A PHASE RULE STUDY OF THE PROTEINS OF THE BLOOD SERUM: A COMPARISON OF THE PROTEINS OF HUMAN, RAT, AND HORSE SERUM*

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(Accepted for publication, May 19, 1936)

In the course of the present investigation an effort has been made to ascertain whether the protein fractions of blood serum may be considered individual proteins. They have been studied in various mammals and in both sexes.

Previous attempts to separate individual proteins from blood serum by classical methods, even when taking the precaution to handle them quickly and at a low temperature, had led to the conclusion that the fractions, with the exception of crystalline serum albumin and possibly one globulin, were either not single proteins or were too unstable to behave as individuals. In order to investigate individual unchanged proteins it seemed necessary to study the whole of the salting-out curve of *fresh* serum at 0° and to interpret it in the light of the phase rule.

The fixing of pH and temperature are necessary prerequisites for the analysis of this question of the individuality of the proteins, since only after eliminating a sufficient number of variables can the phase rule be applied in answering this question.[1]

For the application of the phase rule in its usual form to protein solutions certain assumptions are necessary. Let us consider the mathematical statement C plus 2 = P plus F where C = the number of components, P = the number of phases, and F = the number of degrees of freedom or variance. If the pressure is left at barometric pressure throughout, we have as variables only the composition of the

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

[1] See the work on egg albumin of Sørenson, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, No. 12.

liquid phase and the temperature. There are as possible variables or degrees of freedom one less than the number of components in the liquid phase plus the temperature. In case the pH is left constant, the components are salt, H_2O, and protein. We have then three components and, if we assume one solid protein phase, three phases which give us then, two degrees of variance or freedom. If the protein is a single component and we fix two variables, such as the temperature and the concentration of the salt in the liquid phase, then according to the phase rule the concentration of the protein will be fixed regardless of the amount of protein in contact with the solution.

If, however, more than a single protein is present, fixing the salt content will not fix the composition of liquid until there are as many solid protein phases in contact with the liquid as there are protein components.

Thus, each new solid phase appearing when the salt concentration is increased will give a break in the continuity of the curve representing the liquid. Several such breaks appear on the diagram.

These solid phases must be individual proteins, solid solutions of one or more proteins in another, or continuous series of compounds.

Method

All experiments were carried out at 0° and at a constant pH (6.8 except where otherwise noted) which was checked by the glass electrode in the equilibrium liquid.

Many previous qualitative experiments had raised misgivings as to the advisability of using $(NH_4)_2SO_4$ because of evidence of denaturation as indicated by the gradual clouding of the supernatant liquid and a progressive insolubility of the protein when the solutions are repeatedly brought from 0° to room temperature. Of other salts, potassium citrate, was most satisfactory in that a solution saturated with respect to protein at 0°, remained clear for at least a week, and any protein which separated upon repeated warming to room temperature dissolved when the solution was cooled to 0°. Previous experience in the extraction of pectin from fruit had impressed one of the authors with the specific effect of ions in causing an alteration in a colloidal substance. Such acids as citric or sulfurous could be used to extract an unchanged pectin, while hydrochloric or sulfuric acids at the same pH brought about a loss of its original properties. Potassium citrate is an excellent buffer. It is also sufficiently soluble at 0° to provide nearly complete salting out of the protein.

The pH was brought to 6.8 by the addition of N/2 solution of citric acid. This pH is well within the stability range as found by Svedberg and not too far from the pH of blood. Then, the serum was dialyzed against a cold 5 per cent potassium citrate solution of pH 6.8, and was finally brought to the same pH by further addition of citric acid if necessary. Rapid dialysis was carried out as suggested by Simms and Northrop in rocking Visking cellulose membranes in which a marble was included to stir the serum, until the dialyzing liquid gave no test for chloride and then for at least an half hour more. Usually about 18 liters of dialysate were used for 50 cc. of serum.

All measurements were by weight. The protein was determined by the gravimetric method of Barnett, Jones, and Cohn[2] with the modification that the salt was removed from the heat coagulated samples by washing well with the hot buffer solutions before adding ether and alcohol washes.

