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# The Effect of Carbohydrate Additives in the Freeze-Drying of Alkaline Phosphatase

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**Abstract**—Alkaline phosphatase was used as a model in studies to assess the effects of lyophilization on biological activity and molecular integrity in the presence or absence of added carbohydrate. The stability of the activity of alkaline phosphatase, lyophilized in Tris buffer alone or in the presence of the carbohydrates mannitol, lactose or trehalose was examined. Enzyme activity in formulations with Tris buffer alone or with mannitol was considerably reduced by freeze-drying and further storage at elevated temperatures; freeze-drying with mannitol failed to maintain activity at a temperature of 37°C over 21 days, whilst the loss of activity was more gradual when freeze-dried in buffer alone and stored at higher temperatures. Lactose and trehalose maintained the alkaline phosphatase activity after freeze-drying and, furthermore, preparations containing trehalose retained activity even when the material was subjected to temperatures of up to 45°C for up to 84 days. At 56°C the alkaline phosphatase activity did not show a significant drop until 14 days with the lactose formulation or until 21 days with trehalose. After 84 days at 56°C, 30% of the activity still remained in the formulation containing trehalose. In addition to the changes in the enzyme activity, FPLC chromatographic traces and SDS-PAGE gels demonstrated compositional differences between each formulation after storage.

Lyophilized national or international biological standards have been in use for many decades. Freeze-drying and the subsequent processes of further desiccation and filling ampoules with dry, oxygen-free nitrogen gas before sealing have been shown, in many cases, to maintain the activity of biological preparations over a period of one to two decades (Campbell 1974a, b, c). Generally, the drier the product, the lesser the deterioration in activity (Tarelli & White 1982; Bristow et al 1988).

The aim in lyophilization of biological preparations is to preserve the activity of the substance of interest. Where the biological material is a complex mixture or has a high protein content, such as in serum standards (Reimer et al 1978) or allergen extracts (Ford et al 1985), experience has shown that freeze-drying without additives is usually satisfactory. However, where a pure substance is involved, as in the standard for human insulin, it is usual to formulate to preserve activity (Bristow et al 1988). When the pure substance has high activity relative to the mass and each ampoule need only contain a microgram or less of material, as, for example, in standards for recombinant DNA, derived cytokines (Dawson 1992), formulation with a supposedly inert bulking material is essential. In such cases, the excipients used not only add bulk to the purified material, producing a visible plug on freeze-drying, but can also protect against denaturation and subsequent loss of activity that could result from adsorption to the glass and from various aspects of the freeze-drying process itself.

Examples of excipients used in lyophilization are mannitol, sucrose and trehalose which can be used alone with the active substance or in conjunction with albumin (Tarelli & White 1982). The choice of carbohydrate or protein additives is largely empirical. However, problems such as inadequate plug formation and reconstitution difficulties causing reduction of activity (WHO Expert Committee on Biological Standardization 1990) and instability or batch variability have been encountered and have been shown to be due to high (Baer et al 1986) or variable (Tarelli et al 1987) residual moisture, or oxidation of the final product.

To determine the relative advantages of various excipients used in freeze-drying, we have used the enzyme alkaline phosphatase, freeze-dried in the presence of one of three carbohydrates frequently used in formulations, and exposed the lyophilized material to storage temperatures from −20°C to +56°C for a period of up to 84 days. This procedure is known as 'accelerated degradation' and is recognized as a method of simulating, over a relatively short period of time, the effect of long-term, low temperature storage.

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## Materials and Methods

*Reagents*

All biochemicals including alkaline phosphatase (EC 3.1.3.1. from bovine intestinal mucosa), antisera, immunoblotting and assay reagents, and Trizma base were purchased from the Sigma Chemical Company Ltd, Poole, UK. The carbohydrates mannitol (laboratory reagent), lactose (analytical reagent) and D-(+)-trehalose dihydrate were purchased from BDH (Poole, UK), FSA (Loughborough, UK, Biochemika and Fluka Chemie AG, CH-9470 Buchs (Switzerland) respectively.

*Preparation of materials for freeze-drying*

Eight solutions were prepared: alkaline phosphatase 1 mg $mL^{-1}$, Tris 0·1 M pH 7·3; alkaline phosphatase 1 mg $mL^{-1}$, mannitol 0·06 M, Tris 0·1 M, pH 7·3; alkaline phosphatase 1 mg $mL^{-1}$, lactose 0·03 M, Tris 0·1 M, pH 7·3; alkaline phosphatase 1 mg $mL^{-1}$, trehalose 0·03 M, Tris 0·1 M, pH 7·3;

Tris 0·1 M, pH 7·3; mannitol 0·06 M, Tris 0·1 M, pH 7·3; lactose 0·03 M, Tris 0·1 M, pH 7·3; trehalose 0·03 M, Tris 0·1 M, pH 7·3.

*Freeze-drying*

For each of the eight solutions, some ninety, 1 mL aliquots were dispensed into sterile glass ampoules, which were placed on the shelf of a freeze-drier. The temperature was lowered to −40°C at the rate of approximately −1°C $min^{-1}$. The material was then freeze-dried for 116 h as follows. The shelf temperature of the freeze-dryer was maintained at −40°C for 90 h, raised over a 20 h period under controlled heating to +20°C, then maintained at +20°C for a further 6 h. Throughout the cycle the condenser temperature was between −57°C and −59°C and the vacuum between $4 \times 10^{-2}$–$2{\cdot}5 \times 10^{-2}$ mbars. The ampoules were then fitted with a capillary plug, backfilled with dry nitrogen gas ($O_2$ level ≤10 ppm, $H_2O$ ≤5 ppm) and further desiccated in a vacuum over phosphorous pentoxide for six days at room temperature (20°C). They were again backfilled with dry nitrogen gas and sealed by fusion of the glass. The ampoules were tested for the structural integrity and stored at −20°C before being placed at the experimental temperatures.

