The Heparin-Protein Complex of Ox Liver Capsule

ISOLATION AND CHEMICAL CHARACTERIZATION

By A. SERAFINI-FRACASSINI, J. J. DURWARD AND L. FLOREANI

Department of Biochemistry, University of St Andrews, St Andrews, Fife

(Received 18 September 1968)

1. A procedure for the isolation and purification of the heparin-protein complex from ox liver capsule, based on the solubility properties of mucopolysaccharide-cetylpyridinium complexes, is described. 2. The yield of the heparin-protein complex with this method averages 35 mg./100 g. of dry ox liver capsule. 3. The results of analyses on the polysaccharide show good agreement with values previously published for purified heparin fractions. 4. The amino acid analysis of the protein component shows several similarities to that of chondromucoprotein. 5. The results of β -carbonyl elimination, either with or without catalytic hydrogenation, and column chromatography after β -elimination, show that the polysaccharide is covalently bound to the protein.

Although the glycosaminoglycan-protein complex containing chondroitin 4-sulphate has been extensively characterized (Shatton & Schubert, 1954; Mathews & Lozaityte, 1958) and other complexes having chondroitin 6-sulphate (Buddecke & Schubert, 1961; Mathews, 1962) and hyaluronic acid (Ogston & Sherman, 1958; Pigman, Rizvi & Holley, 1961; Sandson & Hamerman, 1962) as the polysaccharide moieties have been studied, the situation with regard to native heparin-protein complexes has yet to be clarified.

Green, Day & Roberts (1961) reported the presence of ninhydrin-sensitive spots in paper chromatograms of heparin hydrolysates and postulated the presence of amino acids covalently bound to the carbohydrate. These results were confirmed by Lindahl, Cifonelli, Lindahl & Rodén (1965), who demonstrated the presence of peptides bound to heparin in preparations isolated by using either proteolytic enzymes or alkali. Lindahl & Rodén (1965) also proved that the two moieties are covalently bound through a xylosylserine linkage and suggested the existence of a native heparin-protein complex. Korn (1959), however, found no evidence for such covalent complexes in his study of heparin isolated from mast-cell tumours.

Serafini-Fracassini & Durward (1968) outlined a method of extraction of the heparin-protein complex from ox liver capsule, and a preliminary chemical characterization of the complex was given.

The present paper reports a detailed chemical analysis together with the results of β -carbonyl elimination.

MATERIALS AND METHODS

Isolation and purification of the heparin-protein complex of ox liver capsule. The capsule, stripped free from liver tissue, was defatted in acetone, dried and powdered. Portions (20 g.) of this material were each extracted in a blender with 400 ml. of a 1% (w/v) solution of cetylpyridinium chloride in 2M-KCl at 50° for 1 hr. After centrifugation at 38000 g_{av} . for 1 hr. at 40°, the clear supernatant was diluted to give a final concentration of 0.9M-KCl. The fine precipitate that gradually formed at room temperature during 4 hr. was collected by filtration on a cellulose filter pad, the filtrate being discarded. The pad was eluted with 100 ml. of 2M-KCl at 40°, and the cetylpyridinium chloride dissociated from the complex and removed by shaking with 3 vol. of chloroform-pentan-1-ol (5:4, v/v) (Scott, 1960) at room temperature. The aqueous phase, when cetylpyridinium chloride had been extensively eliminated, as verified by the disappearance of its characteristic E_{280} , was dialysed against glass-distilled water for 48 hr. at 4°. After stepwise addition of 4 vol. of ethanol at room temperature, the solution was centrifuged at 38000 g_{av} . for 1 hr. Then 1 vol. of a saturated solution of potassium acetate in ethanol was slowly added to the supernatant. The white precipitate that then formed was collected by centrifugation, washed in acetone and dried. The material was redissolved in water and the ethanol fractionation repeated twice. The final dry powder was stored in a desiccator over P_2O_5 until required for analysis.