Potassium was determined as potassium sulfate by adding an excess of sulfuric acid to the sample of serum, heating to dryness slowly on a hot plate to avoid spattering, igniting at a low red heat in a muffle furnace to remove organic matter, and weighing.

Known quantities of potassium citrate (Kahlbaum's) were added to weighed portions of serum in sufficient amounts to bring the total composition into the range of precipitation. From these values the per cents of protein and potassium citrate in the mixtures were calculated. Both the citrate and the citric acid, added to maintain the same pH, were finely ground to avoid mechanical occlusion of coated undissolved particles in the precipitate, and care was taken that the mixtures were very thoroughly stirred. The tubes were agitated 3 hours at 0° in an ice water mixture to assure complete precipitation. Previous experiments with $(NH_4)_2SO_4$ had shown that the equilibrium satisfied the most stringent test; it is the same whether it is attained by adding salt to the protein solution or by salting out the whole of the protein in a solution, separating and discarding the mother liquor and adding water and salt to the precipitated protein, provided in both cases the final total composition is the same.

Because of the instability of the protein solutions, all processes were carried out as speedily as possible. The solutions were filtered on funnels which were tightly covered with watch glasses or ground glass plates. Weighed samples of filtrate were analyzed for potassium and protein. The precipitated solid phases which separated at each point were rapidly pressed between filter paper to remove excess liquid, mixed as thoroughly and quickly as possible with a spatula, and preserved in glass stoppered bottles. Samples were weighed, dissolved in water, and analyzed for protein and potassium.

The results on the solid phases were necessarily inaccurate because of the difficulty of avoiding evaporation and of mixing the samples properly. However, the fact that the plotted points of solid phase,

[2] Barnett, C. W., Jones, R. B., and Cohn, R. B., *J. Exp. Med.*, 1932, **55**, 633.

total composition, and liquid phase, so nearly fulfill the theoretical requirement that they fall on a straight line when plotted according to per cent composition makes it impossible to disregard their significance. The probable error of the variation from the mean of two determinations of protein as found from about three hundred duplicate analyses of protein solutions is 0.007 parts per part of protein present.

The complete reproducibility of the results is shown in Fig. 3 where the data from two samples of serum, from different groups of 40 rats under the same dietary conditions, are plotted in the same diagram.

All data are recorded upon the Gibb's triangle[3] in terms of per cent

[3] The Gibb's equilateral triangle was employed in which the three corners represent the three pure components. Any point within the triangle denotes a substance or mixture containing all three components.

The perpendicular from each corner (100 per cent of the component) to the side opposite representing substances or mixtures containing none of that component may be used as a scale for measuring the per cent of the said component. The geometrical theorem which states that the sum of all perpendiculars from any point in an equilateral triangle to its sides is equal to the altitude of the triangle, offers the means of measuring the per cents of all three components: the sum is then equal to 100 per cent. For instance 100 per cent protein is found at the apex of the triangle. A point representing 10 per cent protein would fall on a line parallel to the base and cutting the altitude and the two sides 1/10th of their lengths from the base. Similarly, if the analysis showed 10 per cent salt, the point would have to be on a line 10 per cent of the distance from the left hand edge of the triangle to the right corner, measured either on the perpendicular to the side opposite or on one of the two sides which it cuts. In practice the protein in per cent by weight is measured along the left edge and the salt along the base. The graph may then be viewed and the per cents of protein and salt read off just as if rectangular coordinates were used. The per cent of H_2O may be similarly located on a line parallel to the right side of the triangle.

The X's on each diagram represent the per cents by weight of the components in the composition of each mixture of serum, dry potassium citrate, and citric acid. Each point is calculated from an analysis of the serum and the added salt and acid. The boldly drawn curves in the lower parts of the diagrams represent the solubilities of protein at definite salt concentrations, since the curve separates the homogeneous liquid between it and the lower boundary of the diagram from the heterogeneous mixture of liquid and solid above. The distance, then from the X representing the total composition of a mixture and the corresponding O representing the analysis of the liquid separating from it is a measure of the protein precipitated at that point. The triangles represent the analyses of the

potassium citrate, per cent total protein, dried at 110°, and per cent H_2O (including any added citric acid).

