Biochem. J. (1986) 236, 521–525 (Printed in Great Britain)

Concentration and relative molecular mass of hyaluronate in lymph and blood

Anders TENGBLAD,* Ulla B. G. LAURENT,† Karin LILJA,‡ Ross N. P. CAHILL,§ Anna ENGSTRÖM-LAURENT,‖ J. Robert R. FRASER,¶ Hans Erik HANSSON** and Torvard C. LAURENT‡

*Department of Biochemistry, Pharmacia AB, Uppsala, Sweden, Departments of †Ophthalmology, ‡Medical and Physiological Chemistry, ‖Medicine and **Thoracic Surgery, University of Uppsala, Uppsala, Sweden, and Departments of §Veterinary Preclinical Science, School of Veterinary Science, and ¶Medicine, School of Medicine, University of Melbourne, Melbourne, Victoria, Australia

Human lymph was collected from patients with leaking lymph vessels after thoracic surgery. Ovine lymph was obtained from the mesenteric, lumbar, popliteal and prescapular lymph ducts by cannulation. The concentration of hyaluronate varied considerably (between 0.2 and 50 mg/l) and the highest concentrations were found in mesenteric lymph. The M_r of the polysaccharide showed a great polydispersity and variation between individuals and in different regions of the lymphatic system. High-M_r hyaluronate ($> 10^6$) was present in lymph both from man and sheep. Hyaluronate was also isolated by affinity chromatography in 70–80% yield from human serum and plasma obtained from healthy individuals and patients with rheumatoid arthritis and primary biliary cirrhosis. The weight (M_w)- and number (M_n)-average relative molecular masses were roughly the same in the three groups [$(1.4–2.7) \times 10^5$ and $(2.1–5.7) \times 10^4$ respectively]. The low M_r of hyaluronate in blood compared with that in lymph is explained by a preferential uptake of the large molecules by the liver endothelial cells.

INTRODUCTION

Sodium hyaluronate (HA) of low concentration is found in blood (Laurent & Laurent, 1981; Engström-Laurent *et al.*, 1985*a*; Delpech *et al.*, 1985). The circulating HA seems to originate in tissues and is carried by the lymph flow to the general circulation (Laurent & Laurent, 1981). In healthy blood donors the serum concentration is usually < 100 μg/l and the mean value is of the order of 20–40 μg/l (Delpech *et al.*, 1985; Engström-Laurent *et al.*, 1985*a*). Elevated levels are found in patients with rheumatoid arthritis (Engström-Laurent & Hällgren, 1985) presumably owing to an increased production of HA in the peripheral tissues. High levels are also found in patients with liver cirrhosis (Engström-Laurent *et al.*, 1985*b*). In this syndrome the uptake of the polysaccharide is impaired (Fraser *et al.*, 1986).

Intravenously injected radioactively labelled HA is rapidly cleared from the blood with a half-life of 2–5 min (Fraser *et al.*, 1981, 1984). It is taken up, and degraded by, the endothelial cells in the liver sinusoids (Eriksson *et al.*, 1983; Smedsrød *et al.*, 1984; Fraser *et al.*, 1985). The turnover studies have been performed with labelled HA having an M_r of the order of millions (Fraser *et al.*, 1981, 1984), but the M_r of the endogenous HA is not known. Recent work on the binding of HA to liver endothelial cells (Laurent *et al.*, 1986) has shown that the affinity of the receptor for HA is strongly M_r-dependent. It is therefore not certain that the experimental data on turnover are valid for the endogenous polymer. In the present paper we present analyses on the concentration and M_r of HA in human and ovine lymph and the M_r of HA in human blood from healthy subjects and patients with rheumatoid arthritis and primary biliary cirrhosis.

MATERIALS AND METHODS

Human lymph

Case 1. A malignant tumour was resected in the lower part of the oesophagus of a 73-year-old man. Post-operatively he showed an increasing exudation in the left pleura, which was drained. The fluid looked like lymph. A sample was collected and the patient was re-operated on 3 weeks after the original operation. It could then be verified that the liquid originated from a rift in the thoracic duct.

Case 2. A 4-month-old boy with congenital heart disease was operated on at the left side. Post-operatively he showed a lymph leakage, and a sample was collected 1 day after operation. On re-operation the leakage could be stopped by sutures, but it was not possible to identify the vessel that had leaked.

