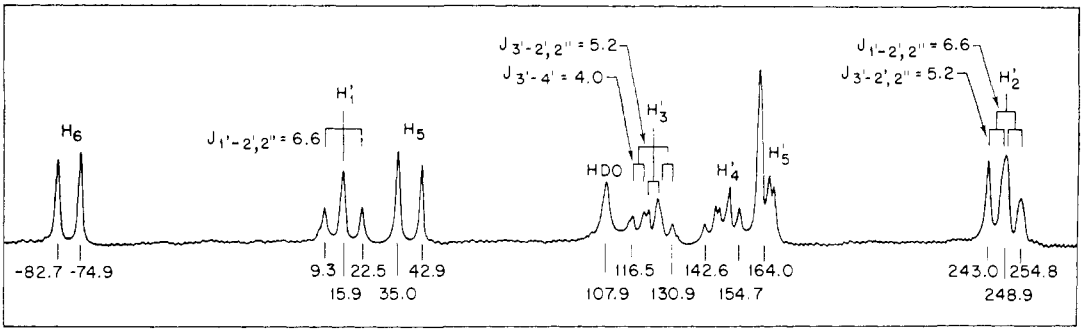

Fig. 1.—Measured and theoretical n.m.r. spectra for deoxyuridine in D_2O at 60 mc. The magnetic field increases from left to right. Primed protons refer to those of the deoxyribose ring, and the unprimed ones to those of uracil. Numbers under the peaks denote the chemical shift in c.p.s. from benzene, the external standard. The peaks due to H_2' and H_2'' (labeled H_2' in the figure) have been analyzed by taking the spacing between the two middle transitions in the AB spectrum (H_2' = A and H_2'' = B) to be less than about 1 c.p.s. This is indeed the case if the difference in chemical shifts of these two protons is of the order of 1 to 3 c.p.s. since J_{AB} = 13 c.p.s. Then spin coupling to H_1' (X) and to H_3' (Y) gives rise to the observed multiplet.

The number of peaks and intensities in the multiplets due to H_1', $H_2' + H_2''$ and H_3' of a number of deoxyribose nucleosides and nucleotides are similar to those of deoxyuridine. With the exception of deoxyguanosine and 5'-thymidylic acid the line widths noted for the other compounds are in good agreement with those predicted on the basis of the O-*endo* conformation, Table II.[8] A more detailed analysis of the multiplet spacings in the spectra of these compounds as well as their temperature dependence will follow.[9]

Acknowledgment.—I am grateful to Prof. J. T. Edsall for his continued interest and support in these studies. I am also indebted to Prof. O. Jardetzky, Department of Pharmacology, Harvard Medical School, for constant encouragement and for the use of the 60 mc. n.m.r. equipment and to Dr. P. Pappas for his expert help in obtaining the spectra.

(8) I am indebted to Dr. R. U. Lemieux for making available to me his manuscript on the configuration and conformation of thymidine prior to publication (*Can. J. Chem.*, in press). The theoretical analysis of the spectra in this case depends on the theory of Richards and Schaefer (*J. Mol. Phys.*, **1**, 331 (1958)) according to which H_2' and H_2'' should have identical chemical shifts. This assumption becomes unnecessary however, if account is taken of the large coupling constant between H_2' and H_2'' (A and B, respectively) and of the small difference between the chemical shifts of these protons. The spectrum may then be treated generally as that of an XABY type ($H_1'H_2'H_2''H_3'$) shown above. Complete analysis of the thymidine spectrum reveals that both O and C_1' are displaced in opposite directions from the plane of the other ring atoms so that the large side groups are more equatorially oriented as compared with those in a completely planar ring (C. D. Jardetzky, *Fed. Proc. Abstracts*, April, 1961). In this case the dihedral angles $H_2'H_3'$ and $H_2''H_3'$ are equal to 5 and 115°, respectively. This conformation is compatible only with an $H_2''H_3'$ dihedral angle of less than 120° and is therefore inconsistent with that suggested by Dr. Lemieux since these angles have been assigned the values of 10 and 130°, respectively. The O-*endo* conformation for deoxyuridine also implies that these angles are equal to 0 and 120°, respectively.

(9) C. D. Jardetzky, Abstracts, Vth International Congress of Biochemistry, Moscow, August 1961.

[CONTRIBUTION FROM THE DEPARTMENT OF MEDICINE AND THE STUDY GROUP ON RHEUMATIC DISEASES, NEW YORK UNIVERSITY SCHOOL OF MEDICINE, NEW YORK, N. Y.]

