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Precipitation and Microflotation of Egg Albumin and Hemoglobin in the Presence of Dioctadecyldimethylammonmin Chloride

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Precipitation and microflotation of egg albumin and of hemoglobin was studied as a function of pH in the presence of dioctadecyldimethylammonium chloride (DOAC). The "DOAC concentration-pH" stability domains for both protein solutions (0.05 wt%) exhibited similar boundaries. Efficient separation by precipitation (more than 80% after 24 h) occurs at pH>5.0 for albumin and at pH>6.3 for hemoglobin. The same amount of removal by microflotation required 5 min under otherwise similar conditions. The DOAC concentration for optimal separation of proteins (from 0.05 wt% solutions) is 0.02 wt% at pH 6—8 for albumin and 0.015 wt% at pH 6.5—8 for hemoglobin. It is noteworthy that both proteins can be precipitated and also foam separated even in strongly alkaline media, such as at pH 12, where the usual flocculents (*e.g.*, aluminum salts) cannot be employed. This work points out the usefulness of infinitely aggregating surfactants as microflotation agents.

Since proteins, such as hemoglobin and albumin, are major components in waste waters of food processing and related industries, their removal and/or recovery is highly desirable. It has been shown that microflotation, which is characterized by the application of small bubbles, could be successfully applied for the separation of inorganic and organic colloidal impurities from their suspensions.[1-4] In this process the materials to be removed must be in flocculated state, which can be accomplished either by the addition of a coagulant or by a change of solution pH. The precipitate is then floated with the help of a surfactant (collector).

Various types of flocculents have been applied to protein solutions, such as lignin[2] and its derivatives,[5] sodium polyacrylate,[6] cationic surfactants (*e.g.*, dodecylpyridinium bromide),[7] anionic surfactants (*e.g.*, sodium alkylbenzenesulfonate or sodium dodecyl sulfate),[7-9] nonionic surfactants,[10,11] and simple inorganic salts.[11,12] Among the listed additives the high molecular weight flocculents (such as lignin) and freshly precipitated metal hydroxides proved to be most suitable for efficient flotation.[2]

The solubility of proteins in water may be reduced by certain reagents which form a less soluble complex with the protein. In this investigation, dioctadecyldimethylammonium chloride, $(C_{18}H_{37})_2N(CH_3)_2Cl$ (DOAC), was used to precipitate and to float egg albumin and hemoglobin. Owing to two long alkyl chains in the DOAC molecules, its solution behavior is quite different from that of ordinary cationic surfactants.[13] Instead of forming micelles these long chain molecules infinitely aggregate yielding multilayer structures in aqueous media over a broad pH range. This property of DOAC makes it a useful precipitant for proteins and it also enhances their floatability by microbubbles.

Experimental

Materials. Egg albumin and hemoglobin were obtained from Tokyo Kasei Kogyo Ltd. and were used without further purification. Dioctadecyldimethylammonium chloride (DOAC), the purity of which with respect to the alkyl chain length was 98.6%, was kindly supplied by Kao Soap Co. Since the proteins used had a certain amount of impurities as shown below, DOAC was also used without further purification. To prepare the stock solutions, the precalculated amount of the protein (either albumin or hemoglobin) was dissolved in 200 cm³ of a NaOH solution (pH 11.0), stirred for 24 h, and filtered through an 1.0 μm pore membrane (Tōyō Roshi Co., TM-100P). A known amount of the filtrate was dried to constant weight *in vacuo* at 70°C from which the weight, obtained by the same procedure using only the solvent, was subtracted. The analysis showed that 1.5% of albumin and 3.2% of hemoglobin remained insoluble in the alkaline media; thus, the concentrations of the solutions of these proteins were corrected accordingly. Stock solutions of DOAC were prepared by dissolving appropriate amount of the solid surfactant in distilled water under stirring at 50 °C for 24 h.

Analyses. Since DOAC scattered light in the visible region and weakly absorbed light in the ultraviolet region, the protein concentrations in solutions containing DOAC were determined by the Lowry method[14] as described previously.[2] The absorbance at 750 nm increased linearly with the DOAC concentration; however, this increase was small and amounted to only 0.08 at 0.05 wt% DOAC, which was the maximum amount of the surfactant in most experiments. The measured absorbance of protein solutions was corrected for the presence of the surfactant.

