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An attempt to freeze-dry haemoglobin in the presence of macromolecules

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Macromolecules are widely used as adjuvants in freeze-drying (Regner 1979). In the course of our research into compounds that prevent the oxidation of haemoglobin to methaemoglobin during desiccation (Labrude & Vigneron 1980a,b), we have examined the anti-oxidant effect of some synthetic or natural polymers and proteins of different molecular weights.

Materials and methods

Haemoglobin solution was prepared from blood that had reached the end of its storage time. Washed red blood cells were haemolysed in demineralized water, and the stromata were eliminated by two centrifugations at 25 000 *g* for 30 min, followed by decantation. Finally the solution was dialysed against demineralized water for 15 h at 4 °C. The concentration of haemoglobin was 80 ± 5 g litre^{-1}.

The following compounds were examined: freeze-dried human serum-albumin more than 95% pure (Centre régional de Transfusion sanguine et d'Hématologie de Nancy); the gelatin hydrolysate used in the synthesis of 'Polygéline' (Hoechst); dextrans T 15–20 and 60–90 (Sigma); 10, 20, 40, 70, 110, 150, 250, and 500 (Pharmacia); hydroxyethylstarch (Polysciences); Polyvidone (Prolabo); Ficolls 70 and 400 (Pharmacia); and polyoxyethylene glycol 10 000 (Merck) (preliminary assays with some PEG of molecular weights 400 to 40 000 showed that PEG 10 000 was the best for methaemoglobin). Each compound was dissolved in 5 ml of demineralized water, and the resulting solution was mixed with 5 ml of haemoglobin solution. The final concentrations of macromolecules were 50 and 100 g litre^{-1}. We went on to study the relationship between the oxidation of the haem and the concentration of protecting substance from 5 to 100 g litre^{-1} with the Ficolls which were the only macromolecules initially found to be effective. Freeze-drying was carried out in an experimental Kreel apparatus (manufacturer at Nancy), in the following conditions: freezing at —40 °C, primary desiccation at —10 °C for 16 h, and secondary desiccation at 5 °C for 8 h. The apparatus was opened to the air, and the flasks, stoppered in air, were immediately analysed.

Analyses were made of the colours and dissolution times of the freeze-dried materials in 10 ml of demineralized water; of the oxyhaemoglobin saturation (using the Hemoximetre OSM 2 Radiometer), of the methaemoglobin concentrations (Evelyn & Malloy 1938); and of the haemoglobin dissociation curves (D.C. Analyzer, Radiometer, as described by Teisseire et al (1975).

Results

The unprotected lyophilizates were brown and poorly soluble, with a methaemoglobin content of 49 ± 10% (n = 30) and a corresponding low oxygen saturation; the oxygen-dissociation curve lost its sigmoidal shape and the p50 was less than 10 torr.

The lyophilizates containing the macromolecules had colouration ranging from brown for the most denatured to red-orange. They dissolved rather slowly; the least soluble were those obtained with hydroxyethylstarch. The samples prepared with polyoxyethylene glycol freeze-dried poorly, showed traces of melting and remained turbid after dissolution.

Table 1 summarizes the methaemoglobin and oxyhaemoglobin rates obtained with the different macro-

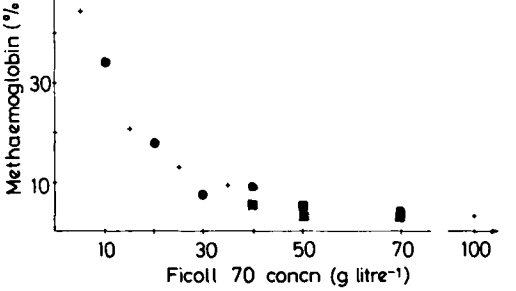

FIG. 1. Relationship between methaemoglobin percent and Ficoll 70 concentrations in freeze-dried haemoglobin samples for three different experiments. Similar results were obtained with Ficoll 400. Without protection, the mean methaemoglobin level is 49%.

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molecules, Ficolls 70 and 400 being the only effective compounds allowing oxidation levels lower than 5% and oxyhaemoglobin saturations reaching 90%.

The dissociation curves obtained with Ficoll 70 were sigmoidal, and the p50s of 14–18 torr were comparable to those obtained with solutions of haemoglobin before freeze-drying. With albumin, dextrans, and hydroxyethylstarch, there was a loss of cooperativity and the p50s were always lower (9–14 torr).

