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PURIFICATION OF SALT-SOLUBLE CROSS-LINKED ELASTIN BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

The hydrophobic protein elastin, which is a major constituent of vascular and lung tissue is fragmented in several pathological conditions. The nature of the soluble fragments is not well understood. Such fragments bind to alkyl chains linked to agarose. Elution, which is effected by dimethylformamide and sodium dodecyl sulphate, is optimal from the decyl-agarose column. Dialysis of the eluates against buffered sodium chloride precipitates elastin, thus further purifying the salt-soluble cross-linked elastin.

INTRODUCTION

Fibrous elastin, which is an important constituent of several tissues, including blood vessels, lung and skin, is one of the most stable mammalian proteins [1—3]. In disease states such as atherosclerosis, emphysema and pseudoxanthoma elasticum the degradation rate of elastin is increased [4, 5]. Also, insoluble elastin is strongly hydrophobic [4, 6]. Therefore it is likely that soluble polypeptide fragments of elastin extracted from tissue might exhibit hydrophobic properties different from the other proteins in the extract. This suggests that hydrophobic interaction chromatography might be useful for isolating soluble elastin from tissue extracts. Additionally, it has been shown that both α-elastin and soluble degraded elastin bind to decyl-agarose [7]. This report presents an analysis of the binding and eluting properties of elastin fragments when such fragments are applied to agarose columns substituted with variable length aliphatic carbon chain ligands.

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MATERIALS AND METHODS

Animal surgery and tissue extraction

Arteriovenous fistulae were fashioned between the right external jugular vein and the right common carotid artery in sheep as described by Stehbens [8]. At various times post-operatively (between 352 and 580 days), the vascular tissue was removed and the loose, perivascular fascia and fat were discarded. The venous tissue in the vicinity of the anastomoses was removed, weighed and stored at −70° C until required.

The cutting of the tissue and the extraction with 0.2 *M* sodium chloride in 0.02 *M* sodium phosphate, pH 7.4 (extraction buffer) were performed as previously described [9]. In brief, this involved cutting the tissue finely, shaking for 16 h at 4° C, followed by centrifugation at 28,000 *g* for 40 min at 4° C. The supernatant was used for the chromatography of soluble elastin.

Hydrophobic interaction chromatography

Tissue extracts (0.5 mg protein) were applied at room temperature to 1-ml columns of alkyl-substituted agarose (Miles-Yeda, Rehovot, Israel) which had been previously equilibrated with extraction buffer. The agarose was substituted with alkyl chains containing either $0(C_0)$, $2(C_2)$, $4(C_4)$, $6(C_6)$, $8(C_8)$, or $10(C_{10})$ carbons. The columns were developed firstly with 2 ml of extraction buffer followed by 2 ml of 50% dimethylformamide (DMF), 2 ml of 1% sodium dodecyl sulphate (SDS) and finally a further 2 ml of extraction buffer.

Electrophoresis

SDS—slab gel electrophoresis using a 10% polyacrylamide running gel and a 4% polyacrylamide stacking gel was performed according to the method of Laemmli [10] with the following modification. The Tris—HCl separating gel buffer was at a pH of 9.5.

The total volume of each eluate was dialysed against water, lyophilized and redissolved in 150 μl of a solution of 1.5 *M* Tris—HCl, pH 8.8, 3.0% SDS, 0.1% bromophenol blue and 15% glycerol. Where indicated, β-mercaptoethanol at a final concentration of 1% was added. These samples were placed in boiling water for 3 min before being electrophoresed on polyacrylamide gels. After electrophoresis gels were stained with 0.1% Coomassie Blue dissolved in 50% methanol—10% acetic acid and then destained with a 10% methanol—7% acetic acid mixture.

Precipitate formation

The formation of an insoluble fraction from the different eluates occurred spontaneously on dialysis against 2 × 1 l of extraction buffer at room temperature for 24 h. Centrifugation at room temperature at 2000 *g* for 20 min sedimented the insoluble material. The supernatant was discarded and the residue was washed twice with extraction buffer.