Interaction in Solution of Lysozyme with Chondroitin Sulfate and its Parent Proteinpolysaccharide[1]

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A study was made of the interaction in aqueous solution between the cationic protein lysozyme and the principal parent proteinpolysaccharide of chondroitin sulfate (PP-L) that occurs in bovine nasal cartilage. A series of salt-like compounds appears to exist whose composition is expressed by the equivalence ratio (e.r.) which is equivalent of polyanion per equivalent of lysozyme. These compounds have been divided roughly into three groups by centrifuging: (a) water insoluble, easily sedimented at 700 × *g*, and with e.r. ranging from 1.0 to 2.0; (b) water soluble, giving strongly opalescent solutions, not easily sedimented at 700 × *g* but easily sedimented at 30,000 × *g*, and with e.r. mainly from 1.9 to 5.2; (c) water soluble, giving grossly clear solutions, not easily sedimented at 144,000 × *g*, but detectable in the analytical ultracentrifuge as distinct from PP-L or lysozyme. This centrifugal method of studying lysozyme-polyanion compounds provides a means to determine the proportions of lysozyme and polyanion in those compounds that are sedimentable.

Polyelectrolytes behave as weak electrolytes and a model for this behavior has been developed by Harris and Rice.[2] In mammalian connective tissues polyelectrolytes occur in the form of anionic polysaccharides associated with simple counterions, principally sodium. These polysaccharides are usually bound to proteins by bonds that are not

(1) This investigation was supported by United States Public Health Service Grants A-2594 (C2) and A-28 (C8), and the Arthritis and Rheumatism Foundation.

(2) F. E. Harris and S. A. Rice, *J. Phys. Chem.*, **58**, 725 (1954).

ionic. Chondroitin sulfate in aqueous solution with sodium counterions behaves as if it were about 50% undissociated, and with calcium counterions it behaves as if about 70% undissociated.[3] Cations of higher charge may behave as if bound to an even greater extent. Among such cations are some complex ferric and cobaltic ions, micellar cationic detergents and polymerizable cationic dyes, the metachromatic dyes.[4] Since proteins may carry multiple cationic charges and also occur in connective tissues, it becomes a matter of concern to know whether, and to what extent, such proteins form undissociated salts with chondroitin sulfate or its naturally occurring proteinpolysaccharides. Lysozyme is of particular interest, since it carries a net cationic charge at *p*H 8, and is widely distributed in the mammalian body, thus making a natural encounter with chondroitin sulfate and similar polysaccharides probable. However, existing information is not sufficient to predict the results of such encounters. In the present work a study is made of the conditions under which lysozyme and chondroitin sulfate or its parent proteinpolysaccharide form insoluble products and the conditions under which there is evidence of the formation of water-soluble but undissociated products. Beyond the specific system studied the results are of concern in many fields of chemistry relating to the interaction in solution of cationic proteins and anionic polyelectrolytes. This problem is of growing interest in enzyme chemistry because of the frequent observation that polyelectrolytes may act as enzyme inhibitors.[5]

Materials and Methods

The lysozyme used was the twice crystallized product supplied by the Worthington Biochemical Corporation, Freehold, N.J. The proteinpolysaccharide was the product containing chondroitin sulfate and protein isolated from bovine nasal cartilage by the method recently described and referred to as PP–L.[6] It contains 85% potassium chondroitin sulfate, all of which appears to be bound to protein by non-ionic bonds. The potassium chondroitin sulfate used was made from PP–L by alkaline degradation as described elsewhere,[6] reprecipitated twice as its barium salt and then converted to the potassium salt by the use of Dowex-50 in the potassium form.

For preparative centrifugation at values over 1000 × *g*, a Spinco Model L ultracentrifuge was used with a Type 30 or a Type 40 rotor. Values of *g* reported are calculated to the center of the centrifuge tube.

Analytical ultracentrifugal studies were performed in a Spinco Model E ultracentrifuge at 20°, using cells with a double sector centerpiece. Most of the studies were carried out in 0.15 *M* KCl. Sedimentation coefficients are expressed using this solvent ($s_{20,\ 0.15\ M\ KCl}$) and are not corrected to distilled water because of the slowing effects of low ionic strength solvents on the sedimentation properties of polyelectrolytes.[7] Quantitative measurements were carried out directly from the photographic plates using a comparator (Gaertner) according to methods described by Trautman.[8]

Preliminary experiments showed that lysozyme when added to solutions of chondroitin sulfate or of PP–L produced either precipitates, strongly opalescent solutions, or perfectly clear solutions, depending on the conditions. Two factors were selected for variation and study, namely, the salt concentration and the ratio of polyelectrolyte to lysozyme. The only salt added to the system was potassium chloride. The proportion of polyelectrolyte to lysozyme in any mixture, solution or sediment is expressed as the number of equivalents of polyelectrolyte per equivalent of lysozyme that is present and is called the equivalence ratio e.r. An equivalent is the relative weight of material associated with a single cationic or anionic charge. The equivalent weight of lysozyme (1670) comes from its molecular weight (14,200) and its net positive charge of 8.5 at *p*H 6 to 7.[9] All the experiments reported were carried out in the temperature range 20–25°.

