THE SOLUBILITY OF THE PLASMA PROTEINS

II. DEPENDENCE ON pH, TEMPERATURE, AND LIPID CONTENT
IN CONCENTRATED SOLUTIONS OF POTASSIUM PHOSPHATE AND APPLICATION TO THEIR SEPARATE PRECIPITATION

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In a paper from this laboratory, describing the dependence of
the solubility of the plasma proteins in concentrated salt solutions
on the salt and plasma concentrations (1), we pointed out the
advantage of using potassium phosphate as a precipitating salt
inasmuch as the salt concentration and pH of such solutions could
be varied independently as described by Cohn (2).

For the specific purpose of ascertaining the optimum conditions
for separating the plasma proteins by single fractional precipita-
tion, we have continued the study of the solubilities of these pro-
teins in concentrated solutions of potassium phosphate and in this
paper report data on the effect of pH, temperature, and removal
of lipids on the solubility curve of the proteins of horse plasma.
Though the dependence of the solubility of protein in salt solutions
on pH is clearly established (3, 4), but few data on the quantita-
tive relation between the solubility of the serum proteins in con-
centrated salt solutions and pH have been presented over the pH
range encountered in the salt solutions commonly used for salting-
out purposes. Csapó and von Klobusitzky (5) observed but little
effect in the precipitating power of sodium sulfate and sodium
chloride solutions on the serum proteins when the pH changed
from 4.8 to 7.4. Wu (6), in studying the solubility of serum pro-
teins in sodium sulfate solutions, observed no appreciable effect
of pH on their solubility over a pH range of from 5.0 to 9. Howe
(7) observed that a greater concentration of phosphate in solutions of mono- and disodium phosphate or mono- and dipotassium phosphate was required to produce equal precipitation of the plasma proteins as the pH of the solutions fell from 8.0 to 5.8, a behavior that might be considered without further analysis contrary to the usually observed decrease in protein solubility as the isoelectric point is approached.

Wu (6) reported a decrease in solubility of the lipid-free serum as compared to the natural serum. His curves, however, are not detailed enough to permit an accurate analysis of the individual protein fractions, particularly the globulin portion, where changes in the curve might be expected since there is evidence that euglobulin is a complex of pseudoglobulin and lipids (8, 9). An analysis of the individual fractions of a lipid-free serum, whose solubility is different from that of natural serum, by such specified salt concentrations as given by Howe (10), may give solubility data that have but little relation to the solubility of the individual serum proteins.

Mellanby (11) observed that ammonium sulfate was slightly more effective as a precipitant of the serum proteins at 0° than at 40°.

EXPERIMENTAL

Effect of pH—Fig. 1 presents the solubility curves of the proteins of horse plasma in potassium phosphate solutions of pH 5.4, 6.5, and 7.7, determined as described in the previous paper (1). All

1 The pH of 5.4 was selected since it was thought that a curve at the isoelectric point of serum globulin might increase the difference in the solubilities of the globulin and albumin fractions. Moreover, this is as acid a pH as can be obtained with a buffer solution of mono- and dibasic potassium phosphates. The pH of 7.7 is approximately the alkaline limit of such a buffer solution. And the pH of 6.5 is a convenient intermediate point. The solutions of varying ionic strength of pH 6.5 were made by dilution of a 3 m solution of phosphate consisting of equimolecular amounts of the dibasic and monobasic potassium salts as described in the paper referred to above (1). The solutions of pH 5.4 and 7.7 required individually constructed solutions for each concentration, since the mole fraction of K₂HPO₄ which provides a constant pH at either of these pH values varies with change in the salt concentration of the solution. The mole fraction of K₂HPO₄ required to give the pH of 5.4 or 7.7 at any desired phosphate concentration was taken from the interpolation tables and graphs of Green (12), the values beyond the concentrations given in that paper being deter-
three curves were made from a single sample of horse plasma collected aseptically. The curve at pH 5.4 was determined the day the plasma was drawn. The plasma was then kept under sterile conditions at 10°, and the curves at pH 6.5 and 7.7 were determined respectively 6 and 15 days after the plasma was collected. Under such conditions the solubility of the proteins remains unaltered. Moreover, the curves at the two latter pH values agree with others obtained at these pH values with fresh plasma.