*Performance of study*

Twelve ampoules for each category were placed at each of the following temperatures −20°C, +20°C, +37°C, +45°C, +56°C for up to 84 days. At 7, 14, 21, 42 and 84 days, the freeze-dried material was examined for macroscopic appearance and the contents of the ampoules were assayed for alkaline phosphatase activity, gel filtration chromatographic profile, SDS-PAGE and immunoblotting patterns. The contents of each ampoule were dissolved in 1 mL of 0·15 M phosphate-buffered saline, pH 7·3 containing 7·7 mM sodium azide (PBS-az). On days 7 and 14, the assays of activity were performed only on the ampoules stored at the higher temperatures together with the −20°C preparation. For 21 days storage and beyond, all preparations from all storage temperatures were assayed.

*Assay for enzyme activity*

A microtitre bioassay was used to determine the enzyme activity after storage at the various times and temperatures; all ampoules from a given time point being tested together in a single assay. The ampoule contents were reconstituted to 1 mg $mL^{-1}$ in PBS-az and doubling dilutions from 1/25 to 1/800 (4 to 0·125 μg $mL^{-1}$) were prepared in PBS-az, in duplicate, in 100 μL volumes. Equal volumes (100 μL) of phosphatase substrate (*p*-nitrophenyl phosphate) at 1 mg $mL^{-1}$ in 1 M diethanolamine buffer containing 0·5 mM $MgCl_2$, pH 9·8, were added. The mixture was incubated at room temperature (20°C) for 12 min for colour development and the reaction was stopped by the addition of 50 μL of 3 M NaOH before absorbance measurement at 405 nM. The mean absorbance for each dilution was determined. The potency of each lyophilized preparation stored at higher temperatures relative to the same lyophilized formulation stored at −20°C was calculated by parallel line assay (Finney 1978).

*Gel filtration chromatography*

Gel filtration chromatography was performed using a Superose 12 (Pharmacia) column attached to an FPLC system controlled by an FPLCManager (Pharmacia). Sterile-filtered PBS-az was the running buffer at a flow rate of 1 mL $min^{-1}$. Samples of 200 μL (200 μg alkaline phosphatase) were separated during a 30 min run, and the absorbance at 280 nm recorded, at a sensitivity of 0·1, unless otherwise stated.

*SDS-PAGE*

The samples were separated by SDS-PAGE after the method of Laemmli (1970) with a 5–15% gradient acrylamide gel using a discontinuous buffer system (Ornstein 1964; Davis 1964) and sample loading of 25 μg. The gels were silver-stained according to the method of Wray et al (1981).

*Immunoblotting*

Material separated by SDS-PAGE was transferred electrophoretically to nitrocellulose membranes (Towbin et al 1979) and antibody binding to alkaline phosphatase was visualized with chloronaphthol (Hawkes et al 1982). Molecular weight markers or original alkaline phosphatase proteins transferred to the nitrocellulose were gold stained with Aurodye Forte (Amersham International plc, UK).

## Results

*Appearance of the freeze-dried material*

After freeze-drying, the formulations with carbohydrates were distinct white plugs whilst those lacking carbohydrate were in the form of friable powders.

Over the period of storage (84 days) at −20°C no macroscopic changes were evident in either carbohydrate or non-carbohydrate formulations (Fig. 1a–c), nor were macroscopic changes evident after storage in any formulations at temperatures up to 45°C for this period. However, gross changes occurred in the formulations containing lactose at temperatures of +56°C; shrinkage became noticeable by 7 days, transformation to a brown, viscous liquid by 14 days and a dark brown liquid by 84 days (Fig. 1e). Shrinkage of the plug to approximately 3 mm diam. by 7 days occurred in the trehalose formulations held at +56°C (Fig. 1f).

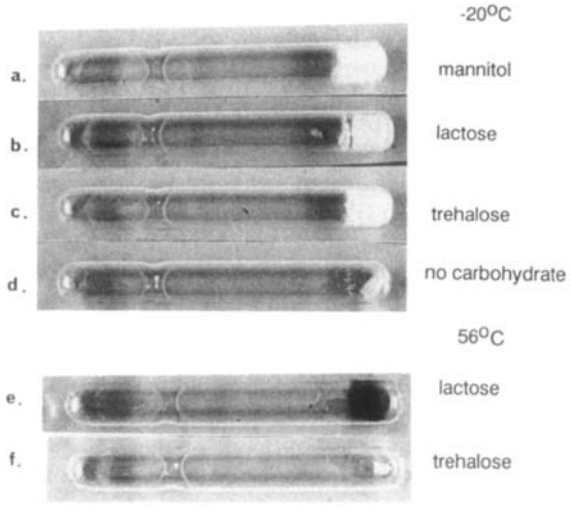


FIG. 1. Appearance of ampoules containing 1 mg alkaline phosphatase prepared in 0·1 M Tris buffer, pH 7·3 with (a) 0·06 M mannitol, (b) 0·03 M lactose, (c) 0·03 M trehalose or (d) no carbohydrate after freeze-drying and storage for 84 days at −20°C; (e) lactose and (f) trehalose after freeze-drying and storage for 84 days at 56°C.