Case 3. A 2-year-old boy was operated on for congenital heart disease. Post-operatively, blood was drained from the mediastinum and the right pleura. The drained fluid gradually changed to become more serous and increase in volume. On days 5 and 6 the fluid looked like lymph and the patient was considered to have a lymph leakage. The fluid was collected on 2 consecutive days. The flow stopped spontaneously.

Blood serum was collected from the three patients at the time of lymph collection or a day later.

Ovine lymph

Samples were obtained from Merino lambs of either sex between 3 and 5 months of age. They were kept in metabolic cages or individual pens and offered lucerne

Abbreviations used: HA, hyaluronate; HABP(s), HA-binding protein(s); M_w and M_n, weight- and number-average relative molecular mass.

chaff, water and salt lick *ad libitum*. A subsidiary intestinal lymph duct was cannulated by a modification of the method of Lascelles & Morris (1961) and the prescapular node was drained by the method of Heitmann (1970). Lymph was collected overnight. The lumbar and popliteal lymph, previously reported by Laurent & Laurent (1981), came from adult sheep. Samples of blood serum were collected at the time of lymph collection.

Handling of lymph

The human lymph was centrifuged for 1 h at 100000 *g* and the top layer, containing chylomicrons, and the bottom layer, containing lymph cells, were removed. The clear lymph fluid was then used for analyses.

The ovine lymph was centrifuged at 1600 *g* for 15 min to remove cells. NaN_3 was added to a concentration of 0.02%. The samples were kept frozen (during transport between Australia and Sweden) until analysed.

Sources of blood samples

Serum from apparently healthy persons was obtained from a woman (age 35 years) and by pooling samples from blood donors (age 20–60 years). Sera from patients with rheumatoid arthritis were also pooled. Plasma from one patient was obtained after medical treatment with plasmapheresis. All the patients met the criteria for definite or classical rheumatoid arthritis (Ropes *et al.*, 1959). None of the patients was taking medication at the time of blood sampling, and the disease was considered to be in an active state. Serum and plasma were collected in the same manner from patients with primary biliary cirrhosis. All the patients met the critiera for this disease, including a positive liver biopsy indicating liver cirrhosis (Scheuer, 1967).

Affinity chromatography on HA-binding-protein-substituted agarose gel (HABP–Sepharose)

HABPs (HA-binding region from proteoglycans and the link proteins) were isolated from nasal cartilage essentially as described previously (Tengblad, 1979). The proteins (~ 40 mg) were mixed with oligosaccharides derived from HA (~ 20 mg of HA-12+HA-14; see Smedsrød *et al.*, 1984). A concentrated solution of the mixture (5 ml) was added to 4 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in 25 ml of 0.1 M-$NaHCO_3$ (pH 8.3)/0.5 M-NaCl and incubated for 5 h at room temperature, essentially as suggested by Pharmacia Fine Chemicals (1974). The gel (total volume 14 ml) was finally washed with 4 M-guanidinium chloride/0.5 M-sodium acetate, pH 5.8, in order to release the oligosaccharides protecting the binding sites.

The capacity of the gel for binding HA was studied by incubating 2 ml of HABP–Sepharose with increasing amounts of HA (Healon; Pharmacia; M_w 1.9×10^6). In the presence of 420 μg of HA, 43% was adsorbed to the gel. 84% of the adsorbed material could be eluted from the gel with 4 M-guanidinium chloride/0.5 M-acetate, pH 5.8. The gel could thus bind more than 75 μg of HA/ml.

Serum or plasma (50–500 ml) was mixed with proteinase inhibitors to the following final concentrations: EDTA (Titriplex III; Merck, Darmstadt, Germany, 8.4 mM; soybean trypsin inhibitor (Worthington, Freehold, NJ, U.S.A.), 0.84 μg/ml; phenylmethanesulphonyl fluoride (Sigma, St. Louis, MO, U.S.A.), 0.84 mM; 6-aminohexanoic acid (Sigma), 84 mM; benzamidine (Sigma), 4.1 mM; pepstatin A (Sigma), 0.84 μg/ml; iodoacetic acid (Sigma), 0.84 mM. A 10 ml portion of HABP–Sepharose was mixed with a batch of serum or plasma and the solution was incubated and turned end-over-end for 40 h. The gel slurry was packed into a column and washed with 160 ml of 0.15 M-NaCl/0.01 M-phosphate buffer, pH 7.4. The HA bound to the gel was eluted from the column with 14 ml of 4 M-guanidinium chloride/0.5 M-acetate, pH 5.8. The eluate was concentrated ten times by ultrafiltration in an Amicon cell with a PM-10 membrane (Amicon Corp., Danvers, MA, U.S.A.) and the solvent was changed to 0.15 M-NaCl/0.01 M-phosphate buffer, pH 7.4.