In some cases, to show detail by increasing the scale, only a small portion of the diagram is included in the figure.

Lines parallel to the left hand side of the triangle represent equal percentages of potassium citrate. Hence it is evident that in every case the salt content of the liquid is quite different from that of the total system and that of the solid phase. In no case are the tie lines parallel to the left hand side of the triangle.

EXPERIMENTAL

The following experiments were carried out (*a*) with horse serum, (*b*) with serum from male rats, (*c*) from female rats, and (*d*) with human serum.

The Results for Horse Serum

Six different samples of horse serum were obtained from the Cutter Laboratory, Berkeley. The blood was freshly drawn, defibrinated, the red blood corpuscles centrifuged off, and the serum chilled immediately and kept at 0°. All six gave the same type of curve, and in two they coincided. Typical sets of analyses are given in Tables I and II.

It will at once be noted that where the potassium citrates have gone fully into solution the analyses check admirably. Where the duplicates in the last column are different, it is seen that this corresponds

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solid phases separating from the mixtures. All the lines connecting phases in equilibrium, that is the corresponding O and triangle must be straight and pass through the X representing the total original composition of the system.

It is important to note that the angle at the corner representing H_2O may be changed to a right angle making a rectangular diagram where protein concentration is plotted against salt concentration and the scales of the ordinates and abscissas may be changed without altering this property of the tie lines, and without losing the advantage of being able to plot all possible compositions of the entire system on such rectangular graphs.

Any discontinuities or changes of direction of the solubility curve in the triangular graphs on any of these diagrams are not straight lines on other diagrams by other authors where composition is not expressed in total weight per cent. Their significance, however, remains the same.

TABLE I

Horse Serum. Precipitation with Potassium Citrate—0°C.—pH 6.9

Total composition by weight		Solid phase by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein	Potassium citrate	Protein
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
12.20	7.753			12.57	7.641
14.02	7.592			14.07	7.532
15.85	7.429			16.14	6.926
17.68	7.264			17.95	6.596
19.51	7.094			19.72	6.104
21.36	6.926	15.15	28.37	22.02	5.171
23.21	6.758	16.82	26.05	24.73	4.047
25.07	6.587	19.42	23.24	25.59	3.748
27.55	6.363	21.63	21.70	28.59	3.223
28.80	6.246	22.41	21.73	29.75	2.906
30.68	6.073	25.30	18.66	31.85	2.452
32.55	5.898	26.80	19.30	34.33	2.080
34.41	5.720	27.63	18.20	35.11	1.744
36.28	5.541	31.34	16.90	38.42	0.952
38.12	5.358			40.60	0.513
39.94	5.172	36.90	12.66	43.10	0.0567

TABLE II

Horse Serum. Precipitation with Potassium Citrate 0°C.—pH 6.8

Total composition by weight		Solid phase by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein	Potassium citrate	Protein
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
15.12	5.60			15.16	5.44
15.32	5.60			15.27	5.56
20.74	5.22	13.37	29.56	21.10	4.32
20.74	5.22	13.51	29.37	21.07	4.32
23.87	5.01	13.10	30.98	21.03	4.34
23.87	5.01	16.29	26.82	24.63	3.06
34.18	4.30	26.76	18.84	35.48	1.09
23.18	4.30	27.06	18.65	34.86	1.10
41.84	3.76	34.75	16.95	43.73	0.059
41.84	3.76	35.21	14.77	43.45	0.045

with the difference in the next to the last column, and upon plotting the data are found to lie upon the same equilibrium curve.

Fig. 1 gives the complete diagram for sample VI of horse serum which had been slightly concentrated by freezing. Some of the detail of this diagram is found in Fig. 2.

