

TABLE I

ALKYL- β -THIOETHYLAMINES, THEIR HYDROCHLORIDES AND UREAS

	RSCH ₂ CH ₂ NH ₂			RSCH ₂ CH ₂ NH ₂ ·HCl			RSCH ₂ CH ₂ NHCONH ₂		
	B. p., °C.	Calcd. % N	Found	M. p., °C.	Calcd. % Cl	Found	M. p., °C.	Calcd. % N	Found
<i>n</i> -Butyl	211	10.53	10.52	118	20.92	20.87	91	15.91	15.83
<i>n</i> -Amyl	231	9.52	9.40	101	14.74	14.70
<i>i</i> -Amyl	231	9.52	9.37	167	19.33	19.26	111	14.74	14.61
<i>n</i> -Hexyl	252	8.69	8.68	131	17.96	18.02	99	13.73	13.80
<i>n</i> -Heptyl	270	8.00	7.99	121	16.77	16.58	95	12.84	12.76

To prepare butyl- β -thioethylamine, 46 g. of sodium was dissolved in 500 cc. of alcohol, 90 g. of butyl mercaptan was added followed by an alcoholic solution of 205 g. of the above salt. After refluxing the solution was decanted from the precipitated sodium bromide, acidified and evaporated to dryness. The base was set free by concentrated sodium hydroxide solution and taken up in ether. This solution was concentrated and distilled, the 200–220° cut being taken and redistilled. The other bases were prepared similarly. The hydrochlorides were precipitated from dried petroleum ether solutions of the bases by passing in hydrogen chloride. They were recrystallized from absolute alcohol and acetone.

The ureas were made by the cyanate method and recrystallized from benzene.

The amines, in dilute hydrochloric acid solution, were oxidized to the sulfoxides by standing two days with the calculated amount of 30% hydrogen peroxide. An excess of this reagent carried them to the sulfones.

The amines are strong bases, insoluble in water but soluble in the usual organic solvents. They form stable hygroscopic hydrochlorides. The ureas are only slightly soluble in water. The properties and analyses are in Tables I and II.

The original purpose in preparing the alkyl- β -thioethylamines was to condense them with chloracetophenone in order to contrast the physiological properties of the expected sulfur com-

TABLE II

HYDROCHLORIDES OF THE SULFOXIDES AND SULFONES FROM THE ALKYL- β -THIOETHYLAMINES

	RSOCH ₂ CH ₂ NH ₂ ·HCl			RSO ₂ CH ₂ CH ₂ NH ₂ ·HCl		
	M. p., °C.	Calcd. % Cl	Found	M. p., °C.	Calcd. % Cl	Found
<i>n</i> -Butyl	112	19.12	19.08	211	17.60	17.50
<i>n</i> -Amyl	121	17.78	17.75	221	16.45	16.38
<i>n</i> -Hexyl	127	16.61	16.60	238	15.46	15.41
<i>n</i> -Heptyl	123	15.59	15.51	230	14.56	14.49

pounds with the corresponding ones containing oxygen. For some unknown reason it was found impossible to effect the desired condensations. Different solvents and different temperatures were tried; either there was no reaction or tarry masses were produced from which nothing could be isolated.

Summary

Several alkyl- β -thioethylamines have been prepared by a new method and from them the corresponding ureas.

The amine hydrochlorides have been oxidized to the sulfoxides and sulfones.

BALTIMORE, MD.

RECEIVED JANUARY 27, 1943

[CONTRIBUTION FROM THE DEPARTMENT OF MEDICINE AND CHEMISTRY DEPARTMENT, STANFORD UNIVERSITY]

Solubility and Electrophoretic Studies of Serum Globulins. I. Gamma Globulin

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Introduction

The object of the present communication is to advance experimental evidence leading to a definite explanation as to why proteins usually do not appear to obey the phase rule, and yet under other conditions can be found to behave in accordance with it. As early as 1937^{1a} we were able to describe a globulin fraction exhibiting definite true solubility in the phase rule sense. We should hesi-

tate to call even this a single pure protein component. Indeed we feel sure that it is not. Our present hypothesis serves to explain not only the solubility relations of globulins but also their otherwise rather complicated electrophoretic behavior. The work reported in this paper constitutes only a sorting or mapping out of this field. It does not attempt a precise specialized purification such as that of Kuntz and Northrop with crystalline enzymes. The main results of our present work stand, regardless of these considerations.

