Heparin Facilitates the Extraction of Tissue Fibronectin

Abstract. *Extraction of fibronectin from two human tissues, lung parenchyma and placental villi, was facilitated by the incorporation of heparin into extraction media. The effect of heparin was additive to the effect of urea which is known to extract fibronectin. These experiments provide further evidence that fibronectin and glycosaminoglycans are associated in connective tissues and the use of heparin forms the basis for a simple method for extraction and quantitation of tissue fibronectin.*

Fibronectin, a glycoprotein of high molecular weight, is a major component of connective tissue matrix, occurring both in loose connective tissue and in association with basement membranes (*1*). In several biological systems fibronectin is associated with glycosaminoglycans (GAG's) and collagen. For example, fibroblasts in culture produce an extracellular matrix that contains fibronectin, heparan sulfate proteoglycans, and hyaluronic acid in addition to collagen (*2*). Fibronectin and GAG's are solubilized from lung tissue by collagenase digestion as collagen is degraded (*3*). Fibronectin and sulfated GAG's as proteoglycans appear to be contiguous on the surfaces of hamster fibroblasts (*4*). The GAG which has been most clearly implicated with fibronectin in systems in vitro is heparin, which interacts with both plasma fibronectin (*5*) and cellular fibronectin (*6*). Heparin in low concentrations enhances the formation of insoluble complexes between fibronectin and collagen types I and III (*7*) and increases the rate of binding of fibronectin to collagen (*8*). Complexes of fibronectin with gelatin are more resistant to dissociation if they are formed in the presence of heparin (*9*). Heparin is also necessary for the fibronectin-mediated uptake of gelatin-coated latex particles by peritoneal macrophages (*10*). These observations suggested that solubilization of fibronectin from tissues could be facilitated by including heparin in extraction media. The added heparin would be expected to compete on the basis of a mass action effect with related tissue GAG's for binding sites on fibronectin.

Table 1 shows the effect of heparin on the solubilization of tissue fibronectin. The amount of fibronectin in extracts containing 10 mg of heparin per milliliter was approximately doubled in first extracts compared to phosphate buffer extracts. As little as 2.5 mg of heparin per milliliter appeared to facilitate extraction of fibronectin from human lung parenchyma; lower concentrations (1 and 0.1 mg/ml) did not increase the fibronectin in tissue extracts (placenta) compared to phosphate buffer extracts.

The effect of heparin (10 mg/ml) in extracting fibronectin was additive to the effect of urea (*11*), and the most efficient extraction mixture contained both heparin and urea. As expected, because of improved mixing of small samples, the efficiency of extraction was inversely related to the size of the tissue sample extracted. The smaller samples, 1 g and 0.5 g of tissue, gave the highest yields of fibronectin. The extraction and analytical procedures were reproducible. In nine experiments on 0.5-g samples of placental villi the fibronectin concentration in tissue extracts containing 10 mg of heparin per milliliter averaged 0.243 mg/ml with a standard deviation from the mean of 0.0494. As one would expect from any extraction procedure, approximately half again as much fibronectin was obtained upon reextraction of a tissue residue after one extraction (Table 2). Therefore, at least three extractions would be required to approach a quantitative removal of tissue fibronectin. Also, the total amount of fibronectin extracted on three sequential extractions with heparin was 2.4- to 4.0-fold greater than that extracted with phosphate buffer alone.

The increase of fibronectin in heparin-containing extracts of both lung and placenta was also apparent in double-diffusion experiments (Fig. 1). In such experiments the fibronectin concentration in extracts obtained by phosphate buffer alone was frequently too low for detection (Fig. 1, well 2), whereas the urea extract (Fig. 1, well 5) and the heparin-containing extracts (Fig. 1, wells 3 and 6) formed a single line of identity with a line formed between plasma fibronectin and the antiserum. Fibronectin in the heparin-containing extracts (10 mg of heparin per milliliter) migrated faster in immunoelectrophoresis (not shown) than did fibronectin in the phosphate buffer extracts, consistent with the presence of a more highly charged fibronectin-heparin complex.

