ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICAL CHEMISTRY, HARVARD UNIVERSITY]

Fractionation of Antibodies from Human γ-Globulins with Zinc Salts[1]

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The solubility of γ-globulins was studied in an aqueous system as a function of *p*H and ionic strength in the presence of different concentrations of zinc ions and chelating agents. The content of antistreptolysin-O, diphtheria antitoxin and influenza A virus antibody was determined in the fractions obtained by extracting a zinc-γ-globulin precipitate with 50 and 100 m*M* glycine (fraction 1 and 2) and 50 and 100 m*M* tartrate (fraction 3 and 4). The antibody activity per unit weight of γ-globulin (specific activity) varied considerably: the largest differences were obtained in the case of diphtheria antitoxin which was concentrated mainly in fraction 1 and in the residue left after the 4 successive extractions. The fractionation of antistreptolysin-O largely depended on the amount of zinc used for the initial precipitation. It appeared that the solubility of the zinc–protein complex varied from one antibody to another.

Considerable attention has been directed to the purification of antibodies from hyperimmune sera. Using normal human plasma as a starting material antibodies have been concentrated in one fraction which consisted mainly of γ-globulin. Little work, however, has been done on the subfractionation of normal human γ-globulin into its antibody constituents. By fractional precipitation with ethanol at specified *p*H and ionic strength, Oncley and coworkers have obtained, with good yields, two subfractions from γ-globulin with partly different antibody composition.[2] Highly purified samples of antibody to influenza A virus have been obtained from human γ-globulin by adsorption with an ion-exchange resin–influenza virus complex and dissociation of the virus antibody complex; with this latter procedure, however, the yields have been uniformly low.[3]

A new method was developed in this Laboratory for the fractionation of plasma proteins, based on the reversible association of metal ions with certain functional groups of the protein.[4,5] Zinc ions, for instance, at a concentration of 15 m*M* will form water-soluble complexes with serum albumin, α-lipoprotein, α-glycoprotein, alkaline phosphatase, serum esterase and β-metal combining protein. Other plasma proteins including the β-lipoproteins, γ-globulin and fibrinogen are insoluble in water under the same conditions. The latter can be fractionated by selectively dissociating the zinc–protein complexes with chelating agents which will compete with the protein for the metal. The association constant of these chelating agents for the metal must only slightly exceed the constants of the ligands on the protein which is to be dissolved. Otherwise complete dissociation occurs and no fractionation is obtained. In the present study this method was extended to the fractionation of antibodies from human γ-globulin. The activity of antistreptolysin-O and diphtheria antitoxin were used as criteria because of the reproducibility and the relative accuracy of their assays. In a first part, the solubility of human γ-globulin was investigated as a function of zinc ion concentration, *p*H, ionic strength and the concentration of various chelating agents. In a second part subfractions of γ-globulin were prepared on a larger scale and tested for antibody activity.

Experimental

1. **Solubility Studies.** (a).—Fraction II prepared from human plasma by methods 6 and 9 was used as a starting material. Electrophoresis in sodium diethylbarbiturate buffer *p*H 8.6, ionic strength 0.1 showed that less than 4% of the preparation had a mobility exceeding -1.5×10^{-5} cm.²/volt sec. The solubility of γ-globulin was measured in 0.15 *N* sodium nitrate at five zinc concentrations ranging from 0.2 to 5 m*M* zinc nitrate. The *p*H was adjusted from 5.6 to 8.5 with 0.02 *N* sodium hydroxide. Fraction II was added to give a final concentration of 10 g. protein/l. Each suspension was equilibrated at 0° overnight and the optical density of the supernatant at 280 mμ was determined after centrifugation. The protein concentrations were calculated on the basis of an extinction coefficient $E^{1\%}_{1\,cm}$ at 280 mμ of 14.5.[6] Figure 1 shows that the solubility minimum was shifted to lower *p*H values (6.5–6.3) with increasing zinc concentration. In absence of zinc, γ-globulins are least soluble in the *p*H range around 7.0. The solubility was less than 1 g./l. in the presence of 1 m*M* zinc nitrate.