Hydrolysis and amino acid analysis

Protein hydrolysis was performed in vacuo in constant boiling hydrochloric

acid for 65 h at 110°C. Amino acid analysis was conducted on a Beckman 119CL amino acid analyzer.

Protein determination

Two methods of determining protein levels were used. The concentration in the tissue extract was estimated by the method of Lowry et al. [11] using bovine serum albumin as the standard. The protein concentration in the column fractions was determined from the amino acid composition of aliquots that had been hydrolyzed and analyzed.

RESULTS

Aliquots of the buffered sodium chloride extracts from the venous extracts were applied to each of the six columns of agarose containing different alkyl ligands. The columns were then eluted sequentially with extraction buffer, DMF, SDS and extraction buffer again as described in Materials and Methods.

SDS—polyacrylamide gel electrophoresis

The distribution of protein eluted by each of the solvents when passed through each of the columns is illustrated in the SDS—polyacrylamide gel patterns in Fig. 1. These gel patterns indicate that all of the column eluates are heterogeneous. All display a number of bands over a wide range of molecular sizes. The gel of extraction buffer eluates confirms that the longer carbon chain ligands retain more protein (Fig. 1A). However this trend does show some variability with different tissue extracts. With each of the extracts examined, DMF consistently elutes the greatest amount of protein from the C_8 and C_{10} columns (Fig. 1B). The gel electrophoresis of the SDS and the second

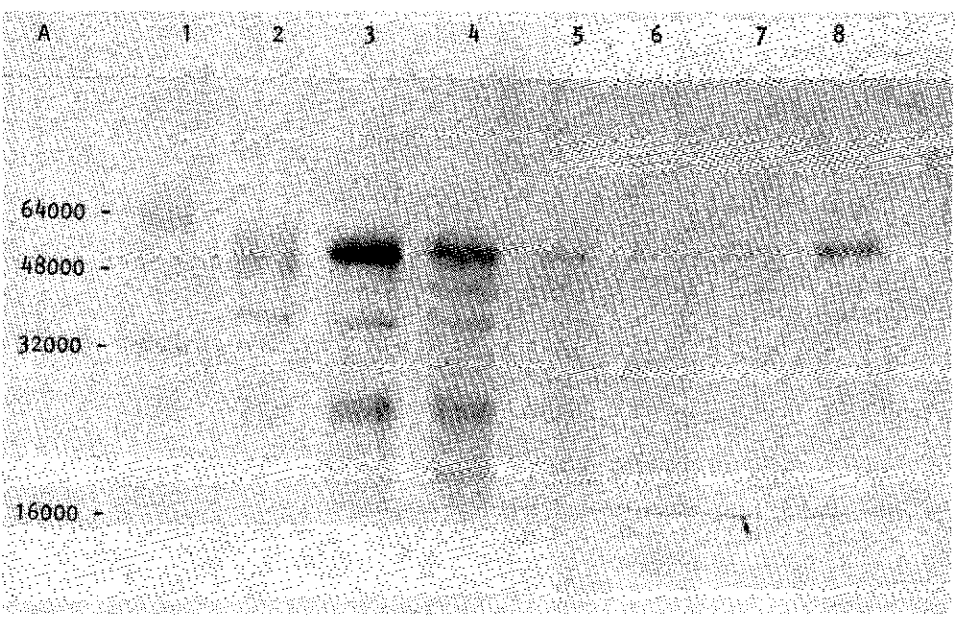

Fig. 1.