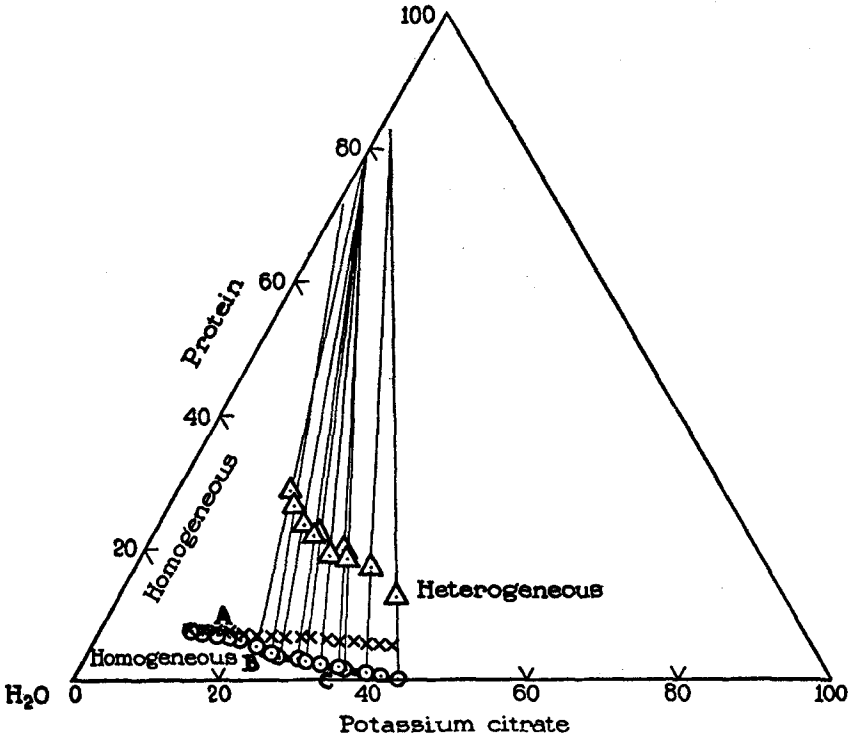

FIG. 1. Horse serum, sample VI. Phase rule diagram at 0°C. at pH 6.8.
In Figs. 1, 2, and 4–6

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

The most obvious feature of the phase rule diagrams is that the tie lines definitely do not point toward anhydrous protein but tend to converge at a protein containing between 21 and 25 per cent of H_2O and quite possibly some salt. For the general shape of the diagram reference should be made to the more elaborately annotated diagram

in the preliminary study of purified horse serum globulin and $(NH_4)_2SO_4$.[4]

The general nature of the equilibrium is surprisingly similar to the liquid boundaries found with soap solutions and gelatin.

The discontinuities in all the solubility curves are taken up in the discussion.

