

BBA 31102

MODE OF ACTION OF HEPARIN LYASE ON HEPARIN

ROBERT J. LINHARDT^a, GERALD L. FITZGERALD^a, CHARLES L. COONEY^a and ROBERT LANGER^{a,b,*}

^a Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139 and ^b Department of Surgery, Children's Hospital Medical Center, Boston, MA 02115 (U.S.A.)

(Received November 10th, 1981)

Key words: Heparin lyase; Heparin; (*Fl. heparinum*)

Heparinase (heparin lyase, EC 4.2.2.7) prepared from *Flavobacterium heparinum* was used to digest heparin. The products of digestion were examined with a viscosometric assay at various stages of the reaction to measure their average molecular weight. By comparison with computer simulations of various models, heparinase was shown to act in a random endolytic mode. The relative abundance of intermediates in heparin degradation catalyzed by heparinase immobilized on Sepharose 4B was measured by high pressure liquid chromatography (HPLC) at various time points. The results obtained using HPLC were consistent with a random endolytic mechanism. The heparin digestion products were separated and identified using gel permeation chromatography. The final distributions of heparin degradation products for free and immobilized heparinase were identical. Contaminating sulfatases and glycuronidases which could have subsequently acted on heparin degradation products were not found in significant amounts in the heparinase preparation studied.

Introduction

Heparinase (heparin lyase, EC 4.2.2.7) is an eliminase which cleaves certain α -glycosidic linkages [1] in heparin, an α,β -linked sulfated polysaccharide with a molecular weight range from 7600 to 19000 [2]. This enzyme is isolated from *Flavobacterium heparinum* [3-5] and presumably serves a role in the initial catabolism of heparin [6]. Heparinase also has found many other applications, including the determination of heparin's structure [1], the preparation of heparin degradation products which exhibit anticoagulant activity [7], the investigation of the coagulation mechanism [8] and the identification of heparin in complex mixtures of mucopolysaccharides in mammalian cells [9]. Recently, heparinase has been used in our laboratory for blood deheparinization in an immobilized enzyme reactor [10,11].

The mechanism of action of heparinase has received little attention. The eliminase mechanism of heparinase, as well as that of chondroitinase (EC 4.2.2.4) and hyaluronidase (EC 4.2.2.1) is unusual; glycosidic linkages are usually cleaved through hydrolysis. The eliminase mechanism is established by the $\Delta 4,5$ site of unsaturation present in the products [5]. However, no one has addressed the question of the order in which heparinase cleaves the α -glycosidic linkages in heparin. It is possible that the reaction occurs in an exolytic or an endolytic fashion. The studies described here were designed to answer this fundamental question. Heparin degradation was followed using both ultraviolet and viscosometric assays and the results were compared with several kinetic models formulated on the basis of an exo- or endolytic cleavage of heparin. The results have been further confirmed through analysis of degradation products using high pressure liquid chromatography (HPLC).

* To whom reprint requests should be addressed at M.I.T.

Theory

1. *General assumptions.* All of the kinetic models for heparin degradation assume that the enzyme operates under saturation kinetics. This was achieved by using a substrate to enzyme ratio of 100 to 1. The models also assume that the substrate is initially a monodisperse homocopolymer. Each substrate molecule is modeled as 20 identical disaccharide units corresponding to a molecular weight of 11000, or as a polydisperse substrate with a polydispersity corresponding to that observed for porcine heparin [34]. All of the models assume that the enzyme can cleave the substrate between any two disaccharide units and that the substrate contains no uncleavable α -linkages. The models further assume that the enzyme will attack any substrate molecule with equal probability, regardless of size.

2. *Likely models of heparin degradation.* Four likely models for heparinase action on heparin were considered.

Model A. Heparinase attacks at only one end of a heparin molecule; the enzyme then cuts all the cleavable linkages in an exolytic fashion before attacking a second heparin molecule.