The concentration of DOAC was determined independently in solutions containing protein by the methyl orange method developed by Wang *et al.*[15] The volume of a sample solution (usually 0.2—1.0 cm³) in a 500-cm³ separatory funnel was adjusted with distilled water to 10 cm³ and then 1.0 cm³ of a buffer solution (pH=3),[16] 0.1 cm³ of a 0.1 wt% Methyl Orange aqueous solution, and 10 cm³ of chloroform were added. After vigorous shaking for 30 sec, the mixture was allowed to stand for about 30 min to achieve complete separation of the chloroform from the water phase. The absorbance of the chloroform solution was then determined spectroscopically at 415 nm. The presence of protein in the sample did not interfere, (even at a 50 fold excess with respect to DOAC) except that it took much longer time for the phase to separate. The absorbance was linearly proportional to the DOAC concentration over the range 0—0.002 wt%; it had a value of ≈1.0 for a 0.002 wt% DOAC solution when a cell of 1.00 cm light path was used.

Stability and Microflotation Measurements. The stability of the protein was very much affected by both the pH and the DOAC concentration. Thus, a series of solutions was prepared at a constant pH and protein concentration (0.05 wt%) whereas the concentration of DOAC was varied systematically.

Desired quantities of stock solutions of the protein and of DOAC were mixed with distilled water to give a final volume of 30 cm^3. The pH was then adjusted with NaOH and HCl, as necessary, and the resulting solution was allowed to settle for 24 h after which time the visual appearance of the system, the relative volume of settled flocs, and the concentrations of both the protein and DOAC in the supernatant solutions were determined.

The microflotation apparatus and the procedure were essentially the same as described earlier[2] except that a 300 cm^3 instead of a 600 cm^3 glass Buchner funnel was employed as the flotation cell. Precalculated amounts of protein and DOAC solutions and of distilled water, which would yield a final volume of 200 cm^3, were introduced into the cell and the pH was adjusted with either NaOH or HCl. As frother/collector served 0.5 cm^3 of ethanol containing 5 mg of lauric acid.

Results

Figure 1 gives the concentration of albumin in supernatant solutions as a function of pH, 24 h after mixing the reacting components. Each curve is for a different initial DOAC concentration whereas the albumin content was kept constant (0.05 wt%) in all systems. In the absence of the surfactant, some precipitation of the protein occurs only over a relatively narrow pH range of 4.4—5.7; the maximum amount of the protein separated by gravity settling after 24 h amounted to only 26% at pH ≈ 5.0. The addition of DOAC affects the precipitation of albumin as follows: (1) no precipitation of albumin is observed at pH<4.4, regardless of the amount of DOAC present; (2) with increasing DOAC concentration, the amount of separated albumin becomes larger and the pH range over which the protein precipitates broadens; (3) although albumin dissolves at high pH, it remains precipitated even at a pH as high as 12, provided the DOAC concentration is ≥0.05 wt%; and (4) a domain of high turbidity exists over the pH region of ≈4.5—5.0 (hatched area in Fig. 1), presumably due to stable albumin–DOAC dispersions.

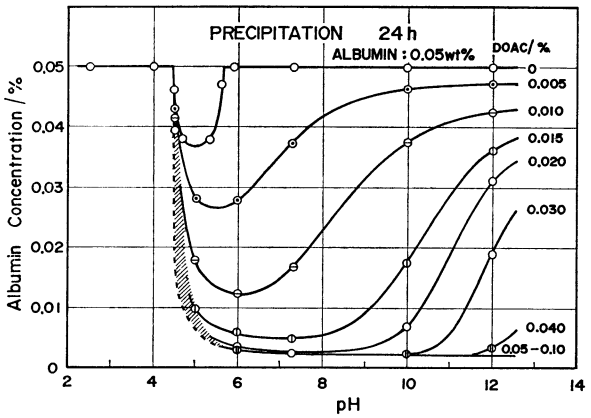

Fig. 1. The concentration of albumin left in the supernatant solutions after gravity settling as a function of pH. Each curve represents systems containing a constant concentration of albumin (0.05 wt%) but different concentrations (0—0.10 wt%) of dioctadecyldimethylammonium chloride (DOAC) 24 h after mixing of the reacting components. Hatched area indicates conditions yielding stable dispersions.

