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The Effect of Hydrotropic Agents on the Heat Coagulation of Bovine Serum Albumin

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Abstract—The effect of two hydrotropic solubilizers on the heat coagulation of bovine serum albumin (BSA) has been investigated. Photon correlation spectroscopy indicated possible unfolding of BSA molecules in solutions of sodium benzoate and sodium salicylate at 25°C. The effect of these hydrotropes on the heat coagulation of BSA was concentration-dependent. Relatively low concentrations stabilized the protein structure as indicated by the increase in the transition temperature (T_m) and induced gelation at temperatures and BSA concentrations lower than those required in the absence of hydrotropes. Higher concentrations of the hydrotropes considerably reduced T_m and inhibited gelation of BSA, the effect of sodium salicylate being more pronounced, as was the lower aggregation rate of BSA. The behaviour of these hydrotropes as protein denaturants differs from that of neutral electrolytes but is similar to that of concentrated solutions of urea.

There is much current interest in the use of particulate colloidal protein carriers, particularly albumin microspheres, as targetable drug delivery systems (Taplin et al 1964; Kramer 1974; Yoshioka et al 1981; Davis et al 1984). Preparation of albumin microspheres either by chemical cross-linking in vegetable oil emulsions (Lee et al 1981; Willmott et al 1985) or coagulation of albumin at elevated temperatures (Widder et al 1981; Gallo et al 1984) is subject to many variables. As the success of such delivery systems may be largely dependent on the characteristics of the carrier matrix conferred by the method of preparation and the preparation variables, much attention has been paid to the pharmaceutical aspects of microsphere preparation (Gallo et al 1984; Burger et al 1985; Gupta et al 1986). Nevertheless, information on the potential effect of the incorporated drugs or formulation additives on the properties of the carrier matrix is still lacking.

In the present contribution, we have been interested in the assessment of the effect of two hydrotropic solubilizers, sodium benzoate and sodium salicylate on the heat coagulation of bovine serum albumin (BSA) under conditions similar to those employed in the preparation of microspheres. This interest has been initiated by our hope to incorporate hydrophobic drug molecules in a solubilized form in albumin microspheres and by the reported inhibitory effect of high hydrotrope concentrations on thermal coagulation of proteins (Neuberg 1916).

Materials and Methods

Materials

Bovine serum albumin was a crystallized and lyophilized, essentially globulin free sample from Sigma Chemical Company, UK. Sodium benzoate, sodium salicylate and sodium chloride were used as received from BDH Chemicals, Poole, UK.

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Photon correlation spectroscopy

A photon correlation spectrometer (Malvern Instruments, Model 2707) with 60 channels was used in conjunction with a He/Cd laser (Liconix) operating at 441·6 nm with a power output of approximately 10 mW. Measurements were carried out at an angle of 90° to the incident beam. Data analysis to give the spherical equivalent hydrodynamic diameter and polydispersity was by the method of cumulants.

For the study of the denaturing effect of the hydrotropic salts, sodium benzoate and sodium salicylate on BSA (2% solution), samples of the solution under study were allowed to equilibrate at 25 ± 0·1°C, while for the effect of hydrotropes on the heat coagulation of BSA, the samples were equilibrated at the required temperature (30–90°C) for 1 h.

pH measurements were carried out using a PT_1-15 Digital pH meter.

Results and Discussion

The action of heat on globular proteins such as BSA can be formulated as a consecutive reaction with denaturation as the first step and aggregation or coagulation as a secondary phenomenon (Putnam 1953; Tanford 1968). In the denatured state, the macromolecular nature of the unfolded protein molecules becomes fully evident leading to changes in physicochemical properties, mainly the molecular size and the spatial arrangements of chemical groups within the molecules. In the present work, the effect of the two hydrotropic solubilizers under study, sodium benzoate and sodium salicylate on the heat denaturation and coagulation of BSA was assessed using photon correlation spectroscopy (PCS) for hydrodynamic diameter (d) measurements.