Model B. Heparinase attacks either end of a heparin molecule once, then releases the substrate and randomly selects a second substrate and again acts exolytically. This second substrate could be the original heparin molecule or a second previously unattacked molecule.

Model C. Heparinase attacks anywhere on the heparin molecule and then continues to cut that molecule until it is completely degraded; it then randomly selects a second molecule on which to act.

Model D. Heparinase attacks anywhere on any heparin chain in a completely random fashion (random endolytic cleavage).

3. *Computer simulation.* On the computer, the above models were described as follows.

Model A. The model is mathematically determined. If the number of cuts is a multiple of 19 (the maximum number of cuts), then the variable S is set to 0. If the number of cuts is not a multiple of 19, then let $S = 20 - C + \text{INT}(C/19) \cdot 19$, where C is the number of cuts and $\text{INT}(\)$ is the greatest integer function. The weight average molecular weight is then calculated by:

$$M_r = \frac{400 \cdot (99 - \text{INT}((C-1)/19))}{20 \cdot (99 - \text{INT}((C-1)/19)) + C + \text{INT}(C/19) + S^2} + C + \text{INT}(C/19) + S$$

The results of this simulation are plotted as curve A in Fig. 1.

Model B. This process is stochastically determined. First the program selects a chain with a length greater than one (disaccharide) unit. It then sets the length of the chain to one less than its prior value. Every 20 cuts, the sum of the lengths of the chains, $\text{Sum}N$, and the sum of the squares of the lengths of the chains, $\text{Sum}N^2$, is taken. Since each cut produces a single disaccharide product, the weight average molecular weight is calculated as:

$$M_r = \frac{\text{Sum}N^2 + C}{\text{Sum}N + C}$$

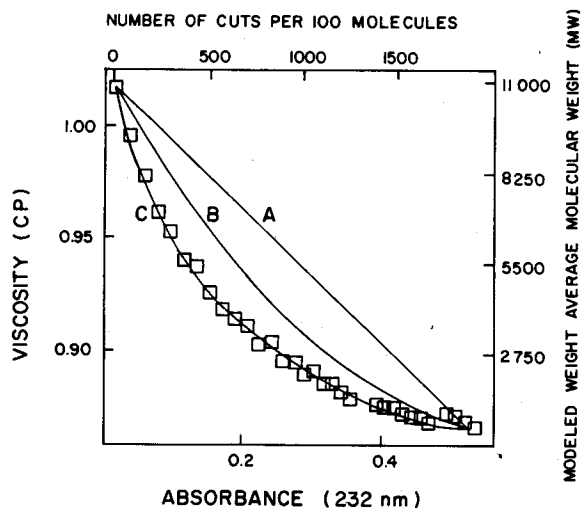


Fig. 1. Theoretical plots of modeled weight average molecular weight versus number of cuts per 100 molecules for four different modes of action of a lyase on a polymeric substrate: A, non-random exolytic and non-random endolytic; B, random exolytic, and C, random endolytic. Data (\square) are viscosity versus change in absorption at 232 nm correlated using time as a parametric variable.

The results of this simulation are plotted as curve B in Fig. 1.

Model C. Every 19 cuts, this model will have the same ensemble of chains as model A. There will be no discernable difference between these models in the plot of weight average molecular weight versus number of cuts.

The results of this simulation are plotted as curve A in Fig. 1.

Model D. This model is also stochastic. The program selects one chain of length greater than one (disaccharide), and determines its length, N . The program then randomly selects the M th of the $N - 1$ bonds and cleaves the chain into two pieces of length M and $N - M$. One of the two pieces is kept on the same spot in the array of substrate molecules; the other is given a new spot. After each cut, the array size increases by one, and the sums are calculated as before and the weight average molecular weight is calculated by:

$$M_r = \frac{\text{Sum } N^2}{\text{Sum } N}$$

The results of this simulation are plotted as curve C in Fig. 1.

