Uronic Acid Composition of Heparins and Heparan Sulfates†

R. L. Taylor,‡ J. E. Shively, H. E. Conrad,* and J. A. Cifonelli

ABSTRACT: The L-iduronic and D-glucuronic acid contents of a variety of heparin and heparan sulfate samples have been determined by measuring the amounts of ^3H recovered in glucose, idose, and idosan when polymers are carboxyl reduced with sodium [^3H]borohydride. The percentage of the total uronic acid represented by L-iduronic acid varied from ~50 to 90% in heparins and from 30 to 55% in heparan sulfates. Ratios of *N*- and *O*-sulfate in these polymers generally increased with increasing L-iduronic acid content. The *N*-sulfate:D-glucosamine ratios varied from 0.7 to 1.0 for heparins and from 0.3 to 0.6 for heparan sulfates. The *O*-sulfate:D-glucosamine ratios ranged from 0.9 to 1.5 for heparins and 0.2 to 0.8 for heparan sulfates.

Following the initial demonstration of the presence of D-glucuronic acid in heparin (Wolfrom and Rice, 1946) and heparan sulfate (Brown, 1957), Brown *et al.* (1961) presented evidence that heparin contained, in addition to D-glucuronic acid, another type of uronic acid, thought to be a ketouronic acid. This heparin component was identified as L-iduronic acid by Cifonelli and Dorfman (1962) who showed that it was present in heparan sulfate and mactins as well. Wolfrom *et al.* (1969a) have isolated L-iduronic acid from heparin as its crystalline brucinium salt, and Perlin *et al.* (1970) have established by nuclear magnetic resonance (nmr) techniques that L-iduronic acid is a major fraction of the total uronic acid in heparin. Heparins, which show anticoagulant or lipoprotein lipase activation activities, are highly N- and O-sulfated, while heparan sulfates, which are less highly sulfated, are diminished in these activities (Grossman *et al.*, 1971). Periodate oxidation studies have shown that the L-iduronic acid, but not the D-glucuronic acid, residues of heparin are O-sulfated at C-2 (Wolfrom *et al.*, 1969b; Lindahl and Axelsson, 1971). Consequently, the L-iduronic acid residues carry a significant fraction of the total *O*-sulfate in heparin. The possibility exists, therefore, that the decreased sulfation (and biological activity) in heparan sulfates might be accompanied by a corresponding decrease in L-iduronic acid contents. This paper reports the analyses of the L-iduronic acid contents of a series of previously described heparin and heparan sulfate preparations and correlates these data with those for other variable constituents of this group of polysaccharides.

Experimental Procedures

Methods. Beef lung heparin and heparin by-products were obtained from Dr. L. L. Coleman, The Upjohn Co. Similar preparations from hog mucosa and heparin from beef mucosa were provided by Dr. H. H. R. Weber, The Wilson Laboratories. Purified heparin fractions described previously (Cifonelli and King, 1970a) include those from beef lung (BLH I), bovine mucosa (BHM I), and whale tissue (WH I). Hog mucosa heparins include those obtained from Sigma (HMH I) and from heparin or heparin by-products after fractionation with cetylpyridinium chloride or on Dowex 1 (chloride) columns (HMH II, BLH II, and BLH III, Rodén *et al.*, 1972). Mactin (M I) was isolated from clam tissues and purified with cetypyridinium chloride (Cifonelli and Mathews, 1972a). Heparan sulfate fractions were isolated from beef lung by-products (HSB I-III) and from hog mucosa by-products (HSH I-III) by fractionation on Dowex 1 columns (Rodén *et al.*, 1972). Umbilical cord heparan sulfate (HSU I) was reported earlier (Cifonelli and King, 1970b). The heparan sulfate degradation fraction (HSD I) was produced by degradation of hog mucosa heparan sulfate (HSH I) with nitrites and isolation of nonreacting N-acetylated glucosamine sections of estimated molecular size 3–4 $\times 10^3$ by gel filtration on Sephadex G-25 as described previously (Cifonelli, 1968). A disulfated disaccharide fraction (D I), composed of uronic acid and 2,5-anhydromannose units, was obtained after reaction of hog mucosa heparin with alkyl nitrites and fractionation of the products on Dowex 1 and Sephadex G-25 as described previously (Cifonelli and King, 1972).

