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The structure of heparin oligosaccharide fragments with high anti-(factor Xa) activity containing the minimal antithrombin III-binding sequence

Chemical and ^{13}C nuclear-magnetic-resonance studies

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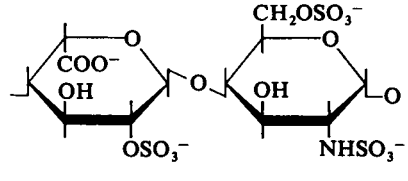
The chemical composition and the ^{13}C n.m.r. spectra of heparin oligosaccharides (essentially octasaccharides), having high affinity for antithrombin III and high anti-(Factor Xa) activity, prepared by three independent approaches (extraction, partial deaminative cleavage with HNO_2 and partial depolymerization with bacterial heparinase), leading to different terminal residues, have been studied and compared with those of the corresponding inactive species. Combined with chemical data, the spectra of the active oligosaccharides and of their fragmentation products afforded information on composition and sequence. The three types of active oligosaccharides were shown to have the common hexasaccharide core I-A_a-G-A_s^*-I_s-A_s, where I = α-L-idopyranosyluronic acid, A_a = 2-acetamido-2-deoxy-α-D-glucopyranose, G = β-D-glucopyranosyluronic acid, I_s = α-L-idopyranosyluronic acid 2-*O*-sulphate, A_s = 2-deoxy-2-sulphamino-α-D-glucopyranose 6-*O*-sulphate. The fourth residue (A_s^*) is an unusually substituted amino sugar resistant to mild deamination. The ^{13}C spectra of the active species are characterized by signals from the above atypical amino sugar, the most evident of which is at 57.7 p.p.m. These signals, compared with those of appropriate synthetic model compounds, are compatible with the recently proposed 3-*O*-sulphation of the residue A_s^* [Lindahl, Bäckström, Thunberg & Leder (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6551–6555].

It is now recognized that the main effect of heparin on blood coagulation is mediated by antithrombin III. Only about one-third of the polysaccharide chains that constitute conventional heparins have high affinity for antithrombin III (Lam *et al.*, 1976; Höök *et al.*, 1976; Andersson *et al.*, 1976). The binding structures are contained in specific oligosaccharide segments (Hopwood *et al.*, 1978).

The structure of heparin can be largely represented by regular disaccharide sequences of α1,4-linked L-idopyranosyluronic acid 2-*O*-sulphate (I_s) and 2-deoxy-2-sulphamino-D-glucopyranose 6-*O*-sulphate (A_s). However, heparin also contains residues of non-sulphated α-L-idopyranosyluronic acid (I), β-D-glucopyranosyluronic acid (G), and 2-deoxy-2-acetamido-α-D-glucopyranose (A_a), these latter being occasionally 6-*O*-sulphated (for a review, see Lindahl & Höök, 1978).

G, I and A_a residues forming an irregular region were found to be significantly more represented in high-affinity than in low-affinity pig mucosal heparin (Rosenberg *et al.*, 1978).



I_s A_s

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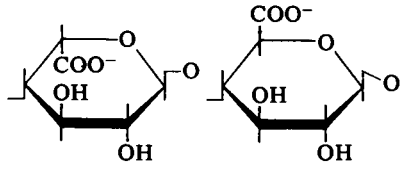
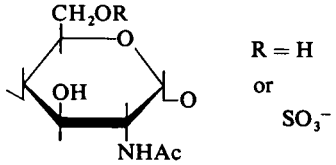




Heparin fragments with high affinity for antithrombin III have been isolated by various processes. Thus, in high-affinity dodecasaccharides (Lindahl *et al.*, 1979) and decasaccharides (Choay *et al.*, 1980) obtained from pig mucosal heparin, the tetrasaccharide fragment I-A_s-G-A_s described by Rosenberg & Lam (1979), was shown to be inserted within 'regular' segments of I_s-A_s units. Further, Lindahl *et al.* (1980) demonstrated that the A_s residue of the above tetrasaccharide fragment bears a 3-*O*-sulphate group.

Heparin has been studied by n.m.r. All the ^{13}C signals of the regular regions as well as some signals originated by residues in the irregular regions of heparin were recently assigned (Gatti *et al.*, 1979). This provides a basis for comparison with the spectra of high-affinity oligosaccharides, thus allowing further insight into the structure of the compounds.

The ^{13}C n.m.r. spectrum of a high-affinity oligosaccharide as reported in preliminary form (Choay *et al.*, 1980) showed that, although a number of signals of this active oligosaccharide were superimposable on those of commercial heparin, others were 'extra signals' that cannot be observed in heparin. More especially, the extra signal attributable to an atypical amino sugar residue was clearly evident in the active oligosaccharide ('active' is hereinafter used to express that the product has been retained on antithrombin III affinity columns and has a high activity on the Yin & Wessler anti-(Factor Xa) assay (Yin *et al.*, 1973).

Most of the heparin oligosaccharides retained on the antithrombin III columns differ in size as well as in internal composition (Lindahl *et al.*, 1979; Choay *et al.*, 1980), indicating that some residues are critical for activity, others are essential, and others are non-essential. The present work deals with parallel studies of oligosaccharides having comparable affinity for antithrombin III obtained by extraction or by cleaving of the heparin chains at different sites. The aim of this comparison, based mainly on n.m.r. spectroscopy, was to detect a common structure containing the minimal active segments.

Experimental

Materials

Heparin. Heparin was a commercial preparation from pig mucosa (Choay, batch no. HH 825); uronic acid 32.3%, hexosamine 30.8%, sulphate/carboxy molar ratio 2.22; anticoagulant activity: 180 US Pharmacopeia units/mg.

Extraction oligosaccharides. Extraction oligosaccharides were prepared as described by Choay *et al.* (1980*a*), by extraction of the low-molecular-weight fractions of heparin, followed by gel filtration and affinity chromatography on agarose-bound bovine antithrombin III. Products retained on the affinity column are referred to as 'active'; products not retained are referred to as 'inactive'. [Biological activity: active extraction oligosaccharides, activated partial thrombin time (APTT), 12 units/mg, anti-(Factor Xa), 1800 units/mg; inactive extraction oligosaccharides, APTT, <2 units/mg, anti-(Factor Xa), <10 units/mg].