The size of the fibronectin-related material in the heparin extracts was exam-

Table 1. Effect of heparin on extraction of tissue fibronectin. For every gram (wet weight) of tissue (*17*), 2 ml of either buffer (0.05*M* phosphate, *p*H 7.1) or buffer containing varying concentrations of heparin was used. The heparin (Sigma) was a preparation from porcine intestinal mucosa of 159 anticoagulant units (U.S. Pharmacopeia) per milligram. All extraction solutions contained 2 m*M* phenylmethylsulfonyl fluoride (PMSF) to prevent proteolysis of fibronectin. In certain experiments 2*M* urea (ultrapure grade from Schwarz/Mann) was included in the extraction solutions since cellular fibronectin can be extracted with urea (*11*). Extraction was carried out by stirring the minced tissue in the extraction fluid for 4 hours at room temperature on a magnetic stirrer. The samples were centrifuged at 7700*g* for 20 minutes and the supernatants were analyzed for fibronectin content in the electroimmunoassay, with plasma of known fibronectin content being used as a standard (*13*, *18*). The antiserum to human plasma fibronectin used in the assay was prepared in rabbits and was monospecific, giving only one arc in an immunoelectrophoresis experiment with human plasma.

Weight of tissue (g)	Heparin concentration (mg/ml)	Fibronectin in supernatant (mg/ml)
	Lung tissue	
1	0	< 0.03
	10	0.09
	5	0.09
	2.5	0.07
1	0	0.05
	10	0.10
	0 (2*M* urea)	0.09
	10 (2*M* urea)	0.13
	Placenta	
0.5	0	0.092
	10	0.20
1	0	0.10
	10	0.22
	1	0.12
	0.1	0.073
5	0	0.05
	10	0.10
	0 (2*M* urea)	0.11
	10 (2*M* urea)	0.26
10	0	0.038
	10	0.070

Table 2. Sequential extraction of tissue fibronectin from placenta. The procedure used was the same as for Table 1 except the tissue samples were extracted sequentially three times with either phosphate buffer or phosphate buffer containing heparin (10 mg/ml). All extraction solutions contained PMSF.

Weight of tissue (g)	Heparin concentration (mg/ml)	Extraction number	Fibronectin in supernatant (mg/ml)
0.5	0	1	0.036
		2	0.026
		3	< 0.026
0.5	10	1	0.200
		2	0.110
		3	0.040
0.5	0	1	0.080
		2	0.038
		3	< 0.026
0.5	10	1	0.236
		2	0.082
		3	0.028

0036-8075/81/1113-0793$01.00/0

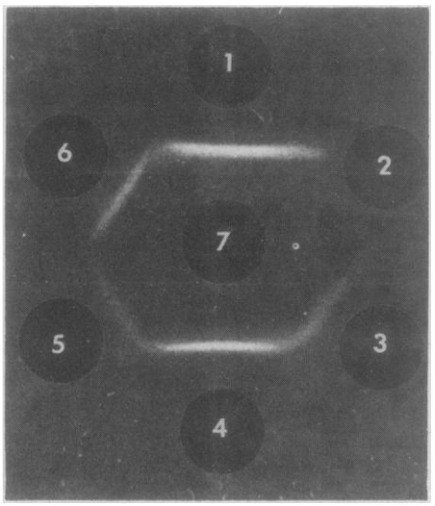

Fig. 1. Double-diffusion reactions of tissue (placenta) extracts with antibody to fibronectin. The contents of the wells were as follows: 1 and 4, human plasma fibronectin as a reference standard; 2, phosphate buffer extract; 3, heparin (10 mg/ml) extract; 5, urea (2*M*) extract; 6, urea (2*M*) and heparin (10 mg/ml) extract; and 7, antiserum to human plasma fibronectin.

ined by crossed immunoelectrophoresis with the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 5 percent disc gel in the first dimension to separate the antigens according to molecular weight (Fig. 2). There were two distinct peaks of fibronectin-related material, one of 440,000 daltons, presumably dimeric fibronectin, and one larger than 440,000 daltons. Thus, the extracted fibronectin was intact.

In addition to the commercial heparin (Sigma) used in the experiments of Table 1, reference heparin (*12*), which was purified from crude beef lung heparin, was as effective as the preparation from Sigma in facilitating fibronectin extraction. This reference heparin had an anticoagulant activity of 183 units (U.S. Pharmacopeia) per milligram.

