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Effect of some drugs and additives on the cross-linking of bovine serum albumin by glutaraldehyde *

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Summary

The effect of sodium cromoglycate and sodium salicylate and a range of diverse pharmaceutical adjuvants: lactose, sodium dodecyl sulphate (SDS), sodium chloride, ammonium sulphate, including sodium benzoate and sodium salicylate as hydrotropic solubilisers, was studied on the cross-linking of bovine serum albumin (BSA) by glutaraldehyde. Photon correlation spectroscopic data indicated that the substances tested affected to different degrees the rate and extent of cross-linking, the effect being most pronounced in the presence of the inorganic salts. The results have been attributed to possible denaturation of BSA and the dehydrating properties of the inorganic salts, as both exposure of additional lysine moieties upon denaturation and dehydration are important factors in the mechanism of cross-linking. Polyacrylamide gel electrophoresis of BSA subjected to cross-linking in the presence of sodium benzoate and sodium salicylate at hydrotropic concentrations indicated a change in the nature of the cross-linked product. The possible effect of drugs and additives on the cross-linking of albumin is a factor that should be taken into consideration in the formulation of albumin microspheres as drug carriers.

Introduction

Albumin microspheres have received considerable attention as targetable drug delivery systems (Davis et al., 1984; Kim and Oh, 1988; Willmott et al., 1988). Stabilization of the albumin matrix in the manufacture of microspheres can be generally achieved by thermal denaturation (Kramer, 1974) or chemical cross-linking of emulsified albumin solution in water-in-oil emulsions using active esters or aldehydes, particularly glutaraldehyde (Widder et al., 1979; Sokoloski and Royer, 1984). Cross-linking of albumin by glutaraldehyde is a rather complex reaction and the chemical nature of the modification has not yet been clearly defined. The various mechanisms proposed, reviewed by Sokoloski and Royer (1984), indicate that the reaction involves lysine side chains and the extremely stable product formed is not a simple Schiff's base. The following scheme illustrates a mechanism suggested by Lubig et al. (1981) which appears to encompass the various speculations:

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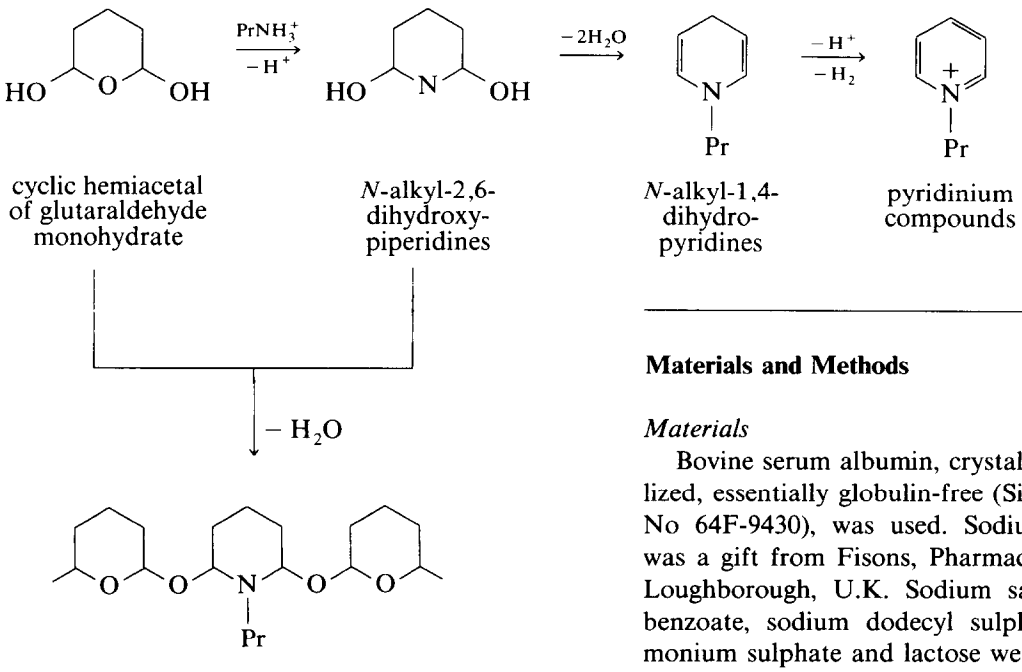

Condensation of the cyclic monohydrate of glutaraldehyde and *N*-alkyl-2,6-dihydroxypiperidines yields linear polymeric cross-links. Accordingly, the number of lysine moieties, intermolecular dehydration and pH appear to be implicated as important factors in the cross-linking reaction.

