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The effect of formaldehyde on the aggregation behaviour of bovine serum albumin during storage in the frozen and unfrozen states in the presence and absence of cryoprotectants and other low molecular weight hydrophilic compounds

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The effect of formaldehyde on the aggregation behaviour of bovine serum albumin (BSA) stored in the frozen and unfrozen states in the presence and absence of cryoprotectants and other low molecular weight compounds was studied by sodium dodecylsulphate electrophoresis. Densitometric determination of the SDS gels was used to quantify the aggregation process. It was shown that, at the concentrations of formaldehyde used in this study (0–100 ppm), aggregation of BSA took place only on frozen storage and that, at concentrations of 300 and 700 mg/g protein, protection against aggregation was obtained with glycine, alanine, tryptophan, sorbitol and lactic acid, while the carboxylic acids, ketogluterate and hydroxypyruvate accelerated the process. Tryptophan appeared to inhibit the aggregation process completely at 700 mg/g protein.

INTRODUCTION

When fish muscle is stored in the frozen state it suffers deteriorative changes which, depending upon time and temperature, lead to toughening and a loss of the succulence associated with fresh unfrozen tissue (Love, 1966; Matsumoto, 1979; Shenouda, 1980). It is now widely accepted that these changes are due to denaturation of the muscle proteins, and in particular the myofibrillar protein myosin (Sikorski *et al.*, 1976). The mechanism of the denaturation process, however, has not been established, although a number of theories have been proposed. These include partial dehydration of the protein due to the formation of ice crystals (Sikorski *et al.*, 1976; Shenouda, 1980), interaction of proteins with oxidised lipids and oxidation products of lipids (Sikorski *et al.*, 1976; Shenouda, 1980) and, in gadoid species, interaction with formaldehyde which, together with dimethylamine (DMA), is formed as a result of enzymic breakdown of trimethylamine oxide (TMAO) on frozen storage (Parkin & Hultin, 1982). It has been suggested that the formaldehyde produced causes crosslinking of the muscle proteins through lysine and cysteine, forming hydroxymethyl derivatives, and that these may crosslink via methylene crosslinks to arginine, asparagine or glutamine (Parkin & Hultin, 1982). However, no studies so far have demonstrated the formation of such links at the concentrations of formaldehyde produced during frozen storage of gadoid species (Ang & Hultin, 1989).

Numerous chemical additives have been listed for their ability to inhibit toughening of fish flesh on frozen storage (Park & Lanier, 1987; Krivchenia & Fennema, 1988). These include amino acids (Noguchi *et al.*, 1975), inorganic salts (Krivchenia & Fennema, 1988), sugars and polyols (Park & Lanier, 1987), hydrocolloids (Da Ponte *et al.*, 1985) and salts of carboxylic acids (Tran, 1975; Parkin & Hultin 1982). Few studies, however, have measured the effect of additives on the formation of formaldehyde from TMAO (Krueger & Fennema, 1989) and none has examined the effect of cryoprotectants on the reaction of formaldehyde with proteins.

This paper describes some studies on the effect of cryoprotectants on the interaction of formaldehyde with protein. Bovine serum albumin (BSA), a globular protein much simpler than myosin, was chosen for this study. Because of its smaller size (molecular weight 66 000), it can be examined by electrophoresis and some estimate can be made of the extent of aggrega-

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tion. It is hoped that further knowledge on how formaldehyde reacts with proteins in the presence of cryprotectants will help the understanding of the mechanism of protein denaturation during frozen storage of fish muscle.

MATERIALS AND METHODS

The following compounds were obtained from the Sigma Chemical Company: BSA as 98% monomer (Sigma A-1900), alanine, glycine, tryptophan, sodium lactate, sodium ketoglutarate, sodium citrate and lithium β-hydoxypyruvate. Sorbitol was obtained from BDH.

Influence of temperature on the reaction of formaldehyde with BSA

Table 1 shows the volumes of a BSA monomer solution of 4 mg ml^{-1} and a formaldehyde solution (F) of 1000 ppm (prepared by diluting commercial formalin of 37% w/w) which were mixed to give concentrations of 0–100 ppm formaldehyde.