We also tested reference heparan sulfate and dermatan sulfate (*12*). The heparan sulfate was as effective as heparin in facilitating extraction of tissue fibronectin, and the dermatan sulfate was only slightly less effective. In contrast, neither chondroitin 4-sulfate (whale cartilage; Sigma) nor chondroitin 6-sulfate (shark cartilage; Sigma) produced the effect. Two dextran sulfates (obtained from Sigma) of molecular weights 5000 and 8000 produced an intermediate effect (*13*).

As anticipated, the incorporation of heparin into phosphate buffer facilitated the extraction of fibronectin from tissues, apparently by the formation of a soluble fibronectin-heparin complex. The fibronectin extracted in the presence of heparin was not degraded. The major peak was 440,000 daltons, the size of the fibronectin dimer (Fig. 2). The presence of dimeric fibronectin and of aggregated fibronectin (material larger than 440,000 daltons) is what one would expect from other reports in the literature (*14, 15*).

That the most effective extraction mixture contained both heparin and urea raises the possibility that there may be more than one type of tissue fibronectin or one type which is incorporated into tissues in at least two different ways. The extensive perfusion of the placenta and thorough washing of the lung tissue ruled out any contribution from plasma fibronectin in these organs. Indeed, the hemoglobin content of the extracts was ≦ 0.2 percent.

The data show that high concentrations of heparin (≧ 2.5 mg/ml) are required for the extraction of fibronectin in soluble form. These results agree with the data of Stathakis and Mosesson (*5*) who showed that the cryoprecipitation of heparin and fibronectin is dependent on the heparin concentration, occurring optimally at 0.25 to 0.5 mg of heparin per milliliter and failing to occur above 2 mg/ml. Therefore, even though extensive disruption of tissue occurred when low concentrations of heparin (1 and 0.1 mg/ml) were used to extract tissue (*16*), the supernatants were not enriched in fibronectin, probably because it was being removed as a precipitate with heparin during centrifugation and storage of samples at 4°C.

The fact that neither chondroitin 4-sulfate nor 6-sulfate facilitated the extraction of fibronectin, whereas heparan sulfate and dermatan sulfate did, suggests that the effect is highly specific, and this specificity agrees in general with that observed in studies on the effect of GAG's on the interaction of fibronectin with collagen (*7, 9*). The ability of heparan sulfate and of heparin, which is chemically similar to heparan sulfate, to dislodge fibronectin from connective tissues is consistent with previous evidence of the association of heparan sulfate with fibronectin in tissues (*2*). Since heparin is commercially available, the experiments reported herein provide the basis for a simple reproducible method of extraction of tissue fibronectin for routine quantitative studies. The procedure can easily be performed on 0.5 g of tissue (wet weight) and will therefore be useful in studies comparing normal tissue with diseased specimens. Since the procedure is carried out at *p*H 7.1 and the extracted fibronectin is intact, it may also lead to methods for the preparation of fibronectin for use in cell culture systems or physiological experiments in vivo.

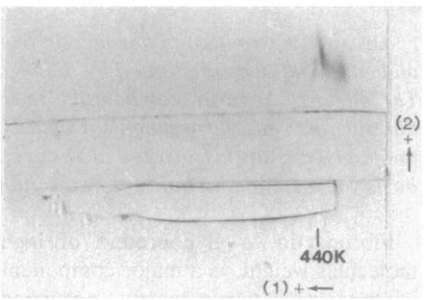

Fig. 2. Crossed immunoelectrophoresis (*19*) of a heparin extract of human placenta. The extract (100 μl) was added to an equal volume of 0.4 percent SDS and 4*M* urea and the mixture was boiled for 5 minutes. The entire sample was electrophoresed on a 5 percent disc gel by SDS-PAGE. The gel was sliced in half and half a gel was positioned so that the separated components would migrate into agarose (12 ml) containing antibody to human plasma fibronectin (100 μl). A layer of agarose containing Triton X-100 was placed between the disc gel and the agarose-antibody layer to avoid artifacts produced by SDS. This second electrophoresis was carried out for 6 hours at 5 V/cm; the plate was then soaked free of proteins that were not involved in the immunoprecipitate, blotted and dried, and stained with Coomassie blue for proteins. For reference, molecular weight markers, including plasma fibronectin (dimer of 440,000 daltons), were run on companion gels and were stained with Coomassie blue to locate the position of intact fibronectin (dimer).

