

[CONTRIBUTION FROM THE STUDY GROUP ON RHEUMATIC DISEASES AND THE DEPARTMENTS OF CHEMISTRY AND MEDICINE, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

The Outer Sphere Association of Chondroitin Sulfate with Polyvalent Complex Cations¹

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RECEIVED JULY 9, 1956

Chondroitin sulfate can be precipitated from aqueous solution by the polyvalent complex cation, $[\text{Co}(\text{NH}_3)_6]^{+3}$. The ion of higher valence, $[\text{Co}(\text{OH})_6(\text{Coen}_2)_3]^{+6}$, precipitates not only chondroitin sulfate but also hyaluronate. The resulting insoluble salts can be dissolved by addition of neutral salts. Salts with divalent ions are more effective than those with monovalent ions. Divalent anions are more effective than divalent cations. Potassium chondroitin sulfate is among the most effective salts in dissolving these precipitates. These results can be interpreted as the result of two factors: binding of neutral salt anions to complex cobalt cations, and binding of neutral salt cations to chondroitin sulfate anions. Experimental evidence for these two factors is presented.

The trivalent cation $\text{Co}(\text{NH}_3)^{+++}$ has been used to precipitate chondroitin sulfate from aqueous solution.² It also precipitates the related anionic mucoprotein of cartilage.³ Such precipitates have been found to have curious solubility properties. They dissolve readily on addition of a small excess of potassium chondroitin sulfate or of neutral inorganic salts. Precipitated hexamminecobaltic chondroitin sulfate is therefore similar in solubility properties to the insoluble products formed from chondroitin sulfate and lysozyme or serum globulins.⁴ The interaction of chondroitin sulfate with polyvalent cations is of concern not only in the formation of euglobulin-like precipitates with proteins but also in the applications and theory of metachromasia,^{5,6} and it may be involved in the calcification of cartilage^{7,8} through what is called the local mechanism. Interest in all these problems stimulated the study of the reaction in solution between chondroitin sulfate and inorganic ions with high positive charge, under conditions where precipitation occurs and also where none occurs. The ions $[\text{Co}(\text{NH}_3)_6]^{+3}$ and $[\text{Co}(\text{OH})_6(\text{Coen}_2)_3]^{+6}$ were chosen because of their high charge and ability to precipitate chondroitin sulfate and because they are coordinately saturated so complications due to inner sphere coordination between metal cation and chondroitin sulfate would be avoided. The hexavalent complex cobalt cation is of additional interest because it can also precipitate hyaluronate from aqueous solution.

Methods.—Hexamminecobaltic chloride was prepared by the method of Bjerrum and McReynolds.⁹ The salt with the polynuclear cation called hexol nitrate, $[\text{Co}(\text{OH})_6(\text{Coen}_2)_3](\text{NO}_3)_6$ was prepared by the method of Werner.¹⁰ Both salts were crystallized from water. Kjeldahl nitrogen determination and constancy of absorption spectrum on recrystallization were used as criteria of purity. Chondroitin

sulfate was made from beef nasal cartilage,¹¹ crystallized as the calcium salt and reconverted to the amorphous potassium salt with the potassium form of Dowex 50. Potassium chondroitin sulfate is represented by the symbol K_2ChS since there are two cations per period, the corresponding anion by ChS^- , and concentration by periods per liter.

Experimental Results

In a series of experiments with the complex cobalt salt at $1.3 \times 10^{-3} M$, and with K_2ChS varying from 0 to 1.4×10^{-3} formal, turbidities were measured as apparent optical density with a Beckman model DU spectrophotometer, at 600 $m\mu$ for hexamminecobaltic chloride and at 700 $m\mu$ for hexol nitrate. At these wave lengths absorption due to the colored complex ions is negligible. The material precipitated appears under the microscope as spherical droplets. Optical density readings plotted against amounts of K_2ChS added gave straight lines which passed through the origin. This data is not presented in detail since it is altogether as expected.

