THE SOLUBILITY OF THE PLASMA PROTEINS

I. DEPENDENCE ON SALT AND PLASMA CONCENTRATIONS IN CONCENTRATED SOLUTIONS OF POTASSIUM PHOSPHATE*

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INTRODUCTION

Since Panum (1) in 1851 found that protein separated from serum on dilution and the addition of acid, the most widely used means of defining the plasma proteins has been by describing their solubility behavior. Between 1859 and 1902 the work of Denis (2), Hammarsten (3), Burckhardt (4), Marcus (5), Kauder (6), Pick (7), and others established the definitions of these proteins as commonly used today. These definitions are based on studies of the solubilities of so called protein fractions separated from plasma or serum by repeated precipitations and re-solution under specified conditions. The terms fibrinogen, euglobulin, pseudoglobulin, and albumin, if strictly used, should, therefore, designate the proteins separated by such procedures.

Between 1890 and 1905 various attempts were made to measure the concentration of the individual proteins of serum by so called single fractional precipitation; that is, by determinations carried out on precipitates or filtrates from the first precipitation at specified salt concentrations.

In 1901 Pinkus (8) published a method for estimating the serum proteins by such fractional precipitation with sodium sulfate at 37°. His method assumed no overlapping of the precipitation

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zones. There was, however, no attempt to analyze the purity of the fractions. Porges and Spiro (9) in 1903 studied such fractional precipitation of serum proteins with ammonium sulfate and found that the concentration of salt solution required to precipitate completely a less soluble fraction extended beyond the concentration at which the more soluble fraction commenced to precipitate out. They state that dilution of serum permits more complete separation, but analyses adequately supporting this claim are not given.

Haslam (10, 11) in investigating the separation of serum proteins by fractional precipitation demonstrated the incompleteness of separations effected by this method. He showed that dilution of serum before precipitation resulted in less of the more soluble protein being carried down in the precipitate of the less soluble protein, and that with sufficient dilution such inclusion caused only a small error. But he also demonstrated that dilution would not lessen the relative concentration of the less soluble protein remaining in solution in the filtrate of the more soluble fraction. The solubility of the less soluble protein in the presence of the soluble protein in salt concentrations at which the latter commenced to be precipitated was such at dilutions of 1:8 or more, that as much as 25 per cent of the euglobulin in ox serum was found in the pseudoglobulin filtrate, and some euglobulin was always found in the albumin filtrate. He interpreted his results as indicating quantitatively the degree of error involved in separations of this kind. If his method of analysis is accepted as applicable to the estimation of errors of single fractional precipitation, his results indicate that the actual solubilities of the less soluble fractions at specified salt concentrations are sufficiently large to vitiate results for the concentrations of the individual protein fractions based on zero solubility of the less soluble fractions at such salt concentrations.

Wiener (12) in 1911 demonstrated the same finding as Haslam concerning the increase in purity of the precipitate as a result of dilution. Apparently unaware of Haslam's finding concerning the amount of the less soluble protein remaining in the filtrate as a result of dilution, Wiener advocated dilution of serum as a means of increasing the accuracy of fractional precipitation.

Haslam's work (11) called attention to a further problem to be
considered in salting out procedures. He observed that purification of the globulin fractions beyond a certain point caused unmistakable changes in the properties of the protein fractions. Chick (13) observed similar changes. Thus arose two questions: are the individual protein fractions separable by salting out procedures distinct individual proteins, and are the fractions so separated similar to the protein complexes existing in plasma? Chick (13) believed that euglobulin was a complex of pseudoglobulin and a phosphorus-containing serum lipid. Analysis of the amino acid composition of the three purified serum protein fractions by Hartley (14) showed no difference in the amino acid composition of euglobulin and pseudoglobulin, but did reveal a difference between globulin and albumin. Woodman (15) in studying the identity of related proteins found no evidence that serum euglobulin and pseudoglobulin were different.

Svedberg and Sjögren (16) concluded from their experiments on the molecular weights of the serum proteins that there are but two proteins in serum, i.e. globulin and albumin. They believed that decomposition of the proteins occurs during such salting out procedures as used by Sørensen (17), and they regarded euglobulin and pseudoglobulin as artificial products.