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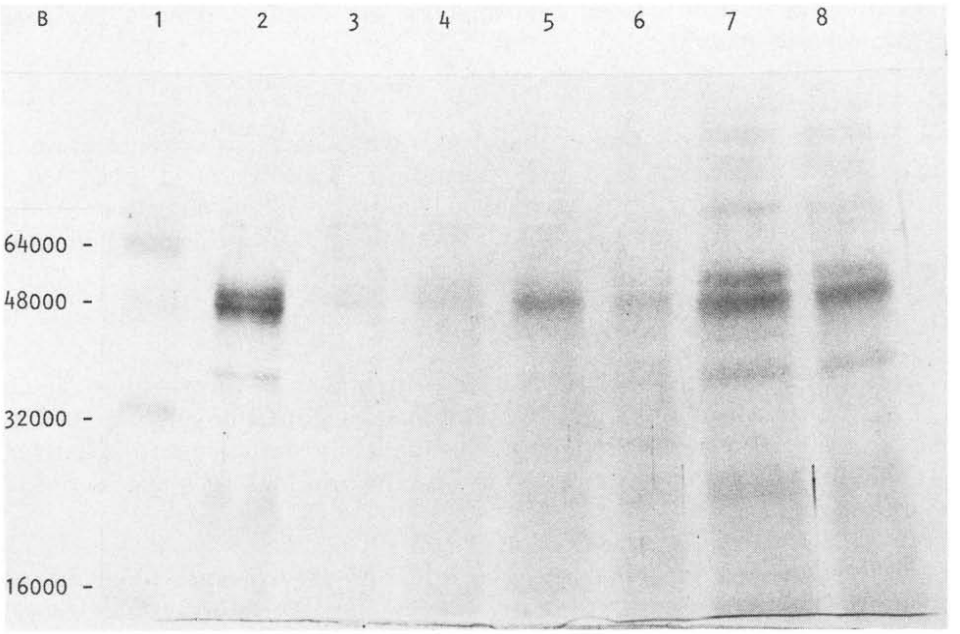
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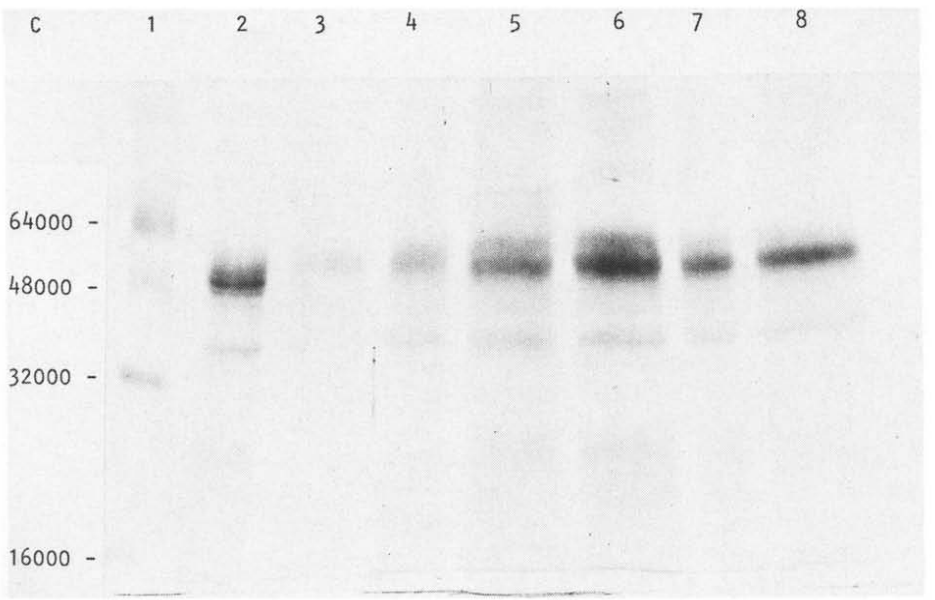
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Fig. 1.

