Kinetic analysis of the heparin-enhanced plasmin–antithrombin III reaction

Apparent catalytic role of heparin

Raymund MACHOVICH, Pál I. BAUER, Péter ARÁNYI, Éva KECSKÉS, Kálmán G. BÜKI and István HORVÁTH

Second Institute of Biochemistry, Semmelweis University Medical School, Puskin u 9, H-1444 Budapest, Box 262, Hungary

(Received 23 March 1981/Accepted 17 July 1981)

Inactivation of plasmin by a 3–4-fold molar excess of antithrombin III follows pseudo-first-order kinetics and the apparent rate constants are proportional to the concentration of the inhibitor. Heparin accelerates the inactivation reaction without changing its pseudo-first-order character, and the apparent rate constants are also proportional to the concentration of the polysaccharide. Heparin results in a minimum 20-fold rate enhancement of the reaction between plasmin and antithrombin III when the concentrations of heparin and plasmin are approx. $0.5\ \mu\text{M}$ and $1\ \mu\text{M}$ respectively. Heparin at a molar concentration well below that of plasmin still accelerates the reaction: one molecule of the polysaccharide is able to facilitate the inactivation of about 100 molecules of plasmin. Heparin must bind to plasmin to accelerate the plasmin–antithrombin III reaction, since the modification of four to five lysine residues of the enzyme inhibits the rate-enhancement effect of heparin and the dissociation of heparin–plasmin complex decreases the inactivation rate of plasmin. Increasing the concentration of antithrombin III, at a constant amount of heparin, results in increase of the inactivation rate. By contrast, the effect of increasing the amount of plasmin in the presence of constant amount of heparin and antithrombin III is such that higher plasmin-to-heparin ratios are associated with lower rates of inactivation. It seems, therefore, that to obtain ‘optimal’ conditions for fast enzyme inactivation, the amount of heparin should be matched to plasmin rather than to antithrombin III. Arrhenius plots of the plasmin–antithrombin III reaction are linear both in the absence and presence of heparin, at concentrations of 1 or $2\ \mu\text{g/ml}$, over a range of 26 K. Under these experimental conditions, heparin increases activation entropy. The findings show that heparin seems to fulfil some criteria that are characteristic for biological catalysis: binding, reaction-rate enhancement (increasing activation entropy), recycling of heparin (effectiveness of non-stoichiometric amounts of the polysaccharide) and specificity.

Heparin accelerates the inactivation of the serine proteinases of blood coagulation by antithrombin III. The mechanism by which heparin enhances the enzyme–inhibitor reaction is not entirely understood at present. Its capability to bind antithrombin III (Rosenberg & Damus, 1973; Einarsson & Andersson, 1977; Nordenman & Björk, 1978; Piepcorn *et al.*, 1978; Holmer *et al.*, 1979; Villanueva & Danishefsky, 1979), thrombin (Machovich, 1975; Machovich *et al.*, 1975, 1977, 1978, 1980a; Hatton & Regoeczi, 1977; Stürzebecher & Markwardt, 1977; Smith, 1977;

Griffith, 1979a,b; Griffith *et al.*, 1979) or both (Danishefsky *et al.*, 1977; Machovich & Arányi, 1978; Pomerantz & Owen, 1978; Laurent *et al.*, 1978) have all been proposed as likely mechanisms. In view of this uncertainty, we decided to investigate the heparin-enhanced inactivation of another enzyme of blood coagulation, plasmin. There is some evidence in favour of the plasmin–heparin interaction in the enzyme inactivation by antithrombin III (Stürzebecher & Markwardt, 1977; Hatton & Regoeczi, 1977; Smith & Sundboom, 1980; Machovich *et al.*, 1980b). This interaction, however, has

not yet been studied in detail. Our knowledge about the kinetics of the plasmin inactivation by antithrombin III is also insufficient.

Therefore, in the present work, we have examined the kinetics of plasmin inactivation by antithrombin III and heparin, and have determined the thermodynamic activation parameters. Furthermore, modification of plasmin with pyridoxal 5'-phosphate has been also used to study the interaction of heparin with plasmin.

The results obtained suggest that heparin must bind to the enzyme to accelerate the plasmin-antithrombin III reaction.