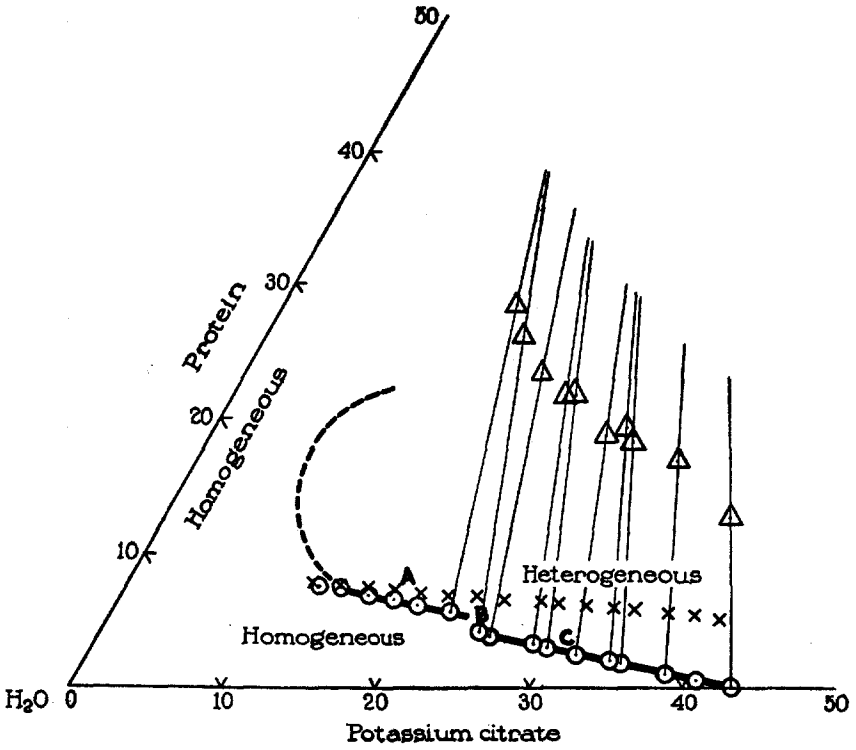

FIG. 2. Horse serum, sample VI. Phase rule diagram at 0°C. at pH 6.8.

Results with Male and Female Rat Serum

The serum was obtained from pools of 30 or more rats of the same sex and age. The blood was allowed to clot and the serum separated by centrifuging. A control experiment with horse serum showed no difference between serum prepared

[4] McBain, J. W., and Jameson, E., *Tr. Faraday Soc.*, 1930, **26**, 769.

in the two manners. The samples for precipitation were much smaller than with horse serum, being from 2 to 15 gm. instead of 40 gm. and, in consequence, the amount of solid phase separating from the smaller samples was not sufficient for accurate analysis.

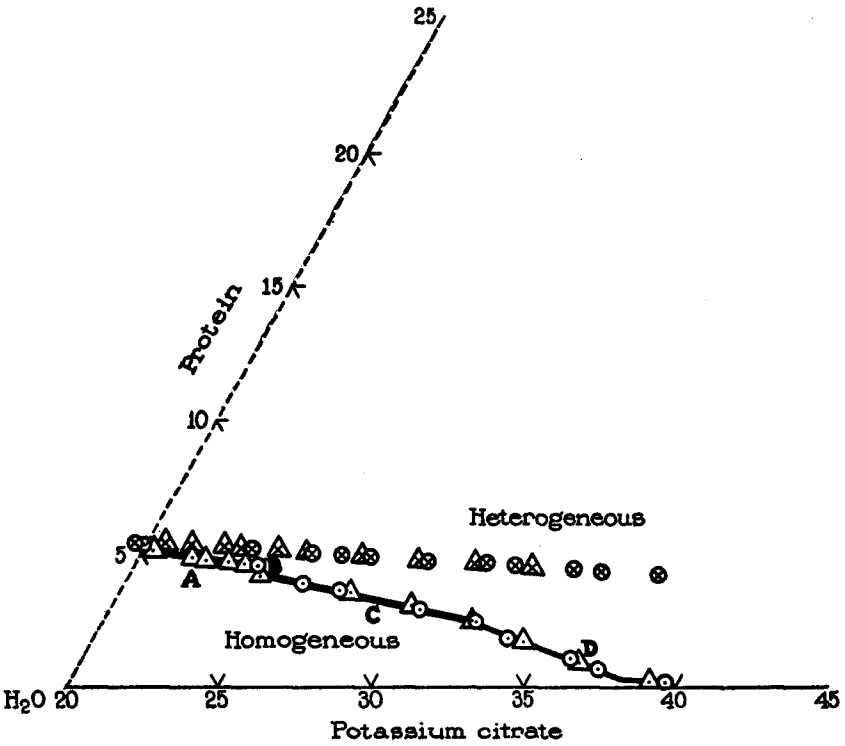

FIG. 3. Stock composite curve. Two samples of serum from 40 male rats each. Phase rule diagram at 0°C. at pH 6.8.

⊗ = sample I, total composition
⊙ = sample I, liquid phase
△ = sample II, total composition
△ = sample II, liquid phase.

Figs. 3 and 4 show the results for male and female rat serum respectively. The sample Table III gives the data for the male serum.

Results for Human Serum

The serum was prepared in the same way as the rat serum and samples similar in size were used. The data in Table IV are repre-

sentative of three similar experiments, two with individuals and one with a pool of serum from six persons. Figs. 5 and 6 show the results at pH 6.8 and pH 5.5 respectively.