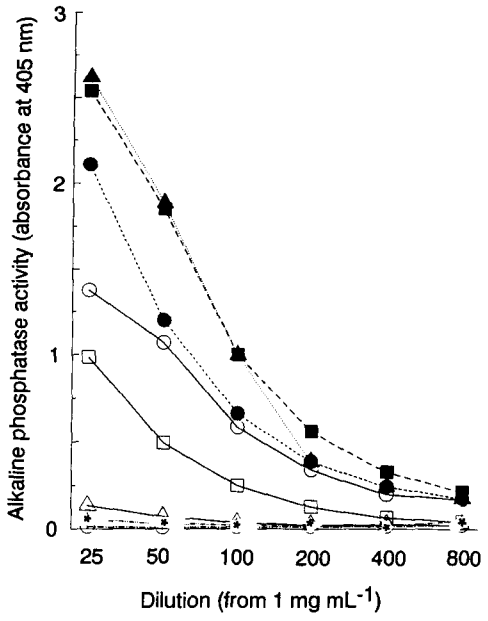


FIG. 2. Activity of alkaline phosphatase freeze-dried in the different formulations after storage for 7 days at −20°C (○ no carbohydrate; ▲ lactose; ● mannitol, ■ trehalose) or storage for 84 days at 56°C (* no carbohydrate, ○ mannitol, △ lactose, □ trehalose).

The loss of activity on storage is shown in Fig. 3 for each preparation. It is seen that whilst the activity of alkaline phosphatase formulated without a carbohydrate (Fig. 3a) was inversely proportional to the storage temperature, and that the activity declined with time, formulations with mannitol (Fig. 3b) had virtually no activity after storage at the three higher temperatures. Alkaline phosphatase preparations formulated with lactose (Fig. 3c) and trehalose (Fig. 3d) retained maximal activity throughout the 84 days at all but the 56°C storage temperature, where decline was more rapid in the lactose formulations (7–14 days) than trehalose (21–42 days). The rate of loss of enzyme activity for the trehalose formulation at each storage temperature is shown in Table 1.

Table 1. Predicted degradation rates for alkaline phosphatase trehalose formulation.

| Temperature (°C) | Loss (% per year) |
|---|---|
| −150 | 0·00 |
| −70 | 0·00 |
| −20 | 0·03 |
| 4 | 1·32 |
| 20 | 10·79 |
| 37 | 58·45 |

*Changes in alkaline phosphatase enzyme activity with time and temperature of storage*

Freeze-drying alkaline phosphatase in Tris-HCl buffer alone or with mannitol, resulted in loss of activity compared with freeze-drying in the presence of lactose or trehalose, where no loss of activity relative to freshly-prepared alkaline phosphatase was seen. The dose-response curves for the alkaline phosphatase preparations after 7 days storage at −20°C and after 84 days at 56°C, where only the trehalose preparations retained any degree of alkaline phosphatase activity, are shown in Fig. 2. The controls, lacking alkaline phosphatase, showed no activity in any of the assays.

*FPLC of alkaline phosphatase*

Separation of freshly prepared alkaline phosphatase (Sigma P-7640) by gel filtration FPLC gave a trace with 5 distinct peaks of UV absorbance (Fig. 4), eluting between 7·5 and 20 min. The alkaline phosphatase activity was associated with the shoulder of the fourth peak at 13·4 min, indicating a mol. wt between 41 000 and 100 000 Da. A purer alkaline phosphatase (Sigma P-8647), not used in the main study, gave a single peak containing the alkaline phosphatase activity at 12·5 min corresponding to a mol. wt of 80 000 Da (Fig. 4, superimposed trace).