(b).—The effect of different chelating agents on the solubility of human γ-globulin in the presence of zinc nitrate was determined using the same technique. A neutral 0.5 *M* solution of the chelating agent was prepared by neutralizing the acid with sodium hydroxide. The final concentration of each chelating agent was varied from 0 to 150 m*M*. The other variables were kept constant: the zinc concentration at 5 m*M*, the ionic strength at 0.15, the protein concentration at 10 g./l. and the *p*H at 7.4. The solubility of γ-globulin under those conditions is represented in Fig. 2. The molar ratio of chelating agent to zinc was plotted on the abscissa; for example, the precipitate of γ-globulin formed by 5 m*M* zinc nitrate was completely dissolved in presence of 150 m*M* sodium diglycolate. The effect of ionic strength was studied in a similar manner; the final concentration of γ-globulin in each tube was 5 g./l. Five different ionic strengths ranging from 0.005 to 0.25 were adjusted with 0.75 *M* sodium chloride. The *p*H range studied was between 6 and 8. The solubility was determined in presence of 5 m*M* zinc nitrate and 150 m*M* glycine. The results, plotted in Fig. 3, show that the effect of sodium chloride was negligible at neutral *p*H. At lower *p*H values increasing ionic strength decreased the solubility. At *p*H 7.5 the usual "salting-in" effect predominated.[7]

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(2) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, THIS JOURNAL, **71**, 541 (1949).

(3) H. C. Isliker, *Ann. N. Y. Acad. Sci.*, **57**, 225 (1953).

(4) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma, THIS JOURNAL, **72**, 465 (1950).

(5) E. J. Cohn, D. M. Surgenor, K. Schmid, W. H. Batchelor, H. C. Isliker and E. H. Alameri, *Faraday Soc. Disc.*, **13**, 176 (1953).

(6) F. R. N. Gurd, unpublished data.

(7) J. L. Oncley, F. H. Gordon, F. R. N. Gurd and R. A. Lontie, Abstract of 119th Meeting of Am. Chem. Soc., Boston, Mass., 1951, p. 25C.

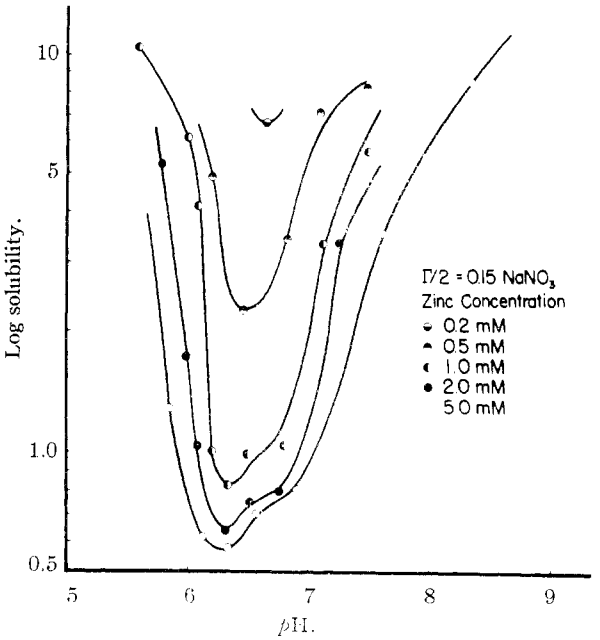

Fig. 1.—The solubility of human γ-globulin in water at 0° as a function of *p*H at different zinc concentrations.