Sørensen (18) has discussed at length both parts of the question raised. He agrees with Svedberg and Sjögren that the changes in the composition of the protein solutions necessitated by salting out procedures alter the composition of the protein complexes. But he makes a sharp distinction between reversible dissociation changes and irreversible decomposition or denaturation. He considers the serum proteins as complexes existing in serum in reversibly dissociable component systems. "Within each complex all the atom groups are interlinked by main valencies, whereas the components are reversibly linked by means of residual valencies." It is such reversible changes that occur during the proper fractionation of the serum proteins to euglobulin, pseudoglobulin, and albumin. Indeed any attempt to separate the proteins from serum probably alters the protein component systems and in all probability the fractions separated out will differ with the procedure used and will be different from the component systems existing in serum.

This brief and incomplete review gives in outline the background
on which salting out methods for the determination of the concentration of the serum proteins, such as that of Howe (19), should be considered and indicates the care with which results so determined should be interpreted.

Cohn (20), in 1925, showed that the solubility of a protein in concentrated salt solution was related to the concentration of the salt according to the equation, \( \log S = \beta - K_\mu \mu \), where \( \beta \) is a constant related to the point of interception between the straight line and the ordinate, where \( \mu \) represents the concentration of salt, in this particular case as represented by the ionic strength, but equally well expressed in terms of molar concentration, and where \( K_\mu \) is a constant representing the slope of the curve and has been shown to be a characteristic of any individual protein for a given salt solution. In 1928, Cohn and Green (21) showed the relationship between this equation and that of Debye and Hückel expressing the solubility of a saturating body in a solution of a strong electrolyte. Cohn’s equation shows that the solubility of a protein plotted against increasing concentration of salt solution results in a logarithmic curve or that the plotting of the logarithm of the solubility against the increasing salt concentration results in a straight line, whose slope, \( K_\mu \), is specific for an individual protein in a given salt solution, and hence is an added means of defining a protein.

If one examines a solubility curve of plasma or serum as presented by Howe (19) or Ruszczynski (22), it is apparent that the applicability of a linear relation between the logarithm of protein solubility and salt concentration to each portion of the curve would permit an estimation of the solubility of the less soluble fraction at points of break in the curve and hence at the salt concentrations selected for fractional precipitation. Mâcheboeuf, Sørensen, and Sørensen (23) have shown that such a linear relation exists for preparations of egg albumin which do not behave strictly as a pure protein; that is, whose solubility is not independent of the amount of protein used in the experiment. And Sørensen (18) has found a similar relation in experiments on serum albumin in which the dissociation tendency and dependence of solubility on total protein in the experiment is greater than for egg albumin. It, therefore, seemed to us that this linear relation might be found to hold for the different portions of precipitation curves of plasma.
So far as we have been able to ascertain the existing data on precipitation curves are unsatisfactory for such an analysis. We have, therefore, for the purpose of supplying such data undertaken a series of experiments on the solubility of the plasma proteins.

Cohn (24) has presented data concerning the variation of the ionic strength of potassium phosphate solutions with the maintenance of constant pH, and *vice versa*. The data make this salt particularly suitable for salting out experiments, as its use enables one readily to vary independently pH, salt concentration, serum concentration, and temperature and thus express the solubility of the saturating body as a function of any one of these variables. Florkin (25) and Green (26) have demonstrated the advantages of such phosphate solutions in protein solubility studies.

*Outline of Experiments*

The experiments reported in this paper deal primarily with the relation between the solubility of the protein complexes of horse and human plasmas and the salt concentration in concentrated potassium phosphate solutions of a constant pH of 6.5 and temperature of 25°.1 Two experiments pertinent to this study in which the plasma concentrations were altered are included. These two experiments present data concerning the relation between plasma concentration and protein solubility.

In spite of the fact that most solubility studies on the plasma proteins have been carried out in ammonium sulfate and sodium sulfate solutions and that the use of a new salt makes the solubility data less comparable, we selected potassium phosphate for the precipitating reagent in our experiments because of the advantages indicated above.

