Laser Raman Inelastic Light Scattering Investigations of Hyaluronic Acid Primary and Secondary Structure

T. W. Barrett† and W. L. Peticolas
Department of Chemistry, University of Oregon, Eugene, Oregon 97403, USA

The solvent-subtracted Raman spectrum of hyaluronic acid is reported. The spectrum is unchanged by (i) variations in the pH of the solution 6.0—8.5, when buffered at constant ionic strength 0.1, and (ii) changes in temperature 10—50 °C.

As rheological, flow birefringence, and linear dichroism studies of hyaluronate solutions indicate a large and abrupt structural transition across the physiological pH range 7.0—7.5, the negative finding reported here is of considerable interest in contrast with those previous studies. When considering the polysaccharides in general, as precise rheological, flow birefringence and linear dichroism data are only available for hyaluronic acid, the present finding is of interest for hyaluronic acid considered as a model polysaccharide system exhibiting elasticity.

The result reported here is discussed within the context of, on the one hand, the possible limitations of the Raman technique which is a probe of *g*-state vibrational modes and, on the other hand, the possible extension of the Raman technique to probing oriented (stressed) samples. As a previous study demonstrated a small limiting birefringence but a large limiting extinction angle with minimum change in monomer anisotropy for these solutions, the suggestion is favored that the abrupt structural transition across the physiological pH range previously reported is due to long-range Van der Waals interactions.

INTRODUCTION

Hyaluronic acid consists of repeating units of *D*-glucuronic acid and *N*-acetyl-glucosamine with alternating β1,4- and β1,3-linkages. It is a polysaccharide present in many body tissues, and is implicated in the molecular dynamics of diverse physiological systems.[1–3] The viscosity and optical properties of hyaluronic acid when oriented by flow are reported to be influenced by the conformation of the polysaccharide chain, which in turn is affected by the degree of ionization of the carboxylic acid group.[4–9] These changes in viscosity and optical properties (flow birefringence and linear dichroism)[7,8] appear to be due to changes in intermolecular long-range ordering, rather than conformational changes in the primary structure.[7,10–16]

EXPERIMENTAL

In a laser Raman inelastic light scattering probe to test this supposition, we examined the spectra of solutions of 4% hyaluronate (Miles Laboratories Inc., Elkhart, Indiana 46514, pig skin hyaluronic acid 97–071–1) buffered in phosphate at six pHs and constant ionic strength 0.1, and also in aqueous solution. The solution pHs studied were 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5.

All Raman spectra were obtained from samples sealed in capillary tubes held horizontally in a brass block thermostated by constant temperature circulating water. About 600 mW of 5145 Å light from a Spectra-Physics Argon ion laser was focussed vertically on the sample. Scattered light was collected at 90° and analyzed in a Spex 1401 double monochromator and phototube, amplified by standard photon counting equipment and recorded on a Varian 620/*i* dedicated computer. Twelve scans were taken of each sample and averaged. During each scan, data were collected for one second at each wavenumber. The Raman spectra of the corresponding phosphate buffers were also taken and computer subtracted from the hyaluronic acid spectra by methods already described.[17] In a second series of experiments, the Raman apparatus consisted of an Argon laser which was tuned to the 5145 Å line and a Cary 81 double monochromator adapted for photodetection.

RESULTS AND DISCUSSION

Figure 1 shows the computer averaged spectra for sodium hyaluronate solutions at various pH and constant ionic strength 0.1. These spectra exhibit no differences.

Figure 2 shows difference spectra for sodium hyaluronate solutions at pH 6.0 and pH 8.0; i.e. these spectra are the computer subtraction of (i) the averaged spectra of phosphate solution buffered at the given pH and ionic strength from (ii) the Fig. 1 spectra, i.e. from the averaged spectra of hyaluronate solutions buffered in those phosphate solutions (i). Thus, the spectra of Fig. 2 are due to the molecular vibrations of the differentially ionized hyaluronic acid alone, without those vibrations due to the phosphate buffer or H_2O, and only traces of

†Visiting professor. Permanent address: Department of Physiology and Biophysics, University of Tennessee Center for the Health Sciences, 894 Union Avenue, Memphis, Tennessee 38163, USA.

