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The Adverse Effect of Glycation of Human Serum Albumin on its Preservative Activity in the Freeze-Drying and Accelerated Degradation of Alkaline Phosphatase

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Abstract—The effects of a glycated protein additive on the stability of freeze-dried biological standards were studied using alkaline phosphatase as a model. Alkaline phosphatase was formulated with artificially glycated albumin, freeze-dried, sealed into glass ampoules and subjected to accelerated degradation studies at temperatures from −20 to 56°C. Alkaline phosphatase, freeze-dried without an additive in neutral buffer, lost over 95% of its activity, but when freeze-dried with human serum albumin it retained approximately 70% of the initial activity. Both deliberately glycated and native albumin protected approximately 70% of the initial activity on freeze-drying and this protection was maintained during storage of the freeze-dried product at temperatures of 20°C or below for up to 16 weeks. At 37°C or above, alkaline phosphatase activity was lost in a time- and temperature-related manner with changes appearing in the SDS-PAGE gels and FPLC chromatograms but, with the artificially glycated albumin formulations, this loss of activity and the changes in the gels and chromatograms happened earlier and at lower temperatures. Formulations with trehalose at 1% w/v and 15% w/v, but without albumin, preserved some 40% of alkaline phosphatase activity following freeze-drying. Further, approximately half of that activity was maintained after 16 weeks' storage at all temperatures up to 56°C by the 15% trehalose without albumin. This study indicates that, in the formulation of freeze-dried biological standards, if albumin is to be used, the initial degree of glycation should be kept to a minimum and combinations of albumin and reducing sugar should be avoided. Trehalose 15% provides an acceptable alternative where exposure to high temperature is likely to occur.

Additives are often required in order to freeze-dry biological material successfully (Tarelli & Wood 1981; Tarelli & White 1982; Calam & Tarelli 1988). This is for a number of different reasons. If the biological material is pure and present in each ampoule in minute quantities additives can act as bulking agents. They can have a protective function preventing the adverse effects of adsorption onto glass or they can serve to maintain the pH or to preserve the activity of the biological materials (Tarelli & Wood 1981; Tarelli & White 1982). Biological materials for use as international standards have been freeze-dried successfully using standardized procedures (Campbell 1974) and, where the materials were not of an already complex nature, such as serum or plasma, this success has been partially due to the use of tried and tested formulations.

Carbohydrates, such as lactose, mannitol and trehalose, or proteins, such as bovine or human serum albumin (HSA), have been used effectively as bulking agents, for protection of the material and the preservation of activity in a number of biological standards (Tarelli & Wood 1981; Tarelli & White 1982). The combination of a carbohydrate and a protein has been employed successfully in preparations of many biological standards; recent preparations of standards for the cytokines which were derived by recombinant DNA technology are examples (Dawson 1992).

Following an earlier study (Ford & Dawson 1993) on the effectiveness of different carbohydrates in protecting the activity and structural integrity of alkaline phosphatase, interest has been aroused in the potential problems that could arise from glycation of proteins when using a combination of protein and carbohydrate as protectants in conjunction with the active biological material, or in the use of already glycated protein as an additive. Glycation, the non-enzymatic covalent binding of sugar to protein with the formation of reactive intermediates, is the first step in the Maillard reaction which leads to the visual effect of browning of proteins (Furth & Harding 1989; Dyer et al 1991) and results in the formation of complexes. A series of reactions occurs whereby the sugar covalently binds via a carbonyl group to an amino group, typically lysine, on the protein to form a Schiff base, which undergoes an Amadori rearrangement resulting in products bearing further reactive carbonyl groups. These react further with other amino groups to form a number of largely uncharacterized products including insoluble, cross-linked protein complexes, decomposition products such as 5-hydroxymethylfurfural and pigments (Calam & Tarelli 1988; Furth & Harding 1989; Njoroge & Monnier 1989; Suarez et al 1991).

Alkaline phosphatase was selected as a model since it loses activity when freeze-dried in the absence of a protective additive, whilst retaining activity when formulated with either lactose or trehalose (Ford & Dawson 1993). The aims of this study were to assess whether albumin formulations afforded protection to alkaline phosphatase in freeze-drying and subsequent storage; to examine the effect of glycation of the albumin on this protection, and to compare the protection with that afforded by trehalose alone.