Materials and Methods

Chemicals

Heparin from porcine intestinal mucosa (Grade II, 159 units/mg), chondroitinase ABC (from *Proteus vulgaris*), chondroitin sulfate A, 4-nitrocatechol sulfate and 4-dimethylaminobenzaldehyde were purchased from Sigma Chemical Company. Sephadex G-15, Sephadex G50 (medium), Blue dextran and CNBr-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals. Glucose and maltose were obtained from the J.T. Baker Company. All inorganics were reagent grade or better.

Equipment

All spectrophotographic measurements were made with a Gilford model 1084 spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, OH). HPLC was performed on a Waters Associates model 6000A Chromatographic pump using a model U6K injector and a Dupont Zorbax

unsilicated silica column with a 7–8 μm particle size. (This column was chosen because of its highly charged nature.) The Gilford spectrophotometer was fitted with an 80- μl flow cell having a 1 mm path length and used to monitor the HPLC effluent at 232 nm. Freeze-drying was done in a Virtis model 10-104-LD freeze-drier. Computer modeling was done in BASIC on a Data General Eclipse operating under the Advanced Operating System.

Methods

Assay for heparinase and chondroitinase activity. Assay solution contains heparin or chondroitin sulfate A at 25 mg/ml in 0.25 M sodium acetate/0.0025 M calcium acetate adjusted with acetic acid to pH 7.0. To 1 vol. assay solution, 2 vol. of the same buffer (without heparin) containing either free or immobilized enzyme at a protein concentration of ≤ 0.01 mg/ml were added. The samples were incubated at 30°C and aliquots (25 μl) were removed at various times, added to 2 ml of 0.03 M hydrochloric acid and the absorbance was measured at 232 nm.

Viscosity measurements. The viscosity assay was carried out according to the procedures in Ref. 12. The time of each assay point was taken as the time of the start of the measurement plus half the duration of the measurement.

Viscosity was measured in assay solution containing 8.33 $\mu\text{g/ml}$ of protein (containing heparinase or chondroitinase) and 8.33 mg/ml heparin (or chondroitin sulfate A). The solution was placed in a capillary viscometer in a $30 \pm 0.05^\circ\text{C}$ water bath. The viscosity was measured as the time required for the level of the solution to fall between two marks above and below a bulb, divided by the time required for distilled water to fall between the marks and multiplied by the viscosity of water at 30°C.

Sulfatase assays. The presence of contaminating sulfatases in heparinase preparations was assayed using 4-nitrocatechol sulfate by the procedure of Otonani et al. [13]. Sulfamidase activity in heparinase was determined by the method of Ototani and Yosizawa [33]. The substrate was a mixture of heparin degradation products (prepared using immobilized heparinase). The formation of de-*N*-sulfated amino sugars was measured

using the Morgan-Elson procedure of Reissig et al. [14].

Glucuronidase assay. Glucuronidase activity was assayed by measuring the stability of the α,β unsaturated ($\Delta 4,5$) carboxylate chromophore at 232 nm. This assay was performed by the method of Warnick and Linker [15] in the assay solution which had been previously used to measure heparinase activity.

Protein assay. Protein was measured by the Biuret method [16] or by the Bio-Rad protein assay [17].

Assay for heparin degradation products. The HPLC effluent containing the digestion products of heparin was read directly at 232 nm, while for the Sephadex column chromatography, the effluent was first diluted 1:100 with distilled water and then read at 232 nm.

Chromatography. Separations by HPLC were performed at 27°C, pH 5.8, in 0.15 M NaCl in degassed double-distilled water at a flow rate of 1 ml/min. Separations by gel permeation chromatography were performed on a 3 × 100 cm G-15 Sephadex column. The temperature was maintained at 4°C while the flow rate was 0.13 ml/min with fractions collected every 10 min. The void volume was 92 ml as determined using Blue dextran. The molecular weight standards were glucose (M_r 180) and maltose (M_r 360) which ran to 173 and 152 ml, respectively. The G-50 Sephadex column was 2 × 40 cm with a void volume of 40 ml measured with Blue dextran. The column was run at 4°C at a flow rate of 1.93 ml/min and fractions were taken at 1-min intervals.