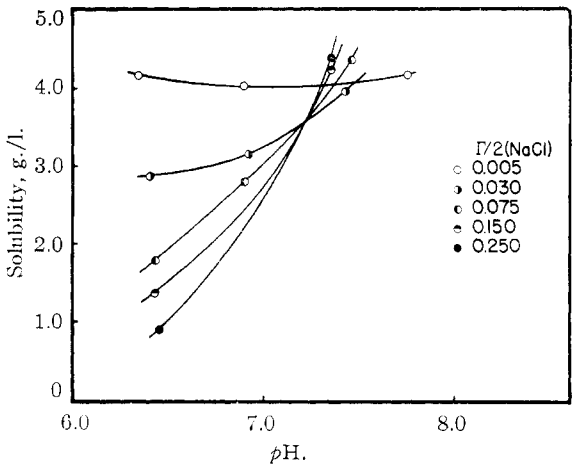

Fig. 3.—The solubility of human γ-globulin in water at 0° in the presence of 5 m*M* zinc nitrate and 150 m*M* glycine.

m*M* glycine (fraction 1 and 2) and 50 and 100 m*M* sodium tartrate (fraction 3 and 4) leaving a final residue (fraction 5). Between each extraction, the precipitate was washed twice with 50 ml. of 0.15 *M* sodium chloride and 0.1 m*M* zinc lactate solution. In each case the two wash solutions were added to the preceding fraction. The residue (fraction 5) was dissolved in 10 ml. of 0.1 *M* sodium citrate.

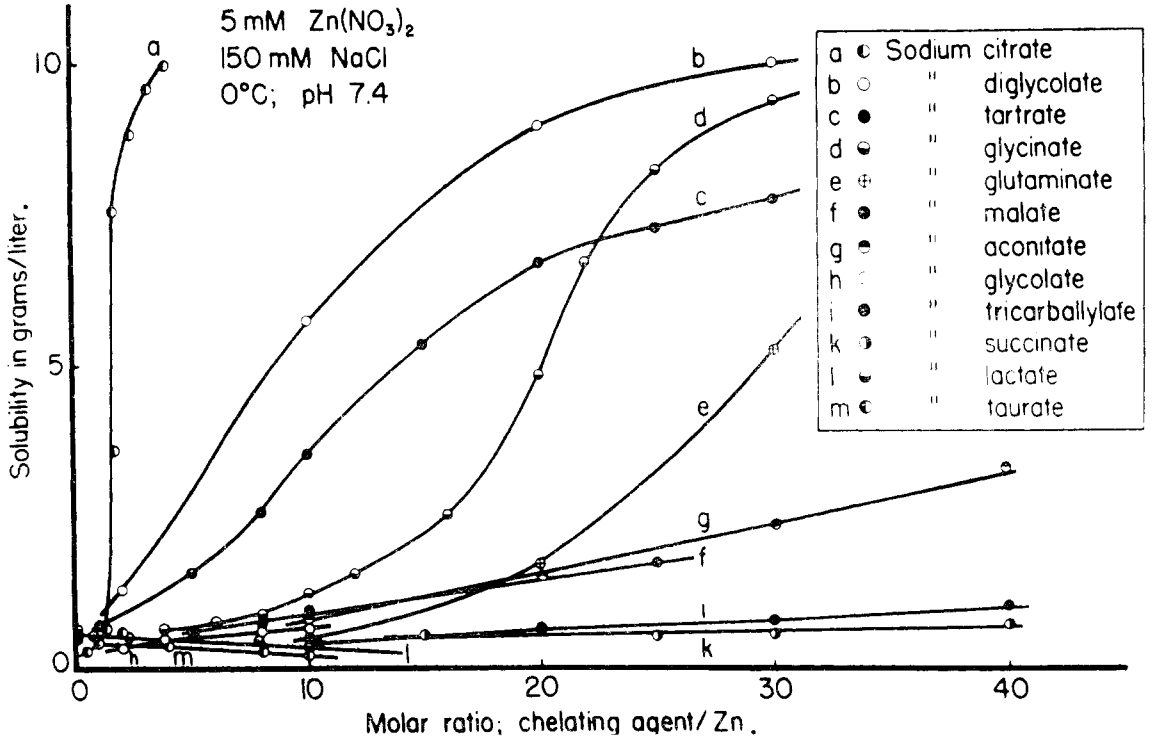

Fig. 2.—The effect of chelating agents on the solubility of human γ-globulin in the presence of zinc nitrate.