The ordinate on the left represents the solubility of protein nitrogen expressed as gm. of nitrogen per liter of filtrate. The ordinate on the right represents the logarithm of the solubility. The abscissa represents the ionic strength of the potassium phosphate per liter of the plasma phosphate solutions, the solutions consisting of 1 cc. of serum to 30 cc. of the various phosphate solutions. The solubility curve at each pH is represented by the solid line, the logarithm curve by the broken line. This latter curve was used to determine the points of break in the solubility curve as described in our previous paper (1). Since no consistent break occurs at the ionic strengths corresponding to 2.50 at pH 6.5 (molar concentration of 1.25), the curves are drawn without any indication of the precipitation of euglobulin. The curve at pH 5.4 could not be extended further because of the limited solubility of potassium phosphate at that pH.

The desired mole fraction and concentration of each solution at pH 5.4 and 7.7 were obtained by weighing out the required amounts of the dried dibasic and monobasic salts or by titrating from standardized solutions of K₂HPO₄ and H₃PO₄, the calculated number of cc. of each solution. The solutions were then checked for total phosphate by the method of Fiske and Subbarow (13) and for approximate pH colorimetrically. The determinations were carried out at 39° in order to increase the solubility of the phosphate in the most acid solutions.

Although the effect of temperature on pH in such concentrated phosphate buffers has not been determined, there is no appreciable change in dissociation constant or activity coefficient with temperature at the concentrations studied (14).

2 Ionic strength per liter of solution is used as a matter of convenience. It may be defined as Γ/2 where Γ = \( \sum C Z^2 \), as used by Debye and Hückel (15), C being the stoichiometrical molarity of each ion and Z its valence. This should be differentiated from ionic strength designated by Lewis and Randall's \( \mu \) (16), where the concentration of each ion is expressed as stoichiometrical molality.
The solubilities of all the fractions increase with increasing pH. Though no two entire curves are superimposable, the corresponding fractions of each of the three curves are very nearly superimposable separately. In the terms of Cohn's equation (17) which defines the solubility of a protein in concentrated salt solutions as

\[ \log S = \beta - K_{s} \frac{\Gamma}{2} \]

where \( \beta \) is an intercept constant, \( K_{s} \) a constant representing the slope of the curve, and \( \frac{\Gamma}{2} \) the salt concentration in terms of ionic strength per liter of solution, the data of Fig. 1 indicate that the \( K_{s} \) constants or slopes of the different fractions are affected but slightly by pH, whereas the \( \beta \) constants are dependent upon pH and for each fraction are affected differently.
In an attempt to obtain more quantitative data on the relationship between pH and the constants $K_s$ and $\beta$, the logarithms of the solubilities of the globulin, probably pseudoglobulin, fraction of the curves were plotted against salt concentration, the zero solubility of this fraction on the curves being made by logarithmic extension as 0.09 mg. of nitrogen per liter (see (1) p. 183). The numerical values for $K_s$ and $\beta$ so obtained at the three pH values are given in Table I.

**Effect of Temperature**—Fig. 2 presents the solubility curves of the proteins of horse plasma at 1°, 20°, and 39° determined on samples from the same horse plasma in concentrated potassium phosphate solutions of pH 6.5. The curve at 20° was determined the day the plasma was drawn. The curves at 1° and 39° were determined 24 hours later, the serum having been kept under aseptic conditions at 10° during the interim.

The presence of a euglobulin break is equivocal, but the curves have been drawn so as to reflect a differential precipitation over the euglobulin range. The three heavy crosses represent the solubility of nitrogen in the filtrate from sodium sulfate solutions at 37.5° according to the method of Howe (10). The ionic strengths of 4.44, 4.56, and 4.87 correspond respectively to 21.0, 21.5, and 23.0 gm. per cent of sodium sulfate.