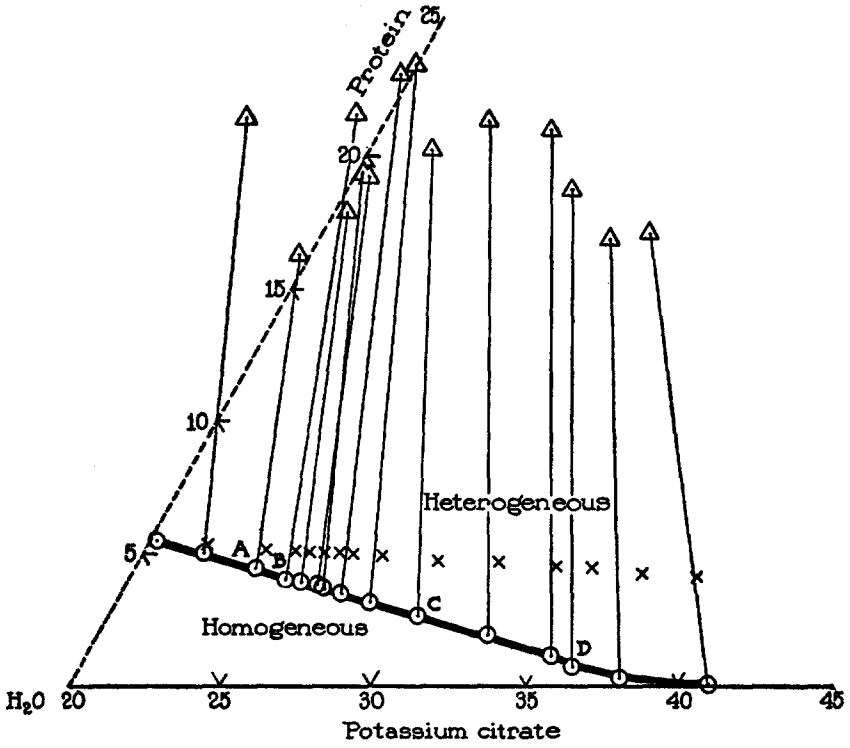

FIG. 4. Diet — stock. Blood serum from 40 female rats. Phase rule diagram at 0°C. at pH 6.8.

DISCUSSION

(*a*) There are four kinds of protein. As may be seen from Figs. 1 to 6, the curves separating the homogeneous and heterogeneous areas are of a similar type.

The first section of the curve before the break at 22–23 per cent salt with horse serum and 24–25 per cent with rat serum may be assumed to represent an equilibrium of the liquid phase in contact with a phase which is probably another liquid, protein A.

The break which follows seems at first to be absent in human

serum and in female rat serum. When the temperature or pH at which precipitation takes place is changed, this break may be made to

TABLE III

Stock Diet.

Blood Serum from 40 Male Rats—100 Days Old. 0°C.—pH 6.8. Precipitation with Potassium Citrate

Total composition by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein
Experiment 1			
per cent	*per cent*	*per cent*	*per cent*
19.61	5.412	19.85	5.314
23.58	5.135	24.03	4.529
25.57	4.997	25.84	3.883
26.57	4.925	27.22	3.605
27.56	4.857	27.84	3.616
29.54	4.718	30.17	2.881
31.51	4.580	32.19	2.371
32.49	4.510	33.69	1.765
34.46	4.372	36.02	1.088
35.43	4.301	37.10	0.734
37.36	4.160	39.54	0.1472
Experiment 2			
20.63	5.339	20.30	5.056
21.58	5.271	21.78	4.778
22.62	5.221	22.30	4.745
23.49	5.140	23.55	4.623
24.45	5.071	24.53	4.135
25.41	5.006	23.02	4.601
27.30	4.872	27.58	3.502
29.24	4.737	29.83	3.062
31.16	4.602	32.13	2.467
33.07	4.468	34.15	1.646
34.97	4.330	36.37	0.883
36.86	4.191	38.99	0.2051
37.81	4.122	39.78	0.0963

appear in the female rat serum.[5] In Fig. 6 showing human serum at pH of 5.5 in a small range of salt concentration, this break is seen to

[5] Unpublished.

occur. This second protein phase, B, appears at about 23 per cent salt. The shape of the curve as it falls abruptly to the curve C has not been determined. The curve C again becomes a straight line showing equilibrium with a third phase, C, containing 20 to 25 per cent water and possibly some salt.