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(1a) E. Jameson and D. B. Roberts, *J. Gen. Physiol.*, **21**, 249 (1937).

The suggestion is that the Tiselius gamma-globulin, that is, the most slowly moving globulin fraction in the electric field at pH 7.7, is in itself a system comprising both a complex and its constituents in true highly mobile equilibrium. This system forms the major part of the serum globulins. Many years of work have led us to the conclusion that globulins, which behave as chemical individuals in that they exhibit true constant solubility, are obtained under certain definite conditions.

These conditions are: First, potassium citrate at 0° is employed rather than ammonium sulfate. Ammonium sulfate as it has been used appears to split or fractionate globulins resulting in heterogeneous products.

Second, the protein must be maintained in high concentration.

Third, to avoid dilution or modification of the system the product is not washed from the potassium citrate mother liquor. It may be dissolved by controlled dialysis or, better, directly at high concentration in undiluted serum. Quite probably, even the accompanying proteins must not be too dilute since they may act as dispersing agents. Many workers have found that proteins change their particle size on mere dilution.

Proteins precipitated from horse serum by potassium citrate, redissolved in high concentration in undiluted serum, and reprecipitated from this solution yield products unlike those obtained by other methods in that the precipitates do behave as *single phases*. There is no reason to believe that proteins that behave as single individuals under the conditions outlined will be electrophoretically homogeneous, particularly since the solution used for electrophoretic measurements is of necessity rather dilute. Each single phase precipitated out may be a complex, containing β and α in much smaller quantities than γ , and become dissociated when diluted for electrophoretic observation. No attempt was made to prepare electrophoretically homogeneous proteins, but rather to study the electrophoretic behavior of the single phases observed by salting out. Doubtless, there may be relationships between different components of the globulin β as well as between the components of the globulin γ in these salted out proteins. It is the principal part or γ which we are discussing.

By our method of precipitation it is possible to separate the serum globulin gamma which appears

to be electrophoretically homogeneous but which has a varying mobility into fractions, two of which at least have definite and different mobilities. These, when redissolved in serum, do not remain as separate entities but reassume the mobility of the original gamma band of serum globulin.

Preparation of Gamma Globulin and its Constituents.—Many, including the authors, have tried without success to isolate serum globulins by precipitation with ammonium sulfate, remove the dispersing agents by washing thoroughly with ammonium sulfate solution, redissolve the globulin in the salt solution, and thus to obtain proteins satisfying the solubility criterion for a chemical individual; that is, of constant solubility regardless of the amount of protein in contact with the solution, when a sufficient number of solid phases are present to satisfy the phase rule. One of the authors^{1a} confirmed by phase rule studies the results of numerous previous investigators that the globulins obtained by this method did not behave as single phases even when the concentration of protein was as high as 10–12%. The “solubility” continues to be dependent upon the amount of globulin in contact with the solution.

Cohn,² *et al.*, have recently separated serum proteins by ammonium sulfate fractionation, dialysis and electro-dialysis. They have found that their preparations fail “to rigorously satisfy the solubility criterion for a chemical individual.” Northrop³ separated antitoxin from a toxin-antitoxin complex without employing a salt and found that it had a constant solubility.

Our method of preparation which yields homogeneous protein precipitates was first reported in 1937.^{1a} The only criterion for homogeneity there used was the phase rule behavior of the precipitates. The amount of protein in solution was the same regardless of the amount of the given protein in contact with the solution provided the concentration was sufficiently high. We have designated the fractions A₁, A₂, B, etc., according to their order of precipitation out of a given protein solution, A₁ being first to precipitate as the salt concentration is increased.

The Solubility Diagrams

We have plotted compositions of original systems and of the saturated solutions resulting therefrom when the precipitate has separated, in terms of percentage protein by weight as ordinates, and of percentage potassium citrate by weight as abscissas, the remaining percentage being water.⁴

Thus, the original composition of the total system in each experiment is indicated by a cross, and the corresponding mother liquor that is left

(2) E. J. Cohn, T. L. McMeekin, J. L. Oncley, J. M. Newell and W. L. Hughes, *THIS JOURNAL*, **62**, 3386 (1940).