Similar experiments in which increasing amounts of a solution of complex cobalt salt were added to a constant amount of chondroitin sulfate gave the very different results shown in curves A, B and C, Fig. 1. The higher the concentration of K_2ChS the wider was the initial zone of cobalt concentration in which no precipitation at all occurred. The zone is thus not simply due to the need to exceed a solubility product for then the zone would be narrower at higher K_2ChS concentrations. The hexol cobaltic salt gave curves similar to those of Fig. 1 except that the zones of no precipitation were not so sharply defined. With either cobalt salt it seems that the presence of larger amounts of K_2ChS tends to inhibit precipitation of the insoluble salts. This inhibition is overcome by addition of more cobalt. Figure 1 also shows that the maximum turbidity reached for each curve is not simply proportional to the concentration of chondroitin sulfate. This may be another aspect of the effect of high potassium chondroitin sulfate concentration increasing the solubility of these salts. Trisethylenediammine cobaltic chloride gave curves like those of Fig. 1, and the *d*- and *l*-forms of this complex cation showed identical behavior in precipitating chondroitin sulfate.

Precipitation of these cobaltic chondroitin sulfate salts is inhibited by simple neutral salts just as by K_2ChS . In a series of experiments with the K_2ChS at 1.1×10^{-3} formal, hexamminecobaltic

(1) This investigation was supported (in part) by Grant No. A-28 (C) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, and (in part) by the Masonic Foundation for Medical Research and Human Welfare.

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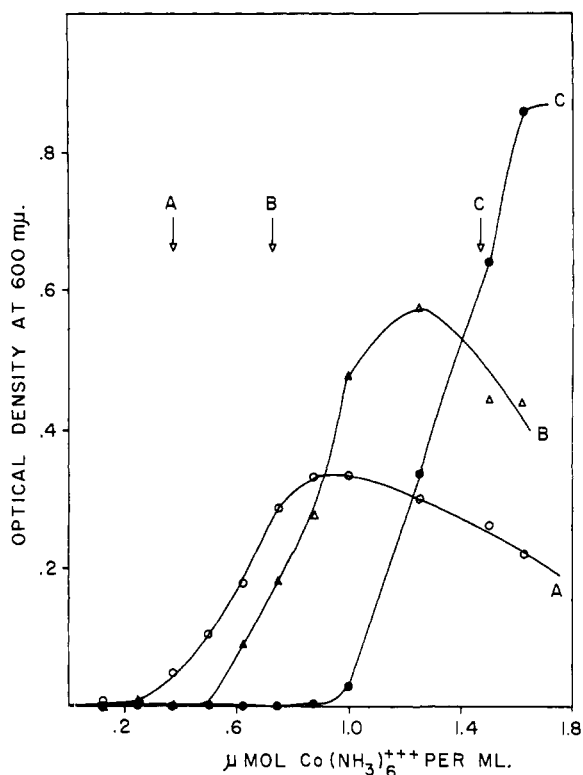


Fig. 1.—Amounts of precipitate produced by mixing increasing amounts of hexamminecobaltic chloride with constant amounts of K_2ChS as measured by the turbidities of the mixtures. Abscissa values are final concentrations of the cobalt salt in mmoles per l. Ordinate values are turbidities measured as the apparent optical densities. Each curve represents these values at a single concentration of K_2ChS as follows: A, 0.55; B, 1.10; C, 2.20 mperiods per l. The arrows A, B and C mark abscissa values where cobalt complex and K_2ChS are equivalent for each curve.

chloride at $1.1 \times 10^{-3} M$, and various neutral salts in concentrations up to 0.02 N , turbidities were read as before. Representative results are shown in Fig. 2 and similar results with hexol nitrate in Fig. 3.

The best way to compare the added salts is in terms of the concentration needed to reduce the amount of precipitate formed to half, that is, the abscissas at which each curve crosses the dotted lines in the figures. Uni-univalent salts are all similar and are least effective. Bi-univalent salts are more effective, and of these calcium and barium chloride are almost identical. Most effective are salts with bivalent anions and K_2ChS itself. The hexol cobaltic precipitate requires over ten times as much uni-univalent or bi-univalent salt to bring it half into solution as is required by the hexamminecobaltic precipitate, but the concentration of K_2ChS required is almost exactly the same. That chondroitin sulfate so readily dissolves both the cobalt precipitates seems to be the reason for the failure to get linear precipitation with increasing cobalt in curves A, B and C of Fig. 1.