The upper part of Fig. 2 gives the concentration of albumin in the supernatant solutions, 24 h after mixing the reacting components as a function of the initial concentration of DOAC. The two curves refer to systems containing 0.05 wt% albumin at constant pH of 4.5 and 5.0, respectively. The center part of the figure shows the corresponding concentrations of DOAC in the supernatant solution. Finally, the lower part gives the percent of albumin removed after 5 min of microflotation in the same systems at pH 5.0. In Fig. 3 analogous plots refer to samples at pH 7.3 and 12.0

At pH 4.5 the addition of DOAC to protein solutions causes high turbidities. Obviously, a stable protein–DOAC precipitate is formed, which shows little tendency to settle, especially if the concentration of the surfactant exceeds 0.04 wt%. At somewhat higher pH (5.0) the removal of albumin by precipitation with DOAC and gravity settling is considerably enhanced. In these systems most of the added surfactant molecules combine with albumin leaving, for example, 0.0014 wt% and 0.0011 wt% DOAC in the supernatant solutions, at initial concentrations of 0.010 wt% and 0.015 wt%,

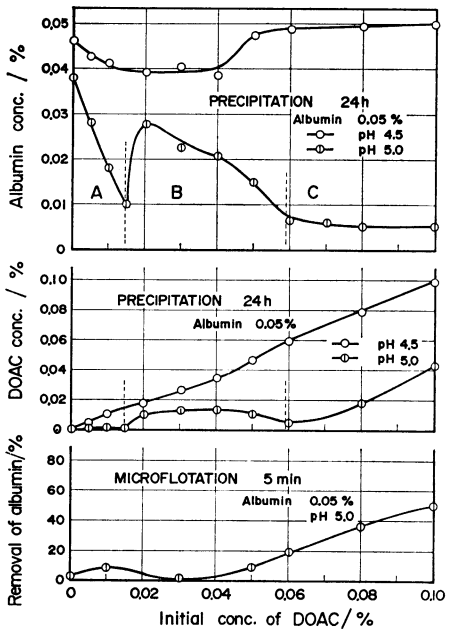

Fig. 2. The concentrations of albumin (upper part) and of dioctadecyldimethylammonium chloride (DOAC) (center part) remaining in the supernatant solutions above the precipitate separated by gravity settling 24 h after mixing of the reacting components. Lower part: the percentage removal of albumin by microflotation after 5 min. All systems contained a constant concentration of albuimn (0.05 wt%) at a constant pH (○, 4.5; ⓘ, 5.0), by varying amounts of added DOAC.

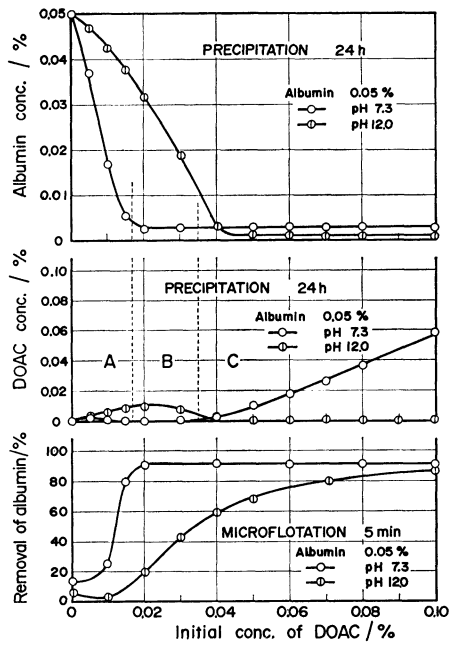

Fig. 3. Same plot as Fig. 2 for systems at pH 7.3 (○) and pH 12.0 (◑).

respectively. At intermediate amounts of DOAC a part of the precipitated albumin is peptized, giving somewhat higher concentrations of albumin and DOAC in the supernatant liquids. However, in contrast to the systems at pH 4.5, the separated albumin–DOAC phase at pH 5.0 coagulates and settles when the initial concentration of DOAC is $\geq \approx 0.06$ wt%.

The removal of albumin after 5 min of microflotation is much lower than by gravity settling after 24 h. For example, at the initial concentration of DOAC of 0.01 wt% only 8% of albumin is separated, whereas more than 60% settles out. Although the floatability of albumin improves with increasing DOAC concentration, only 50% is removed at best (in the presence of 0.10 wt% DOAC); under the same conditions $\approx$ 90% of the protein settles out.