The pH of 6.5 was determined by using phosphate solutions consisting of equimolecular amounts of dibasic and monobasic potassium phosphate and was selected merely as a matter of convenience, as dilutions could be made from a 3 molal solution2

1 Experiment 1 was an exception as this was carried out at a temperature of 20°.
2 This solution was made up in 2 liter lots as follows: KH2PO4 is ground in a mortar and dried. From this 817 gm. of salt are accurately weighed out and poured dry into a 2 liter volumetric flask. Any salt adhering to the beaker in which the weighing was carried out is dissolved and washed quan-
with the constant pH remaining in all dilutions. 0.5 cc. of plasma\(^3\) was added to 15 cc. of phosphate solution in order to provide a large dilution of the plasma so that the effect of the total protein or the individual constituents of serum upon the solubility of the individual proteins would be reduced as much as possible, and also so that the inclusion of a more soluble fraction in a less soluble precipitate would be minimal. The salt concentration to which the plasma was added varied from 0.6 molar to 3.0 molar. A period of at least 12 hours was allowed for equilibrium to be reached,\(^4\) during which time the solutions immersed in a constant temperature water bath at 25° were gently shaken by a mechanical shaker. The solutions were then filtered at 25°, precautions being observed to prevent evaporation and filtrations being repeated until the filtrate was clear. The nitrogen in the filtrates was determined by Kjeldahl's macro method.

**Experimental Solubility Curves**

In describing the solubility curves we have found it convenient to identify the several portions of the curves with the protein fractions, i.e. fibrinogen, euglobulin, pseudoglobulin, and albumin, that can be separated from the precipitates obtained from the

\(^3\) 0.5 cc. of saturated sodium citrate per 100 cc. of whole blood. Immediately following collection, the cells were separated by centrifuging. In the experiments on horse plasma the plasma as soon as separated was added to the phosphate solutions. In the experiments on human plasma the plasmas were placed in the ice box overnight and added to the phosphate solution the following morning after being brought to 25°.

\(^4\) Samples of filtrates from the precipitation flasks were allowed to remain at 25° for an added 48 hours without a further precipitation occurring.
different portions of the curves. In so doing we wish to avoid intimating that the protein complexes precipitated in our experiments are the purified proteins obtained by repeated fractionation. We, therefore, where there is any likelihood of ambiguity, have referred to the fractions of the curves as being identified with the fibrinogen, euglobulin, etc., complex or fraction.

The procedure involved in the experiments probably alters the protein complexes so that we are not dealing with the component systems existing in serum. As the procedure is milder and occupies a lesser time interval than that employed in the experiments of Svedberg or Sørensen, we may assume with reason that such changes in the component systems that have occurred are reversible and do not represent denaturation.

Experiments on Horse Plasma

Graphs I and II present the solubility curves of the plasma proteins from two different horses when portions of plasma were added to phosphate solutions of equimolecular amounts of dibasic and monobasic potassium phosphate. The procedure was carried out as given above in the outline of the experiments. The abscissa represents the molar concentrations of phosphate in the plasma phosphate solutions calculated from the dilutions made from the original 3 molar phosphate solution and the dilution resulting from the addition of the plasma to each particular phosphate solution. In two experiments the plasma phosphate solutions were analyzed for phosphate by the method of Fiske and Subbarow (27) and the results agreed within the expected experimental error with the concentrations as calculated. To simplify the experimental work we, therefore, eliminated the phosphate analyses except as checks on the 3 molar solutions and have throughout used the calculated concentrations in the graphs. The ordinate on the left represents the solubility of protein nitrogen expressed as gm. of nitrogen per liter of filtrate. In Graphs I and II the curves through the outlined circles repres
sent the solubility curves. The broken line curves through the solid black dots represent the logarithms of the solubilities. It will be noted that there are breaks in this logarithmic curve not only at the points of break in the solubility curve, but also in the case of the pseudoglobulin portion at other points; for curves representing individual fractions are logarithmic only when the

![Graph I. Horse plasma, 20; dilution 1:31; Experiment 1. O = solubility curve; □ = pseudoglobulin curve corrected by subtracting euglobulin nitrogen; ● = logarithm of solubility.](http://www.jbc.org/)

Debye's $\Gamma/2$. Green (26) has shown that hemoglobin solubility expressed in such terms yields, within the experimental error, values of $K_s$ identical to those obtained when the molar terms here used are employed. In our systems the molar concentrations recorded are always one-half the ionic strength per liter, and hence should cause no confusion.
the precipitation of another protein fraction. This, we feel, has enabled us to detect with greater accuracy than otherwise possible the points of significant break in the solubility curves of the plasmas examined.