Heparinase preparation and immobilization. Heparinase was produced fermentatively from *F. heparinum* [4] and was purified using batch hydroxyapatite chromatography [10,11]. Immobilization to CNBr-activated Sepharose 4B was performed by the method described in Ref. 18 at pH 7.0 in 0.2 M sodium phosphate buffer at a protein concentration of 0.5 mg/ml and in the presence of 60 mg heparin/mg protein.

Results and Discussion

The computer simulations of heparin molecules' weight change as a function of the number of cleavages for the different models are represented

by plots A, B, and C in Fig. 1 (the polydisperse case gives results identical to within < 2%). These curves are easily distinguished from one another; the greatest difference arises in the initial stages of substrate degradation.

The computer simulations of heparinase action assume total degradation of heparin and do not explain the larger polysaccharide products observed experimentally. These models best approximate the actual enzymatic system in the initial stages of substrate degradation where the lower proportion of uncleavable sites have little influence on the computer simulations.

To obtain experimental data on the change in substrate molecular weight as a function of the number of cleavages, heparin degradation with heparinase was monitored simultaneously with a viscosometric and an ultraviolet (232 nm) assay. The viscosity assay provides a measure of the average molecular weight of mucopolysaccharides such as heparin which behave as newtonian solutions at the concentrations being studied [12,19]. Lasker and Stivala [35] correlated the molecular weight (measured by sedimentation) of bovine heparin fractions (isolated using gel chromatography) to the viscosity of these heparin fractions in solution. From these data, we find that the molecular weight of heparin can be estimated (to within 2%) by a linear transform of the relative viscosity over a molecular weight range from 4000 to 10000. Viscosometric measurements have been used to examine enzymatic depolymerizations catalyzed by cellulase [20], by DNAase [21] and by hyaluronidase [22] and to determine a specific endolytic mode of action [22,23]. The ultraviolet 232 nm assay provides a measure of the number of cuts made by heparinase, since each such cut results in the formation of a new site of unsaturation.

By comparing the experimental data with the computer simulations the curve generated for the random endolytic model (model D represented by curve C in Fig. 1) correlates almost exactly with the data (see Fig. 1). The results suggest that heparinase acts on heparin in a random endolytic fashion.

Controls were performed by measuring the viscosity of enzyme in assay solution containing no substrate, and assay solution with substrate only. No change in viscosity was observed over the

time of the assay for these solutions. In preliminary studies, we have also extended our methodology to a second polysaccharide α -eliminase, chondroitinase ABC acting on chondroitin sulfate A, and have found that it gives a curve nearly identical to that obtained for heparinase action on heparin.

To test further the validity of the random endolytic model, heparin digestion products were prepared using immobilized heparinase and fractionated by both gel permeation chromatography (GPC) and HPLC. GPC has been used in the sizing of enzymatic degradation products derived from both heparin [1] and chondroitin sulfates [24]. Polysaccharide products obtained from chondroitin sulfates using chondroitinase [25–27] and disaccharides prepared by the action of nitrous acid on heparin have also been studied using HPLC [28].

Heparinase was immobilized on Sepharose 4B with a 91% recovery of activity. Immobilized heparinase is thermally more stable ($t_{1/2} = 40$ h at 25°C) [10,11], allowing preparation of products and easy separation of these products from the immobilized enzyme. The enzyme does not leach off the beads at a significant rate (<2%/month) based on protein assay.

