through a Watson-Biemann separator (Watson and Biemann, 1965) to the mass spectrometer, while the remainder of the effluent went to the flame ionization detector of the gas chromatograph.

Retention indices (I_E values) were determined by the method of van den Dool and Kratz (1963) using standards of ethyl esters of *n*-aliphatic acids and programmed temperature glc. The I_E values for the unknown compounds in the glc–ms runs were obtained by interpolation between peaks of unambiguously identified compounds of known I_E values. For this reason and because of the influence of other components on retention times, the I_E values for some of the later eluting peaks could not be determined precisely as for the earlier eluting ones. The I_E values obtained on the open tubular columns were, in most cases, very close to those obtained on standard packed columns.

RESULTS AND DISCUSSION

The compounds identified from all the trappings and methylated acid fraction are listed in Table I. Identification was made by matching the mass spectra of the unknown with reference spectra and verified by comparing the retention index values of the unknown compounds in the glc–ms runs to I_E values for known compounds. Tentative identifications are indicated where the mass spectra were weak or mixed, or reference compounds were not available for comparison. Many compounds that were used for reference were commercially purchased samples. In some cases, the chemicals were synthesized by the authors. When sufficient material could be trapped out, nmr and ir spectra were obtained to aid in the identification.

Many of the major constituents of the tar of hickory wood smoke have been identified. Although there has been no previous work on the tar of hickory wood smoke, several of these compounds have been previously reported in smoke condensate and extracts. This is noted in Table I. There were also several compounds found in smoke condensate which were not found in the tar. A possible reason is that the smoke condensate was obtained in a laboratory under controlled conditions while the material for this investigation was a commercially purchased sample of which little is known.

SUMMARY

As a result of this work on the tar of hickory wood smoke, we have identified 81 of the major constituents. Although many of the identified constituents can be considered to contribute to the hickory smoke aroma, it is not possible on the basis of this study to pinpoint one or more chemicals which could be described definitely as hickory smoke.

ACKNOWLEDGMENT

The authors thank Anne Sanderson for her help in the mass spectral interpretation and Mort Jacobs for his assistance in obtaining and interpreting the nmr spectra.

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Received for review May 7, 1973. Accepted October 31, 1973.

Solubility Behavior of Soybean Globulins as a Function of pH and Ionic Strength

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The solubility behavior of partially purified soybean globulins as a function of pH and ionic strength of the dispersing medium was investigated. It appeared that, even at the isoelectric point, the soy protein could be dissolved easily up to very high concentrations, provided that the ionic strength of the solution exceeded a critical value which, at pH 4.5, was about 0.7 for NaCl and Na_2SO_4 and 0.25 for $CaCl_2$. Below the critical ionic strength a two-phase system was formed, consisting of a protein-poor upper layer and a viscous protein-rich lower layer. At pH 7.0 no phase separation was observed at very low ionic strength but, as the salt concentration was increased, a region was passed in which the solution demixed. Outside the regions of immiscibility only homogeneous systems were obtained. The composition of the protein-poor layers in the two-phase systems was in agreement with the well known protein extractability curves for soybean meal.

The majority of the soy proteins are insoluble at their isoelectric point. Their solubility in dilute aqueous solution increases as the pH diverges from this value or, at constant pH, as the salt concentration is increased (salting-in). Therefore these proteins have been classified as globulins. The typical solubility behavior of globulins has been clearly demonstrated on β-lactoglobulin (Grönwall, 1942).

It is also well known that salting-in of proteins may be followed by salting-out when the ionic strength is in-

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creased sufficiently. Little, however, is known about the maximum solubility that can be reached before salting-out starts. Some work on carboxyhemoglobin has been published by Green (1932) and Cohn (1936).

As for the absolute solubility of soy protein, only some incomplete values obtained by salting-in purified soy protein at the isoelectric point have been published by Eldridge and Wolf (1967). In general, however, the solubility of soy protein is expressed as a percentage of the total amount present in aqueous mixtures containing relatively little protein. These data yield no information about the maximum solubility.