Before thermal denaturation experiments, the effect of both hydrotropic agents on the native structure of BSA at 25 ± 0·1°C was examined. Fig. 1a shows the change in d as a function of hydrotrope concentration. The diameter increased, reaching a maximum (with ≃ 36% increase) at around 0·16 M with no further increase at higher concentrations. Such effects can be explained by possible unfolding of

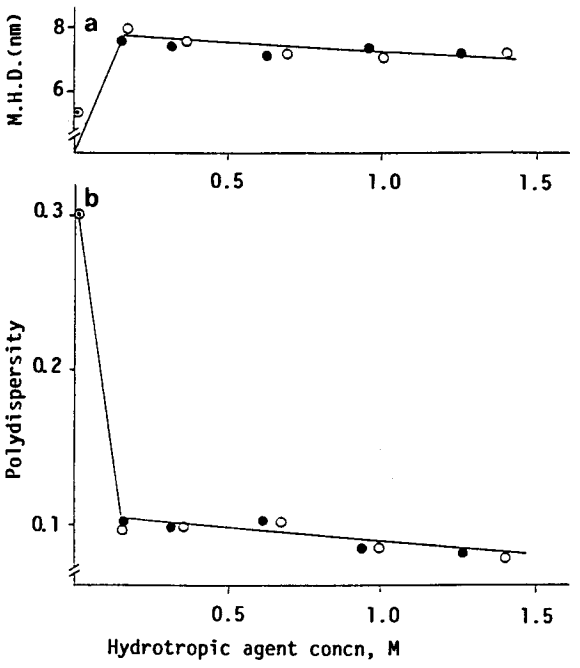

FIG. 1. Effect of sodium benzoate (○) and sodium salicylate (●) on the mean hydrodynamic diameter (a) and polydispersity (b) of BSA in 2% aqueous solution at 25±0·1°C. M.H.D. = mean hydrodynamic diameter.

Table 1. pH changes in aqueous solutions of BSA 2% w/v (pH = 6·93), in presence of sodium benzoate and sodium salicylate.

Concn of Hydrotrope g/100mL	Sodium benzoate	Sod. benz. + BSA	Sod. salicylate	Sod. salicylate + BSA
2	—	—	6·49	7·19
2	7·03	7·19	6·53	7·22
5	7·11	7·20	6·60	7·26
10	7·23	7·35	6·80	7·33
15	7·36	7·46	6·91	7·38
20	7·47	7·56	7·07	7·41

* pH of distilled water used was 6·44.

BSA molecules as a result of binding of the hydrotropes. This view is supported by the increase in pH of 2% BSA solutions upon the addition of sodium benzoate or salicylate (Table 1). Unfolding of the BSA molecules may bring to the periphery more basic groups originally buried in the folded native structure. In addition, both agents reduced the polydispersity of BSA in solution as shown in Fig. 1b. Hydrotropic agents appear to reduce the tendency of BSA to partial polymerization, a phenomenon occurring normally in commercial albumin preparations (Foster 1977; Harvey et al 1979).

The effect of sodium benzoate on the heat denaturation of BSA in aqueous solution (2%) is shown in Fig. 2. In the absence of sodium benzoate, BSA molecules aggregate at a transition temperature (T_m) of 65°C which compares favourably with that reported in the literature (Jaenicke 1967), the mean hydrodynamic diameter (d) reaching a maximum at