Products prepared using immobilized heparinase were fractionated by size with GPC on

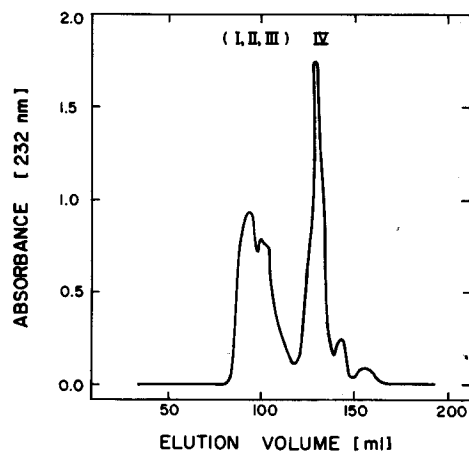


Fig. 2. The Sephadex G-15 column was run by adding 1.5 ml of 0.03 M hydrochloric acid containing 500 mg of heparin degraded by immobilized heparinase on to the bed and eluting with 0.03 M hydrochloric acid. The fractions were combined as peaks (I, II, III) IV and concentrated by freeze-drying

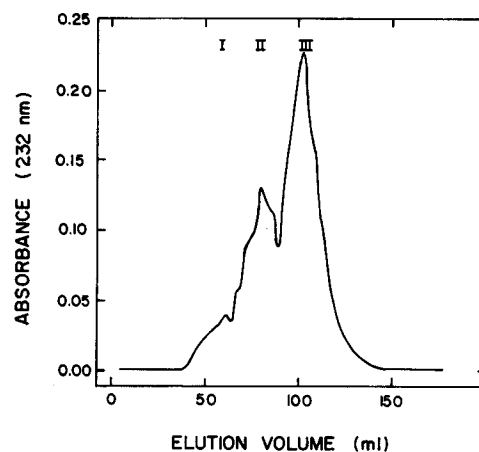


Fig. 3. The solids obtained from freeze-drying peaks I, II, and III from the G-15 column were redissolved into 1.5 ml of 0.03 M hydrochloric acid. This solution was loaded onto a Sephadex G-50 column and eluted with 0.03 M hydrochloric acid. The fractions were combined as peaks I, II and III and concentrated by freeze-drying.

a G-15 Sephadex column. (The same elution profile was obtained with either 0.03 M hydrochloric acid or distilled water solvent systems.) The peaks were combined into fractions (I, II, III) and IV (Fig. 2) and freeze-dried. The large molecular weight components (I, II, and III) were applied together to a G-50 column (Fig. 3) and eluted in a similar fashion.

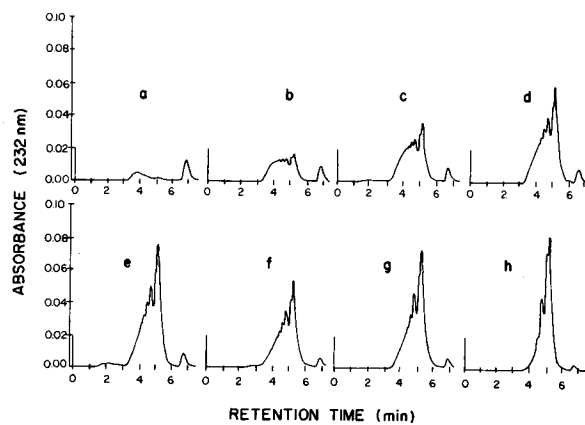


Fig. 4. HPLC chromatograms of samples taken at various times from a mixture containing immobilized heparinase and heparin (8.33 mg/ml) in assay solution. The samples shown (a–h), the % time completion of the reaction of each sample point, and the injection size (μ l) are as follows: a, 0, 50; b, 4, 40; c, 16, 30; d, 21, 30; e, 26, 30; f, 30, 30; g, 38, 20; h, 100, 10.

The kinetics of product formation were measured during heparin degradation with immobilized heparinase; samples were taken as a function of time and injected into the HPLC. The results are shown in Fig. 4. The area under the chromatograms 4a through 4h increases when these traces are corrected for decreasing injection size.