Chondroitin sulfate consists of repeating disaccharide units or periods. Each period contains one carboxylate anion, one ester sulfate anion and two potassium cations. The equivalent weight of potassium chondroitin sulfate (303) is therefore half its period weight (606). Likewise PP–L can be regarded as consisting of such periods, each associated statistically with 1.0 amino acid residues of the protein portion of the compound. PP–L as its potassium salt thus has an equivalent weight of 340, half its period weight of 680.[6] This neglects any contribution of the protein component of PP–L to the net charge of the molecule. Since the protein content of PP–L is only 15%, its charge contribution calculated per equivalent of chondroitin sulfate probably is small.

For the analysis of solutions containing both lysozyme and polyelectrolyte in 0.15 *M* KCl the following method was found suitable. It is based on the fact that lysozyme can be removed completely from solution by carboxymethylcellulose, while neither chondroitin sulfate nor PP–L is adsorbed at all. Samples in 0.15 *M* KCl were diluted with 0.15 *M* KCl to contain not more than 0.7 mg. lysozyme per ml. and the absorbancy at 280 mμ was measured. About 10 ml. of such a solution was stirred 10 minutes with carboxymethylcellulose (150 mg.) and centrifuged. The supernatant was again stirred 10 minutes with carboxymethylcellulose (50 mg.), centrifuged, and the absorbancy of the solution at 280 mμ was measured. From the drop in absorbancy the mg. lysozyme per ml. and the total mg. lysozyme in the original sample were calculated by comparison with similar solutions of known lysozyme content. After removal of the lysozyme a part of the solution containing no more than 0.8 mg. of chondroitin sulfate or PP–L was diluted to 5 ml. with 0.15 *M* KCl. To this was added 1 ml. of a 1% solution of cetylpyridinium chloride in 0.15 *M* KCl. The turbidity produced was measured at 600 mμ in a 1-cm. cell about 15 minutes after adding the cetylpyridinium chloride. Comparison with a standard curve of turbidity made for chondroitin sulfate or PP–L treated similarly yielded the mg. of polyelectrolyte in the samples analyzed.

Results

Table I summarizes the results of a preliminary qualitative survey of the conditions under which precipitates, opalescent solutions or clear solutions were formed. All solutions contained lysozyme (1 mg./ml. or 0.60 μeq./ml.) and an amount of chondroitin sulfate indicated by the equivalence ratio (e.r.), as well as the indicated concentration of potassium chloride. The *p*H of all solutions lay between 6 and 7. At potassium chloride concentration of 0.2 *M* or above there was no grossly visible indication of any interaction, all solutions remaining perfectly clear. At 0.1 *M* potassium chloride the amount of precipitate was small, but the solutions were very strongly opalescent, except in the presence of rather large excesses of chondroitin sulfate. At 0.04 *M* potassium chloride, dense precipitates formed except in the presence of a large excess of chondroitin sulfate. In the absence of added salt a dense precipitate appeared only up to an equivalence ratio of one. With some excess of chon-

(3) S. J. Farber and M. Schubert, *J. Clin. Invest.*, **36**, 1715 (1957).

(4) M. Schubert and D. Hamerman, *J. Histochem. Cytochem.*, **4**, 159 (1956).

(5) J. Coleman and H. Edelhoch, *Arch. Biochem. Biophys.*, **63**, 382 (1956).

(6) B. Gerber, E. C. Franklin and M. Schubert, *J. Biol. Chem.*, **235**, 2870 (1960).

(7) L. Varga, A. Pietruszkiewicz and M. Ryan, *Biochim. Biophys. Act.*, **32**, 155 (1959).

(8) R. Trautman, *J. Phys. Chem.*, **60**, 1211 (1956).

(9) C. Tanford and M. L. Wagner, *J. Am. Chem. Soc.*, **76**, 3331 (1954).