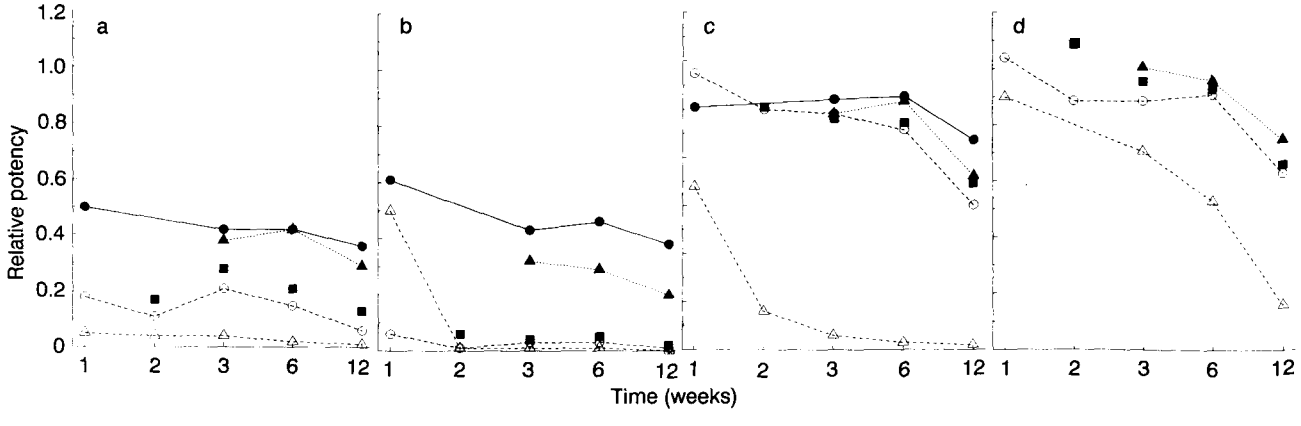


FIG. 3. Activity of freeze-dried alkaline phosphatase with temperature and time. The figure shows the activity of alkaline phosphatase freeze-dried in different formulations, stored at temperatures of ● −20°C, ▲ +20°C, ■ 37°C, ○ 45°C, △ 56°C and tested at intervals from 7 to 84 days. The activity is expressed as a potency relative to the most stable preparation, alkaline phosphatase + trehalose, stored at −20°C. The reproducibility of the assay as a geometric coefficient of variation was 2·75%. a. Alkaline phosphatase in Tris buffer, 0·1 M, pH 7·3. b. As (a) + mannitol, 0·06 M c. As (a) + lactose, 0·03 M. d. As (a) + trehalose, 0·03 M.

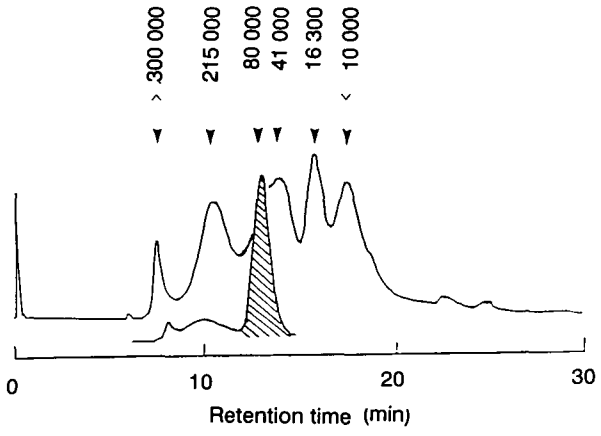


FIG. 4. Chromatographic trace of bovine intestinal alkaline phosphatase (Sigma P-7640) separated by gel filtration. The superimposed shaded peak represents the trace of the purer alkaline phosphatase (Sigma P-8647). The mol. wt of the peaks are indicated. Conditions: column = Superose 12 (Pharmacia), running buffer = PBS (0·15 M, pH 7·3) + 7·7 mM $NaN_3$, sample = 200 μg alkaline phosphatase in 200 μL running buffer, flow rate = 1 mL $min^{-1}$. Measurement of absorbance at 280 nm, sensitivity = 0·1.

*Changes in the chromatograms with time and temperature*

After 7 days storage, very few changes from the original appearance of the alkaline phosphatase chromatogram (as shown in Fig. 4) occurred for any of the formulations at any storage temperature with the exception of the lactose-alkaline phosphatase formulation stored at 56°C, where peaks 3 and 4 shifted to the left, peak 5 enlarged and four additional components eluted after 20 min (not shown).

By 84 days, the appearance of the chromatographic traces for alkaline phosphatase-lactose formulations at all storage temperatures had changed (Fig. 5a). At 56°C storage, peaks 2–4 had merged into the shoulder of a large, broad peak and numerous components eluted after 20 min (Fig. 5a, top trace).

By 84 days, the lactose controls (lacking alkaline phosphatase) exhibited similar changes at the higher storage temperatures (Fig. 5b), with marked enlargement of the peaks between 17·5 and 25 min, which culminated in an extremely broad peak and tall subsidiary ones at 56°C (Fig. 5b, top trace).

In contrast, virtually no changes in the chromatograms occurred after storage for 84 days at any temperature in the trehalose formulations (Fig. 5c) apart from small, late peaks present at all storage temperatures and a slight flattening of the shoulder on peak 3 at the higher temperatures. For alkaline phosphatase in Tris-HCl buffer alone (not shown) or with mannitol (Fig. 5d), the loss of the shoulder on peak 3 was more marked and late peaks occurred at all storage temperatures.

*SDS-PAGE and immunoblotting*

Silver-staining of alkaline phosphatase (P-7640) separated by SDS-PAGE under reducing conditions showed numerous bands between approximately 10 000 and 150 000 Da with the most heavily stained at 24 000 and 28 000 Da and gold-staining of the material electrophoretically transferred to nitrocellulose showed a similar pattern of multiple bands. However, immunoblotting of alkaline phosphatase gave predominant antibody binding at 66 000 Da (Fig. 6, lane 1) which was where the purer alkaline phosphatase (Sigma P-8647) showed sole binding (Fig. 6, lane 2) and where the active chromatographic fractions of both forms of alkaline phosphatase (P-7640 and P-8647) bound. No strong immunostaining on the immunoblot was seen to correspond with the silver-stained bands at 24 000 and 28 000 Da.

*Changes in SDS-PAGE and immunoblotting with time and temperature*

*SDS-PAGE.* Freshly prepared alkaline phosphatase (i.e. non freeze-dried) and alkaline phosphatase freeze-dried in all the formulations and examined shortly after freeze-drying were identical by silver-stained SDS-PAGE. After storage at the higher temperatures, marked changes were apparent in the freeze-dried lactose formulations. These changes were most marked after 56°C storage (Fig. 7a, lanes 3–7) but similar changes occurred more slowly at 45 and 37°C (gels not shown).

No similar changes by SDS-PAGE gels were seen for the other formulations throughout the course of the experiment (e.g. trehalose, Fig. 7a, lanes 9–14).