Bioassay. Blood anticoagulant activity was determined by the protamine titration method of Jaques (1943) as modified by Sharp, Excell, Salzman & Thorup (1961).

Chemical assays. Ash and moisture were determined as described by Eastoe & Courts (1963).

Samples of the preparation were hydrolysed for amino acid analysis in constant-boiling HCl (2 ml./mg. of material) under N_2 in sealed tubes at 110° for 24, 36 and 72 hr. Excess of acid was removed from the hydrolysates in a rotary film evaporator at 30°. Owing to the presence of cyst(e)ine,

separate portions of the material were oxidized with performic acid (Schram, Moore & Bigwood, 1954) before hydrolysis. The amino acid analyses were carried out in triplicate with a Technicon AutoAnalyzer. The reproducibility of the results was about $\pm 1.5\%$. Superimposition of valine and glucosamine peaks was prevented by using an initial buffer of pH 2.75 and by replacing 10 and 7 ml. of buffer in the first and second chambers of the Autograd with methanol.

The hydrolysis for hexosamine analysis was carried out in 4M-HCl (2 ml./mg. of material) at 100° for 10 hr. (Wolfson, Weisblat, Karabinos, McNeely & McLean, 1943) under N₂ in sealed tubes. Excess of acid was neutralized and total hexosamine was determined by the method of Cessi & Piliago (1960). The concentrations of glucosamine and galactosamine were determined after chromatographic separation (Partridge & Elsdon, 1961).

Quantitative determination of uronic acid was performed by the method of Bitter & Muir (1962).

Xylose was determined by the anthrone method of Tsiganos & Muir (1966).

The sulphur content was determined by the method of Giellman & Tölg (1960).

Identification of neutral sugars. Samples of the complex were hydrolysed in 2M-HCl (2 ml./mg. of material) at 100° for 8 hr. The identity of the neutral sugars present in the hydrolysate was determined by thin-layer chromatography on kieselguhr G, by the method of Stahl & Kaltenbach (1961), with a Desaga S-Chamber (Desaga Co., Heidelberg, Germany).

Alkali-induced β -elimination. A 40 mg. sample of the material was dissolved in 10 ml. of 0.5M-NaOH and stirred under N₂ for 19 hr. at room temperature. A 5 ml. portion of this solution was then acidified to give a final concentration of 6M-HCl and hydrolysed for 24 hr. in sealed tubes, under N₂, at 110° before amino acid analysis. The remainder was neutralized and used for chromatography on AE-cellulose* (see below).

A separate 20 mg. sample was hydrogenated by the method of Anderson, Hoffman & Meyer (1965) with 20 mg. of Adams catalyst (Adams, Voorhees & Shiner, 1941) in 5 ml. of 0.5M-NaOH for 19 hr. at room temperature. The catalyst was removed by filtration and washed several times with small amounts of conc. HCl. The washings were added to the initial filtrate and, after adjustment of the HCl concentration to 6M, 30 ml. of constant-boiling HCl was added. The hydrolysis for amino acid analysis was carried out for 24 hr. under reflux. The presence of α -aminobutyric acid in the above hydrolysate was detected by two-dimensional thin-layer chromatography on silica gel G by the method of Fahmy, Niederwieser, Pataki & Brenner (1961) with a Desaga S-Chamber.

Chromatography on AE-cellulose. The adsorbent, 1m-equiv. of cationic groups per g. (Whatman; W. and R. Balston Ltd., Maidstone, Kent), after repeated washings with 0.5M-HCl and 0.5M-NaOH, was suspended in 0.1M-NaCl, degassed and poured into a 1 cm.-diameter glass column to a height of 30 cm. Water was passed through until a chloride-free effluent was obtained. The flow rate was adjusted to 12 ml./hr. After application of the sample, the column was washed with water for 1 hr. Stepwise elution was carried out with 50 ml. volumes of different NaCl

concentrations in 0.01M-HCl; 0.25M increments in NaCl concentration were used. Fractions (2 ml.) were collected and tested for polysaccharide content by using a 1% (w/v) solution of cetylpyridinium chloride (Scott, 1960). Chondroitin sulphate (lot no. 15B-0010; Sigma Chemical Co., St Louis, Mo., U.S.A.) and heparin (lot no. 136651; Wilson Laboratories, Chicago, Ill., U.S.A.) were used as standards for the determination of the relative retention volumes.