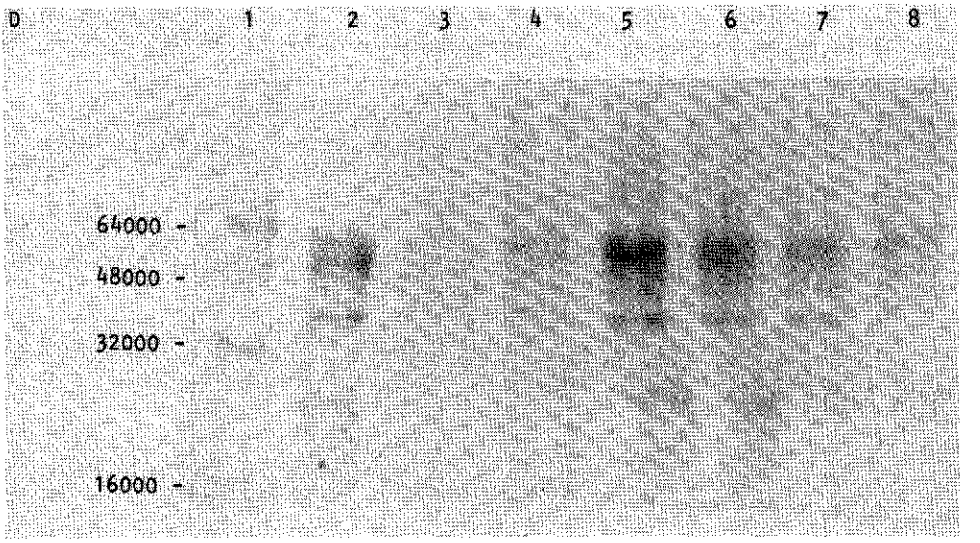

Fig. 1. SDS—polyacrylamide gel electrophoresis of eluates from agarose column substituted with alkyl chains. The total eluate volume was concentrated before preparation for electrophoresis. (A) Extraction buffer eluates; (B) DMF eluates; (C) SDS eluates; (D) second extraction buffer eluates. See Materials and Methods for conditions of electrophoresis. Lane 1: cross-linked haemoglobin; Lane 2: tissue extract; Lane 3: C_0 eluate; Lane 4: C_2 eluate; Lane 5: C_4 eluate; Lane 6: C_6 eluate; Lane 7: C_8 eluate; Lane 8: C_{10} eluate.

extraction buffer eluates suggest that these solvents are eluting, non-specifically, the remaining bound protein (Fig. 1C and D).

When electrophoresis is performed on samples that have been treated with β-mercaptoethanol, much of the slowly migrating material appears to diminish quantitatively. Concomitantly there is an increase in faster migrating species. This is illustrated for the eluates from the C_{10} column (Fig. 2).

Amino acid analyses and protein elution profiles

The protein hydrolyzates were analyzed so as to determine the protein concentration of each eluate. These analyses also provided a comparison of the amino acid composition of each fraction. Fig. 3 indicates that, of the extraction buffer eluates, the highest protein concentration is in that from the C_0 column. DMF is most effective at removing bound proteins from the C_8 column while of the SDS eluates, that from the C_{10} had the highest protein concentration. For the second extraction buffer elution, the highest protein concentrations were from the agaroses with the shorter chain ligands.

Elastin elution

As a measure of the binding and eluting behaviour of soluble cross-linked elastin which is known to be present in the extracts [7], the desmosine plus isodesmosine concentrations of the eluates (as determined by amino acid analyses) are shown in Fig. 4. There was no cross-link detectable in the extraction buffer eluate. The highest concentration amongst the DMF eluates is found in the C_{10} eluate whilst amongst the SDS eluates only the C_2 and C_{10}