Oligosaccharides from partial deaminative cleavage of heparin ('deaminative-cleavage oligosaccharides'). (*a*) Depolymerization of heparin. Commercial pig mucosal heparin was dissolved in water at room temperature (concn. 0.25%); H_2SO_4 was added to obtain a final concentration of 0.1 M, followed by $NaNO_2$ (final concn. 0.2 M). The reaction was stopped after 15 min by raising the pH to 7–7.2 with NaOH. The depolymerization products were precipitated with ethanol.

(*b*) Chromatography: the degradation products (2 g) were placed on a column (5 cm × 18 cm) containing the affinity material (10 mg of antithrombin III/ml of Sepharose) and equilibrated with 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.5. Washing with the above buffer eluted the oligosaccharide fraction not retained by antithrombin III. The fraction retained by antithrombin III (referred to as 'active') was eluted with a 1 M-$CaCl_2$ solution, pH 7.2. The oligosaccharides in the two fractions were recovered by precipitation with ethanol and centrifugation. Yields: non-retained fraction, 1.6 g; retained fraction, 8 mg.

(*c*) Gel filtration: both fractions were passed through a column (200 cm × 0.6 cm or × 2.5 cm) of Sephadex G-50 (Superfine grade), 0.2 M-NaCl being used as eluant. The collected fractions were desalted on Sephadex G-25 and freeze-dried. [Biological activity: active deaminative-cleavage oligosaccharides, APTT, 10 units/mg, anti-(Factor Xa), 1500 units/mg; inactive deaminative-cleavage oligo-

saccharides, APTT, <2units/mg, anti-(Factor Xa), <10units/mg].

Oligosaccharides from partial cleavage of heparin by heparinase ('heparinase-cleavage oligosaccharides'). (*a*) Preparation of heparinase. The strain of *Flavobacterium heparinum* was grown as described by Payza & Korn (1956). The freeze-dried cells were dry-ground in the presence of alumina, and extracted with an acetate buffer at neutral pH. The solution was passed through columns of DEAE-cellulose, CM-Sepharose and Ultrogel AcA 54. The yield was 20mg of heparinase from 20g of freeze-dried cells. The enzyme activity was determined as described by Linker & Hovingh (1972).

(*b*) Enzymic degradation of heparin. A solution of heparin (20mg/100ml of acetate buffer, pH6.9) was incubated at 30°C with heparinase (0.4mg of protein). Two new additions of 0.2mg each were made after 7 and 24h. Further addition of the enzyme after 36h did not cause any further increase in A_{232}. The products were precipitated with ethanol. The yield was 90%.

(*c*) Chromatography on Sepharose–antithrombin III and (*d*) gel filtration. These were conducted as described for deaminative-cleavage oligosaccharides above. [Biological activity: active heparinase-cleavage oligosaccharides, APTT, 15units/mg, anti-(Factor Xa), 1800units/mg; inactive heparinase-cleavage oligosaccharides, APTT, <2units/mg, anti-(Factor Xa), <10units/mg.]

All the active extraction, deaminative-cleavage and heparinase-cleavage oligosaccharides were electrophoretically homogeneous.

N,O-Desulphated, re-N-sulphated heparin. This was prepared by selective re-*N*-sulphation (Lloyd *et al.*, 1964) of *N,O*-desulphated heparin, prepared as described by Nagasawa *et al.* (1977).

Synthetic model compounds. The four model compounds used in the present study (Fig. 4 below) were synthesized by methods, full details of which will be forwarded by M. P. or P. S. on request. Briefly, the two *N*-sulphated model compounds were prepared from benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside (Pfanstiehl, Waukegan, IL, U.S.A.). Alkaline *N*-deacetylation gave the amino derivative, which was selectively *N*-sulphated (trimethylamine/sulphur trioxide complex) to provide, after appropriate work-up, the sodium salt of benzyl 2-deoxy-2-sulphamino-α-D-glucopyranoside. Catalytic hydrogenolysis then afforded the sodium salt of 2-deoxy-2-sulphamino-D-glucopyranose. The two *N*- and 3-*O*-sulphated model compounds were obtained from benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (Pfanstiehl). Alkaline *N*-deacetylation was followed by *N*- and *O*-sulphation (trimethylamine/sulphur trioxide complex) to provide benzyl 4,6-*O*-benzylidene-2-deoxy-2-sulphamino-α-D-glucopyranoside 3-*O*-sulphate. The benzylidene group was then removed (acid hydrolysis) to give benzyl 2-deoxy-2-sulphamino-α-D-glucopyranoside 3-*O*-sulphate. Catalytic hydrogenolysis then afforded 2-deoxy-2-sulphamino-D-glucopyranose 3-*O*-sulphate.

Methods

Analytical methods. Uronic acids. These were determined by the carbazole-borate method (Bitter & Muir, 1962).

Hexosamines. These were determined as described by Smith & Gilkerson (1979); *N*-acetylated hexosamines were determined by difference to total hexosamines, omitting the acid-hydrolysis step.

Sulphate groups/carboxy groups ratio. This was determined by conductimetry as described by Casu & Gennaro (1975).

Electrophoretic analyses. These were performed on cellulose acetate, both at pH1.1 in HCl/KCl (Casu *et al.*, 1979) and (at low amperage) at pH5.7 in barium acetate (Oreste & Torri, 1980).

Paper chromatography (descending). This was performed on Whatman no. 1 paper in 1M-NH_3/acetic acid/butan-1-ol (1:3:2, by vol.) (Yamagata *et al.*, 1968).

G.l.c. analyses. These were made on SE-30 columns (for trimethylsilylated compounds) or OV-221 columns (for acetylated compounds), with N_2 as carrier gas. In one set of experiments (*a*), the oligosaccharides were carboxy-group-reduced, *N*-desulphated, re-*N*-acetylated, hydrolysed with methanol/HCl and trimethylsilylated; in a second set (*b*), they were periodate-oxidized, reduced with borohydride, hydrolysed and acetylated (Lindberg & Lönngren, 1978).

Gel chromatography. This was performed on Sephadex G-50 (Superfine grade), either in 0.2M-NaCl buffer or in salt-free aqueous solutions. Desalting was done with Sephadex G-25. Effluents were monitored with a u.v. detector at 206 and 254nm and analysed colorimetrically for uronic acid and/or 2,5-anhydromannose; heparinase-cleavage oligosaccharides and any products from heparinase cleavage were also detected by their absorbance at 230nm. The column was calibrated with inactive oligosaccharides (di- to dodeca-saccharide).