2. **Preparation of Fractions for the Determination of Antibody Content.**—Two aqueous solutions of fraction II, each containing 6 g. of γ-globulin, were precipitated at 2° with zinc lactate to give a final zinc concentration of 5 and 20 m*M*, respectively. The *p*H was adjusted with diethylbarbiturate buffer to 7.2 and each solution was made up to 200 ml. with 0.15 *M* sodium chloride. The suspensions were equilibrated overnight at 2° and centrifuged. The precipitate was washed twice with 0.15 *M* sodium chloride solution containing 0.1 m*M* zinc lactate. Fractional extraction of the zinc–γ-globulin complexes was then carried out by four successive extractions with 50 ml. of 50 and 100

In order to remove the zinc, each fraction was dialyzed for 24 hours against 0.01 *M* ethylenediaminetetraacetate (EDTA) and 0.1 *M* sodium chloride solution. EDTA was removed by another 24 hours of dialysis against 0.1 *M* sodium chloride solution. The amount of protein in each fraction was determined by optical density measurement at 280 mμ.

The total amounts of protein extracted into each fraction are summarized graphically in Fig. 4. A striking difference was observed between the preparations precipitated at 5 and 20 m*M* zinc lactate. In the former case, large amounts of protein were extracted with glycine, whereas in the latter the excess of zinc present in the precipitate competed with the

chelating agents. In both cases the total amount of protein extracted into each fraction equaled the amount of protein carried down by zinc in the initial precipitation. Since our methods of antibody determination require fairly concentrated γ-globulin solutions, each fraction was freeze-dried and redissolved in small volumes of distilled water to give a final protein concentration of about 10%. Before testing for antibody the fractions were dialyzed for 24 hours against 0.15 *M* sodium chloride.

3. Immunologic Assay of the Subfractions.—The testing for antibody was performed according to methods previously used.[2,8-10] Antibody levels were expressed in the conventional units or titers and compared to a reference standard. To account for the protein concentration the term "specific activity" was introduced expressing the titer per mg. protein per ml. of the solution tested. The "total antibody activity" was obtained by multiplying the specific activity with the mg. of protein in the whole fraction. Consequently the sum of the total antibody activities of each fraction resulting from processing γ-globulin should be equal to the total antibody activity of the original γ-globulin. The ratio of the specific activity of a fraction tested to that of the original γ-globulin is an index for the fractionation achieved (fractionation index).

Diphtheria antitoxin was determined using the rabbit skin test described by Enders.[8] The titers were expressed in units per ml. Compared to the reference standard (II G 141) the starting material had a ratio of 0.75. The test has an experimental error of ±10%.

Antistreptolysin-O titers were obtained according to the technique developed by B. F. Massell, J. M. Miller and M. Kaplan.[9,10] The procedure is based on the inhibition of the lysis of rabbit red blood cells by reduced streptolysin-O.[11]

Since zinc salts are known to precipitate red blood cells, it was thought that the antistreptolysin test might be affected by the presence of zinc in the fractions. Accordingly the titer of a constant amount of γ-globulin was tested in the presence of various zinc concentrations: 4 samples of 0.5 ml. of 15% γ-globulin were treated with zinc lactate to give concentrations of 1, 3, 5 and 20 m*M* zinc. In each case the final volume was 5 ml. and the *p*H 7.2. A control was treated with 0.15 *M* sodium chloride. After 60 hours standing at 0° the precipitates were redissolved in 2 drops of 0.1 *M* EDTA solution. In a similar experiment the γ-globulin precipitate was redissolved five minutes after the addition of 5 m*M* zinc lactate. In each case the titer for antistreptolysin-O amounted to the same value: 100. The activity tests were therefore independent of the zinc concentration.