The relationship between the methaemoglobin level and Ficoll 70 concentration is given Fig. 1. Satisfactory results were obtained from 30 g litre^{-1}.

Table 1. Oxyhaemoglobin and methaemoglobin concentrations in haemoglobin samples freeze-dried with the various polymers (n = 2).

		Concentration			
		50 g litre^{-1}		100 g litre^{-1}	
Macromolecule	mean mol. wt	MetHb %	HbO_2 %	MetHb %	HbO_2 %
Albumin	69 000	30	66	28	68
Hydrolysed gelatin	12 000	26	71·5	20	78
Dextran 10	10 000	13·5	74	8	81
,, 15–20	15–20 000	17	67	7	82
,, 20	20 000	18·5	67	11	77
,, 40	40 000	13·5	76	11	77
,, 70	70 000	23	63	15	72
,, 60–90	60–90 000	21	64	14	79
,, 110	110 000	20	62	20	66
,, 150	150 000	22	61	22	65
,, 250	250 000	20	66	22	65
,, 500	500 000	24	72	23	67
Hydroxyethyl starch	400 000	15	79	11·5	82·5
Polyvidone	40 000	22	76	20	78·5
Ficoll 70	70 000	4·2	90	3·2	90·5
,, 400	400 000	6	82	4	80
PEG 10 000	10 000	23	65	25·5	59

Logarithmic plots (log MetHb % vs Ficoll g litre^{-1}) between 5 and 50 g litre^{-1} are almost linear and allow a regression line to be drawn: $y = -0{\cdot}0244\,x + 1{\cdot}76$ ($n = 20$; $r = -0{\cdot}982$; $P < 0{\cdot}001$).

The results obtained with Ficoll 400 were less satisfactory the line being defined by $y = -0{\cdot}0198\,x + 1{\cdot}64$ ($n = 14$; $r = -0{\cdot}935$; $P < 0{\cdot}001$). The amounts of oxyhaemoglobin plus methaemoglobin reach respectively 94 ± 4% and 88 ± 6% for all the concentrations of Ficoll 70 and 400 studied.

Discussion

Of the macromolecules examined in identical conditions, only the Ficolls enabled haemoglobin to be satisfactorily freeze dried. This result confirms our earlier experiment (Labrude et al 1976a), and those of Pristoupil et al (1978) with dextran 70. The poor results obtained with polyoxyethylene-glycols may be the result of their rather low melting points.

Ficolls, which are synthetic polymers, to our knowledge have not been used in the freeze-drying of haemoglobin, but the lack of elucidation of their chemical structure makes the understanding of these results difficult. Our experiments show that the molecular weight is not important because a large molecule like

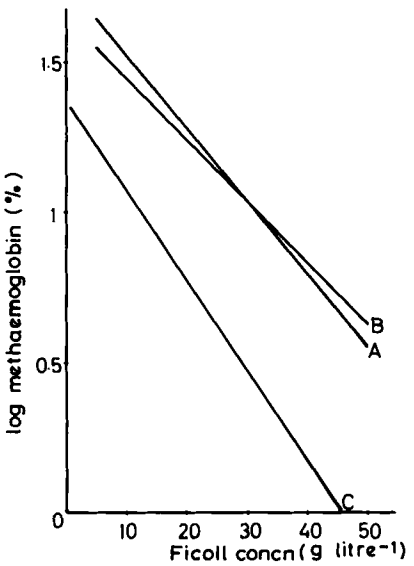

FIG. 2. Relationship between the logarithm of methaemoglobin percent and the concentration of Ficoll 70 (A) and Ficoll 400 (B). The C line is obtained with sucrose in the same conditions. For other details, see text.

Ficoll 400 is better than albumin or dextran 10. Such compounds cannot enter the haem-pocket to reach the iron during the desiccation. Stereospecific interactions involving suitably directed hydroxyl functions of such compounds and water may stabilize the hydration shell around the haemoprotein during freeze-drying (Suggett 1975; Finney 1977; Pristoupil et al 1978).

Although the polymeric nature of Ficoll is unknown, a possible unit is

$$\left[\text{-sucrose-O-CH}_2\text{-CH(OH)-CH}_2\text{O-}\right]_n$$

where the hydroxyl groups are almost as numerous as in the sucrose monomer which is very effective towards haemoglobin during freeze-drying. In studies in the same conditions as the Ficolls, sucrose effected a methaemoglobin level below 10% at a concentration of 12 g litre^{-1} (30 g litre^{-1} for Ficoll 70) and gave a line $y = -0{\cdot}0293\,x + 1{\cdot}36$ ($n = 28$, $r = -0{\cdot}897$, $P < 0{\cdot}001$) between 2 and 50 g litre^{-1}.