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Materials and Methods

Materials

The following were obtained from Sigma Chemical Co. Ltd, Poole, Dorset: alkaline phosphatase, EC 3.1.3.1., from bovine intestinal mucosa, affinity-purified, 10,000 DEA units per vial (product no P-8647), D-(+)-glucose (G-8270), HEPES (H0891), EDTA (ED-255) and the glucose assay kit (115a). KH_2PO_4, K_2HPO_4, NaH_2PO_4 and NaCl (Analar quality) were obtained from British Drug Houses Ltd, Poole, Dorset; sorbitol from FSA Laboratory Supplies, Loughborough, and D-(+)-trehalose dihydrate (Biochemika) from Fluka Chemie AG, Buchs, Switzerland. Matrex PBA screening columns were obtained from Amicon, Danvers, MA, USA. For the thiobarbituric acid assay, 5-hydroxymethylfurfural, oxalic acid and thiobarbituric acid were obtained from Aldrich Chemical Co. Ltd, Dorset, and trichloroacetic acid from FSA. The HSA was kindly provided as a 20% w/v solution by the Bio-Products Laboratory, Elstree, Hertfordshire.

Glycation of HSA

A 1 M solution of glucose in 20% w/v HSA was prepared, incubated for two weeks at 37°C, dialysed against three changes of 5 L phosphate-buffered saline (PBS) (0·14 M, pH 7·4)+0·02% w/v sodium azide at 4°C over 3 days, then finally against 5 L PBS without azide at +4°C for a further day. HSA (20%), containing no glucose, as a control was prepared similarly. The absence of free glucose after dialysis was confirmed using Sigma Diagnostics glucose assay with a sensitivity of 0·1 mg mL^{-1}. The degree of glycation of the control HSA and the deliberately glycated HSA (GHSA) was determined by the binding to Matrex PBA-10 and PBA-30 screening columns following the manufacturer's protocol and confirmation of glucosylation of the albumin was obtained by the thiobarbituric acid reaction following acid hydrolysis (McFarland et al 1979; Dolhofer & Wieland 1980).

Preparation of the alkaline phosphatase solutions

The following formulations were prepared in 0·1 M potassium phosphate buffer, pH 7·4: alkaline phosphatase (25 units mL^{-1}), alkaline phosphatase+0·1% HSA, alkaline phosphatase+0·1% GHSA, alkaline phosphatase+1% trehalose, alkaline phosphatase+15% trehalose. Control preparations, lacking alkaline phosphatase, were also prepared. The solutions were dispensed into neutral glass ampoules. Some ampoules from each category were frozen and maintained at −70°C as frozen controls; the remainder were placed within a freeze-dryer precooled to −40°C, and the contents frozen at the rate of approximately −1°C min^{-1}. Freeze-drying was performed as previously described (Ford & Dawson 1993) with all the formulations being processed by the same freeze-drying conditions in one session. The ampoules were filled with nitrogen gas, sealed by glass fusion and individually tested for the integrity of the seal.

Accelerated degradation tests

Ampoules of each alkaline phosphatase formulation and controls were placed at the following temperatures for accelerated degradation studies: −20, 4, 20, 37 and 56°C. Two ampoules of the active formulations and two of the controls were removed from each temperature at intervals over a period of 16 weeks. Following assay of alkaline phosphatase activity, the stability of the various formulations was calculated as predicted rate of degradation based on the potency relative to that of the same preparation stored at −20°C (Jerne & Perry 1956; Kirkwood 1977).

Assay of alkaline phosphatase activity

The assay has been described previously (Ford & Dawson 1993). The contents of the ampoules (two ampoules of each formulation from each storage temperature) were reconstituted with 1 mL distilled water containing 0·02% sodium azide. These were left overnight at ambient temperature to allow for dissolution of the solids and any difficulties with reconstitution were noted. All samples removed from storage on a given day were assayed in a single assay. The enzyme activity for each of the samples was determined by parallel line assay (Finney 1978) and expressed as a relative potency in terms of the most active preparation included in the assay (usually alkaline phosphatase+HSA). The reproducibility of the assay, as expressed by the geometric mean coefficient of variance, was 2·75%.