Analysis of HA concentration

HA was analysed by a specific binding assay (Tengblad, 1980; Laurent & Tengblad, 1980; Engström-Laurent *et al.*, 1985*a*). Analyses on serum and plasma were made by the modification described by Engström-Laurent *et al.* (1985*a*), and analyses of chromatograms were done by the modification described by Laurent & Tengblad (1980). The between-assay S.D. in the determination was ±15%.

Analysis of the M_r distribution of HA

Calibrated gel chromatography was used to fractionate the HA and determine the M_r distribution (Wik *et al.*, 1979; Laurent & Granath, 1983; see also the legend to Fig. 1). By using a radioassay for analysis of the eluate, it was possible to determine a complete M_r distribution on as little as 10 μg of HA. When necessary, the samples were concentrated by ultrafiltration before application on the column. Previous experience has shown that M_r values determined by this technique are reproducible within 10%.

Test for degradation of HA during affinity chromatography

Uniformly ^{3}H-labelled HA was obtained in cultures of human synovial cells as described by Fraser *et al.* (1981). Its specific radioactivity was approx. 3×10^5 d.p.m./μg. The labelled HA was added to serum batches after they had been collected. Its M_r was determined by gel chromatography after its isolation by affinity chromatography and compared with data obtained with the original preparation. ^{3}H was determined in a Searle–Nuclear Chicago Isocap 300 liquid-scintillation counter, with Packard 299 emulsion (Packard–Becker, Breda, The Netherlands) as scintillant.

RESULTS

Concentration of HA in lymph

The analytical results are shown in Table 1.

M_r of HA in lymph

M_r distributions were determined on thoracic lymph from Case 1 and from the lymph of Case 2, which presumably originated from the thoracic duct. The concentration of HA in lymph from Case 3 was too low to allow an analysis of M_r.

Two samples of intestinal lymph from sheep and one sample of prescapular lymph were also analysed. The chromatograms of the five samples are given in Fig. 1,

Table 1. Concentration of HA in lymph

		Concentration of HA (mg/l) in:	
		Lymph	Blood serum
Human			
Adult male (Case 1)	Thoracic duct	8.5	0.055
Child			
Case 2	Thoracic duct (?)	18	0.7
Case 3	Right pleura		
	Day 5	0.13	0.12
	Day 6	0.20	0.14
Sheep			
3–5 months	Intestinal*	47.3 (40.0–53.3)	0.4 (0.2–0.7) ($n = 3$)†
	Prescapular	1.0±0.8	0.3±0.1 ($n = 8$)‡
Adult	Lumbar*	2.6	0.4 ($n = 1$)
	Popliteal*	11	0.4 ($n = 1$)

* One sample from intestinal lymph and lumbar and popliteal lymph were previously reported by Laurent & Laurent (1981).
† Mean (range).
‡ Mean ± S.D.