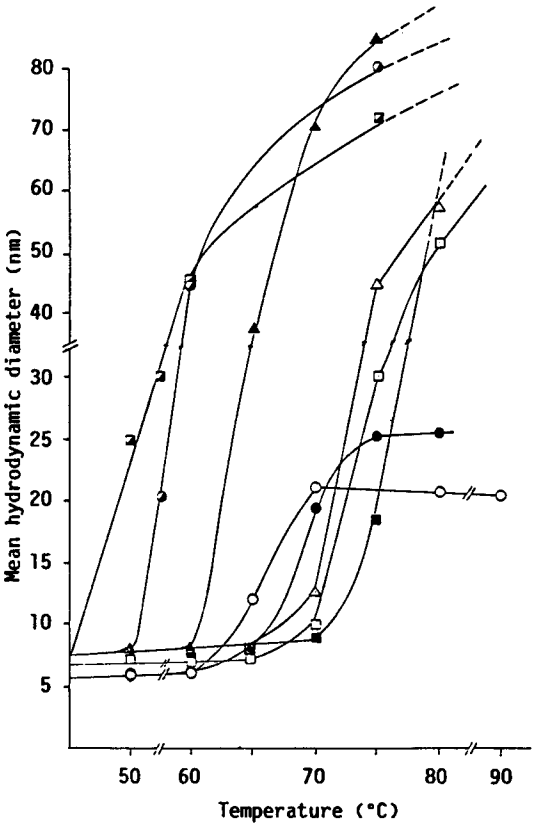

FIG. 2. Effect of sodium benzoate concentration (M) on the heat denaturation of BSA in 2% aqueous solution. (○) control, (●) 7×10^{-3} M (□) 35×10^{-3} M, (■) composite curve for 0·07, 0·17 and 0·35 M, (△) 0·70 M, (▲) 1·04 M, (◑) 1·40M and (◪) 1·74 M. N.B. The dotting denotes gelation of the system upon heating at 90°C.

70°C. Addition of sodium benzoate at relatively low concentrations ($7–70 \times 10^{-3}$ M) resulted in an increase in T_m to a maximum value of 75°C indicating stabilization of the protein to heat denaturation. However, a gradual change of the heat aggregation patterns compared to that of BSA in water was observed (Fig. 2). The heat denaturation curves obtained over a higher concentration range (0·07–0·35 M) were almost superimposed. A further increase in sodium benzoate concentration resulted in a considerable reduction in T_m probably associated with a strong destabilizing effect. A similar behaviour was observed with sodium salicylate systems (Fig. 3), nevertheless, the heat denaturation pattern at a relatively high salicylate concentration (0·94 M) was obviously different from those obtained at lower concentrations. The change in T_m as a function of concentration of both hydrotropes is shown in Fig. 4.

Further, it was found that heating the BSA-hydrotropic agent systems at temperatures higher than the respective T_m generally resulted in a marked increase in the mean hydrodynamic diameter (Figs 2, 3) with aggregation proceeding to the extent of gel formation at 90°C (dotted curves). The gel obtained was transparent and homogeneous. However, gelation of BSA was not observed at very low concentrations of either hydrotrope (Figs 2, 3) or relatively high concentrations (0·94 M) of sodium salicylate in particular (Fig. 3).

In principle, denaturation and aggregation of proteins result from the ability of the peptide chains to participate in

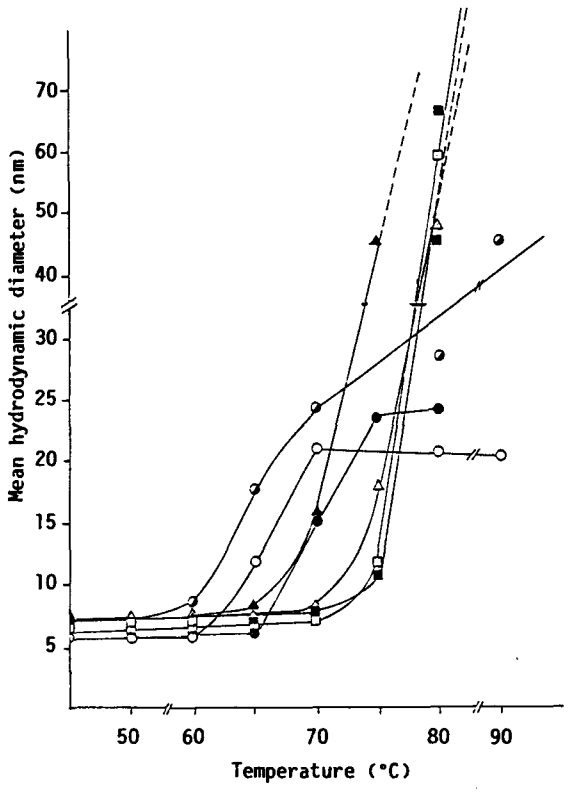

FIG. 3. Effect of sodium salicylate concentration (M) on the heat denaturation of BSA in 2% aqueous solution. (○) control, (●) 6×10^{-3} M, (□) 0·03 M, (■) composite curve for 0·06 and 0·13 M, (△) 0·31 M, (▲) 0·63 M and (◕) 0·94 M. N.B. The dotting denotes gelation of the system upon heating at 90°C.