Experimental

Materials

Lysine-Sepharose 4B and pyridoxal 5'-phosphate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. D-Val-Leu-Lys *p*-nitroanilide dihydrochloride (S-2251) was obtained from Kabi AB, Stockholm, Sweden. Sephadex G-25, streptokinase (Kabikinas) and urokinase were the products of Pharmacia Fine Chemicals (Uppsala, Sweden), Kabi AB, (Stockholm, Sweden) and Koch-Light (Colnbrook, Bucks., U.K.) respectively. Heparin (from bovine intestine, with a specific activity of 165 units/mg) was obtained from G. Richter Pharmaceuticals (Budapest, Hungary): 1 unit is defined as 7.6 μ g of the International Standard Heparin Preparation (Jaques *et al.*, 1973). Other chemicals were purchased from Reanal Fine Chemicals (Budapest, Hungary).

Methods

Plasminogen was purified from human plasma by the method of Deutsch & Mertz (1970), desalted, freeze-dried and stored at -18°C . (This preparation contained approx. 60% Glu- and 40% Lys-plasminogen.) Immediately before experimentation, plasminogen was activated by streptokinase (10 μ g/mg of plasminogen) at 37°C for 30 min in 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.1 mM-6-aminohexanoic acid. Thereafter, plasmin was gel-filtered on Sephadex G-25, equilibrated with 0.1 M-sodium phosphate buffer, pH 7.4. The specific activity of the plasmin preparation was 7.8 units/mg determined with Cagtest (Kabi AB) as a standard. [One proteolytic unit was arbitrarily taken as the amount of enzyme producing an increase of 450 μ g of acid-soluble tyrosine in a medium of 4% casein in 1 h (Remmert & Cohen, 1949).] In some experiments, plasmin was isolated from citrated human plasma by activation with urokinase on Sepharose-lysine as detailed by Hatton & Regoeczi (1977).

Antithrombin III, purified from human plasma (Wickerhauser *et al.*, 1979), was the product of the American Red Cross Fractionation Center, Bet-

hesda, MD, U.S.A. The final product of the inhibitor protein, over 95% purity, was freeze-dried and before experimentation was dissolved in 0.15 M-NaCl containing 0.01 M-sodium phosphate, pH 7.0. No contaminants were detected in the preparation by immunoelectrophoresis against an antiserum raised to whole human serum.

Protein concentrations were determined as described by Lowry *et al.* (1951), with human serum albumin as a standard.

Molar concentrations of plasmin, antithrombin III and heparin were calculated by using mol.wts. 76 500 (Sodetz *et al.*, 1972), 65 000 (Miller-Andersson *et al.*, 1974) and 11 000 (Hilborn & Anastasiadis, 1971) respectively.

Modification of plasmin with pyridoxal 5'-phosphate. A portion (0.1 ml) of pyridoxal 5'-phosphate (4 mg/ml) was added to 1 ml of plasmin (1 mg) solution. After 8 min incubation at 22°C in the dark, 0.1 ml of NaBH_4 (10 mg/ml) was pipetted into it. After incubation for 1 more min, 0.1 ml of lysine (4 mg/ml) was added and the reaction mixture placed immediately on ice. In the reaction mixture, every reactant was dissolved in 0.1 M-triethanolamine, at pH 8.0. Thereafter, it was dialysed overnight at 0°C against 3 litres of 0.1 M-sodium phosphate buffer, pH 7.4, containing 1 mM-6-aminohexanoic acid. The bound pyridoxal 5'-phosphate was determined by absorbance at 322 nm, a molar absorption coefficient of $9000\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Griffith, 1979a) being used. As a control, the same procedure was performed without pyridoxal 5'-phosphate addition.

Assay of plasmin activity. A portion (0.5 ml) of D-Val-Leu-Lys *p*-nitroanilide (0.1 mg) dissolved in 0.05 M-sodium phosphate buffer, pH 7.4, was preincubated at 22°C for 30 s. Thereafter, 0.05 ml of plasmin solution was pipetted and the change in absorbance at 405 nm was monitored with a Beckman Model 25 spectrophotometer.

Measurement of plasmin inactivation. Distilled water (0.1 ml), heparin (0.1 ml; dissolved in 0.1 M-NaCl) and plasmin (0.1 ml) were preincubated for 1 min at the appropriate temperature. Thereafter 0.1 ml of antithrombin III solution was added and the reaction mixture incubated at $0-37^{\circ}\text{C}$ for 0-60 min. At various times, 0.05 ml portions were withdrawn and assayed for plasmin activity as described above.