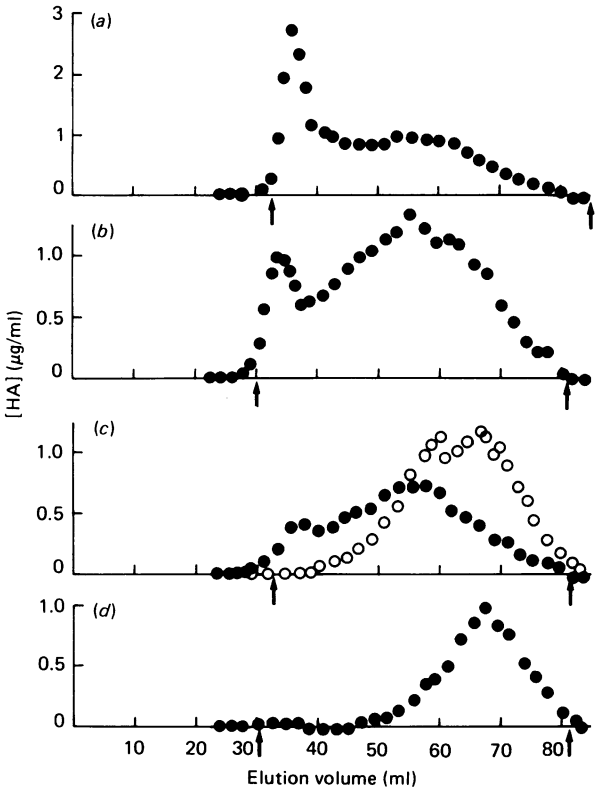

Fig. 1. Gel chromatograms of HA from lymph

The sources were: (*a*) Thoracic lymph (?) from a child (Case 2); (*b*) thoracic lymph from adult male (Case 1); (*c*) intestinal lymph from two different sheep; and (*d*) prescapular lymph from sheep. A 1 ml lymph sample (if necessary concentrated) containing > 10 μg of HA was applied on a 1 cm × 110 cm column consisting of a mixture of Sepharose 4B and cross-linked 0.5% agarose (1:2, v/v) as described by Laurent & Granath (1983). The polysaccharide was eluted with an alkaline buffer (pH 11.6). The column was calibrated with HA fractions of known M_r and the elution diagram was translated into M_r distributions by a computer. The M_w and M_n values are given in Table 2. V_0 and V_t of the column are marked by arrows.

and the calculated M_w and M_n values are given in Table 2. All samples showed a considerable polydispersity. Furthermore, the M_w values varied more than 10-fold, depending on source and individuals.

Isolation of HA by affinity chromatography

HA was isolated from two batches of serum from healthy individuals (500 ml each), from one batch of serum and one sample of plasma from rheumatoid patients (150 ml each) and from one batch of serum and one sample of plasma from patients with primary biliary cirrhosis (50 ml each). The recovery of HA in the isolation procedure is given in Table 3 and varied between 53 and 86% with the exception of one 'normal' serum, where the recovery was 167%. It is probable that the latter value is due to an error in the determination of HA in the original sample where the HA level was low.

Test for the degradation of HA during the isolation procedure

When [^{3}H]HA was added to various serum batches, the HA isolated by affinity chromatography and the M_r distribution of the radioactive material determined, there were only minor changes in the M_r values from those obtained for the original material (Fig. 2 and Table 4). The variations in M_r data seen in Table 4 can in some cases be ascribed to a minor degradation of HA during the isolation procedure, but also to a certain selection of HA molecules during the affinity chromatography and uncertainties in the analysis of the M_r distributions. There could not, however, have been any pronounced degradation of the radioactive material.

The M_r of circulating HA

Chromatograms of HA from serum taken from the three categories are seen in Fig. 2 and the M_r values are given in Table 3. There is a good reproducibility within each group. Furthermore, in all three conditions the M_r of HA is low compared with that of HA found in most tissues. There is, however, a tendency for a slight increase in M_w with increasing concentration of the polysaccharide in serum or plasma.

Table 2. M_w and M_n values for HA in lymph

Species	Source	M_w	M_n
Human			
Adult male (Case 1)	Thoracic duct	1.4×10^6	6.4×10^4
Child (Case 2)	Thoracic duct (?)	3.3×10^6	2.1×10^5
Sheep	Intestinal duct	2.0×10^6	1.7×10^5
	Intestinal duct	2.9×10^5	7.1×10^4
	Prescapular	1.6×10^5	1.9×10^4

DISCUSSION

The present results show that the concentration of HA in lymph varies depending on the source (Table 1). Of the human samples, that from Case 1 was verified as thoracic lymph and that from Case 2 as *probably* thoracic lymph as judged from appearance and site of leakage. The thoracic duct collects the main part of the lymph from the body and the analytical values are therefore representative mean values. The lymph leaking into the right pleural cavity of Case 3 is difficult to trace. It may have been lymph from the upper-right part of the body, mainly pulmonary lymph from an overloaded pulmonary circulation, or merely transudation caused by heart failure. The HA content in the fluid was similar to that of blood serum.

Intestinal lymph from sheep had persistently high concentrations of HA (Table 1). It arises from the stomach, the intestines and the mesentery, which contain large amounts of connective tissue. The second highest concentration was found in popliteal lymph vessels, which drain the lower part of the hindlimb. Lumbar lymph is formed from a conglomerate of lymphatic vessels draining the pelvis and the hindlimb. The prescapular lymph, which has a consistently low concentration of HA, comes from the superficial soft tissues of the entire forequarter region, together with lymph from the forelimb and brisket.