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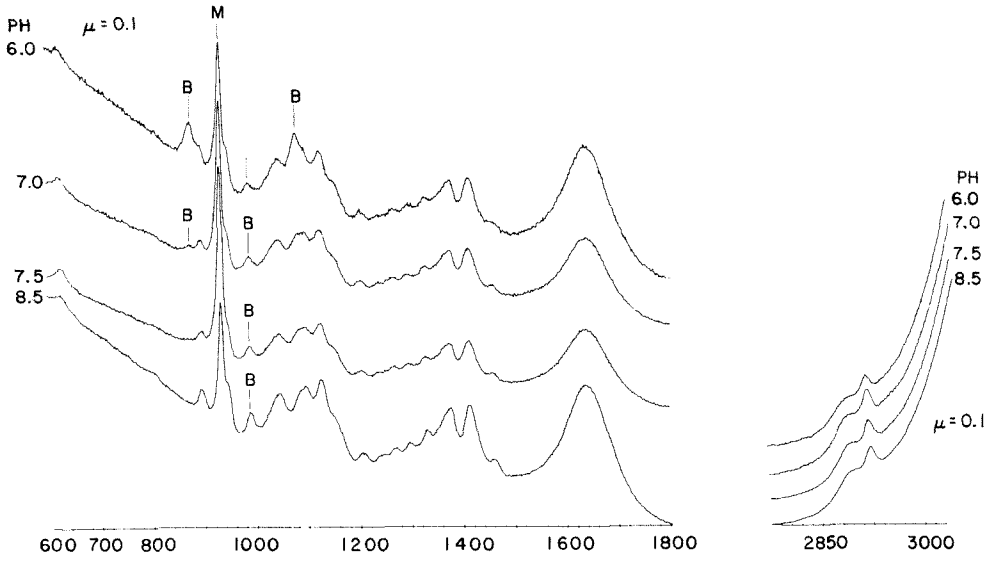

Figure 1. Averaged Raman spectra of hyaluronic acid at various pH and constant ionic strength 0.1. 'M' indicates the band due to the perchlorate 'marker' at ~928 cm^{-1}, and 'B' indicates those bands due to the phosphate buffer. Each spectrum is the average of 12 spectra, each of which was a scan with a dwell time at each wavenumber of one second.

the sodium perchlorate 'marker' vibrations remain at ~930 cm^{-1}. The sodium perchlorate 'marker' which was present in both the hyaluronic acid buffered in phosphate and also in the phosphate solution in equal amounts provided a gauge for the computer subtraction of the latter solution from the former. Thus, the aim was to eliminate every trace of sodium perchlorate from the final subtracted solution, although in practice some traces always remained. The differences in the height of the two spectra shown are due solely to differences in percentages (pH 6.0 versus pH 8.0) of phosphate present with respect to a constant volume exposed to the laser light. The spectra shown in Figs 1, 2, and 3 were recorded at 20 °C. There are no differences between the spectra of the hyaluronate solutions recorded at this temperature and others recorded between 10 and 50 °C.

The hyaluronic acid used in the present study was of more than 95% purity by infrared spectroscopic and electrophoretic analyses. A previous study[18] reported the Raman spectrum of human umbilical cord hyaluronic acid (Sigma Chemical Co.) and also the potassium salt of hyaluronic acid (Calbiochem) in hydraulically-pressed pellet form and deuterated samples. The hyaluronic acid used in the latter study is, however, of less than 90% purity and, under identical conditions to the present study, fluoresces too much for a spectrum to be taken. Thus, the present study reports considerably more resolution and signal-to-noise ratio in the hyaluronic acid spectrum for the region 600–1800 cm^{-1} and 2750–3100 cm^{-1}, and we have been able to resolve the previously reported broad band spectral lines at ~1100 and ~1365 cm^{-1} into multiple peaks at 1050, 1100, 1129 and 1145, and at 1376 and 1413 cm^{-1},