BONNIE ANDERSON BRAY
Department of Medicine, Columbia University, New York 10032
INES MANDL
Department of Obstetrics and Gynecology, Columbia University
GERARD M. TURINO
Department of Medicine, Columbia University

References and Notes

1. A. Vaheri and D. F. Mosher, *Biochim. Biophys. Acta* **516**, 1 (1978); K. M. Yamada and K. Olden, *Nature (London)* **275**, 179 (1978); E. Pearlstein, L. I. Gold, A. Garcia-Pardo, *Mol. Cell. Biochem.* **29**, 103 (1980).
2. K. Hedman, M. Kurkinen, K. Alitalo, A. Vaheri, S. Johansson, M. Höök, *J. Cell Biol.* **81**, 83 (1979).
3. B. A. Bray, *J. Clin. Invest.* **62**, 745 (1978).
4. M. E. Perkins, T. H. Ji, R. O. Hynes, *Cell* **16**, 941 (1979).
5. N. E. Stathakis and M. W. Mosesson, *J. Clin. Invest.* **60**, 855 (1977).
6. K. M. Yamada, D. W. Kennedy, K. Kimata, R. M. Pratt, *J. Biol. Chem.* **255**, 6055 (1980).
7. F. Jilek and H. Hörmann, *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 597 (1979).
8. S. Johansson and M. Höök, *Biochem. J.* **187**, 521 (1980).
9. E. Ruoslahti and E. Engvall, *Biochim. Biophys. Acta* **631**, 350 (1980).
10. P. W. Gudewicz, J. Molnar, M. Z. Lai, D. W. Beezhold, G. E. Siefring, Jr., R. B. Credo, L. Lorand, *J. Cell Biol.* **87**, 427 (1980).
11. K. M. Yamada, D. H. Schlesinger, D. W. Kennedy, I. Pastan, *Biochemistry* **16**, 5552 (1977).

12. The reference heparin, heparan sulfate, and the dermatan sulfate were supplied by M. B. Mathews and J. A. Cifonelli, University of Chicago, under contract with NIH.
13. To be certain that the presence of heparin did not affect the quantitation of fibronectin by electroimmunoassay, we ran a control test as follows. To a phosphate buffer extract of tissue which contained fibronectin, we added enough heparin to bring the concentration of heparin to 10 mg/ml. Two aliquots of this mixture were run side by side with two aliquots of phosphate buffer extract to which no heparin was added. The analytical values for fibronectin were not affected by the addition of heparin; these values were 0.051 and 0.045 mg/ml for the phosphate buffer extract and 0.051 and 0.045 for the phosphate buffer extract containing heparin. Similar comparisons showed no effect when heparan sulfate or dermatan sulfate was used in place of heparin. However, the dextran sulfates of 5000 and 8000 molecular weight showed a slight effect toward increasing the height of the precipitin peaks and thus the fibronectin values of these same phosphate buffer extracts.
14. J. Keski-Oja, D. F. Mosher, A. Vaheri, *Biochem. Biophys. Res. Commun.* **74**, 699 (1977).
15. R. O. Hynes and A. Destree, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2855 (1977).
16. B. A. Bray, I. Mandl, G. M. Turino, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 769 (Abstr. No. 3066) (1981).
17. Normal lung tissue was obtained at autopsy from a 32-year-old female and was stored at −70°C. Dissected parenchyma, consisting mostly of alveolar-capillary tissue, was washed free of blood with 0.15*M* NaCl containing 2 m*M* PMSF. A term placenta was perfused immediately after birth with phosphate-buffered, citrated saline until it was free of blood. It was then perfused with a mixture of 1 m*M* PMSF, 1 m*M* iodoacetamide, 100 m*M* ε-aminocaproic acid, 5 m*M* benzamidine hydrochloride, 10 m*M* disodium ethylenediaminetetraacetic acid, and soybean trypsin inhibitor (50 μg/ml) at physiological *p*H and ionic strength. The perfused cotyledons were teased off and stored frozen.
18. C.-B. Laurell, *Anal. Biochem.* **15**, 45 (1966).
19. C. A. Converse and D. S. Papermaster, *Science* **189**, 469 (1975); F. H. Kirkpatrick and D. J. Rose, *Anal. Biochem* **89**, 130 (1978).
20. We thank Anita Hersh for technical assistance and Dr. Karl Meyer for review of the manuscript. Supported by NIH grant HL 15832. A preliminary report of the work was presented at the FASEB meeting in Atlanta, 1981 (*16*).