These solutions were transferred to Eppendorf tubes and stored for 24 h at the following temperatures: 40, 26, 6, −15, −30 and −70°C.

The solutions, after thawing where appropriate, were then diluted for electrophoresis in a ratio of 1:4 with 80 mM Tris-HCl containing 12% glycerol and 0·01% Bromophenol Blue.

Influence of ionic strength on the reaction of formaldehyde with BSA

In order to study the effect of ionic strength on the reaction of formaldehyde, the BSA monomer was dissolved in the following potassium chloride/phosphate buffer solutions at pH 7·0: 0·18 μ (8·95 mM KH_2PO_4, 41·05 mM Na_2HPO_4, 32·00 mM KCl); 0·37 μ (17·90 mM KH_2PO_4, 82·10 mM Na_2HPO_4, 66·00 mM KCl); 0·70 μ (17·90 mM KH_2PO_4, 82·10 mM Na_2HPO_4, 0·40 M KCl); 1·0 μ (17·90 mM KH_2PO_4, 82·10 mM Na_2HPO_4, 0·70 M KCl).

The formaldehyde and BSA solutions were then combined as above, to give the same final concentrations as for the storage experiment in water. The samples were stored for 4 days at −15°C in Eppendorf tubes. They were then thawed and prepared immediately for electrophoresis.

Table 1. Volumes of solutions of BSA and formaldehyde combined

Formaldehyde concentration (ppm)	Volume of BSA solution (μl)	Volume of formaldehyde solution added (μl)
0	1 000	0
10	990	10
50	950	50
100	900	100

Table 2. Volumes of solutions of BSA, formaldehyde and additive combined

Solution	BSA	F1	F2	A1	A2	F1A1	F1A2	F2A1	F2A2
BSA	1 000	950	900	900	800	850	750	800	700
Formaldehyde		50	100			50	50	100	100
Additive				100	200	100	100	100	200

Influence of cryoprotectants and other compounds on the interaction of formaldehyde with BSA

Table 2 shows the volumes of a BSA solution of 4 mg ml^{-1} in phosphate buffer (8·95 mM KH_2PO_4, 41·05 mM Na_2HPO_4, pH 7·0), formaldehyde solution of 1000 ppm and additive solution of 10 mg ml^{-1} which were combined to give concentrations of 0–100 ppm formaldehyde with and without additives.

The additives were present at essentially two concentrations of 300 and 700 mg/g protein respectively. The additives evaluated were tryptophan, glycine, alanine, sodium pyruvate, lithium β-hydroxypyruvate, sodium ketoglutarate, sodium citrate, sodium lactate and sorbitol. After storage for one week at −15°C the extent of polymerisation of BSA was examined by electrophoresis and densitometry.

Electrophoresis of BSA samples

After the storage study had been completed the protein solutions were diluted as previously described and analysed by polyacrylamide gel electrophoresis (PAGE) on separating gels (140 × 120 × 1·5 mm^3) of 10% total acrylamide concentration (10% T, 2·6% C) with 5·3% stacking gels (5·3% T, 2·6% C) as described by Laemmli (1979) but without sodium dodecylsulphate (SDS). Electrophoresis was carried out in a Biorad Protean Cell (Biorad Laboratories, Watford, UK) at 40 mA per slab with cooling to approximately 10°C until the tracking dye reached the bottom of the gel.

Following electrophoresis, the gels were stained with Coomassie Blue 0·04% in 25% ethanol v/v. and 8% acetic acid for 30 min at 60°C. Excess stain was removed with several washes of destaining solvent (ethanol 25% v/v, acetic acid 8% v/v).

Densitometry

The density of staining of the Coomassie Blue-stained protein bands obtained after electrophoresis was measured by an LKB Ultrascan Laser Densitometer.

RESULTS AND DISCUSSION

Influence of temperature on the reaction between formaldehyde and BSA

The extent of aggregation of BSA after storage for 24 h at the various temperatures in the presence of different concentrations of formaldehyde was determined by

measuring the density of the aggregate zones on the electrophoretograms. Aggregates were not observed in the unfrozen samples, and were observed in the frozen samples only in those which were stored at the higher temperatures of −15 and −30°C (Fig. 1). In those samples, too, the extent of aggregate formation was dependent on the formaldehyde concentration. It could also be seen that more aggregation took place at −15°C than at −30°C.