Analytical procedures for estimating uronic acid, hexosamine, total sulfate, *N*-sulfate, neutral sugars, and amino acids have been described previously (Lindahl *et al.*, 1965). Anticoagulant assays were performed by Dr. L. W. Van Ness, the Wilson Laboratories. Molecular weights were approximated by measuring elution volumes obtained by gel filtration of samples on a Sephadex G-75 column (0.85 $\times$ 185 cm) and comparing the values with a molecular weight diagram given by heparin and heparan sulfate fractions of known sizes, ranging from ~4.5 to 11 $\times 10^3$, as described by Constantopoulos *et al.* (1969) and Wasteson (1969). The molecular weights of HSU I and M I were estimated from η measurements.

Quantitation of D-*Glucuronic and* L-*Iduronic Acid Contents.* The reaction sequence used for the quantitation of uronic acids is based upon a recently described procedure for quantitative carboxyl reduction and stoichiometric depolymerization of glycosaminoglycuronans (Taylor and Conrad, 1972) and is illustrated in Figure 1. In this procedure the polymers (I) are allowed to react with 1-ethyl-3-(dimethylaminopropyl)-carbodiimide and the carboxyl-activated polymers are reduced with sodium [^3H]borohydride (reaction 1). The reduction

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801 (R. L. T., J. E. S., and H. E. C.), and the Departments of Pediatrics and Biochemistry, The LaRabida-University of Chicago Institute, and The Joseph P. Kennedy, Jr., Mental Retardation Center, University of Chicago, Chicago, Illinois 60637 (J. A. C.). Supported by grants from the U. S. Public Health Service, National Institute of Allergy and Infectious Diseases (AI 09551), National Institute for Arthritis and Metabolic Diseases (AM 05996), and National Heart Institute (HE-12927).

‡ Present address: University of Chicago Medical School, Chicago, Ill. 60615.

product, which is labeled with two tritiums per reduced carboxyl group, is then stoichiometrically depolymerized by acid hydrolysis (reaction 2) followed by deaminative cleavage of the remaining glucosaminides with nitrous acid (reaction 3) as described earlier. The monosaccharides thus formed are reduced with unlabeled sodium borohydride (reaction 4) and quantitated by radiochromatography (Conrad *et al.*, 1973). This reaction sequence yields a mixture of L-[^{3}H]idosan (II) and L-[^{3}H]iditol (III) from L-iduronic acid (Perlin and Sanderson, 1970), D-[^{3}H]glucitol (V) from D-glucuronic acid, and unlabeled anhydromannitol (IV). The total ^{3}H counts appearing in these three peaks is equivalent to the total uronic acid; the ^{3}H counts in the L-iditol and L-idosan peaks are summed as a measure of the total equivalents of L-iduronic acid. Quantitative recoveries of the monosaccharides from the starting polysaccharide are obtained on the radiochromatograms (Taylor and Conrad, 1972).

A typical reaction sequence is carried out as follows. A mucopolysaccharide sample (7 mg) is allowed to react at room temperature with 19.2 mg of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide in 1 ml of water at pH 4.75 for 1 hr. An aliquot (125 μl) of the reaction mixture is added to 250 μl of 3 M sodium [^{3}H]borohydride (14.3 mCi/mmol, New England Nuclear Corporation) and the mixture is heated at 50° for 2 hr). The sample is then cooled and excess borohydride is destroyed by acidification with 3 N sulfuric acid. Salts are removed by dialysis and the dialyzed sample is transferred to a test tube and evaporated to dryness in a stream of air at 50°. The [^{3}H]carboxyl-reduced product is taken up in 50 μl of water, 25 μl each of [^{14}C]glucose solution (20,000 cpm/μl, 200 mCi/mmol) and 4 N sulfuric acid is added, and the sample is hydrolyzed at 100° for 6 hr. An aliquot of the hydrolysate is deaminated by addition of sodium nitrite, aldehyde reduced with unlabeled sodium borohydride, and analyzed by radiochromatography (Shively and Conrad, 1970).