Observations by M. P. Tombs, B. Newsom, and D. Hall of Unilever Research Colworth/Welwyn Laboratory, Sharnbrook, Bedford, England, over the period 1965–1972, which led to a patent (Tombs, 1972) showed that soy protein and other proteins could exhibit liquid–liquid phase separation under certain conditions and that crude soy protein isolate, even at the isoelectric point, could form liquid systems containing more than 50% protein in the presence of sufficient salt. This observation caused us to investigate the solubility behavior of soy protein in more detail.

Protein isolates made by isoelectric precipitation from aqueous soybean meal extracts always contain denatured irreversible insoluble protein (Nash and Wolf, 1967). Since the latter hinders the observation of phase separation in solubility experiments, we removed the insoluble material from the isolated protein. From the partially purified protein thus obtained we made mixtures with salt and water. Type of salt, ionic strength, and pH were varied and the resulting phases were analyzed.

EXPERIMENTAL TECHNIQUES

Preparation of Purified Isoelectric Soy Protein. Protein isolate was extracted from defatted soybean meal (Soyafluff-200W, ex Central Soya) with 0.1% Na_2SO_3 in water at a solvent-to-meal ratio of 4:1 for 30 min at room temperature. The sulfite was added in order to prevent the formation of disulfide aggregates (Tombs, 1972). The insoluble carbohydrates were removed by centrifuging and the supernatant solution was acidified to pH 4.8 with 4 *N* HCl. The curd was separated by centrifuging and subsequently mixed with 4% NaCl to give a turbid viscous apparently homogeneous system containing about 25% protein. When centrifuged for 1 hr at 40,000 × *g*, this mixture separated into a putty-like lower layer and a clear oily upper layer. The lower layer was discarded since it was a mixture of the supernatant solution and the insoluble obviously denatured material described by Nash and Wolf (1967). This followed from the fact that repeated washings with 4% NaCl gave a residue (amounting to about 15% of the starting protein) which was only slightly soluble at pH 7.0.

The upper layer was dialyzed against running tap water for 2 days. During dialysis the protein separated from the solution as a pale-yellow precipitate, which was centrifuged, dispersed twice in an excess of distilled water, and centrifuged again. The purified protein was stored at −25°. Under these conditions no change in solubility properties could be detected even after 3 months of storage.

The protein preparation contained 46% water. The composition, calculated on dry matter, was 104% protein (N × 5.70; Tkachuk, 1969), 0.1% Ca, 0.2% P, 0.1% Na, and 0% Cl.

Analytical Procedure. Dry matter was determined by drying an aliquot of the material for 16 hr at 105° in air. Chloride was determined by potentiometric titration, calcium and sodium by atomic absorption, phosphorus by molybdenum-blue method, and nitrogen by Kjeldahl analysis. The NaCl and $CaCl_2$ contents were calculated from the chloride and calcium analyses, respectively. Na_2SO_4 content was calculated from sodium determination. Salt contents were expressed in terms of molarity, *i.e.*, moles per 1000 ml of the salt solution, excluding the protein. The protein percentages (g/100 g) of the test mixtures were estimated from the dry matter content after correction for the salt content.

Solubility Experiments. Mixtures with final concentrations of 10, 20, or 30% protein were prepared. Purified protein was dispersed in water and the pH was adjusted to the desired value by careful addition of 0.15 *N* NaOH or HCl. Solid salt was then dissolved in the mixture until required concentration had been reached. After mixing, the pH was readjusted with some drops NaOH or HCl solution. The change in ionic strength, resulting from the addition of NaOH or HCl, was not corrected for.

Portions of about 15 g of each mixture were allowed to equilibrate in well stoppered bottles by moderate shaking for 16 hr and, unless otherwise stated, at 20°. The pH was readjusted again if necessary and the samples were centrifuged for 45 min at 40,000 × *g*. The separate phases were analyzed for protein and salt content.