Periodate oxidation of extraction oligosaccharides. This was performed as described by Cifonelli & King (1975) for heparin.

Analytical degradation of extraction oligosaccharides with heparinase. A 0.1ml portion of a solution of heparinase from *Flavobacterium heparinum*, containing 8×10^{-3} units (see Linker & Hovingh, 1972) of enzyme, was added to 0.02ml of a 1% solution of oligosaccharide. A portion (0.02ml) of 10mM-calcium acetate was added and the final volume made up to 3.2ml with 0.1M-sodium acetate,

pH 7. The solution was incubated at 30°C in the dark in a 1 cm cell. The absorbance at 232 nm was determined at regular intervals. The enzymic reaction was complete after 8 h.

Mild deaminative cleavage of extraction oligosaccharides. This was performed essentially as described by Perlin *et al.* (1972). A sample (60 mg) of oligosaccharide was dissolved in 1 ml of water and 30 mg of $NaNO_2$ was added. The pH was adjusted to 3.5 with 0.1 M-HCl. The solution was incubated for 24 h at 4°C in the dark, then neutralized with 0.1 M-NaOH. For reduction of the primary products of deamination, 65 mg of $NaBH_4$ was added with stirring. After being left for 2–3 h at room temperature, the solution was adjusted to pH 3 with HCl, with vigorous stirring, and then to pH 7 with 0.5 M-NaOH.

Exhaustive deaminative hydrolysis. This was performed as described by Shively & Conrad (1976).

Hydrogenation of inactive heparinase-cleavage oligosaccharide. This was performed in water by using a palladium/carbon catalyst.

^{13}C n.m.r. measurements. The spectra were obtained at 35°C with a Varian CFT-20 Fourier-transform spectrometer at 20 MHz, with a 5 mm or (for the spectrum of Fig. 3*c* below), a 1.7 mm probe. Samples (50–100 mg) were dissolved in 0.4 ml of ^2H_2O (99.7% ^2H) for spectra in the 5 mm probe, and 3–5 mg in 30 μl of ^2H_2O for the microprobe. Experimental conditions: 70° pulse; 8 K or 2 K data points, with Fourier number 8 K; 80–500 K pulses. The chemical shifts were measured with reference to internal methanol (50.0 p.p.m. with respect to external trimethylsilane, 51.75 p.p.m. with respect to internal sodium 3-trimethylsilyl[2,2,3,3-^2H]-propionate).

Biological assays. The anticoagulant activity was determined by the APTT assay (Caen *et al.*, 1968) and the anti-(Factor Xa) activity was assayed by the method of Yin *et al.* (1973).

Results

Extraction oligosaccharides

The high-affinity extraction oligosaccharides eluted from Sephadex G-50 in the region of octa- to deca-saccharides. The hexosamine/uronic acid molar ratio was close to 1:1, with some variations in the internal composition of different preparations of these oligosaccharides. The iduronic/glucuronic acid ratio (g.l.c. determination, method *a*) was ~3; we found (g.l.c., method *b*) an approx. 1:1 ratio between non-sulphated iduronic acid and glucuronic acid residues.

On periodate oxidation under conditions that split free diol groups of both glucuronic and iduronic acid residues (Cifonelli & King, 1975), a typical extraction oligosaccharide consumed 1.15 μmol of IO_4/mg, i.e. an amount approximately equivalent to 3 mol/mol of oligosaccharide of assumed average degree of polymerization 8.5. Degradation with heparinase afforded 1.1 μmol of unsaturated uronic acid/mg of oligosaccharide, also approximately equivalent to 2.5 mol/mol of oligosaccharide.

An active extraction oligosaccharide and its inactive counterpart were treated with HNO_2 under mild conditions that were nonetheless sufficient for converting the 'regular' I_s-A_s segments of heparin to the disulphated disaccharide 4-*O*-(α-L-ido-

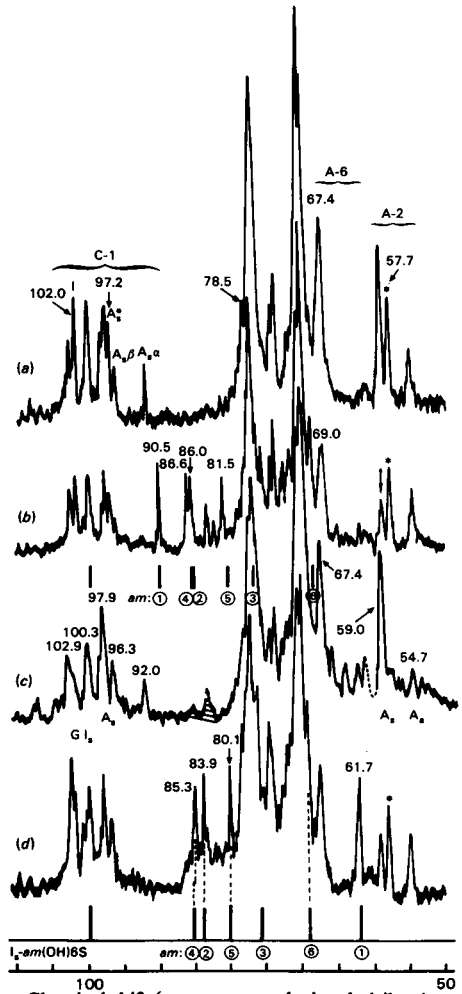


Fig. 1. *^{13}C n.m.r. spectra (ring-carbon atoms region) of (a) an active extraction oligosaccharide, (b) the same, after mild deamination, (c) the inactive counterpart of the extraction oligosaccharide and (d) the active deaminative-cleavage oligosaccharide*

Stick diagrams: C-1 signal of I_s and *am* pattern of the reference disaccharides I_s-*am*-6S (below spectrum *b*) and I_s-*am*(OH)6S (below spectrum *d*).

pyranosyluronic acid 2-*O*-sulphate)-2,5-anhydro-D-mannose-6-*O*-sulphate [I_s-*am*(6S); Perlin *et al.*, 1972). Whereas the inactive oligosaccharides afforded mainly the above disaccharide, the active species did not produce any mono- or di-saccharide fragments, the size of the oligosaccharide remaining substantially unaltered (paper-chromatographic analysis).

