The Properties of Bovine Serum Albumin and Chymotrypsinogen A in Solvent Mixtures containing Phenol

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1. The optical rotation and reduced viscosity of bovine serum albumin and chymotrypsinogen A in solvents containing phenol, acetic acid and water were studied. 2. The changes brought about in the properties of the proteins by varying the composition of the solvent or by heat treatment in these solvents were established to be reversible. 3. A method for returning the proteins to aqueous media, based on these observations, was worked out. 4. The recovered proteins were shown to be very similar to, if not identical with, the native proteins on the basis of measurements of optical rotation, viscosity, sedimentation, ultraviolet spectroscopy, immunochemical behaviour (serum albumin) and proteolytic activity (chymotrypsinogen A, after activation with trypsin). 5. The importance of the findings for partitioning of polyelectrolytes in the phenol-aqueous buffer systems is discussed.

Phenol has long been known to be an excellent and selective solvent for proteins (for references see Pusztai, 1966). Its use for the preparation of proteins, however, has been limited as it was commonly held that phenol irreversibly denatured proteins and abolished enzymic activity (e.g. Cooper, 1912; Cooper & Woodhouse, 1923; Labes & Jansen, 1931; Grassmann & Deffner, 1953). The recent demonstration of the reversible inhibition of ribonuclease by phenol (Huppert & Pelmont, 1962; Hsu & Wang, 1964) and the subsequent successful isolation of the active enzyme from various sources by extraction with phenol (Kickhöfen & Bürger, 1962; Rushizky, Greco, Hartley & Sober, 1963; Glitz & Dekker, 1964) have revived the interest in phenol as a preparative protein solvent. Pusztai (1966) studied the essential features of the interactions between proteins and other macromolecules and demonstrated the relative ease of separating proteins from various other polyelectrolytes in phenol-containing solvents on partitioning, in contrast with the strong interactions occasionally found between these macromolecules in purely aqueous solvent systems. This, taken together with the known inhibitory effect of phenol on enzyme activities, offers some promise for the development of techniques for the isolation of enzymically unchanged cellular constituents and for their analysis. A study of the properties of a few proteins in solvent mixtures containing phenol as a function of the composition of the solvent was therefore undertaken to gain an insight into their

stability and to test the reversibility of any changes induced. No effort was made, however, to conduct a complete physical study, because of the inherent difficulties of the theoretical interpretation of the interactions between complex macromolecules like proteins and the organic solvents used. It was hoped, however, that, by measuring simple physical parameters of the proteins, like $[\alpha]_D$ and η_{red} , under conditions likely to be encountered when utilizing both the selectivity of phenol as a protein solvent and its other advantageous properties, the principles of the recovery of proteins essentially unchanged from phenol-containing solvents could be found. By utilizing these principles, the proteins were recovered and their properties carefully compared with those of samples of proteins not exposed to organic solvents.

The following is an account of the experimental work carried out with bovine serum albumin and chymotrypsinogen A.

MATERIALS AND METHODS

Bovine serum albumin. A crystalline commercial preparation was purchased from Armour Pharmaceuticals Ltd., Eastbourne, Sussex, and used without any further purification. For a few selected experiments a fatty acid-free serum albumin was prepared by treating the protein with 0.1N-HCl (Williams & Foster, 1959). The protein was recovered after extensive dialysis against water by drying from the frozen state. The results obtained with this sample were, however, essentially the same as those

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obtained with the commercial untreated preparation and the procedure was therefore abandoned.

Chymotrypsinogen A. A commercial crystalline preparation (type II; lot no. 35B-2040) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and was used without any further purification.

Trypsin. The crystalline enzyme was also obtained from Sigma Chemical Co. (type I; lot no. 95B-2620).

N-Acetyl-L-tyrosine ethyl ester monohydrate. A chromatographically pure preparation was purchased from British Drug Houses Ltd., Poole, Dorset.

Phenol and other chemicals. These were of A.R. quality.