Influenza A virus antibody was determined using the Hirst test based on the titration of the substance inhibiting the agglutination of chicken red blood cells by influenza virus. Some inconsistencies were encountered in zinc-treated fractions even when the zinc had been removed by chelating agents. The titers were therefore compared with the activities of neutralizing antibody as determined by the mouse protection test.[8]

Mumps virus antibody was estimated by hemagglutination inhibition techniques only. In the case of mumps and influenza A virus antibody the presence of non-specific inhibitors of hemagglutination was excluded by heating the samples to 57° for 30 minutes and by treatment of the latter with extracts of **vibrio cholerae** (receptor destroying enzyme).

Results

Antistreptolysin-O.—Depending on the zinc concentration used for the inital precipitation different results were obtained. The γ-globulin precipitated in presence of 5 m*M* zinc yielded fractions with unchanged antistreptolysin-O activity (fractionation index 0.9–1.18). When 20 m*M* zinc was used, a fraction 1 was obtained containing only one-half the antistreptolysin-O activity of the starting material. The specific activity of fraction 2 was increased 2.5 times as compared with fraction 1 (Table I).

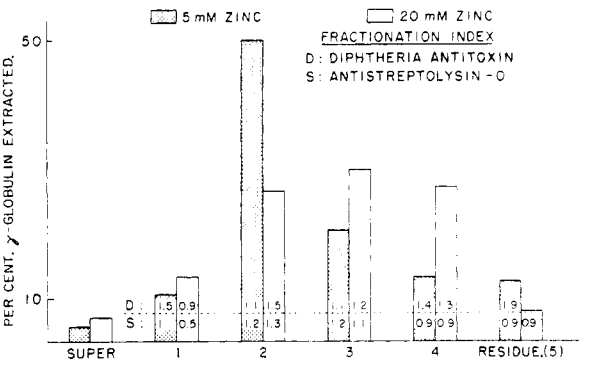

Fig. 4.—Distribution of antistreptolysin-O and diphtheria antitoxin in subfractions of γ-globulins. The numbers indicate the ratio of the specific activity of a fraction to that of the original γ-globulin (fractionation index). The height of the columns shows the amount of protein extracted into each subfraction on a percentage basis.

TABLE I

DISTRIBUTION OF ANTISTREPTOLYSIN-O IN SUBFRACTIONS OF γ-GLOBULIN

	Fraction II pptd. with 5 m*M* zinc lactate			Fraction II pptd. with 20 m*M* zinc lactate		
Subfraction	Specific activity	Total activity	Fract. index	Specific activity	Total activity	Fract. index
1	8.5	3,833	1.04	4.0	1,600	0.49
2	9.7	29,100	1.18	10.7	21,935	1.30
3	9.7	10,851	1.18	9.0	13,032	1.10
4	7.4	4,824	0.90	7.4	8,717	0.90
5	7.8	4,992	0.95	7.8	4,368	0.95
Starting material (fraction II)	8.2	49,200	1.00	8.2	49,200	1.00

γ-Globulin stored for several weeks in the presence of 50 m*M* zinc showed the highest antistreptolysin-O activity in fraction 3 (Fractionation index 1.4).

Further Subfractionation.—Fraction 2 containing 256 mg. of protein was reprecipitated in a final volume of 17 ml. of 5 m*M* zinc lactate at *p*H 7.2 and ionic strength 0.15. The precipitate was washed twice with 5 ml. of 0.15 *M* sodium chloride solution containing 0.1 m*M* zinc lactate.

Fractional extraction was carried out with 100, 50 and 100 m*M* glycine using for each extraction 5 ml. of the solution. The subfractions were tested for antistreptolysin-O. No significant increase of activity was observed.

Diphtheria Antitoxin.—When 5 m*M* zinc lactate was used for the initial precipitation, fraction 1 and 5 (residue) showed highest specific activity of diphtheria antitoxin. The increase in activity amounted to 54% for fraction 1 and to 92% for fraction 5.