*Immunoblotting.* Fig. 7b and 7c show the appearance by immunoblotting of the freeze-dried alkaline phosphatase preparations after storage at the various temperatures and indicate the activity of the alkaline phosphatase. Where the formulation was in Tris buffer alone (Fig. 7b), the appearance after 7 days at −20°C (Fig. 7b, lane 2) closely resembled that of the fresh alkaline phosphatase (Fig. 7b, lane 1). However, from 7 days onwards at 56°C the prominent alkaline phosphatase bands at 66 000, 100 000 and 150 000 Da were missing (Fig. 7b, lanes 3–6). Similarly, the freeze-dried mannitol formulation after 7 days at −20°C (Fig. 7b, lane 7) appeared similar to fresh alkaline phosphatase, but had lost the prominent bands by 7 days at 56°C (Fig. 7b, lanes 8–10).

The appearance of the immunoblots of alkaline phosphatase formulated and freeze-dried with lactose echoed that of the SDS-PAGE stained gel; after 7 days at −20°C (Fig. 7c, lane 4) the binding pattern appeared identical to freshly-prepared alkaline phosphatase (Fig. 7c, lane 3) but, after the same period at 56°C, the prominent band at 66 000 Da had moved to a higher mol. wt position (Fig. 7c, lane 5); by 21 days at this temperature the band had become extremely faint and moved to a higher mol. wt (Fig. 7c, lane 6) and by day 42 had disappeared (Fig. 7c, lane 7).

The freeze-dried alkaline phosphatase formulation containing trehalose presented a more stable picture with banding patterns in immunoblotting after 7 or 21 days at 56°C (Fig. 7c, lanes 9, 10) or after 7 days at −20°C (Fig. 7c, lane 8) resembling those of the fresh alkaline phosphatase (Fig. 7c, lanes 3, 13). By 42 days at 56°C (Fig. 7c, lane 11); however, the prominent band at 66 000 Da had become fainter and by 84 days had virtually disappeared (Fig. 7c, lane 12).