Conversion of chymotrypsinogen into chymotrypsin and measurement of its activity. The chymotrypsinogen samples were dissolved in 0.001 N-HCl (1–2 mg./ml.) and adjusted to about pH 8 by the addition of an equal volume of 0.01 M-tris-HCl buffer, pH 8.0. Trypsin dissolved in the same buffer (1 mg./ml.) was added in a final chymotrypsinogen/trypsin ratio 100:1–2 and the mixtures were incubated at 0–4° for times up to several days. To samples of these solutions, taken at intervals of 2, 16 and 48 hr., suitable amounts of 0.05 N-HCl were added to adjust their pH to about 3 and these mixtures, after dilution with 0.005 M-tris-HCl buffer, pH 8.0, were tested for chymotrypsin activity in a pH-stat (Cunningham, 1954).

Optical rotation. The readings were made with a Hilger visual-reading polarimeter at the fixed wavelength of 589 m μ in a 1 dm. tube in a thermostatically controlled room at 20 $\pm$ 2°. To improve the accuracy of the readings the concentration of the protein in the various solvents was usually 2% (w/v) and the results are given as $[\alpha]_D$ uncorrected for changes in the refractive index or the dielectric constant of the solvent. The accuracy of the readings was usually better than $\pm$ 2% and only occasionally, with solutions showing slight scatter or spurious reflexion, did it decrease to about $\pm$ 4%.

Viscosity. For the measurement of the relative viscosity of the proteins in solvent mixtures containing organic solvents, relatively fast-flow Ostwald viscometers (flow times with water about 70 sec. at 20°) and high temperatures, 40 $\pm$ 0.02°, were employed. The viscosity of aqueous solutions of the protein was measured at 19.85 $\pm$ 0.02° in a somewhat finer Ostwald viscometer (flow times with water about 240 sec. at 20°). The viscometers were always cleaned with chromic acid before the introduction of each individual sample and the reproducibility of the readings was better than $\pm$ 1%.

Sedimentation. The sedimentation coefficient of the proteins before exposure to organic solvents and that of a few selected samples of recovered material were estimated from the results of sedimentation-velocity experiments at 59780 rev./min. in a Spinco model E ultracentrifuge equipped with a temperature-control unit and a schlieren optical system. All experiments were performed at 20° and at a protein concentration of 0.5% (w/v).

Ultraviolet spectroscopy. The spectra in the range 240–340 m μ were recorded on a Beckman DB spectrophotometer in 1 cm. cells. In the recording of difference spectra the proteins not exposed to organic solvents were used in the place of the solvent blank after all the concentrations were carefully adjusted to the same value (N estimations).

Immunoelectrophoresis and double diffusion. These were carried out as described before (Pusztai, 1965). In these experiments a commercial rabbit anti-(bovine serum

albumin) serum by Burroughs Wellcome and Co., Beckenham, Kent (series K4316), was employed. Chymotrypsinogen samples were not investigated by these immunochemical methods.

RESULTS

It has been established (Pusztai, 1966) that as a result of the action of phenol the molecular conformation taken up by a number of proteins becomes incompatible with their solubility in the aqueous phase on partitioning between phenol and aqueous buffers. When the removal of phenol from solutions of proteins in phenol-containing solvents is attempted by dialysis against water or dilute aqueous buffers, phase separation occurs and the proteins become more and more concentrated in the ever-decreasing phenol-rich phase and are precipitated out when the phenol is dialysed out. In most cases these precipitates remain insoluble in aqueous buffers. It was thought that the transfer of proteins from phenol to aqueous solutions could be best achieved by the introduction of a third solvent, completely miscible with both phenol and aqueous buffers. This would eliminate the dangers of phase separation during dialysis and would at the same time provide a means of continuous transfer of the proteins from phenol back into water. The only other prerequisite is that the third component should be a good solvent for proteins. Acetic acid at or above about 5% (w/v) concentration is known to render phenol and water fully miscible (Ust-Kachkintsev, 1937; Golubkova, Petin & Topchieva, 1941) and is also a good solvent for proteins. This makes it possible to study the behaviour of proteins in almost the full range of phenol–water mixtures at various constant acetic acid concentrations. To make an allowance for the contribution that acetic acid may make, the optical rotation and viscosity of bovine serum albumin (Fig. 1a) and chymotrypsinogen A (Fig. 1b) were measured in the acetic acid–water system at 2% (w/v) protein concentration as a function of the relative composition of the solvent. To keep the concentration of the protein constant throughout the experiment, two stock solutions of serum albumin were used, each 2% (w/v), one in water and the other in acetic acid. The second stock solution of chymotrypsinogen A was made up in an aq. 83% (w/v) acetic acid since the solubility of this protein decreased rapidly at higher acetic acid concentrations. All measurements were made on solutions obtained by mixing the two stock solutions in various proportions as quickly as possible after mixing, and then at various time-intervals until constant readings were obtained. All solutions of serum albumin up to about 20% (w/v) acetic acid concentration made up by this

