A PHASE RULE STUDY OF THE PROTEINS OF BLOOD SERUM

III. GLOBULIN*

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(Accepted for publication, July 15, 1937)

INTRODUCTION

For many years the authors have been engaged in determining the composition of phases which occur in the system, globulin, salt, and water. In this communication a new method is described for applying Gibbs' phase rule to the characterization of the serum globulins as single proteins. The method may be applied to a study of a system of any number of protein components. It takes a small fraction of the time usually needed to carry out the experiments. Experience of our own and others has shown that the methods previously used fail to prove these globulins to be homogeneous substances. The principal difficulties found by other investigators have been overcome by keeping the protein to be studied in its original form in the medium in which it occurs.

It had been observed by several early workers that the amount of globulin remaining in solution after a definite amount of salt had been added to its solution is dependent upon the concentration of the protein in the original mixture (1). There was, as they said, a fractionation taking place. If one considered the protein as a single substance the phase rule did not apply, or considering Gibbs' phase rule to hold with proteins, globulin was not a single substance (2).

The application of this procedure has not only shown that the phase rule may be used to show that certain globulins are homogeneous substances but may also be used for a like study of each protein component.

* This work was aided by The Rockefeller Foundation Fluid Research Fund.

EXPERIMENTAL

Early Work

In 1930 an attempt was made to indicate the general nature of the phase rule equilibria obtainable and reference may be made to the diagram then published (3).

More detailed studies of portions of the phase rule diagrams for horse serum and rat serum are given in Figs. 1 and 2 (4).

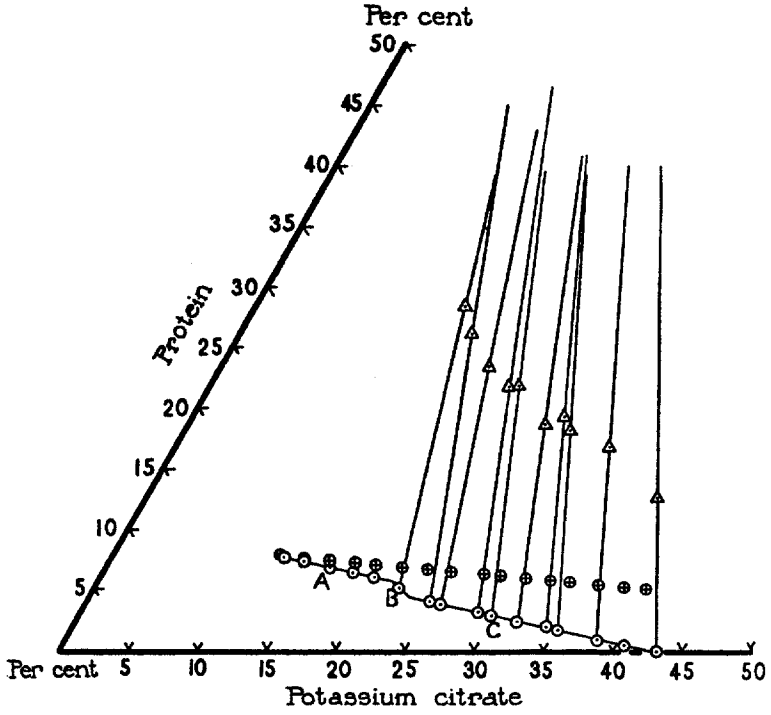

FIG. 1. Horse serum.

⊕ = total composition
⊙ = liquid phase
△ = solid phase.

The results are plotted as per cent by weight on Gibbs' triangular phase rule diagrams. Pure protein, pure salt, and water are to be found at the apeces of the triangles. Only a portion of the diagram is given as may be seen from the percentages along its sides.

A is considered the first fraction to appear as a solid phase on adding

potassium citrate; B is then precipitated on further addition, coming down in a very narrow range of salt concentration; and C is precipi-

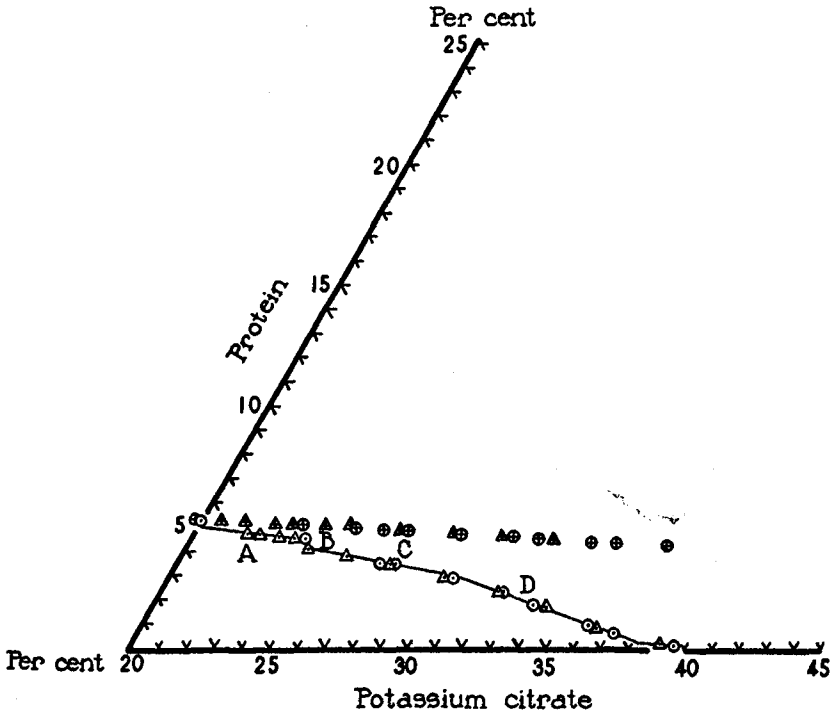

FIG. 2. Rat serum. Composite curve from two pools of rat serum from 30 rats each.

