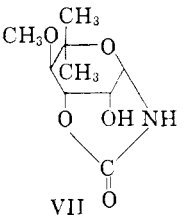
TABLE I

Compound hydrolyzed	Moles IO_4^- consumed[12] per mole cpd. 15 min.	60 min.
α-Methyl 4-O-methyl-5,5-dimethyl-L-lyxopyranoside (IV)	1.76	1.84
α-Methyl 3-O-carbamyl-4-O-methyl-5,5-dimethyl-L-lyxopyranoside (II)	0.76	0.77
β-Methyl 3-O-carbamyl-4-O-methyl-5,5-dimethyl-L-lyxopyranoside (III)	0.68	0.68
α-Methyl 2-O-carbamyl-4-O-methyl-5,5-dimethyl-L-lyxopyranoside (VI)	0.03	0.03

dimethyl-L-lyxopyranoside (IV), m.p. 69.5–71°, $[\alpha]^{25}D$ −45° (*c* 1.5 in water), was 0.65 g. (80%). When this product was mixed with an authentic sample of IV, the mixture melted at 69–71°.

Periodate Oxidation Studies.—Samples of the following glycosides were hydrolyzed on the steam-bath for an hour with 0.1 *N* hydrochloric acid (approximately 0.033 mmole/ml.). The hydrolysates were neutralized with equivalent amounts of 1 *N* sodium hydroxide and aliquots (containing approximately 0.1 mmole of aldose) were oxidized with excess 0.1 *M* sodium metaperiodate. The results are shown in Table I.

(12) Hinman, Caron and Hoeksema have shown (ref. 4) that acidic hydrolysis of α-methyl 3-O-carbamyl 4-O-methyl-5,5-dimethyl-L-lyxopyranoside (II) yields in addition to 3-O-carbamyl-4-O-methyl-5,5-dimethyl-L-lyxose a by-product which they have formulated as the bicyclic compound VII. Formation of such a by-product would account for the low consumption of periodate in the oxidations of II and III.



VII

RAHWAY, N. J.

[CONTRIBUTION NO. 2501 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY OF THE CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Dissociation of Antigen–Antibody Precipitates in Alkali Chloride Solutions

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The dissociation of antigen–antibody precipitates in solutions of alkali chlorides was studied by measuring the solubility of specific precipitates of purified bovine serum albumin and rabbit anti-bovine serum albumin in solutions of LiCl, NaCl, KCl and CsCl over the concentration range 0.08 to 1.6 molar. At electrolyte activities below 0.25 the solubility of the precipitate at a given activity has the order CsCl > KCl > NaCl > LiCl. Above an activity of 0.25 different results are obtained. The order of the results below an activity of 0.25 is most readily explained by the interaction of the hydrated cations with negative sites involved in the antigen–antibody bonds.

Introduction

The properties of precipitates of antigen (Ag) and antibody (Ab) have been shown to depend on the *p*H and the concentration of the inorganic salt solutions in which the Ag–Ab reaction takes place. The rate of flocculation and the amount of precipitate formed in these reactions are influenced by the *p*H and the electrolyte concentration of the medium in a variety of Ag–Ab systems, including polysaccharide:protein as well as protein:protein precipitin reactions.[2–8] In general, these experiments show that the amount of precipitate formed between antigens and rabbit antibodies is reduced at $5 > pH > 8$ and is also reduced by increasing electrolyte concentration up to 2 *M*. Furthermore, a portion of the Ag–Ab precipitate is rendered soluble by resuspension in NaCl solutions.[9,10] The evidence currently available indicates that treatment of such precipitates with saline solutions preferentially extracts antibody.[4,5,11]

Although it has been pointed out that different inorganic salts vary in their ability to reduce the amount of an Ag–Ab precipitate at equivalent ionic strength, there has been no systematic study of this effect with respect to the members of the alkali metal ions series compared at equivalent activities.[4,8] A comparison of this kind might disclose different degrees of interaction between the inorganic ions and the surfaces of the antigen and antibody molecules or, alternatively, might indicate that the dissociative effect of the electrolytes is a non-specific function of electrolyte activity. In the former case, one might expect that the smaller the ion the closer the distance of approach to the protein surface (perhaps to the specific combining site of the antigen or antibody) and the greater the dissociation of the precipitate. In the latter case, all of the univalent:univalent salts would have the same dissociative effects at the same activities and temperatures.

This paper reports the dissociative effects of the alkali chlorides (Li, Na, K, Cs) on precipitates formed from bovine serum albumin and rabbit antiserum at 3°.