It is of interest that BSA, a very stable globular protein, is denatured by concentrations of formaldehyde believed to be produced in gadoid species of fish under some conditions of frozen storage (Ang & Hultin, 1989). It is significant, too, that aggregates formed only in the frozen samples and only at the higher temperatures.

In the other studies on model systems of formaldehyde and BSA, the reaction was found to take place at 37°C but conditions were rather different: formaldehyde concentrations were higher (~100 mg/g of protein, compared with 25 mg/g protein in the present study) and incubation times were for 5 days (Tome *et al.*, 1985). In model studies using amino acids the conditions were even more drastic: the reaction was obtained by allowing the amino acids to react with an excess of formaldehyde in an ethanolic solution at 37°C for several days (Dewar *et al.*, 1975).

The dependence of denaturation, as measured by aggregate formation, on the temperature of incubation was reported by Singh and Wang (1977) for the reaction between myosin and malonaldehyde; the rate of reaction decreased as the temperature was reduced from 45°C to 0°C. However, when the solution was frozen, the reaction rate increased once again to the level found at 45°C and the maximum reaction rate for the system was found to be −24°C. It would appear that, under the conditions used in this study, some kind or prior change in the protein, such as that arising from the freezing of the associated water, is necessary before formaldehyde can act as an aggregating agent. A similar suggestion was made by Ang and Hultin (1989) who stated that formaldehyde accelerated the denaturation of cod myosin when the protein was 'stressed' by the freezing–thawing process as measured by loss of solubility of the protein was well as by the increase in surface hydrophobicity.

However, these results show that the degree of freezing is important. If freezing involves a change in the conformation of the protein, because of the removal of surrounding water, then lower temperatures permit less liquid water than higher temperatures. The effect of temperature on the degree of denaturation would therefore appear to be a compromise between (1) 'stressing' the protein by concentrating solute molecules in the protein micro-environment through the freezing process (Fennema, 1976); (2) retaining sufficient liquid water around the protein to permit attack of the protein target sites of formaldehyde; and (3) reducing the rate of reaction between formaldehyde and its targets.

Influence of ionic strength on reaction of formaldehyde with BSA

The electrophoretic patterns of samples which had been stored for 4 days at −15°C at different ionic strengths in concentrations of formaldehyde ranging from 0 to 100 $\mu g\ g^{-1}$ showed that the formation of aggregates was dependent on the concentration of formaldehyde only when the ionic strength was low (Fig. 2). At high ionic strength, it was found that, even in the absence of formaldehyde, aggregation took place.

To explain these effects it is necessary to consider the three-dimensional structure of a protein molecule being determined by hydrogen bonding, electrostatic interactions and hydrophobic forces. For a hydrophobic protein in its native state, the interior of the folded molecule is not saturated with water and so hydrogen bonds and non-polar forces predominate. On lowering the temperature, the entropy of water is reduced, thus promoting the solvation of non-polar amino acid residues. As a consequence the equilibrium between the folded and unfolded states moves towards the latter (Suelter, 1985) and, at low ionic strengths, dependence of denaturation on the concentration of formaldehyde can be envisaged. However, at high ionic strength the effect of salts surrounding the protein has to be

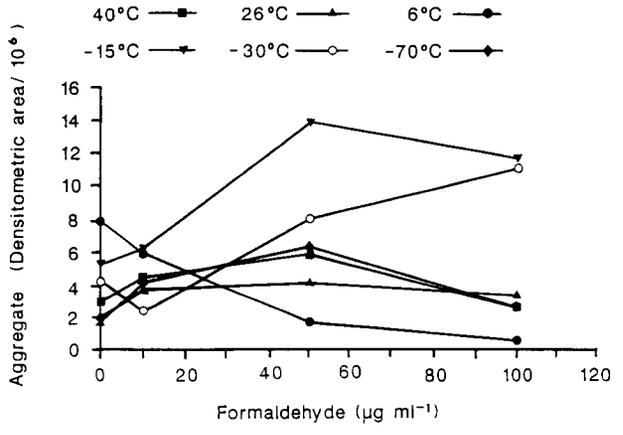


Fig. 1. Densitometric values for BSA aggregates formed on storage of the protein in the presence of formaldehyde over a range of temperatures.