In the two experiments shown here and in three more on horse plasma the points of significant breaks occurred at phosphate concentrations of between 1.20 to 1.35, 1.5 to 1.6, and 2.4 to 2.5 molar. Assuming that horse plasma contains protein complexes from which fibrinogen and the three serum proteins, euglobulin, pseudoglobulin, and albumin, can be salted out, the data present curves very similar to a hypothetical solubility curve constructed

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\[ \text{Graph II. Horse plasma, } 25^\circ; \text{ dilution 1:31; Experiment 2. } \circ = \text{ solubility curve; } \square = \text{ pseudoglobulin curve corrected by subtracting euglobulin nitrogen; } \bullet = \text{ logarithm of solubility.} \]

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\[ \text{6 Howe (19) working with calf sera reports evidence for two pseudoglobulins. We have determined the protein solubility on horse and human plasmas in 17.7 per cent Na}_2\text{SO}_4\text{ and find no evidence of breaks in the phosphate curves at the corresponding protein solubility. Haslam (11) working with ox serum was unable to separate the pseudoglobulin into two proteins. Sørensen (17) divides the serum globulins of the horse into euglobulin and pseudoglobulin.} \]
on the assumption that the individual protein complexes separated out similarly to pure proteins in concentrated salt solutions.

The first portion of the solubility curve in each graph corresponding to phosphate concentrations from 0.7 to 1.1 molar we believe represents the precipitation of the fibrinogen complex. Its identification as fibrinogen does not rest solely on its being the first fraction to precipitate out. Its salting out range agrees with that of fibrinogen in similar phosphate solutions reported by Florkin (25). Furthermore, it represents in our experiments an average of 7 per cent of the total plasma proteins, which agrees with the reported values for fibrinogen in horse plasma of from 5 to 10 per cent. As no measurable precipitation occurs from 1.1 molar phosphate to 1.2 molar, an extension of the fibrinogen curve logarithmically indicates a negligible solubility of the fibrinogen complex in 1.2 molar phosphate solutions, and therefore a negligible amount of fibrinogen precipitated at higher phosphate concentrations.

Unfortunately there are no studies available for comparing the precipitation ranges of the serum protein complexes in phosphate solutions with the other breaks in the solubility curves. We, therefore, for the time being assume that the other breaks represent the precipitation of component systems from which euglobulin, pseudoglobulin, and albumin complexes, in the order corresponding to their known relative solubilities, can be separated.

From the data presented in Graphs I and II together with slight modifications to include the three experiments not presented graphically, the next protein fraction, the euglobulin complex, precipitates over the phosphate concentrations from 1.2 to 1.6 molar. The pseudoglobulin complex precipitates over the phosphate concentrations from 1.5 to 2.5 molar. And the albumin complex precipitates over the concentrations from 2.4 to 3.0 molar. From the analytical data the single phosphate concentrations which would approach with minimum error the points of break in the solubility curve of a given horse plasma are 1.1 molar, 1.5 molar, and 2.4 molar. But it is clear from the nature of the solubility curves that, with the exception of the concentration 1.1 molar, solubility determinations carried out at these concentrations do not represent the points of zero solubility of individual fractions.
The broken line curves continuing the individual portions of the solubility curves corresponding to the precipitation of the euglobulin and pseudoglobulin fractions represent the extension of these fractional curves. Assuming that these extensions approximate the solubilities of the respective fractions, we see that the solubility of the euglobulin complex in the filtrate of 1.5 molar phosphate is considerable, roughly 25 per cent, and that the solubility of the pseudoglobulin complex in the filtrate of 2.4 molar phosphate, though less, is not negligible.

The analytical solubility curve over the pseudoglobulin range is not the true solubility curve of the pseudoglobulin complex because of the inclusion of euglobulin nitrogen in these determinations. The lower curve over the pseudoglobulin range in each graph represents the corrected pseudoglobulin complex solubility, the euglobulin complex nitrogen having been subtracted from the analytical data. It is, of course, this lower curve that theoretically is extendible logarithmically.