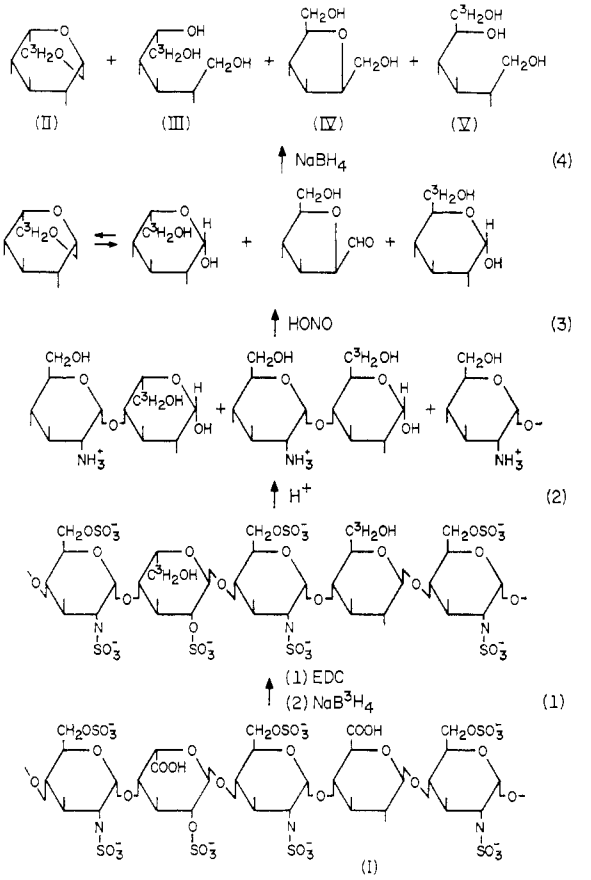

FIGURE 1: The reaction sequence used in uronic acid analyses of heparins and heparan sulfates.

Results and Discussion

Table I presents the analytical data for those constituents of heparin and heparan sulfate which are relatively invariant for all fractions. The compositions of these preparations are in agreement with those generally reported for heparin and heparan sulfate fractions. For all samples the ratios of uronic acid to hexosamine are very similar regardless of source. The colorimetric methods used to obtain the percentages of uronic and hexosamine yield molar ratios of uronic acid to hexosamine considerably in excess of 1.0 in spite of a large accumulation of data which has led to the belief that the basic heparin structure is best represented by the repeating disaccharide, hexuronosylglucosamine (Jeanloz, 1970). Such a structure should give a hexuronic acid:glucosamine ratio of 1.0, after correction for the D-glucuronic acid residue of the linkage region (see below). As shown in Table I, ratios of hexuronic acid:hexosamine ranging from 1.2 to 1.5 are obtained in the radiochromatographic analyses of this series of heparins and heparan sulfates. Values of hexuronic acid in excess of hexosamine have also been reported by Perlin *et al.* (1970) on the basis of the proton magnetic resonance spectrum of heparin. It may be noted that our previous application of these radiochromatographic procedures in the analysis of mucopolysaccharides (Taylor and Conrad, 1972) yielded the expected hexuronic acid:hexosamine ratios of 1.0 for both hyaluronic acid and chondroitin sulfate. In these earlier studies it was also shown that quantitative recoveries of monosaccharides were obtained from a measured weight of mucopolysaccharide in the sequence of reactions shown in Figure 1.

The very low levels of D-galactosamine in these preparations indicate lack of significant contamination with dermatan sulfate, commonly found in varying concentrations in less purified preparations. In the mactin fraction, however, approximately 15% of the hexosamine was found to be D-galactosamine. This type of preparation was found to have a backbone similar to beef lung heparin, with 98–99% of its glucosamine residues substituted with *N*-sulfate groups (Cifonelli and Mathews, 1972b). Since most of the galactosamine appears to be derived from spisulan (Cifonelli and Mathews, 1972b), in which the amino sugar is not associated with uronic acid, estimation of the percentage of each uronic acid in mactin is not appreciably affected by this contaminant.

The presence of D-galactose and D-xylose in all of these samples reflects the presence of the linkage region. The linkage region also includes a D-glucuronic acid moiety (Lindahl and Rodén, 1965). This residue is determined as part of the total D-glucuronic acid in analysis for the relative amounts of D-glucuronic and L-iduronic acids and must be taken into consideration when it is wished to estimate the L-iduronic and D-glucuronic acid contents solely of the heparin backbone. Estimations of the D-glucuronic acid content originating from the linkage region were made on the basis of molecular weights, which ranged from 5.0 to 27 $\times$ 10^3. These data are presented in Table II. The values listed for linkage region D-glucuronic acid are maximal and assume that all molecules possess D-glucuronic acid at the linkage position. This is a reasonable assumption since most of the preparations contain appreciable amounts of D-galactose, which can only be derived from the linkage region, even in samples in which D-

TABLE I: Composition of Heparin and Heparan Sulfate Preparations.