Precipitation of the original γ-globulin with 20 m*M* zinc yielded a fraction 2 with 46% higher activity as compared with the starting material. The residue showed no increase in titer. The sum of the total activities of the fractions did not deviate significantly from the activity of the starting material (Table II).

Influenza A Mouse Protective Antibody.—Fractions 1, 2 and 4 obtained from 4 g. of γ-globulin precipitated in presence of 5 m*M* zinc lactate were tested for influenza A antibody, by the mouse pro-

(8) J. F. Enders, *J.Clin. Invest.*, **23**, 510 (1944).

(9) B. F. Massell, J. M. Miller and M. Kaplan, unpublished data.

(10) B. F. Hodge and H. F. Swift, *J. Exptl. Med.*, **58**, 277 (1933).

(11) The titers were expressed in units of antistreptolysin-O and may vary from 50 to 4000 units in human plasma.

TABLE II

DISTRIBUTION OF DIPHTHERIA ANTITOXIN IN SUBFRACTIONS OF γ-GLOBULINS

Fractions	Fraction II pptd. with 5 m*M* zinc lactate[12] Specific activity × 10^{-3}	Total activity	Fract. index	Fraction II pptd. with 20 m*M* zinc lactate[13] Specific activity × 10^{-3}	Total activity	Fract. index
1	20	9,200	1.54	12	3,300	0.90
2	14	42,000	1.08	19	38,900	1.46
3	15	17,500	1.15	16	23,100	1.23
4	18	11,700	1.38	17	20,000	1.30
5	25	16,300	1.92	0.5	280	0.035
Starting material (fraction II)	13	82,800	1.00	13	82,800	1.00

tection test. As shown in Table III, fraction 2 exhibited a 69% higher specific activity than the original γ-globulin. Fractions 1 and 4 had lower specific activities. The titers could be reproduced within ±20%.

TABLE III

DISTRIBUTION OF INFLUENZA A MOUSE PROTECTIVE ANTIBODY IN SUBFRACTIONS OF γ-GLOBULIN

Subfractions	Specific activity	Total activity	Fract. index
1	1.00	280.0	0.89
2	1.93	3744.2	1.69
3	Not tested	....	..
4	0.76	357.2	0.67
5	..	....	..
Starting material (fraction II)	1.14	4460	1.00

Hemagglutination Inhibition (HAI) Test for Mumps and Influenza A Antibody.—The specific activity of HAI antibody to mumps and influenza virus varied greatly from one fraction to another. In the case of influenza virus antibody, the sum of the total activities from each fraction greatly exceeded the total activity of the starting material.

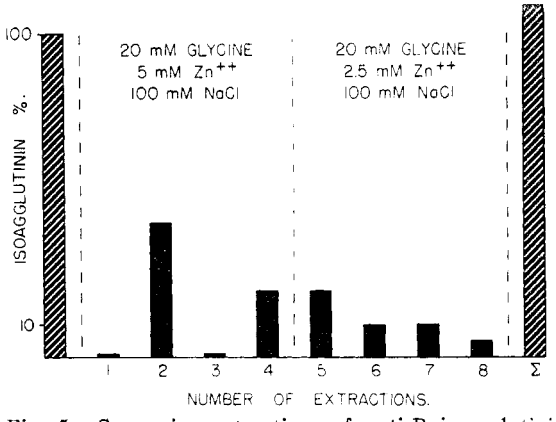

Fig. 5.—Successive extractions of anti-B isoagglutinins from a zinc–globulin precipitate (prepared from a pool of A- and O-plasma) with 20 m*M* glycine and 0.1 *M* sodium chloride. The first four extractions were carried out in presence of 5 m*M*, the last extractions in presence of 2.5 m*M* zinc ions.

(12) The antitoxin activity in fractions 1 and 5 was determined by injection into one rabbit only. Fractions 2, 3 and 4 were tested on two rabbits.

(13) The antitoxin activity in all fractions (precipitated with 20 m*M* zinc lactate) was determined by injection into one rabbit only.