SDS-PAGE

The samples were separated by SDS-PAGE by the method of Laemmli (1970) using a 5–15% w/v gradient acrylamide gel with a discontinuous buffer system (Ornstein 1964; Davis 1964) and sample loading of 25 μg in 25 μL. The gels were stained with Coomassie brilliant blue or were silver-stained (Wray et al 1981).

FPLC

Size exclusion FPLC using a Superose 12 (Pharmacia) column, 200 μL samples, PBS+0·02% w/v sodium azide as running buffer and a recording sensitivity of 0·1 full scale deflection, has been described previously (Ford & Dawson 1993).

Results

Glycation of HSA

The degree of the glycation of the HSA, following incubation with glucose, as determined by binding to Matrex PBA-30 columns, was found to be between 93 and 100% whereas that of the control HSA, treated in the same manner without glucose, was between 13 and 14%. Dialysis of the glycated HSA (GHSA) resulted in the removal of all free glucose to below the level of detection (0·1 mg mL^{-1}) and no free glucose was found in the control HSA solution. The thiobarbituric acid reaction demonstrated that the deliberately glycated HSA at 10 mg mL^{-1} released the equivalent of 0·11 mM of 5-hydroxymethylfurfural following acid hydrolysis compared with control HSA which released only 0·01 mM.

GHSA had a higher relative molecular mass (M_r) than HSA by SDS-PAGE and absorbed both Coomassie brilliant blue and silver stains less readily (not shown).

Appearance of the freeze-dried material

The formulations containing 0·1% w/v HSA, 0·1% w/v GHSA or 15% w/v trehalose, with or without alkaline

Table 1. Activity of freeze-dried alkaline phosphatase preparations immediately after freeze-drying and sealing the ampoules and of their frozen controls expressed as potencies relative to the frozen controls of alkaline phosphatase + HSA.

	Relative potency (95% confidence limits)	
Preparation	Frozen controls	Freeze-dried
Alkaline phosphatase + HSA	1·0 (0·89–1·12)	0·69 (0·62–0·77)
Alkaline phosphatase + GHSA	1·0 (0·90–1·12)	0·69 (0·62–0·77)
Alkaline phosphatase + 15% trehalose	0·38 (0·34–0·43)	0·28 (0·25–0·31)
Alkaline phosphatase + 1% trehalose	0·13 (0·12–0·15)	0·24 (0·21–0·26)
Alkaline phosphatase	0·19 (0·17–0·22)	0·033 (0·029–0·038)

Abbreviations as given in the text.