RESULTS

When the material obtained from the first ethanol fractionation was resuspended in water, 40% remained insoluble after stirring for 12 hr. The insoluble fraction was found to have a sulphur content of approx. 0.25%. The soluble fraction, however, once precipitated and dried, gave a preparation that was readily soluble. Its yield averaged 35 mg. of protein-polysaccharide/100 g. of dry capsule. This is the fraction that was subjected to biological and chemical characterization.

The anticoagulant activity of this material averaged 55 B.P. units/mg.

The results of amino acid analysis are reported in Table 1, column (A), where the concentrations are expressed as μ g. of anhydro amino acid/10 mg. of dry and ash-free material. The corrected concentrations of the various amino acids were calculated by either extrapolating to zero hydrolysis time those values showing linear kinetics of hydrolytic destruction, or by selecting the greatest value for those showing increased concentration during hydrolysis. In Table 1, column (B), the corrected amino acid composition of the protein component is reported, whereas column (C) shows the percentage zero-time recovery for each amino acid after 24 hr. hydrolysis. A protein content of 13.2% was derived by summation of the corrected values.

Thin-layer chromatography for neutral sugars revealed that only xylose and galactose were present in the hydrolysate. The quantitative determination of xylose showed that more than one chromogen was formed during the reaction with anthrone. The main one had an absorption maximum at 615 m μ , but at least another two were present, with absorption maxima at 560 and 505 m μ respectively.

The results of all quantitative analyses on the complex are summarized in Table 2.

The amino acid compositions of the two samples subjected to alkaline treatment either with or without catalytic hydrogenation are shown in Table 3, where they are compared with the corresponding hydrolysate of the untreated material.

Owing to the superimposition of glucosamine and α -aminobutyric acid peaks, precise identification of the latter in the hydrolysate of the material sub-

* Abbreviation: AE-cellulose, aminoethylcellulose.

Table 1. *Amino acid analysis of heparin-protein complex*

(A) Wt. of anhydro amino acid ($\mu\text{g./10 mg.}$ of heparin-protein complex, ash- and moisture-free); (B) wt. of anhydro amino acid (g./100 g. of the protein component); (C) recovery after 24 hr. hydrolysis (%).

Time of hydrolysis (hr.) Amino acid	(A)				(B)	(C)
	24	36	72	Corrected values	Corrected values	
Cysteic acid	62.3	60.4	67.3	63.3*	4.79	98.3
Hydroxyproline	1.5	Trace	Trace	1.5†	0.11	100.0
Aspartic acid	128.8	128.6	128.6	128.8†	9.75	100.0
Threonine	64.9	61.8	51.6	71.7‡	5.43	90.5
Serine	53.9	49.0	33.5	64.3‡	4.87	83.8
Glutamic acid	183.5	190.3	191.2	191.2†	14.48	96.0
Proline	80.9	76.5	75.9	89.7§	6.79	90.2
Glycine	83.7	85.8	88.3	88.3†	6.69	94.8
Alanine	73.9	75.2	80.0	80.0†	6.06	92.4
Valine	66.6	68.6	68.8	68.8†	5.21	96.8
Methionine	0.0	0.0	0.0	0.0	0.00	
Isoleucine	40.6	42.2	42.7	42.7†	3.23	95.1
Leucine	105.7	109.5	109.3	109.5†	8.29	96.5
Tyrosine	6.3	4.2	4.6	10.5§	0.79	60.0
Phenylalanine	69.5	70.7	68.6	70.7†	5.35	98.4
Hydroxylysine	0.0	0.0	0.0	0.0	0.00	
Ornithine	5.1	5.1	6.2			
Lysine	111.0	112.2	112.2	112.2†	8.49	98.9
Histidine	31.2	31.3	28.7	31.3†	2.37	99.7
Arginine	90.3	89.9	82.0		7.29	
Arginine + ornithine	(95.4)	(95.0)	(88.2)	96.3§		99.1
Totals	1259.7	1261.3	1239.5	1320.8	99.99	

* Average value (24 hr., 36 hr. and 72 hr. hydrolysates).

