discussion

Rat hepatocytes were the first liver cell type reported to bind and internalize HA in vitro (Truppe et al., 1977). Subsequently, Laurent and co-workers reported that endothelial cells in liver are the major site of HA clearance in vivo and that these cells also endocytose and degrade exogenous HA in vitro (Smedsrod et al., 1984). We also investigated the ability of cultured liver endothelial cells to metabolize HA and found that there is a large intracellular pool of HA receptors involved in a recycling endocytic receptor system (Raja et al., 1988; McGary et al., 1989). The high activity of the liver endothelial HA receptor system could mask a weaker endocytic system in hepatocytes in vivo. Therefore, the present study was undertaken to clarify the possible ability of hepatocytes to bind and internalize HA.

Our results confirm the initial studies by Truppe et al. (1977) that hepatocytes can specifically bind and internalize HA, although the rate of internalization of HA that we observe is considerably lower. There are at least two possible explanations for this difference. First, the hepatocyte cultures used by Truppe et al. (1977) may have contained a small percentage of contaminating endothelial cells, which could explain their results. Our procedure of isolating pure viable hepatocytes over discontinuous Percoll gradients (Dalet, 1982) ensures that endothelial cells are not present. Second, it is possible that the lower molecular weight HA we employed does not bind to hepatocytes as effectively as larger HA, and thus little internalization is observed. However, even if hepatocytes bind and internalize native HA, this may not be physiologically relevant because HA found in the blood stream is relatively small, M_r ~150 000 (Tengblad et al., 1986). Since our HA probe (M_r ~60 000) is closer in size to the HA in the circulation, the present results suggest that HA would not be internalized by hepatocytes in vivo.

Although hepatocytes have a modest surface binding capacity for HA, there is a surprisingly large intracellular pool of HA binding sites. Greater than 90% of the total HA binding sites in hepatocytes are intracellular. The HA binding site(s) may be a membrane protein, since HA binding to

membrane extracts of hepatocytes is trypsin sensitive but the activity is not released from membranes by washing with sodium carbonate at pH 11 (Frost et al., 1988). Polyanionic glycosaminoglycans compete for ^{125}I-HA binding, while other anionic polysaccharides do not. Equilibrium binding studies with ^{125}I-HA (M_r ~30000) on permeabilized rat hepatocytes in suspension show there are 1.3×10^6 binding sites/cell. This is a very large number of sites. Also the K_d of 1.8×10^{-7} M ($K_a = 5.6 \times 10^6$ M^{-1}) indicates that the affinity is reasonably strong for a such a small HA. The binding of even smaller HA (M_r ~7000) to liver endothelial cells has a $K_d \sim 2 \times 10^{-7}$ M (Laurent et al., 1986).

A 50% decline in the total number of ^{125}I-HA binding sites/cell was observed after 24 h in culture with no apparent change in affinity for HA. This decrease could be due to (i) downregulation of the binding sites upon cell culture or (ii) masking of ^{125}I-HA binding sites, for example, by endogenously synthesized HA. A similar decrease was observed in the activity of liver endothelial cell HA receptors when these cells were cultured (Raja et al., 1988). Both of the above characteristics would be consistent with a cytoskeletal component present in large amount and reorganized when hepatocytes adhere to and spread on a culture dish. This possibility is consistent with the previous conclusion that the HA binding site is either an integral membrane or cytoskeletal protein (Frost et al., 1988).

Low pH increased binding as compared to physiologic pH. At pH 5.0, binding was ≥10-fold greater than at pH 7.4. This is unlike many receptor–ligand complexes that dissociate at low pH (Yamashiro and Maxfield, 1984). When the pH was >7, ^{125}I-HA binding decreased. Low ionic strength also increased HA binding two to four times over that at physiologic ionic strength. These results indicate that ionic interactions between HA and its intracellular binding site(s) are important. It is important to note that even though physiologic pH and ionic strength do not give maximal HA binding to permeable hepatocytes, the number of HA binding sites detected under these conditions is still >10^6/cell. The increased HA binding observed under nonphysiologic conditions could be due to an increased affinity of the same sites or to the appearance of additional or different binding sites. They may have no physiologic significance.

The sensitivity to ionic strength of HA binding to the intracellular binding sites in hepatocytes is unique among known cellular HA binding proteins. The HA binding protein or proteins in hepatocytes are apparently different from those reported on SV3T3 cells (Underhill & Toole, 1980) and liver endothelial cells (Raja et al., 1988). At physiologic pH, SV3T3 cells and liver endothelial cells are not affected by high ionic strength (0.4 M NaCl), whereas the hepatocyte binding protein loses ~90% of its HA binding activity.

