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Note

Amino acid content of heparins

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The configuration of "native" or "macromolecular" heparin, as released from (presumably) mast cell granules, has received attention over the past twenty years[1–6], but as yet its nature has not been settled. Whatever it may be, it is generally agreed that the heparin of commerce consists of unbranched single-chain fragments of the macromolecular form, which is not stable to commercial isolation procedures. These fragments are believed to derive from side-chains attached in some numbers to core molecules which have variously been thought to be polypeptide[3] or polysaccharide[4] chains. The side-chains have a molecular weight which is several times that of commercial heparin, so only a proportion of the molecules of the latter can retain anything of the side-chain-to-core linkage region; most are the product of mid-chain scission by an endoglycosidase[7]. As with other glycosaminoglycans the linkage region consists of a glucuronyl-galactosyl-galactosyl-xylosyl-O-serine sequence, of which the serine is regarded as part of the core. Recent work[6] concludes that the core is probably a peptidase-resistant poly(glycylserine). However, other amino acids have been persistently reported as being present in heparin hydrolysates[1,3,8], and Metcalfe *et al.*[9] have acknowledged that a number, chief among them aspartic and glutamic acids, appear to form part of the structure of larger proteoglycans constituting the native heparin of rat peritoneal mast cells. Since none of these amounted to more than a tenth of the serine or glycine present, Metcalfe *et al.* regarded the poly(glycylserine) model as largely valid.

In the course of collaborative work on the possible origin of small nuclear magnetic resonance signals from heparins, we were asked to check on the presence of amino acids in the samples. We found that, when using conventional amino acid analyser equipment, it was very difficult to be sure of the identity of ninhydrin-reacting compounds from heparin hydrolysates, and therefore investigated two more recent analytical procedures using high-performance liquid chromatography with pre-column derivatisation and fluorimetric detection[10,11]. The nature of the problem then became clear: with the higher resolution large numbers of peaks which could not be identified with known amino acids appeared on the chromatograms at all levels of intensity. It was reasonable to attribute them to basic breakdown products of glucosamine, since it had previously been reported that hexosamine yield, as observed on amino acid analyser traces, decreases with increase in the severity of hydrolysis conditions[8]. The interference so caused was sufficient to make it impossible

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to quantify, and in some cases even to detect with certainty, many amino acids, including the ubiquitous serine and glycine, even though two different procedures were available.

The greater part of the hexosamine was therefore destroyed before hydrolysis by reaction with nitrous acid, yielding, principally, anhydromannose residues from all except the N-acetylated glucosamines. This procedure yields much low-molecular-weight material, and to remove this the product was fractionated on Sephadex G-25; remaining peptide bound to the linkage region will be among the larger fragments. Hydrolysis of this material did in fact yield "cleaner" chromatograms on which identification of amino acids was usually unequivocal, though N-terminal amino acids and unprotected lysine would have been destroyed by the nitrous acid treatment. The proportions therefore were not necessarily those present in the heparin sample.

These procedures were applied to five samples of unbleached heparin, three porcine mucosal and one each porcine duodenal and bovine lung, and one sample each of bleached pharmacopoeial-quality porcine mucosal and bovine lung heparin.

EXPERIMENTAL

The porcine mucosal heparin samples were, in origin, as follows: (1) Evans Medical batch 630/2, unbleached; (2) Riker lot N4234; (3) Laboratori Derivati Organici "Fraction 8"; (4) Bioiberica lot F4. The porcine duodenal sample (5) was from Crinos Fraction F5. The bovine lung samples were: (6) Upjohn lot 730EH; (7) from a mixed glycosaminoglycan fraction, no reference, from Riker. Nos. 2, 3, 5 and 7 were more or less crude mixed products from which the heparins were isolated by fractional barium salt precipitation from aqueous solution using isopropanol[12] followed by precipitation as the potassium salts from strong potassium acetate solution[13]. Sample 1 was dissolved in 2 *M* sodium chloride solution and exhaustively dialysed to remove possible electrostatically bound peptide material, and samples 4 and 6, of pharmaceutical quality, were used as received. Sulphate-to-carboxylate ratios[14] ranged from 2.1 (sample 4) to 2.45 (sample 6), and in all samples glucosamine constituted 99% or more of the total hexosamine[15]. The detailed application of these purification and analytical procedures is discussed in ref. 16.