A valuable suggestion that may account for the potent effect of divalent anions in dissolving the complex cobaltic chondroitin sulfate precipitates

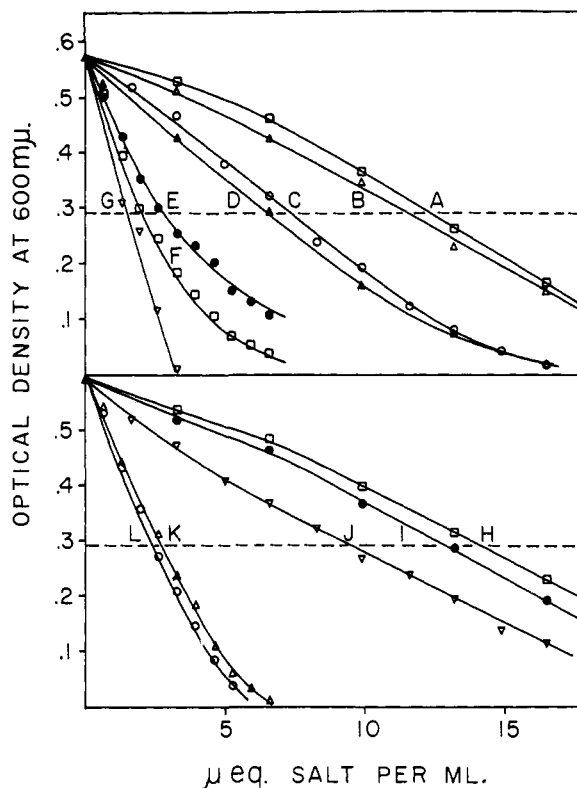


Fig. 2.—Inhibitory effects of increasing amounts of various salts on the amounts of precipitate produced in a mixture containing hexamminecobaltic chloride ($1.1 \times 10^{-3} M$) and K_2ChS (1.1×10^{-3} mperiods per l.) as measured by the turbidities remaining. Abscissas are concentrations of salt in eq. per l. Ordinates are turbidities measured as apparent optical densities. The dotted line represents the ordinate corresponding to half the turbidity produced in the absence of added salts. The added salts are A, KCl; B, NaCl; C, $BaCl_2$; D, $CaCl_2$; E, $K_2C_2O_4$; F, K_2SO_4 ; G, K_2ChS ; H, NaI; I, KI; J, $CdCl_2$; K, $CdSO_4$; L, $ZnSO_4$.

was found in a recent study of Posey and Taube¹² citing spectrophotometric evidence for the combination in solution of the ions $Co(NH_3)_6^{+++}$ and $SO_4^{=}$ to form reversibly dissociable ion pairs which they prefer to call outer sphere complexes. The evidence was based on the increment in absorption at 235 $m\mu$ produced on mixing hexamminecobaltic ion and sulfate and the quantitative relation between the amount of this increment and the concentration of sulfate. If sulfate ions can form such outer sphere complexes with hexamminecobaltic ions it seemed not unreasonable to think that the half ester sulfate ions of K_2ChS might also do so and produce a similar change in ultraviolet absorption. It is not possible to set up experiments similar to those of Posey and Taube, adding gradually increasing amounts of K_2ChS to the hexamminecobaltic ion since precipitation begins immediately, as described above. Only when K_2ChS is in excess, as in the zones of curves A, B and C (Fig. 1) at low cobalt to K_2ChS ratio where no precipitation occurs can the absorption at 235 $m\mu$ be measured and compared with that produced in the presence of simple sulfate. Results

(12) F. A. Posey and H. Taube, *THIS JOURNAL*, **78**, 15 (1956).