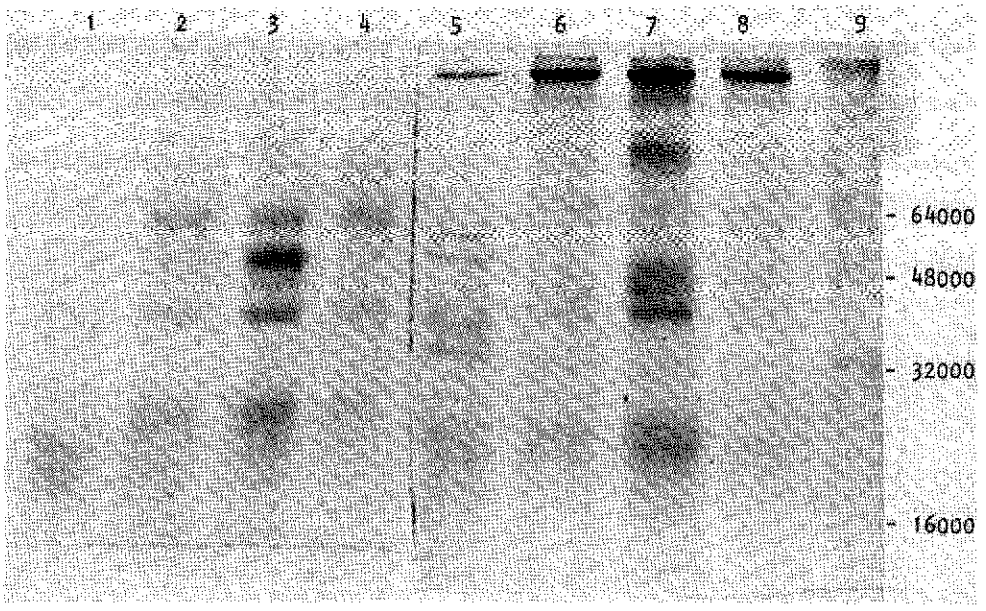

Fig. 2. Polyacrylamide gel electrophoresis of eluates from C_{10} column. Lanes 1 and 5: extraction buffer eluates; Lanes 2 and 6: DMF eluates; Lanes 3 and 7: SDS eluates; Lanes 4 and 8: second extraction buffer eluates; Lane 9: cross-linked haemoglobin; Lanes 1—4: in presence of β-mercaptoethanol; Lanes 5—9: in absence of 1% β-mercaptoethanol.

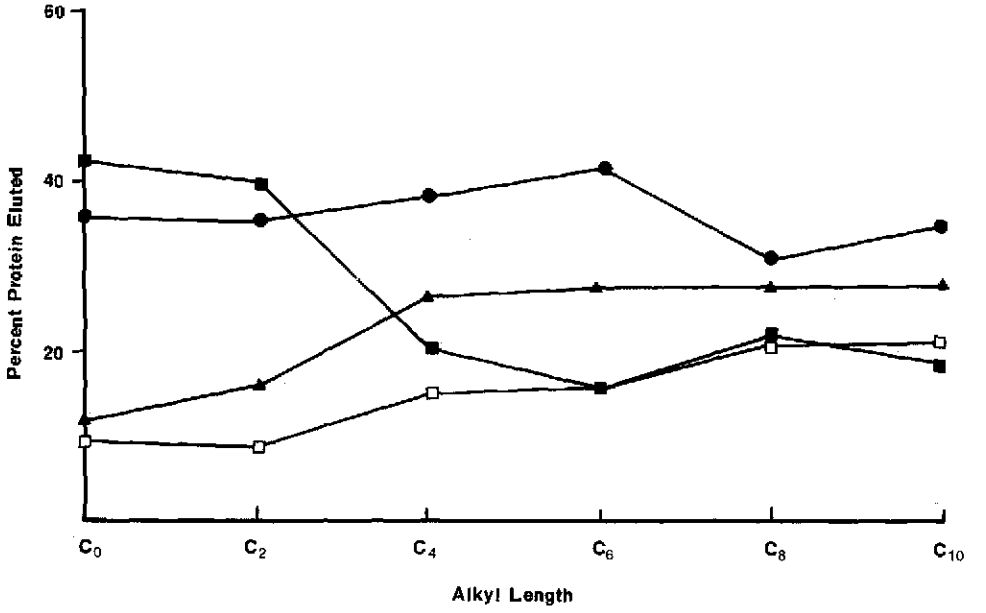

Fig. 3. Distribution of protein in eluates from alkyl-substituted agarose columns. (■—■) Extraction buffer eluate; (□—□) DMF eluate; (▲—▲) SDS eluate; (●—●) second extraction buffer eluate.