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Experimental

Antigen.—Crystallized bovine serum albumin (BSA) (Armour) was the antigen in all instances. The production of anti-BSA serum was induced in rabbits by serial inoculation of 0.01 g. BSA per ml. of 1% NaCl. The same antigen was used to form specific precipitates by dissolving BSA in 0.08 *M* LiCl in one set of experiments and in 0.04 *M* LiCl in a second set.

Antisera.—Rabbits were immunized by seven serial intravenous injections of 2 ml. of 1% BSA over a period of two weeks, rested for one week and then further immunized by six 1 ml. injections of 1% BSA on alternate days. The animals were bled by heart puncture six days after the last injection. Sera of medium and high precipitin titer from three to five rabbits were pooled and stored frozen. Each pool was processed by removal of lipids, decomplementation and dialysis. Clarified sera were aspirated with syringe and needle after flotation of lipid particles by centrifugation at ~ 50,000 g. for thirty minutes in the cold. Decomplementation was achieved by thorough mixing of the clarified rabbit antiserum with washed precipitates of pneumococcus polysaccharide types I and II and refined horse antisera (Lederle) in a ratio of 1 ml. of rabbit anti-BSA serum to 0.13 mg. of precipitate nitrogen, incubating the mixture at 3° for 24 hr. and recovering the serum by centrifugation in the cold. This process was repeated twice to insure thorough decomplementation.[12] Following this procedure, 300 ml. of serum was dialyzed in Visking sausage casing against 5 to 7 l. of 0.08 *M* LiCl for 96 hr. with five changes of the external solution. An additional 300 ml. of serum was similarly dialyzed against 0.04 *M* LiCl. The clarified, decomplemented, dialyzed sera were stored in the frozen state until used. These are designated processed sera in this report.

Dissociation of the Precipitates—Set No. 1.—The point of maximum precipitation was determined by duplicate precipitin curves in 0.08 *M* LiCl solutions of both antigen and antibody. A maximum precipitate of 0.57 mg. of nitrogen was obtained with 1.0 ml. of 1:2000 dilution of BSA and 1.0 ml. of processed antiserum. The antigen dilution refers to g. per volume of final solution. A dilution of 1:2000, therefore, corresponds to a BSA concentration of 0.5 mg./ml. The dissociation of this precipitate in the presence of the alkali chlorides was studied by the following procedure: 1.00 ml. of 1:2000 dilution of BSA in 0.08 *M* LiCl plus 1.00 ml. of processed antiserum (dialyzed to equilibrium in 0.08 *M* LiCl) was added to each of twenty-eight 13 × 100 mm. test tubes, mixed and allowed to stand at room temperature for 2 hr. All tubes were then remixed and incubated at 3° for an additional 48 hr. The precipitates were harvested by centrifugation in the cold at ~ 1500 g. for thirty minutes, decanting the supernatants and resuspending in 0.08 *M* LiCl. This procedure was repeated three times. Four ml. of salt solution was then added to each tube. The salt solutions were made up to the following molarities: 0.16, 0.32, 0.60, 0.80, 1.20 and 1.60, with LiCl, NaCl, KCl and CsCl. Glass encased, magnetized micro-wires were added to each tube and the tubes were placed on magnetic stirrers and incubated with continuous stirring of the precipitates at 3° for 48 hr. The supernatant salt solutions were collected free of the precipitates by decantation after centrifugation in the cold, followed by filtration of the decanted solution through No. 2 Whatman paper. A 2.00 ml. aliquot of each supernatant was added to 2.0 ml. of 20% trichloracetic acid (TCA), allowed to stand in the cold for 5 hr. after mixing, centrifuged and then decanted and drained on filter paper. The TCA precipitable material was analyzed for nitrogen content by the micro-Nessler procedure.[13] The TCA method was found to give quantitative recoveries of protein in control studies with dilute solutions of BSA (1:2000 or 1:4000). To obtain representative mean values, the entire protocol was replicated four times.

Set No. 2.—The same procedure was followed as outlined for Set No. 1 except that both the BSA and the antisera were in 0.04 *M* LiCl at the time of formation of the precipitates. The precipitates were washed three times with 0.04 *M* LiCl, and salt solutions of 0.08 and 0.12 *M* were included in the dissociation studies. The point of maximum precipitation was determined in a 0.04 *M* LiCl medium. The precipitate at the point of maximum precipitation formed from 1.0 ml. of a 1:2000 dilution of BSA plus 1.0 ml. of processed serum contained 0.60 mg. of nitrogen.