Fraction	Source	Uronic Acid[a] (%)	Hexosamine[a] (%)	HexA[b] HexN	Mol/mol of Glc-N Gal	Xyl	Gal N	Ser	Gly
BLH I	Beef lung	39	23		0.007	0.003	Trace	Trace	Trace
BLH II	Beef lung	38	21	1.4	0.005	0.001	0.004	0.013	0.009
BLH III	Beef lung	40	22	1.5	0.030	0.021	Trace	Trace	Trace
HMH I	Hog mucosa			1.3	0.031	0.020	0.010	0.006	Trace
HMH II	Hog mucosa	36	21	1.4	0.080	0.042	0.022	0.005	0.003
BMH I	Beef mucosa	49	24	1.5	0.050	0.035	0.025	0.044	0.024
WH I	Whale	43	23		0.011	0.004	Trace	Trace	Trace
M I	Clam	39	26	1.4	0.080	0.020	0.150	0.028	0.011
HSH I	Hog mucosa by-product	38	21	1.4	0.042	0.022	0.016	0.017	0.011
HSH II	Hog mucosa by-product	45	30	1.2	0.011	0.006	0.012	0.018	0.017
HSH III	Hog mucosa by-product	34	26	1.3	0.010	0.005	0.008	0.006	Trace
HSB I	Beef lung by-product	38	21	1.3					
HSB II	Beef lung by-product	38	25	1.3	0.042	0.024	0.010	Trace	Trace
HSB III	Beef lung by-product	44	25	1.2	0.028	0.018	0.002	Trace	Trace
HSU I	Human umbilical cord	45	29	1.2	0.042	0.020	0.018	0.019	0.009
HSD I	Heparan sulfate by-product	39	26						
D I	Heparin disaccharide	13	30[c]						

[a] Determined as described by Lindahl *et al.* (1965). [b] Molar ratio of total hexuronic acid to hexosamine determined by radiochromatographic analysis (Conrad *et al.*, 1973). [c] Present as 2,5-anhydromannose.

xylose and serine are present in low or negligible concentrations.

The percentage of linkage region D-glucuronic acid is subtracted from the percentage of total uronic acid represented by D-glucuronic acid to obtain the value for the per cent D-glucuronic acid in the backbone of these polymers. The relative amounts of D-glucuronic and L-iduronic acids in the backbone may then be compared with the other variable parameters of this series of polysaccharides as shown in Table II. For convenience the undegraded samples are grouped into heparin and heparan sulfate fractions, based primarily on the marked difference in their anticoagulant properties. When both groups are considered together, it is seen that the percentage of L-iduronic acid varies from 27 to 84, with values in the higher part of this range characterizing the heparins.[1] Similarly, the *O*-sulfate values increase with increasing *N*-sulfate, and both *N*- and *O*-sulfate increase with increasing L-iduronic acid. Curiously, the sharp delineation between heparins and heparan sulfates reflected in the anticoagulant activities is not so sharply defined in terms of sulfate content or per cent L-iduronic acid.

Several fractions merit special comment. The mactin fraction (MI) has a relatively low L-iduronic acid content in spite of its high anticoagulant activity. This low level of L-iduronic acid was suggested previously (Cifonelli and Mathews, 1972a) on the basis of paper chromatographic evidence and ratios of uronic acid to 2,5-anhydromannose obtained from degradation products formed upon reaction with mactin with nitrous acid. Several degraded polymer fractions have been examined for their uronic acid content. Fraction BLH III was obtained as a minor fraction after chromatography of heparin on Dowex 1, chloride. This fraction was of unusually low molecular size, corresponding to a molecular weight of $\sim 5.2 \times 10^3$ daltons, and is estimated to have D-glucuronic acid in the linkage region amounting to 10% of the total uronic acid. Since this product possesses both *N*- and *O*-sulfate in the range found for heparins, it appears that the fraction represents a polysaccharide derived from heparin. The presence of appreciable D-xylose in the sample indicates that the linkage region is not degraded to any major extent. Furthermore, the proportion of L-iduronic acid in this material is 75% of the total uronic acid, a value at the higher end for heparins, adding support for viewing this product as a heparin type.