## Discussion

The aim of this study was to examine the effect of formula-

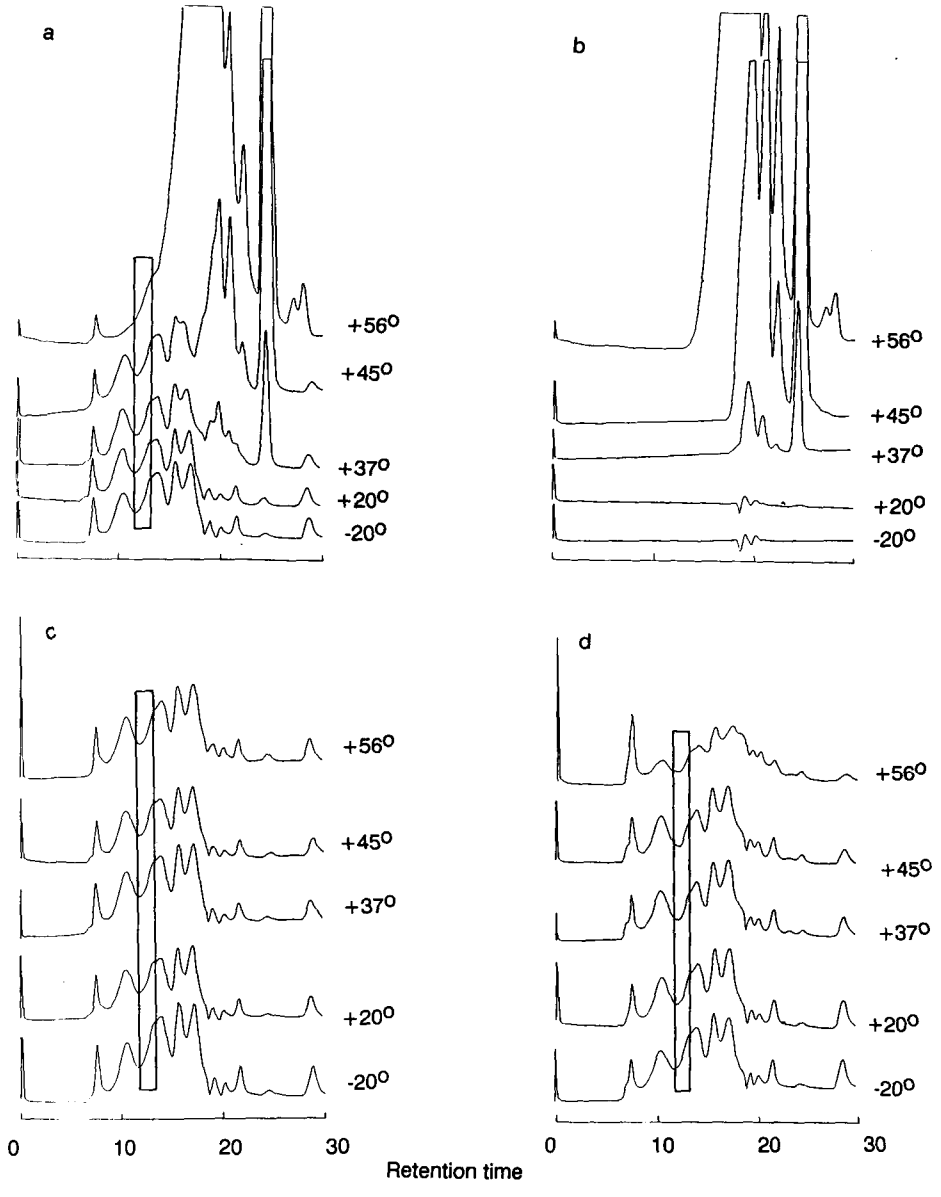


FIG. 5. Changes of FPLC chromotograms with temperature and time. Appearance of chromatograms of alkaline phosphatase freeze-dried and stored in sealed glass ampoules for 84 days at temperatures of −20°C, +20°C, +37°C, +45°C and +56°C for 84 days. a. Alkaline phosphatase in Tris buffer, 0·1 M, pH 7·3+0·03 M lactose. b. Tris buffer, 0·1 M, pH 7·3+0·03 M lactose control. c. Alkaline phosphatase in Tris buffer, 0·1 M, pH 7·3+0·03 M trehalose. d. Alkaline phosphatase in Tris buffer, 0·1 M, pH 7·3+0·06 M mannitol. The regions of alkaline phosphatase activity are outlined.

tion and freeze-drying using a model protein, alkaline phosphatase. A fairly impure preparation was selected for its ready availability, assayable activity and its partial loss of activity upon unprotected freeze-drying. In order to accelerate the effects of freeze-drying and that of continuous storage at a low temperature in the dark (the usual method of storage of a freeze-dried material prepared as a standard (Campbell 1974 a, b)), the sealed ampoules were subjected to storage at elevated temperatures. An indication of changes before reduced alkaline phosphatase activity could be shown were gained from the chromatographic profiles and SDS-PAGE. Although the changes observed were occurring in the additive (as with the lactose as seen by gel permeation chromatography) or in the impurities present in the alkaline phosphatase (as seen by SDS-PAGE), they preceded or accompanied loss of alkaline phosphatase activity and thus acted as indicators of deterioration.

Few studies have been performed to date on the effects of formulation in stabilizing biological activity on freeze-drying. The carbohydrates chosen for use in this study had been used previously as cryoprotectants in freeze-drying experimentally (Tarelli & Wood 1981; Tarelli & White 1982; Tarelli et al 1987; Calam & Tarelli 1988; Carpenter et al 1988), for standards (WHO Expert Committee on Biological Standardization 1987) or in freeze-thaw experiments (Carpenter & Crowe 1988). Mannitol has been used as an

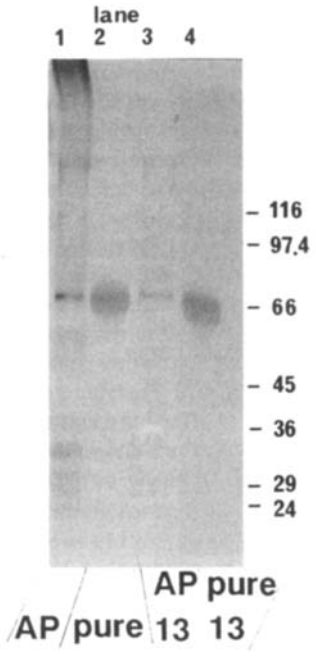


FIG. 6. Immunoblotting of freshly prepared alkaline phosphatase and chromatographic fractions. Lane 1, freshly prepared alkaline phosphatase (P-7640); lane 2, freshly prepared purer alkaline phosphatase (P-8647); lane 3, Fraction 13 of alkaline phosphatase (P-7640); lane 4, Fraction 13 of purer alkaline phosphatase (P-8647).

excipient for freeze-drying for decades. It was shown to be superior to trehalose in the protection of the peptide calcitonin (derived from salmon) and the WHO Expert Committee on Biological Standardization (1987) proposed the replacement standard for calcitonin be prepared with mannitol. More recently, the first International Standard for eel calcitonin, freeze-dried with mannitol and albumin, was found to have adequate stability (WHO Expert Committee on Biological Standardization 1990). In freeze-thaw experiments with the enzyme, lactate dehydrogenase, which loses all activity at 25 $\mu$g mL$^{-1}$ if frozen and thawed without cryoprotectants, the enzyme was protected with mannitol concentrations of 0·1–1·0 M, the degree of protection increasing with the concentration (20% at 0·1 M to 60% at 1 M; Carpenter & Crowe 1988). In our experiments, the concentration of mannitol (0·06 M) was below this range, which could account for the observed lack of protection of alkaline phosphatase activity. Freeze-dried mannitol formulations have been shown to differ in their appearance, by scanning electron microscopy, from formulations with other carbohydrates showing areas of crystallinity (Tarelli et al 1987). When freeze-dried to a low residual moisture content (< 0·2%), mannitol preparations were not hygroscopic but batch to batch variability of moisture content after freeze-drying under apparently identical conditions has been reported (Tarelli et al 1987). A low level of residual moisture (0·1–0·3%, close to the limit of detection) was achieved in our study and may account for the integrity of the freeze-dried cake in the sealed ampoules.