A series of attempts were made to identify the materials released into the supernatants from the Ag–Ab precipitates in strong salt solutions. The supernatants were concentrated by pervaporation or by dialysis against polyvinylpyrrolidone or by ultrafiltration. Subsequent paper electrophoresis in barbital buffer (pH = 8.6, μ = 0.055) or acetate buffer (pH = 5.0, μ = 0.1) revealed only non-migrating protein at the point of application. A similar component was found in the electrophoresis of gamma globulin purified by ammonium sulfate fractionation. However, this does not allow one to conclude that the supernatant protein is antibody.

Results

Reproducibility of the BSA–anti-BSA precipitates with respect to the total amount exposed to each salt solution at each concentration was determined by analyzing replicates of the total amount of precipitate in control tubes prior to the addition of salt solutions. The precipitates formed in 0.08 *M* LiCl averaged 0.569 mg. of nitrogen with a range of 0.566 to 0.573 mg. in five samples. Those formed in 0.04 *M* LiCl averaged 0.604 mg. with a range of 0.579 to 0.618 mg. in eleven samples. These data indicate that the constancy of total precipitate yield from tube to tube, as well as between the two sets of experiments, was satisfactory.

The results of the two sets of experiments are depicted in Figs. 1 to 4. Figure 1 demonstrates a positive curvilinear relation between TCA precipitable nitrogen, presumably protein, in the salt solution supernatants and the molarity of the alkali metal chloride solutions. It is apparent by inspection that with NaCl and KCl solutions the protein in the supernatant attains a plateau at concentrations of about 1.0 *M* but that the dissociative effects of LiCl and CsCl on the Ag–Ab precipitate are maintained throughout the range of concentrations which were tested (0.16 to 1.6 *M*). The second set of experiments covered a greater range of salt solution concentrations (0.08 to 1.6 *M*); these data are depicted in Fig. 2. The similarity of these results to those obtained in the first set of experiments is readily apparent. These results extend and confirm the earlier observations on the dissociative or solubilizing effects of inorganic salt solutions at neutral pH on Ag–Ab precipitate.[4,9–11] To compare the intensity of the dissociative effects of the members of this series of chlorides, the mean nitrogen concentration of the supernatants is plotted as a function of the activity of the salt solutions $[(M \cdot \gamma_{\pm})^2$, where $\gamma_{\pm}$ is the mean activity coefficient at 25°[14]] in Figs. 3 and 4. The results obtained with precipitates prepared in 0.08 *M* LiCl are depicted in Fig. 3 and those prepared in 0.04 *M* LiCl in Fig. 4. Both sets of experiments reveal that at any given activity below 0.25 the magnitude of precipitate dissociation is in the order CsCl > KCl > NaCl > LiCl. At activities greater than 0.25, however, this order is no longer preserved, the LiCl, NaCl and KCl lines crossing each other so that at an activity of 0.6 the strength of the dissociation is in the order CsCl > LiCl > NaCl ≅ KCl. The order of the effects of the members of the alkali chloride series on precipitate dissociation suggests that specific interactions between

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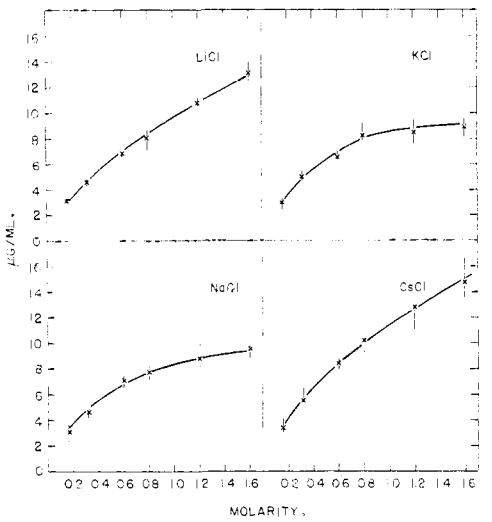

Fig. 1.—Mean nitrogen concentration in μg./ml. of supernatant salt solutions after equilibration of specific Ag–Ab precipitates with the salt solutions *vs.* molarity of the salt solutions. Specific precipitates were formed in 0.08 *M* LiCl. Vertical lines represent the range of nitrogen values obtained.