If all of the L-iduronic acid is O-sulfated at C-2 as found for heparin (Wolfrom *et al.*, 1969b; Lindahl and Axelsson, 1971; R. L. Taylor and H. E. Conrad, unpublished results) and for at least some samples of heparan sulfate (J. E. Shively and H. E. Conrad, unpublished results), the data presented here show that in none of the samples is there sufficient *O*-sulfate for all of the glucosamine residues to be O-sulfated at C-6. That *O*-sulfate may vary on the *N*-acetyl-D-glucosamine-containing sections of heparan sulfates has been reported previously for heparan sulfates from umbilical cords (Cifonelli and King, 1970b) and beef lung (Cifonelli, 1968) and from rat brain (Margolis and Atherton, 1973). The present data suggest that the variability of the O-sulfation of glucosamine residues is also observed in the heparin fractions.

The two degraded fractions, HS DI and DI, recovered after nitrite treatment of heparan sulfate and heparin, respectively, show a further correlation of sulfate and L-iduronic acid contents. HS DI is a segment of heparan sulfate in which the glucosamine residues are N-acetylated. The low level of sulfate in this fraction suggested that D-glucuronic acid should predominate in this preparation; this is confirmed by the results in Table II. Fraction DI, a di-O-sulfated disaccharide obtained by nitrite cleavage at the N-sulfated glucosamine residues of heparin (Cifonelli and King, 1972), consists of uronic acid and 2,5-anhydromannose only. Evidence from several sources indicates that both of these residues may be sulfated

[1] Dr. Ulf Lindahl (personal communication) has determined the relative amounts of D-glucuronic acid and L-iduronic acid in a purified heparin from hog mucosa and a heparan sulfate from human aorta and has obtained results comparable to those presented in Table II.

TABLE II: Variation of Iduronic Acid Content with Other Variable Physical, Structural, and Biological Parameters.

Fraction	$[\alpha]_D^{24}$[a] (deg)	Mol Wt ($\times 10^{-3}$)	DP[b]	% of Total Uronic Acid: Glc A Linkage[c]	Glc A Backbone	Id A Backbone	Sulfate (mol/mol of GlcN): *N*-	Sulfate (mol/mol of GlcN): *O*-	Anticoag Act. (IU/mg)
Heparins									
BLH I	43	11	16	6	19	75	0.98	1.48	180
BLH II	38	11	16	6	10	84	0.96	1.14	109
BLH III	44	5.2	10	10	17	73	0.86	1.12	
HMH I	41	10.5	16	6	21	73	0.89	1.41	177
HMH II	41	9	17	6	36	58	0.81	1.04	114
BMH I		11	16	6	28	66	0.86	1.56	152
WH I	67	11	18	5	35	60	0.74	1.06	176
M I	47	17	31	3	50	47	0.83	0.86	132
Heparan sulfates									
HSH I	61	6.2	12	9	39	52	0.63	0.64	16
HSH II		16	33	3	64	33	0.34	0.38	7
HSH III	67	14	28	3	70	27	0.48	0.42	<10
HSB I	62	15.5	31	3	56	41	0.49	0.53	
HSB II	54	17	32	3	57	40	0.56	0.82	13
HSB III	73	16	32	3	60	37	0.51	0.48	40
HSU I	65	27	58	2	63	35	0.32	0.22	
Degradation products									
HSD I	73	3–4	6–8		96	4	0.06	0.08	
DI	7		1		13	87	(1.00)[d]	2.11	

[a] All rotations, measured at a concentration of 1% in water, are positive. [b] Degree of polymerization of the disaccharide repeating unit; *i.e.*, moles of GlcN/mole of polysaccharide backbone. [c] Estimated assuming 1 mol of linkage region D-glucuronic acid/mol of polysaccharide. [d] This fraction is derived from regions in heparin in which all of the amino sugar residues are N-sulfated; deamination yields this disaccharide fraction with loss of the *N*-sulfate group originally present.

(Foster *et al.*, 1963; Wolfrom *et al.*, 1969b; Perlin *et al.*, 1971; Danishefsky *et al.*, 1969) and that L-iduronic acid may predominate in this fraction. The data in Table II are consistent with this conclusion and with previous paper chromatographic analysis of acid hydrolysates of this disaccharide.