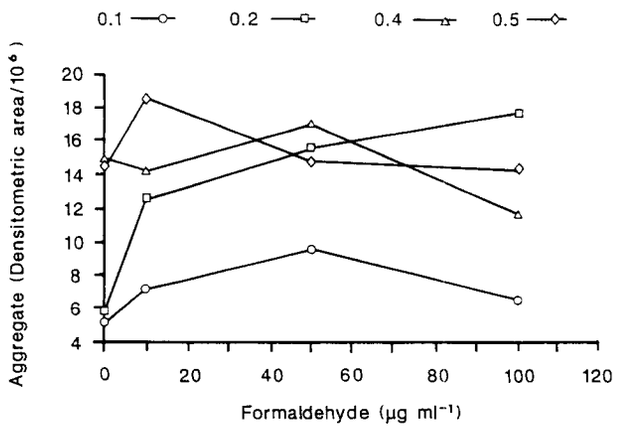


Fig. 2. Densitometric values for BSA aggregates formed on storage of the protein in the presence of formaldehyde at different ionic strengths.

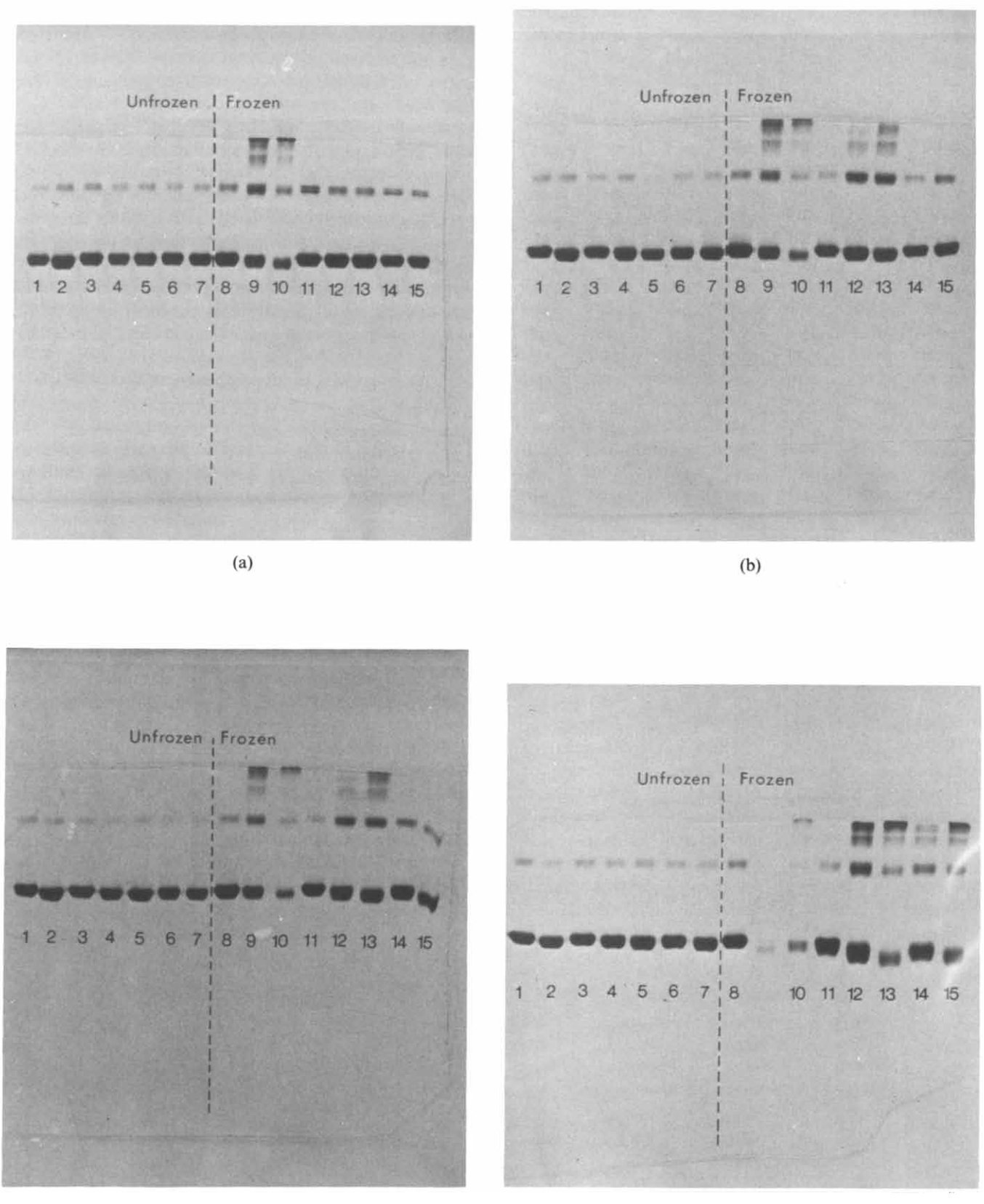
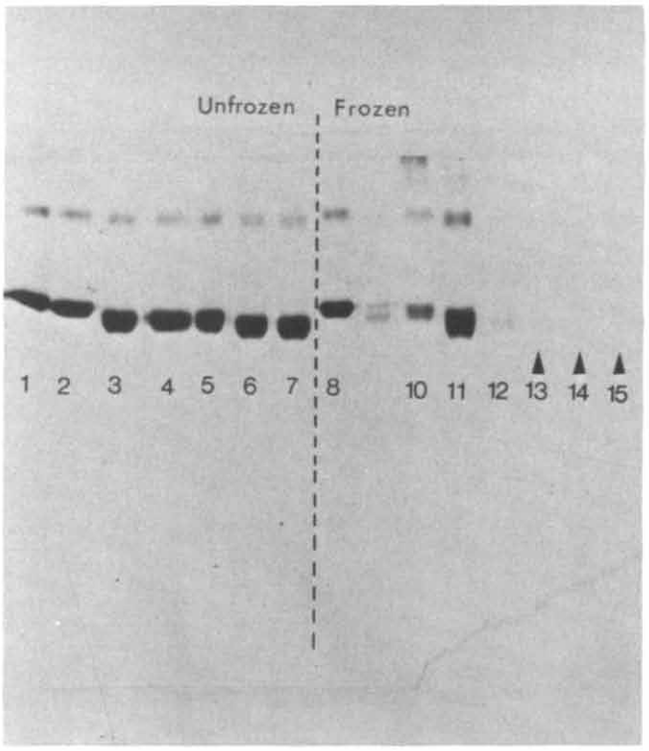
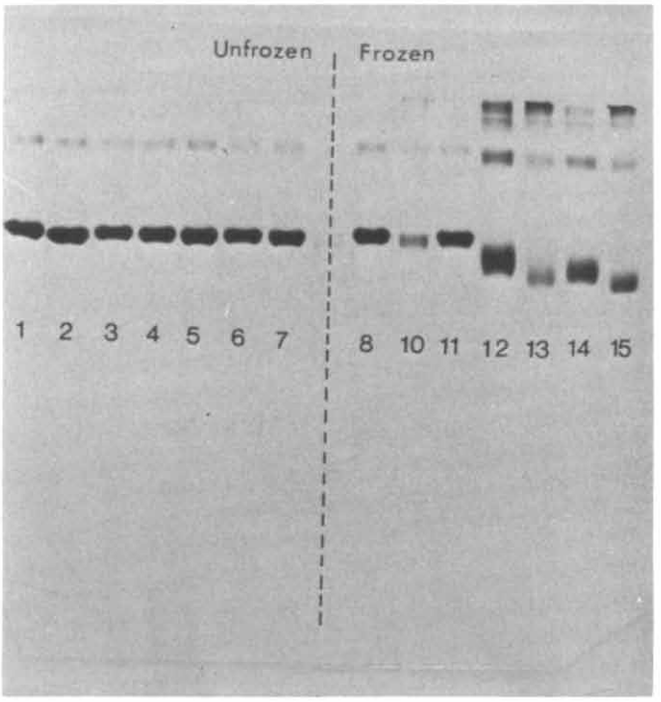