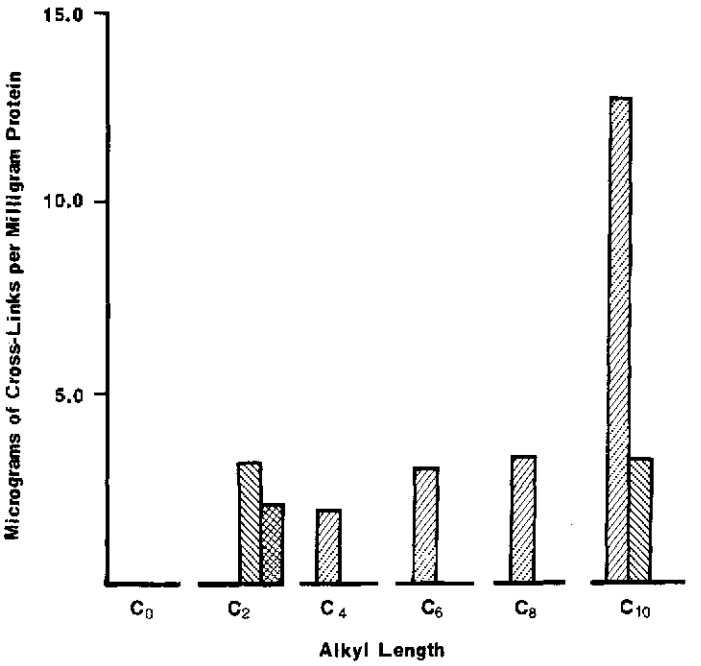

Fig. 4. Distribution of elastin cross-links (isodesmosine and desmosine) in eluates from alkyl-subtituted agarose columns. (▨) DMF eluate; (▧) SDS eluate; (▩) second extraction buffer eluate.

contained significant concentrations of these cross-links. There was cross-link also present in the second extraction buffer eluate from the C_2 column.

Precipitate formation

After dialysis of the eluents against extraction buffer at room temperature, some formed precipitates. Varying amounts of precipitates formed from the DMF and SDS eluates and the extent of these seemed to parallel the distribution patterns of the cross-links (data not shown). When these precipitates were washed with extraction buffer, dried, hydrolyzed and analyzed, there was a noticeable compositional change. As an example, the composition of the DMF eluate from the C_{10} column is compared with the precipitate obtained from the same eluate (Table I). As determined by their difference indices [12], precipitate resulting from dialysis of the DMF eluate has greater similarity than the DMF eluate itself to α-elastin [7] and to fibrous elastin [9].

DISCUSSION

The recent detection of polypeptides containing elastin cross-links in the salt extracts from vascular tissue has important implications with regard to the turnover of fibrous elastin [7]. Analyses of these polypeptides indicated the presence of both desmosine and isodesmosine. Thus it would appear that the protein(s) containing these is a fragment of the insoluble fibrous molecule and that it is not essential to cleave to pyridinium cross-links per se so as to

TABLE I

AMINO ACID COMPOSITION OF SOLUBLE ELASTIN FRACTIONS COMPARED WITH FIBROUS ELASTIN

Cross-links expressed as leucine equivalents.

	Residues per 1000 residues			
	DMF eluate from C_{10} column	Precipitate from DMF eluate from C_{10} column	α-Elastin [7]	Fibrous elastin [9]
Lysine	82.0	22.3	30.7	7.9
Histidine	33.9	11.2	13.6	3.0
Arginine	30.9	18.7	6.8	11.1
Hydroxypyroline	—	—	—	22.4
Aspartic acid	102.1	34.9	1.8	15.2
Threonine	—	19.0	9.2	16.6
Serine	14.8	33.2	9.0	17.8
Glutamic acid	146.3	67.2	20.4	25.8
Proline	62.0	85.8	105.3	103.0
Glycine	162.9	265.2	241.1	304.5
Alanine	104.8	215.2	288.7	214.7
Valine	86.0	80.9	111.7	118.3
Methionine	—	—	—	1.7
Isoleucine	16.3	26.2	17.5	23.2
Leucine	95.4	58.4	57.5	58.9
Tyrosine	7.5	11.8	13.4	13.9
Phenylalanine	38.4	34.1	34.5	29.7
Isodesmosine	—	6.3	29.3	4.4
Desmosine	16.7	9.9	7.2	6.2
Lysinonorleucine	—	—	2.2	1.3
Difference index [12] (comparison with α-elastin)	36.6	16.7		
Difference index [12] (comparison with fibrous elastin)	38.4	12.3		

solubilize the fibre. The ability to isolate such soluble elastin would assist the characterization of it, which in turn, may aid the elucidation of the mechanism of cleavage. By taking advantage of the strong hydrophobic nature of elastin [4, 6], it has been possible, by chromatography on decyl-agarose, to purify partially the soluble cross-linked elastin [7]. This hydrophobic characteristic has been used previously for the purification of soluble elastin by means of relatively higher concentrations of alcohols [13]. This report provides further data on the behaviour of cross-linked elastin when chromatographed on agaroses substituted with alkyl chains.