15 May 1981; revised 26 August 1981

Lamellar Twinning Explains the Nearly Racemic Composition of Chiral, Single Crystals of Hexahelicene

Abstract. *Solvent etching of single crystals of hexahelicene grown from a racemic solution reveals an unusual layer-like pattern in which pure (+)- and pure (−)-layers alternate through the crystal; this arrangement results in a nearly racemic composition although the crystal is ostensibly chiral, space group* $P2_12_12_1$. *Etched crystals of enantiomerically pure hexahelicene display no such pattern. The two kinds of crystal are indistinguishable by x-ray diffraction.*

Crystals of hexahelicene grown from racemic solution show an unusual behavior: although the crystals are apparently chiral ["spontaneously resolved"; enantiomorphic space group $P2_12_12_1$ (*1*), structure refined to $R = 4$ percent (*2*)], dissolved single crystals display optical rotations corresponding to enantiomeric excesses of only ~ 2 percent instead of 100 percent expected for chiral, single crystals. Similar results have also been obtained with other, related materials (*3*). This puzzling phenomenon is relevant to the general problem of spontaneous resolution (*4*) and to the use of crystal-state reactions for asymmetric synthesis (*5*) and may be of fundamental importance for understanding the nature of chiral interactions. We have therefore examined the situation in more detail.

Substitutional solid solutions of (+)- and (−)-enantiomers seemed unlikely on the basis of the packing arrangement of the molecules (*2*), their marked steric differences (Fig. 1), and the significant difference in the melting points of racemic (231° to 233°C) and optically pure (265° to 267°C) material (*6*). The x-ray study showed no evidence of molecular disorder.

Ordinary twinning (*7*) along a growth direction was initially considered but rejected on the basis of the following experiment. A large single crystal grown from a racemic solution was cleaved in two, each half was further cleaved in two, and the four fragments were allowed to partially dissolve in *n*-hexane until small crystals, each ~ 10 percent of the original crystal, remained. Each of these crystals contained the same, ~ 2 percent, enantiomeric excess and had the same sign of rotation as the original crystal (*8*). Had the fragments contained opposite enantiomers in excess, ordinary twinning along a growth direction would have been indicated.

However, when single crystals were allowed to dissolve only slightly in *n*-hexane or in carbon tetrachloride, an interesting lamellar pattern was readily discerned under the light microscope (Fig. 2). The layers, 10 to 30 μm thick, could be carefully cleaved from the crystal, and individual layers were now found to display optical purities of ~ 100 percent (*9*). When adjacent layers in the same crystal were examined, the signs of rotation alternated. Although all crystals grown from racemic hexahelicene dis-

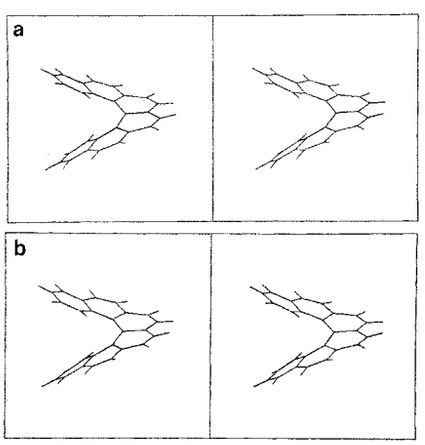

Fig. 1 (left). Stereodrawings of enantiomers of hexahelicene: (a) P-(+)-hexahelicene; (b) M-(−)-hexahelicene.

Fig. 2 (right). (a) Crystal of nearly racemic hexahelicene (measured enantiomeric excess 2 percent), grown from an ether–*n*-hexane solution of racemic hexahelicene, after partial solution (etching) in *n*-hexane. (b) Crystal of optically pure hexahelicene (measured enantiomeric excess > 99 percent) grown from ether-*n*-hexane solution after etching in *n*-hexane.

0036-8075/81/1113-0795$01.00/0