(3) J. H. Northrop, *Science*, **93**, 92 (1941).

(4) The total diagram, representing all ranges of concentration, would be a right angled triangle and would constitute a phase diagram when the phase rule is obeyed. All points on the diagram would then obey the requirements of Gibbs, such as, that tie lines between two condensed phases are straight and pass through the total composition of the original system. These tie lines converge in a point or area which gives the true composition of the solid phase.

in contact with the resultant precipitate is indicated by a triangle for the solution coming from the more concentrated systems and by a circle for the solution resulting from the system less concentrated in protein. Only that portion of the phase diagram dealing with fractions A_2 and B is given. The points in the curves were obtained by adding weighed portions of potassium citrate to the protein solution, the pH being maintained constant by addition of citric acid. The precipitated proteins were separated on filters and the liquid phase analyzed for protein and potassium.

Phase Rule Studies

The total concentration of serum proteins in serum has a profound effect on the homogeneity of globulins salted out of serum. Not only so, but the total concentration of such globulins in a potassium citrate solution has a similar effect upon maintaining the homogeneity of the proteins separating therefrom.

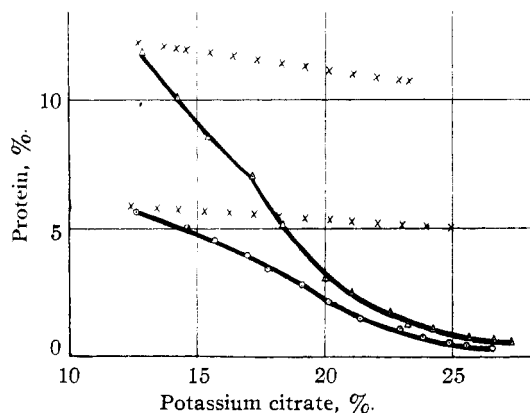


Fig. 1.—Gamma serum globulins not appearing to obey the phase rule. Curves made from two solutions containing different amounts of A_2 and B. Concentration of other serum proteins is different: X, total compositions of the systems in per cent. by weight Series I and Series II; Δ , compositions of final solutions Series I; O, compositions of final solutions Series II.

This may be seen in Fig. 1. A solution was prepared by dialyzing against water the globulins $A_2 + B$ which had been precipitated from horse serum at 20.5% potassium citrate by weight, and which had been left unwashed. This solution was compared with one made by adding to the original solution an equal weight of water. The concentrations of the proteins are thus reduced in one solution to half those in the other. The two curves (Fig. 1) give no evidence of constant solubility of the globulins, although the total pro-

tein concentration of the more dilute solution was above 5%. The curves are divergent and do not coincide as they would for constant true solubility of a single individual species. Thus, at about 17% concentration of potassium citrate where A_2 is precipitating the curves show 7.0% protein in the liquid phase from the more concentrated solution, and 3.75% protein from the more dilute one. The protein remaining dissolved is roughly proportional to that in the solutions which had been used for precipitation. Where B is precipitating at 20% potassium citrate the percentages of protein in solution are 3.5 and 2.5, respectively.

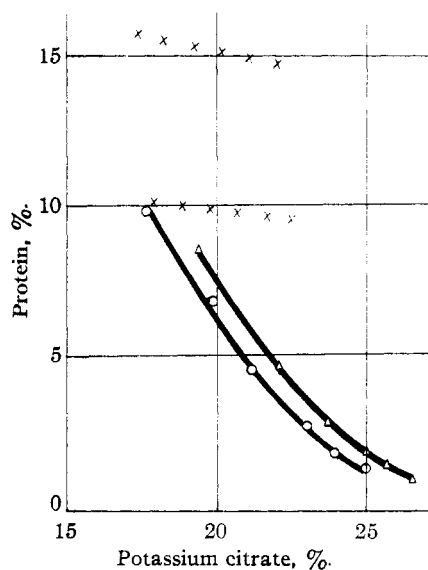


Fig. 2.—Gamma serum globulins approaching true solubility: curves made from two solutions containing different amounts of B; concentration of other proteins different: X, total compositions of the system in per cent. by weight Series I and Series II; Δ , compositions of final solutions Series I; O, compositions of final solutions Series II.