Fig. 1. Optical rotation and viscosity of bovine serum albumin (a) and bovine chymotrypsinogen A (b) in acetic acid-water mixtures as a function of the composition of the solvent. For (a), two stock solutions of serum albumin, 2% (w/v) each, one in water and another in acetic acid, and for (b), two stock solutions of chymotrypsinogen A, 1-90% (w/v) each, one in water and another in aq. 83% (w/v) acetic acid, were made up and mixed in various proportions. The optical rotation (○) and the reduced viscosity (●) of these solutions were measured at 20°. To eliminate the time-dependence (slight in b) of the readings only the final values are given.

procedure gave time-dependent increases in the properties examined; the lower the acetic acid content the longer it took to reach stable readings (0-48 hr.). The values obtained at higher acetic acid concentrations showed no time-dependence and were similar to other values at similar acetic acid concentrations described before (e.g. Steinrauf & Dandliker, 1958a; Harrap & Woods, 1965). With chymotrypsinogen A the $[\alpha]_D$ and the η_{red} values also showed a slight upward drift for a few days. For this reason only final readings are given.

Fig. 2. Optical rotation of bovine serum albumin (a) and bovine chymotrypsinogen A (b) in phenol-water mixtures at various constant acetic acid concentrations as a function of the composition of the solvent. Two stock solutions of each protein, 2% (w/v) each, one in aqueous acetic acid and another in a phenol-acetic acid mixture containing the same amount of acetic acid as the aqueous solution, were made up and mixed in various proportions. The symbols for the various acetic acid concentrations used are as follows: In (a): x, 1.4% (w/v); □, 3% (w/v); ■, 10% (w/v); △, 15% (w/v); ▲, 20% (w/v); ○, 37% (w/v); ●, 70% (w/v). In (b): ○, 10% (w/v); △, 20% (w/v); □, 35% (w/v).

The changes in $[\alpha]_D$ and η_{red} values were found to be reversible; the experimental curves obtained were identical regardless of whether the concentration of the acetic acid increased or decreased in the reaction mixtures.

Next, the optical rotation of serum albumin (Fig. 2a) and chymotrypsinogen A (Fig. 2b) at 2% (w/v)

protein concentration in phenol-water systems containing various constant amounts of acetic acid were measured. The concentration of the protein was again kept constant by a procedure similar to that described above. With serum albumin, time-dependence of the $[\alpha]_D$ values was again observed at low acetic acid concentrations and Fig. 2(a) contains the final readings only. Fig. 3(a) shows

Fig. 3. Viscosity of solutions of bovine serum albumin (a) and bovine chymotrypsinogen A (b) in phenol-water mixtures at constant acetic acid concentrations as a function of the solvent composition. For experimental details see Fig. 2 and the text. The reduced viscosity values (dl./g.) were measured at 40°. The solutions contained the following constant amounts of acetic acid: In (a): ■, 10% (w/v); △, 15% (w/v). In (b): ○, 10% (w/v); △, 20% (w/v); □, 35% (w/v). In (b), where time-dependence was observed the continuous lines connect the final readings, and the broken experimental curves give the same 5 min. after mixing the solutions and placing them in the water bath.