### Table I

*Total nitrogen concentration per liter where no precipitation occurred.*

<table>
<thead>
<tr>
<th>Fig. No.</th>
<th>Source of sample</th>
<th>Total N concentration gm. per l.</th>
<th>pH</th>
<th>Temperature °C.</th>
<th>$K_s$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Horse plasma</td>
<td>0.345</td>
<td>5.4</td>
<td>39</td>
<td>0.52</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>0.345</td>
<td>6.5</td>
<td>39</td>
<td>0.51</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>0.345</td>
<td>7.7</td>
<td>39</td>
<td>0.48</td>
<td>1.06</td>
</tr>
<tr>
<td>2</td>
<td>&quot; &quot;</td>
<td>0.325</td>
<td>6.5</td>
<td>1</td>
<td>0.47</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>0.315</td>
<td>6.5</td>
<td>20</td>
<td>0.49</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>0.320</td>
<td>6.5</td>
<td>39</td>
<td>0.49</td>
<td>0.70</td>
</tr>
<tr>
<td>3</td>
<td>Serum powder</td>
<td>0.310</td>
<td>6.5</td>
<td>20</td>
<td>0.49</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>0.620</td>
<td>6.5</td>
<td>20</td>
<td>0.49</td>
<td>0.90</td>
</tr>
</tbody>
</table>
The solubilities in the phosphate solutions at 20° and 39° are practically identical, while the solubility at a given salt concentration is slightly greater at 1° than at the two higher temperatures.

The $K_s$ constants of the different fractions appear to be independent of temperature over this range. The $\beta$ constant of a given fraction apparently increases when the temperature approaches 0°. The numerical values for $K_s$ and $\beta$ for the pseudoglobulin fraction at each temperature were estimated and are given in Table I.

**Effect of Lipids**—Fig. 3 presents the solubility curve of lipid-free horse serum proteins at pH 6.5 and a temperature of 20°. Lipid-free serum protein powder was prepared according to the method of Hewitt (18). These proteins were readily soluble in 0.7 gm. per cent saline, forming a clear solution with a protein concentration as high as 25 gm. per cent. The composition of the lipid-free serum protein solution, which was added to the various phosphate solutions, was: serum protein 6.1 gm. per cent, non-protein nitrogen 6 mg. per cent, sodium 137 milli-equivalents per liter, chloride

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\(^3\) In a few instances the antitoxic titer of antitoxin horse serum, as measured by skin tests on guinea pigs, was correspondingly raised; i.e., to 5 to 10 times the titer of the original horse serum.
116 milli-equivalents per liter, calcium 12 mg. per cent, and cholesterol 0.

The upper curve represents the solubilities from an experiment in which 1 cc. portions of lipid-free serum protein solution were added to 15 cc. of the phosphate solutions; i.e., a dilution of the serum protein solution of 1:16 instead of the 1:31 dilution used in the majority of our experiments. The lower curve represents the solubilities of the same lipid-free serum protein solution diluted 1:31 in the phosphate solutions.

The character of the curve is changed but little from that of...
fresh horse serum. From the data of the lower curve at the usual dilution there is a questionably significant precipitation between the ionic strengths of 1.55 and 2.60 as might be expected for serum from which fibrinogen has been withdrawn as fibrin. Between the ionic strengths of 2.60 and 2.90 there is a precipitation which may be attributed to euglobulin. The major portion of the globulin is precipitated between the ionic strengths of 2.90 and 4.40. The albumin begins to precipitate at an ionic strength of 4.50 instead of at that of 4.80 as observed with natural serum, thus indicating a slight decrease in solubility in this protein fraction. The break in the albumin portion of the curve occurs at an ionic strength of 5.15 and corresponds to that with natural serum.

Without a correction for the precipitation of euglobulin with pseudoglobulin (1) the $K_s$ and $\beta$ constants of the second globulin fraction (pseudoglobulin) have been estimated and are given in Table I. The numerical values of the $K_s$ and $\beta$ constants are in close agreement with those obtained with pseudoglobulin of natural plasma at the same pH and similar protein concentration. The increase in protein nitrogen solubility with increase in serum concentration is shown by the $K_s$ and $\beta$ constants for pseudoglobulin (Table I) to be due to an increase in the $\beta$ constant, thus confirming the relationship suggested in our previous paper (1).