A non-specific inhibition seemed to be involved: the presence of the two non-specific inhibitors of virus hemagglutination known to occur in plasma was eliminated by heating each fraction before testing for 30 minutes at 57° and by treatment with receptor destroying enzyme. In a further series of experiments excess virus fixed on formalinized red blood cells was added to each fraction and the red cell–virus–antibody complex was removed by centrifugation.[14] Tests of the supernatant revealed the persistence of non-specific virus hemagglutination inhibitor. The reagents used in the fractionation procedure: zinc lactate, glycine, tartrate, EDTA and citrate by themselves or in combination with each other did not show inhibition of hemagglutination. However, the HAI titer increased with the amount of zinc used for the initial precipitation. Also the hemagglutination inhibition was more pronounced when the EDTA–zinc complex was removed from the protein by dialysis against sodium citrate and saline. This suggests that the addition and removal of zinc ions brings about a physico-chemical alteration of the γ-globulin which inhibits the agglutination of red blood cells by virus.

Isoagglutinins.—Although isoagglutinins are not γ-globulins a similar fractionation could be carried out using a zinc globulin precipitate from pooled human plasma as starting material. Eight extractions were performed with 20 m*M* glycine solution. The fractions were freed from zinc, concentrated as described in Section 2 and tested for anti-B isoagglutinins. The results shown in Fig. 5 suggest the presence of at least two anti-B isoagglutinins with different solubility characteristics. Those components may be related to the agglutinins found by Wurmser[15] in A plasma of individuals with different genotypes.

Discussion

Zinc ions were found to interact strongly with γ-globulins. Over 90% of the protein was precipitated in the presence of 1 m*M* zinc nitrate. These data indicate that less than 50 zinc ions are necessary to precipitate one molecule of γ-globulin. The minimal number required for precipitation is considerably smaller since only a part of the available zinc ions are bound by the protein. The consistent assymmetry of the solubility curves at different zinc concentrations suggests the presence of more than one γ-globulin with different solubility minimum. The ability of various chelating agents to dissolve the zinc–γ-globulin precipitate parallels the association constant of the chelating agent for zinc.[16] This effect is striking for α- and β-alanine: a zinc–γ-globulin complex completely dissolved in the presence of 250 m*M* α-alanine; using the same concentration of β-alanine, a much weaker chelating agent, only 8.7% of the γ-globulin passed into solution. The S-shape of the curve in the case of amino acids such as glycine might be due to the

(14) J. E. Jensen and T. Francis, *J. Exptl. Med.*, **98**, 619 (1953).

(15) R. Wurmser, *Advances in Enzymol.*, **15**, 49 (1954).

(16) Association constants[17] (log *k*) of different anions for zinc: tartrate, 2.68; malate, 2.8; glycolate, 1.92; lactate, 1.86; succinate, 1.78.

(17) R. K. Cannan and A. Kibrick, THIS JOURNAL, **60**, 2314 (1938).

initial formation of a protein–zinc–glycine complex. Only at higher concentrations of glycine does this complex dissociate to form the stable zinc diglycinate and soluble protein.

Tartrate has been found to decrease the solubility of γ-globulin in the absence of heavy metals. In the presence of zinc this effect was largely counteracted by the chelating properties of the anion. The effect of ionic strength on the solubility of a zinc–γ-globulin complex was very small at *p*H 7.4. In contrast, at *p*H values below 7, where the protein carries a positive net charge, addition of sodium chloride strongly decreased the solubility of a zinc–γ-globulin complex even in the presence of glycine.[7]

On the basis of the immunological assays performed the antibody composition of extracted γ-globulins differed from the composition of the original γ-globulin. In the case of antistreptolysin-O and influenza A protective antibody these differences did not exceed 60%. Diphtheria antitoxin was concentrated by a factor of 2 in one of the residues. The lowest specific activity was found in the supernatant of the precipitate formed in the presence of 5 m*M* zinc. A fairly high activity in fraction 1 (52% higher than in the starting material) may be related to the existence of two different fractions in diphtheria antitoxin.[18]

(18) W. J. Kuhns, *J. Exptl. Med.*, **99**, 577 (1954).