the changes in $\eta_{red.}$ of serum albumin and Fig. 3(b) gives the results for chymotrypsinogen A. The experimental curves obtained for chymotrypsinogen A at 10% (w/v) acetic acid concentration (Figs. 2b and 3b) were found to be discontinuous owing to precipitation of the protein. No precipitation occurred at the higher acetic acid concentrations studied although, in about the same phenol concentration range, the viscosity values showed time-dependent changes. When solutions made up at room temperature were placed in the 40° water bath their viscosity decreased to reach stable values in about 1-2 hr. As it was found impossible to extrapolate the viscosity values to zero time, Fig. 3(b) contains viscosity-phenol concentration curves drawn from the approximate $\eta_{red.}$ values obtained after about 5 min. from the time of immersing the viscometers in the water bath (broken line) together with the curves constructed from the final $\eta_{red.}$ values (continuous line). When these solutions were left at room temperature overnight their viscosity increased. If now the temperature was increased to 40° the viscosity values showed a time-dependent decrease very similar to that found before. It was also found that the attainment of the final readings could be accelerated by exposing these solutions to higher temperatures (60-80°) for a short time (2-3 min.) before measuring their viscosity at 40°. The heating, however, did not abolish the reversibility, since the viscosity values of the solutions rose again on standing at room temperature overnight and showed the time-dependent fall in viscosity on exposing them to 40°. Solutions of chymotrypsinogen A in solvent mixtures containing a high proportion of phenol, on the other hand, showed very little or no time-dependence in any of the properties examined. More detailed information on the effects of phenol on the behaviour of serum albumin in aqueous and dilute acetic acid solutions was obtained from the results of experiments in which the changes in $[\alpha]_D$ and $\eta_{red.}$ values were measured on adding small increments of phenol to these solutions (Figs. 4a and 4b). The abrupt termination of the various curves was due to precipitation of the protein. At or above 8% (w/v) acetic acid concentration in the solvent, the protein was no longer precipitated out by the addition of phenol and showed very little time-dependent changes similar to those observed with chymotrypsinogen A. All experimental curves were duplicated by performing the experiments either by increasing or decreasing the proportion of phenol in the solvent mixtures. Because these experiments took a long time to complete, and because some of the properties of the proteins studied showed changes with time, the two experimental curves were identical only when aged stock solutions were used in which most, or all, of these

Fig. 4. Optical rotation (a) and reduced viscosity (b) of bovine serum albumin in dilute aqueous acetic acid solutions on addition of phenol. The experiments were performed at the following constant acetic acid concentrations: ●, 2% (w/v); ○, 3% (w/v); ▲, 4% (w/v); △, 15% (w/v). Experimental details are given in the text.

changes had already taken place. The time-dependent changes could also be decreased substantially by exposing the stock solutions to 60–80° for a short time (2–3 min.). Under these conditions all the experimental curves were established to be reversible. Finally, as a comparison, the optical rotation of serum albumin dissolved in phenol (2%, w/v, protein concentration) was measured as a function of the added amount of a strongly protic solvent, dichloroacetic acid (Fig. 5).

The stability of the proteins dissolved in aq. 90% (w/v) phenol to heat treatment was tested by exposing these solutions to 40–90° for up to 30 min.

Fig. 5. Optical rotation of bovine serum albumin in phenol-dichloroacetic acid mixtures as a function of the composition of the solvent. Experimental details are given in the text.

at each particular temperature. The solutions were then allowed to cool to 20° and their optical rotation was measured. There was little change in $[\alpha]_D$ with increasing temperature; its value, in fact, decreased slightly from –57° (at 20°) to –55° (at 90°) for serum albumin, whereas for chymotrypsinogen A both $[\alpha]_D$ (–56°) and $\eta_{red.}$ values (0.186 dl./g.) were found to be unchanged within experimental error.