The chemical cross-linking process may have marked effects on the characteristics and performance of the microspheres obtained. The influence of various physicochemical factors such as the type and concentration of the cross-linking agent, cross-linking time and site of cross-linking have been considered in several investigations (Widder et al., 1979; Longo et al., 1982; Willmott et al., 1985; Burgess et al., 1987). However, information on the effect of incorporated drugs and formulation additives likely to be used in the manufacture of microspheres on the process of cross-linking is still lacking. In the present work, the effects of some drugs and pharmaceutical additives that do not react chemically with aldehydes on the cross-linking of bovine serum albumin (BSA) by glutaraldehyde were investigated.

Materials and Methods

Materials

Bovine serum albumin, crystallized and lyophilized, essentially globulin-free (Sigma, A-7638, lot No 64F-9430), was used. Sodium cromoglycate was a gift from Fisons, Pharmaceutical Division, Loughborough, U.K. Sodium salicylate, sodium benzoate, sodium dodecyl sulphate (SDS), ammonium sulphate and lactose were obtained from BDH, Poole, U.K. and glutaraldehyde was purchased from Sigma.

Photon correlation spectroscopy

A Malvern Instruments, model 2707 photon correlation spectrometer with 60 channels was used in conjunction with an He/Cd laser (Liconix) operating at 446.1 nm with a power output of approx. 10 mW. BSA solutions (2% w/v) cross-linked in the absence or presence of additives were measured at an angle of 90° to the incident beam after equilibration at 25 ± 0.1° C. All measurements were carried out at least in triplicate. The densities and viscosities of solutions of the drugs and additives under study were determined at 25 ± 0.1° C. The data were processed to give values for the equivalent hydrodynamic diameters and the average diffusion coefficients.

Polyacrylamide gel electrophoresis

Gel electrophoresis of 4% BSA solution subjected to cross-linking by 1% glutaraldehyde in the absence and presence of 10% sodium salicylate or sodium benzoate was carried out using 5.5% polyacrylamide gels in a rod gel apparatus similar to that described by Hames (1981). Cross-linking was allowed to proceed for 5 min and excess

glutaraldehyde was quenched with 1 ml of 10% glycine solution. A 10 ml portion of 1% SDS was added and the mixtures heated in a boiling water bath until the cross-linked albumin completely dissolved. Aliquots of these BSA solutions (50 μl) were added to 0.2 ml of 0.01 M phosphate buffer (pH 7.2) containing 1% SDS, 10% glycerol, 0.002% bromophenol blue as tracking dye and 0.01 M mercaptoethanol. The mixtures were boiled at 100 °C for 3 min. Samples (50 μl each) were loaded onto separate gel rods and the reservoirs were filled with 0.05 M phosphate buffer containing 0.1% SDS. Electrophoresis was conducted at 8 mA per gel. After completion of the process, the gels were stained with 0.25% Coomassie brilliant blue in trichloroacetic acid for 1 h before washing with 7% acetic acid. The R_f values of the various BSA bands were calculated using the mobility of the tracking dye.

Preparation of BSA microspheres

The method of Willmott et al. (1985) was used for the preparation of BSA microspheres. For the preparation of microspheres loaded with the hydrotropic salts, sodium benzoate and sodium salicylate, these salts were added at a concentration of 10% to the disperse phase containing BSA prior to emulsification.

Results and Discussion

In the present investigation, the effects of sodium cromoglycate (2%) and sodium salicylate (2%) and the additives: lactose (5%), SDS (2%), sodium chloride (0.9 and 4%) and ammonium sulphate (4 and 10%) as inorganic salts and sodium benzoate (2 and 10%) and sodium salicylate (10%) as hydrotropic solubilisers, on the cross-linking of BSA by 1% glutaraldehyde were examined using photon correlation spectroscopy (PCS). The values of the hydrodynamic diameter (d_g) of BSA molecules determined immediately following the addition of glutaraldehyde to 2% solutions of BSA in the absence and presence of these substances are listed in Table 1. With the exception of lactose, all substances used induced a higher degree of cross-linking as indicated by the increase in d_g, the effect being more pronounced at higher concentration of the additive, particularly in systems containing sodium chloride and ammonium sulphate.