The ^{13}C n.m.r. spectrum (ring-carbon-atoms region) of a typical extraction oligosaccharide is compared in Fig. 1(*a*) with the spectrum of the same product after mild deamination (Fig. 1*b*) and with that (Fig. 1*c*) of the unmodified inactive oligosaccharides. The spectrum of the latter is roughly similar to that of typical heparins (Gatti *et al.*, 1979), except for being somewhat more complex, and showing, among other things, signals clearly attributable to reducing end-groups. These signals are especially noticeable at 92.0 and 96.3 p.p.m. in the high-field side of the region 90–105 p.p.m., characteristic of the anomeric (C-1) carbons.

The active oligosaccharides show extra signals that are clearly absent or very weak in their inactive counterparts. Most prominent among these are the C-1 signals at 102.0 and 97.2 p.p.m., and the C-2 signal (labelled with an asterisk) at 57.7 p.p.m. On the basis of a comparison with the spectrum of *N*,*O*-desulphated *N*-re-sulphated heparin, the signal at 102.0 p.p.m. was assigned to unsulphated L-iduronic acid (I) residues. [A downfield shift of C-1 (~2 p.p.m.) on *O*-desulphation of I_s residues was also reported by Fransson *et al.* (1978) for *N*,*O*-desulphated, re-*N*-acetylated heparins.] The signals at 97.2 and 57.7 p.p.m. are from an unusual amino sugar residue referred to in the Discussion section as 'A_s^*'.

Minor signals are present in both active and inactive oligosaccharides, especially in the inactive species, which also contained non-carbohydrate contaminants (shaded areas in Fig. 1). Taking into account only the major signals, integration in the C-1 region indicates a (reducing carbon atoms)/(total anomeric carbon atoms) ratio of approx. 1:8. As found for the monomer (2-deoxy 2-sulphamino-D-glucopyranose), the α-anomer was assumed to account for ~80% of the reducing A_s residue.

In the C-2 region of the amino sugar (48–60 p.p.m.), the active product shows signals from A_s (59.0 p.p.m.), A_s^* (57.7 p.p.m.) and A_a (54.7 p.p.m.), with area ratios of approx. 2.2:1:1. The area of the methyl-carbon signal (at 23.4 p.p.m., not shown in the spectra) is practically the same as that of A_a-2.

Except for somewhat different intensities of signals, the spectra of the other two preparations of active extraction oligosaccharides were substantially similar to the one of Fig. 1(*a*), particularly as concerns the extra A_s^* signal. Especially evident, in the C-1 region, were the variations of relative

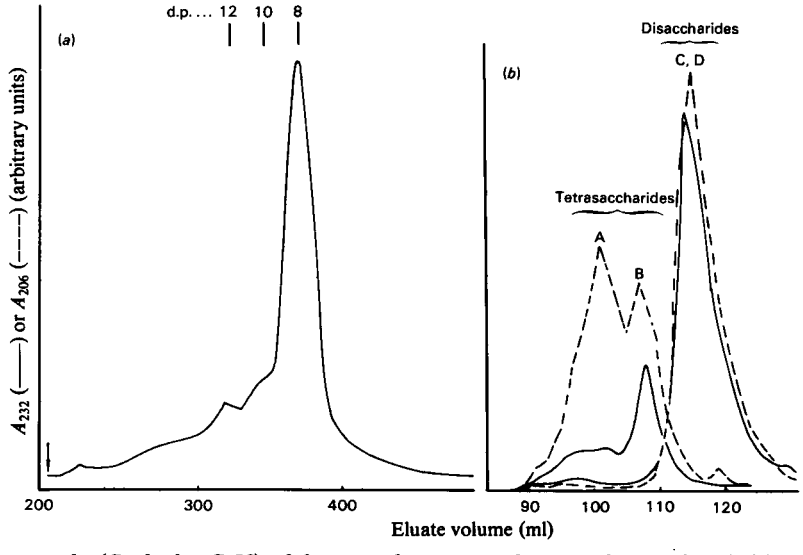


Fig. 2. *Gel chromatography (Sephadex G-50) of the active heparinase-cleavage oligosaccharide (a) and of products of its exhaustive deaminative degradation (b)*

Conditions are described in the text. Columns were calibrated with oligosaccharides obtained by heparinase cleavage of unfractionated heparin. The degree of polymerization (d.p.) of these oligosaccharides was determined by ^{13}C n.m.r. end-group analysis.

intensity of signals from G and I residues. In all cases, the A-6 signal of 6-*O*-sulphated hexosamines (67.4 p.p.m.) was much stronger than signals in the 60–61 p.p.m. region, attributable to residues non-sulphated at C-6.

As shown in the A-2 region of Fig. 1(*b*), the product of mild deamination of an active extraction oligosaccharide left substantially unaltered the A_s^* and the A_a signal, while decreasing (to half its original value) the A_s signal. [The pattern in the A-2 region was practically unmodified on borohydride reduction of the anhydromannose (*am*) residues.] Typical signals accounting for 1 *am* residue (mostly 6-*O*-sulphated) are evident at 90.5, 96.6, 86.0, 81.5 and 69.0 p.p.m. The assignments reported in the Figure are based on those reported for the disaccharide I_s-*am*(6S) (Mushayakarara, 1977), the signals of which are shown as a stick diagram below spectrum (*b*). It can be noted that the *am* signals in the deaminated oligosaccharide, except C-1, are somewhat shifted with respect to those of I_s-*am*(6S).