TABLE I

RESULTS OF MIXING, IN AQUEOUS SOLUTION, LYSOZYME (0.60 μEQ./ML.) AND CHONDROITIN SULFATE (0.54 TO 25 μEQ./ML.) IN THE PRESENCE OF THE INDICATED CONCENTRATIONS OF KCl

Equiv.[a] ratio	0.00 *M* KCl			0.04 *M* KCl			0.10 *M* KCl			0.20 *M* KCl	
	Absorb. 600 mμ	Appearance[b] Ppt.	Soln.	Absorb. 600 mμ	Appearance Ppt.	Soln.	Absorb. 600 mμ	Appearance Ppt.	Soln.	Appearance Ppt.	Soln.
0.9	0.53	++	0	0.99	++	0	0.14	+	++	0	0
1.6	.18	0	++	.69	++	0	.26	+	++	0	0
2.5	.13	0	++	.65	++	0	.30	+	++	0	0
5.0	.04	0	+	.81	++	0	.44	+	++	0	0
8.2	.02	0	0	.77	++	+	.48	+	++	0	0
16.5	.02	0	0	.80	+	++	.19	0	+	0	0
41.2	.02	0	0	.10	0	++	.02	0	+	0	0

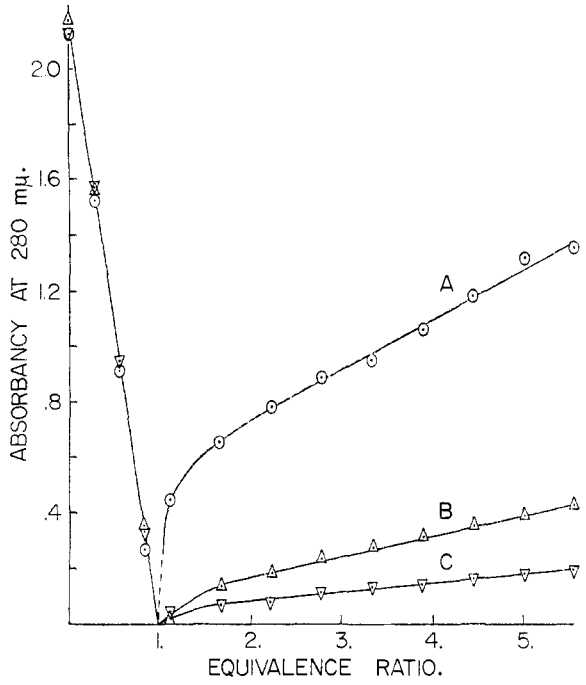
[a] The equivalence ratio is the ratio of equivalents of chondroitin sulfate to equivalents of lysozyme added in each case. [b] In ppt. column: ++ is dense, + is slight and 0 is no ppt. In solution column: ++ is dense opalescence, + is slight opalescence and 0 is a clear solution, in each case referring to the solution remaining after any ppt. has settled.

droitin sulfate, no precipitate formed, but the solutions were opalescent, and even this disappeared at higher excess of chondroitin sulfate. The purpose of the summary in Table I is to indicate the ranges of the salt concentration and of the equivalence ratio in which grossly visible interaction occurred. The data are not readily susceptible of quantitative treatment. An attempt was made to make the qualitative descriptions in Table I somewhat objective by measuring the absorption of each mixture at 600 mμ in a 1-cm. cell as a measure of the turbidity (column headed Abs. 600 mμ).

Another and more quantitative way to study this reaction was to measure the amount of lysozyme left in solution after centrifuging series of solutions made as those of Table I for 20 minutes at 25,000 × *g*. This was sufficient to remove the precipitates and to clarify the opalescence in those ranges of salt concentration and equivalence ratio where opalescence occurred and yielded in all cases perfectly clear supernatant solution. Measure of their absorbancy at 280 mμ gave directly the amount of lysozyme left in the solution since absorbancy of the chondroitin sulfate or of the PP-L at the concentrations involved was always small. The curves of Figs. 1 and 2 show the results. At low salt concentrations (0 to 0.06 *M*) the amount of lysozyme left in solution decreases linearly as the amount of chondroitin sulfate of PP-L added increases up to an equivalence ratio of one. At values of this ratio just over one, there is a rather abrupt change in the direction of the curve and the amount of lysozyme left in solution then increases with the amount of chondroitin sulfate or PP-L added. At higher salt concentrations (0.06 to 0.14 *M*), similar trends are observed but the amount of lysozyme left in solution at the equivalence ratio of one progressively increases until at concentrations of salt over 0.15 *M* the entire initial amount of lysozyme remains in solution and none is sedimented under the conditions used. Another curious effect observable in Fig. 1 is that when the equivalence ratio is over one and the salt is increased in the range 0 to 0.03 *M*, the amount of lysozyme sedimented increases, but when the salt is increased in the range 0.06 to 0.14 *M*, the amount of lysozyme sedimented decreases. The same is true in Fig. 2, except for the odd shape of the curve at 0 salt, which cuts across other curves.