Graph III presents the logarithms of the corrected pseudoglobulin complex solubilities of the two experiments on horse plasma (Graphs I and II) plotted against the salt concentrations. In determining the pseudoglobulin solubilities a correction for the euglobulin complex precipitation over the pseudoglobulin range had to be introduced. In doing this we used the same logarithmic curve for the euglobulin fraction in each experiment. This coincided with the experimental data, and had to be done were a comparison of the pseudoglobulin curves to be made, as the limited length of the euglobulin curve and the distribution of the experimental points allowed considerable variation in drawing the curves of this fraction. The slope of the lines determines the $K_s$ for each experiment. As $K_s$ is slightly varied by altering the points of zero solubility for the pseudoglobulin complex, the significance of the data must be considered in terms of a first approximation. The closeness with which the data fall along the individual straight lines favors the validity of the logarithmic extensions as made in Graphs I and II and indicates that the selected points of zero solubility are not far from that which would result from a correct logarithmic extension and that the $K_s$'s are not grossly far from correct values.

Graph III shows that the slopes of the two lines or $K_s$'s agree
closely. We should not expect the solubilities to be the same, as Experiment 1 was carried out at 20° and Experiment 2 at 25°. Green (26) has shown that the solubility of several proteins varies with temperature because of the dependence of Cohn's intercept constant, β, on temperature.

Graph III. Logarithmic plot of pseudoglobulin of horse plasma from Experiments 1 and 2.

The albumin curve has not been corrected because of the small correction as compared to the analytical errors involved over the albumin portion of the solubility curve.

In Graph II we have indicated the protein solubilities determined on that plasma at one-third and one-half saturation by volume with ammonium sulfate at a plasma dilution of 1:31.

7 The protein nitrogen determinations were carried out by heat-coagulating 10 cc. of the ammonium sulfate plasma filtrate, by washing the coagulated protein precipitate with hot water until the washings contained no sulfate as measured by the precipitation of barium sulfate, and by determining the nitrogen of the protein precipitate by the macro-Kjeldahl method.
This is higher than the usual dilution of plasma when working with ammonium sulfate solutions, and will be commented on later.

Experiments on Human Plasma

Graphs IV and V present solubility curves on human plasmas as determined in Experiments 6, 7, 8-A, and 8-B.

Graph IV. Human serum; Experiments 6 and 7. ○ = solubility; ● = log S; Experiment 6; dilution 1:31 (without chloroform). × = solubility; □ = log S; Experiment 7; dilution 1:16 (with chloroform).

The plasmas of Experiments 6 and 7 clotted slightly so that in these experiments the fibrinogen fraction of the curve is not correct. Experiment 8-B, Graph V, gives the fibrinogen complex content of this human plasma as 8.2 per cent of the total plasma protein. Examination of the curves of Experiment 6, Graph IV, shows that no break can be detected at or near the phosphate concentration of 1.5 molar. The logarithmic plot also gives no con-
Vincing indication of a break over the entire globulin range. This plasma also differs from the horse plasmas examined by its larger content of albumin complex. This increase in albumin complex with a lesser globulin complex results in a higher albumin to globulin ratio than in the horse plasmas.

Graph V. Human plasma; Experiment 8. ○ = solubility; ● = log S; Experiment 8-A; dilution 1:31. × = solubility; □ = log S; Experiment 8-B; dilution 1:16.

The decreased globulin content we felt might have been responsible for the curve not showing any break corresponding to passing from the precipitation of euglobulin complex to pseudoglobulin complex. So in Experiment 7, we changed the experimental method by adding 1 cc. of plasma to 15 cc. of the phosphate...
solution. This, of course, gave a greater range of protein concentrations in the solubility curve. But examination of the solubility curve or the logarithmic curve of Experiment 7 gives no convincing evidence of the precipitation of two protein fractions between 1.1 and 2.4 molar phosphate concentrations. The solubility curves of Experiments 8-A and 8-B, Graph V, confirm the absence of positive evidence of this type for two globulin fractions. Experiments 8-A and 8-B were performed simultaneously, with plasma from the same patient. In Experiment 8-A 0.5 cc. of plasma was added to 15 cc. of phosphate solution; while in Experiment 8-B 1 cc. of plasma was added to 15 cc. of phosphate solution. In these two experiments, in which the plasma contained more albumin than previously encountered, the break in the solubility curve caused by what we assume is the precipitation of albumin complex comes at a phosphate concentration slightly less than 2.4 molar. This coincides with what might be expected.
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theoretically from Cohn's equation. The fact necessitates our moving the phosphate concentration that approaches the globulin to albumin point of break with minimum error to 2.3 molar when dealing with human plasma.