Although sheep were kept in metabolic cages when the lymph was collected, they were able to move quite freely, whereas the humans were confined to bed. Increased lymph flow might be expected in freely ranging sheep and in actively mobile humans, and the present data are therefore not representative for the active individual. It is noteworthy that Engström-Laurent (1985) recently demonstrated that physical activity increases the HA level in serum, especially in patients with rheumatoid arthritis, and drew the conclusion that this was due to an increased lymph flow from joints and muscles.

The M_r of lymphatic HA varies considerably between different individuals and has a considerable polydispersity

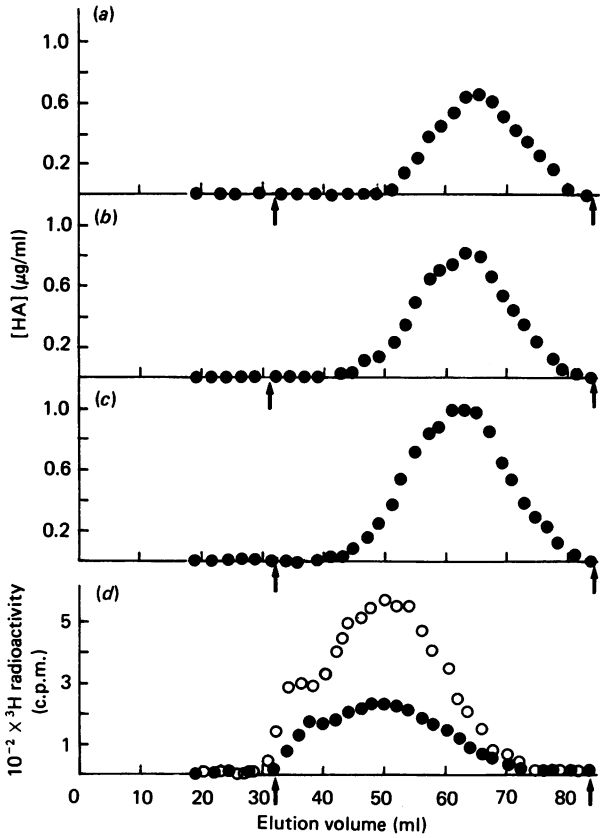

Fig. 2. Chromatography of HA isolated from serum of (*a*) apparently healthy blood donors, (*b*) patients with rheumatoid arthritis and (*c*) patients with primary biliary cirrhosis

Also shown, in (*d*), is the gel chromatogram of [^{3}H]HA before (○) and after (●) isolation from 'normal' serum by affinity chromatography. For technical details, see the legend to Fig. 1.

Table 3. Recoveries of, and M_w and M_n values for, HA isolated from serum (or plasma) by affinity chromatography

Source	Serum (S) or plasma (P)	Original concn. of HA in serum or plasma (μg/l)	Volume of serum or plasma (ml)	Recovery (%)	M_w	M_n
Healthy subjects	S	19	500	86	1.4×10^5	2.9×10^4
	S	23	500	167	1.6×10^5	5.7×10^4
Rheumatoid arthritis	S	169	150	85	2.0×10^5	2.1×10^4
	P	240	150	53	2.1×10^5	5.1×10^4
Primary biliary cirrhosis	P	553	50	76	2.7×10^5	4.5×10^4
	S	705	50	68	2.3×10^5	5.1×10^4

Table 4. M_w and M_n values for [^{3}H]HA recovered from serum by affinity chromatography

Preparation	M_w	M_n
Original [^{3}H]HA	1.9×10^6	0.8×10^5
[^{3}H]HA added to and subsequently isolated from:		
50 ml of serum from healthy subjects	1.4×10^6	1.6×10^5
500 ml of serum from healthy subjects	0.6×10^6	1.2×10^5
150 ml of serum from patients with rheumatoid arthritis	2.4×10^6	2.1×10^5
50 ml of serum from patients with primary biliary cirrhosis	1.1×10^6	1.7×10^5

in the same individual. HA may be synthesized with different degree of polymerization in the tissues, but it is more probable that the HA in lymph is a degradation product from the tissues and that the M_r distribution is affected by the mode and the extent of degradation. It is, however, apparent that the polysaccharide in lymph can be of high M_r. The thoracic lymph in man and one of the intestinal lymph samples from sheep contained HA with M_w values in the millions (Table 2).