The reversal in direction of the curves of Figs. 1 and 2 in the neighborhood of an equivalence ratio of one shows that progressively more lysozyme was left in solution, as excess polyelectrolyte was added. This suggests that in the presence of excess polyelectrolyte there exist soluble undissociated salts of lysozyme and polyelectrolyte. This is more marked with PP-L than with chondroitin sulfate, as is apparent from the steeper slopes of the curves of Fig. 2.

The composition of the sedimentable products formed in the range of polyelectrolyte excess was studied at concentrations of lysozyme ranging from 3–10 mg./ml. and amounts of PP-L so that the equivalence ratio ranged from 1.5–6.5. The usual procedure was to start with the appropriate solution of PP-L in 0.15 *M* KCl and add a constant amount of lysozyme, also in 0.15 *M* KCl. The final solution volumes and the amounts of lysozyme and PP-L were those given in the first four columns of Table II. The resulting turbid solutions were stirred for several hours to equilibrate since on the addition of lysozyme a precipitate formed where local excesses of lysozyme occurred and equilibration was slow. The resulting solutions were turbid, though less so the higher the content of PP-L. They were then centrifuged at 700 × *g* for ten minutes giving a sediment called R_1 and an opalescent supernatant solution called F_1. A sample of F_1 (0.5 ml to 1.0 ml.) was removed and diluted with 0.15 *M* KCl for analysis and the remaining F_1 was centrifuged for one hour at 100,000 × *g* giving a sediment called R_2 and a relatively clear supernatant solution called F_2. The sediments R_1 and R_2 were not washed as they dispersed or dissolved in water of KCl solution. They were simply drained by inversion of the centrifuge tubes, and the sides of the tubes were wiped. The method of assay of these sediments for content of PP-L and lysozyme depends on the fact that they could be dissolved completely in 0.15 *M* KCl if stirred sufficiently long (2 to 4 hr.) again illustrating the long equilibration time needed. If sufficient solvent (1 to 3 ml. per mg. of sediment) was added, the solutions produced were perfectly clear indicating that the sedimented compounds were largely dissociated under these conditions. Analysis of these solutions and of the diluted samples F_1 and F_2 for lysozyme and PP-L were carried out by the method described above. Since the sediments could not be washed before analysis, their compositions were calculated in two different ways, (a) directly from the solutions of R_1 and R_2 in 0.15 *M*



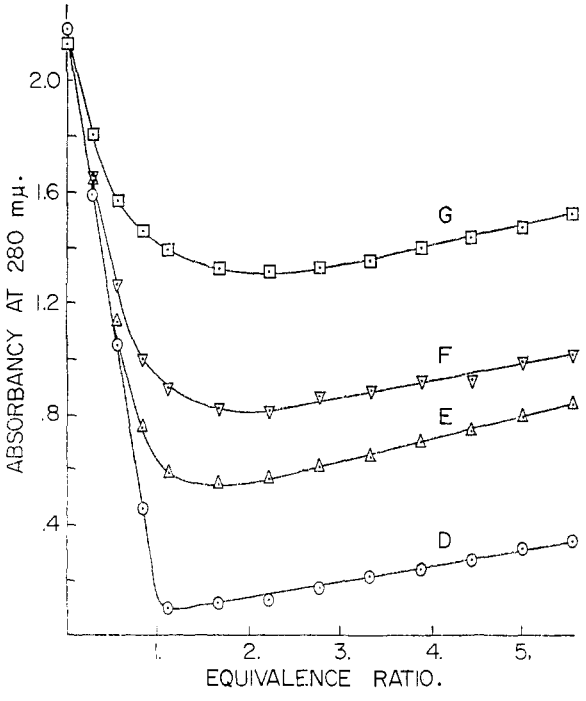
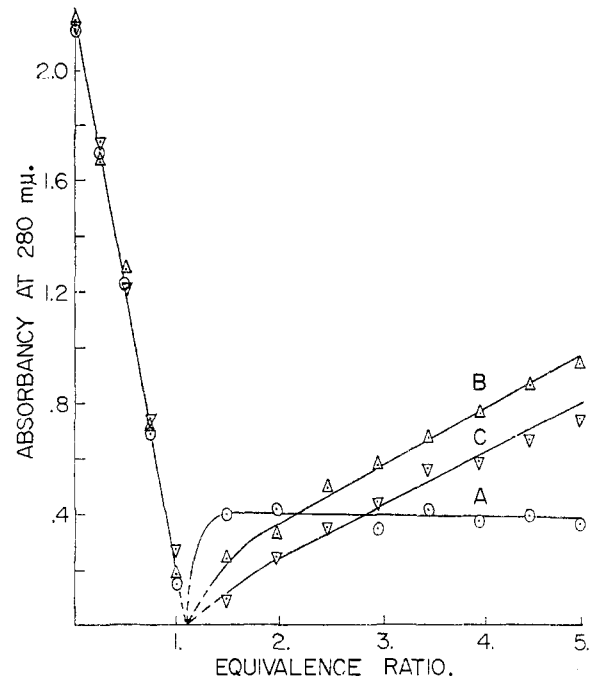