Pseudo-first-order rate constants for plasmin inactivation were calculated by the method of least squares.

Activation enthalpy and entropy, as well as free energy was calculated from the rate constants (Tanford, 1970) as:

$$k = A e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R}$$

where H is activation enthalpy, S is activation entropy, R is the gas constant. For A , the value RT/Nh

was used, where N is Avogadro's number, h is Plank's constant and T is absolute temperature.

Results

Catalyst-like function of heparin in the plasmin-antithrombin III reaction

Inactivation of plasmin by severalfold molar excess of antithrombin III followed pseudo-first-order kinetics and the apparent rate constants were proportional to the concentration of the inhibitor (Fig. 1). Heparin, at a molar concentration well below that of enzyme, accelerated the inactivation reaction without changing its pseudo-first-order character, and the apparent rate constants were also proportional to the concentration of the polysaccharide (Fig. 2). Under our experimental conditions, heparin caused an approx. 3.4-fold rate enhancement of the reaction when the molar concentrations of the reactants were: antithrombin III, $4.6\mu\text{M}$; plasmin, $0.99\mu\text{M}$; heparin, 91 nM . In another series of experiments (Fig. 2, inset) 11 nM -heparin was

found to be sufficient to accelerate the inactivation of $1\mu\text{M}$ -plasmin by excess antithrombin III. The semi-logarithmic plot was linear down to about 35% remaining plasmin activity, with no sign of a fraction of plasmin being inactivated via a mechanism different from that of the inactivation of the rest. The heparin-to-plasmin molar ratio was about 1:100 in this case.

Dependence of the rate of the plasmin-antithrombin III-heparin reaction on the relative concentrations

Plasmin inactivation was measured in the presence of heparin at various concentration of antithrombin III or plasmin.

In the absence of heparin, the apparent rate constants were proportional to the antithrombin III concentration in the range used, in agreement with the result obtained above. The rate of inactivation of plasmin by antithrombin III increased with the inhibitor concentration in the presence of heparin too (results not shown). By contrast, increasing the

Fig. 1. Dependence of apparent first-order rate constant of the plasmin-antithrombin III reaction on the inhibitor concentration

Plasmin ($75\mu\text{g}$ of protein/ml) was incubated in 25 mM -sodium phosphate buffer, $\text{pH } 7.4$, containing 37 mM - NaCl , at 16°C in the presence of various concentrations of antithrombin III. Remaining enzyme activity was determined at different times for estimation of the rate constants as described in the Experimental section.

Fig. 2. Time course of plasmin inactivation by antithrombin III in the presence of heparin

Plasmin ($75\mu\text{g}$ of protein/ml) and antithrombin III ($300\mu\text{g}$ of protein/ml) in 25 mM -sodium phosphate buffer, $\text{pH } 7.4$, containing 37.5 mM - NaCl and various concentrations of heparin was incubated at 16°C for 0–10 min. Remaining plasmin activity was determined as described in the Experimental section. In the inset, heparin ($\mu\text{g/ml}$) was added as follows: $\circ$, none; $\blacktriangle$, 0.125; $\blacksquare$, 5.

concentration of plasmin relative to heparin resulted in a decreased inactivation rate (Table 1).

Characterization of heparin-plasmin binding

Effect of ionic strength. The ionic bonds participating in the plasmin-heparin interaction are apparently easier to disrupt than those involved in the antithrombin III-heparin interaction. Thus, the plasmin-heparin complex was dissociable in solution containing slightly more than 0.25 M-salt (Machovich *et al.*, 1980b), whereas the antithrombin III-heparin complex required 1 M-NaCl for dissociation (Miller-Andersson *et al.*, 1974). Therefore it was hoped that the significance of the plasmin-heparin interaction in the inactivation reaction of the enzyme by antithrombin III could be judged by studying the effect of ionic strength, similarly to the case of the thrombin-antithrombin III-heparin system (Machovich *et al.*, 1979). As shown in Table 2, the inactivation of plasmin by antithrombin III is not significantly dependent on ionic strength in the absence of heparin. On the other hand, the rate-enhancing effect of heparin was manyfold in the presence of 25 mM-salt but barely significant in more concentrated NaCl solutions.