TABLE 1

Effect of the drugs and additives used on the hydrodynamic diameter of BSA immediately following the addition of glutaraldehyde (d_g), gradients of the cross-linking time dependence and initial hydrodynamic diameter of BSA (d_i)

Substance	Concentration (% w/v)	d_g (nm)	Gradient * ($\times 10^{-3}$)	d_i (nm)
Control (water)	–	7.8	1.3	5.0
Sodium cromoglycate	2.0	14.4	13.0	8.0
Sodium salicylate	2.0	10.3	5.6	7.4
	10.0	10.7	6.1	7.6
Sodium benzoate	2.0	13.5	9.5	7.9
	10.0	16.4	10.4	7.9
SDS	2.0	13.1	0.0	8.8
Lactose	5.0	6.6	4.8	4.4
Sodium chloride	0.9	17.1	47.8	7.6
	4.0	21.9	–	7.8
Ammonium sulphate	4.0	10.7	36.0	8.1
	10.0	40.1	–	7.7

* Gradient of the second phase of the cross-linking time dependence (Figs. 1 and 2).

The time dependence of cross-linking of BSA at 25 °C in the presence of drugs and additives used was examined in comparison to that of BSA in water. Semi-logarithmic plots of the diffusion coefficients of BSA determined in 2% solutions vs. time are shown in Figs. 1 and 2. A biphasic relationship was observed in all systems, suggesting that cross-linking takes place at a relatively high rate in the first stage of the reaction (approx. 5 min) with about 40–50% reduction in the values of the diffusion coefficient. No further reduction as a function of time was observed in the control system or in the presence of 2% SDS (Fig. 1). This may explain an earlier observation (Burgess et al., 1987) that drug release from albumin microspheres was not affected by the time of exposure of microspheres to glutaraldehyde. In the other systems, however, cross-linking proceeded at different slower rates over the 25 min study period. For the purposes of illustrating the relative effects of the additives under study on the rate of cross-linking of BSA, gradients of the second phase of the cross-linking time dependence are shown in

Table 1. Maximum rates were obtained in systems containing the inorganic salts sodium chloride (0.9%) and ammonium sulphate (4%). A gel was formed at the higher concentrations of these salts. These findings clearly indicate that drugs or additives present in conjunction with albumin during chemical cross-linking may affect the extent and rate of reaction as well as the time required to reach completion.

According to the cross-linking mechanism proposed by Lubig et al. (1981), the enhancement of BSA cross-linking observed in most systems could be attributed to a possible effect of the drug or additive involved on the structural conformation of BSA, thus probably exposing more lysine moieties and/or its dehydrating properties. A pH effect could be excluded as the pH of solutions of all the substances used ranged from pH 6.9 to 7.5. PCS measurements carried out in the absence of glutaraldehyde showed that all of the drugs and

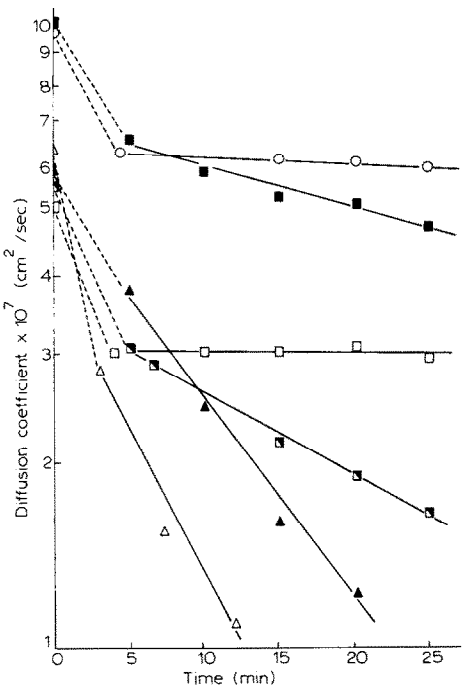

Fig. 1. Semilogarithmic plots of diffusion coefficient vs. time for BSA cross-linked by 1% glutaraldehyde, in water (○), 5% lactose (■), 2% SDS (□), 2% sodium cromoglycate (◪), 4% ammonium sulphate (▲) and 0.9% sodium chloride (△) at 25 °C.