The HPLC chromatogram in Fig. 4h and the final product distribution examined by GPC (Fig. 2) appear similar in that high molecular weight polysaccharides are eluted before the low molecular weight polysaccharides. Taking samples from each of the pooled peaks (Figs. 2 and 3), the identities of the HPLC peaks were confirmed as eluting from high molecular weight to low molecular weight, with HPLC retention times for the gel permeation peaks of 4.3, 4.6, 4.8 and 5.1 min for peaks I, II, III and IV, respectively. The HPLC peak showing a retention time of 6.8 min, present in traces 4a-h and not acted upon by heparinase, has been shown to be the acetate from the assay solution as determined by separate injection.

The results of the HPLC assays clearly substantiate the conclusion arrived at from the viscosity data. Fig. 4 shows transient accumulation of high molecular weight sugars (as indicated by the area between 3.0 and 4.7 min). This is consistent with the random endolytic mechanism. The exolytic mechanisms described above (see Theory Section) would give rise to a large amount of disaccharide product (at 5.1 min) early in the reaction. Similar HPLC studies with free heparinase gave the same profile of product formation, suggesting that free and immobilized heparinase have the same mechanism of action.

Heparinase, obtained from *F. heparinum* [3-5] acts with high specificity [6,29] on heparin, producing a complex mixture of $\Delta 4,5$ unsaturated polysaccharides [1,30]. Crude extracts of *F. heparinum* also contain glycuronidase [15], sulfoesterase [31] and sulfamidase [31] which subsequently [6,32] act on the heparinase-derived polysaccharides. Heparinase can be purified to a point where these contaminating activities are either no longer present or are greatly reduced [13,33].

We have examined the enzymatic activities in the heparinase preparation which are expected to act on heparin or the products derived from

heparin. By the time the heparinase assay is complete, less than 0.01% (with immobilized enzyme) or 1.7% (with free enzyme) of the chromophore has been degraded. Assuming that the glycuronidase is specific for β -glycosidic linkages adjacent to the α, β unsaturated end, as has been previously reported [32], only 0.01% (with immobilized enzyme) or 1.7% (with free enzyme) of these linkages are cleaved during the heparinase assay. The sulfatase activity, in both the free and immobilized hydroxyapatite preparations, is only capable of 3.6 or 3.3% desulfation, respectively, during the duration of the heparinase assay as determined by two different assays (see Methods section).

In summary, the results obtained suggest that the mode of action of heparinase is random endolytic. A new way to separate the products of these reactions using HPLC has been developed and we have established new simplified computer models to determine endolytic versus exolytic mechanisms for soluble polymeric substrates.

Acknowledgements

This work was supported by Public Health Service Grant 1 RO1 GM25810-01 from the National Institutes of Health. We thank Arthur Grant for his help in the early stages of this work.

References

- 1 Linker, A. and Hovingh, P. (1979) Enzymatic Degradation of Heparin as a Tool for Structural Analysis, in Heparin: Structure, Cellular Functions and Clinical Applications, (McDuffie, N.M., ed.), pp. 3-24, Academic Press, New York
- 2 Jaques, L.B. (1979) *Science* 206, 528-533
- 3 Korn, E.D. and Payza, A.N. (1956) *Nature* 177, 88-89
- 4 Galliher, P.M., Cooney, C.L., Langer, R. and Linhardt, R.J. (1981) *J. Appl. Env. Microbiol.* 41, 360-365
- 5 Linker, A. and Hovingh, P. (1972) *Methods Enzymol.* 28, 902-911
- 6 Dietrich, C.P., Silva, M.E. and Michelacci, Y.M. (1973) *J. Biol. Chem.* 248, 6408-6415
- 7 Lasker, S.E. and Chiu, M.L. (1973) U.S. Patent No. 3766167
- 8 Lindahl, U., Backstrom, G., Hook, M., Thunberg, L., Fransson, L. and Linker, A. (1979) *Proc. Natl. Acad. Sci. USA* 76, 3198-3202
- 9 Kanwar, Y.S. and Farquhar, M.G. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1303-1307
- 10 Langer, R., Linhardt, R.J., Klein, M., Galliher, P.M., Cooney C.L. and Flanagan, M.M. A System for Heparin Re-