We purposely refrain from describing in this paper the albumin portion of the curves in detail. In the experiments on horse plasma the albumin complex concentrations are so low that the curves are too short to warrant analysis. In the experiments on human plasmas the solubility data of the albumin portion of the curves do not warrant the drawing of definite conclusions due to the divergence of many of the points in this portion of the curve of Experiment 8-B from a curve similar to that of the other experiments.

### TABLE I

**Effect of Serum Dilution on Serum Protein Precipitation at \( \frac{1}{3} \) and \( \frac{1}{2} \) Volumes Per Cent Saturation by Volume with Ammonium Sulfate (Horse Serum)**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Volumes per cent saturation</th>
<th>N per sample of filtrate (mg.)</th>
<th>Solubility of protein N as gm. per liter solution</th>
<th>Soluble protein N per 100 cc. serum (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>33.3</td>
<td>12.93</td>
<td>1.293</td>
<td>1.263</td>
</tr>
<tr>
<td>1:16</td>
<td>33.3</td>
<td>8.36</td>
<td>0.836</td>
<td>1.308</td>
</tr>
<tr>
<td>1:31</td>
<td>33.3</td>
<td>4.54</td>
<td>0.454</td>
<td>1.378</td>
</tr>
<tr>
<td>1:5</td>
<td>50.0</td>
<td>6.20</td>
<td>0.620</td>
<td>0.280</td>
</tr>
<tr>
<td>1:10</td>
<td>50.0</td>
<td>3.36</td>
<td>0.336</td>
<td>0.306</td>
</tr>
<tr>
<td>1:16</td>
<td>50.0</td>
<td>2.10</td>
<td>0.210</td>
<td>0.306</td>
</tr>
<tr>
<td>1:31</td>
<td>50.0</td>
<td>1.08</td>
<td>0.108</td>
<td>0.305</td>
</tr>
</tbody>
</table>

Graph VI presents the solubilities of the globulin fractions of the four experiments on human plasma plotted as described for Graph III.

It is seen that the agreement as regards slope is close. Though the closeness with which the experimental points follow the straight lines is not as good as in Graph III, it is sufficient to warrant the construction of the lines as drawn.

Table I shows the effect of plasma concentration on the protein solubilities of solutions of one-third and one-half volumes saturation with ammonium sulfate. The determinations were carried out on a single specimen of horse serum. In the fourth column is shown the change in protein nitrogen solubility in the plasma salt.
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solutions. In the last column is shown the effect on the protein solubility calculated as gm. of soluble protein nitrogen per 100 cc. of serum.

DISCUSSION

In Graph II the solubilities at one-third and one-half saturation with ammonium sulfate are designated as a means of identifying points on the phosphate curve with the commonly used concentrations of this standard salting out salt. Ordinarily, however, precipitation with ammonium sulfate is carried out with a plasma dilution of not more than 1:10. We, therefore, examined the effect of dilution of the plasma on the solubilities at these ammonium sulfate concentrations. The data of Table I show that at one-third saturation a change from a dilution of 1:10 to one of 1:31 causes an increase in the soluble protein per 100 cc. of serum. The solubility at one-third saturation with ammonium sulfate on Graph II cannot, therefore, be considered as the solubility at a standard ammonium sulfate concentration. At one-half saturation, however, changes from a plasma dilution of 1:10 up to one of 1:31 did not alter the soluble protein per 100 cc. of serum. Hence the solubility at this saturation with ammonium sulfate may be considered as a standard of reference. That the solubility at one-half saturation with ammonium sulfate is greater than that at the globulin to albumin point of break on the phosphate curve (Graph II) does not necessarily mean that the fractionation in the two solutions is different. For Ruszczynski (22) presented evidence indicating that 60 per cent saturation with ammonium sulfate corresponds to the beginning of albumin precipitation in horse serum ammonium sulfate solubility curves.