Fig. 3. Electrophoretograms of BSA after storage over a range of concentrations of formaldehyde (0–100 ppm) and in the presence and absence of low molecular weight additives. Unfrozen samples: (1) BSA, (2) F2, (3) A2, (4) F1Al, (5) F2Al, (6) F1A2, (7) F2A2. Frozen samples: (8) BSA, (9) F1, (10) F2, (11) A2, (12) F1A1, (13) F2Al, (14) F1A2, (15) F2A2. (a) Tryptophan, (b) glycine, (c) alanine, (d) sodium pyruvate, (e) lithium β-hydoxypyruvate, (f) sodium ketoglutarate, (g) sodium citrate, (h) sodium lactate, (i) sorbitol (for samples d–i, lane 9 has been omitted).



(e)



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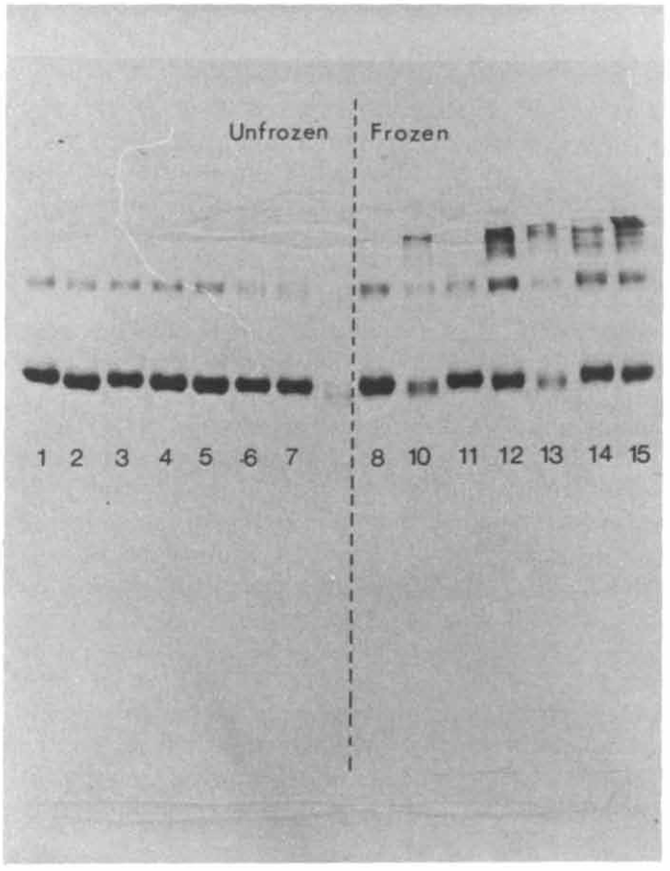
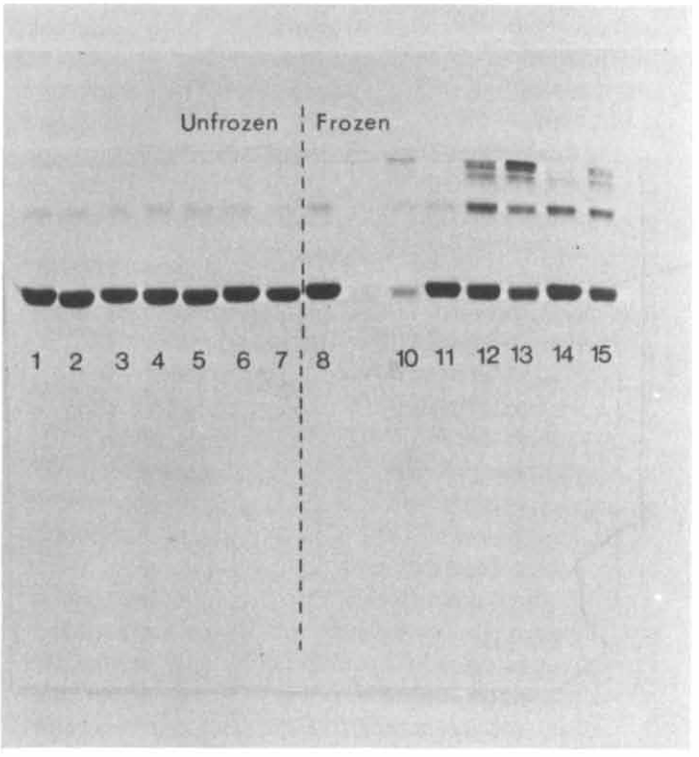
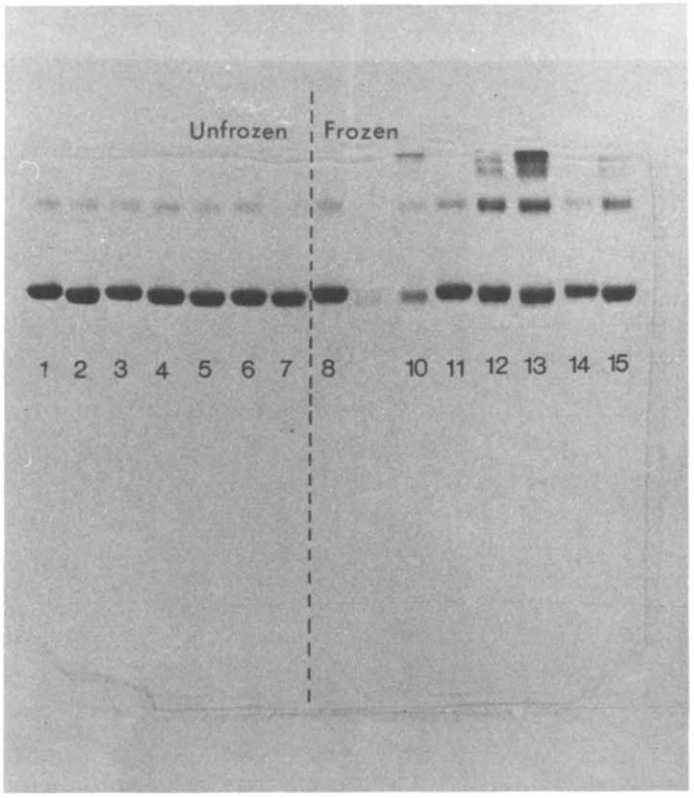