- moval, in *Advances in Chemistry*, (Cooper, S., Hoffman, A., Peppas, N. and Rattner, B., eds.), in the press.
- 11 Linhardt, R.J., Langer, R., Cooney, C.L., Galliher, P.M., Flanagan, M.M. and Hoffberg, S.M. (1981) Blood Deheparinization Using Immobilized Microbial Heparinase, in *Proceedings of the Second World Congress of Chemical Engineering*, Vol. I, Montreal
 - 12 Roden, L., Baker, J.R., Cifonelli, J.A. and Mathews, M.B., (1972) *Methods Enzymol.* 28, 73-140
 - 13 Ototani, N., Kikuchi, M. and Yosizawa, Z. (1981) *Carbohydr. Res.* 88, 291-303
 - 14 Reissig, J.L., Strominger, J.L. and Leloir, L.F. (1955) *J. Biol. Chem.* 217, 959-966
 - 15 Warnick, C.T. and Linker, A. (1972) *Biochemistry*, 11, 568-572
 - 16 Gornal, A.G., Bardwill, C.W. and David, M.M. (1949) *J. Biol. Chem.* 177, 751-766
 - 17 Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254
 - 18 March, S.C., Parikh, I. and Cuatrecasas, P. (1974) *Anal. Biochem.* 60, 149-152
 - 19 Liberti, P.A. and Stivala, S.S. (1967) *Arch. Biochem. Biophys.* 119, 510-518
 - 20 Levinson, H.S. and Reese, E.T. (1950) *J. Gen. Physiol.* 33, 601-628
 - 21 Thomas, C.A. (1956) *J. Am. Chem. Soc.* 78, 1861-1868
 - 22 Anderson, S.O. and Grace, J. (1955) *Acta Chem. Scand.* 9, 1431-1437
 - 23 Bak, T.A. and Bak, K. (1959) *Acta Chem. Scand.* 13, 1997-2008
 - 24 Ulrich, H.P., Klein, U. and Figura, K.V. (1979) *Z. Physiol. Chem.* 360, 1457-1463
 - 25 Lee, G.J.L. and Tieckelmann, H. (1979) *Anal. Biochem.* 94, 231-236
 - 26 Delaney, S.R., Conrad, H.E. and Glaser, J.H. (1980) *Anal. Biochem.* 108, 25-34
 - 27 Lee, G.J.L., Evans, J.E., Tieckelmann, H., Dulaney, J.T. and Naylor, E.W. (1980) *Clinica Chimica Acta* 101, 65-75
 - 28 Delaney, S.R., Leger, M. and Conrad, H.E. (1980) *Anal. Biochem.* 106, 253-261
 - 29 Linker, A. and Hovingh, P. (1972) *Biochemistry* 11, 563-567
 - 30 Hovingh, P. and Linker, A. (1970) *J. Biol. Chem.* 245, 6170-6175
 - 31 Dietrich, C.P. (1969) *Biochem. J.* 111, 91-95
 - 32 Yamagata, T., Saito, H., Habuchi, O. and Suzuki, S. (1968) *J. Biol. Chem.* 243, 1523-1525
 - 33 Ototani, N. and Yosizawa, Z. (1979) *Carbohydr. Res.* 70, 295-306
 - 34 Laurent, T., Tengblad, A., Thunberg, L., Hook, M. and Lindahl, U. (1978) *Biochem. J.* 175, 691-701
 - 35 Lasker, S. and Stivala, S. (1966) *Arch. Biochem. Biophys.* 115, 360-372