In Graph V the curves of Experiments 8-A and 8-B show the effect of the change in plasma concentration on protein solubility. Sørensen (17) observed a similar dependence of the globulin solubility on total globulin employed in his studies on the solubility of horse globulins. Because of this effect one cannot hope by increasing the plasma concentration to increase significantly the accuracy of estimating the concentration of an individual plasma protein complex by reducing the relative amount of that protein complex left in solution at the salt concentration selected for precipitation. This is presented quantitatively very clearly by plotting
the data of Experiments 8-A and 8-B so that the abscissa represents gm. per cent of soluble protein nitrogen in the plasma itself, not plasma-phosphate solutions. The data so plotted are presented in Graph VII.

Since the resulting curves of the two experiments coincide within the probable limit of experimental error, no advantage has resulted from the analysis at the higher plasma concentration.

Graph VII. Human plasma; Experiment 8. ◦ = from precipitations at 1:31 dilutions. × = from precipitations at 1:16 dilutions.

In Experiments 8-A and 8-B we wished to detect breaks in the globulin curve at points generally accepted as being near the beginning precipitation of pseudoglobulin and albumin and we therefore used a 1:10 dilution of the plasma for the ammonium sulfate precipitations. The soluble protein nitrogen per 100 cc. of serum at one-third and one-half saturation by volume with ammonium sulfate and in 17.7 and 22 per cent sodium sulfate solutions (these latter being the solutions used by Howe (19) as coinciding respectively with the beginning precipitation of his Pseudoglobulin II and albumin) is designated on Graph VII. The data show no convincing evidence of a significant break at
the solubilities corresponding to that found in any of these solutions.

Green (26) in describing the solubility behavior of egg albumin and carboxyhemoglobin in concentrated solutions of strong electrolytes showed that the $\beta$ term of Cohn's equation depends upon temperature, pH, and particular protein, whereas the $K_s$ term varies with the electrolyte and protein. In the solutions of purified proteins, such as are considered in Green's paper, the solubility is independent of the amount of protein used in the experiment. In our experiments the solubility of the plasma protein complexes is dependent on the concentration of plasma. As already mentioned, Sørensen, in experiments on serum albumin, in which the solubility varied with the amount of albumin used in the experiments, found a linear relation between the logarithm of the solubility and the salt concentration. The data of Experiments 8-A and 8-B, as plotted in Graph VI, suggest that the plasma concentration affects the $\beta$ rather than the $K_s$ term.

Insofar as Cohn's $K_s$ can be considered as a characterization of a plasma protein fraction, Graph VI presents evidence that but one protein complex is being precipitated over the globulin range. And insofar as the slopes of the straight lines of Graph VI are different from the slopes of the lines of Graph III, the data suggest that the globulin of human plasma is different from the pseudoglobulin of horse plasma. The need for calling attention to such differences in the solubility behavior of the globulin fractions from sera of the two species is shown by the frequency with which values for euglobulin and pseudoglobulin in human serum determined by methods based on calf or other sera analyses have been reported in medical literature.

Since our experiments give no evidence that the linear relation between the logarithm of the solubility of an individual protein fraction and salt concentration does not apply to the precipitation of the protein complexes as separated in fractional precipitation, and since these experiments and those of Sørensen present evidence favoring the applicability of the relation, we have calculated the concentrations of the protein fractions in plasma by logarithmic extension of each fraction's curve to zero solubility for that fraction.

Table II shows the comparison of the estimated concentrations
Comparison of Serum Protein Concentrations As Determined by Precipitation with 1.5 and 2.4 M $\text{KH}_2\text{PO}_4$ and $\text{K}_2\text{HPO}_4$ of pH 6.5 and by Logarithmic Extension of Phosphate Solubility Curves and by Precipitation with $\frac{1}{3}$ and $\frac{1}{2}$ Volumes Per Cent Saturation with $(\text{NH}_4)_2\text{SO}_4$ at 25° and with 14.5 and 22 Per Cent $\text{Na}_2\text{SO}_4$ at 87°