RESULTS

After centrifuging, the experimental mixtures were either homogeneous or separated into a protein-poor upper layer and a protein-rich lower layer. A very small amount of obviously denatured material settled on the bottom of all tubes. The results of the analyses of the phases and the homogeneous mixtures are plotted in Figure 1.

Figure 1a shows the phase relations in the system soy protein–NaCl–H_2O (pH = 5.0) at ambient temperature (24 ± 1°), and for the total protein contents of 10, 20, and 30%. The data obtained at salt concentrations above 0.55 *M* (up to 0.9 *M*) are omitted from the figure, as only homogeneous mixtures were obtained in this region.

The imaginary boundaries between the blank and black symbols in Figure 1a indicate a critical NaCl concentration for phase separation at pH 5.0 which is about 0.40–0.45 *M*. Above this concentration (up to 0.9 *M*) no phase separations were observed. The tie-lines drawn in the figure indicate the approximate position of the boundaries. As the imaginary boundaries are fairly flat, no plait point could be detected. Moreover, the shape of the diagrams appears to depend on the total protein concentration. At salt contents near the critical concentration, the separated phases were clear, yellowish, viscous liquids, but at lower salt contents the upper layer resembled a normal protein solution, whereas the lower layer was a nonadhesive, elastic, opaque semisolid.

Figure 1b illustrates the solubility behavior of soy protein as a function of pH and NaCl concentration for a total protein content of 10% (the curve at pH 5.0 is copied from Figure 1a). It appears that the region of the coexisting phases becomes smaller with increasing pH. At pH 6.0 and 7.0 there is no longer any phase separation at low ionic strength and an "island" of immiscibility is formed. At pH 7.0 the phases separated with some difficulty; at room temperature only a turbid mixture was formed and cooling overnight at 5° was therefore applied to ensure complete phase separation. The lower layer thus formed is presumably the cold-insoluble fraction described by Briggs and Mann (1950).

Attempts to draft a phase diagram at pH 3.0 failed. The soy protein dissolved readily in water at this pH but precipitated in an insoluble form when NaCl was added. When soy protein–NaCl–H_2O systems were acidified to pH 3.0 the situation improved but reliable experiments were still not possible.

The influence of the $CaCl_2$ concentration on the solubility behavior of soy protein at pH 4.5 and 7.0 in mixtures