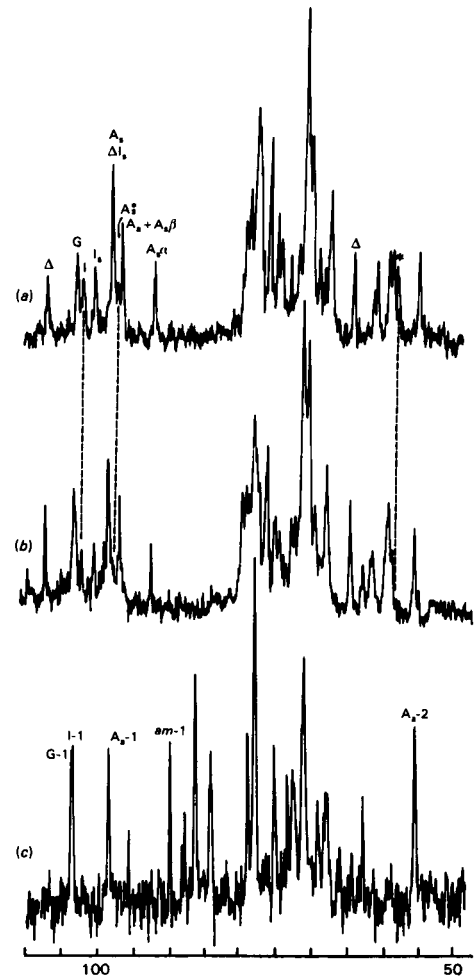
Deaminative-cleavage oligosaccharides

Partial deaminative cleavage of heparin, followed by reduction with borohydride, gel filtration and affinity chromatography on antithrombin III afforded an active oligosaccharide, eluted from Sephadex G-50 mainly in the octasaccharide region. As determined by colorimetry, the molar ratio (hexosamine + 2,5-anhydromannose)/(uronic acid) was ~1:1, the ratio *N*-sulphated/*N*-acetylated amino sugar being 2:1.

The ^{13}C n.m.r. spectrum of the deaminative-cleavage oligosaccharides (Fig. 1*d*) is closely reminiscent of that of the mildly deaminated extraction oligosaccharides (Fig. 1*b*), especially in the A-2 region, where the extra signal of A_s^* is present. The terminal residue of deaminative-cleavage oligosaccharides gives rise to a pattern of signals similar to that of the main reduced disaccharide I_s-*am*(OH)(6S) obtained by exhaustive deamination of heparin (Mushayakarara, 1977). These signals are especially prominent in the products (mainly disaccharides) obtained by mild deamination of the inactive oligosaccharides. However, C-4 of the *am*(OH) residue of the active deaminative-cleavage oligosaccharides is slightly shifted upfield with respect to the products from the inactive species.

In the active deaminative-cleavage oligosaccharides, each of the signals originated by secondary carbon atoms of the terminal *am*(OH) residue accounts for approximately one *am*(OH) residue per seven anomeric carbon atoms. As usually observed for primary as compared to secondary carbon atoms, the area of the C-1 signal of *am*(OH), at 61.7 p.p.m., is somewhat higher than for the other signals of this residue. In addition, also C-6 of partially 6-*O*-desulphated amino sugar residues contributes to the intensity of this signal.

Advantage was taken of the different ^{13}C patterns of *am* and *am*(OH), and of variations in these patterns observed when *am* [and *am*–OH)] are glycosidically bound (at C-4) to I_s or G residues, for characterizing the fragments of exhaustive deamination (Shively & Conrad, 1976) of the deaminative-cleavage oligosaccharides. The spectrum of this pool of products, where the A_s signals (including the one from A_s^*) were no longer present, corresponded to that of a ~ 1:1:1 mixture of the two disaccharides I_s-*am*(OH)6S and I_s-*am*(6S), and of a tetrasaccharide containing 1 I, 1



Chemical shift (p.p.m., external trimethylsilane)

Fig. 3. *^{13}C n.m.r. spectra of heparinase-cleavage oligosaccharides*

(*a*) Active; (*b*) inactive; (*c*) major tetrasaccharide fragment from exhaustive deaminative degradation.

A_a, 1 G and 1 *am* (the asterisk on *am* indicates the possibility of some peculiar feature of this residue).

Heparinase-degradation oligosaccharides

Cleavage of heparin by heparinase, followed by affinity chromatography on antithrombin III and gel filtration on Sephadex G-50, afforded an octasaccharide as the major active product (Fig. 2*a*). Chemical analysis of this oligosaccharide indicated 1 A_a per three A_s residues, A_s being at the reducing end.

The ^{13}C n.m.r. spectrum of the heparinase-cleavage octasaccharide reported in Fig. 3(*a*) is sharper and cleaner than those of the active extraction and deaminative-cleavage oligosaccharides (Figs. 1*a* and 1*d*). The spectrum clearly shows signals characteristic of both terminal residues. The C-1 signals of the reducing residue absorb at 92.1 p.p.m. (α-anomer) and 96.8 p.p.m. (β-anomer), i.e. at about the same frequencies as the monomer 2-sulphamino-2-deoxy-D-glucopyranose. Signals labelled Δ arise from the 4,5-unsaturated uronic acid residue at the non-reducing end. These signals were also observed in the spectra of the inactive oligosaccharides (di- to octa-saccharides) obtained by heparinase cleavage of heparin, and disappeared on hydrogenation of the $C_{(4)}=C_{(5)}$ double bond of these oligosaccharides.

Characteristic signals of the inactive octasaccharide are shown in Fig. 3(*b*). A few signals of the active species are clearly shifted with respect to its inactive counterpart. The area ratio of each Δ signal to the total of C-1 signals is about 1:8. Signals in the A-2 region are four: one A_a and three A_s.

The most upfield of the A_s signals is the extra signal corresponding to the unusual A_s^* residue, as also found in active extraction and deaminative-cleavage oligosaccharides (Fig. 1*a* and *d*). The other two A_s-2 carbon atoms give separate signals, one of which may be attributed to the terminal reducing amino sugar. The area ratio A-6-*O*-sulphated/A-6-non-sulphated (signals at 67.2 and 60.5 p.p.m.) is slightly lower than for the oligosaccharides (~3:1 as against ~7:1). This comparison is, however, complicated by new unassigned signals at about 60.6 p.p.m., also observed, with varying intensities, in inactive oligosaccharides from heparinase cleavage of heparin.