**DISCUSSION**

The increase in solubility of the plasma proteins over the globulin and albumin portions of the curves with increase in pH from 5.4 to 7.7 as shown in Fig. 1, where the salt concentration is expressed as ionic strength, conforms to the expected behavior of proteins whose isoelectric points are 5.4 and 4.9. The increase in solubility of the fibrinogen portion over the same increase in pH indicates an isoelectric point of fibrinogen below 5.4 and agrees with Florkin's (19) evidence on this point. Our data are in agreement with Howe's findings in the experiments with phosphate solutions already mentioned (7). For, if our salt concentrations are plotted as molar concentrations, the solubility of nitrogen in the filtrate of a given molar concentration decreases as the pH rises from 5.4 to 7.7. Conversely, if Howe's phosphate concentrations are expressed in terms of ionic strength, the solubility of the plasma proteins increases as the pH rises from 5.8 to 8.0. The conformity
of the data when salt concentrations are expressed as ionic strengths with the expected behavior indicates the appropriateness of using this unit of concentration when expressing the solubility of proteins as a function of salt concentrations. It is for this reason that we have used in this paper units of ionic strength rather than molarity in expressing the concentrations of the phosphate solutions.

Our finding that the $K_s$ constants of the various fractions are affected but little by pH or temperature, while the $\beta$ constants are dependent upon both these factors, agrees with the findings of Florkin for fibrinogen (19) and Green for carboxyhemoglobin (20). A slight decrease in $K_s$ with increasing pH, as shown in Fig. 1 (see Table I) may be experimental error, but may be due to the specific ion effects of monovalent and divalent phosphate ions. However, evidence from the effect of pH on the solubility of fibrinogen in sulfate and phosphate solutions (18) indicates that the specific ion effect of these two ions would account for only a small fraction of the observed change in solubility and that we are justified in assuming that the greater part of the solubility change is due to a relationship between pH and $\beta$.

The numerical values of the $K_s$ and $\beta$ constants over the globulin or pseudoglobulin portions of the curves are presented for the purpose of comparing and characterizing changes in solubilities rather than for the purpose of obtaining accurate numerical values for these constants. However, the order of magnitude of the $K_s$ obtained in our experiments, approximately 0.50, may have further significance when it can be compared with the $K_s$ of purified horse pseudoglobulin in phosphate solutions. At the present time comparison can be made only with the $K_s$ of 1.47, calculated from Sørensen’s data4 on purified horse pseudoglobulin in concentrated solutions of ammonium sulfate. Though these $K_s$ values are not directly comparable, the difference seems more than is to be expected from specific ion effects, since we have obtained pseudoglobulin $K_s$ values of approximately 0.50 from solubility curves in sodium sulfate solutions and Florkin for fibrinogen (19) and Green for carboxyhemoglobin (20) have observed lesser $K_s$ values in sulfate solutions than in phosphate solutions. Such an increase in the $K_s$ of purified pseudoglobulin above that obtained for the

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4 Taken from Cohn (21).
native pseudoglobulin of plasma again raises the question of whether procedures of purification alter the protein molecular complex.

Estimation of Individual Plasma Proteins from Single Fractional Precipitation with Potassium Phosphate Solutions

The results presented in Fig. 1 indicate that the effect of pH on the solubility of the plasma proteins over the pH range from 5.4 to 7.7 may be large enough to introduce significant errors in salting-out procedures as commonly used today, for commercial preparations of ammonium sulfate and sodium sulfate are notoriously variable in the pH of their solutions. Furthermore, the pH of a solution of these unbuffered salts and plasma may depend not only upon the pH of the salt solution but also on the dilution of the plasma, since this may affect the pH of the plasma salt solution. The data presented in Fig. 1 therefore suggest the advisability of using effectively buffered solutions in procedures of fractional precipitation where a particular salt concentration is specified.

Fig. 2 shows that ordinary variations of room temperature do not affect the solubility of the plasma proteins in these phosphate solutions beyond the limit of experimental error. In this respect potassium phosphate solutions as the precipitating reagent in salting-out procedures provide an advantage over solutions of sodium sulfate whose temperature must be maintained at approximately 37.5°C.

Since the maximum buffer effect of potassium phosphate is obtained at a pH of 6.5 and since concentrations of this salt at this pH may be made by dilution of a 3 M solution together with the fact that no improvement in the separation of the protein fractions results at pH 5.4 or 7.7, available data suggest the use of potassium phosphate solutions of pH 6.5 in the precipitation of individual plasma proteins.