Deamination was carried out essentially as described by Shively and Conrad[17]. The heparin (200 mg) was dissolved in *ca.* 1 ml of water and mixed with *ca.* 5 ml of cold nitrous acid solution obtained by mixing equivalent volumes of chilled 0.5 *M* sulphuric acid and 0.5 *M* barium nitrite and centrifuging. After *ca.* 1 h at room temperature most of the excess of nitrogen oxides was removed by agitating under water pump vacuum, the product was precipitated with 2–3 volumes of ethanol, redissolved and reprecipitated with ethanol containing some sodium acetate, washed with ethanol and dried; the yield was *ca.* 130 mg.

Fractionation on Sephadex G-25 was carried out with 100-mg loads on a column (73 × 2.2 cm I.D.) with 0.1 *M* sodium chloride as eluent, flow-rate 30 ml/h. The detector was a Waters R403 differential refractometer. Four complex peaks eluted from each deamination product, of which the first two gave strong carbazole reactions for uronic acid. The first peak, K_{av} = 0–*ca.* 0.3, was collected for amino acid estimation. Desalting was performed using a column of Sephadex G-15, 37 × 1.4 cm I.D., with a Thorn-NPL type 243 polarimeter as detector.

Hydrolysis for amino acid determination was carried out in 4 *M* hydrochloric acid at 100°C for 16 h. The derivatisation and chromatographic procedures were essentially those of De Jong *et al.*[10] using 1-dimethylaminonaphthalene-5-sulphonyl chloride (Dns), and of Jones *et al.*[11] using *o*-phthaldialdehyde (OPTH). In both cases the column, 200 × 5 mm I.D., was packed with ODS-Hypersil (Shandon Southern). For the Dns procedure β-alanine was added to the sample before hydrolysis as an internal standard, and chromatography was run at ambient temperature. For the OPTH procedure α-aminooctanoic acid (Fluka) served as internal standard, solvent A was modified to tetrahydrofuran–0.05 *M* sodium acetate (pH 5.9) (1:99), solvent B remained methanol–sodium acetate (80:20), and the gradient was linear from 0% to 85% solvent B.

RESULTS AND DISCUSSION

The greater part of any tryptophan and cysteine present, and much of the methionine, are likely to have been destroyed during hydrolysis, though small peaks in the position of methionine were often observed. The tyrosine content was always insignificant. These amino acids are not listed in Table I.

Figs. 1 and 2 show that the reduction in interference by non-amino acid material after deamination is more pronounced with the OPTH system than with the Dns one, though still useful with the latter. Unfortunately, but not surprisingly, the elution sequence is rather similar with the two systems; a principal difference is that the basic arginine and histidine elute much earlier in the Dns sequence than in the OPTH one. With OPTH heparin hydrolysates yield an intense peak precisely in the arginine position (asterisked in Fig. 2), though after deamination its intensity is considerably, and disproportionately, reduced. In contrast, the Dns records show that arginine, which should elute immediately before serine, is substantially absent. Histidine estimation presents difficulties; in the Dns traces it is unidentifiable in the initial clutter of peaks, and with OPTH it is more or less obscured at the foot of the most intense of all the non-amino acid peaks, between serine and glycine. It is thus obscured in Fig. 2B, but other runs indicate that sample 7 does in fact contain a trace of histidine. With our OPTH system threonine was poorly resolved from glycine; it

TABLE I

AMINO ACID CONTENT OF HEPARIN SAMPLES

Sample No.	*Amino acid content (μmol/g)*												
	Asp	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Val*	*Ile*	*Leu*	*Phe*	*His*	*Lys*
1	1.5	2	20	2	1	4	1	0.5	0.5	1	0.5	tr*	tr
2	3	2	15	2	1	5	2	0.5	tr	0.5	tr	tr	tr
3	2	2	25	4	1.5	4	2	2	0.5	0.5	0.5	tr	tr
4	0.5	1	18	1	tr	2	0.5	0.5	tr	tr	tr	tr	tr
5	8	5	25	10	5	20	5	3	2	4	2	tr	5
6	1	1	1	1	tr	1	tr	tr	tr	tr	–	tr	tr
7	3	1	13	4	2	8	3	1.5	1	1.5	1	0.5	2

* tr = trace (*i.e.* less than 0.5).