Figure 2 shows solubility curves made in the same manner with a precipitate separated from horse serum, between 20.6 and 23.5% potassium citrate by weight. This precipitate was packed tightly in a cellophane bag and dissolved by being dialyzed against water. In this case the concentration of the proteins in one of the solutions was diluted to two-thirds of that in the other solution. A comparison of the curves made with these solutions shows that the phase rule curve has hardly been modified by this limited dilution of all the protein present. If the difference in concentration between the two solutions precipitated had been only in the B fraction, the curves

would have coincided. There is no longer a proportionality between the protein in the original mixture and that remaining in solution.

The phase rule was then applied to solutions of different quantities of the same protein in the serum or in serum from which part of the globulin had been removed by precipitation (Fig. 3).

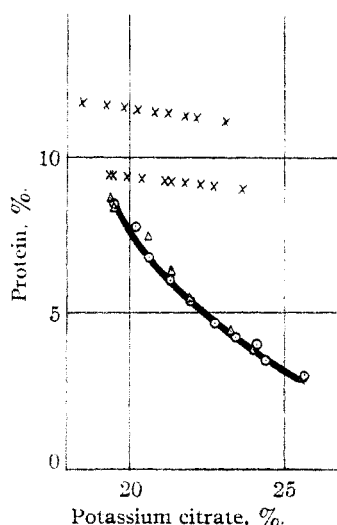


Fig. 3.—Gamma serum globulins showing true solubility according to the phase rule: Curves made from two solutions containing different amounts of B; concentration of other proteins the same: X, total composition of the system in per cent. by weight Series I and Series II; Δ , compositions of final solutions Series I; O, compositions of final solutions Series II.

These solutions were prepared in a similar manner to the solutions used in obtaining Fig. 2. In this case, however, the solvent was serum, from which the globulins precipitating at 20.6% potassium citrate had been removed. Some dialysis was also necessary to remove enough salt to bring the proteins into solution.

Two solutions of different concentration were made by dissolving twice as much precipitate in a given amount of serum in one case as in the other. Thus the concentrations of all the proteins not precipitated at 23.5% potassium citrate by weight were the same (Fig. 3).

As far as was possible the lowest salt concentrations at which any precipitation occurred were studied. At the left-hand side of the solubility curve representing the solutions separating from the weaker protein solution, it may be seen that almost all of the protein remains in solution. The precipitation from the more concentrated solution is so sudden and great on adding a very small increment of salt (one half of one per cent.)

that it was impossible to get an analysis on a solution approaching the point of total composition in protein content. If there is any phase separating before the one shown in Fig. 3, it must be very small. Since the protein which occurs in different concentrations in two solutions is the first to precipitate on adding salt, the fact that the curves coincide with each other shows a constant solubility of the given protein regardless of its concentration.

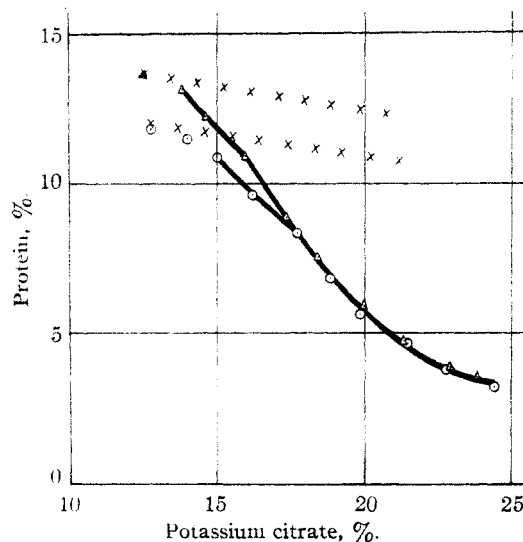


Fig. 4.—Globulins showing constant solubility above 17.5% potassium citrate, curves made from two solutions containing different amounts of both A_2 and B; concentration of other proteins the same: X, total compositions of the systems in per cent. by weight Series I and Series II; Δ , compositions of final solutions Series I; O, compositions of final solutions Series II.