Fig. 1.—Absorbance at 280 mμ of the supernatant solution after being centrifuged at 25,000 × *g*; each solution before centrifuging contained lysozyme (1 mg./ml. or 0.6 μeq./ml.), an amount of potassium chondroitin sulfate indicated by the equivalence ratio and KCl of the concentrations: A, 0.00; B, 0.01 *M*; C, 0.03 *M*; D, 0.06 *M*; E, 0.10 *M*; F, 0.12 *M*; G, 0.14 *M*.



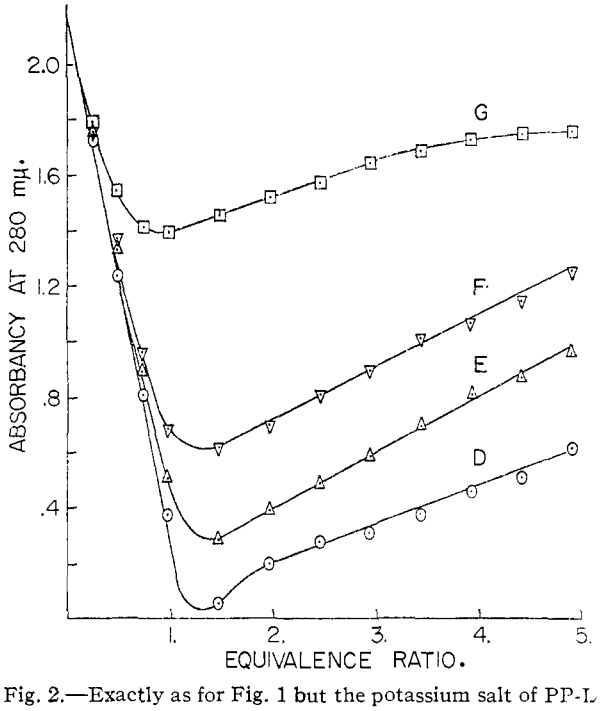


Fig. 2.—Exactly as for Fig. 1 but the potassium salt of PP-L was used instead of potassium chondroitin sulfate.

KCl, and (b) indirectly from the differences between the initial mixture and F_1 and the differences between F_1 and F_2. The results were in fair agreement and are summarized in Table II. The sediments R_1 and R_2 show no constancy in composition, but their equivalence ratio increases with a rise in the e.r. of the initial solutions, and the e.r. of R_2 is always greater than that of R_1. In such sediments containing more polyanion than lysozyme, electrical neutrality is maintained with potassium cations.

To study the state of the lysozyme and PP-L remaining in the supernatant solutions, F_1 and F_2, after removal of the insoluble and/or more rapidly sedimenting compounds, these solutions were examined in the analytical ultracentrifuge. The appearance of each of the patterns was similar in that each consisted of a major broad heterogeneous

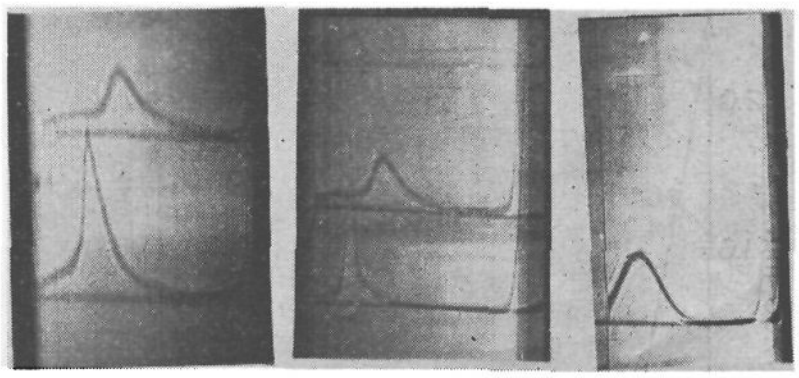


Fig. 3.—Ultracentrifugal patterns of: left, solution of F-2 formed at an e.r. of 4 (bottom) and an e.r. of 5 (top). Each solution contains a major heterogeneous peak and small amounts of more slowly sedimenting material; center, solution of PP-L 1% (bottom). For comparison, the soluble complexes obtained at an e.r. of 5 are shown on top. Right, solution of lysozyme, 1%. All photographs are taken after 96 minutes at 52640 r.p.m., sedimentation proceeds from left to right.