On exhaustive deaminative cleavage, the active heparinase-cleavage oligosaccharide afforded two major fractions on Sephadex G-50, corresponding to tetrasaccharides and disaccharides, in the ratio of approx. 1:1 (Choay *et al.*, 1981*a*). Under better resolving conditions (Fig. 2*b*), the tetrasaccharide fraction was further divided into two tetrasaccharides, A and B [the latter (B) a minor

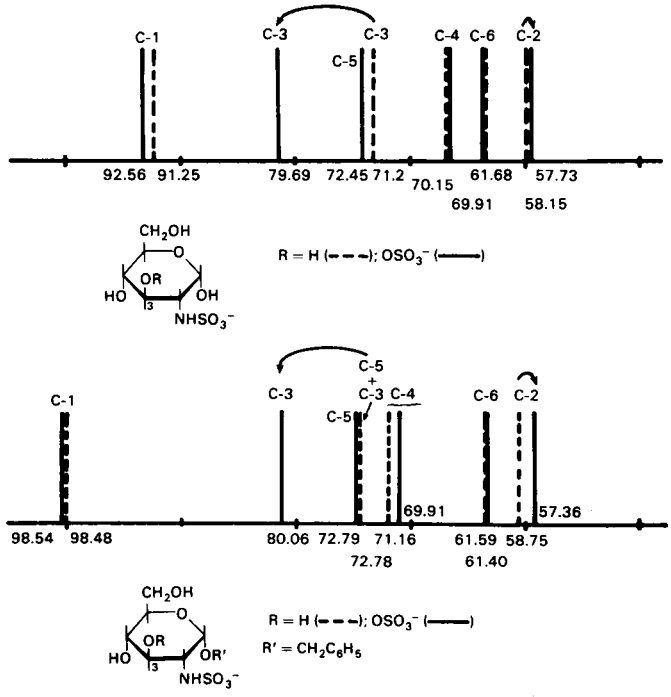


Fig. 4. *^{13}C n.m.r. chemical shifts (external trimethylsilane) of 2-deoxy-2-sulphamino-α-D-glucose and benzyl 2-deoxy-2-sulphamino-α-D-glucoside and their 3-O-sulphated counterparts*

component showing absorption at 232nm]. By working in salt-free solution, the disaccharide fraction was also divided into two subfractions, C and D, the latter (D) with strong absorption at 232nm.

The ^{13}C spectrum of the major tetrasaccharide fragment (A), showing typical C-1 signals from G, I, A_s and *am** residues in the approximate ratio 1:1:1:1, is shown in Fig. 3(*c*). Signals from the *am** pattern, some of which not clearly shifted with respect to those observed for the mildly deaminated extraction oligosaccharides (Fig. 1*b*), are evident in the spectrum. I-1 is shifted to lower field with respect to its position in the original octasaccharide, as also observed for the active deaminative-cleavage octasaccharides compared with the active extraction and heparinase-cleavage octasaccharides. Another notable feature of the tetrasaccharide fragment A with respect to the original octasaccharide is the disappearance of the A_s^*-2 signal. No attempts were made to assign minor signals, a few of which correspond to major signals of the tetrasaccharide B and of disaccharides C and D (spectra not shown).

As anticipated from their absorption at 232nm, fragments B and D show typical ^{13}C signals from 4,5-unsaturated I_s residues; these signals are especially strong for the disaccharide D. In addition, the disaccharide C shows strong signals from terminal (reducing) A_s residues, and disaccharide D the characteristic pattern from *am*-type residues. In all the deamination fragments, the area ratio of A-6(S) to [A-6(S)+A-6(OH)] signals is slightly lower than 1.

Model compounds

As models for the 3-*O*-sulphated amino sugar residue proposed as a typical component of the antithrombin III binding site of heparin (Lindahl *et al.*, 1980), the two monosaccharides 2-deoxy-2-sulphamino-D-glucopyranose 3-*O*-sulphate and benzyl 2-deoxy-2-sulphamino-D-glucopyranoside 3-*O*-sulphate was synthesized.

The ^{13}C chemical shifts of the two products are compared in Fig. 4 with those of their non-3-*O*-sulphated counterparts. In both cases, 3-*O*-sulphation brought about a large downfield shift of C-3 (+8.5 and +7.3p.p.m.) and a smaller but significant upfield shift of C-2 (+0.4 and +1.4p.p.m.). The C-2 signal observed for 2-deoxy-2-sulphamino-D-glucopyranose 3-*O*-sulphate (57.73 p.p.m.) is practically at the same field as observed for the A_s^*-2 signal for all the active oligosaccharides.

Discussion

The compositional analysis and the ^{13}C n.m.r. of all the active heparin oligosaccharides studied in the present work show common features pointing to a common hexasaccharide core containing 1 G, 1 I, 1 I_s, 1 A_s, 1 A_a and 1 A_s^* residues, the A_s^* residue being an atypical amino sugar giving a characteristic C-2 signal at 57.7p.p.m., already observed in our previous work (Choay *et al.*, 1980*a*). [This signal was also recently observed by Meyer *et al.* (1981) in a *d*-decasaccharide, and attributed to C-2 of a 3-*O*-sulphated residue.] This signal is practically absent from all the inactive species. I signals, too, are frequently absent or very weak in inactive oligosaccharides.

In addition to the above hexasaccharide core, the active extraction oligosaccharides contain one or two I_s-A_s units (*e*, Fig. 5). In principle, extraction oligosaccharides can have either an amino sugar or a uronic acid as the terminal reducing residue. However, the chemical and ^{13}C n.m.r. data indicate A_s as the reducing residue. In fact, the most prominent signals in the region of reducing carbons (at 92.0 and 96.3p.p.m., Fig. 1) correspond to those of 2-deoxy-2-sulphamino-D-glucose. This is obviously the only residue that was converted into *am* (mostly 6-*O*-sulphated) on mild treatment with HNO_2 (Fig. 1*b*).

The I_s and A_s residues that, together with the hexasaccharide core common to the other active (*d* and *h*, Fig. 5) octasaccharides would complete an extraction octasaccharide, are tentatively placed at the non-reducing end of the octasaccharide. This is suggested by the C-1 signal of I being at a field higher than observed for the major fragment obtained by deaminative cleavage of the active heparinase-cleavage octasaccharide, i.e. the tetrasaccharide I-A_s-G-*am*, where the I residue is quite certainly terminal at the non-reducing end. Also, reaction of extraction oligosaccharides with HNO_2 under rather mild conditions (Perlin *et al.*, 1972) did not affect two amino sugar residues: the A_s^* residue giving rise to the atypical C-2 signal at 57.7p.p.m., and one other A_S residue. Since the I–A_s bonds belonging to 'regular' sequences are usually cleaved under the conditions used for mild deamination, it seems more probable than the A_s residue (other than A_s^*) that is resistant to deamination is next to an I rather than to an I_s residue. This supports the assumption that I is internal rather than at the terminal non-reducing end. The periodate consumption (about 3 mol per average mol of oligosaccharide) is compatible with both possibilities.