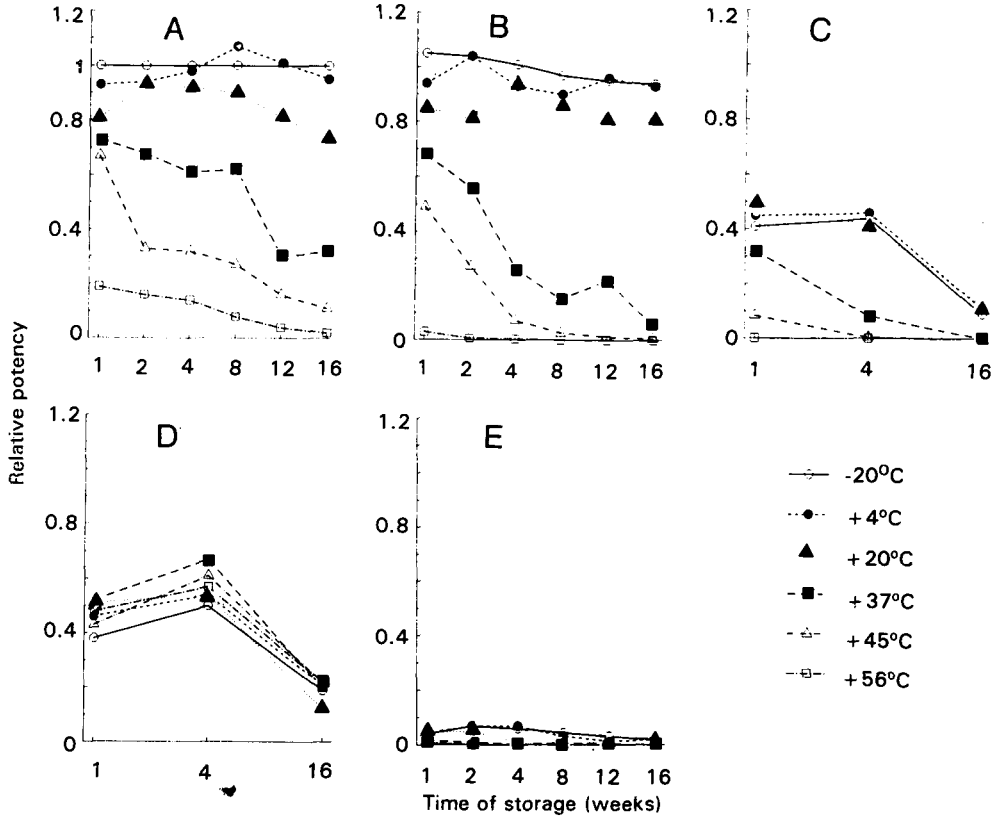

FIG. 1. Activity of pure freeze-dried alkaline phosphatase (P-8647) formulations during 16 weeks of accelerated degradation tests. A. Human serum albumin + alkaline phosphatase. B. Glycated HSA + alkaline phosphatase. C. Alkaline phosphatase + 1% trehalose. D. Alkaline phosphatase + 15% trehalose. E. Alkaline phosphatase alone. The potency, based on the mean of two ampoules, is expressed relative to that of the most stable preparation (alkaline phosphatase + HSA) stored at −20°C.

phosphatase, produced solid freeze-dried plugs, whereas the remainder, lacking these excipients at these concentrations, produced a granular or powdery deposit in the ampoule.

Accelerated degradation studies

Appearance and reconstitution. Apart from the alkaline phosphatase + 1% trehalose formulation, which became brown after storage for eight weeks at 56°C, 12 weeks at 45°C or 16 weeks at 37°C, there were no apparent changes in the appearance of the remainder of the freeze-dried material on storage for up to 16 weeks at any temperature.

Difficulty was encountered in the reconstitution of the GHSA formulations stored at temperatures of 37°C and above after four weeks. In these, though not apparent before addition of the water, an insoluble skin had formed on the surface of the freeze-dried plug, which had not dissolved 24 h later when stored at ambient room temperature or even after storage at 4°C for the remainder of the 16 week course of the experiment. It proved to be insoluble in cold 8 M urea or after boiling for 5 min in the SDS-PAGE reducing sample buffer. Only 15 s was required for the apparent complete dissolution of the other freeze-dried formulations.

Alkaline phosphatase activity. Table 1 compares the alkaline phosphatase enzyme activities of the freeze-dried formulations tested immediately after freeze-drying with those of

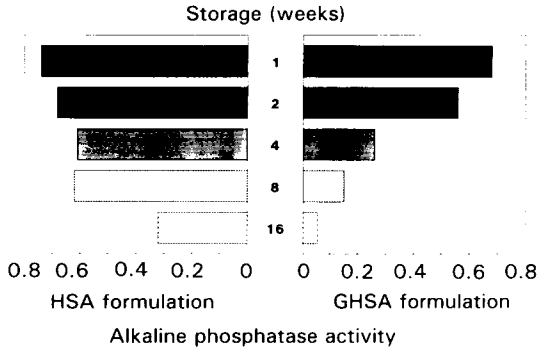

FIG. 2. Paired bar histogram to compare the activity of pure alkaline phosphatase (P-8647) freeze-dried in formulation with HSA or GHSA and held at 37°C for up to 16 weeks. The potency, based on the mean of two ampoules, is expressed relative to that of the most stable preparation (alkaline phosphatase + HSA) stored at −20°C. The difference in the activity of the formulations was not significant by analysis of variance after 1 week ($P > 0.05$) but was significant after 2 weeks ($P < 0.05$) and after 4 weeks onwards ($P < 0.01$).

their frozen controls. Generally, the enzyme activity of the freeze-dried preparations was approximately 70% that of their frozen controls. Alkaline phosphatase freeze-dried with trehalose had only 40% of the activity of that freeze-dried with either HSA or GHSA, whereas that freeze-dried in buffer without an additive had less than 5% of that activity.