The third change in direction and appearance of yet a different fourth solid phase, D, is obvious in human serum and in the rat serum

TABLE IV

Human Blood Serum from a Normal Person. Precipitation with Potassium Citrate—0°C.—pH 6.8

Total composition by weight		Solid phase by weight		Liquid phase by weight	
Potassium citrate	Protein	Potassium citrate	Protein	Potassium citrate	Protein
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
17.94	6.422			18.14	6.252
19.93	6.264	13.78	28.7	20.22	5.814
21.91	6.101	17.32	21.0	22.27	5.284
22.90	6.021	17.54	23.85	23.26	5.157
23.89	5.941	21.15	20.55	24.30	4.934
24.88	5.862	20.22	20.65	25.41	4.655
25.87	5.782	22.16	18.7	26.43	4.601
27.85	5.622			28.61	4.138
29.82	5.462	23.04	19.2	30.51	3.674
31.78	5.302	25.70	17.4	32.29	3.335
32.76	5.220	20.33	18.5	33.39	2.976
33.74	5.141	28.36	15.95	34.73	2.491
35.68	4.978	30.81	17.0	36.51	1.726
37.61	4.815	32.90	19.1	39.82	0.558
39.52	4.652	33.43	20.3	43.30	0.014

(especially in the male) but not in any of the six horse serum samples. It is evident from the work of others that this change does appear in horse serum after dilution and in the presence of $(NH_4)_2SO_4$ at room temperature.

(*b*) There is a straight line relationship between the protein and salt concentration except where fraction D appears when it becomes a curve asymptotic to the base of the triangle.

There is a rough proportionality between amount of protein in a

mixture and that remaining in solution, as may be shown by plotting all the horse serum curves on one diagram.

One fraction does not cease to precipitate when another begins. The question as to whether the fractions are individual proteins or not cannot be answered from these curves. The straight line relationship

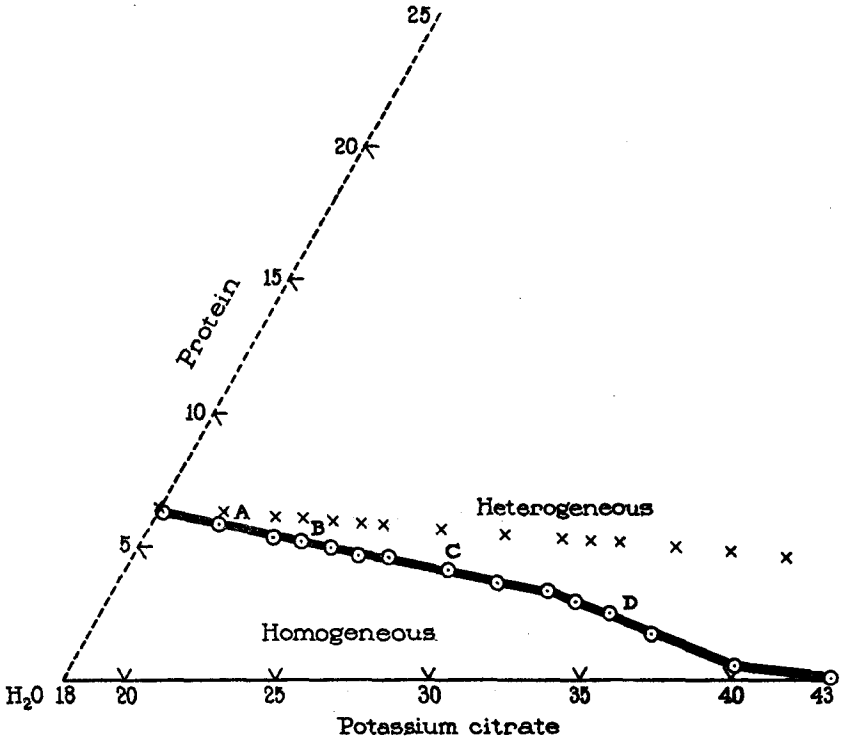

FIG. 5. Human serum. Phase rule diagram at 0°C. at pH 6.8.

