HAEMOGLOBIN STABILIZATION DURING LYOPHILIZATION WITH SACCHARIDES. PERTURBATION EFFECT OF POLYETHYLENE GLYCOLS

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The stabilizing effect of glucose and sucrose upon haemoglobin molecules against oxidation during lyophilization was perturbated by the presence of fluid or greasy polyethylene glycols (m.w. 300—600 daltons) but not of the rigid ones (m.w. 1 500—6 000 daltons). The results corroborate the idea of a simple mechanical nature of haemoglobin stabilization under study.

Lyophilization of haemoglobin solutions can be achieved with good results only when appropriate stabilizing substances have been added to protect haemoglobin from spotaneous methaemoglobin formation and aggregation[1-6]. Among the stabilizing substances, low-molecular saccharides especially the physiologically innocuous glucose and sucrose are most feasible with regard to the problems of long time storage and *in vivo* testing of stroma-free haemoglobin[1-4,6-9]. However, the mechanism of the protective effect of saccharides remains unclear[6-8].

In an attempt to elucidate the mechanism of haemoglobin stabilization[3,4] we came to a hypothetical conclusion that during lyophilization the saccharide molecules form a rigid, dry network which immobilizes mechanically the spatial architecture of the haemoglobin molecules[3,4]. In the present work we tested the validity of the above hypothesis by perturbation of the assumed rigid saccharide network through the addition of low-molecular fluid polyethylene glycols to stroma-free haemoglobin solutions before lyophilization.

EXPERIMENTAL

Stroma-free haemoglobin was prepared by a standard method from outdated banked human erythrocytes[9]. Polyethylene glycols of average molecular weights 300, 600, 1 500, 4 000 and 6 000 daltons (Chemische Werke, Hüls) were used as perturbating agents. Their respective approximate melting points were estimated as −10, 0, 46, 56 and 61°C. The solutions under study contained equal concentrations (60 g/l) of haemoglobin, glucose or sucrose and varying concentrations of polyethylene glycols ranging from 0 to 50 g/l. The solutions were buffered to pH 7·3 ± 0·1 by the presence of 12·2 g/l of Na_2HPO_4 and 4·1 g/l of $NaH_2PO_4.2\,H_2O$. 5 ml samples were frozen at −40°C within 1 h after mixing and lyophilized at standard conditions[3,4]. The temperature was monitored from −20 to +30°C during freeze-drying. The dry samples were stored at 25°C

for one week, then redissolved in distilled water to reach the original volume and analysed immediately. Methaemoglobin was estimated photometrically as its cyano derivative[10].

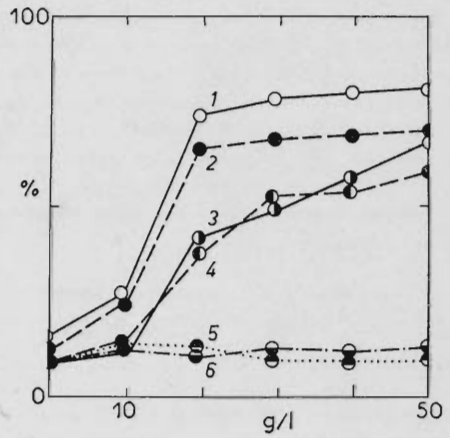
RESULTS

In orientation experiments the buffered stroma-free haemoglobin samples were lyophilized alone or with the fluid polyethylene glycol of m.w. 300 daltons or the rigid one of the m.w. of 6000 daltons. No significant stabilizing effect was exhibited by the sole polyethylene glycols in the absence of saccharides. On the contrary, the methaemoglobin concentration rose from the original 5–10% to high values of 44–73% after lyophilization. A similar rise was found in comparative lyophilizates of the sole stroma-free haemoglobin. A part of the samples was aggregated.