† Greatest value.

‡ Extrapolation to zero time (24 hr., 36 hr. and 72 hr. hydrolysates).

§ Extrapolation to zero time (24 hr. and 36 hr. hydrolysates).

Table 2. *Composition of heparin-protein complex*

Values are expressed as percentages of dry ash-free material.

Protein	13.21
Total hexosamine (as free base)	14.82
Glucosamine	14.28
Galactosamine	0.54
Hexuronic acid (as free acid)	27.83
Hexuronic acid (as potassium salt)	33.39
Xylose	0.68
Sulphur (as S)	8.90
Sulphur (as ester sulphate, potassium salt)	33.09
Total	95.19

jected to β -elimination and hydrogenation proved unfeasible by automatic amino acid analysis, although the marked asymmetry of the glucosamine peak implied its presence. This, however, was conclusively demonstrated by thin-layer chromatography carried out on the hydrolysate.

Column chromatography of commercial heparin and chondroitin sulphate was performed on AE-cellulose as described in the Materials and Methods section. Chondroitin sulphate and heparin were eluted by 1.0M- and 1.5M-sodium chloride respectively. In this system, concentrations of sodium chloride in excess of 3M failed to displace any fraction of the untreated complex. After β -elimination, however, the polysaccharide was eluted by 1.5M-sodium chloride.

DISCUSSION

Many authors have used the term 'heparin-protein complex' to describe various associations of heparin with proteins, including protein contaminants present in their heparin-isolation media. In this paper this terminology refers to a macromolecular complex of heparin with protein through covalent linkage, in keeping with the model described by Lindahl & Rodén (1965).

Table 3. *Amino acid analysis of heparin-protein complex after β -carbonyl elimination*

Values are reported as wt. of anhydro amino acid ($\mu\text{g./10 mg. of dry ash-free protein-polysaccharide complex}$). All hydrolyses were carried out for 24 hr. (A) Untreated sample; (B) sample after β -elimination; (C) sample after β -elimination and reduction in the presence of Adams catalyst. N.D., Not determined.

Amino acid	(A)	(B)	(C)
Cysteic acid	62.3	N.D.	N.D.
Hydroxyproline	1.5	0.0	0.0
Aspartic acid	128.8	120.4	89.3
Threonine	64.9	52.5	43.0
Serine	53.9	52.6	50.8
Glutamic acid	183.5	182.7	148.2
Proline	80.9	80.5	62.3
Glycine	83.7	83.8	72.9
Alanine	73.9	76.1	57.0
Valine	66.6	64.3	47.1
Methionine	0.0	0.0	0.0
Isoleucine	40.6	37.7	29.1
Leucine	105.7	99.9	72.4
Tyrosine	6.3	32.7	35.2
Phenylalanine	69.5	65.6	45.1
Hydroxylysine	0.0	0.0	0.0
Ornithine	5.1	9.0	15.3
Lysine	111.0	105.5	72.1
Histidine	31.2	26.6	24.9
Arginine	90.3	86.7	57.3
Arginine + ornithine	(95.4)	(95.7)	(72.6)

Perhaps the most significant previous attempt to isolate the native heparin-protein complex is that of Julén, Snellman & Sylvén (1950). In the supernatant of a phosphate-buffer extract of ox liver capsule, after centrifugation at 60000g, protein and lipid, in addition to heparin, were detected. From these findings Julén *et al.* (1950) concluded that the native heparin-protein complex exists in a lipoprotein form. However, the protein component was dissociated from the complex and precipitated by saturation with phosphate ions (Snellman, Sylvén & Julén, 1951), indicating the absence of covalent linkage. We therefore consider, in agreement with Korn (1959), that this complex is a methodological artifact, due to the established affinity of heparin for lipoproteins (Bernfeld, Donahue & Berkowitz, 1957).