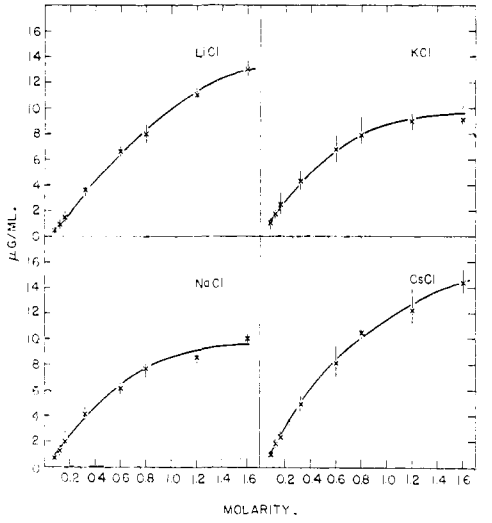

Fig. 2.—Mean nitrogen concentration in μg./ml. of supernatant salt solutions after equilibrium of specific Ag–Ab precipitates with the salt solutions *vs.* molarity of the salt solutions. Specific precipitates were formed in 0.04 *M* LiCl. Vertical lines represent the range of nitrogen values obtained.

the inorganic ions and the proteins rather than non-specific charge effects are involved in the disruption of the Ag–Ab precipitates.

Discussion

A thorough description of the interactions between the alkali chloride ions and the components of an antigen–antibody precipitate is at present impossible. The metal and chloride ions may be bound to specific antigen or antibody sites and also to non-specific sites on the protein molecules or may affect the Ag–Ab combination in a more complex way. The positive correlation between salt activities and supernatant-protein concentration,

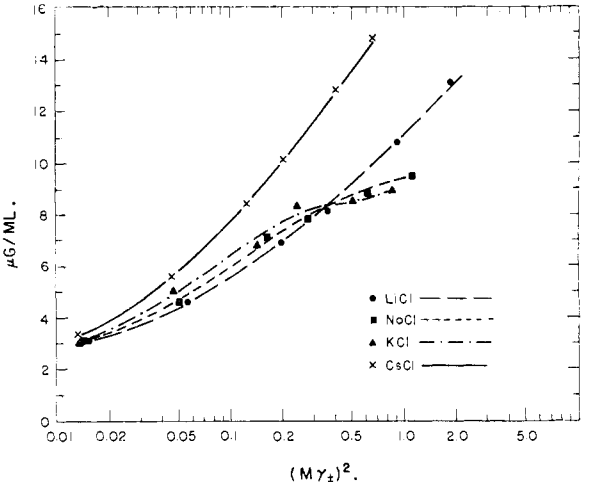

Fig. 3.—Mean nitrogen concentration in μg./ml. of supernatant salt solutions after equilibration of specific Ag-Ab precipitates with the salt solutions *vs.* activity $(M\gamma_{\pm})^2$ of the salt solutions. Specific precipitates were formed in 0.08 *M* LiCl.

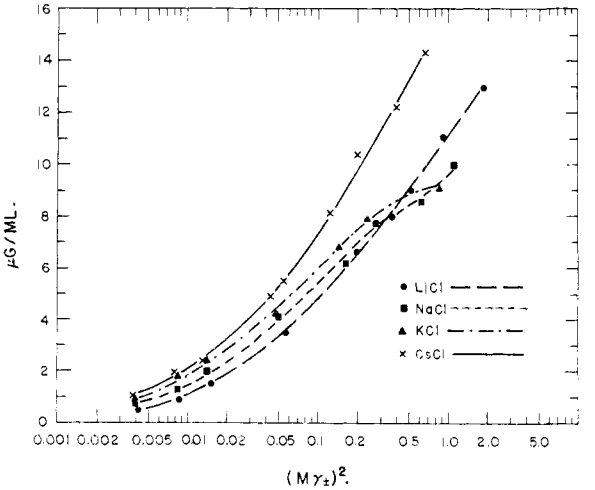

Fig. 4.—Mean nitrogen concentration in μg./ml. of supernatant salt solutions after equilibration of specific Ag–Ab precipitates with the salt solutions *vs.* activity $(M\gamma_{\pm})^2$ of the salt solutions. Specific precipitates were formed in 0.04 *M* LiCl.

however, can be most easily explained by weak binding of the elementary ions to the specific combining sites. In the case of the BSA–anti-BSA system, a negatively charged carboxyl group is thought to be active at the antigenic determinant site, and a corresponding positive group is thought to be involved in the specific antibody site.[15,16] The binding of a metal ion near the antigenic site and/or of a chloride ion near the antibody site would thus tend to interfere with recombination of antigen and antibody and result in preferential solubilization of antibody.[11]

Another problem which must be considered in a thorough description of this system is heterogeneity