Lactose, like mannitol, has also been used in the formulation of biological materials for freeze-drying (Tarelli & White 1982; WHO Expert Committee on Biological Standardization 1987) and in the formulation of pharmaceutical preparations (Tarelli et al 1987). Although affording protection in freeze-drying, it is not an ideal excipient since, being a reducing sugar, it is reactive with proteins by the Maillard reaction (Tarelli & Wood 1981; Carpenter & Crowe 1988; Njoroge & Monnier 1989; Furth & Harding 1989) whereby proteins are glycated, intermediate Schiff's bases are formed, which react further to form reactive Amadori products, inter-reacting with intermediates to form large and inactive complexes known as advanced glycation end-products (AGEs). This reaction appeared to have occurred in the lactose preparations stored at 56°C in this study. Its occurrence is supported by SDS-PAGE and immunoblotting where, at the early stages of glycation and the apparent formation of Amadori products, the prominent low mol. wt bands (by SDS–PAGE) and the 66 000 Da alkaline phosphatase bands (by immunoblotting) moved to higher mol. wt positions. Later (84 days at 56°C), if this supposition is correct, when AGEs had formed, creating large molecules, the complexes were too large to enter the pores of the gel resulting in no stained bands being seen. Gel filtration chromatography contributed information on the accumulation of lower mol. wt entities on higher temperature storage of the lactose formulations but was insufficiently discerning to demonstrate the small increase in mol. wt shown by SDS-PAGE and immunoblotting. However, the increase in the size of peak 1 with increase in storage temperature up to +45°C for 84 days could be evidence of dimer formation and the absence of this peak at 56°C, its removal by filtration before chromatography.

Wherever the late peaks were observed in the lactose alkaline phosphatase preparations, they were also in the lactose controls and, if anything, they appeared slightly larger (i.e. may have developed earlier). This, too, could have been the result of the Maillard reaction occurring in the presence of the amine-containing Tris buffer and their earlier appearance could have been due to a more favourable (in terms of the Maillard reaction) molar ratio of reducing sugar (lactose) to amine (Tris) than in the enzyme protein formulations.

For the other preparations, not containing lactose, the changes in the chromatography proved to be less dramatic. Careful examination and comparison, however, showed a loss of the active shoulder on the third peak, which accompanied loss of enzyme activity of the freeze-dried preparation. There was, however, no advance warning by chromatography of imminent change as in the lactose preparations.

Although lactose is used as a cryoprotectant and also used pharmaceutically as an inert bulking agent (Calam & Tarelli 1988) the balance of advantages and disadvantages in freeze-dried standards should be carefully evaluated because of its ability to participate in the Maillard reaction.

Another possible explanation for the loss of activity in the lactose formulations, which should be explored if only to be dismissed, is that of $Tg^1$, the temperature at which the freezing composition curve meets the glass transition curve (Franks et al 1991). Freeze-dried materials have a percentage of water in the form of glass. Elevated temperatures, above the $Tg^1$, can lead to the unlocking of this water from the glass resulting in the collapse of the freeze-dried cake due to dissolution of the protein.