inter- and intra-chain interactions according to the different kinds of functional groups in the protein molecule (Kauzmann 1959; Jaenicke 1967). It appears that unfolding of BSA molecules induced by the two hydrotropes brings more functional groups to the periphery, thus enhancing intermolecular interactions leading to aggregation. Lack of gel formation at comparatively low hydrotrope concentrations implies that a minimum degree of unfolding or disruption of the native BSA conformation is required for gel formation.

On the other hand, inhibition of gelling of BSA upon heating to 90°C (and even boiling) in a relatively concentrated sodium salicylate solution (0·94 M, Fig. 3) is in accordance with an early observation reported by Neuberg (1916) in his original work on hydrotropy. The phenomenon was not discernable in sodium benzoate systems over the concentration range tested (up to 1·75 M) due to its less pronounced hydrotropic behaviour. It has been recently postulated (Saleh et al 1983, 1986) that hydrotropic agents, at relatively high concentrations, increased the structuredness and hydrophobicity of water resulting in a medium with a marked solubilizing power (Badwan et al 1983; Huttenrauch & Fricke 1982). Such solvent characteristics appear to allow more complete denaturation of BSA as well as solubilization of the hydrophobic regions of the molecules. Consequently, the BSA coagulation inhibitory effect of fairly concentrated hydrotropic agent solutions seems to concern a system with strongly modified protein structure characterized by increased solubility and probably marked inhibition of hydrophobic interactions leading to coagulation.

The time dependence of the heat coagulation of BSA was further determined at 80°C at two concentration levels of sodium benzoate and sodium salicylate. The results (Fig. 5) indicated that while BSA underwent spontaneous aggregation at the temperature of the study, aggregation proceeded at a much lower rate at the higher hydrotrope concentration probably due to partial hydrotropic solubilization of the protein molecules.

For a better understanding of the hydrotropic behaviour, the heat denaturation pattern of BSA in solutions of a non-hydrotropic sodium salt, NaCl (Fig. 6a) and a potent protein denaturant, urea (Fig. 6b) at two concentration levels was compared with those obtained with sodium benzoate and sodium salicylate in Figs 2 and 3, respectively. Contrary to the behaviour of hydrotropes, both relatively low and high

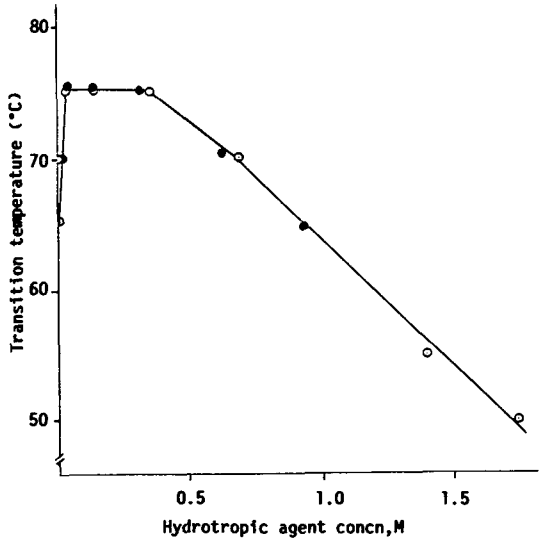

FIG. 4. Effect of concentration of sodium benzoate (○) and sodium salicylate (●) on the transition temperature of BSA.