The data in Table II show a close structural relationship among heparins and heparan sulfates, whether derived from the same source or from a variety of sources. These relationships are consistent with earlier suggestions that heparan sulfate is a biosynthetic precursor of heparin. Biosynthesis studies indicate that heparin synthesis proceeds *via* a reaction sequence initiated by polymerization of *N*-acetyl-D-glucosamine and D-glucuronic acid from UDPGlcNAc and UDPGA, respectively (Sibert, 1963). This polymer is then N-deacetylated and N- and O-sulfated (Silbert, 1967a,b; Balasubramanian *et al.*, 1968; Lindahl, U., personal communication), and epimerized at C-5 of the D-glucuronic acid residue to form the L-iduronic acid residues in these polymers (Lindahl *et al.*, 1972). Although the exact metabolic sequence in which the unsulfated polymer matures remains to be established, the data presented here show that heparins and heparan sulfates at varying stages of maturity may accumulate in a single tissue.

References

Balasubramanian, A. S., Joun, N. S., and Marx, W. (1968), *Arch. Biochem. Biophys. 128*, 623.

Brown, D. H. (1957), *Proc. Nat. Acad. Sci. U. S. 43*, 783.

Brown, K. O., Rosenthal, W. A., and Helbert, J. R. (Sept, 1961), Abstracts of the 140th National Meeting of the American Chemical Society, Chicago, Ill.

Cifonelli, J. A. (1968), *Carbohyd. Res. 8*, 233.

Cifonelli, J. A., and Dorfman, A. (1962), *Biochem. Biophys. Res. Commun. 7*, 41.

Cifonelli, J. A., and King, J. (1970a), *Carbohyd. Res. 12*, 391.

Cifonelli, J. A., and King, J. (1970b), *Biochim. Biophys. Acta 215*, 273.

Cifonelli, J. A., and King, J. (1972), *Carbohyd. Res. 21*, 173.

Cifonelli, J. A., and Mathews, M. B. (1972a), *Connect. Tissue Res. 1*, 121.

Cifonelli, J. A., and Mathews, M. B. (1972b), *Connect. Tissue Res. 1*, 231.

Conrad, H. E., Varboncoeur, E., and James, M. E. (1973), *Anal. Biochem. 51*, 486.

Constantopoulos, G., Dekaban, A. S., and Carroll, W. R. (1969), *Anal. Biochem. 31*, 59.

Danishefsky, I., Steiner, H., Bella, Jr., A., and Friedlander, A. (1969), *J. Biol. Chem. 244*, 1741.

Foster, A. B., Harrison, R., Inch, T. D., Stacey, M., and Webber, J. M. (1963), *J. Chem. Soc.*, 2279.

Grossman, B. J., Cifonelli, J. A., and Ozoa, A. K. (1971), *Atherosclerosis 13*, 103.

Jeanloz, R. W. (1970), *in* The Carbohydrates, Vol. IIB, Pigman, W., and Horton, D., Ed., New York, N. Y., Academic Press, p 590.

Lindahl, U., and Axelsson, O. (1971), *J. Biol. Chem. 246*, 74.

Lindahl, U., Bäckström, G., Malmström, A., and Fransson, L.-A. (1972), *Biochem. Biophys. Res. Commun. 46*, 985.

Lindahl, U., Cifonelli, J. A., Lindahl, B., and Rodén, L.

(1965), *J. Biol. Chem. 240*, 2817.
Lindahl, U., and Rodén, L. (1965), *J. Biol. Chem. 240*, 2821.
Margolis, R. U., and Atherton, D. M. (1972), *Biochim. Biophys. Acta 273*, 368.
Perlin, A. S., Casu, B., Sanderson, G. R., and Johnson, L. F. (1970), *Can. J. Chem. 48*, 2260.
Perlin, A. S., Mackie, D. M., and Dietrich, C. P. (1971), *Carbohyd. Res. 18*, 185.
Perlin, A. S., and Sanderson, G. R. (1970), *Carbohyd. Res. 12*, 183.
Rodén, L., Baker, J., Cifonelli, J. A., and Mathews, M. B. (1972), *Methods Enzymol. 28*, 73.
Shively, J. E., and Conrad, H. E. (1970), *Biochemistry 9*, 33.
Silbert, J. E. (1963), *J. Biol. Chem. 238*, 3542.
Silbert, J. E. (1967a), *J. Biol. Chem. 242*, 5146.
Silbert, J. E. (1967b), *J. Biol. Chem. 242*, 5153.
Taylor, R. L., and Conrad, H. E. (1972), *Biochemistry 11*, 1383.
Wasteson, A. (1969), *Biochim. Biophys. Acta 177*, 154.
Wolfrom, M. L., Honda, S., and Wang, P. Y. (1969a), *Carbohyd. Res. 10*, 259.
Wolfrom, M. L., and Rice, F. A. H. (1946), *J. Amer. Chem. Soc. 68*, 532.
Wolfrom, M. L., Wang, P. Y., and Honda, S. (1969b), *Carbohyd. Res. 11*, 179.