peak with a prominent shoulder on the leading edge. In addition, small amounts of more slowly sedimenting materials were usually present. Figure 3A illustrates the ultracentrifugal patterns obtained with the supernatant solutions of two mixtures formed at equivalence ratios of 4 and 5, following the removal of R_2. The observed s rates were 4.9 and 6.3 S, respectively. In these experiments, the observed s rates, rather than the sedimentation coefficients, extrapolated to infinite dilution, are given, because these compounds exist in a state of equilibrium and therefore do not lend themselves to studies to determine the $s^\circ_{20,w}$. However, in all instances the observed s rates were significantly greater than those observed with PP-L at similar concentrations. Comparison of the appearance of these peaks with those obtained with PP-L or lysozyme in a 1% solution (Fig. 3B and 3C) indicates that the shape of the peak representing the compounds also differed from the sharp narrow band characteristic of PP-L at these high concentrations, as well as the slow, broad peak due to lysozyme. When F_1 was examined under similar conditions, the same major peaks were visible. In addition, a component could be seen to sediment rapidly to the bottom of the cell during the period of acceleration. This sedimented at speeds as low as 12,590 r.p.m. and probably corresponds to R_2 prepared by centrifugation in the preparative ultracentrifuge.

TABLE II

WEIGHT AND COMPOSITION OF THE SEDIMENTS PRODUCED BY CENTRIFUGING SOLUTIONS OF THE COMPOSITION INDICATED, FIRST AT 700 × g TO GIVE SEDIMENT R_1, AND THEN AT 100,000 × g TO GIVE SEDIMENT R_2

Start				R_1		R_2		Total wt. sediment $R_1 + R_2$, mg.
Lys., mg.	PP-L, mg.	Vol., ml.	E.r.	Wt., mg.	E.r.	Wt., mg.	E.r.	
30	9.2	10	1.5	20.4	1.3	5.9	1.6	26.3
30	15.3	10	2.5	12.4	1.3	13.1	1.9	25.5
30	21.4	10	3.5	5.5	1.5	14.4	2.2	19.9
30	24.5	3	4.0	5.6	1.3	14.3	2.8	19.9
30	27.5	10	4.5	2.7	2.0	14.9	2.9	17.6
30	30.5	3	5.0	2.3	3.6	14.1	3.7	16.4
30	33.6	10	5.5	..	..	12.2	4.2	12.2
30	39.7	10	6.5	..	..	6.5	5.2	6.5

The effect of salt concentration on compound formation between PP-L and lysozyme was studied from 0 M to 1.2 M KCl and e.r. from 6.5 to 8. No complexes were detectable in distilled water. In the range near physiologic salt concentration (0.06–0.18 M) the amount and nature of the complex was rather constant. The amount of complex decreased somewhat when the mixing was carried out at progressively higher salt concentrations of 0.3, 0.6 and 1.2 M KCl with a concomitant decrease in the amount of insoluble material.

When chondroitin sulfate and lysozyme were mixed in proportions similar to those used with PP-L, no new peaks in addition to those of lysozyme and chondroitin sulfate were visible after removal of precipitate. The mixture sedimented as a broad peak with s rates ranging from those of pure chondroitin sulfate at low concentrations of lysozyme to those approaching the s rate of lysozyme at higher concentrations. While this finding does not preclude the formation of soluble complexes, it does not offer evidence of their existence. Studies are currently in progress to attempt to detect such complexes by electrophoresis.

Discussion

It appears that just as chondroitin sulfate and PP-L show the typical polyelectrolyte property of behaving as weak electrolytes and binding small counterions, they may also bind cationic proteins. Because of the high charge of lysozyme, its binding with the polyanion would be expected to be favored over the binding of univalent counterions, while at sufficiently high concentrations of small counterions their competitive effect would probably displace lysozyme from association with the polyanion.