Figure 4 shows solubility curves made from solutions which had been formed by dissolving different amounts of a mixture of two proteins, A_2 and B, in serum. Two different quantities, one twice the weight of the other, of protein precipitated from horse serum at 19.5% potassium citrate by weight were dissolved in the same quantity of serum, making the two solutions used. The composition of the liquid phase is fixed when it is saturated with respect to all proteins, that is, at concentrations higher than 17.5% potassium citrate. From here on, the curves coincide. B commences to precipitate from the more concentrated solution at about 16% and from the more dilute at about 17.5% salt. At salt concentrations too low for the precipitation of B, while only A_2 is precipitating, the curves are parallel and not divergent. If the amount of B added to

the two solutions shown in Fig. 4 had been equal, the curves representing the precipitation of A_2 would have coincided. Therefore from the criterion of the phase rule both A_2 and B are homogeneous. Each precipitates as one solid phase and shows a definite solubility. Under these definite conditions proteins behave as entities.

Preparation of Proteins for Electrophoresis.—Preparations were made from eight different samples of horse serum. As a precaution it would have been desirable to have made a preliminary phase rule study, so as to effect a more nearly perfect separation of the proteins when salting out from horse serum, because the concentrations of the different protein constituents vary from serum to serum. Since we can show a true solubility under the conditions outlined, the amount of salt necessary to bring about the beginning of precipitation of a given protein depends upon the concentration of this fraction in solution. It was impossible to do this and still use the proteins when freshly prepared, so the curves already made were used to find the approximate salt concentrations necessary to get as homogeneous proteins as possible. These proteins were used for electrophoretic measurements usually within ten days and always within three weeks after the date of collection of the serum.

Gamma globulin A_2 containing a small amount of A_1 as an impurity was obtained by precipitating, at 12% potassium citrate, a solution prepared in the same manner as the more concentrated one in Fig. 4. The addition of the citrate brings the concentration of the mother liquor to about 15% potassium citrate. The precipitate was separated by centrifugation and pressed free from mother liquor.

A_1 was prepared by washing this precipitate free from A_2 with serum containing 13% potassium citrate or by adding potassium citrate to the original solution from which A_2 was prepared until the first faint precipitate occurred. A_1 is then the most insoluble protein.

Gamma globulin B was prepared from a solution made in the same manner as the more concentrated one in Fig. 3. The addition of further citrate to a total concentration of 18–20% citrate brings about the precipitation of B. The mother liquor contains from 20–23% of citrate. The precipitate was pressed free from mother liquor. The precipitates of B were obtained as anisotropic soft masses. They showed no more definite crystalline form than broken plates and an occasional rod. They may be liquid crystals.⁵ No further attempt was made to purify A_2 or B. (A_2 of course contained small amounts of A_1 .)

Two other gamma-globulin fractions, contained in C and D were separated from horse serum from 23–31% and 31–43% potassium citrate, respectively. The first portion of the C to precipitate, that is, from 23–25% potassium citrate, was further purified by redissolving in serum from which A and B had been separated, and precipitating at 20% citrate. This was called C_1 , and was only partly gamma-globulin.

These preparations were dissolved in a 0.02 *M* sodium

phosphate buffer of pH 7.7, containing 0.15 *M* sodium chloride. The solutions were dialyzed against the same buffer until they were in equilibrium.

Electrophoresis

An electrophoretic study of each of the fractions of serum, salted out with potassium citrate and shown to be homogeneous as judged by phase rule, that is, globulin fractions A_2 and B, as well as the other serum fractions A_1 , C and D, was carried out in the Tiselius apparatus using Töpler's schlieren method as modified by Longsworth. A greater ratio of rate of movement of photographic film to speed of diaphragm than that employed by Longsworth was used in order to increase the heights of the peaks and thereby facilitate detection and mobility measurements. Measurements were made with potential gradients of 2 and 2.5 volts/cm.

Tiselius has named the proteins of serum in order of their decreasing mobilities in a buffer of pH 7–8; albumin and alpha, beta and gamma globulins, respectively. In our preparations, in each case except A_1 and D, we have a fraction the mobility of which falls in the range of that of normal horse serum gamma. The mobility of the slowest fraction of D is not great enough to be measurable.

In addition to the gamma component comprising the main constituent of A_2 and B, there is an appreciable amount of beta and alpha in all the fractions. Some samples of B have very little beta or alpha. A_1 and C are the least homogeneous, C containing all of Tiselius' serum fractions and A_1 containing principally beta globulin and a fraction with a mobility between that of gamma and beta globulin. D is chiefly albumin with some alpha and beta. It is with the gamma serum globulin and its true constituents that we are concerned in this paper.