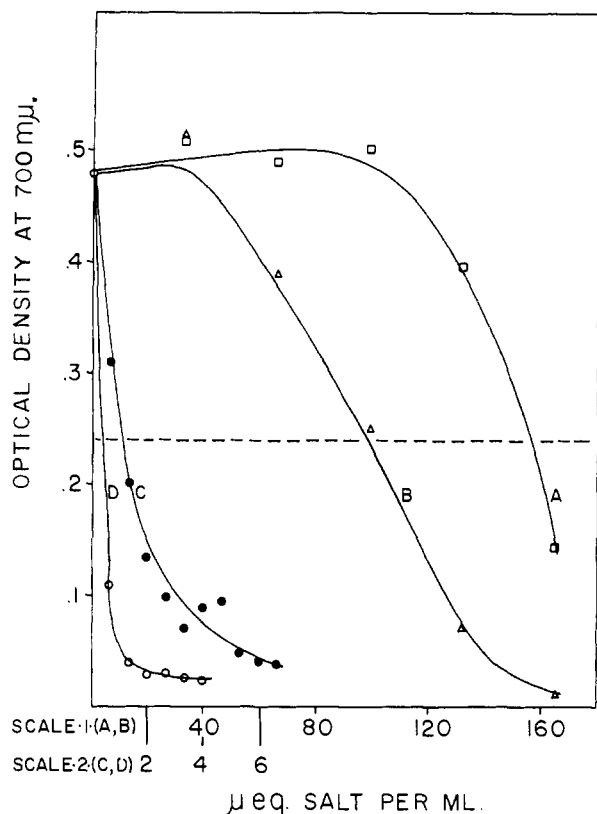


Fig. 3.—Inhibitory effects of increasing amounts of various salts on the amounts of precipitate produced in a mixture containing hexol nitrate ($2.5 \times 10^{-4} M$) and K_2ChS (5.5×10^{-4} periods per l.) as measured by the turbidities remaining. Abscissae, ordinates and dotted line as in Fig. 2. The added salts are A, KCl ; B, $CaCl_2$; C, K_2ChS ; D, K_2SO_4 . Abscissa scale 1 is for curves A and B; scale 2 is for curves C and D.

are given in Table I. The increment in absorption, Δ , at $235 m\mu$, is the difference between the observed absorption of the mixture, corrected for the sulfate or K_2ChS in solution, and the absorption of the cobalt salt alone. With increasing concentration of sulfate the increment in absorption increases toward a value about 0.300 at sulfate over 20μ moles per ml. With K_2ChS the least concen-

TABLE I
INCREMENT IN OPTICAL DENSITY AT $235 m\mu$ (Δ) PRODUCED IN A SOLUTION OF HEXAMMINECOBALTIC CHLORIDE ($7.5 \times 10^{-4} M$) IN THE PRESENCE OF SODIUM SULFATE OR POTASSIUM CHONDROITIN SULFATE (CONCENTRATION OF LATTER, P , IS PERIODS PER LITER)

Concn. $M \times 10^3$	Na_2SO_4		[Potassium chondroitin sulfate] Correction due to K_2ChS		
	Optical density	Δ	Concn. $P \times 10^3$	Optical density	Δ
0.0	0.404	...	0.0	0.389	...
1.5	.509	0.105	1.8	.707	0.060
3.8	.560	.156	2.2	.716	.075
5.6	.593	.189	3.1	.767	.104
9.4	.603	.199	4.4	.809	.149
14.1	.626	.222	5.7	.857	.194
19.7	.652	.248	7.0	.909	.238
37.7	.674	.270	8.8	.981	.298
56.3	.697	.293

tration that will keep the insoluble cobalt salt from precipitating is about 1.8μ periods per ml. which gives an increment in absorption about 80% of the limiting value or about the same as that with sulfate ten times more concentrated. Increasing the K_2ChS concentration causes a further increase in Δ . Because of the high optical density of the chondroitin sulfate alone at $235 m\mu$ it is not feasible to carry its concentration beyond 9μ periods per ml. The results serve as some evidence that in solution, the hexamminecobaltic cations are bound to ChS^- as they are to simple sulfate anions to form ion pairs or outer sphere complexes. Parallel evidence has been found for the binding of SO_4^{2-} and ChS^- to the tris-ethylenediammine cobaltic cation but with the hexavalent cobalt hexol cation no such evidence could be obtained in the accessible ultraviolet.