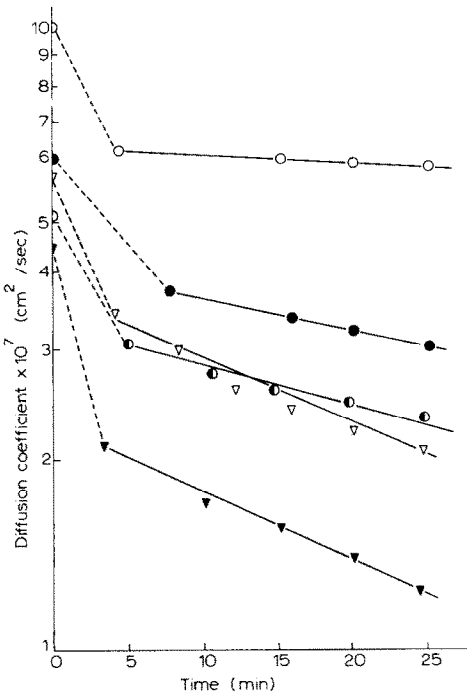

Fig. 2. Semilogarithmic plots of diffusion coefficient vs time for BSA cross-linked by 1% glutaraldehyde, in water (○), 2% sodium salicylate (●), 10% sodium salicylate (◐), 2% sodium benzoate (▽) and 10% sodium benzoate (▼) at 25 °C.

additives studied, except lactose, induced some degree of unfolding of native BSA as indicated by the increase in the initial hydrodynamic diameter (d_i) determined in 2% BSA solutions (Table 1). This may bring to the periphery more lysine residues originally buried in the folded BSA structure.

Denaturation of proteins by inorganic salts and detergents is well documented (Haschemeyer and Haschemeyer, 1973; Lapanje, 1978). Hydrotropic agents, namely sodium benzoate and sodium salicylate, have also been shown in a recent study (Saleh et al., 1989) probably to induce unfolding of BSA molecules, associated with an increase in the hydrodynamic diameter to a maximum at about 0.16 mol · l^{-1} of the hydrotrope and a concentration-dependent, slight increase in the pH of 2% BSA solutions. However, the reported slight increase in the diffusion coefficient of BSA monomers as a result of salicylate binding at much lower concentrations (Harvey et al., 1979) may

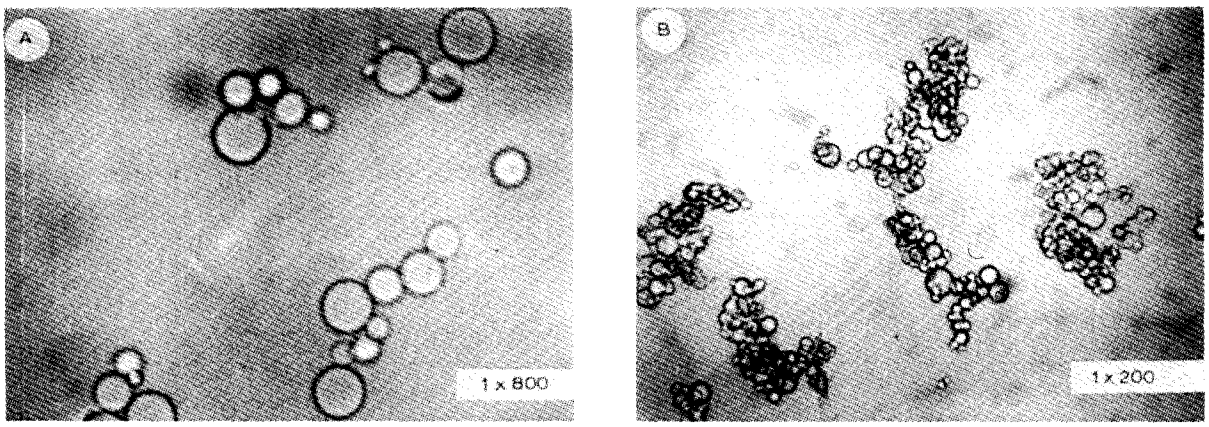

Fig. 3. Photomicrographs of BSA microspheres prepared in the absence of hydrotropes (A) and in the presence of 10% sodium benzoate (B).

point to the importance of the degree of self-association of BSA and the albumin : salicylate ratio in data interpretation. The much more pronounced effect of sodium chloride and ammonium sulphate on cross-linking can probably be attributed to their dehydrating properties, intermolecular dehydration being an essential step in the mechanism of cross-linking (Lubig et al., 1981).