The electrophoretic diagrams, Fig. 5, show electrophoretic patterns of A_1 , A_2 , B and C_1 in the buffer, of normal serum, and of A_1 , A_2 , B and C in serum. The mobility of the gamma-globulin of serum, expressed as usual in cm. per sec. per volt per cm. $\times 10^5$ varies from 1.1–1.7. The gamma-globulin in A_2 in buffer always has a constant mobility corresponding to the highest value of the gamma band of serum, average 1.7.

When, however, A_2 is dissolved in the same serum from which it was separated, the mobility of the gamma band becomes less, approaching that of the gamma serum globulin of that particular

(5) E. Jameson, "Symposia on Quantitative Biology," 6, 331 (1938), Cold Spring Harbor.

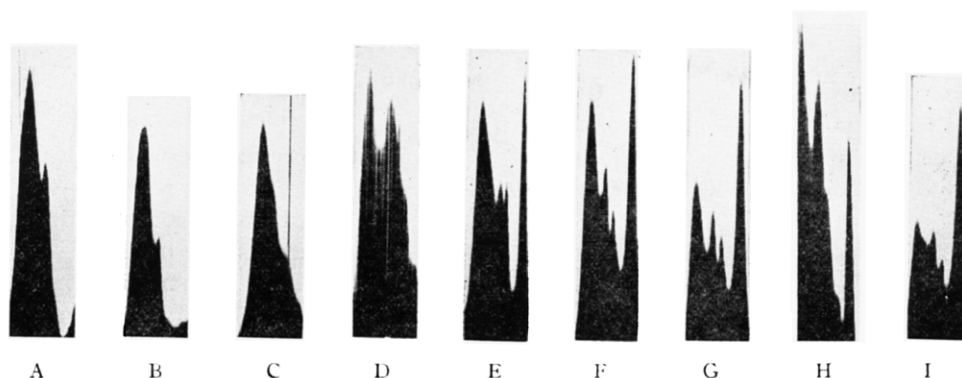


Fig. 5.—Electrophoretic analyses of serum proteins: (A) A_1 in buffer; (B) A_2 ; (C) B; (D) C; (E) A_1 in serum; (F) A_2 ; (G) B; (H) C; (I) serum control.

serum, even though the concentration may be increased ten-fold.

The mobility of the gamma band of B has a range of values corresponding to that of the gamma globulin of the serum. When redissolved in serum the mobility of the gamma is not changed appreciably. The values vary from 1.0–1.7.

The mobility of the gamma portion of C corresponds to that of the lowest values of serum, giving a second constant value, with an average of 1.2.

The mobility of the fraction with the lowest mobility in A_1 falls outside the values of serum gamma and corresponds to the value of T^6 (antibody globulin) found in the literature, that is, 2.2. The gamma fraction of D moves too slowly to have a measurable mobility. It may be seen that the gamma part of each fraction merely adds itself to the serum globulin gamma when dissolved in serum. In no case does the gamma band show two peaks. The mobilities of the gamma part of A_2 and of serum gamma are at times so different that unless a complex had been formed two separate peaks would have appeared.

Discussion

We have fractionated gamma serum globulin into its separate true constituents, appearing in A_1 , A_2 , B and C. These components of the gamma globulin of serum may thus be salted out, identified by differing mobilities and after resolution in serum, they may spontaneously become parts of the original equilibrium system which is included within the whole electrophoretic gamma band. The values for the electrophoretic mobilities of the components have been given.

(6) A. Tiselius and E. A. Kabat, *J. Expt. Med.*, **69**, 119 (1939).

Both the phase rule and solubility evidence on the one hand, and the electrophoretic data on the other, support our suggestion that in the gamma-globulin system of serum there obtain very mobile equilibria of the type $nP^1 + mP^2 \rightleftharpoons P_n^1P_m^2$.

We recognized in 1937 that the B fraction, itself, although homogeneous because it acted as a single solid phase, must be formed by the interaction of two or more components occurring in serum. The gamma part of A_2 would then represent the component P^1 , and would be precipitated until its curve was cut by that of B representing the solubility of $P_n^1P_m^2$. Then the phase precipitated would be the compound. The gamma part of C or P^2 would only appear when in excess of the amount necessary to combine with P^1 ; that is, after all the complex had been precipitated.