HA binding to permeabilized hepatocytes occurred when EGTA was present. Nonetheless, divalent cations cause an increase in specific HA binding. Though the physiologic concentrations of Mn^{2+} and Ca^{2+} are much lower than the millimolar concentrations used in our experiments, subcellular stores of both divalent cations may be in the millimolar range. Also, Mg^{2+}, which stimulates HA binding 2-fold (Figure 7), is the major intracellular divalent cation and its concentrations, which can approach 30 mM, exceed those used here. Therefore, it is possible that the stimulation of HA binding by divalent cations is physiologically relevant and that there may be two distinct HA binding sites, one divalent cation dependent and one divalent cation independent. This would be similar to the situation with the two mannose-6-phosphate receptors, where there is a small divalent cation-dependent receptor and a larger cation-independent receptor (Kornfeld, 1987).

Although there are detectable HA binding sites on the cell surface, these sites do not mediate endocytosis of HA by a coated-pit pathway. There is, therefore, presently no reason to believe that this HA binding activity is a receptor. The internalization rate for HA by hepatocytes is extremely slow when compared to accumulation of asialoorosomucoid by hepatocytes (36000 molecules cell^{-1} min^{-1}), the accumulation of HA by liver endothelial cells (15000 molecules cell^{-1} min^{-1}) (McGary et al., 1989), or the endocytosis of mannose-containing ligands by macrophages (33000 molecules cell^{-1} min^{-1}) (Stahl et al., 1984). Since the HA binding site in hepatocytes is membrane bound (Frost et al., 1988), recycling of plasma membrane by a noncoated-pit pathway (i.e., by fluid-phase pinocytosis or general membrane recycling) is the most reasonable explanation for the slow rate of HA internalization.

What is the function of this HA binding protein(s) in hepatocytes if the majority of the binding sites are not involved with internalization of extracellular HA? Since there is such a large intracellular pool of HA binding sites, it is very likely that they must have an important cellular function. Recently, HA has been localized intracellularly in adult neuronal tissue (Ripellino et al., 1988) and cultured rat testicular cells (Rodriguez & Minguell, 1989). Proteoglycans have been localized intracellularly in rat serosal mast cells (Katz et al., 1986) and HL-60 human leukemic promyelocytes (Lohmander et al., 1990). HA and other glycosaminoglycans including heparan sulfate have also been localized to the nucleus in hepatocytes (Fedarko & Conrad, 1986; Furukawa & Terayama, 1979) and other cell types (Sames, 1979; Margolis et al., 1976). Therefore, HA and other glycosaminoglycans may be important intracellular molecules as well as extracellular molecules.

Possible functions for an intracellular HA binding activity could be in the biosynthesis of HA or other glycosaminoglycans or in proteoglycan and HA export from the cell. Since the binding sites have a broad specificity for polyanionic glycosaminoglycans (Frost et al., 1988), these sites may bind to HA or glycosaminoglycan chains on proteoglycans. The binding sites could be involved in transferring HA or proteoglycans to different intracellular compartments or the extracellular matrix.

If cells contain internal cytoplasmic HA, then a structural role is another possible function for intracellular HA and HA binding protein(s). This role might be similar to the function of structural proteins, such as laminin, fibrin, or fibronectin, that can form polymers and bind HA in the extracellular matrix. HA exhibits two important physical properties, high viscosity and viscoelasticity, that are important in cartilage and skin, where HA is a major component in the extracellular matrix (Fraser & Laurent, 1989). These characteristics are enhanced by the noncovalent binding of HA to macromolecules such as proteoglycan core and link proteins (Hascall, 1977). Perhaps HA plays a similar role inside cells. The intracellular HA binding protein(s) could organize with HA in the cell and enhance cytoplasmic viscoelasticity and viscosity. These properties could help the cell retain its shape and maintain its integrity under external pressure.

ACKNOWLEDGMENTS

We thank Deborah Baudy and Paige Smith for preparing rat hepatocytes, Janet Oka for help preparing the figures, and Glenda M. Romero for help preparing the manuscript. We also thank Maryanne Herzig for helpful discussions.

Registry No. HA, 9004-61-9; Ca^{2+}, 7440-70-2; Mn^{2+}, 7439-96-5; Mg^{2+}, 7439-95-4; Sr^{2+}, 7440-24-6; Ba^{2+}, 7440-39-3.

REFERENCES

Asou, H., Brunngraber, E. G., & Delpech, B. (1983) *J. Neurochem. 40*, 589–591.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Clarke, B. L., Oka, J. A., & Weigel, P. H. (1987) *J. Biol. Chem. 262*, 17384–17392.

Dalet, C., Fehlmann, M., & Debey, P. (1982) *Anal. Biochem. 122*, 119–123.

Fedarko, N. S., & Conrad, H. E. (1986) *J. Cell Biol. 102*, 587–599.

Fraker, P. J., & Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.

Fraser, J. R. E., & Laurent, T. C. (1989) in *The Biology of Hyaluronan* (Evered, D., & Whelan, J., Eds.) Ciba Foundation Symposium 143, pp 41–59.

Fraser, J. R. E., Laurent, T. C., Pertoft, H., & Baxter, E. (1981) *Biochem. J. 200*, 415–424.

Frost, S. J., McGary, C. T., Raja, R. H., & Weigel, P. H. (1988) *Biochim. Biophys. Acta 946*, 66–74.