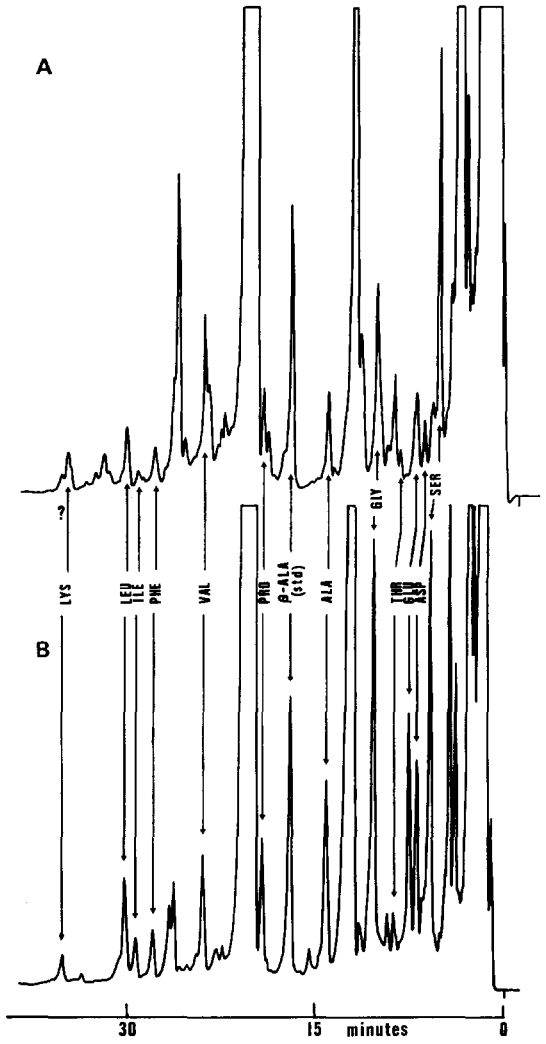

Fig. 1. Recorder traces (Dns procedure) of amino acids from sample 7. (A) Original sample. (B) First Sephadex G-25 fraction after deamination.

appears as a small spike on the tail of the glycine peak in Fig. 2B (and Fig. 2A). The two procedures are seen to be complementary.

None of the heparin samples contained more than 1% or so of peptide material, and no attempt is made to give values in Table I with precision. For some amino acids levels of less than 2 μmol/g may depend on somewhat subjective interpretations of recorder traces; sample 7 (Figs. 1 and 2) was richer than most in peptide material. The samples were all ultimately products of commercial processing, and they will all have undergone either treatment with strong alkalies (which cleave xylose-serine linkages[5]), autolysis, or treatment with proteolytic enzymes, or combinations of these, all resulting in the breakdown of peptide material whether covalently

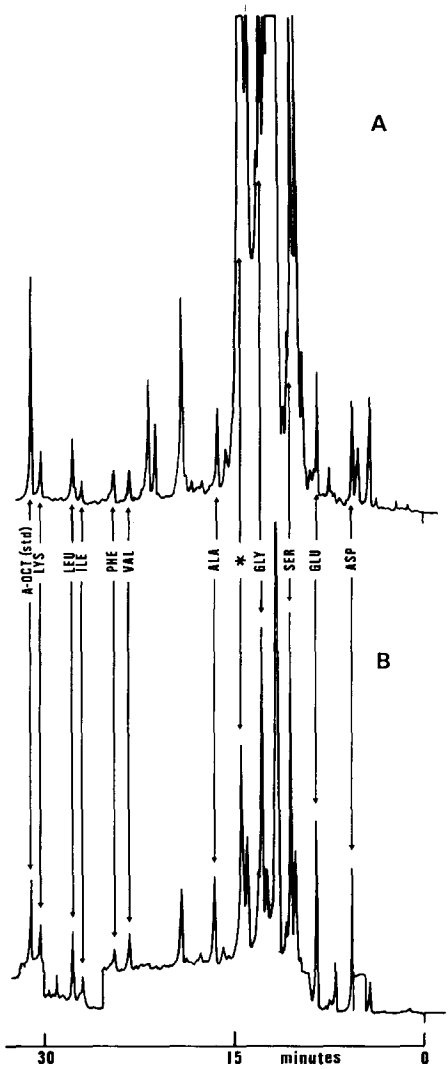

Fig. 2. As Fig. 1, OPTH procedure. For * see text.