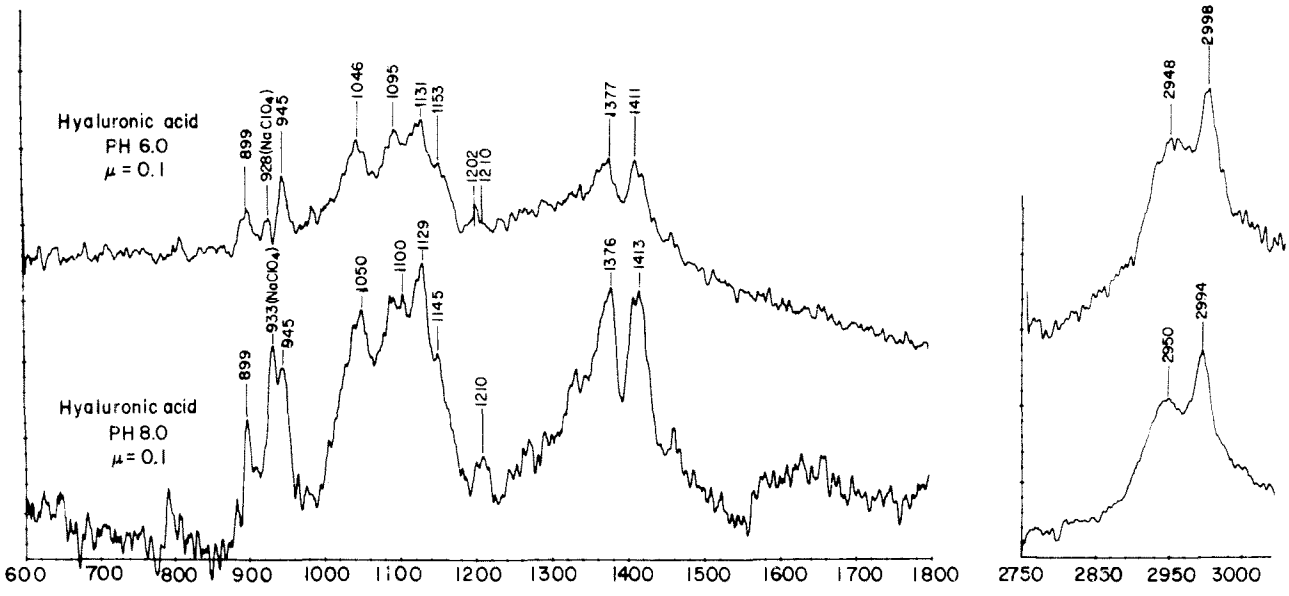

Figure 2. Solvent subtracted Raman spectra of hyaluronic acid at pH 6.0 and 8.0 and constant ionic strength 0.1. Vibrations due to traces of the sodium perchlorate 'marker' at ~928 cm^{-1} remain in these spectra.

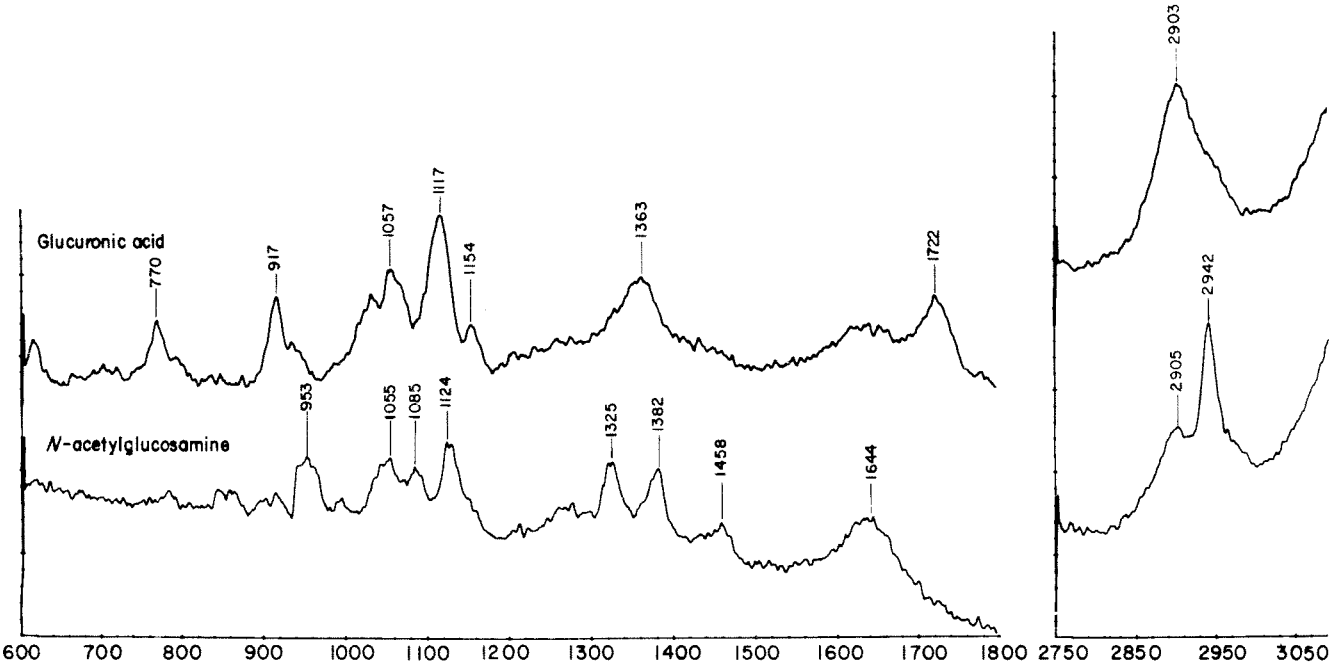

Figure 3. Raman spectra of the monomers: *D*-glucuronic acid (10% in H_2O) and *N*-acetyl-*D*-glucosamine (10% in H_2O). The Raman band at ~1650 cm^{-1} is due to H_2O.