and continuous curve of D would indicate that they must either be single proteins or continuous series of compounds or solid solutions.

The existence in the case of a rat serum of a high salt concentration where there is a definite solubility regardless of the amount of protein in the original mixture supports the view that the protein fractions are definite individuals.[5] However, it is probable that the high concentrations of the salt used, may break existing labile bonds. Also

the salt concentration is so high that only the solubility of the D fraction is appreciable. Further work is necessary to definitely decide this question.

(*c*) The convergence of the tie lines at the upper left side of the diagram indicates that all the solid protein phases contain from 20 to

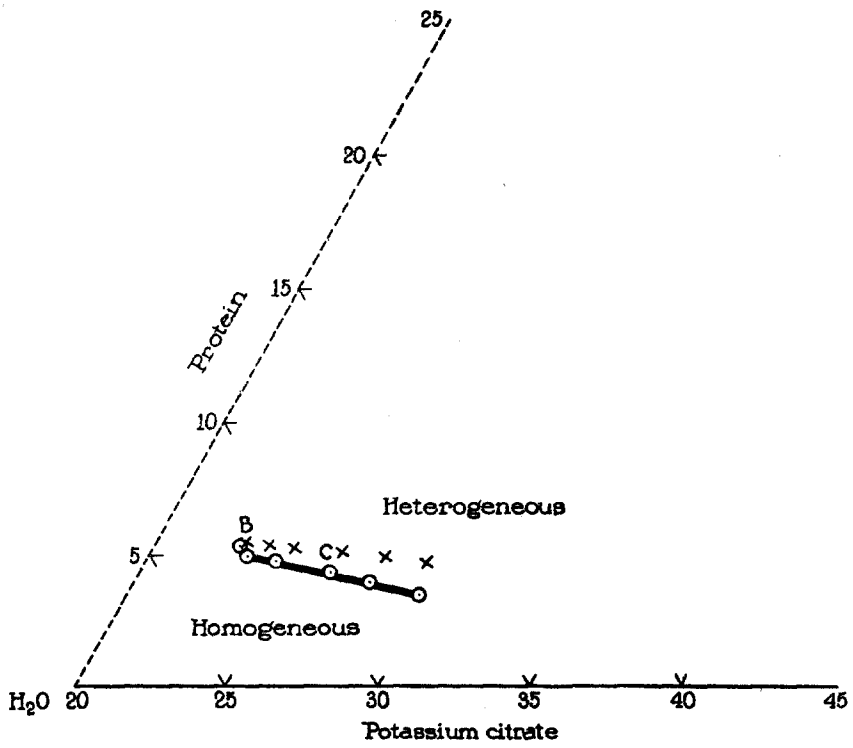

FIG. 6. Human serum. Phase rule diagram at 0°C. at pH 5.5.

25 per cent of water. Consequently, they have a definite hydration even when separating from a wide range of concentrated salt solutions.

(*d*) The salt concentrations at which the different protein fractions begin to be precipitated vary for different species. The relative per cents of the four fractions not only vary for different species but also for the two sexes.

When plotted on a single diagram all of the solubility curves of a single species tend to converge at the same concentration of salt;

namely, about 45 per cent with horse serum, 38 per cent with rat serum, and approximately 40 per cent with human serum.

SUMMARY AND CONCLUSIONS

There are four different kinds of protein in blood serum as shown by the solubility curves.

They must be either single proteins, several continuous series of compounds, or solid solutions.

The solid protein phases are hydrated.

There are definite sex and species differences.

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.