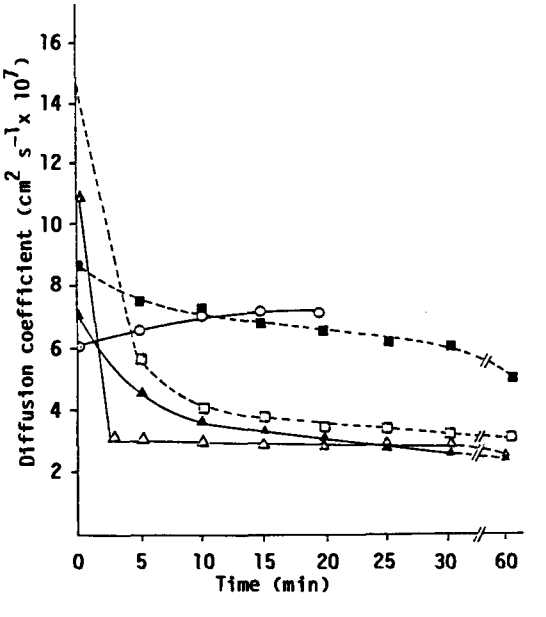

FIG. 5. Time dependence of the heat denaturation of BSA at 80°C in the absence of hydrotropes (○), in the presence of 0·13 M (△) and 1·04 M (▲) sodium benzoate or 0·14 M (□) and 0·94 M (■) sodium salicylate.

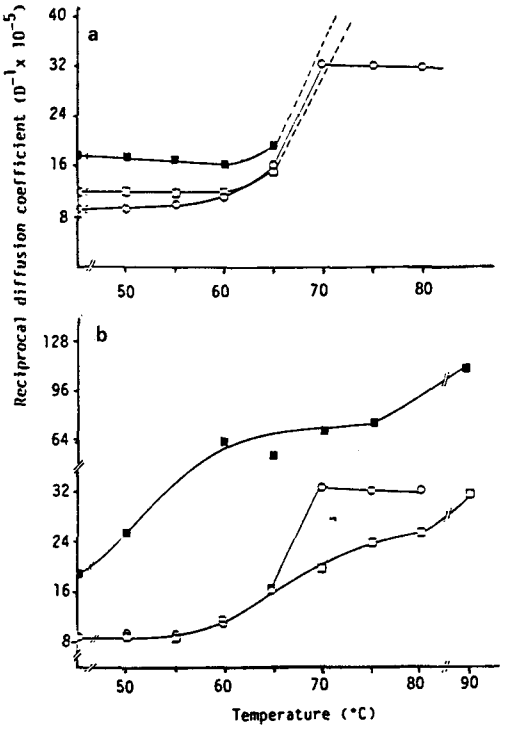

FIG. 6. Effect of NaCl (a), 0·34 M (□) and 3·40 M (■) and urea (b), 0·40 M (□) and 7·00 M (■) on the heat denaturation of BSA in 2% aqueous solution measured by the reciprocal diffusion coefficient. (○) control.

concentrations of NaCl (0·34 and 3·40 M) affected the transition temperature of BSA only slightly and a granular and opaque gel was formed at 70°C, a much lower temperature than that required for gelling in hydrotropic systems (90°C). On the other hand, urea was shown to be an efficient denaturant only at the higher concentration used, 7 M (Fig. 6b), an observation reported earlier using techniques other than PCS (Tanford 1968; Lapanje 1978). Urea exhibited a denaturation behaviour similar to that of hydrotropes at relatively high concentrations with respect to the lowering of T_m and the inhibition of gel formation upon aggregation. These results point to a similarity of the solvent characteristics of concentrated solutions of hydrotropes and urea.

In conclusion, hydrotropic agents affect considerably the heat coagulation of BSA. While relatively low concentrations of sodium benzoate and sodium salicylate induced aggregation and gelling at much lower temperatures and BSA concentrations than those required in their absence, higher concentrations can inhibit gelling of BSA as a result of the change of the solvent characteristics of the medium and hydrotropic solubilization. The nature of the effect of hydrotropic agents on heat coagulation of BSA compares more closely to that of urea than that of NaCl. From a practical standpoint, inclusion of hydrotropes in the preparation of albumin microspheres by heating would allow the use of much lower protein concentrations and relatively lower coagulation temperatures, in addition to the possibility of incorporation of insoluble drugs in solubilized form.

In view of the present results, the effects of foreign materials, either drugs or formulation additives, incorporated in proteinaceous drug carrier systems should be assessed individually. Such effects may be beneficial or detrimental to the desirable characteristics of the carrier matrix prepared.

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