The results of the present work are in general conformity with this expected behavior. Some of the salt-like compounds of chondroitin sulfate or PP-L with lysozyme precipitate spontaneously (Table I), and some can be sedimented centrifugally. When e.r. is less than one, the lysozyme is precipitated spontaneously and quantitatively if the KCl concentration is not over 0.06 M. The amount precipitated is a linear function of the amount of chondroitin sulfate or PP-L added and becomes essentially complete when e.r. is one (Fig. 1 and 2). At higher concentrations of KCl, or in the presence of excess polyelectrolyte (e.r. > 1), less lysozyme is precipitated spontaneously (Table I), and the total amount of material sedimentable at higher values of g also progressively decreases. This is paralleled by an increase in the value of e.r. for these sediments (Table II and Fig. 1 and 2). Finally, even after centrifuging at values of g up to 100,000, it is possible to detect in the clear supernatant solutions entities different from lysozyme and PP-L; entities that are presumably undissociated compounds of PP-L and lysozyme.

A major advantage of the centrifugal method of studying protein-polyanion compounds of a salt-like character is that, whenever it is applicable and yields compact sediments, these can be analyzed

to determine the proportions in which protein and polyanion are actually combined. The evidence shows the existence of a series of compounds of lysozyme with PP-L with a continuously varying composition (e.r.). These compounds can be roughly sorted into three groups by their centrifugal properties; (a) those formed at low salt concentration, in the presence of either excess lysozyme or only a small excess of PP-L, which are insoluble in water, easily sedimentable spontaneously or at 700 $\times$ g, and which generally have e.r. between 1.0 and 2.0; (b) those formed in solutions either at higher salt concentration or at higher values of e.r., which are not easily sedimented at 700 $\times$ g but easily sedimented at 30,000 $\times$ g, and generally have e.r. from 1.9 to 5.2; (c) water soluble compounds which give almost clear solutions and are not easily sedimented at 144,000 $\times$ g, but are detectable in the analytical ultracentrifuge by the shapes and sedimentation rates of their peaks as distinct from either PP-L or lysozyme.

Bernfeld and his associates[10] have cited evidence that serum β-lipoproteins interact with a variety of polyanions and that there exist three types of complex distinguished on the basis of precipitability and electrophoretic behavior. The systems and the techniques used for their study are altogether different from the work presented here. Their three types of complex are described as: (I) insoluble products; (II) soluble but with only a little tendency to dissociate; (III) soluble and readily dissociable. While there is an interesting parallel between the three types of lipoprotein–polyanion complex and the rough sorting by centrifugal means of lysozyme–polyanion compounds in the present work, there is also a difference. The three types of compounds sorted centrifugally are all lysozyme-polyanion salts and differ only in the proportion of polyanion to lysozyme (e.r.). The lipoprotein–polyanion complexes are not clearly salt-like in character.

Of the many examples of enzyme inhibition by polyelectrolytes already reported, only a recent one need be cited. Mora and Young[11] studied the effects of polyglucose sulfate on a number of enzymes which were known to be inhibited by polyanions. Among these enzymes was lysozyme. Their conclusion was that inhibition appeared to be due to electrostatic forces and could be reversed by the presence of a polycation that could bind the polyanion more effectively than the lysozyme. In none of these enzyme systems has it been possible to estimate the proportions of protein and polyanion involved in the interaction.

In a survey of the many naturally occurring products which appear to be compounds containing both protein and polysaccharide, Bettelheim-Jevons[12] considered the nature of the forces associating protein and polysaccharide. In the case of the interaction of "acid aminopolysaccharides" with proteins, he considered these forces to be "weaker than covalent bonds" and said that, "In all probability, most complexes of this type are predominantly ionic ones...." This view of the salt-like character of those compounds of protein and polysaccharide which appear to be easy to split into protein and polysaccharide by treatment with dilute alkali is common. It is based on the thought that the effect of the alkali is to suppress the cationic charge on the protein. One difficulty with this view is that there exist many kinds of labile covalent bonds, as for instance those involved in semiacetal formation. Another difficulty is that we do not know of a clear case of a compound of protein and polysaccharide which is of a salt-like character and which requires alkali for its dissociation. In the present work there is little reason to doubt that the sedimented lysozyme-polyanion compounds are simply salts, since they are separated readily by dissolving in 0.15 M KCl and shaking with carboxymethyl cellulose which binds the lysozyme and leaves the polyanion in solution.

Also of interest in the present connection is a centrifugal study of the metachromatic compound of PP-L or chondroitin sulfate with methylene blue.[13] While parallel to the present work in technique, the results differ in that even when a large excess of polyanion is present, the e.r. of the sedimented metachromatic compound is one.

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