The M_r distributions of all the HA samples isolated from blood showed significantly lower mean M_r values than that of HA from human lymph (Tables 2 and 3). There are at least three possible explanations. Firstly, there could be other major sources for serum HA than lymph. Although this cannot be excluded, available data indicate that the amount of HA carried by lymph to the general circulation is of the same order as the turnover of HA in blood. Assuming a lymph flow of 2 litres/day in an adult man and an HA concentration of 10–20 mg/l (Table 1), the total HA in lymph should be in the order of 20–40 mg/day. The total turnover in serum calculated from tracer studies (Fraser *et al.*, 1984) and the serum levels (Engström-Laurent *et al.*, 1985*a*) was 10–100 mg/day. Secondly, HA could be degraded when circulating in blood and during the isolation procedure. This is unlikely in view of our experiments with ^{3}H-labelled HA added to serum after it had been collected. The HA was not significantly degraded during our preparative procedure. Thirdly, there could be an enrichment in blood of the low-M_r fraction of lymphatic HA. This is the most probable explanation. The lymphatic HA is very polydisperse (Fig. 1) and contains an appreciable fraction of low M_r. The higher affinity of the liver endothelial cells for the high-M_r species (Laurent *et al.*, 1986) will leave the low-M_r species circulating for a longer time.

We thank the Department of Polymer Physical Chemistry at Pharmacia AB for help with the M_r determinations. This project was supported by grants from the Swedish Medical Research Council (03X-4), The Swedish Board for Technical Development (84-4706), Gustaf V:s 80-årsfond, the National Health and Medical Research Council of Australia and Pharmacia AB.

Received 28 October 1985/13 January 1986; accepted 23 January 1986

REFERENCES

Delpech, B., Bertrand, P. & Maingonnat, C. (1985) Anal. Biochem. **149**, 555–565

Engström-Laurent, A. (1985) Circulating Sodium Hyaluronate (Acta Universitatis Upsaliensis, Dissertation no. 525), Faculty of Medicine, University of Uppsala

Engström-Laurent, A. & Hällgren, R. (1985) Ann. Rheum. Dis. **44**, 83–88

Engström-Laurent, A., Laurent, U. B. G., Lilja, K. & Laurent, T. C. (1985*a*) Scand. J. Clin. Lab. Invest. **45**, 497–504

Engström-Laurent, A., Lööf, L., Nyberg, A. & Schröder, T. (1985*b*) Hepatology **5**, 638–642

Eriksson, S., Fraser, J. R. E., Laurent, T. C., Pertoft, H. & Smedsrød, B. (1983) Exp. Cell Res. **144**, 223–228

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

Fraser, J. R. E., Laurent, T. C., Engström-Laurent, A. & Laurent, U. B. G. (1984) Clin. Exp. Pharmacol. Physiol. **11**, 17–25

Fraser, J. R. E., Alcorn, D., Laurent, T. C., Robinson, A. D. & Ryan G. B. (1985) Cell Tissue Res. **242**, 505–510

Fraser, J. R. E., Engström-Laurent, A., Nyberg, A. & Laurent, T. C. (1986) J. Lab. Clin. Med. **107**, 79–85

Heitmann, H. H. (1970) Zentralbl. Veterinärmed. Reihe A **17**, 517–526

Lascelles, A. K. & Morris, B. (1961) Q. J. Exp. Physiol. **46**, 199–205

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

Laurent, U. B. G. & Granath, K. A. (1983) Exp. Eye. Res. **36**, 481–492

Laurent, U. B. G. & Laurent, T. C. (1981) Biochem. Int. **2**, 195–199

Laurent, U. B. G. & Tengblad, A. (1980) Anal. Biochem. **109**, 386–394

Pharmacia Fine Chemicals (1974) Affinity chromatography, Principles and Methods, Pharmacia, Lund, Sweden

Ropes, M. W., Bennet, G. A., Cobb, S., Jacox, R. & Jessar, R. A. (1959) Ann. Rheum. Dis. **18**, 49–53

Scheuer, P. J. (1967) Proc. R. Soc. Med. **60**, 1257–1260

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

Tengblad, A. (1979) Biochim. Biophys. Acta **578**, 281–289

Tengblad, A. (1980) Biochem. J. **185**, 101–105

Wik, K. O., Andersson, T., Jacobsson, J. Ö. & Granath, K. (1979) in Wik, K. O. (1979) Physicochemical Studies on Hyaluronate (Acta Universitatis Upsaliensis, Dissertation no. 334), Faculty of Medicine, University of Uppsala