Further evidence for the binding of sulfate and hexamminecobaltic ions comes from plots of $\Delta/(SO_4^{2-})$ against observed optical density. Such plots should be linear and Posey and Taube showed this to be so though at high sulfate concentration they found deviations indicating the binding of a second sulfate ion to the complex cobalt ion. Curve A of Fig. 4 is such a plot. A similar plot

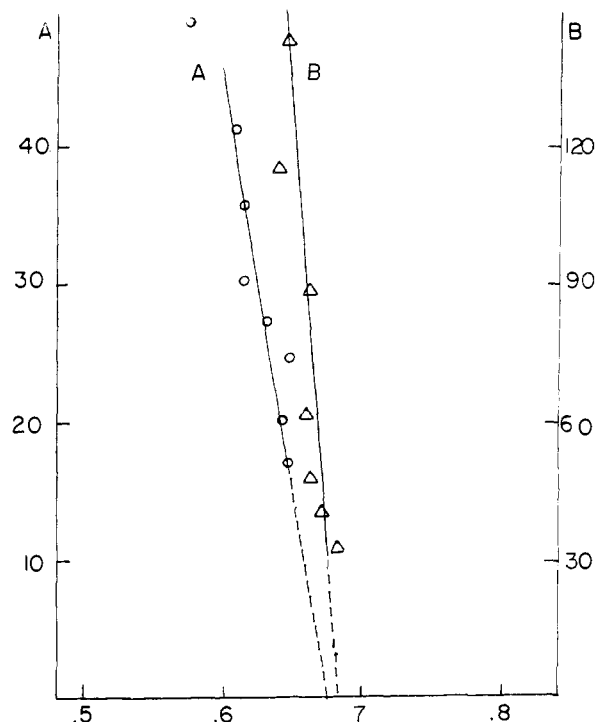


Fig. 4.—Variation of $\Delta/(SO_4^{2-})$ or $\Delta/(ChS^-)$ with observed optical density at $235 m\mu$. Abscissas are the observed optical densities, in the case of curve B corrected for optical density of added K_2ChS . Ordinates are values of $\Delta/(SO_4^{2-})$ for curve A and scale A, or $\Delta/(ChS^-)$ for curve B and scale B.

of $\Delta/(ChS^-)$ against observed optical density corrected for the optical density of the chondroitin sulfate has been made. This gives curve B of Fig.

4, which also appears to be a straight line and may be considered as supporting evidence that ChS^- binds hexamminecobaltic ion in a way similar to that of SO_4^{2-} . That curve B is steeper than curve A indicates that the binding constant of ChS^- for CoA_6^{+3} is greater than that of SO_4^{2-} . The near coincidence of the intercepts of curves A and B at 0.680 on the abscissa axis may also be of significance as this would be the optical density of the outer sphere complex. Similar study of the binding of chloride and acetate ions to the hexamminecobaltic ion indicates that though it occurs it is much weaker.

That both the hexamine and the hexol cobaltic cations are bound by ChS^- even when no precipitate is formed was also shown by equilibrium dialysis. An excess of K_2ChS in the dialysis bag was used so that even when all the cobalt ion outside the bag was drawn inside no precipitation occurred. All solutions contained $4.5 \times 10^{-3} M$ hexamine cobaltic chloride or $4.7 \times 10^{-4} M$ hexol nitrate and in addition the concentration of neutral salt listed in Table II. At the start there were inside the bag 3 ml. of such a solution and outside 5 ml. of the same solution. Inside the bag there were in addition 60 mg. of K_2ChS . After equilibration by shaking for 24 hours the concentrations of cobalt inside and outside were measured by reading optical densities at $475 m\mu$ for the hexamine complex and $490 m\mu$ for the hexol complex. Chondroitin sulfate has only a slight effect on the extinction coefficients of the cobalt salts at these wave lengths. Results are given in Table II

TABLE II

RESULTS OF EQUILIBRIUM DIALYSIS STARTING WITH THE INDICATED CONCENTRATIONS OF COBALT COMPLEX AND NEUTRAL SALT INSIDE AND OUTSIDE THE BAG

Values tabulated are the ratios of the concentrations of cobalt ion inside to that outside after equilibrium was reached. K_2ChS (60 mg.) was inside the bag.