⊕ = total composition, pool 1
⊙ = liquid phase, " "
▲ = total composition, " 2
△ = liquid phase, " ".

tated gradually as the salt concentration becomes higher. Finally D, an albumin fraction, appears in some serums[1] (Fig. 2).

[1] The experimental procedure used in these experiments was described in a previous communication. To weighed portions of dialyzed serum or protein solutions at pH 6.8, potassium citrate was added to make the desired salt concentration. The pH was maintained by added citric acid. The precipitated protein was separated on filters and the liquid phase analyzed for protein and potassium. All analyses were made on weighed samples—so the results are in per cent by weight. All processes were carried out as rapidly as possible at 0°C.

A clear illustration of the application of the phase rule may be seen in Figs. 3 and 4 (4). One fraction after another has been salted out from the rat serum by increasing the potassium citrate concentration. The curves of undiluted and diluted serum converge showing that the protein in solution is independent of its concentration in the

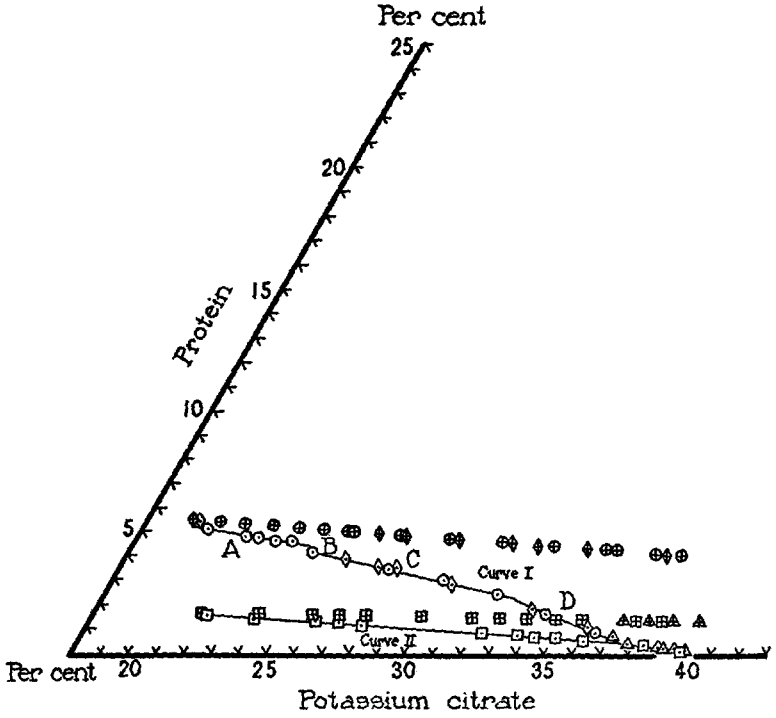

FIG. 3. Comparison of diluted and undiluted rat serum.

⊕ and ⊕ = total compositions, undiluted
⊙ and ◇ = liquid phases, undiluted
⊞ and ▲ = total compositions, diluted
⊡ and △ = liquid phases, diluted.

mixture at high salt concentrations. Enough solid phases are present to fix the concentration of the protein in solution.

Preliminary experiments were made on globulin prepared from horse serum by the method described by Svedberg. A phase rule study was carried out with this substance, $(NH_4)_2SO_4$, and water.

An early experiment showed a definite solubility of this globulin fraction in the same concentration of $(NH_4)_2SO_4$ regardless of the amount of protein in the mixture. In this case the protein precipitated from a solution of globulin was added to another portion of the original solution to make the concentrated solution (3).

New experiments were tried under a variety of conditions in an

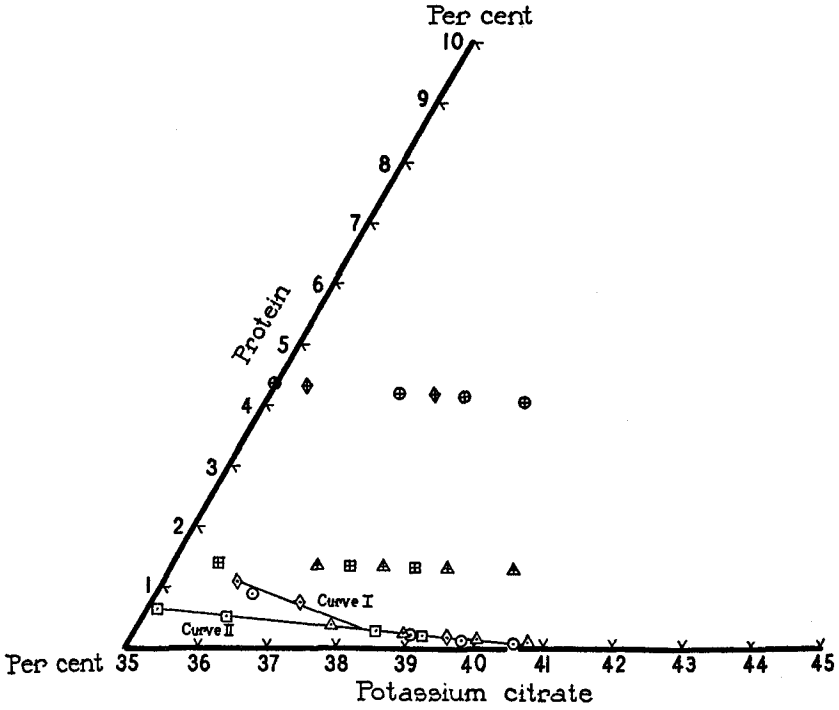

FIG. 4. Enlargement of a section of Fig. 3.