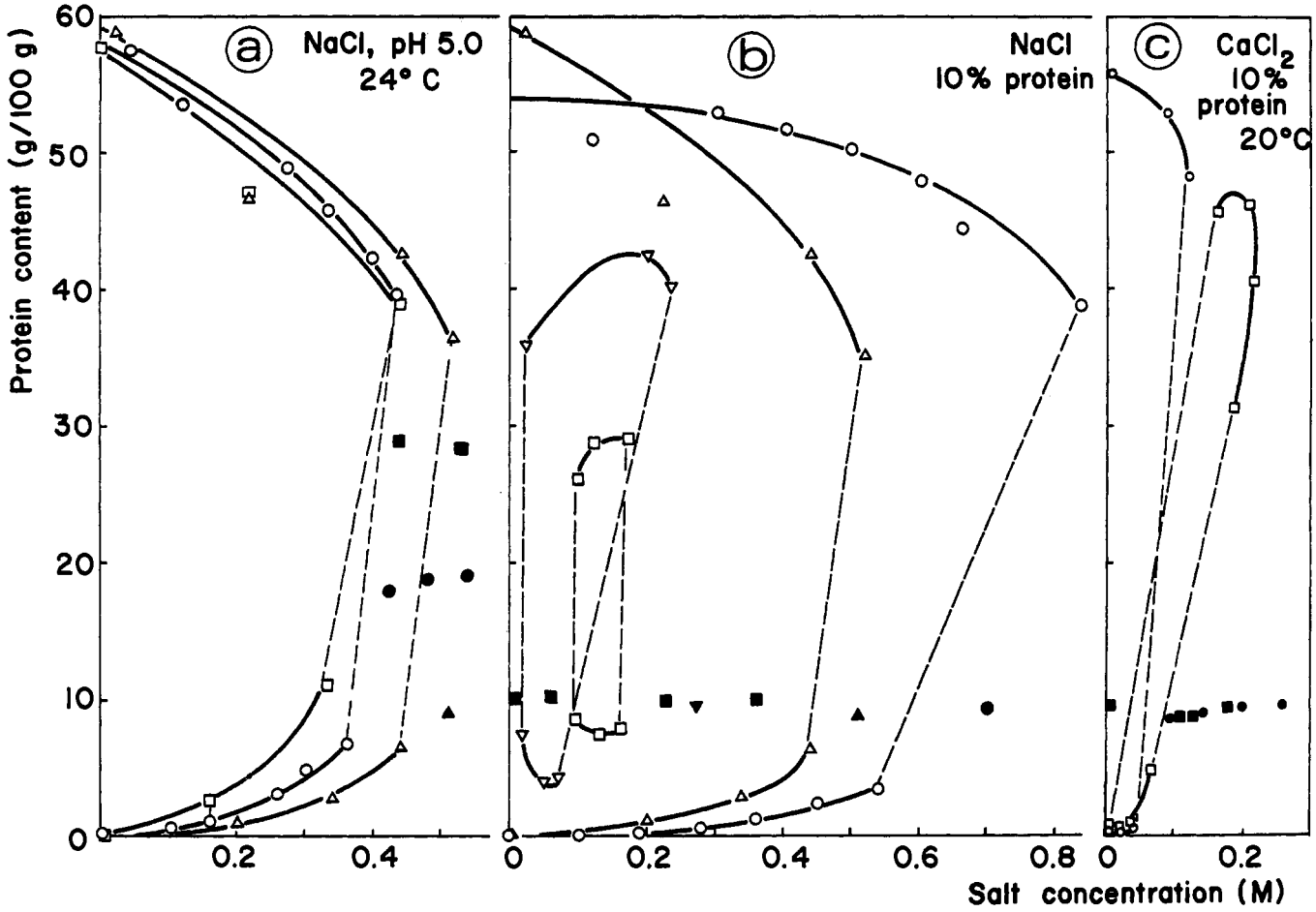

Figure 1. Phase separation in the system soy protein–salt–water. (a) (△) 10, (○) 20, (□) 30% total protein; (b) (○) pH 4.5/20°, (△) pH 5.0/24°, (▽) pH 6.0/20°, (□) pH 7.0/5°; (c) (○) pH 4.5, (□) pH 7.0. No phase separation is indicated by ▲, ●, ■, and ▼.

containing 10% protein is shown in Figure 1c. At pH 7.0 an island of immiscibility was also formed although its shape was quite different from that of the analogous case for NaCl in Figure 1b. In contrast to the mixtures with NaCl at this pH, phases separated spontaneously without cooling.

At $CaCl_2$ concentrations over 0.1 *M*, both at pH 7.0 and 4.5, an aggregate-like precipitate, comprising 5–10% of the protein present, was formed which did not dissolve upon further addition of salt. At pH 7.0 and a total $CaCl_2$ concentration of 0.2 *M*, the wet precipitate contained about 25% protein in (apparently) 0.6 *M* $CaCl_2$. The points representing the composition of this obviously denatured material are not included in the figure.

A few experiments, performed at pH 4.5 with Na_2SO_4 and 10% soy protein, also revealed a region of two coexistent liquid phases. The critical concentration for phase separation was about 0.25 *M*.

DISCUSSION

The results show that the solubility behavior of soy proteins at room temperature is characterized by the occurrence of liquid–liquid phase separations. Below a certain salt content, which we termed "critical salt concentration," a protein-poor upper layer and a protein-rich lower layer are formed; the extent of the area of immiscibility (Figure 1) depends on the type of salt present and the pH. As the composition of both phases varies with the total composition of the system, we presented the results of our investigations in diagrams in which the composition of both the protein-poor and the protein-rich layers could be displayed.