The gel electrophoretic patterns suggest that the lowest protein binding for vascular tissue extracts is to the C_0- and C_2-substituted matrices (Fig. 1A). This is supported by the quantitative protein determinations (Fig. 3). As a protein eluent, DMF is most effective for the material bound to the longer

alkyl chain columns (C_8 and C_{10}) (Figs. 1B and 3) while SDS appears to be most suitable for the C_6 (Fig. 1C) and C_{10} matrix (Fig. 3). The second extraction buffer elution was most effective for the shorter chain ligands.

DMF and SDS eluates, when electrophoresed in the absence of β-mercaptoethanol, indicate a predominance of high molecular weight species. As this material is reducible by the addition of β-mercaptoethanol and as elastin contains neither free thiols nor disulphide linkages, it would appear that there are disulphide-containing proteins associated with the soluble elastin. Although the nature of these is unknown, one possibility is that they might be microfibrillar proteins which contain disulphides and which are known to associate with fibrous elastin [14]. The predominant bands in the reduced samples comigrate with proteins of approximately 40,000 to 50,000 daltons. The protein species eluted by each solvent appear to be rather similar for each of the six columns — it is the quantities that vary.

Difficulties were experienced in determining the protein concentration of the eluted fractions by colorimetric techniques such as the method of Lowry et al. [11], the biuret method [15] and the method according to Bradford [16]. Components in the eluents interfered with colour development in each case. Hydrolysis and amino acid analysis of an aliquot of each fraction not only yielded the composition but also provided a very accurate determination of the protein concentration [17]. Such data provide quantitative confirmation that the greatest binding capacity for vascular proteins is provided by the C_6, C_8 and C_{10} columns (Fig. 3). DMF is most effective at removing protein from the C_8 and C_{10} columns (about 20% of the bound protein) while SDS removed between 24% and 27% of that bound to the C_4—C_{10} columns. There is further elution of protein when a second extraction buffer elution is performed. As indicated in Fig. 3, the amount eluted by this solvent is rather constant (between 34% and 42%) for all columns except for the C_8 where the level is only about 28% of the applied protein.

The yield of cross-links (desmosine plus isodesmosine) as determined by amino acid analysis can be used as an index of the elastin fragments present in an extract [18]. The absence of cross-links in any of the first extraction buffer eluates indicates that elastin binds to these substituted agaroses. DMF elutes cross-link containing material from the C_4—C_{10} columns. By far the highest level of cross-link per mg of protein is in the DMF eluate from the C_{10} column. This indicates that the best preparation of soluble elastin from hydrophobic interaction chromatography is in this eluate. There is some elution of cross-link by SDS from both the C_2 and C_{10} columns. There is no cross-link apparent in any of the eluates from the unsubstituted agarose (C_0) column. A possible explanation is that there are reduced quantities of protein (including elastin) bound to this column as indicated by the relatively high concentration in the extraction buffer eluate (Fig. 2). The low level of cross-links in such a heterogeneous protein mixture would make detection difficult.

When the DMF and the SDS eluates from these substituted agarose columns are dialyzed against extraction buffer, varying amounts of precipitates form. The weights of precipitate as determined gravimetrically closely parallel the elastin content of the fractions. As shown in Table I, these precipitates have a composition more akin to elastin than do the eluates from which they arise.

This suggests that precipitation further purifies the elastin fragments in the extracts. The nature of the precipitate which appears to be fibrous and the mechanism of its formation are currently under investigation.

Hydrophobic interaction chromatography can be used for the isolation of salt-soluble cross-linked elastin from extracts of vascular tissue. Alkyl chain ligands have been used with the optimal appearing to be 10 carbons long. Little difference is detected between the use of alkyl chains and alkyl chains with ω-amino groups (data not shown).

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