Concn. of neutral salt N	Hexamminecobaltic chloride $4.5 \times 10^{-3} M$			Hexol nitrate $4.5 \times 10^{-4} M$
	KCl	CaCl ₂	Na ₂ SO ₄	KCl
0.00	20	a
.02	11.4	6.39	3.93	...
.05	7.18	4.51	3.04	...
.10	3.82	2.66	1.74	10.6
.20	2.16	5.68
.50	1.76
1.00	1.19	1.32

^a The optical density inside the bag could never be well measured in this case because the solution was always somewhat turbid. However, most of the cobalt was obviously inside the bag.

as the equilibrium ratios of cobalt concentration inside to that outside. In the absence of added neutral salt almost all the cobalt and a considerable portion of the water were pulled into the bag. A great excess (100 to 1000-fold) of potassium chloride over the cobalt salt is needed to displace the cobalt from the ChS^- so the equilibrium ratio approaches unity. Calcium chloride is more effective than potassium chloride in displacing cobalt from ChS^- and sodium sulfate is still more effective. This is the same order as the solubilizing effects of these salts shown in Figs. 2 and 3.

Discussion

Two lines of evidence, the increment in absorption at $235 m\mu$ and equilibrium dialysis, show that chondroitin sulfate polyanion binds hexamminecobaltic cation. The absorption increment indicates in addition that the binding site is specifically at the ester sulfate group. The effect of added neutral salts is to decrease this binding as measured by equilibrium dialysis. In the case of Na_2SO_4 this can be explained as the result of competition between ChS^- and SO_4^{2-} for the cobalt cation to form diffusible from non-diffusible ion pairs. On the other hand the difference between the results of dialysis in the presence of KCl and CaCl_2 cannot be due to anion binding to the cobalt cation, but another competition may account for it. Since the hexamminecobaltic cation can bind to ChS^- , competitive binding of the latter with other cations may also be assumed to occur. This might be expected to be weaker the lower the charge on the cation. Though more weakly bound these mono- or divalent cations at sufficiently high concentration could displace the more firmly bound hexamminecobaltic cation. These assumptions can account not only for the equilibrium dialysis data of Table II, the absorption data of Table I, but also the solubility data of Figs. 1, 2 and 3.

Precipitated hexamminecobaltic chondroitin sulfate can be dissolved as the result of three different equilibria. (1) In the presence of sulfate equilibria are shifted in the direction of formation of outer sphere complexes of hexamminecobaltic ion with sulfate which are soluble and dialyzable. (2) In the presence of extra K_2ChS similar complexes are formed with ChS^- which are soluble and non-dialyzable. (3) In the presence of simple cations at sufficient concentration the latter competitively bind ChS^- and the displaced cobalt becomes dialyzable.

A similar set of equilibria may hold also for the hexol cobaltic complex. In this case binding to chondroitin sulfate seems to be much firmer as judged by the much higher concentration of neutral salt needed either to dissolve the precipitates or to displace the cobalt from combination as shown in equilibrium dialysis. The data presented indicate binding of cations to chondroitin sulfate which is firmer the higher the valence of the cation; $(\text{Co hexol})^{+6} > (\text{Co hexamine})^{+3} > \text{Ca}^{+2} > \text{K}^{+1}$.

The interpretation suggested does not take account of the possible effects of changing ionic strength. Because of the very pronounced solubility effects of low salt concentrations and the high charges of both chondroitin sulfate and the cobalt complexes experiments have not yet been devised in which significant variations could be studied at constant ionic strength. The interpretation of these results in terms of cation binding by chondroitin sulfate is similar to that of Wall and Grieger¹³ in studies on sodium polyacrylate in transference cells citing evidence for the reversible binding of sodium cations to polyacrylate anions.

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