respectively. A single discrepancy remains between the previously reported hyaluronate spectrum[18] and the present, namely, the intense and sharp band at 745 cm^{-1} present in the former study, but which is absent in the spectra reported here. Due to the greater purity of the samples used in the present study and the known impurities used in the former study which result in intense fluorescence under the conditions used in the present study, we assume that this spectral line is due to an impurity.

The choice of hyaluronic acid used in the present study was dictated for reasons of purity. This hyaluronic acid exhibits viscometric properties similar to the human umbilical cord hyaluronic acid and, apart from the 745 cm^{-1} 'impurity' band present in the previously reported study,[18] gives the same Raman spectrum, but with increased signal-to-noise ratio. The use of this extremely pure hyaluronic acid in a Raman light scattering investigation of a structural transition demonstrated on three instruments—rheological,[7] flow birefringence,[7] and linear dichroism[8]—but with hyaluronic acid from a different source (human umbilical cord), is therefore justified by the similarity of the spectra reported here and those reported for human umbilical cord hyaluronic acid.[18] No qualitative differences in the hyaluronic acids from different sources have been demonstrated by any technique.[4]

Figure 3 shows unsubtracted Raman spectra of the monomers *D*-glucuronic acid (Sigma Chemical Co., St. Louis, Mo.) and *N*-acetyl-*D*-glucosamine (Mann Research Laboratories, Inc., New York) with a water band at about 1650 cm^{-1}. These spectra are comparable to those obtained for the hydraulically pressed pellet form of these substances.[19]

Table 1 shows tentative Raman peak assignments based on previous assignments in carbohydrates[20] such as glucose,[21–26] amylose,[22] and lactose.[27] There appears to be no Raman line which cannot be assigned to primary structure vibrations. We therefore conclude that the pH-induced changes in viscosity and optical properties of hyaluronic acid previously reported[4–9] are dependent upon changes in cooperative structure which forms network-like associations, as witnessed by the presence of large concentration effects discernible in extinction angle measurements.[7]

It is likely that network-like associations in hyaluronic acid random coil chains are due to either hydrogen bonds or ionic bonds or Van der Waals forces. However, it is also unlikely that such bonding in randomly coiled chains, if it is due to some kind of bonding, provides *g* (gerade) vibrational states. As Raman spectroscopy is *g*-state vibrational spectroscopy, the absence of any change in the Raman spectrum under conditions in which changes in viscosity, flow birefringence and linear dichroism are produced, indicates either (i) the structural forms underlying these changes produce only *u* (ungerade) vibrational states, or (ii) these structural forms require an energy perturbation so great that their existence is discernible only in rotational and vibrational modes of large wavenumber. Postulate (ii) would be true in the case of long-range interactions of the Van der Waals kind.

Addressing suggestion (i): a study was made of the infrared spectra (i.e. *u*-state spectra) of the six hyaluronate solutions, but no differences were detected. However, due to the considerable infrared absorption of water, no definite conclusions can be drawn from such a study as the hyaluronic acid may be absorbing in the region where the solution is absorbing. Thus, suggestion (i) still stands; but, in the light of the discussion below, we consider it unlikely to be true.

Addressing suggestion (ii): due to the unique viscoelastic properties of hyaluronic acid,[6] it is likely that the pH-induced transition detected by viscometry[7] will also be detected by ultrasound absorption methods.

There is also the complication that the structural transition has been identified by three instrumentation methods which stress the system studied, e.g. in

Table 1. *Tentative assignment of Raman spectral lines*

Glucuronic acid	*N*-Acetyl-glucosamine	Hyaluronic acid
620		
770		
		899 *β-linkages*
917		945 C—C stretch
		C—O—C stretch
	953	
1030		
		~1050 C—O, C—C
	1055	
1057		
	1085	
		~1100 C—O—H bend, acetyl group
1117		
	1124	
		~1130 C—C
1154		~1150 C—O, C—C, oxygen bridge
		1210 CH_2 twist
	1325	
		1330 CH bend, Amide III
1363		
		~1380 CH_3, ionized carboxyl
	1382 CH_3	
		~1410 CH bend, ionized carboxyl, Amide II
	1485 CH_3, C(6)—H_2, COH	
		1460 CH_2 bend
1722 Carboxyl group		
2903		
	2905	
	2942	
		2950 CH_3 stretch
		~3000 NH stretch, CH_2 stretch

rheological tests and in optical instruments in which the molecule is oriented by flow in a Couette cell.[7,8] The present result, therefore, may have a simple explanation, namely: that structural differences that result in differences in the molecule's ability to store or dissipate energy from a shearing force are only apparent in such shearing situations. If this third and final suggestion is valid, the Raman spectra for the six hyaluronate solutions of the present study might differ if the solutions were stressed or sheared during data collection.