Chemical modification of plasmin. The apparent role of ionic interactions in the plasmin-heparin complex, the chemical structure of heparin and assumed analogy with the thrombin-heparin interaction, led us to suspect that lysine side chains of plasmin might be involved in the binding of the polysaccharide to the enzyme. We tried to block lysine residues with pyridoxal 5'-phosphate, a reagent that proved most useful in the case of thrombin (Griffith, 1979a).

When four to five lysine residues (if *N*-terminal amino acids were not involved in the reaction) of plasmin were modified, the interaction of enzyme by antithrombin III was not affected (Fig. 3, inset). However, the rate of inactivation of modified plasmin in the presence of antithrombin III was less influenced by heparin than that of the control plasmin (Fig. 3). This finding supports also the pivotal role of the heparin-plasmin interaction in the inactivation of the enzyme by inhibitor.

Thermodynamic characterization of the plasmin-heparin-antithrombin III reaction

Plasmin inactivation by antithrombin III was measured in the range 4–30°C. The actual values of the rate of inactivation were slightly dependent on plasmin concentration and specific activity. They showed also batch-to-batch variation.

In the presence of heparin at low concentration (1 µg/ml) the rate of inactivation increased at any temperature and the time course still followed pseudo-first-order kinetics within the measurable range. Arrhenius plots of the plasmin-antithrombin III reaction were linear in both the absence and presence of heparin over a range of almost 26 K (Fig. 4). Furthermore, the plots are parallel to each other, with $\Delta H^\ddagger = 43 \text{ kJ} \cdot \text{mol}^{-1}$. Absolute values of activation entropy as well as free energy are somewhat arbitrary. Substituting RT/Nh for A (see under 'Methods'), we have $\Delta G^\ddagger = 8.9 \text{ kJ} \cdot \text{mol}^{-1}$, $\Delta S^\ddagger = -152 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ at 15°C for the plasmin-antithrombin III reaction. Variation of $\Delta S^\ddagger$ with heparin concentration can be reliably derived from the data. Heparin (1 µg/ml) increases activation

Table 1. *Influence of various plasmin concentrations on the heparin-enhanced plasmin-antithrombin III reaction rate* Plasmin (at the concentrations shown) and antithrombin III (300 µg of protein/ml) were incubated at 16°C in the presence of 25 mM-sodium phosphate buffer, pH 7.4, containing 25 mM-NaCl, for 0–10 min. The rate constants were determined as described in the Experimental section.

Heparin added (µg/ml)	[Plasmin] (µg/ml) ...	<i>k</i> (inactivation rate) (min ⁻¹)		
		100	150	200
None		0.07	0.055	0.050
0.5		0.25	0.115	0.10
1.0		0.57	0.30	0.24

Table 2. *Effect of NaCl on the heparin enhancement of the plasmin-antithrombin III reaction* Plasmin (75 µg of protein/ml) and antithrombin III (350 µg of protein/ml) dissolved in 25 mM-sodium phosphate buffer, pH 7.4, were incubated in the presence of different concentrations of NaCl and heparin. After incubation at 16°C for various times, portions were taken and determined for remaining plasmin activity. Pseudo-first-order rate constants were calculated as described in the Experimental section.

Heparin added (µg/ml)	[NaCl] (mM) ...	<i>k</i> (inactivation rate) (min ⁻¹)		
		25	75	125
None		0.090	0.086	0.080
0.5		0.28	0.113	
1.0		0.86	0.19	
2.0		>1.0	0.36	0.17

Fig. 3. Effect of heparin on the inactivation of phosphopyridoxylated plasmin in the presence of antithrombin III

Plasmin (125 μg of protein/ml), antithrombin III (250 μg of protein/ml) and heparin (at the concentration shown) were incubated at 16°C for 1 min in the presence of 25 mM-sodium phosphate buffer, pH 7.4, containing 25 mM-NaCl and 0.25 mM-6-aminohexanoic acid. Thereafter remaining plasmin activity was determined. In the inset, the time-dependence of plasmin inactivation by antithrombin III (without heparin) is shown. Modification of plasmin (approx. four to five lysine residues per mol of enzyme) was performed as described in the Experimental section. O, Control plasmin; Δ, modified plasmin.

entropy of the plasmin-antithrombin III reaction by $7.95 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ and results in an approx. 2.6-fold acceleration of the reaction.