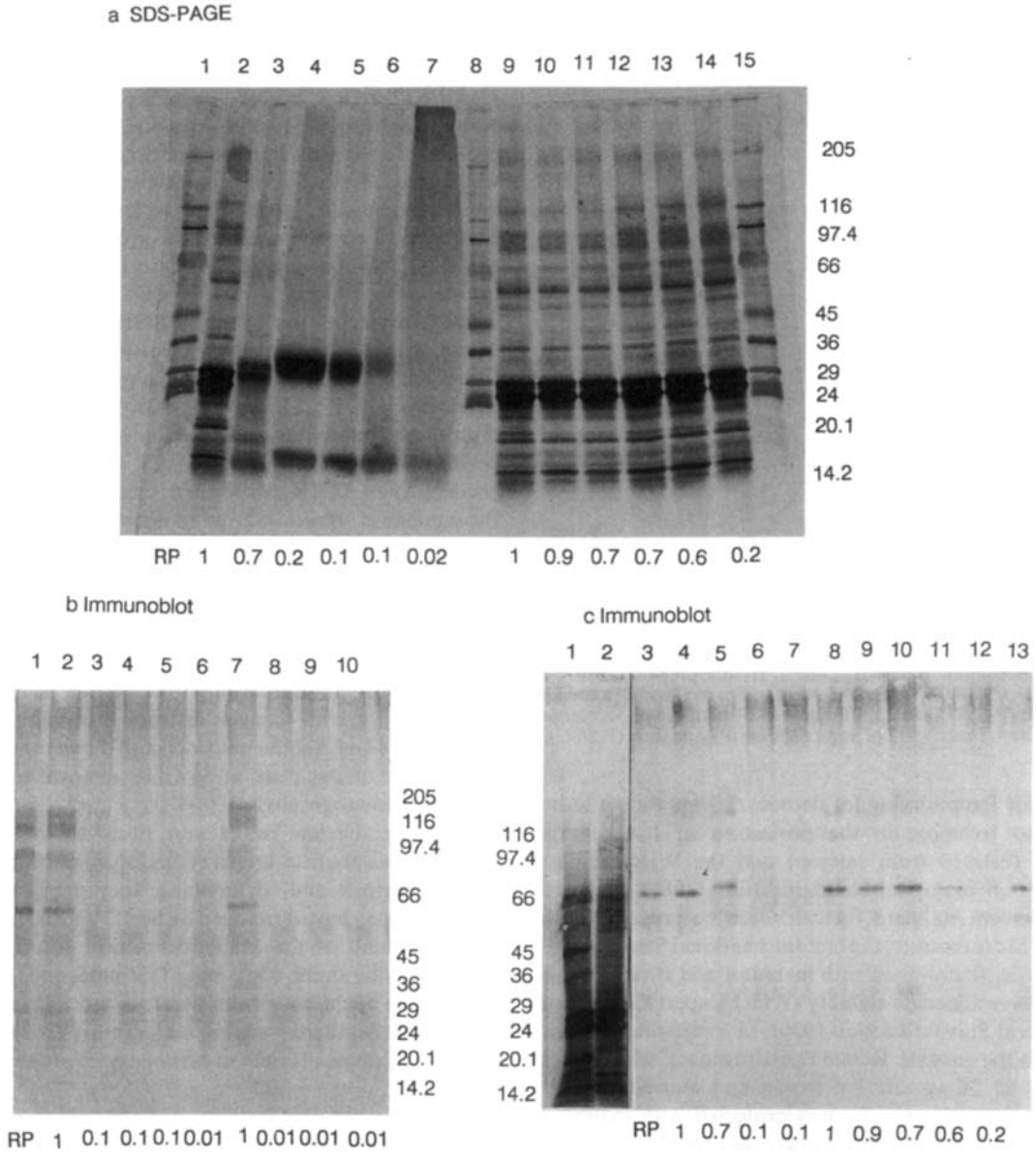


FIG. 7. SDS-PAGE and immunoblotting of freeze-dried alkaline phosphatase in lactose and trehalose formulations after accelerated degradation in sealed ampoules. The activity of alkaline phosphatase, as a relative potency (RP), is shown beneath each lane. a. SDS-PAGE of alkaline phosphatase formulated with lactose or trehalose and silver-stained. Lane 1, mol. wt markers; lane 2, alkaline phosphatase freeze-dried with lactose, stored at −20°C and sampled at 7 days; lanes 3–7, alkaline phosphatase freeze-dried with lactose, stored at +56°C and sampled at 7, 14, 21, 42 and 84 days, respectively; lane 8, mol. wt markers; lane 9, alkaline phosphatase freeze-dried with trehalose, stored at −20°C and sampled at 7 days; lanes 10–14, alkaline phosphatase freeze-dried with trehalose, stored at +56°C and sampled at 7, 14, 21, 42 and 84 days respectively; lane 15, mol. wt markers. b. Immunoblotting of alkaline phosphatase formulated with Tris or mannitol. Lane 1, freshly prepared alkaline phosphatase; lane 2, alkaline phosphatase freeze-dried in Tris-HCl, stored at −20°C and sampled at 7 days; lanes 3–6, alkaline phosphatase freeze-dried in Tris-HCl, stored at +56°C, and sampled at 7, 21, 42 and 84 days, respectively; lane 7, alkaline phosphatase freeze-dried with mannitol, stored at −20°C and sampled at 7 days; lanes 8–10, alkaline phosphatase freeze-dried with mannitol, stored at +56°C and sampled at 7, 21 and 42 days, respectively. c. Immunoblotting of alkaline phosphatase formulated with lactose or trehalose. Lane 1, mol. wt markers; lanes 2 and 3, freshly prepared alkaline phosphatase; lane 4, alkaline phosphatase freeze-dried in lactose, stored at −20°C and sampled at 7 days; lanes 5–7, alkaline phosphatase freeze-dried in lactose, stored at +56°C and sampled at 7, 21 and 42 days, respectively; lane 8, alkaline phosphatase freeze-dried in trehalose, stored at −20°C and sampled at 7 days; lanes 9–12, alkaline phosphatase freeze-dried in trehalose, stored at +56°C and sampled at 7, 21, 42 and 84 days, respectively; lane 13, freshly prepared alkaline phosphatase. Lanes 1 and 2 were gold-stained and the remainder were immunoblotted.

The glass transition temperature (Tg) for sucrose, in rigorously dried samples, is 56°C and that for trehalose, 77°C (Franks et al 1991). The lower the moisture content, the higher the Tg. Carbohydrates are used in freeze-drying because their presence raises the Tg of a protein solution. The presence of salts and other low mol. wt substances will likewise raise the Tg. The possibility remains that, at 56°C, the Tg[1] was exceeded and loss of activity could be attributed to that. This explanation is unlikely because the residual moisture content was extremely low (< 0·2%). Additionally,

the brown colour formation and the fact that this occurred only with the reducing sugar indicate very strongly that a Maillard reaction had occurred.

Trehalose appeared to be the carbohydrate of choice in the formulation of alkaline phosphatase in this particular study. It provided protection against loss of activity upon freeze-drying and maintained the activity even at high storage temperatures. Trehalose is currently the carbohydrate most frequently used in the formulation of biological materials requiring long-term stability for use as international standards, especially where the active material to be processed is present in microgram quantities or less as in the products of rDNA technology, such as the current cytokine standards (Dawson 1992). It is interesting that trehalose is found in a number of organisms (such as yeasts, nematodes, cysts of the brine shrimp and *Selagenella lepidophylla* (the desert resurrection plant) capable of surviving virtually complete dehydration where it can account for up to 20% of the dry weight of the organism (Crowe et al 1987).

The mechanism of cryoprotection afforded by carbohydrates and other groups of chemicals to proteins during freeze-drying is beyond the scope of this study. However, this study has demonstrated that carbohydrates do differ in their ability to protect the activity of the enzyme, alkaline phosphatase, on freeze-drying and in their ability to continue this protection under storage at elevated temperatures. The visual appearance of the freeze-dried material was not a good guide to the protection afforded and, for the enzyme alkaline phosphatase, trehalose was superior to either mannitol or lactose and deserves more recognition in its role as a cryoprotectant in freeze-drying.

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