effort to preserve globulin in its original form. They differed from previous experiments since in all cases comparisons were made between concentrated solutions and others diluted from them. Horse serum globulin which had been precipitated by half saturation with $(NH_4)_2SO_4$, washed repeatedly, reprecipitated, and rewashed twice, was used for the concentrated solutions. Figs. 5 and 6 give illustrations of the data. As may be observed in Fig. 6 when the total

composition represents 10.5 per cent of protein and 14.8 per cent salt, the liquid phase separating contains 4.6 per cent protein. When the total compositions on the same tie line show 8.4, 6.25, and 2.0 per cents protein respectively, the liquid phases contain 3.7, 2.9, and 1.05

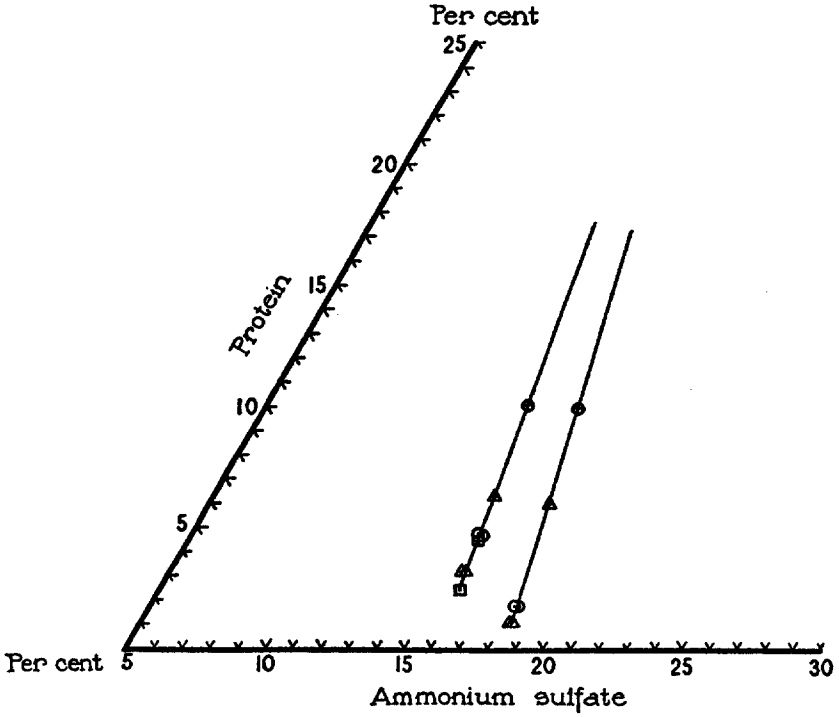

FIG. 5. Globulin separated by ammonium sulfate.

⊕ = total composition, concentrate
⊙ = liquid phase, concentrate
⧋ = total composition, diluted sample 1
△ = liquid phase, " " "
⊞ = total composition, " " 2
⊡ = liquid phase, " " ".

per cents protein. No matter what the conditions of the experiment (changes in temperature, etc.) the results were the same. The observations of earlier workers on the dependence of the solubility of globulin on the amount in the mixture under these conditions were corroborated. An explanation of these apparently conflicting results

will appear in the discussion. (The failure to understand these observations at the time led to a study of the whole salting-out curve of a very fresh undiluted serum at 0°C., to make certain how many globulin fractions were present in the native state.)

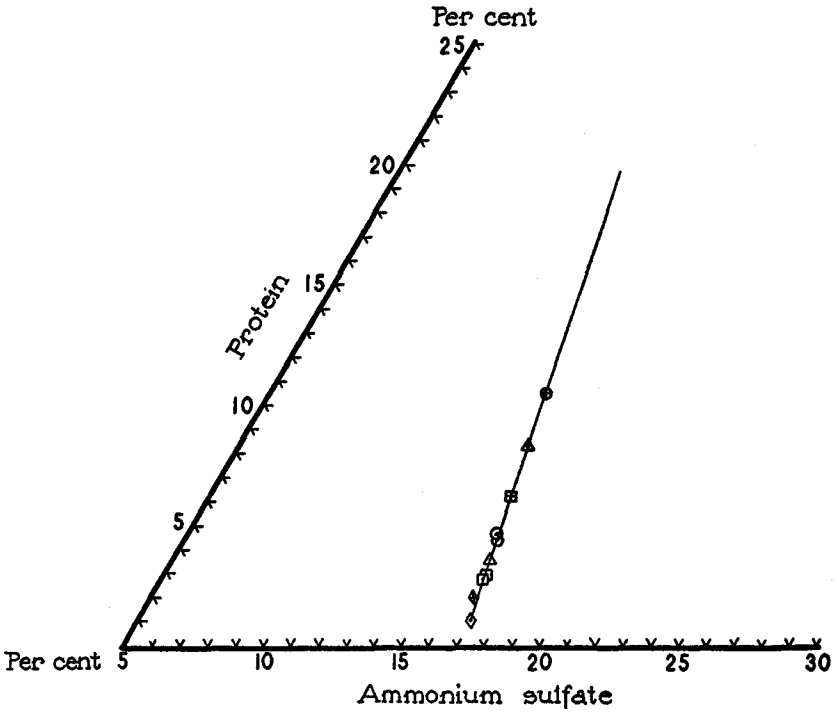

FIG. 6. Globulin separated by ammonium sulfate.