Recycling of heparin in the plasmin-antithrombin III reaction

The low heparin-to-plasmin ratio in the plasmin inactivation by antithrombin III (Fig. 2) strongly suggests that heparin may be released from the plasmin-antithrombin III complex formed, this way eliciting a catalyst-like effect. This possible mode of action is analogous to the mechanism that is apparently operating in the thrombin-antithrombin-heparin system (Machovich & Arányi, 1978; Pomerantz & Owen, 1978; Machovich *et al.*, 1979). To test this interesting possibility, we wanted to check if the heparin was exhausted in the reaction or not, and therefore the following experiments were done. An incubation mixture was set up by mixing heparin, plasmin and antithrombin III, and was supplemented at regular intervals by further additions of the same amount of freshly mixed enzyme and inhibitor but no heparin. The results shown in Table 3 reveal no apparent loss of heparin activity,

Fig. 4. Arrhenius plot of the plasmin-antithrombin III reaction

Plasmin (125 μg of protein/ml) and antithrombin III (350 μg of protein/ml) were incubated in 25 mM-sodium phosphate buffer, pH 7.4, containing 50 mM-NaCl for 0–60 min at various temperatures. Thereafter, remaining plasmin activity was measured. Apparent first-order rate constants were determined from the curves. Heparin added (μg/ml): O, None; Δ, 1; ■, 2.

even after five 'turns' of complete inactivation of fresh plasmin.

Discussion

It is generally believed that heparin accelerates the inactivation of enzymes of blood coagulation by interacting with antithrombin III. Namely, heparin acts as if it were a co-inhibitor of the antithrombin III ('inhibitor-fitting' hypothesis). Several other results, however, suggest the primary importance of the heparin binding to the serine proteinase of blood coagulation ('enzyme-fitting' hypothesis). The present findings support the 'enzyme-fitting' hypothesis, i.e. the enhancing effect of heparin on the plasmin inactivation by antithrombin III correlates with the binding of heparin to the enzyme. In this respect, dependence of the heparin-enhanced plasmin inactivation on ionic strength and on the chemical modification of certain plasmin side chains are particularly relevant. In these cases, experimental conditions that could influence the enzyme-heparin interaction without affecting the interaction between heparin and antithrombin III substantially decreased the effect of the polysaccharide on the reaction between the enzyme and its inhibitor.

Minute amounts of heparin were effective in the reaction. Kinetics proved that one molecule of heparin could enhance the inactivation as much as about 100 molecules of plasmin, or perhaps many more, considering that only about one-fifth of the heterogeneous heparin preparation was active (Rosenberg *et al.*, 1978). Besides, heparin activity

Table 3. *Availability of residual heparin activity in the plasmin-antithrombin III reaction*

First sample (62.5 µg of plasmin/ml, 86 µg of antithrombin III/ml, 6.25 µg of heparin/ml, 25 mM-sodium phosphate buffer, pH 7.4, 25 µM-6-aminohexanoic acid and 87.5 mM-NaCl) incubated for 5 min at room temperature, was mixed with an equivalent volume of supplement containing the same additives as first sample but no heparin (first supplement). After 5 min incubation, a portion of the reaction mixture was mixed again with equivalent volume of a fresh supplement. Thereafter these procedures were repeated several times. During the 5 min incubation periods, 0.1 ml portions of reaction mixture were taken exactly at 3 min for determination of remaining plasmin activity as described in the Experimental section. In the control assay (second line), the first sample did not contain heparin, but to the first, second, third and fourth supplements, 6.25, 3.12, 1.56 and 0.78 µg of heparin/ml was added respectively. Values (means of the two determinations) are percentages of the enzyme activity obtained after incubation of plasmin and antithrombin III for 3 min without heparin.

and antithrombin III for 3 min without heparin.		Percentage of enzyme activity						
Additives	No. of plasmin/ antithrombin supplements	...	0	1	2	3	4	No heparin
Heparin (in recycling)			0	18	32	40	50	—
Heparin added (in dilution)			0	19	30	52	71	100

could be recovered without loss after the reaction between plasmin and antithrombin III. Activation parameters of the enzyme-inhibitor reaction were also determined. Heparin apparently influenced only activation entropy.

These facts could be visualized by assuming that heparin exerts an ordering function on the reactants. After the heparin binds plasmin, and antithrombin III formed an inactive complex, heparin could be released and become available for other plasmin and antithrombin III molecules.