It must be noted that extraction oligosaccharides are rather non-homogeneous in size, their average chain length being ~8.5. If it is assumed (see above) that the reducing residues are essentially A_s, these oligosaccharides are most probably mixtures of octasaccharides (major components) and decasaccharides. This is further suggested by the extent of degradation by heparinase (~2.5 mol of unsaturated uronic acid per 'mol' of oligo-

saccharide), which is somewhat higher than expected for an octasaccharide with no more than two cleavable bonds. The contaminating decasaccharide is therefore assumed to have an extra I_s-A_s disaccharide unit at its reducing end, with respect to the octasaccharide.

Non-integral signals in the ^{13}C spectra suggest some degree of heterogeneity also at the level of the hexasaccharide 'core'. The variable relative intensity of G and I signals in three different preparations of extraction oligosaccharides may imply some interchangeability of these residues to be possible without a substantial loss in affinity for antithrombin III. The same concept applies for the degree of *O*-sulphation at C-6 of the amino sugar residues, which appears nonetheless higher than for commercial heparins.

The ^{13}C spectrum of the deaminative-cleavage oligosaccharide clearly indicates a size not exceeding an octasaccharide, with three A-2 signals (attributable to 1 A_s, 1 A_s^* and 1 A_a), and one *am*(OH)6S signal. This pattern in the anomeric region and in the C-4, C-2 and C-5 of *am*(OH) is quite complex, and includes a few non-integral signals. This indicates some degree of internal heterogeneity. However, the most prominent signals correspond to the residues suggested as major components of the octasaccharide sequenced by Thunberg *et al.* (1980) also prepared by a deamination procedure. I-1 is somewhat downfield with respect to the active extraction oligosaccharides, a feature associated with a terminal I residue (see above).

The spectrum of the heparinase-cleavage octasaccharide (Fig. 3*a*) is fully compatible with the structure proposed by Choay *et al.* (1981*a*). It can be noted that I-1 is at about the same field as in extraction oligosaccharides, as expected for the 'internal' arrangement A_s-I-A_a.

Also, the spectra of the major fragments (A, C and D) obtained by exhaustive deaminative cleavage of the heparinase-cleavage octasaccharide are compatible with the structure of the tetrasaccharide I-A_a-G-*am**, and of the two disaccharides (unsaturated-I_s-*am* and I_s-*am*) expected from the cleavage of the octasaccharide having the structure proposed by Choay *et al.* (1981*a*).

The structure proposed for the active *e*-, *d*- and *h*-octasaccharides are reported in Fig. 5. Residues 1–4 are those that give rise, on exhaustive deamination, to the 'Rosenberg tetrasaccharide' (Rosenberg *et al.*, 1978; Rosenberg & Lam, 1979), implying that the unique A_s^* residue is at position 4 of the original octasaccharide. The ^{13}C signals from the 'regular' disaccharide sequence I_s-A_s should therefore account for residues 5 and 6, and those for the sequence I_s-*am* for residues 7 and 8.

Residues 7 and 8 are probably present also (as I_s-A_s, at the reducing end in the extraction oligosaccharide, especially in the decasaccharide. However, they are not part of the heparinase-cleavage octasaccharide, which terminates with the I_s-A_s unit next to the 'Rosenberg tetrasaccharide' (on exhaustive deamination, this disaccharide unit produced an I_s-*am* fragment). At the non-reducing end, the extraction or the heparinase-cleavage oligosaccharides have the extra disaccharide units I_s-A_s and unsaturated I_s-A_s respectively, the latter producing the unsaturated I_s-*am* fragment obtained by exhaustive deamination.

It is therefore concluded that the hexasaccharide I-A_a-G-A_s^*-I_s-A_s is common to the three types of

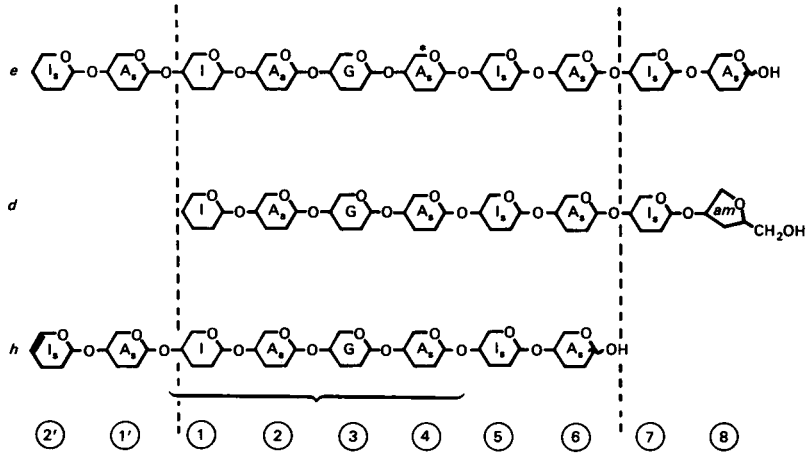


Fig. 5. *Proposed structures for the active heparin oligosaccharides*
The brace encompasses the major tetrasaccharide fragment from exhaustive deaminative degradation *e*, extraction polysaccharide; *d*, deaminative-cleavage oligosaccharide; *h*, heparinase-cleavage oligosaccharide.

active oligosaccharides. This implies, as we already suggested (Choay *et al.*, 1981*a*) that the minimum sequence for binding to antithrombin III is confined within these six residues. An active fragment corresponding to the above hexasaccharide (with unsaturated I at the terminal non-reducing end) was recently isolated (Choay *et al.*, 1981). The unsaturated tetrasaccharide, B, obtained as a by-product of the active heparinase-cleavage oligosaccharide is obviously a fragment from this hexasaccharide.

The ^{13}C signals of the atypical A_s^* residue were prominent in all the active oligosaccharides and practically absent in the inactive species, thus being an essential feature of the hexasaccharide core.