As the pH is raised to 7.3 (upper part of Fig. 3) $\approx$ 94% of the protein is removed by settling at DOAC concentration $\geq$0.02 wt%. The surfactant content in the supernatant solutions (middle part of Fig. 3) remains low at initial concentrations <0.035 wt%, but increases nearly linearly when more DOAC is added. At this higher pH the separation of albumin by microflotation is comparable to that of settling (92% after 5 min) except that the time required is considerably shorter. The time dependence of the removal of albumin by microflotation in the absence and in the presence of different concentrations of DOAC at a constant pH of 7.3 is shown in Fig. 4.

Figure 3 also shows that at still higher pH (12.0) the precipitation and the microflotation of albumin in the presence of DOAC becomes again less efficient; thus, a minimum of 0.042 wt% of surfactant is needed for complete removal of the protein in contrast to 0.017 wt% at pH 7.3. Furthermore, at pH 12 no peptization in the presence of DOAC is detected over the concentration range studied (0—0.26 wt%).

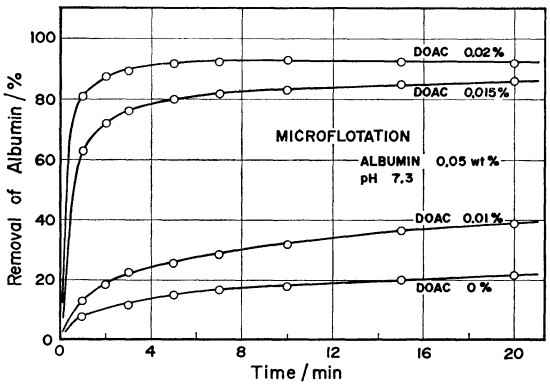

Fig. 4. Percentage removal of albumin from a 0.05 wt% protein solution at a pH of 7.3 as a function of time, in the absence and in the presence of DOAC in different concentrations.

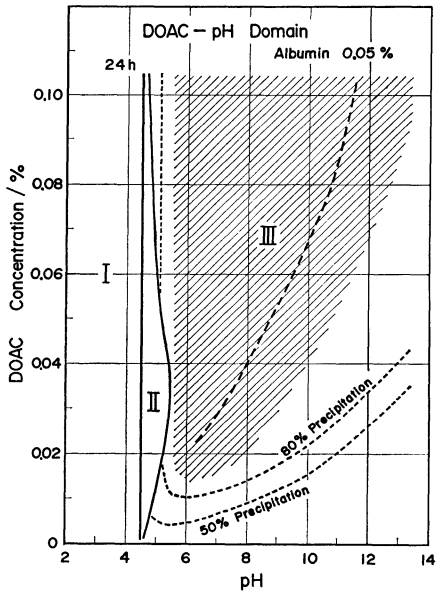

Fig. 5. DOAC-pH domain for the systems containing 0.05 wt% albumin. The solid lines delineate regions of various degree of stability, 24 h after mixing of the components. In I no precipitation takes place; in II the sols are stable; in III the albumin–DOAC floc readily settles. Dotted lines indicate the boundaries over which 50 and 80% albumin, respectively, is separated by gravity settling. Hatched area gives the conditions of 80% or greater removal of albumin after 5 min of microflotation.

In the system containing 0.20 wt% DOAC, for example, the surfactant concentration in the supernatant solution, 24 h after preparation, is only 0.0009 wt% although the settled precipitate occupies >50% of the total volume of the system. When DOAC concentration is >0.24 wt%, the entire system is solidified; *i.e.*, no supernatant solution appears 24 h after mixing the components. However, if the latter system is kept undisturbed for a longer period of time (more than 3 d), the precipitated phase gradually separates out from a transparent solution and floats as a jelly-like phase.

Figure 5 gives the entire DOAC concentration-pH

stability domain for a system containing a constant concentration of albumin (0.05 wt%), 24 h after mixing the reacting components. This plot is constructed from the data shown in Figs. 2 and 3 as well as from the analogous information for systems at pH 6.0 and 10.0. The heavy solid lines delineate conditions of different stability of albumin: in region I no precipitation is observed; in II stable albumin–DOAC aggregates are found (high turbidity); in III the complex precipitates settle by gravity. The dotted lines drawn within region III and labeled "50% precipitation" and "80% precipitation" indicate the boundary above which more than 50% (or 80%) of albumin is removed by settling 24 h after mixing. The hatched area indicates the conditions which result in 80% or better removal of albumin after 5 min of microflotation. Finally, above the dashed line an excess of DOAC is detected in the supernatant liquids (separated from the settled precipitates).