The change in activity with time and temperature of storage is shown for the five alkaline phosphatase preparations in Fig. 1. There was a marked temperature-dependent loss in activity with time in the two albumin formulations and in the 1% trehalose formulation, but the loss of activity of the 15% trehalose formulation appeared to be similar at all the storage temperatures. The loss of activity seen in the two albumin formulations occurred earlier and at lower temperatures with GHSA than with HSA. When the materials were stored at 37°C (Fig. 2) or above, statistically significant ($P < 0.05$) differences were seen after two weeks between the activity of the HSA and GHSA formulations. This difference is highly significant ($P < 0.01$) after four weeks storage at 37°C.

After storage for 16 weeks, albumin preserved activity in the formulations stored at the lower temperatures (at and below 20°C), but was less effective at 37°C and above, with GHSA preserving activity to a lesser extent than HSA, whilst the 15% trehalose preserved activity, albeit at a lower level, throughout the temperature range of the experiment. The predicted rates of degradation of freeze-dried alkaline phosphatase in the various formulations are given in Table 2.

SDS-PAGE

SDS-PAGE of the alkaline phosphatase formulated with albumin and stored for two weeks at various temperatures showed marked differences in the position and appearance of the stained bands depending on whether the formulations contained native or glycated HSA (Fig. 3). With lower temperature storage (−20, 4 or 20°C) a single stained band was seen having a higher M_r for the GHSA than the HSA formulations. With storage at 37°C the main band in both the formulations appeared unchanged but additional stained bands of higher M_r from 100 to 205 kDa were present in the GHSA formulation. With 45°C storage, both HSA and GHSA formulations showed bands of higher mol. wt, with the GHSA formulation showing these more markedly. After 56°C storage, the HSA formulation showed bands of higher mol. wt whereas the GHSA formulation appeared not to enter the gel; no Coomassie brilliant blue-stained bands were seen.

FPLC

The difference in the behaviour of the two albumin formulations after storage at 37°C was clearly indicated by the difference in appearance of their FPLC gel filtration chromatograms (Fig. 4). Traces produced over the first 12 weeks of the study by the alkaline phosphatase + HSA formulation

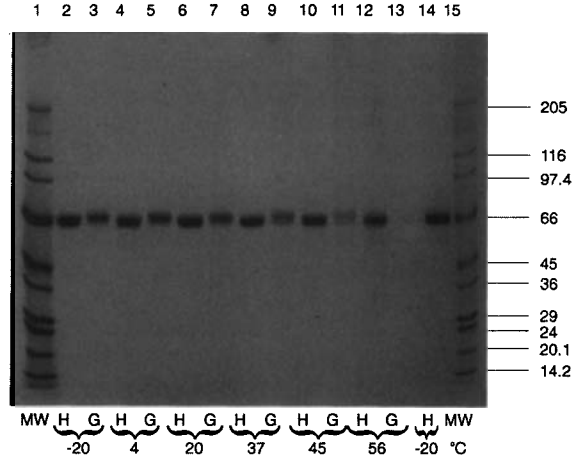

FIG. 3. SDS-PAGE of samples of alkaline phosphatase formulated with natural human serum albumin (H) or glycated human serum albumin (G) after storage of freeze-dried material in sealed ampoules at temperatures of −20–56°C for 2 weeks. Note, in the glycated human serum albumin formulations, the higher M_r of the albumin, the appearance of additional bands at higher M_r after storage at the higher temperatures and the reduced uptake of the Coomassie stain.

Table 2. Predicted rates of degradation of alkaline phosphatase freeze-dried with natural human serum albumin (HSA), glycated human serum albumin (GHSA), 1% trehalose or without an additive.