Bovine Fibrinogen. I. Effects of Amidination on Fibrin Monomer Aggregation†

H. M. Phillips and J. Lyndal York*

ABSTRACT: Eighty-four to ninety per cent of the lysine residues in bovine fibrinogen were accessible to ethyl acetimidate modification at pH 8 to 8.5. The pseudo-first-order plot for the amidination reaction was biphasic with 42% of the lysines reacting at the faster rate and 30% at the slower rate. All lysines were trinitrophenylated at pH 9–9.5 in 50% urea. The lysine residues accessible to ethyl acetimidate and distinguishable by their reactivity were also distinguishable on the basis of the effect of their amidination on the pH profile of clot opacity and syneresis. Opacity–per cent amidination studies indicated that some or all of the first 16% of the residues amidinated and that some or all of the residues modified between 57 and 84% play a role in the aggregation of fibrin monomers. The amidination of the middle 40% of the lysine residues had no observable effect on the properties of the clot. We have suggested that the pH shifts observed in the clot opacity–pH transition, clot syneresis–pH transition, and breaking weight maximum are a result of a change in hydrogen-bonding ability due to the change in basicity of lysine and/or amino terminal upon amidination.

Fibrinogen is a large protein involved in blood clotting; however, relatively little is known about its structure, particularly the topography of its functional groups. The biological role of fibrinogen involves the formation of a gel induced by proteolysis by thrombin to form fibrin monomers and subsequent aggregation of the fibrin monomers to form the gel. Mihalyi (1970a) has suggested that the surface features of the molecule should reflect this mechanical function (clot formation) with specific structural details in certain regions on the surface of the fibrin monomer determined by the nature of the forces, or the specific bonds, which are necessary for the aggregation phenomena.

In an attempt to elucidate the mechanism of fibrin polymerization, Mihalyi (1954) observed pH changes accompanying polymerization and suggested that hydrogen bonding may be involved. Sturtevant *et al.* (1955) and Scheraga (1958) have postulated that the links holding the individual fibrin monomers together were of the hydrogen-bond type, based on the observation that the reversible fibrin aggregation is exothermic. Endres *et al.* (1965) found that hydrogen bonding could not account for the magnitude of the heat of polymerization and suggested that reversible fibrin polymerization could be the result of covalent-bond formation between ionizable groups. More recently, Endres and Scheraga (1968) have reported that the concept of reversible covalent-bond formation is no longer tenable based on the ionization changes and heat evolution in the polymerization of an acceptor-modified fibrin monomer.

Many attempts have been made to modify fibrinogen in order to determine the effect on clot formation. Zieve and Solomon (1966) found that photooxidation of fibrinogen renders it unclottable. Also extensive iodination of fibrinogen (Laki and Mihalyi, 1949) and 35% acetylation of fibrinogen (Caspary, 1956) have been found to render fibrinogen nonclottable even though fibrinopeptides A and B were removed by thrombin. These data have been used to suggest a role for lysine and/or tyrosine in the polymerization of fibrin monomers. However, the reagents used in the experiments just quoted are either nonspecific or introduce a large change in charge on the protein which could alter its properties drastically. More recently Fuller and Doolittle (1966) have found that amidination of the amino groups in fibrinogen does not affect clottability or clotting time but does prevent cross-linking of fibrin. Amidination does not change the charge on amino groups but does shift the pK from about 10.5–11.0 to about 12–12.5 (Hunter and Ludwig, 1962).

Thus since amidination is specific for amino groups and

† From the Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas 72207. *Received April 13, 1973*. Supported by the National Science Foundation Grant Gb-30742 X. This work forms part of a dissertation presented by H. M. P. in partial fulfillment of the requirements for the Doctor of Philosophy.