The diphtheria antitoxin activity of some fractions[12,13] was determined by injection into one rabbit only instead of two rabbits. The experimental error should therefore be higher than ±10%. However even allowing for an experimental error of 15–30% the extreme values of specific activities of the antibodies tested indicated a small but consistent fractionation.

The discrepancy between the activity of the starting material and the sum of the subfractions in the case of virus HAI antibodies might be explained on the following basis: The purification procedure might uncover combining sites on the antibody molecule which in the starting material were unavailable due to complex formation with other antibodies. There is no reason, however, why this effect should only occur in the case of the tested virus antibodies. It is more likely to be an artifact related to the presence of traces of zinc.

Acknowledgment.—The authors wish to express their appreciation to Drs. J. L. Oncley, D. M. Surgenor and P. E. Wilcox for their interest and assistance in this study. They are also indebted to Dr. M. Kaplan, Miss J. C. Sullivan and Miss D. McCarthy for help in the immunologic assay of the fractions.

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[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

Synthesis of Cytosines

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Cyclization of substituted β-ureidoacrylonitriles yielded 5-carboxy-, 5-carbethoxy-, 5-cyano- and 5-carbamylcytosines. The facility in which cyclization occurred was dependent upon the nature of substituent groups on the β-ureidoacrylonitriles. Acid hydrolysis converted 5-cyanocytosines to 5-cyanouracils and 5-carbethoxycytosines to 5-carboxycytosines. Decarboxylation of 5-carboxycytosines yielded homologs of cytosine.

Current interest in the pharmacological properties of pyrimidines motivated an investigation, in this Laboratory, of possible new methods for their synthesis. This present paper contains a description of methods that were employed in the synthesis of 5-cyano-, 5-carboxy-, 5-carbethoxy- and 5-carbamylcytosines (II, X = CN, CO_2H, $CO_2C_2H_5$ and $CONH_2$), and of their conversion to other new pyrimidines.

Synthesis of the cytosines reported here involved cyclization of an intermediate β-ureidoacrylonitrile. Ureidoacrylonitriles (I) of the type RNHCONHCH=CXCN (R = alkyl, aralkyl, cycloalkyl or aryl and X = CO_2H, $CO_2C_2H_5$, $CONH_2$ and CN) were prepared by methods previously described.[1,2]

Cyclization of the ureidoacrylonitriles (I) was effected by heating the open chain intermediate or by treating it with a basic catalyst. It was found that under these conditions ring closure occurred more readily with some derivatives than with others. When R was phenyl or substituted phenyl and X was CN, cyclization occurred readily either by heating the ureidoacrylonitrile or by treating it with sodium methylate. Alkylureidoacrylonitriles (I, R = alkyl) did not cyclize when heated and were, therefore, treated with the base in alcoholic solution. Here the ease in which the alkylureidoacrylonitrile cyclized was apparently dependent upon the nature of both groups R and X. The ring closure could not be effected when R was an alkyl group branched at the α-carbon and X was a carbethoxy group, as in the examples of β-(3-cyclohexylureido)-α-carbethoxyacrylonitrile (I, R = cyclohexyl, X = $CO_2C_2H_5$) and β-(3-isopropylureido)-α-carbethoxyacrylonitrile (I, R = isopropyl, X = $CO_2C_2H_5$). Ring closure did occur when X was a cyano group and R was an α-branched alkyl group. Thus, β-(3-cyclohexylureido)-α-cyanoacrylonitrile, β-(3-isopropylureido)-α-cyanoacrylonitrile and β-(3-*t*-butylureido)-α-cyanoacrylonitrile all yielded the corresponding cytosines. The ease with which cyclization occurred was apparently determined mostly by the nature of the particular

(1) C. W. Whitehead, THIS JOURNAL, **75**, 671 (1953).
(2) C. W. Whitehead, *ibid.*, **74**, 4267 (1952).