⊕ = total composition, concentrate
⊙ = liquid phase, concentrate
⧋ = total composition, diluted sample 1
△ = liquid phase, " " "
⊞ = total composition, " " 2
⊡ = liquid phase, " " "
⊕ = total composition, diluted sample 3
◇ = liquid phase, " " ".

Early experiments confirmed observations already made by Hardy (5). The fraction precipitated by saturation with NaCl proved to be extremely unstable, separating readily into a soluble fraction and a

highly pigmented insoluble one. Ammonium sulfate seemed to have a deleterious effect on one of the globulin fractions. In consequence, the method of precipitation with potassium citrate was developed.

The proportion of the total serum protein precipitated by 31 per cent citrate is similar to that precipitated by half saturated $(NH_4)_2SO_4$ solution (Fig. 1). It is obvious that there are at least two changes in direction with increasing salt concentration up to 31 per cent citrate, indicating three different fractions separating. From a statement of the phase rule we could not expect to have a constant per cent of protein in solution when keeping the salt concentration and temperature constant and varying the protein until there were three precipitated phases, or until the salt concentration was nearly 31 per cent of potassium citrate or half-saturated with $(NH_4)_2SO_4$. It was in such a part of the curve with $(NH_4)_2SO_4$ after nearly all globulin was removed from salt solution, that the point of constant solubility was found in our first experiments.

In the results with salting out of fraction "A" with potassium citrate, it was noted that the more of this fraction present, the lower the salt concentration at which it began to separate. This fact suggested a definite solubility. A comparison may be made with the albumin fraction "D" in Figs. 3 and 4. It is evident that it does not begin to separate on addition of salt until its point of saturation is reached. Of course, when the concentration of D is less a higher salt concentration is required for its first appearance on the salting-out curve.

During some experiments carried out by Dr. T. Addis on the effect of diet on body proteins of rats, depletion of the proteins was produced by fasting and then restored by refeeding proteins. The blood serum from these rats was studied. On regeneration certain protein fractions were replaced before the others, one of these being a globulin fraction C′. In newborn calves Howe (6) had reported that the globulins were formed very quickly upon feeding colostrum. We decided to study these fractions, particularly as it was observed that the globulin of calf serum so rapidly formed was anisotropic and had a definite crystalline form. Experience had proved to us that certain fractions of globulin are extremely unstable. Therefore, it seemed essential, in so far as it was possible, to study them without removing

them from the original serum. This idea was carried out by dissolving protein which had been precipitated from serum by a certain per cent of potassium citrate in another portion of the same serum and then comparing the solubility curves of the serum and the concentrate so made. If the phase rule applies in its usual form and if only the first fraction, A, has been added, precipitation should begin at a lower

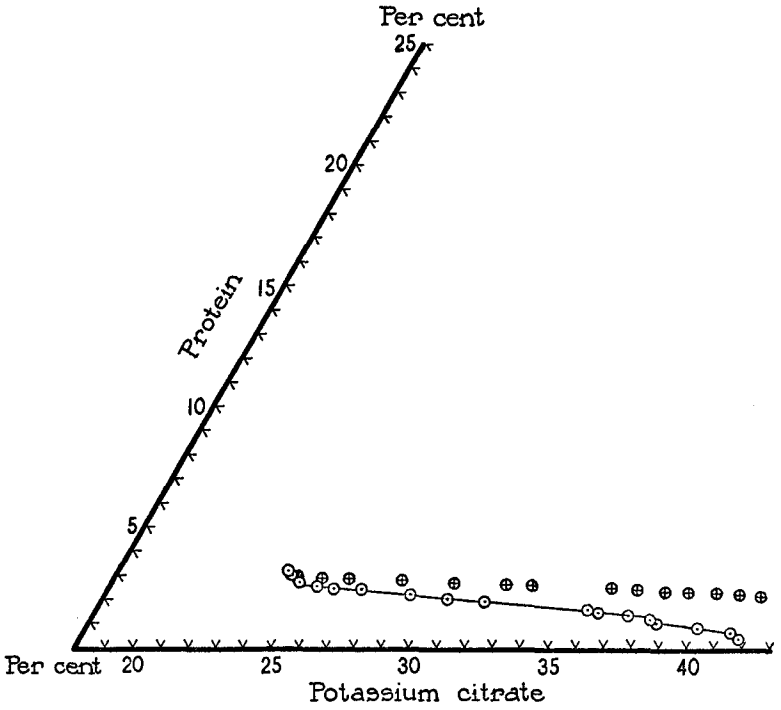

FIG. 7. Newborn calf serum. In Figs. 7 and 8
⊕ = total composition
⊙ = liquid phase.

salt concentration in the concentrate than in the serum and the two curves should coincide beginning with the point of first precipitation of A in the serum. The thermodynamical potential of the phases would not have been changed. If A and B have been added the solubility curve of the latter part of B and subsequent fractions should coincide, but not that of A.

A solubility curve with potassium citrate was made from the blood serum of a newborn calf. Fig. 7 shows that precipitation does not begin until 24 per cent citrate has been added. The first two fractions to be precipitated from other serums, namely A and B, are not visible in this calf serum. (See Howe.)