(g)



(h)

Fig. 3.—*Contd.*



(i)

Fig. 3.—*Contd.*

considered. Salts at a concentration greater than 0·2 M not only neutralise the electrostatic forces but also interact with protein structural elements affecting the final three-dimensional structure and stability of proteins, leading to a denatured state. This mechanism could explain the higher amount of aggregates found in the high ionic strength samples regardless of the presence of formaldehyde. The influence of salts on protein denaturation has been reported previously (Luijpen, 1957; Linko & Nikilä, 1961).

Influence of cryoprotectants and other compounds on the interaction of formaldehyde with BSA

Again it was observed that the detrimental effect of formaldehyde only took place on frozen storage of the BSA solutions (Fig. 3(a)–(i)). In the absence of cryoprotectants, aggregate zones were invariably observed in all the frozen samples but with the various compounds present some degree of protection was offered.

Of the three amino acids tests (Fig. 3(a)–(c)), tryptophan appeared to completely inhibit the formation of aggregates. Glycine and alanine, although less effective, prevented aggregation when present at the higher concentration and appeared to exert comparable protection. As tryptophan is a diamine and is known to act preferentially with formaldehyde (Paverrgou & Clifford, 1992) it may be that its greater effectiveness against aggregation of BSA can be attributed to the removal of formaldehyde from the system.

When the salts of the carboxylic acids were present, very variable effects were observed. Pyruvic acid only slightly inhibited aggregate formation (Fig. 3(d)). As a keto acid, pyruvic acid might be expected to compete with formaldehyde and block target groups on the protein, and with a carboxyl group it might be expected to increase the stability of BSA by decreasing the hydrophobicity.

Following this reasoning, a compound with more hydroxyl groups and with the ability to bond to targets on proteins could further improve the stability of proteins. Such a compound is hydroxypyruvate. However, the results showed that hydroxypyruvate also modified frozen BSA, resulting in greatly increased aggregate formation. The combination of formaldehyde and hydroxypyruvate reduced the solubility of BSA to such an extent that almost no protein could be seen on the electrophoresis gels (Fig. 3(e)).