Furukawa, K., & Terayama, H. (1979) *Biochim. Biophys. Acta 585*, 575–588.

Gerke, E., Meyer-Schwickerath, G., & Wessing, A. (1985) in *Surgical Pharmacology of the Eye* (Sears, M., & Turakkanen, A., Eds.) pp 411–415, Raven Press, New York.

Hascall, V. C. (1977) *J. Supramol. Struct. 7*, 101–120.

Katz, H. R., Austen, K. F., Caterson, B., & Stevens, R. L. (1986) *J. Biol. Chem. 261*, 13393–13396.

Knudson, W., Biswas, C., & Toole, B. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6767–6771.

Kornfeld, S. (1987) *FASEB J. 1*, 462–468.

Labarca, C., & Paigen, K. (1980) *Anal. Biochem. 102*, 344–352.

Laurent, T. C., Fraser, J. R. E., Pertoft, H., & Smedsrod, B. (1986) *Biochem. J. 234*, 653–658.

LeBoeuf, R. D., Raja, R. H., Fuller, G. M., & Weigel, P. H. (1986) *J. Biol. Chem. 261*, 12586–12592.

Lohmander, L. S., Arnljots, K., & Yanagishita, M. (1990) *J. Biol. Chem. 265*, 5802–5808.

Love, S. W., Shannon, B. T., Myrvik, O. N., & Lynn, W. S. (1979) *J. Reticuloendothel. Soc. 25*, 269–282.

Margolis, R. K., Crockett, C. P., & Kiang, W.-L. (1976) *Biochim. Biophys. Acta 451*, 465–469.

McGary, C. T., Raja, R. H., & Weigel, P. H. (1989) *Biochem. J. 257*, 875–884.

Oka, J. A., & Weigel, P. H. (1987) *J. Cell. Physiol. 133*, 243–252.

Oka, J. A., Christensen, M. D., & Weigel, P. H. (1989) *J. Biol. Chem. 264*, 12016–12024.

Raja, R. H., LeBoeuf, R. D., Stone, G. W., & Weigel, P. H. (1984) *Anal. Biochem. 139*, 168–177.

Raja, R. H., McGary, C. T., & Weigel, P. H. (1988) *J. Biol. Chem. 263*, 16661–16668.

Ripellino, J. A., Bailo, M., Margolis, R. V., & Margolis, R. K. (1988) *J. Cell Biol. 106*, 845–855.

Rodriguez, J. P., & Minguell, J. J. (1989) *Cell Biochem. Funct. 7*, 293–300.

Sames, K. (1979) *Acta Anat. 103*, 74–82.

St. Onge, R., Weiss, C., Denlinger, J. L., & Balazs, E. A. (1980) *Clin. Orthop. 146*, 269–275.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Scott, J. E. (1960) *Methods Biochem. Anal. 8*, 145–197.

Seglen, P. O. (1973) *Exp. Cell. Res. 82*, 391–398.

Smedsrod, B., Pertoft, H., Eriksson, S., Fraser, J. R. E., & Laurent, T. C. (1984) *Biochem. J. 223*, 617–626.

Stahl, P. D., Wileman, T. E., Diment, S., & Shepherd, V. L. (1984) *Biol. Cell 51*, 215–218.

Tengblad, A., Laurent, U. B. G., Lilja, K., Cahill, R. N. P., Engstrom-Laurent, A., Fraser, J. R. E., Hansson, H. E., & Laurent, T. C. (1986) *Biochem. J. 236*, 521–525.

Toole, B. P. (1981) in *Cell Biology of the Extracellular Matrix* (Hay, E. D., Ed.) pp 259–294, Plenum Press, New York.

Toole, B. P., Biswas, C., & Gross, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6299–6303.

Truppe, W., Basner, R., von Figura, K., & Kresse, H. (1977) *Biochem. Biophys. Res. Commun. 78*, 713–719.

Underhill, C. B. (1982) *J. Cell Sci. 56*, 177–189.

Underhill, C. B., & Dorfman, A. (1978) *Exp. Cell Res. 117*, 155–164.

Underhill, C. B., & Toole, B. P. (1980) *J. Biol. Chem. 255*, 4544–4549.

Weigel, P. H., & Oka, J. A. (1982) *J. Biol. Chem. 257*, 1201–1207.

Weigel, P. H., Ray, D. A., & Oka, J. A. (1983) *Anal. Biochem. 133*, 437–449.

Weigel, P. H., Fuller, G. M., & LeBoeuf, R. D. (1986) *J. Theor. Biol. 119*, 219–234.

Weiss, C., Balazs, E. A., St. Onge, R., & Denlinger, J. L. (1981) *Semin. Arthritis Rheum. 11*, 143–144.

West, D. C., Hampson, I. N., Arnold, F., & Kumar, S. (1985) *Science 228*, 1324–1326.

Yamashiro, D. J., & Maxfield, F. R. (1984) *J. Cell. Biochem. 26*, 231–246.