	Percentage loss of activity per annum (alkaline phosphatase)			
Temperature (°C)	+HSA	+GHSA	+1% Trehalose*	Alone
−70	0	0	0	0·005
−20	0·7	0·4	0·002	6·3
4	13·4	13·6	1·00	55·5
20	55·5	68·0	32·8	96·9
37	98·7	100·0	100·0	100·0

* It was not possible to predict rates of degradation for alkaline phosphatase + 15% trehalose as, after the initial loss brought about by freeze-drying, there was no temperature-dependent loss.

were similar to one another; the only change with time being a small increase in the height of the dimer peak (arrow) at 11·6 min (Fig. 4A). On the other hand, the traces produced by the alkaline phosphatase + GHSA preparation changed considerably with time. By four weeks, both polymer (7·6 min) and dimer peaks had enlarged and the monomer peak diminished in size. After eight weeks storage at 37°C, reconstitution and filtration difficulties were encountered; the small amount of sample that was filterable produced a trace resembling that at four weeks with a reduction in the height of the polymer peak presumably due to the formation of complexes too large to pass through the filter (Fig. 4B). After 12 or 16 weeks of storage it was impossible to filter the reconstituted material and FPLCs could not be performed after this time for the GHSA formulations.

Discussion

This study has used exaggerated conditions of high temperature storage and albumin glycated artificially to a high degree in order to demonstrate the possible effects of glycation of an additive on a biological standard. The effect of the glycation of the albumin to approximately 90 to 100% was to reduce the protective effect of albumin on storage at high temperatures.

The evidence presented here strongly supports the view that the adverse effects seen were due to glycation of the albumin and not due to the presence of free glucose. Extensive dialysis of the glycated albumin was used and tests on the 20% albumin proved negative for free glucose within the sensitivity of the test. This was then diluted 200-fold for use with the alkaline phosphatase in freeze-drying, thus making it highly unlikely that the observations were due to the presence of free glucose. There was a marked difference in the binding of the glycated vs the natural HSA to PBA columns and an increase in the mol. wt of the GHSA over HSA was shown by SDS-PAGE and by FPLC. Finally, there was an eleven-fold increase in 5-hydroxymethyl furfural released by acid hydrolysis of the glycated over the natural HSA.

Alkaline phosphatase proved to be a good model for demonstration of the effects of additive glycation. It lost all but 5% of its activity when freeze-dried without an additive in the buffer but retained approximately 70% of it when freeze-dried with albumin. Both natural and glycated albumin afforded this protective effect on both freezing and freeze-drying processes and appeared to do so to the same