After the completion of the experiments described above, all materials subjected to the various treatments in the organic solvents studied were recovered by dialysis against aqueous acetic acid solutions of various concentrations. Later it was found that, with bovine serum albumin, dialysis against water was as satisfactory; although the protein was precipitated at one stage in the dialysis, when the phenol had been completely removed, all the protein went back into solution and could be recovered by freeze-drying. Solutions (2%, w/v) of the recovered materials and of the original protein were made up in 0.05M-sodium acetate buffer containing sodium chloride (0.1M), pH 5.08, on the basis of N estimations and these were used for measuring $[\alpha]_D$, $\eta_{red.}$ and the sedimentation coefficient (Table 1). All materials gave identical spectra and, further, the albumin samples showed no difference spectra when the recovered and original materials were compared. The proteins when tested in immunoelectrophoresis behaved as the original protein, by giving one precipitin line only. When the immune serum used was first absorbed with serum albumin that had not been

Table 1. *Properties of bovine serum albumin before and after exposure to organic solvents*

Experimental details are given in the text.

Protein sample	$-\alpha_D$	$[\eta]$ (dl./g.)	$S_{20,w}$ (s) at 0.5% (w/v) protein concn.
Original	60.6°	0.041	4.48
Exposed to acetic acid	61.8	0.040	4.44
Recovered from phenol containing 10% (w/v) of acetic acid	61.9	0.042	4.48
Recovered from phenol containing 37% (w/v) of acetic acid	61.1	0.041	4.50
Heated in aq. 90% (w/v) phenol at 90° for 30 min. and recovered	62.1	0.043	4.48
Recovered from phenol-dichloroacetic acid mixtures	61.3	0.042	4.49

Table 2. *Recovery of the amount and proteolytic activity (after treatment with trypsin) of chymotrypsinogen A dissolved in aq. 90% (w/v) phenol and dialysed under various conditions*

Experimental details are given in the text. The results are calculated as percentages of (a) soluble protein (w/w) and (b) chymotrypsin activity (total activity/original total activity) after trypsin treatment of original chymotrypsinogen recovered. The control experiments were performed under identical conditions but phenol was omitted from the solvents.

Conditions of dialysis	Experimental		Control	
	(a)	(b)	(a)	(b)
Water	15.2	16.3	90.9	92.5
0.001N-HCl	45.9	47.1	98.0	98.7
2% (w/v) Acetic acid	68.0	68.8	94.8	93.8
10% (w/v) Acetic acid	85.6	87.3	97.0	95.6
20% (w/v) Acetic acid	96.4	96.0	97.8	96.2
40% (w/v) Acetic acid	99.1	97.8	97.5	96.2

exposed to organic solvents, it gave no further reaction with any of the recovered proteins and vice versa. In the double-diffusion tests all materials gave one line only and a reaction of complete identity. Chymotrypsinogen A, on the other hand, became almost totally insoluble in aqueous solvents if its solutions in solvent mixtures containing phenol had been dialysed against water or aqueous buffers. Preliminary experiments were therefore performed to investigate the factors influencing the recovery of the native protein. Chymotrypsinogen (35mg.) was dissolved in aq. 90% (w/v) phenol (35ml.) by standing at 40° overnight and samples (5ml. each) of this solution were dialysed against water, 0.001N-hydrochloric acid, 2%, 10%, 20% and 40% (w/v) acetic acid solutions respectively for 4 days. As a final step the 10%, 20% and 40% (w/v) acetic acid solutions were replaced with a more dilute (2%, w/v) acetic acid solution to decrease the acid concentration in the protein solution to a low level. After the phenol had been removed, all solutions were centri-

fuged to remove insoluble material. The precipitates were washed twice in the centrifuge and the supernatants combined with the appropriate washings were adjusted to pH 8 with 0.1N-sodium hydroxide. The protein concentration in these solutions was estimated from their extinction values at 282m μ based on a value of 20.7 for $E_{1cm}^{1\%}$. (Eisenberg & Schwert, 1951). The chymotrypsinogen was then converted into the active enzyme and assayed. Recoveries of protein and enzymic activity (Table 2) were poor when the protein solutions containing phenol had been dialysed against water or dilute acids. Dialysis against more concentrated acetic acid solutions, on the other hand, kept the protein in solution throughout the dialysis and the recovery of the amount and the activity of the protein was practically complete (Table 2). In the control dialysis experiments (solutions of proteins in water) the recovery of the protein and its enzymic activity were quantitative in all instances except those in which the solutions were dialysed against water. When the protein was

Table 3. *Properties of chymotrypsinogen A before and after exposure to organic solvents*

Experimental details are given in the text.