The presence of the polyethylene glycols (50 g/l) in buffered haemoglobin solutions, both with or without saccharides, did not change significantly the normal rate of spontaneous methaemoglobin formation at 25°C during 24 h. As it is shown in Fig. 1, the stabilizing effect of sucrose during lyophilization remained unchanged in the presence of polyethylene glycols of m.w. 1 500, 4 000 or 6 000 daltons which all are rigid at temperatures used during freezing (−40°C), drying (up to 30°C) and storage (25°C). However, a marked perturbation of the stabilizing effect of sucrose was caused by both polyethylene glycols of m.w. 300 and 600 daltons which are rigid only during freezing period but fluid or greasy during the conditions of drying and storage. Similar results were obtained with haemoglobin samples stabilized with

FIG. 1

Methaemoglobin formation (%) during lyophilization of stroma-free haemoglobin with glucose, sucrose and different concentrations of polyethylene glycol (g/l). The concentrations of haemoglobin and of the saccharides were 60 g/l. The methaemoglobin concentrations are expressed as % of the total haemoglobin. The curves relate to the following combinations of glucose (G), sucrose (S) and polyethylene glycols (PEG) before lyophilization of buffered (pH 7·3) haemoglobin solutions: *1*, G + PEG 300; *2*, G + PEG 600; *3*, S + PEG 300; *4*, S + PEG 600; *5*, G + + PEG 1 500 or 4 000 or 6 000; *6*, S + PEG 1 500 or 4 000 or 6 000. The curves *5* and *6* represent average values; the standard deviations did not exceed ±18%



glucose, although a higher methaemoglobin formation was observed here. The critical parts of the curves *1 – 4* (Fig. 1) showing the highest increase of methaemoglobin, corresponded to the same concentrations of polyethylene glycols of m.w. 300 and 600 daltons with both saccharides.

DISCUSSION

The advantage of using polyethylene glycols of different molecular weight as perturbating agents in the above experiments is in their chemical similarity, low reactivity and unlimited solubility in water. In parallel experiments the samples contained equal concentrations of glycol residues although with a different degree of polymerization. With regard to their perturbation effects the polyethylene glycols may be divided into two groups: Effective (low-molecular, fluid or greasy at temperatures above 0°C), and non-effective (high-molecular, rigid at temperatures up to 46°C). During drying and storage of stroma-free haemoglobin above 0°C the molecules of the low molecular polyethylene glycols (when present above a critical concentration) act as plasticizers hindering the formation of a rigid structure stabilized by an integral system of adhesive forces (mostly H-bonds) involving both saccharide–saccharide and saccharide–haemoglobin interactions[3,4]. In such destabilized environment the haemoglobin molecules may behave like partly solubilized and easily undergo intramolecular deformations due to thermal vibrations. Consequently, the denaturation of the globin molecule begins and the quaternary structure favouring the protection of the ferrous form of the heme iron[11] breaks down. This is followed by an accelerated oxidation of haemoglobin to methaemoglobin similarly as if no stabilizators were present. However, an assumed interaction of the dry haemoglobin molecules with the low molecular polyethylene glycols may influence the denaturation of haemoglobin molecules.

A similar explanation based on the consistence of the stabilizators can be used when considering the negligible protecting effect of some other fluid substances[2], *e.g.* glycerine, ethylene glycol, alcohols, *etc.* The perturbation of the stabilizing effect of saccharides by elevating the content of water over 2·4% in the lyophilized haemoglobin samples as observed earlier[4] seems to be also connected with a partial solubilization of the lyophilizate. On the other hand, the molecules of the rigid high-molecular polyethylene glycols imbeded randomly into the supporting saccharide network during freeze drying and storage do not influence the rigid consistence of the system and thus the stabilizing effect of glucose and sucrose upon the haemoglobin molecules remains unchanged.

The results of the present investigation seem to corroborate the idea of a simple mechanical nature of the stabilization of haemoglobin by glucose and sucrose during lyophilization.

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