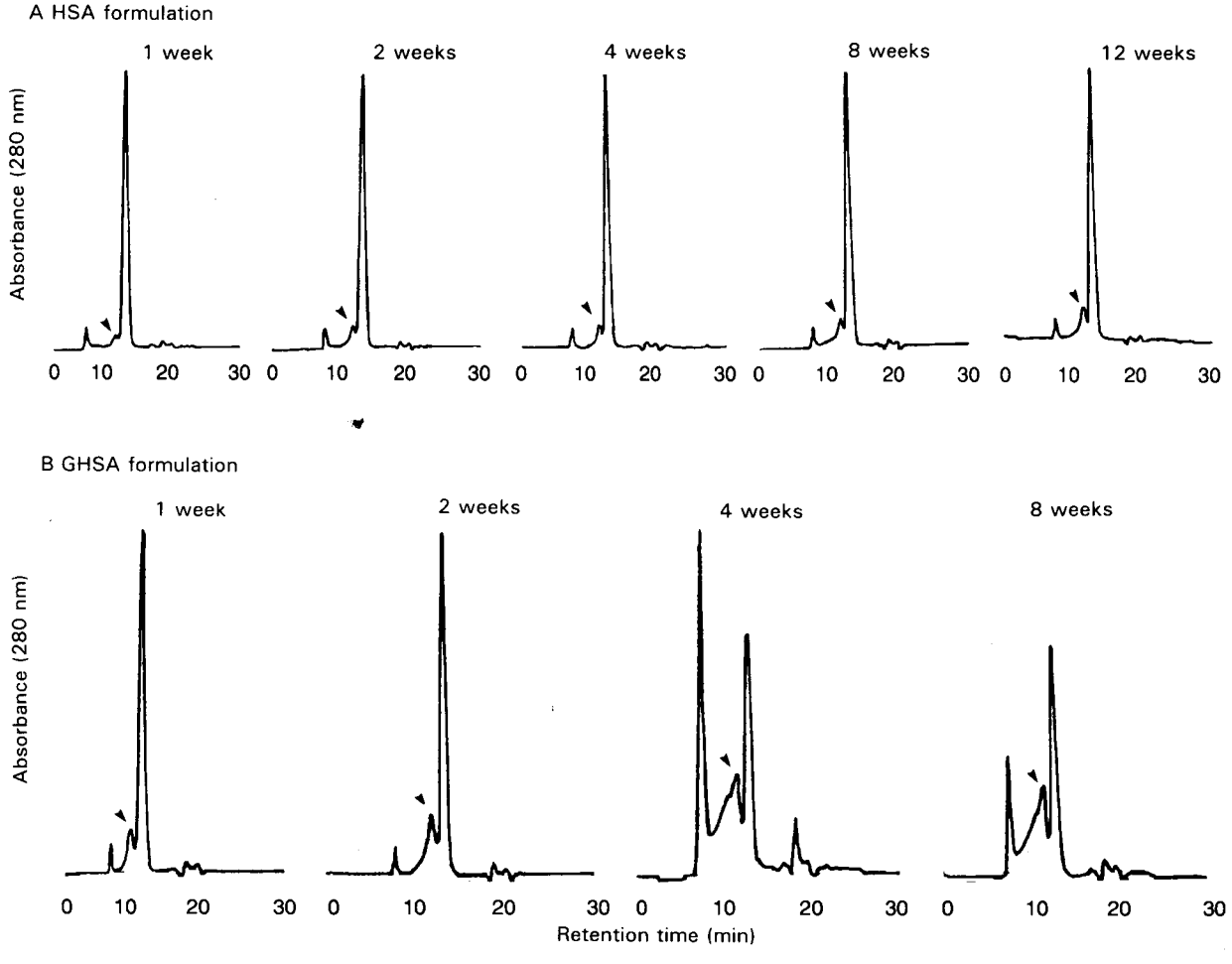

FIG. 4. Gel filtration FPLC chromatograms after storage for up to 12 weeks at 37°C. A. Alkaline phosphatase + HSA. B. Alkaline phosphatase + GHSA. The arrow indicates the position of the dimer.

extent, but subsequent storage at higher temperatures revealed a faster loss of activity in alkaline phosphatase when formulated with glycated albumin than with natural albumin.

That this loss of activity was a result of involvement of the alkaline phosphatase in the Maillard reaction, was demonstrated by changes in the mol. wt distribution, as shown by SDS-PAGE and FPLC, and by the relative insolubility of the GHSA formulations after high temperature storage, indicating the possible formation of large molecular complexes. Previous studies using sugars in the formulation of alkaline phosphatase also showed slower migration of the alkaline phosphatase through SDS-PAGE gels on immunoblotting (Ford & Dawson 1993).

The exaggerated conditions of 100% glycation and high temperature storage should be put in context. Although the level of glycation and thus the availability of reactive carbonyl groups on the albumin were far higher than would normally be used in the preparation and storage of biological standards, the following points should be considered.

Firstly, glycation of proteins is a natural phenomenon and the importance of the Maillard reaction has long been established in the food industry, where it produces changes in the appearance and taste of foodstuffs (Kaanane & Labuza 1989), and in medicine where the various complications of diabetes, such as lens cataracts and cardiovascular disorders are viewed as consequences of increased glycation of proteins (Guthrow et al 1979). Albumin, too, is naturally glycated. The levels observed in the control albumin of 13 to 14% were similar to levels reported previously of 6 to 15% (Day et al 1979) and 10 to 12% (Garlick & Mazer 1983). They also agree with the level of 9% glycation measured in a batch of HSA used in the formulation of certain cytokine standards (A. Furth, personal communication).

Second, often there is a high molecular ratio of additive, such as albumin, to the active biological material, so that even if only 10% of the additive is glycated then there could well be a molecular excess of the glycated substance. This is often the case where the active material is the result of recombinant DNA technology when the material is highly purified and is dispensed to each ampoule in nanogram amounts. A molecular ratio of additive material of the order of 1000:1 is not unusual.