Protein sample	$-\left[\alpha\right]_D$	$[\eta]$ (dl./g.)	$S_{20,w}$ (s) at 0.5% (w/v) protein concn.	Recovery of soluble protein (% of original protein)	Recovery of chymotrypsin activity (% of original activity)
Original	76.5°	0.034	2.60	—	—
Exposed to acetic acid	74.8	0.035	2.60	77.2	75.0
Recovered from phenol containing 10% (w/v) of acetic acid	77.2	0.035	2.58	85.0	88.2
Recovered from phenol containing 20% (w/v) of acetic acid	78.0	0.036	2.59	91.0	90.5
Recovered from phenol containing 35% (w/v) of acetic acid	76.8	0.034	2.60	87.5	90.5
Heated in aq. 90% (w/v) phenol at 90° for 15 min.	77.2	0.033	2.58	94.5	97.0
Material precipitated on dialysis against water and subsequently resolubilized from phenol	76.0	0.034	2.62	80.1	81.0
Material that remained in solution when dialysed against water	75.8	0.035	2.60	12.3	11.2

recovered from the above solutions by freeze-drying some aggregated material [insoluble in 0.005M-tris-hydrochloric acid buffer, containing sodium chloride (0.5M), pH 8.0] usually formed. The amount of this depended on the solvent, the concentration of the protein and the thoroughness of the removal of phenol. It was found that the amount of aggregated material was kept to a minimum (usually less than 5%, w/w, of total protein recovered) if dilute solutions (less than 0.1%, w/v, protein) of chymotrypsinogen dissolved in 0.001N-hydrochloric acid or in acetic acid of moderate strength (about 10%, w/v) were freeze-dried. All aggregated materials, however, could be reconverted into soluble form by dissolving in phenol-containing solvents, dialysing first against aq. 20% (w/v) acetic acid, then against 0.001N-hydrochloric acid and, finally, freeze-drying the solutions (Table 3).

In view of the above experiments, all proteins exposed to phenol or acetic acid were recovered by the dialysis and freeze-drying procedure described above. The thoroughly dried protein samples from the various experiments and a sample of original chymotrypsinogen A were dissolved in a 0.025M-glycine-hydrochloric acid buffer, containing sodium chloride (0.125M), pH 3.17, to give a final protein concentration of 2% (w/v). These solutions were used for measuring $[\alpha]_D$, $[\eta]$ and the sedimentation coefficient (Table 3) and were tested, after activation with trypsin, for chymotrypsin activity. All samples gave identical spectra and the chymotrypsinogen samples showed no difference spectra when the recovered and unexposed materials were compared.

DISCUSSION

Much interesting and fundamental information has been obtained on the conformation and the properties of proteins and other biologically important macromolecules by studying their solutions in various non-aqueous solvents (Singer, 1962). The present study, however, is essentially much more limited in scope; it is not so much concerned with attempting to elucidate the possible conformations of proteins that result from the interactions between the solvent molecules and the proteins but rather with the direction and the reversibility of the changes induced by the organic solvents studied. The aim was to investigate the conditions that govern the transfer, if possible, of various proteins from phenol to aqueous solutions unchanged and in their native state. The direction of the changes in the physical parameters studied in the phenol-acetic acid-water system was somewhat similar to that found with other proteins and in different solvent systems (e.g. Bresler, 1958; Weber & Tanford, 1959; Tanford, De & Taggart, 1960; Tanford & De, 1961; Van Holde & Sun, 1962; Bigelow & Krenitsky, 1964). It is to be noted, however, that even the two proteins investigated in the present work showed certain dissimilarities (compare, for example, Fig. 3a with Fig. 3b) as well as agreements in their behaviour, although the theoretical significance of these differences are difficult to interpret. At least one of the changes found in the phenol-acetic acid-water solvent system, however, merits some further discussion. It can be seen (Figs. 2a, 2b, 3a, 3b, 4a and 4b) that the addition of small amounts of phenol to aqueous