Fig. 8 illustrates the effect of feeding colostrum for 2 days to a newborn calf. Since precipitation begins now at 19 per cent citrate it is

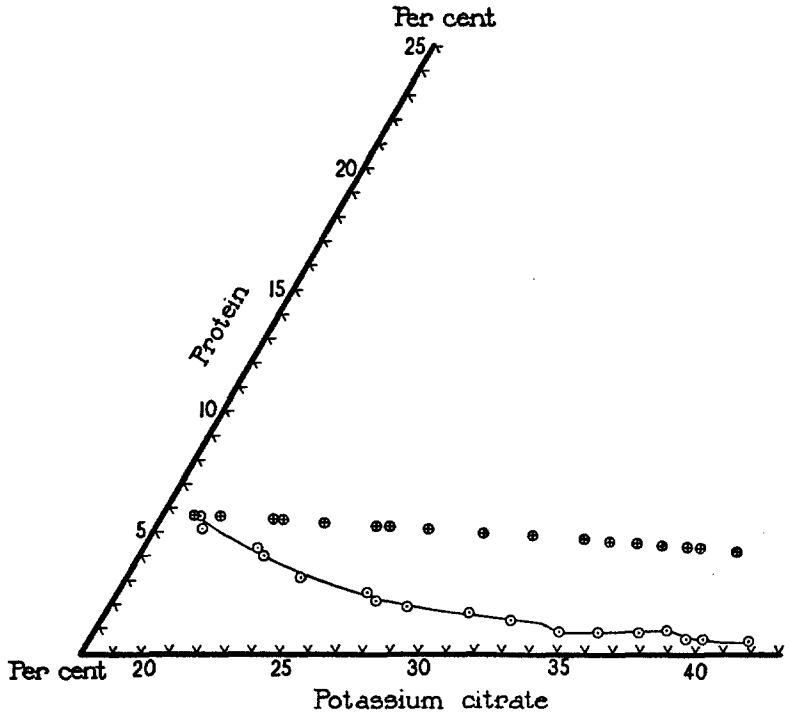

FIG. 8. Colostrum fed calf serum.

evident that fraction B has been formed rapidly. From the appearance of this curve the fraction up to 26 per cent citrate was provisionally assumed to be a single protein.

Application of the Phase Rule

Another calf was fed colostrum for 2 days and its serum globulin studied. In order to maintain the concentration of the other proteins

constant, unwashed globulin precipitated from 240 cc. of serum at 26 per cent citrate was added to the 100 cc. of the original serum. In this calf serum protein production had proceeded further and fraction A had appeared as seen in Fig. 9. The curve is drawn to follow the direction of that in Fig. 7 at 23–24 per cent citrate although the points

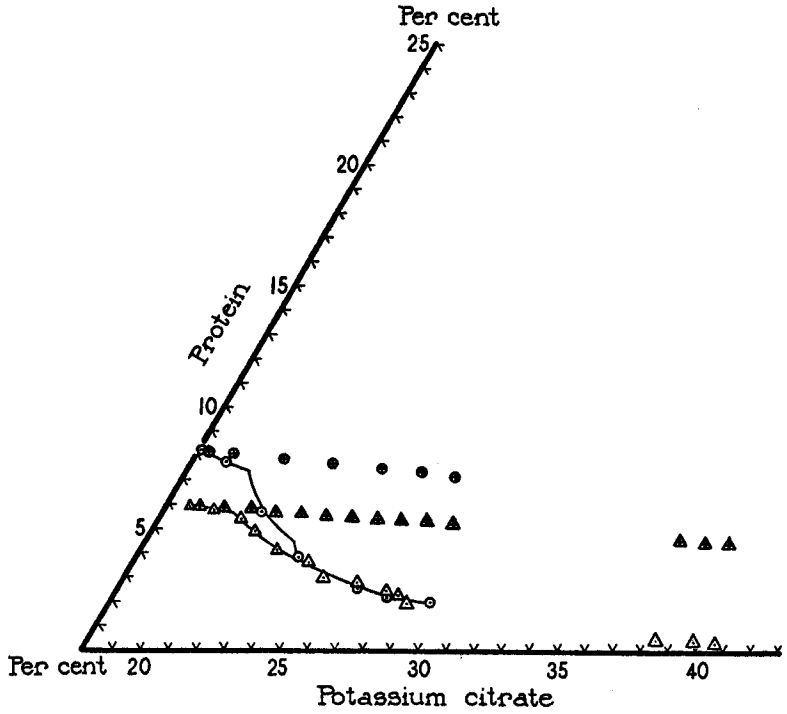

FIG. 9. Colostrum fed calf serum.