Finally, although the temperatures used in this study were higher than those that would normally be encountered and the periods of exposure to them longer, cases of exposure of biological standards to temperatures exceeding 20°C during shipment have been recorded. A recent study (Ford 1989) examining the temperatures to which biological standards were exposed during dispatch, revealed that 5% of those issued from the UK during its winter months were exposed to temperatures of 33°C or greater and 100% to temperatures above 22°C. The duration of transit was between 3 to 44 days and, although the 44 days (to Australia) was exceptional, mean transit times to the various overseas destinations ranged between 7 and 12 days. The finding that a significant loss of activity was seen in the GHSA formulation after just two weeks at 37°C indicates that formulations with a high ratio of albumin to the standard biological material could be at risk of being adversely affected during transit.

Since glycation is a natural phenomenon, there is a limit to the number of ways in which it is possible to minimize the adverse effects in biological standards. Screening of individual serum donations and the use of only those sera with low levels of glycated albumin offers one possible solution. The use of young, healthy donors is another, as the extent of glycation tends to increase with age (Furth & Harding 1989). The combination of selection of albumin with a low degree of glycation and the use of inhibitors of the Maillard reaction could also be considered. Various substances have been used in the food industry and in medical research for inhibiting the Maillard reaction. These include sulphites, effective antioxidants, commonly used in packaged foods, though now banned in the USA (Kaanane & Labuza 1989) and aminoguanidine which has been shown to prevent or inhibit the formation of advanced glycation end products or the cross-linking of proteins in model systems in-vitro and in-vivo in diabetic rats (Brownlee 1989). Glutathione has also been shown to inhibit glycation and cross-linking of lens proteins induced by ascorbic acid in-vitro (Ortwerth & Olesen 1988) and lysine has been used to inhibit the reaction between glucosylated and native ribonuclease in the absence of free glucose (Eble et al 1983). Aspirin, too, has been shown to have a role in the prevention of glycation and cross-linking of lens and other proteins (Furth & Harding 1989). The addition of new chemicals into formulations of biological standards would, however, require further research to ensure that they did not interact with the biological materials or interfere in the assays for which the standards were intended. It is also extremely unlikely that a single inhibitor of the Maillard reaction would have universal application.

Several side issues have come to light in this study. The finding that, under conditions of accelerated degradation, 15% trehalose was protective but that 1% trehalose was not, was unexpected. Trehalose at 1% had proved to be an excellent protectant in previous studies using impure alkaline phosphatase and the same range of temperatures (Ford & Dawson 1993) and is routinely used successfully at concentrations of between 0·1 and 1% in formulations for biological standards (Tarelli & White 1982). A possible explanation for its lower protective capacity in this study could be the use of different buffer salts to those used in the earlier study (potassium phosphate instead of Tris) or the greater purity of the alkaline phosphatase, hence the smaller amount per ampoule required, and the possible loss of protection afforded by some of the impurities. Trehalose was used at 15% in this study, since this is closer to the level at which it occurs naturally in some plants, where it offers protection against dehydration (Crowe et al 1987). At this concentration, although only approximately 40% of the initial activity was retained on freeze-drying and loss of approximately 80% occurred during the 16 week course of the experiment, the loss did not appear to be temperature-dependent, with samples stored at 56°C having virtually the same level of activity as those stored at −20°C. After 16 weeks of higher temperature storage, alkaline phosphatase formulated with 15% trehalose had a higher level of activity than any other formulation. The reason for this initial loss on freeze-drying then subsequent relative stability is not immediately apparent.

In conclusion, it is unlikely that, in the preparation of biological standards, a single additive such as albumin or

trehalose would be found to be suitable for all the various materials freeze-dried. Both albumin and trehalose, used both individually and together, have proved useful in the past, but evidence from this study has shown that, when albumin is selected for use, its glycation level should be kept to the minimum and that the mixture of albumin and a reducing sugar in the formulation should be avoided. Accelerated degradation studies on the freeze-dried final formulation of each biological standard should be performed to assess its stability under conditions of long-term storage.

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