At the present time, there is no evidence eliminating any of the three suggestions given here concerning the inability of the laser Raman inelastic light scattering to detect a transition observed with three different techniques. However, we favor suggestion (ii) as we have demonstrated[7] that the hyaluronate solutions exhibit a small limiting birefringence concentration dependence, but a large limiting extinction angle concentration dependence, with minimum change in anisotropy as solution pH is changed. This strongly implies interchain long-range Van der Waals interactions. The action of the phosphate buffer may be to electronically shield the carboxyl groups,[29] resulting in a wide divergence of the true and apparent pK as previously suggested.[8] The structural change as solution pH is changed across the physiological range 7.0–7.5 might then be explained by a change in long-range Van der Waals interactions at a pH determined by the apparent and not the true pK.

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REFERENCES

1. A. C. Guyton, *Pflügers Archiv. Suppl.* **336**, S1–S20 (1972).
2. A. C. Guyton, *Fed. Proc.* **35**, 1861 (1976).
3. T. W. Barrett, *Biosystems* **8**, 103 (1976).
4. E. A. Balazs, *Fed. Proc.* **25**, 1817 (1966).
5. D. A. Rees, *Adv. Carbohydr. Chem. Biochem.* **24**, 267 (1969).
6. D. A. Gibbs, E. W. Merrill, D. A. Smith and E. A. Balazs, *Biopolym.* **6**, 777 (1968).
7. T. W. Barrett and R. E. Harrington, *Biopolym.* **16**, 2167 (1977).
8. T. W. Barrett, *Biopolym.* **17**, 1567 (1978).
9. B. Chakrabarti, *Archiv. Biochem. Biophys.* **180**, 146, (1977).
10. B. Chakrabarti and E. A. Balazs, *J. Mol. Biol.* **78**, 135 (1973).
11. L. A. Buffington, E. S. Pysh, B. Chakrabarti and E. A. Balazs, *J. Am. Chem. Soc.* **99**, 1730 (1977).
12. E. A. Balazs, A. A. McKinnon, E. R. Morris, D. A. Rees and E. J. Welsh, *J. Chem. Soc. Chem. Comm.* **2**, 44 (Jan. 1977).
13. A. L. Stone, *Biopolym.* **3**, 617 (1965).
14. A. L. Stone, *Biopolym.* **7**, 173 (1969).
15. A. L. Stone, *Biopolym.* **10**, 739 (1971).
16. A. L. Stone, *Biopolym.* **11**, 2625 (1972).
17. T. W. Barrett, W. L. Peticolas and R. M. Robson, *Biophys J.* **23**, 349 (1978).
18. A, T. Tu, N. D. Dinh, C. Y. She and J. Maxwell, *Stud. Biophys.* **63**, 115 (1977).
19. C. Y. She, N. D. Dinh and A. T. Tu, *Biochim. Biophys. Acta* **372**, 345 (1974).
20. J. Koenig, *J. Polym. Sci.* **D6**, 59 (1972).
21. F. H. Spedding and R. F. Stamm, *J. Chem. Phys.* **10**, 176 (1942).
22. J. J. Cael, J. L. Koenig and J. Blackwell, *Carbohydr. Res.* **29**, 123 (1973).
23. J. J. Cael, J. L. Koenig and J. Blackwell, *Carbohydr. Res.* **32**, 79 (1974).
24. P. D. Vasko, J. Blackwell, and J. L. Koenig, *Carbohydr. Res.* **19**, 297 (1971).
25. P. D. Vasko, J. Blackwell and J. L. Koenig, *Carbohyd. Res.* **23**, 407 (1972).
26. A. G. Walton and J. Blackwell, *Biopolymers*, Academic Press, New York, 1973.
27. H. Susi and J. S. Ard, *Carbohydr. Res.* **37**, 351 (1974).
28. A. Darke, E. G. Finer, R. Moorhouse and D. A. Rees, *J. Mol. Biol.* **99**, 477 (1975).
29. R. Potenzone and A. J. Hopfinger. *Polym. J.* **10**, 181 (1978).

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