The results, except for the albumin-globulin ratio, are expressed as gm. per cent protein derived by multiplying the protein nitrogen by 6.25.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Protein fraction</th>
<th>$\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$ At 1.5 and 2.4 M</th>
<th>By logarithmic extension</th>
<th>$(\text{NH}_4)_2\text{SO}_4$ precipitation</th>
<th>$\text{Na}_2\text{SO}_4$ precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Horse, diphtheria</td>
<td>Euglobulin</td>
<td>1.28</td>
<td>2.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pseudoglobulin</td>
<td>4.60</td>
<td>4.35</td>
<td></td>
<td></td>
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<td>2. Horse, scarlet fever</td>
<td>Euglobulin</td>
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<td>0.15</td>
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<td>6. Human, cardiac failure</td>
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<td>0.97</td>
<td>0.58</td>
<td>1.76</td>
<td>1.45</td>
</tr>
</tbody>
</table>

* For the experiments on human serum 2.3 M was taken instead of 2.4 M.
† Dilution of plasma 1:31.
‡ Dilution of plasma 1:10.
§ Dilution of plasma 1:3.
∥ Dilution of plasma 1:4.
of the protein complexes of several horse and human plasmas according to whether the concentrations of the individual protein fractions were determined by precipitation with 1.5 and 2.48 molar KH$_2$PO$_4$ and K$_2$HPO$_4$ of pH 6.5 or by logarithmic extension of the phosphate solubility curves or by precipitation with one-third and one-half saturation by volume with ammonium sulfate or with 14.5 and 22 per cent sodium sulfate according to the method of Howe (19).

By the method of logarithmic extension of the phosphate curves Experiments 3 and 4 give an albumin to globulin ratio for the normal horse of 0.33. In Experiment 4 the ratio by Howe's method is 1.1. Van Slyke, Hastings, Hiller, and Sendroy (28), using Howe's method, give a ratio of 0.8 for a normal horse. Analysis of Ruszczynski's curves in ammonium sulfate solutions by logarithmic extensions indicates a ratio of approximately 0.43. Mellanby (29), using alcohol-water mixtures, concluded that in horse serum about 3 per cent of the total protein was euglobulin, 85 per cent what he called albumin A and 12 per cent albumin B. If we assume the first two fractions represent globulin and the last fraction albumin, the ratio from his method of analysis is 0.14. Sørensen (18), in determining the albumin and globulin content of horse serum protein powder by repeated fractionation, obtained an albumin to globulin ratio of 0.37.

It is interesting to note the low albumin to globulin ratios in the plasmas of the horses producing diphtheria and scarlet fever antitoxin.

**SUMMARY**

In this paper we have outlined the evolution of our knowledge concerning the separation and identification of the plasma proteins, and have pointed out advantages of using phosphate solutions in experiments concerning their solubility behavior.

Solubility curves of the plasma proteins of horse and human plasmas in concentrated phosphate solutions of pH 6.5 and temperature of 25° are presented.

The effect of plasma concentration on the protein solubility in phosphate and ammonium sulfate solutions is demonstrated and discussed.

*For the experiments on human serum 2.3 M was taken instead of 2.4 M.*
Solubility of Plasma Proteins. I

By assuming for each portion of the protein solubility curve of plasma a linear relation between the logarithm of protein solubility and the salt concentration, an attempt has been made to determine the solubilities of the less soluble plasma protein fractions at the concentrations selected for fractional precipitation and to estimate the concentrations of the individual plasma protein fractions.

A comparison is made of the individual protein content of several horse and human plasmas according to whether the concentrations of the individual protein fractions were estimated by precipitation with selected phosphate solutions or by logarithmic extension of the phosphate solubility curves or by precipitation with the commonly used ammonium sulfate and sodium sulfate solutions.

The difference between the precipitation curves of horse and human plasmas is demonstrated.

We wish to acknowledge our indebtedness to Dr. E. J. Cohn, who in conversation with one of us called attention to the desirability of such a study as presented here, and who advised us throughout the early part of the work.

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THE SOLUBILITY OF THE PLASMA PROTEINS: I. DEPENDENCE ON SALT AND PLASMA CONCENTRATIONS IN CONCENTRATED SOLUTIONS OF POTASSIUM PHOSPHATE
Allan M. Butler and Hugh Montgomery


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