The results obtained with 0.05 wt% hemoglobin solutions are summarized in Figs. 6 and 7. A comparison with the corresponding data for albumin shows that DOAC reacts with both proteins in essentially the same manner. Minor differences noted are: (1) DOAC precipitates hemoglobin more efficiently than albumin, (2) the domain of stable dispersion (region II in Fig. 7) for hemoglobin system extends over a wider pH range than for albumin, and (3) the effective precipitation (and corresponding microflotation) region of hemoglobin is shifted by about one pH unit towards the higher pH as compared with that for albumin.

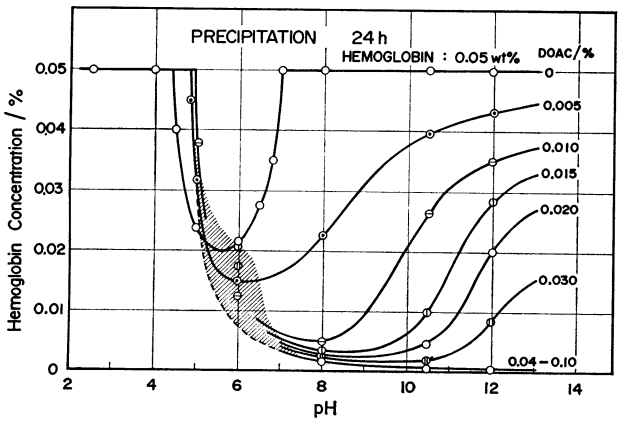

Fig. 6. Same as Fig. 1 for the hemoglobin–DOAC system.

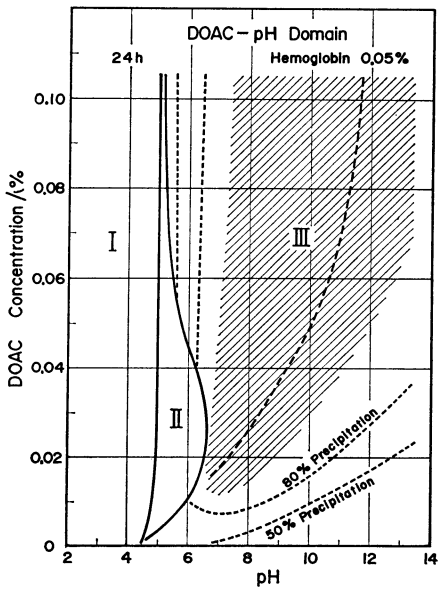

Fig. 7. Same as Fig. 5 for the hemoglobin–DOAC system.

Discussion

Dioctadecyldimethylammonium chloride (DOAC) was chosen because of its property to form very stable dispersions of liquid crystal particles that could interact with the proteins. There dispersions can be produced in aqueous solutions above or below the melting point (37 °C) of the hydrated solid DOAC. The positively charged particulate system is readily supercooled and remains unchanged for long time (over one month), even at room temperature. Thus, liquid crystals are expected to be suitable flocculents for the negatively charged proteins. The very low saturation concentration of the monomeric DOAC (2.7×10^{-4} wt% at 30 °C)[13] and the insensitivity of its liquid-crystalline phase to pH (except at a rather high concentration of base) are additional convenient properties of this surfactant for the intended purpose.

The data presented here show that the interactions between albumin (or hemoglobin) and DOAC depend strongly on the concentration of the surfactant and on the pH. Typical effects of the added DOAC on the stability of albumin are distinguished in Fig. 3 as regions *A*, *B*, and *C*. In *A* the mixing of albumin and DOAC yields rapidly settling flocs and the amount of removed albumin by gravity settling is approximately proportional to the quantity of DOAC added. The DOAC concentration in the supernatant solution is very low; the slight turbidity of the latter is most likely due to finally dispersed systems which did not settle during the 24 h of aging. In region *B* nearly the entire amount of albumin is precipitated and settled, and very little DOAC (less than 0.001 wt%) is left in the supernatant solution, even though there is an excess of the surfactant over what is necessary to react with the protein. The precipitate in this region is more voluminous than under conditions of region *A*. Finally, in *C* most of albumin is in the settled floc, but some of the DOAC can be found in the supernatant solution, the amount of which becomes larger with increasing concentration of the added surfactant.