The studies performed on model compounds show that the extra signal, first observed in extraction oligosaccharides (Choay *et al.*, 1980), may be related to C-2 of A_s^*. The shift observed for this signal (compared with C-2 of A_s) may indeed be explained by the presence of an *O*-sulphate group at C-3. This observation fits the recent suggestion based on enzymic studies that a 3-*O*-sulphated glucosamine unit might play a crucial role in heparin physiology (Leder, 1980); this hypothesis is strongly supported by recent work (Lindahl *et al.*, 1980).

We have observed in model compounds that the major shift on 3-*O*-sulphation is at C-3 (about −8 p.p.m.), as expected on the basis of known substituent effects (Perlin, 1976). Since C-3 of A_s of the 'regular' heparin sequence is at 70.6 p.p.m. (Gatti *et al.*, 1979; value corrected for the different internal reference), C-3 of A_s^* has to be expected somewhere around 78.5 p.p.m. This is unfortunately a region where severe overlap by other signals prevents unambiguous observation of a relevant signal. However, we suggest that the signal at 78.5 p.p.m., clearly observable in the spectrum of the active extraction oligosaccharides (Fig. 1) might be associated with the above atypical carbon. The 3-*O*-sulphate group should also strongly affect the spectrum of the major tetrasaccharide fragment (A), in which it is expected to be incorporated as terminal *am**. The *am* pattern of this fragment (Fig. 3*c*) differs from that reported (Mushayakarara, 1977) for the disaccharide G-*am*, more than anticipated on the only basis of this unit being part of a tetrasaccharide. The possibility that *O*-sulphation at C-3 of the *am** residue is responsible for these differences is being evaluated with appropriate model compounds.

It should be noted that the resistance to cleavage of the A_s^*–I_s bond under conditions of mild deamination can be explained on a chemical basis by the electronegativity of an *O*-sulphate vicinal to the leaving group generated at C-2 on reaction with HNO_2.

The present data are compatible with the proposal that A_s^* is an *N*-sulphated glucosamine unit that bears an *O*-substituent at C-3. Although this is not strictly proven, this substituent is most probably a sulphate group that is responsible for the extra signal observed at 57.7 p.p.m. in the active oligosaccharides so far studied. Such a signal could be used as a physical characteristic of this kind of active oligosaccharide.

References

Andersson, L. O., Barrowcliffe, T. W., Holmer, E., Johnson, E. A. & Sims, G. E. C. (1976) *Thromb. Res.* **9**, 575–583

Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334

Caen, J. Larrieu, M. J. & Samama, M. (1968) *L'Hémostase*, pp. 133–135, Expansion Scientifique Française, Paris

Casu, B. & Gennaro, U. (1975) *Carbohydr. Res.* **39**, 168–176

Casu, B., Torri, G. & Vercellotti, J. R. (1979) *Pharmacol. Res. Commun.* **11**, 297–310

Choay, J., Lormeau, J. C., Petitou, M., Sinaÿ, P., Casu, B., Oreste, P., Torri, G. & Gatti, G. (1980) *Thromb. Res.* **18**, 573–578

Choay, J., Lormeau, J. C. & Petitou, M. (1981*a*) *Ann. Pharm. Fr.* **39**, 37–44

Choay, J., Lormeau, J. C., Petitou, M., Fareed, J. & Sinaÿ, P. (1981*b*) *Ann. Pharm. Fr.* in the press

Cifonelli, J. A. & King, J. (1975) *Connect. Tissue Res.* **3**, 97–104

Fransson, L. A., Huckerby, T. N. & Nieduszynski, I. A. (1978) *Biochem. J.* **175**, 299–309

Gatti, G., Casu, B., Hamer, G. K. & Perlin, A. S. (1979) *Macromolecules* **12**, 1001–1007

Höök, M., Björk, I., Hopwood, J. & Lindahl, U. (1976) *FEBS Lett.* **66**, 90–93

Hopwood, J., Höök, M., Linker, A. & Lindahl, U. (1978) *FEBS Lett.* **69**, 51–54

Lam, L. H., Silbert, J. E. & Rosenberg, R. D. (1976) *Biochem. Biophys. Res. Commun.* **69**, 570–577

Leder, I. G. (1980) *Biochem. Biophys. Res. Commun.* **94**, 1183–1289

Lindahl, U. & Höök, M. (1978) *Annu. Rev. Biochem.* **47**, 385–417

Lindahl, U., Bäckström, G., Höök, M., Thunberg, L., Fransson, L. Å. & Linker, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3198–3202

Lindahl, U., Bäckström, G., Thunberg, L. & Leder, I. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6551–6555

Lindberg, B. & Lonngren, J. (1978) *Methods Enzymol.* **50**, 3–33

Linker, A. & Hovingh, P. (1972) *Biochemistry* **11**, 563–568

Lloyd, A. G., Wusteman, F. S., Tudball, N. & Dodgson, K. S. (1964) *Biochem. J.* **92**, 68–72

Meyer, B., Thunberg, L., Lindahl, U., Larm, O. & Leder, I. G. (1981) *Carbohydr. Res.* **88**, C1–C4

Mushayakarara, E. (1977) M. Sci. Thesis, McGill University

Nagasawa, K., Inoue, Y. & Kamata, T. (1977) *Carbohydr. Res.* **58**, 47–55
Oreste, P. & Torri, G. (1980) *J. Chromatogr.* **195**, 398–401
Payza, N. & Korn, E. D. (1956) *J. Biol. Chem.* **223**, 853–858
Perlin, A. S. (1976) *Int. Rev. Sci. Org. Chem. Ser. 2*, **7**, 1–34
Perlin, A. S., Ng Ying Kin, N. M. K., Bhattacharjee, S. S. & Johnson, L. F. (1972) *Can. J. Chem.* **50**, 2437–2441
Rosenberg, R. D. & Lam, L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1218–1222
Rosenberg, R. D., Armand, G. & Lam, L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3065–3069
Shively, J. E. & Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
Smith, R. L. & Gilkerson, E. (1979) *Anal. Biochem.* **98**, 478–480
Thunberg, L., Bäckström, G., Grundberg, H., Riesenfeld, J. & Lindahl, U. (1980) *FEBS Lett.* **117**, 203–206
Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535
Yin, E. T., Wessler, S. & Butler, J. V. (1973) *J. Lab. Clin. Med.* **81**, 298–310