We have found that solutions of c.p. ammonium sulfate in distilled water vary from an approximate pH of 4.5 to 5.5. Some preparations even after recrystallization have been so acid as to cause complete precipitation of the serum proteins when 0.5 cc. of serum was added to 7.25 cc. of water and 7.75 cc. of saturated ammonium sulfate solution. Dr. Scriven and Dr. Berglund (personal communication) found such variations in the pH of sodium sulfate solutions that they felt it was necessary to buffer their sodium sulfate solutions for use as a precipitating reagent.
In our previous paper (1) we give the reasons for believing that at pH 6.5 the first portion of the curve from 0.8 to 1.25 M represents the precipitation of fibrinogen, while the second portion from 1.25 to 1.50 M in the horse plasma curves of that paper reflects the precipitation of euglobulin. For human plasmas no differential precipitation of euglobulin and pseudoglobulin was observed, and this observation has been confirmed by Kydd (22). With the accumulation of further data many horse plasmas fail to show any differential precipitation of two globulin fractions, and the question arises as to whether some of the euglobulin is precipitated with the fibrinogen or is present in such small amounts as to escape detection. Because of the uncertainty as to whether the first fraction is entirely fibrinogen and our knowledge that no other portion of the curve represents the complete precipitation of a pure fraction, it would be safer to refer to the portions of the curves as Fractions I, II, III, IV, etc. But where a fraction over a particular salt range does not appear such as in the curves of Fig. 1, a numerical nomenclature of the fractions might be confusing. Therefore, though aware of the uncertainty, we may for convenience refer to the portions of the curves as representing the fibrinogen, euglobulin, pseudoglobulin, Albumin I, and Albumin 2 fractions. The finding of a break in the albumin portion of the curve indicative of two albumin fractions has been repeatedly confirmed.

With potassium phosphate solutions of pH 6.5, the single fractional precipitation of individual protein fractions at specific concentrations may be summarized as follows:

Fraction I, containing fibrinogen, is precipitated at a molar concentration of phosphate of 1.25.6

Fraction II, probably largely euglobulin, but no more than two-thirds of the total amount, begins to precipitate at a molar concentration of phosphate of 1.25 and continues to be precipitated without the active precipitation of pseudoglobulin up to a molar concentration of 1.50.

Fraction III, representing approximately 80 per cent of the pseudoglobulin and 30 per cent of the euglobulin, precipitates between the molar concentrations of 1.50 and 2.40.

6 At pH 6.5 the ionic strength of potassium phosphate per liter of solution = 2 × molar concentration of phosphate.
Fraction IV, consisting of Albumin 1 with some pseudoglobulin, begins to precipitate at a molar concentration of phosphate of 2.40 and continues to be precipitated without the active precipitation of Albumin 2 up to a molar concentration of 2.58.

Fraction V, consisting largely of Albumin 2 with a trace of pseudoglobulin and Albumin 1, begins to precipitate at a molar concentration of phosphate of 2.58 and is completely precipitated at a 3.00 M concentration of phosphate.

From the nature of the precipitation curves it is clear that an estimation of the euglobulin fraction by single fractional precipitation in potassium phosphate solution over a pH range from 5.4 to 7.7 is subject to such an error as to make a quantitative estimation of euglobulin concentration unwarranted. Complete precipitation curves of plasma in sodium sulfate solutions at 37.5° according to the method of Howe (10) indicate that an error of the same order of magnitude applies to euglobulin determinations by that method.

The inability to make a quantitative estimation of certain of the plasma proteins by single fractional precipitation does not mean that they cannot be separated by repeated fractional precipitation.

**SUMMARY**

The effect of pH, temperature, and removal of lipids on the solubility of the plasma or serum proteins in concentrated solutions of potassium phosphate is described. Neither the variations of pH or temperature over the ranges studied nor the removal of lipids affects the completeness of separation of these proteins by single fractional precipitation.

The significance of the observed dependence of the solubility of the plasma proteins on pH in the determination of plasma protein fractions by the commonly used salting-out procedures is discussed.

The concentrations of potassium phosphate in plasma phosphate solutions of pH 6.5 that may be used in estimating the concentration of certain plasma protein fractions or in accomplishing their separation by repeated fractional precipitation are given.
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