The isolation procedure described in this paper has the advantage, over those currently employed for the extraction of heparin, that it does not involve the use of either alkali or enzymes causing degradation or modification of both protein and polysaccharide components. There is a possibility, however, that during the initial manipulation of the capsule material some degradation of the heparin-protein complex could occur, due to the presence of

trypsin- and chymotrypsin-like proteases that are known to be present in mast cells (Benditt & Arase, 1959; Lagunoff & Benditt, 1963). But, since trypsin is known to be inhibited by low concentrations of soaps (Peck, 1942), and the stability of both enzymes is temperature-dependent, especially at neutral pH (Anson & Mirsky, 1934; Eisenberg & Schwert, 1951), it is considered that the combination of a high concentration of detergent and potassium chloride, at a temperature of 50°, which is used in the extraction procedure, would prevent hydrolytic damage of the complex.

It is known that heparin has the property of binding to collagen (Serafini-Fracassini, 1967), lipids (Bernfeld *et al.* 1957) and other materials (Korn, 1959). In this study this was prevented by the use of cetylpyridinium chloride, which effectively blocks the sulphate groups on the polysaccharide without decreasing its solubility, if in the presence of 2M-potassium chloride (Scott, 1960). Moreover, cetylpyridinium chloride is a well-known deproteinizing agent and allows fractionation of heparin, from other mucopolysaccharides and DNA present in the extraction medium, by stepwise dilution of the initial supernatant. The dissociation of the cetylpyridinium chloride-heparin complex involves the use of organic solvents, which would remove any residual contamination (Blix & Snellman, 1945).

Serafini-Fracassini & Durward (1968) reported that the material isolated behaves, during ultracentrifugal analysis, as a single peak characterized by a sedimentation coefficient of 1.85s at a concentration of 0.95% (w/v). It is considered that the ultracentrifugation pattern alone does not exclude the possibility of contamination by either non-covalently bound protein or glycoprotein-like materials. However, the preparation examined, once freed from cetylpyridinium chloride, behaves as a single compound during ethanol fractionation and requires high concentrations of both ethanol and potassium acetate before precipitation. It is considered that in such an environment it is highly unlikely that any protein or peptide non-covalently bound to heparin would not be removed. Further, the results of chromatography for neutral sugars show that only xylose and galactose are present, in conformity with the accepted structure of the linkage region of heparin to protein (Lindahl & Rodén, 1965).

In view of the overall similarity of the protein moieties of the complexes of heparin and chondroitin 4-sulphate (Serafini-Fracassini, Peters & Floreani, 1967), a comparison of their amino acid composition is of interest. In both cases, proline, glycine and glutamic acid are the most frequent residues. On the other hand, the serine concentration is decreased in the heparin-protein complex

and becomes equimolar with those of threonine and valine. Many of the other amino acids show slight variations between the two complexes, the major differences being the exceptionally low tyrosine content and the appreciable cysteine or cystine content of the heparin-protein complex. The presence of the latter amino acids suggests that the a protein moiety of the complex could play some structural role in the organization of the mast-cell granules.

The recovered polysaccharide constitutes approx. 82% of the complex and contains 18.1% hexosamine, 33.9% hexuronic acid and 10.9% sulphur. These values are in close agreement with the analyses of purified heparin fractions reported by Lindahl *et al.* (1965). It has been generally accepted that heparin gives high colour yields in the carbazole reaction, but Perlin, Mazurek, Jaques & Kavanagh (1968) suggested, from the results of proton-magnetic-resonance studies on commercial heparin preparations, that the polysaccharide may be composed of three sugar moieties, namely L-iduronic acid, glucuronic acid and glucosamine in approximately equimolar proportions. If this is the case, the values reported in Table 2 for hexuronic acid would be absolute and the sum of all analytical values for the complex would be correct. The high sulphur content of our preparation indicates that the polysaccharide undergoes very little, if any, modification during the extraction procedure.