Further, it was of interest to assess the effect of the two hydrotropes, sodium benzoate and sodium salicylate, as non-surfactant solubilisers, on the properties of albumin microspheres prepared by chemical cross-linking. The method described by Willmott et al. (1985) was used for microsphere preparation, with sodium benzoate or sodium salicylate (10% w/v) added to the aqueous phase of the water/oil emulsion. Preliminary investigations indicated instantaneous 'solidification' of BSA upon addition of glutaraldehyde (1.25%) and the microspheres obtained showed surface deformities and a tendency towards aggregation as observed in Fig. 3B. Attempts to reduce such defects by varying the concentrations of BSA, glutaraldehyde and a nonionic surfactant stabilizer (Span 60) resulted in marked improvements. Nevertheless, the relatively rapid 'solidification' of the protein pointed to a possible effect of the hydrotropes on the nature of the BSA cross-linking product. This was investigated using polyacrylamide gel electrophoresis at pH 7.2.

Cross-linking of BSA by glutaraldehyde (1%) in the absence of hydrotropes resulted in 4 electrophoretic bands (Fig. 4B), the characteristics of which are given in Table 2. Band 1 (also shown by untreated BSA, Fig. 4A) corresponds to BSA monomers whilst bands 2–4 correspond to BSA polymeric crosslinks. Electrophoretic analysis of BSA cross-linked in the presence of 10% sodium salicylate or sodium benzoate resulted in the appearance of an additional band close to the origin

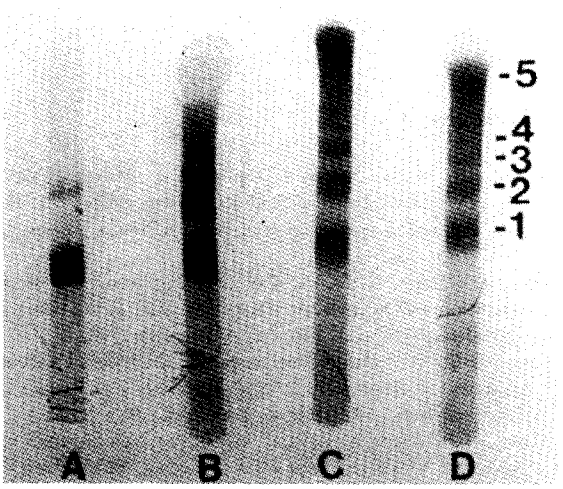

Fig. 4. Polyacrylamide gel electrophoresis patterns of BSA, not subjected to cross-linking (A); cross-linked, in water (B), 10% sodium salicylate (C) and 10% sodium benzoate solution (D).

TABLE 2

Characteristics of the gel electrophoresis bands of BSA cross-linked in water, 10% w/v sodium salicylate or 10% w/v sodium benzoate

Band no.	Water		10% sodium salicylate		10% sodium benzoate	
	R_f	Thickness (mm)	R_f	Thickness (mm)	R_f	Thickness (mm)
1	0.71	5	0.72	4	0.66	2
2	0.53	4	0.52	3	0.48	2
3	0.39	2	0.40	2	0.36	2
4	0.29	1	0.30	1	0.30	1
5	–	–	0.20	10	0.20	10

(Fig. 4C and D, respectively) with R_f values = 0.2 (Table 2), corresponding to higher BSA polymers. These results are consistent with the PCS data obtained in this study (Fig. 2) and may explain, at least in part, the irregularities exhibited by microspheres prepared in the presence of these hydrotropes.

It could thus be concluded that some drugs and additives may influence the rate, extent and time for completion of cross-linking of albumin by glutaraldehyde and possibly the nature of the cross-linking product. From a practical standpoint, this would affect to varying degrees the characteristics of albumin microspheres manufactured by chemical cross-linking, a factor that should be taken into consideration for a better engineering of the performance of microspheres as a drug delivery system.

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