bound or not. Some of the remaining bound serine might retain other linked amino acids, and it is to be expected that the amounts of these would bear some kind of inverse relation to their distance from the terminal serine in the chains. From the present work the order seems to be, with fair consistency, Ser > Gly > Glu > Asp > Thr = Ala, with Pro, Val, Leu, Lys, Ile and Phe almost always present as well. Contamination might be suspected, but the results on the two pharmaceutical-grade samples, 4 and 6, show that "bleaching" (with permanganate, peroxide or hypochlorite) is effective in removing all but traces of amino acids, except for serine and some glycine in sample 4. As far as can be judged, the deamination procedure does not much affect the relative proportions of amino acids, and the mass percentage in

the first fraction from Sephadex G-25 does not differ greatly from that in the original sample.

Some of these results are not readily interpretable. Nevertheless, it is quite difficult to reconcile the amino acid distributions found here —which are generally in good accord with those reported for similar samples by Lindahl *et al.*[1] nearly 20 years ago— with a poly(glycylserine) model, either in its simple form or with relatively minor variations as envisaged by Metcalfe *et al.*[9]. Samples such as we have examined, of various and largely unknown origins, may not be regarded as suitable material with which to develop ideas about the nature of the heparin "core", but nevertheless they are products of chain-shortening processes which would be unlikely to result in such radical rearrangement of the relative amino acid proportions. A structure which could yield such a result would consist of a chain, essentially of poly(glycylserine), linked by side-chains of other aminoacids to the "linkage region" serine (or glycylserine) —an indication of the problem rather than a serious speculation. It may be that the tissues from which commercial heparins are obtained contain heparin proteoglycans which differ from those of rat peritoneal mast cells or skin. Certainly it is easier to reconcile the rather consistent pattern of residual amino acids in commercial-type samples with the results of Serafini-Fracassini *et al.*[3] from ox liver capsule heparin, or even those of Oldberg *et al.*[18] from a heparan proteoglycan. Metcalfe *et al.*[9] were unable to estimate four amino acids because of hexosamine interference, and it may be that such difficulties have contributed to the variety of results reported.

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REFERENCES

1 U. Lindahl, J. A. Cifonelli, B. Lindahl and L. Roden, *J. Biol. Chem.*, 240 (1965) 2817.
2 A. G. Lloyd, G. D. Bloom and E. A. Balasz, *Biochem. J.*, 103 (1967) 76P.
3 A. Serafini-Fracassini, J. J. Durward and L. Floreani, *Biochem. J.*, 112 (1969) 167.
4 A. A. Horner, *J. Biol. Chem.*, 246 (1971) 231.
5 R. W. Yurt, R. W. Leid, K. F. Austen and J. E. Silbert, *J. Biol. Chem.*, 252 (1977) 518.
6 H. C. Robinson, A. A. Horner, M. Höök, S. Ögren and U. Lindahl, *J. Biol. Chem.*, 253 (1978) 6687.
7 L. Jansson, S. Ögren and U. Lindahl, *Biochem. J.*, 145 (1975) 53.
8 C. C. Wang and L. B. Jaques, *Arzneim.-Forsch.*, 24 (1974) 1945.
9 D. D. Metcalfe, J. A. Smith, K. F. Austen and J. E. Silbert, *J. Biol. Chem.*, 255 (1980) 11753.
10 C. de Jong, G. J. Hughes, E. van Wieringen and K. J. Wilson, *J. Chromatogr.*, 241 (1982) 345.
11 B. N. Jones, S. Pääbo and S. Stein, *J. Liquid. Chromatogr.*, 4 (1981) 587.
12 S. Gardell, *Methods Carbohydr. Chem.*, 5 (1965) 9.
13 J. D. Scott, *Brit. Pat. 1,221,784 (1971).*
14 B. Casu and U. Gennaro, *Carbohydr. Res.*, 39 (1975) 168.
15 J. D. Navratil, E. Murgia and H. F. Walton, *Anal. Chem.*, 47 (1975) 122.
16 E. A. Johnson, *Pharmacol. Res. Commun.*, 14 (1982) 289.
17 J. E. Shively and H. E. Conrad, *Biochemistry*, 15 (1976) 3932.
18 Å. Aldberg, L. Kjellén and M. Höök, *J. Biol. Chem.*, 254 (1979) 8505.