α-Ketoglutate (Fig. 3(f)) also seemed to modify the protein, but less severely. The mobility of the BSA monomer changed after freezing in the presence of this additive. Citrate (Fig. 3(g)) only slightly improved the solubility of BSA while lactate (Fig. 3(h)), amongst the carboxylic acids, was the most effective in preventing aggregate formation.

Finally sorbitol (Fig. 3(i)) showed a marked ability to inhibit the effect of formaldehyde on BSA during frozen storage. It was not so effective as tryptophan, but slightly better than lactate and pyruvate.

These results show that different kinds of low molec-

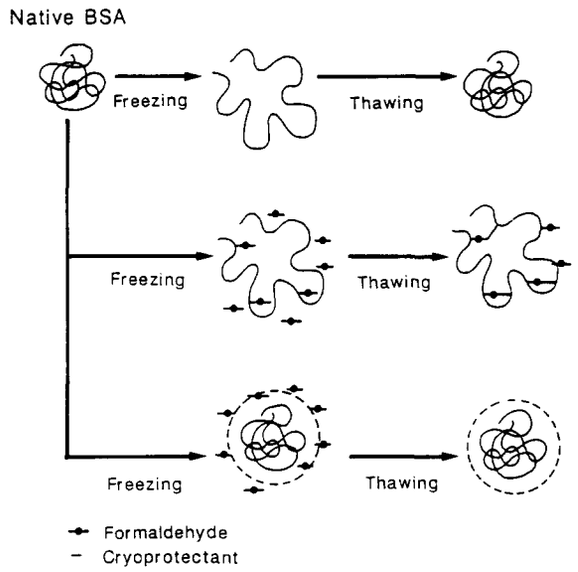


Fig. 4. Possible mechanism of the protective effect of cryoprotectants over BSA in the presence of formaldehyde during freezing and frozen storage.

ular weight compounds can inhibit the aggregation effect of formaldehyde on BSA. Sorbitol is a well-known cryoprotetant (Gekko & Morikawa, 1981; Hanafusa, 1985; Park & Lanier, 1987; Park *et al.*, 1987) while alanine has been found in relatively high concentrations in the antifreeze peptides of Antarctic fish (De Vries, 1982).

The proposed mechanism of protection of proteins by solutes during freezing and frozen storage is through a preferential hydration of proteins, so that solutes are excluded from the surface of the proteins (Arakawa & Timasheff, 1982; Carpenter & Crowe, 1988). The native structures of monomers and the polymerised form of oligomeric proteins are stabilised because denaturation or dissociation, respectively, could lead to a greater contact surface between the protein and the solvent and, therefore, could augment this thermodynamically unfavourable effect. Compounds that can bind to proteins alter the net surface charge and hydrophobicity of the protein and so can foster the unfolding of the polypeptide chains (Carpenter & Crow, 1988).

The present findings show that agents like formaldehyde need the frozen protein to bind to. It is known that formaldehyde binds to amino acids such as tryptophan and histidine and amines such as asparagine and glutamine (Tome *et al.*, 1985). As some of these amino acids are in the inner parts of proteins when they are in the folded state, it is likely that, when freezing causes the proteins to partially unfold, these 'hidden' amino acids become exposed to formaldehyde. Formaldehyde-mediated bridges can then be formed, preventing the refolding of the protein when the solution is thawed. In the absence of formaldehyde, BSA can refold, recovering its initial native state (Fig. 4).

In the studies on the effect of temperature on the aggregation process associated with formaldehyde, the electrophoretic pattern showed that aggregation takes place only in the frozen state. On the other hand, when the protein is frozen in the presence of a substance with cryoprotective effect, then partial unfolding does not take place and the protein can resist the effect of frozen storage even in the presence of a denaturing agent. It is possible that, as stated by Carpenter and Crowe (1988), such compounds are excluded preferentially from the surface of the protein, leaving a 'layer' of water surrounding the protein. This probably enables the protein to maintain the folded state, thus preventing the exposure of target sites to formaldehyde.

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