We are indebted to Mrs. Theresa Fazekas and Mrs. Vera Pichler for their excellent technical assistance. This work was supported by the Hungarian Ministry of Health (grant no. ETK.3). Antithrombin III used in these studies was provided by the American Red Cross National Fractionation Center under N.I.H. (National Institutes of Health) grant HL 13881.

References

- Danishefsky, I., Ahrens, M. & Klein, S. (1977) *Biochim. Biophys. Acta* **498**, 215–222
- Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170**, 1095–1096
- Einarsson, R. & Andersson, L.-O. (1977) *Biochim. Biophys. Acta* **490**, 104–111
- Griffith, M. J. (1979a) *J. Biol. Chem.* **254**, 3401–3406
- Griffith, M. J. (1979b) *J. Biol. Chem.* **254**, 12044–12049
- Griffith, M. J., Kingdon, H. S. & Lundblad, R. L. (1979) *Biochem. Biophys. Res. Commun.* **87**, 686–692
- Hatton, M. W. C. & Regoeczi, E. (1977) *Thromb. Res.* **10**, 645–660
- Hilborn, J. C. & Anastasiadis, P. A. (1971) *Anal. Biochem.* **31**, 51–57
- Holmer, E., Söderström, G. & Andersson, L.-O. (1979) *Eur. J. Biochem.* **93**, 1–5
- Jaques, L. B., Kavanagh, L. W. & Kuo, S. H. (1973) *Thromb. Res.* **3**, 295–306
- Laurent, T. C., Tengblad, A., Thunberg, L., Höök, M. & Lindahl, U. (1978) *Biochem. J.* **175**, 691–701
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Machovich, R. (1975) *Biochim. Biophys. Acta* **412**, 13–17
- Machovich, R. & Arányi, P. (1978) *Biochem. J.* **173**, 869–875
- Machovich, R., Blaskó, Gy. & Pálos, Á. L. (1975) *Biochim. Biophys. Acta* **379**, 193–200
- Machovich, R., Borsodi, A., Blaskó, Gy. & Orakzai, A. S. (1977) *Biochem. J.* **167**, 393–398
- Machovich, R., Staub, M. & Patthy, L. (1978) *Eur. J. Biochem.* **83**, 473–477
- Machovich, R., Regoeczi, E. & Hatton, M. W. C. (1979) *Thromb. Res.* **15**, 821–834
- Machovich, R., Regoeczi, E. & Hatton, M. W. C. (1980a) *Thromb. Res.* **17**, 383–391
- Machovich, R., Regoeczi, E. & Hatton, M. W. C. (1980b) in *Chemistry and Biology of Heparin*, (Lundblad, R. L., Mann, K. G., Brown, W. & Roberts, H. R., eds.), pp. 289–296, Elsevier/North-Holland, Amsterdam
- Miller-Andersson, H., Borg, H. & Andersson, L.-O. (1974) *Thromb. Res.* **5**, 439–452
- Nordenman, B. & Björk, I. (1978) *Biochemistry* **17**, 3339–3344
- Piepkorn, M. W., Laquonoff, D. & Schmer, G. (1978) *Biochem. Biophys. Res. Commun.* **85**, 851–856
- Pomerantz, M. W. & Owen, W. G. (1978) *Biochim. Biophys. Acta* **535**, 66–77
- Remmert, L. F. & Cohen, P. P. (1974) *J. Biol. Chem.* **181**, 431–448
- Rosenberg, R. D. & Damus, P. S. (1973) *J. Biol. Chem.* **248**, 6490–6505.
- Rosenberg, R. D., Armand, G. & Lam, L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3065–3069
- Smith, G. F. (1977) *Biochem. Biophys. Res. Commun.* **77**, 111–117
- Smith, G. F. & Sundboom, J. L. (1980) in *The Chemistry and Biology of Heparin* (Lundblad, R. L., Mann, K. G., Brown, W. & Roberts, H. R., eds.), pp. 287–288, Elsevier/North-Holland, Amsterdam
- Sodetz, I. M., Brockway, W. J. & Castellino, F. J. (1972) *Biochemistry* **11**, 4451–4458
- Stürzebecher, I. & Markwardt, F. (1977) *Thromb. Res.* **11**, 835–846
- Tanford, C. (1970) *Adv. Protein Chem.* **24**, 1–95.
- Villanueva, G. B. & Danishefsky, I. (1979) *Biochemistry* **18**, 810–817
- Wickerhauser, M., Williams, C. & Mercer, J. (1979) *Vox Sang.* **36**, 281–293