⊕ = total composition, serum with added globulin
⊙ = liquid phase, " " " "
⧋ = total composition, serum unchanged
△ = liquid phase, " " .

are not close enough to determine the line. It is now apparent that the break in the continuity of the line is between 23 and 24 per cent instead of at 26 per cent. When the precipitate made at the latter salt concentration was added three fractions A, B, and C were added. Theoretically the curves should then coincide at about 24 per cent if

the proteins are homogeneous. This actually took place. If C had been fractionated by precipitation from the original serum, the more insoluble precipitated part should have displayed a different solubility from the original C and there would be a divergence up to 26 per cent

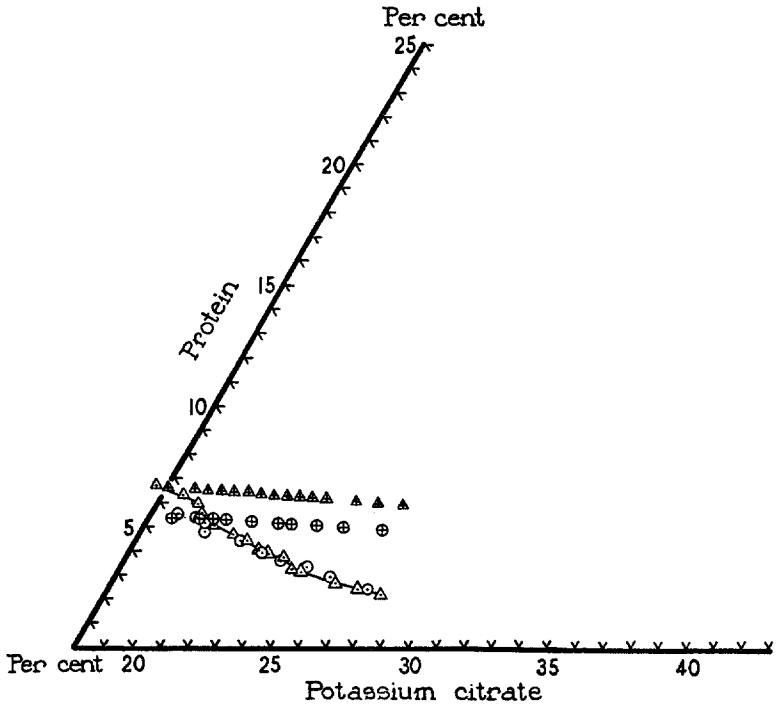

FIG. 10. Colostrum fed calf serum.

⧋ = total composition, concentrate I
△ = liquid phase, " "
⊕ = total composition, unchanged serum
⊙ = liquid phase, " " .

or a different curve entirely along the line of C, unless after fractionation the same equilibrium had been established.

Serum from another colostrum fed, $2\frac{1}{2}$ day old calf was utilized in the next experiment. The precipitate from 90.4 gm. of serum at 24.15 per cent potassium citrate dissolved in 100 cc. of serum formed con-

centrate No. 1. During dialysis of this solution and the original serum some dilution took place, unfortunately slightly more in the concentrate than in the serum. The coincidence of the two salting-out curves, compared in Fig. 10 is obvious although not perfect. There is an appreciable divergence only in the part of the curves representing the precipitation of A and the first part of B. Both A and B begin to precipitate at lower salt concentrations in the concentrate than in the serum. There is no evidence whatsoever of anything like a proportionality between the protein in the original mixture and that remaining in solution as may be seen from comparing the points of total composition of the two series. Protein is precipitated from the concentrated solution until the concentration of the protein in the liquid separated from the precipitate is equal to that of the serum. Here the two curves join. Comparing the curve made from concentrate I with that made from concentrate II (Fig. 11) formed by dissolving protein precipitated from 224.5 gm. of serum at 21.4 per cent potassium citrate in 60 cc. of the same serum, the salting-out curves are found to be in the main part parallel although the protein content of the two concentrates is very different. Concentrate I would contain unfractionated A and B if the assumption is correct that practically all of A and B have been precipitated at 24.15 per cent of citrate. The precipitation of A undoubtedly overlaps that of B. Since a greater volume of the serum was precipitated for concentrate II than for concentrate I, there is a greater concentration of A in the former. Also at 21.4 per cent citrate only part of the fraction B has been precipitated and added to make concentrate II. If a fractionation took place during the precipitation of B only the most insoluble fraction could have been added. There is no proportionality between the per cents of protein in the mixtures and those in the solutions. The mixtures with higher per cents of total protein (concentrate II) give solutions with less per cents of protein at the same potassium citrate content. This is because concentrate II is more dilute with respect to C and the fractions of protein precipitating at greater concentrations of citrate than is concentrate I. The convergence of the curves in Figs. 9 and 10 at greater concentrations of potassium citrate than 24 per cent is evidence of this fact. When the

concentrations of C were the same, the solubility curves of B coincided (Fig. 10). The solubility curve of A in the second concentrate is not well defined but probably falls sharply (Fig. 11).

Horse serum globulin was studied in the same manner as the calf serum globulin. Potassium citrate was added to the serum to make

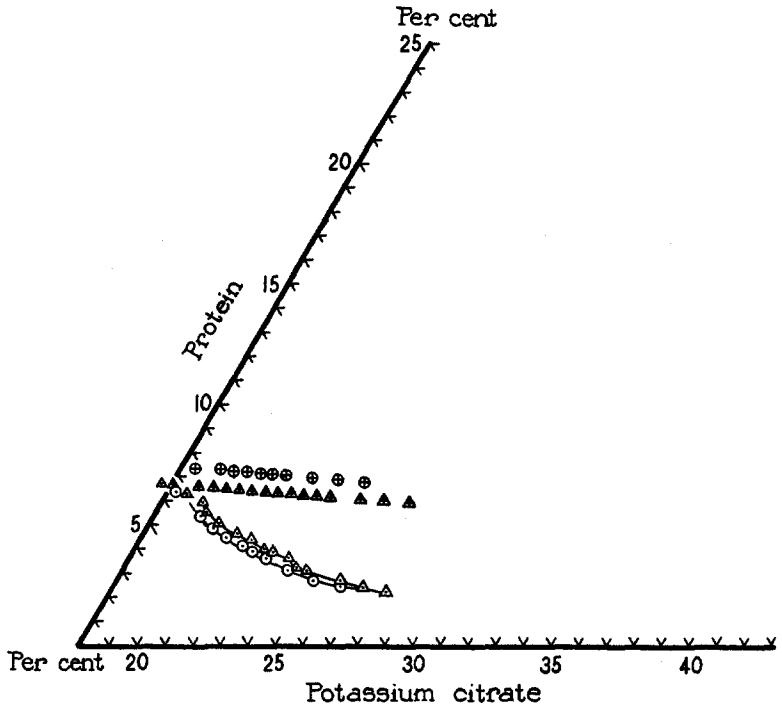

FIG. 11. Colostrum fed calf serum.