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in the strengths of the specific combining sites. Nisonoff and Pressman[17] have summarized the evidence for antibody heterogeneity and presented data which indicate a wide distribution of equilibrium constants for the combination of hapten with anti-hapten antibody obtained from a single animal. Furthermore, Aladjem and Lieberman[6] found quantitative differences in the reduction of precipitate by increasing concentrations of NaCl when pseudoglobulin antibody was compared to euglobulin antibody as reactants with the same antigen. The effect of increasing salt concentration in the present work would be expected to be the solubilization of weak antibody at low salt concentrations and stronger antibody at high salt concentrations. Above about 2 *M* salt concentration salting-out effects begin to occur, and the dissociated antibody may no longer be soluble. In any event in the BSA–anti-BSA system the maximum solubilization of specific precipitate is small compared to the total amount of precipitate (only ~10%) so that the ions may dissociate antibody from only a comparatively small fraction of the antigenic sites.

It is obvious from an inspection of Figs. 1 and 2 and of Figs. 3 and 4 that preparation of the antiserum as a 0.04 *M* LiCl compared with 0.08 *M* LiCl solution did not modify the results except that more nitrogen appeared in the supernatants at salt concentrations of 0.16 to 0.32 *M* in the 0.08 *M* LiCl system. At higher salt concentrations, however, there was no discernible difference in the two sets of experiments. Dialysis of the antisera against 0.04 *M* LiCl resulted in the formation of a fine precipitate. This precipitate was redissolved in 0.16 *M* NaCl and was found to give a precipitin curve with BSA. Therefore, preparation in 0.04 *M* LiCl removes a portion of the euglobulin antibody fraction, whereas preparation in 0.08 *M* LiCl results in no loss of globulin. The removal of a portion of euglobulin antibody presumably accounts for the lesser amounts of nitrogen eluted by the dilute salt solutions in the 0.04 *M* LiCl system.

The order in which the alkali chlorides solubilize components of the Ag–Ab precipitate at activities below 0.25 suggests that there are weak binding reactions between the hydrated cations and the negative sites involved in the antigen–antibody bonds.[18] The degree of hydration of the alkali ions increases in the order Cs < K < Na < Li.[14] This is the reverse of the order of effectiveness of the salts in releasing protein from the precipitates CsCl > KCl > NaCl > LiCl. Hence, in the more dilute electrolyte solutions, the quantity of precipitate dissociation appears to be inversely related to the size of the hydrated cation or directly related to the distance of closest approach of such a hydrated cation to the negative site involved in an antigen–antibody bond.

At activities > 0.25 the nitrogen curve for LiCl crosses the curves for KCl and NaCl, and the solubilizing effectiveness of the salts falls in the order CsCl > LiCl > NaCl ≅ KCl. We do not have a completely convincing explanation for this effect, but the following tentative explanation can be submitted: The concept of "localized hydrolysis" has been introduced by Robinson and Harned[19] to explain the tendencies of certain alkali salts to have activity coefficient values in which CsX > KX > NaX > LiX, where X represents an anion capable of hydrogen bonding such as formate, acetate or fluoride. The activity coefficients for the alkali salts of non-hydrogen bonding anions such as chlorides fall in the reverse order. "Localized hydrolysis" involves the polarization of water molecules by the strong electric field at the surface of an ion of small crystallographic radius such as Li^+. The positive end of the water dipole in an inner hydration shell would then show an increased tendency to form bonds with proton acceptors such as carboxylate ions. It would be expected that the ions most hydrated in solution (such as Li^+) would tend to show this "localized hydrolysis" if the energy necessary to break down the large outer hydration shell could be compensated for by the bond energy of the interaction of the polarized water molecules with the proton acceptor ions in the system. In the case of the antigen–antibody system, carboxyl groups are present on the protein surfaces, and some of these groups may be involved in specific Ag–Ab bonds. As the concentration of an electrolyte such as LiCl is increased, the amount of hydration per Li^+ ion may decrease; the tendency of the Li^+ to be associated with a carboxyl group on the protein through "localized hydrolysis" may become greater. Therefore, LiCl would become relatively more effective in disrupting Ag–Ab bonds as the salt activities increased, and the nitrogen curve for LiCl might rise above the curves for KCl and NaCl.

It is also possible that the curves at activities >0.25 may be explained by an increased tendency of KCl and NaCl to salt out protein over that exhibited by LiCl. This possibility is supported by the data depicted in Figs. 3 and 4 where the NaCl and KCl curves appear to deviate from the CsCl, LiCl curves rather than the converse.

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