A further proof of the covalent nature of the complex was obtained by demonstrating that during β -carbonyl elimination the glycosaminoglycan is cleaved from the protein moiety, as shown by the results of chromatography on AE-cellulose. Moreover, a comparison of the amino acid analyses reported in Table 3, columns (A) and (B), reveals that, during β -carbonyl elimination with 0.5M-sodium hydroxide alone, only the threonine content is significantly diminished (by 19.1%). When the material is subjected to β -elimination and hydrogenation in the presence of Adams catalyst (Table 3, column C), many other component amino acids are destroyed to an appreciable extent. In view of the chemical similarity of threonine and serine on the one hand, and glycine, alanine, leucine, isoleucine and valine on the other, it would be expected that each of these two groups, under identical conditions, would be degraded in similar proportions. However, in fact, the threonine content is more drastically decreased (33.8%) than that of serine (5.7%), and the destruction of glycine is only 12.9%, whereas that of alanine, leucine, isoleucine and valine averages 28.0%. Threonine therefore suffers a great deal more destruction than would be expected from catalytic hydrogenation alone, whereas glycine shows a relative increase in proportion to its related compounds. Further,

α -aminobutyric acid was detected in the hydrolysate of the treated material. These results suggest the conversion of threonine into glycine and α -aminobutyric acid, and such a conversion would be in keeping with the β -elimination mechanism for threonine involved in an O-glycosidic linkage (Adams, 1965; Seno, Meyer, Anderson & Hoffman, 1965). Lindahl & Rodén (1965), from their study of commercial heparin preparations isolated mainly from pig tissues by using either alkali or proteolytic enzymes, concluded that serine is involved in the heparin proteoglycan linkage. Although our results appear to be completely at variance with these earlier findings, it is important to point out that the complex examined in this paper was isolated, by a different procedure, from a different tissue and animal species.

The anticoagulant activity of the complex is appreciably lower than that of commercial heparin preparations of similar sulphur content. However, it is recognized that other molecular features such as size and shape of the polysaccharide influence its biological properties (Jensen, Snellman & Sylvén, 1948; Laurent, 1961; Stivala & Liberti, 1967). Since the heparin chains, once arranged in a macromolecular complex with protein, presumably change their conformation, it is impossible, at the present time, to correlate the chemical composition of the heparin-protein complex and its anti-coagulant activity.

We thank Dr A. S. Todd and Mr R. Henderson, Department of Haematology, Dundee Royal Infirmary, for anti-coagulant activity determinations. We are grateful to the Scottish Home and Health Department for financial support. J. J. D. is in receipt of a Science Research Council grant.

REFERENCES

- Adams, J. B. (1965). *Biochem. J.* **94**, 368.
Adams, R., Voorhees, V. & Shiner, R. L. (1941). In *Organic Syntheses*, collective vol. 1, p. 463. Ed. by Gilman, H. New York: John Wiley and Sons Inc.
Anderson, B., Hoffman, P. & Meyer, K. (1965). *J. biol. Chem.* **240**, 156.
Anson, M. L. & Mirsky, A. E. (1934). *J. gen. Physiol.* **17**, 393.
Benditt, E. P. & Arase, M. (1959). *J. exp. Med.* **110**, 451.
Bernfeld, P., Donahue, V. M. & Berkowitz, M. E. (1957). *J. biol. Chem.* **226**, 51.
Bitter, T. & Muir, H. (1962). *Analyt. Biochem.* **4**, 330.
Blix, G. & Snellman, O. (1945). *Ark. Kemi.* **19A**, no. 32.
Buddecke, E. & Schubert, M. (1961). *Hoppe-Seyl. Z.* **325**, 189.
Cessi, C. & Piliego, F. (1960). *Biochem. J.* **77**, 508.
Eastoe, J. E. & Courts, A. (1963). *Practical Analytical Methods for Connective Tissue Proteins*, p. 32. London: E. and F. N. Spon Ltd.
Eisenberg, M. A. & Schwert, G. W. (1951). *J. gen. Physiol.* **34**, 583.