The diagrams represent a state of equilibrium: no difference in phase composition could be detected whether the total composition of the two-phase systems was obtained by adding salt to a protein–water mixture or by diluting a homogeneous protein–salt–water mixture with water to below the critical salt concentration.

As shown in Figure 1a, the composition of the phases also depends on the total protein content of the system. This is most probably due to the inhomogeneity of our protein preparation, which is a mixture of different protein species. Moreover, the free salt concentration may change as more protein is added, since protein binds water and salt from the solution; this in turn, may influence the composition of the phases.

If the salt concentration exceeds the critical value, the solubility of the proteins appears to be independent of the salt concentration, although at high salt contents salting-out may start. At pH 4.5 the critical value, expressed as ionic strength, is about 0.7 for NaCl and Na_2SO_4 and 0.25 for $CaCl_2$.

The lower branches of the phase diagrams represent in fact the solubility of the protein preparation as a function of the salt concentration, be it that the amount of protein in these protein-poor phases is plotted as a function of their own salt content. More usual presentation of such solubility data is obtained by plotting the protein contents of the protein-poor phases and the homogeneous mixtures as a function of the overall salt concentration of the systems. Figure 2 shows a set of solubility curves thus drafted from the experiments with NaCl (Figure 2a) and $CaCl_2$ (Figure 2b) at a protein concentration of 10%.

These curves invite comparison with the literature data on the extractability of proteins from defatted soybean meal, although it must be borne in mind that differences in protein concentration and the presence of meal components, which have been removed from our protein prepa-