In region *A* the negatively charged albumin reacts most likely with positively charged liquid-crystalline fragments of DOAC, as evidenced by the following observations: (1) no precipitation of albumin with DOAC takes place at pH < the isoelectric point[17] (Figs. 1 and 5); (2) the amount of DOAC which is needed to precipitate a certain amount of albumin

increases with increasing pH, *i.e.*, with increasing net negative charge on the albumin molecules (Figs. 1 and 3); and (3) at a constant pH, the amount of DOAC necessary to completely precipitate albumin is proportional to the initial protein concentration. For example, at pH=12.0, the systems containing 0.015, 0.030, and 0.05 wt% albumin, required 0.013, 0.025, and 0.042 wt% DOAC, respectively.

Although the pH dependence of the net charge on an albumin molecule (Z) is not fully established, one may assume Z to be approximately 14, 18, and 30 at pH 7.3, 10.0, and 12.0. These values are estimated by averaging the data obtained from electrophoretic mobilities[17] and from titrations.[18] Using these Z values and assuming that 60% of the positive charge on a liquid-crystalline aggregate of DOAC is electrostatically shielded by gegenions (such as Cl^- and OH^-),[19] one may calculate the DOAC content which is necessary to neutralize the negative charge on a definite amount of albumin molecules. The calculated values for a 0.05 wt% albumin solution are 0.015, 0.02, and 0.033 wt% at pH 7.3, 10.0, and 12.0, respectively. These results are in reasonable agreement with the observed DOAC concentrations (0.017, 0.022, and 0.042 wt%), which correspond to the boundary between the regions *A* and *B*.

In region *B* liquid-crystalline aggregates of DOAC may adhere to the electrically neutral albumin–DOAC floc; the onset of region *C* could, therefore, correspond to the saturated adsorption of the DOAC aggregates. The efficient multi-layer adsorption of a lammelar liquid-crystalline phase of DOAC to a surface, such as fabrics or hair, is noted elsewhere.[13] The resultant aggregate still remains in a settled state and is efficiently removed by microflotation if pH is appropriately adjusted ($\approx$6—$\approx$10). Ahmad[11] showed that the liquid-crystalline phase formed by surfactants and glycerides in association with protein was effective in the foam separation of albumin although a direct comparison with the experiments described in this work is not possible. Furthermore, some preliminary experiments indicated that the addition of $C_{18}H_{37}N(CH_3)_3Cl$, which forms micelles but does not form liquid crystalline phase in dilute aqueous solution, could also cause precipitation and settling of albumin at pH$>\approx$5. However, in excess of this surfactant, over what was needed to neutralize electric charge of the protein, peptization occurred due to the charge reversal of particles. The finely dispersed system could not be floated with microbubbles.

Stable dispersions in region *B* at pH 5 may also consist, at least in part, of small fragments of DOAC in liquid crystalline form. Since the aggregates of this surfactant contain up to 96% water, their density is very close to that of the liquid medium. Furthermore, the slight positive charge of these fragments would prevent an interaction with the finely dispersed albumin–DOAC floc of the same sign of charge. The addition of a small amount of electrolyte (*e.g.*, 1 wt% NaCl) causes a decrease in the amount of water in the swollen liquid crystals of DOAC from 96 to 70%, so that a separation of the surfactant aggregates occurs more readily. This result would indicate that the use of hard tap water rather than distilled water would be beneficial in the removal of albumin by DOAC.

Region *B* at pH 12 is considerably wider, which may be due to destabilization of liquid-crystalline aggregates of DOAC in the medium of high ionic strength. For instance, if a 0.10 wt% DOAC solution at pH 12 was aged for 24 h, a white creamy layer appeared in the upper part of the liquid; the amount of the separated phase increased with time and with increasing concentration of the surfactant.

Figure 5 shows that the conditions for an effective removal of albumin by microflotation ($\geq$80%) in the presence of DOAC coincide fairly well with those giving a floc that readily settles. The exception are either systems containing lesser amounts of the surfactant or systems at higher pH values of the media. Indeed, the higher the pH the more DOAC is needed to float the protein. The failure to separate the floc by microbubbles in this region may be due to the nature of its composition, which consists predominantly of liquid-crystalline DOAC fragments. Apparently, bubbles do not adhere to these solids sufficiently well unless the ionic strength is high enough. The addition of electrolytes (or using tap water) could improve the efficiency of microflotation under these conditions.

Since hemoglobin behaved in a very similar manner in the presence of DOAC as albumin, analogous explanation of the observed phenomena apply.

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