The polymerization of sucrose with the disappearance of some hydroxyl functions appears only to induce a decrease of the lyoprotective activity of the monomer. This fact, not noticed with dextrans that also contain many hydroxyl functions and proceed from glucose, a good protector (Labrude et al 1976b), confirm that it remains difficult to relate the stabilization of haemoglobin during freeze-drying to a definite chemical structure.

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A fluorimetric investigation of the binding of drugs to lysozyme

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The binding of drugs in tears influences the pharmacoinetics of drugs used for ophthalmic purposes. The major proteins in human tears are albumin, globulins and lysozyme. Although there is much literature on the binding of drugs to albumin (Meyer & Guttman 1968; Vallner 1977) and lipoproteins (Vallner & Chen 1977) there is little on the binding of drugs to lysozyme (Chrai & Robinson 1976). This is partially due to the small molecular weight of the protein (14 400) making dialysis and ultrafiltration unreliable, due to leakage of the small quantities of protein through the cellulose membranes frequently used.

Lysozyme is a basic protein and is able to bind to anions of inorganic origin. It tends to associate into dimers and higher polymers, as the pH and concentration are increased and the temperature decreased. At the concentrations, ionic strength and pH of tears, it exists entirely as the monomer.

Three of the six tryptophan residues in lysozyme seem to be located near the binding site for substrates on lysozyme (Johnson & Phillips 1965; Phillips 1966). Residues 62 and 108 are readily accessible to specific oxidizing agents (Hayashi et al 1965; Imoto et al 1971) and appear to be in an aqueous environment whereas residue 63 is buried in the interior of the molecule (Hayashi et al 1965). This together with a low tyrosine content and minimal (Johnson & Phillips 1965; Phillips 1966) lysozyme to tryptophan energy transfer means that the fluorescence of emission of lysozyme is readily quenched by certain inorganic ions including nitrate and iodide (Altekar 1977) as well as by substrates such as tri- and di-*N*-acetyl-D-glucosamine (Lehrer & Fasman 1967). Such observations make the interaction of certain drugs and lysozyme by fluorescence a possibility.

Hen lysozyme, twice crystallized, batch numbers D2-3246 and E2-3359 were obtained from Schwarz/Mann, Orangeburg, New York. Solutions of lysozyme were aged for at least 6 h (Attallah et al 1968) and filtered through a 45 μm Millipore filter (Millipore Bedford, Ma). Sulphisoxazole, methyl paraben, ethylparaben and propylparaben, cortisone, hydrocortisone were all obtained from Sigma, St Louis, Mo, chloramphenicol from Aldrich, Milwaukee, WI; phenyl mercuric acetate and nitrate from Eastman Kodak, Rochester, N.Y. Sulphathiazole sodium from Pfaltz and Blauer, Stanford, CT, atropine sulphate and phenobarbitone from Mallinkrodt, St Louis, MO; chlorobutanol from Fischer, Fair Lawn, NJ and sulphathiazole from Nutritional Biochemical Corp., Cleveland, OH. Sulphaethidole was a gift of Smith Kline and French Laboratories, Philadelphia, PA. All other materials were reagent grade. Deionized water was used.

Fluorescence was measured at 22 °C using a Perkin Elmer MPF-44A spectrofluorimeter (Perkin-Elmer, Norwalk, CT). The excitation wavelength was 305 mm with a slit width of 6 nm and emission was scanned using a slit width of 7 nm. The emission peak was near 337 nm for lysozyme and lysozyme-drug solutions. The solutions were prepared in 0·1 M phosphate, the pH being adjusted with a Beckman Digital pH meter model 4500 (Beckman, Fullerton, CA).

Relative fluorescence was plotted as a function of drug concentration at fixed lysozyme concentration. The terminal slope was extrapolated to zero drug concentration, and the intercept reading subtracted from a given value on the terminal slope line, to correct for absorbance of the drug (Attallah & Lata 1968; Velick et al 1960). This value was then subtracted from the value on the experimental curve to give the corrected curve (Fig. 1). No drug caused a significant shift in the emission spectrum of the lysozyme. The binding constants (Table 1) determined at pH 7·4 are calculated assuming a 1 : 1 interaction, from the equation

$$L + D \rightleftharpoons LD$$

$$\text{and } K = \frac{[LD]}{[L][K]}$$

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