or dilute acetic acid solutions precipitated the proteins. This precipitate, however, redissolved on increasing the acetic acid concentration. No precipitation occurred with bovine serum albumin above 8% (w/v) and with chymotrypsinogen A above 12% (w/v) acetic acid concentration, an observation that might have some practical importance in fractionating proteins as the transition between precipitation and dissolution was sharply different for the two proteins. The amount of phenol added to the aqueous solutions of the proteins in these experiments was small, so that it is unlikely that the significant changes taking place in the properties of the two proteins can be explained by the changes in the character of the solvent alone. It is more likely that the experimentally well-established uptake of phenol by proteins (Reichel, 1909; Cooper, 1912; Cooper & Woodhouse, 1923; Cooper & Treadgold, 1934) is responsible for the reversible changes in the physical properties of serum albumin and chymotrypsinogen A in this precipitation stage, and this explanation receives further support from the results of recent studies on the solubility of model compounds in phenol and in other organic solvents (Robinson & Jencks, 1965).

It has been found (Pusztai, 1966) that, when proteins and other polyelectrolytes were partitioned in two-phase phenol-aqueous buffer systems, heating the reaction mixtures to about 70° accelerated all the changes taking place and, at the same time, increased the efficiency of the extraction. It has now been demonstrated that, at least for serum albumin and chymotrypsinogen A, only small changes, if any, occurred in the physical properties examined as a result of heating solutions of these proteins in phenol-containing solvents. With bovine serum albumin, even in unbuffered solutions, heating did not bring about any irreversible aggregation through a thiol-disulphide exchange reaction, in contrast with that found in aqueous solutions of this protein near or above neutral pH values (Steinrauf & Dandliker, 1958b; Warner & Levy, 1958). Further, with chymotrypsinogen A, exposure to moderate temperatures (70–90°) was clearly shown to accelerate the attainment of the final viscosity values (Fig. 3b) and also facilitated the dissolving of the protein in the solvent mixtures containing phenol. These observations could have an important bearing on efforts of utilizing the potentials of the phenol-partitioning technique (Pusztai, 1966).

In these studies great emphasis was laid on the problem of reversibility of the changes taking place. The picture was somewhat complicated by the time-dependence of some of the changes observed. These were, however, largely overcome by the use of aged or heated stock solutions of the proteins.

The experiments were usually performed by either increasing or decreasing the proportion of phenol in the solvent mixtures, a very useful test for reversibility. The curves produced in this way were the same within experimental errors. Solutions exposed to high phenol concentrations for weeks were brought to lower phenol content and the physical properties were compared with those in solutions made up directly at the same phenol concentration. Again no significant differences were found. The returning of the two proteins to purely aqueous systems via the dialysis procedure against acetic acid solutions is clearly based on these studies and, at the same time, gives the most convincing evidence to support the reversibility. The physical constants of the recovered protein samples were sufficiently similar to those of the untreated proteins to exclude the possibility of any major changes in their molecular conformation as a result of the exposure to phenol (Tables 1 and 3). With serum albumin the near-quantitative recoveries of the monomer as calculated from the area under the sedimenting boundary excluded the possibility of the formation of significant amounts of aggregated material. The rather restricted pH range of stability of chymotrypsinogen in aqueous solutions (Eisenberg & Schwert, 1951), the easily induced irreversible changes in the conformation of this protein on the addition of organic solvents (Brandts & Lumry, 1963; Brandts, 1964) and the aggregation noticed on freeze-drying necessitated a detailed study of the exact conditions of the recovery. The conditions established in the present work yielded materials of negligible aggregate content. The lack of difference spectra and the reaction of immunochemical identity may indicate an even closer similarity, if not complete identity, between the native proteins and the samples exposed to phenol and other organic solvents.

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