If our explanation is correct, then in Fig. 3 there may be a very small amount of P^1 separating before the solubility curve of $P_n^1P_m^2$ cuts that of P^1 . The very great viscosity of these solutions makes separation of the precipitate very difficult when it is small. It may be that the complex is different with an excess of P^2 and a very small amount of P^1 . The actual composition of the complex probably depends on the relative concentrations of P^1 and P^2 . A similar type of combination has been found in antibody-antigen reactions. It is also dissociated by salt.

The complex is not resolved electrophoretically into its components at any of the pH values investigated, even when the pH value was low enough to reverse the direction of motion.

The salt must enter into the combination in some manner because straight tie lines connect-

(7) S. P. L. Sørensen, *Kolloid-Z.*, **53**, 102 (1930).

ing the points representing the total composition, the liquid phase and solid phase in any case converge in a point or area inside of the phase rule diagram, showing the presence of salt in the precipitate. This criterion does not admit of occluded solution as the source of the salt.

The gamma in B as well as the gamma bands observed in serum, in accordance with this theory, are due to the whole equilibrium system and do not correspond to any one of the three components involved but are a resultant of all. The free fast component if present in excess increases the apparent mobility, the free slow component decreases it. There is a constant dissociation and reassociation. The spreading of the boundary which occurs would be expected. The effect may well be related to that on sedimentation of a system in mobile equilibrium where the sedimentation rate is decreased by the slow component and increased by the faster one, with a resultant sedimentation velocity which does not correspond to that of the compound or either of its components but is a resultant of all. It may be noted also that the component of gamma with the greater mobility is salted out at the lower salt concentration. This is not in accordance with the generalization of Cohn² that with increasing salt concentration proteins of higher and higher mobilities are separated.

It is possible that the appearance of the T

fraction seen in some immune sera and not in others, may indicate that in the former sera the complex is not held together in its usual manner. Its mobility is that of the gamma part of A₁.

The relationships of the various components of serum to each other are undoubtedly very complex. They are under further investigation.

We wish to acknowledge the helpful advice of Dr. J. W. McBain and Dr. Thomas Addis.

Summary

1. A method for the preparation of homogeneous constituents of gamma globulins has been described, in which emphasis is laid upon the use of potassium citrate in place of ammonium sulfate. The latter induces heterogeneous mixtures when used in the usual manner as shown by the solubility behavior.

2. Constant solubilities were found for two of our globulin fractions designated as A₂ and B.

3. Electrophoretic measurements were made on four different fractions of gamma globulin (range 1.1-1.7). The mobility of the gamma globulin of A₁ was 2.2; A₂, 1.7; B, 1.0-1.7; and C, 1.2, expressed in cm. per sec. per volt per cm. $\times 10^5$.

4. Both solubility behaviors and electrophoretic results are explained in terms of a mobile equilibrium between a complex and its components.

RECEIVED OCTOBER 27, 1941

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF SOUTHERN CALIFORNIA]

The Phase Behavior of Lithium Palmitate with Water and with Lithium Chloride and Water

BY MARJORIE J. VOLD

This paper presents the results of a study of the phase behavior of lithium palmitate and water over the complete composition range at temperatures ranging from the melting point of the lithium palmitate down to about 100°; the solubility of lithium palmitate in water below 100° is inconsequential. The effect on the phase diagram of small additions of lithium chloride has also been determined.

Phase studies of binary systems of soap and water are now available for several sodium soaps in detail sufficient to show the effect of simple variations in the anion of a soap on its solubility

in water. Vold, Reivere and McBain¹ have discussed this effect, pointing out that it is a complex one since the decrease in lattice stability (m. p.) and the increase in non-polar character with increasing carbon content of a soap molecule are factors opposing each other in their influence on its solubility in water. Similar study of a series of alkali soaps of the same fatty acid provides the simplest analog in which the cationic portion of the soap is varied systematically. In this case also, as with variations in the anion, the results

(1) R. D. Vold, R. Reivere and J. W. McBain, *THIS JOURNAL*, **63**, 1293 (1941).