⧋ = total composition, concentrate I
△ = liquid phase, " "
⊕ = total composition, " II
⊙ = liquid phase, " ".

it 20.8 per cent citrate. The precipitated fraction, separating, was freed from the greater part of its adhering liquid by being pressed between filter paper. It was then dissolved in serum to make two concentrates of different strengths (I and II). Citrate was added to the above filtrate to bring about a second precipitation at 23.5 per

cent citrate. This second precipitated fraction was now partially dried with filter paper and added to small portions of the first filtrate to make concentrates III and IV.

TABLE I

	Protein	Potassium citrate
	per cent	*per cent*
Concentrate I	12.8	7.33
Concentrate II	14.3	8.50
Concentrate III	9.95	15.04
Concentrate IV	12.0	16.6

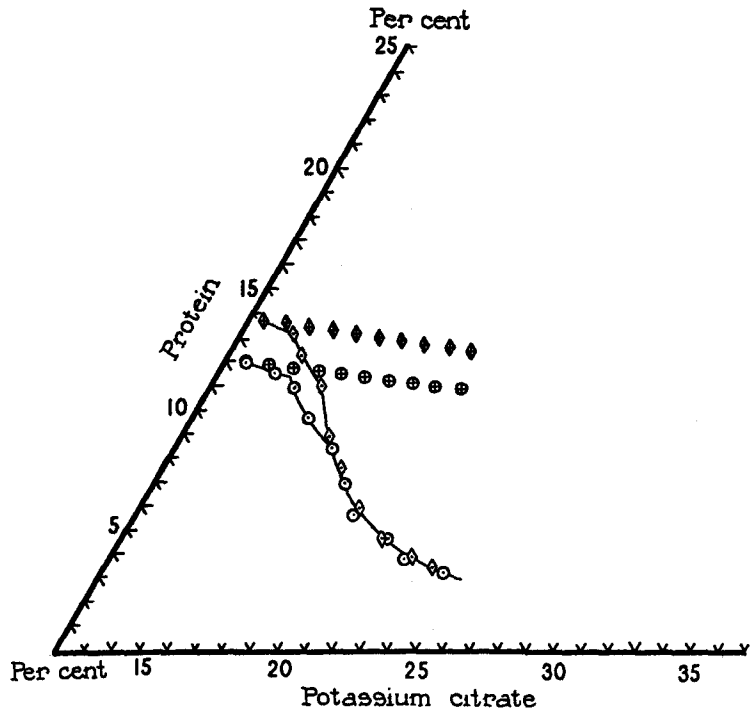

FIG. 12. Horse serum.

⊕ = total composition, concentrate I
⊙ = liquid phase, " "
⊕ = total composition, " II
◇ = liquid phase, " ".

To several weighed portions of each of these four solutions potassium citrate and citric acid were added as usual at 0° and pH 6.8 and the filtrates from the precipitates analyzed. From these analyses the curves in Figs. 12 and 13 were constructed. The curves have the same general nature as those from calf serum. Those from concentrates

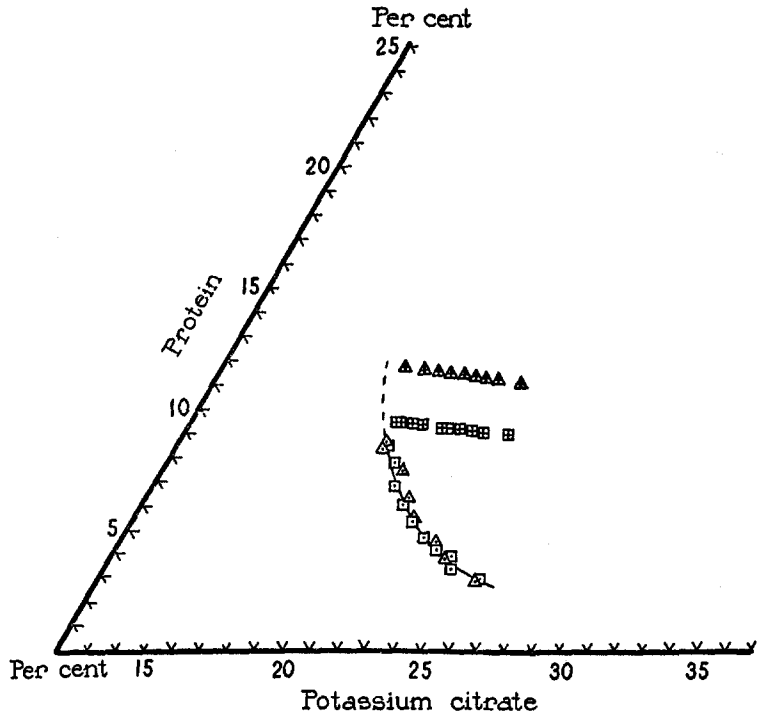

FIG. 13. Horse serum.