- Fahmy, A. R., Niederwieser, A., Pataki, G. & Brenner, M. (1961). *Helv. chim. Acta*, **44**, 2022.
- Giellman, W. & Tölg, G. (1960). *Glastech. Ber.* **33**, 332.
- Green, J. P., Day, M. & Roberts, M. (1961). *J. Pharmacol.* **132**, 58.
- Jaques, L. B. (1943). *Biochem. J.* **37**, 189.
- Jensen, R., Snellman, O. & Sylvén, B. (1948). *J. biol. Chem.* **174**, 265.
- Julén, C., Snellman, O. & Sylvén, B. (1950). *Acta physiol. scand.* **19**, 289.
- Korn, E. D. (1959). *J. biol. Chem.* **234**, 1325.
- Lagunoff, D. & Benditt, E. P. (1963). *Ann. N.Y. Acad. Sci.* **103**, 185.
- Laurent, R. L. (1961). *Arch. Biochem. Biophys.* **92**, 224.
- Lindahl, U., Cifonelli, J. A., Lindahl, B. & Rodén, L. (1965). *J. biol. Chem.* **240**, 2817.
- Lindahl, U. & Rodén, L. (1965). *J. biol. Chem.* **240**, 2821.
- Mathews, M. B. (1962). *Biochim. biophys. Acta*, **58**, 92.
- Mathews, M. B. & Lozaityte, I. (1958). *Arch. Biochem. Biophys.* **74**, 158.
- Ogston, A. G. & Sherman, T. F. (1958). *Nature, Lond.*, **181**, 482.
- Partridge, S. M. & Elsdon, D. F. (1961). *Biochem. J.* **80**, 34 p.
- Peck, R. L. (1942). *J. Amer. chem. Soc.* **64**, 487.
- Perlin, A. S., Mazurek, M., Jaques, L. B. & Kavanagh, L. W. (1968). *Carbohydr. Res.* **7**, 369.
- Pigman, W., Rizvi, S. & Holley, H. (1961). *Biochim. biophys. Acta*, **53**, 254.
- Sandson, J. & Hamerman, D. (1962). *J. clin. Invest.* **41**, 1817.
- Schram, E., Moore, S. & Bigwood, E. J. (1954). *Biochem. J.* **57**, 33.
- Scott, J. E. (1960). *Meth. biochem. Anal.* **8**, 145.
- Seno, N., Meyer, K., Anderson, B. & Hoffman, P. (1965). *J. biol. Chem.* **240**, 1005.
- Serafini-Fracassini, A. (1967). *Biochem. J.* **104**, 13 p.
- Serafini-Fracassini, A. & Durward, J. J. (1968). *Biochem. J.* **109**, 693.
- Serafini-Fracassini, A., Peters, T. J. & Floreani, L. (1967). *Biochem. J.* **105**, 569.
- Sharp, A. A., Excell, B., Salzman, E. & Thorup, O. (1961). In *Thrombosis and Anticoagulant Therapy*, p. 88. Ed. by Walker, W. Edinburgh: E. and S. Livingstone Ltd.
- Shatton, J. & Schubert, M. (1954). *J. biol. Chem.* **211**, 565.
- Snellman, O., Sylvén, B. & Julén, C. (1951). *Biochim. biophys. Acta*, **7**, 98.
- Stahl, E. & Kaltenbach, U. (1961). *J. Chromat.* **5**, 351.
- Stivala, S. S. & Liberti, P. A. (1967). *Arch. Biochem. Biophys.* **122**, 40.
- Tsiganos, C. P. & Muir, H. (1966). *Analyt. Biochem.* **17**, 495.
- Wolf from, M. L., Weisblat, D. I., Karabinos, J. V., McNeely, W. H. & McLean, J. (1943). *J. Amer. chem. Soc.* **65**, 2077.