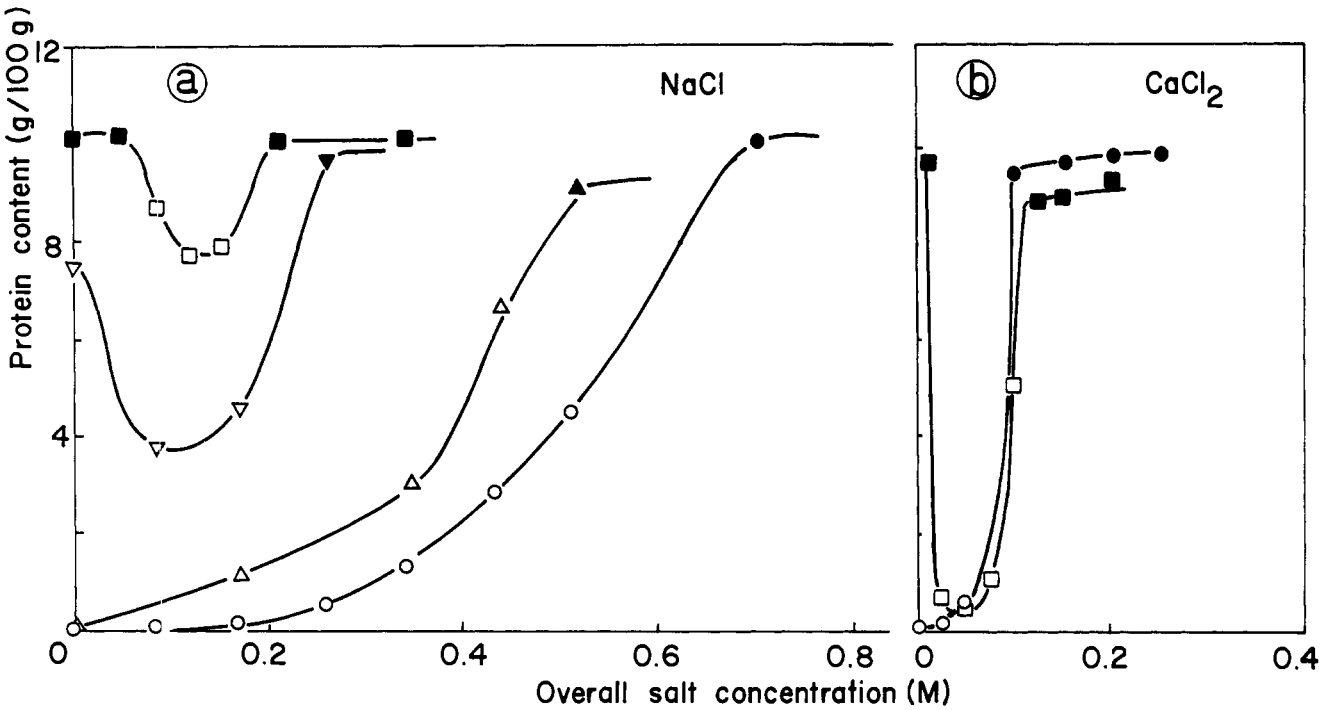

Figure 2. Solubility curves of soy protein as a function of overall NaCl (a) and $CaCl_2$ (b) concentration, derived from phase separations as plotted in Figure 1b and 1c. (○) pH 4.5, (△) pH 5.0, (▽) pH 6.0, (□) pH 7.0. No phase separation is indicated by ●, ▲, ▼, and ■.

ration, may affect the results considerably. Nevertheless, the solubility minimum at 0.1 *M* NaCl at neutral pH is in agreement with the minimum in extractability found by Smith *et al.* (1938). Wolf and Briggs (1956) have demonstrated that the 11*S* and 15*S* protein fractions in particular are involved in this remarkable salting-out effect at low ionic strength. The maximum solubility at 0.7 *M* NaCl and pH 4.5 is in accordance with the extraction data of Anderson *et al.* (1973).

With $CaCl_2$, very effective salting-out occurs at pH 7.0, a salt concentration of 0.05 *M*. At pH 4.5 maximum solubility is obtained at 0.1–0.15 *M* $CaCl_2$. The results of Smith *et al.* (1938) and Smith and Circle (1938) show a minimum in extractability at about 0.01 *M* $CaCl_2$ at pH 5.7, while according to Anderson *et al.* (1973) maximum extractability is reached at 0.15 *M* $CaCl_2$, pH 4.5.

The separation of globulin solutions into liquid phases has in the past been observed by Palmer (1934) for β-lactoglobulin and by Holwerda (1935, 1936) for edestin. The process is undoubtedly related to the liquid–liquid phase separations (*e.g.*, coacervation) which are often observed in polymer solutions. The phase separation which occurs in mixtures containing soy proteins may be initiated by association of some of the proteins; association of soy protein fractions (7*S*, 11*S*) upon lowering of the ionic strength of the solution has been demonstrated by Naismith (1955) and Roberts and Briggs (1965). Investigation of the partition of soy protein fractions (7*S*, 11*S*, 15*S*) between the phases would be interesting, as the solubilities of these components are quite different (Eldridge and Wolf, 1967). In particular the extraction experiments of Anderson *et al.* (1973) suggest that fractionation of the proteins plays an important role in our observations.

From Figure 1 it appears that the salt content of the protein-rich layer still exceeds that of the protein-poor layer which indicates binding of the salt ions to the protein. The electrostatic nature of this binding is evident from the pH dependence and the different effects of the monovalent sodium and the divalent calcium (Figures 1b and 1c).

From the composition of the lower layer in Figure 1c, it can be estimated that at pH 7.0 soy protein binds an amount of calcium in the order of 3 mmol/g, which is in agreement with the data of Saio *et al.* (1968) for the maximum binding capacity for calcium to the "cold-insoluble fraction." The aggregate-like material which was precipitated at 0.2 *M* $CaCl_2$ contained as much as 1.2 mmol of calcium/g, calculated on dry matter. This suggests that the protein (partially) unfolds, thus exposing active sites for calcium binding.

Summarizing, our results reveal at which pH's and ionic strengths highly concentrated soy protein solutions can be made at room temperature. Provided the ionic strength is about 0.7 (in the case of NaCl), these conditions even include pH values down to the isoelectric point. This knowledge will be advantageous for applications in food products (Tombs, 1972).

ACKNOWLEDGMENT

The author is indebted to Wout Kalkman for his technical assistance.

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Received for review June 18, 1973. Accepted October 26, 1973.