⊞ = total composition, concentrate III
⊡ = liquid phase, " "
⧋ = total composition, " IV
△ = liquid phase, " ".

I and II coincide at about 17.5 per cent citrate when both solutions are saturated with respect to the third globulin fraction. This fraction begins to separate at a lower salt concentration from the more concentrated protein solution. The curves of III and IV nearly coincide. As this fraction is separated as a crystalline substance it

was more difficult to get the solubility curve before the fractions decomposed. Although the coincidence of the lines is not perfect there is no evidence of fractionation. This fraction shows the characteristics of a single homogeneous protein.

DISCUSSION

The method described here is different from those used previously, in that the globulin has not been removed from its original medium and has been in so far as is possible retained in its original state. It may be that albumin or other substances normally present in the serum are necessary to prevent changes in the aggregation of the globulins. Only 3 to 4 days elapsed before all the precipitations were made, since the processes of purification are eliminated. A temperature of 0°C. was maintained thus retarding denaturation. The pH is within the stable zone.

The curves in Figs. 9, 10, 12, and 13 are those which were predicted by applying the phase rule to systems of several simple protein components.

Then, in all probability, the principal globulin that precipitated between 19 per cent and 24 per cent potassium citrate from calf serum is a homogeneous substance (either a single substance or a solid solution), a dispersion of particles of the same size, or an equilibrium system if made up of unit particles. It could not be a dispersion of particles of different sizes by a stabilizing agent, nor a compound of varying composition such as $mx.\ ny$. In each of the latter cases there should not have been a parallelism between the two curves plotted in Fig. 11 nor a coincidence of the curves in Figs. 9, 10, 12, and 13 unless indeed the fractions which might make up the protein had practically the same solubility, which is unlikely. With, for example, two component parts of different solubilities we should have obtained a greater concentration of the more insoluble part in the higher concentrate (II) in Fig. 11. Hence one of two different solubility curves would have been found, either a change in direction at about 21 per cent, if a series of dispersed particles of varying sizes constituted the protein "B," or a gradual divergence throughout the curve from 19 per cent to 24 per cent citrate if B were made up of a varying complex of $mx.ny$.

It does not necessarily follow that other fractions of serum are homogeneous because this fraction has been found to be so. In fact, there are indications that this fraction may be of a simpler nature than some of the others occurring in serum.

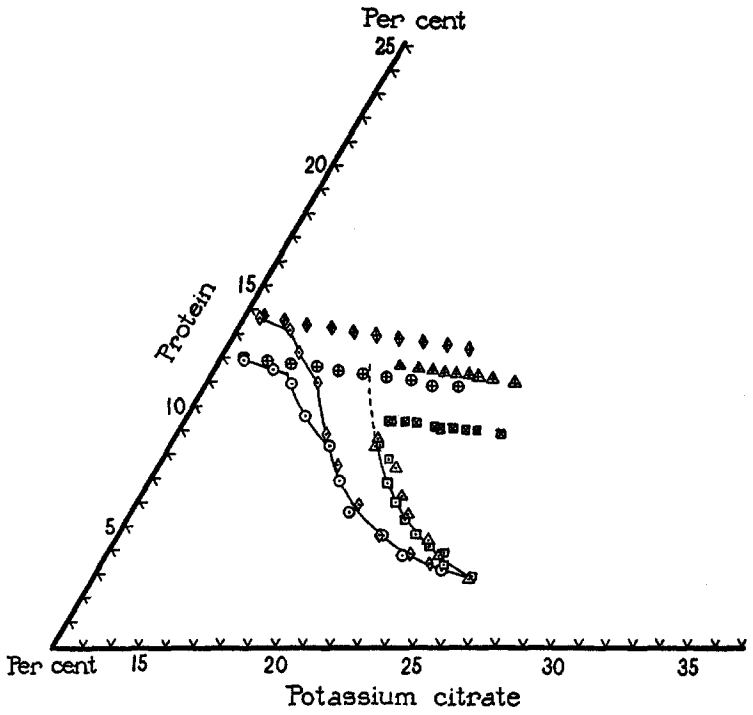

FIG. 14. Horse serum. Combination of Figs. 12 and 13.

⊕ = total composition, concentrate I
⊙ = liquid phase, " "
⊕ = total composition, " II
⊙ = liquid phase, " "
⊠ = total composition, " III
⊡ = liquid phase, " "
⚠ = total composition, " IV
△ = liquid phase, " ".

The case for a solid solution becomes very strong when the four curves in Fig. 14 are compared. The four curves do not coincide until the potassium citrate becomes about 26 per cent although the two

curves in each pair converge during the separation of the fraction from 17 per cent to 26 per cent salt. It may be that we have a *solidus-liquidus* curve with a minimum at 26 per cent salt when the descending salt concentration is plotted against the composition at a constant temperature. In such a case the curves in Fig. 12 fall on one arm while those in Fig. 13 fall on the other.

Since a solid solution is a homogeneous substance we seem justified in calling the principal globulin fractions, which have been considered in this paper, homogeneous.

CONCLUSIONS

A method has been developed for applying the phase rule to systems of several protein components in serum.

The globulin fractions which have been investigated appear to be homogeneous substances.

Throughout the duration of this borderline study of the application of the phase rule to a physiological problem, I have been indebted to Dr. T. Addis and Dr. J. W. McBain for helpful criticism.

The calves for these experiments were kindly furnished by Mr. H. H. Sortor of Tulare, California.

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