MODIFICATION AND *IN VITRO* EVALUATION OF STROMA-FREE HAEMOGLOBIN

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ABSTRACT

Stroma-free haemoglobin solution was prepared from outdated blood. Red blood cells were washed with normal saline, and lysed with distilled water and ether. The lysate was clarified by centrifugation and all the stromal particles were removed by filtration through 0.22 μ millipore membrane. Pyridoxal phosphate was added in a 4:1 molar ratio to deoxygenated stroma-free haemoglobin to link covalently across the polyphosphate binding site of the haemoglobin tetramer by reduction with sodium borohydride under nitrogen. Excess reagents were removed by dialysis. Subsequent cross-linking was done using glutaraldehyde in the molar ratio of 7:1. Excess glutaraldehyde was removed by dialysis and sterility was assured by final filtration through 0.22 μ millipore filter. Favourable *in vitro* results indicate better *in vivo* performance.

INTRODUCTION

EXPERIMENTS on replacement of blood by a substitute began as early as in 1859[1]. In the development of haemoglobin solution as a blood substitute, two predominant limitations of the present product are higher oxygen affinity and shorter intravascular retention time of free haemoglobin as compared to intracellular haemoglobin. Several investigators have devised different approaches including chemical modification to overcome these two limitations. Benesch *et al*[2] demonstrated that pyridoxal 5′ phosphate can be attached to the N-terminal valine of haemoglobin B chains by forming a Schiff base and that the resulting pyridoxalated haemoglobin has an oxygen affinity lower than the unmodified haemoglobin. It is suggested that polymerization of the pyridoxylated haemoglobin will increase the intravascular retention time[3].

The preparation of stroma-free haemoglobin solution in our laboratory is evolved through several phases. In an earlier publication[4], the preparation of haemoglobin solution by a method of crystallization was described. This paper reports a simpler method for large scale production and experimental condition for pyridoxylation and subsequent polymerization of human haemoglobin.

MATERIALS AND METHODS

Stroma-free haemoglobin solution was prepared from outdated human blood obtained from the Blood Bank of this Institute. All experiments were conducted in a sterile atmosphere under a laminar flow system. The red cells were centrifuged at 3000 rpm at 4°C for 10 min and washed with normal saline to remove all plasma components. These cells were lysed using distilled water and ether and overnight freezed. After lysis, the larger stroma particles were removed by filtration and the stroma was spun down into a tight button by repeated centrifugation at 12,000 rpm at 4°C, till the supernatant was clear of all stroma. The stroma was completely removed by subsequent filtration through millipore pre-filters AP15, 0.45 μ and 0.22 μ respectively. The solution was subjected to dialysis against normal saline for maintenance of normal electrolytes. Sterility of the solution was further assured by a second filtration through 0.22 μ millipore filter. The solution was further modified by pyridoxylation with pyridoxal phosphate[2] followed by polymerization with glutaraldehyde.

Polymerized pyridoxalated haemoglobin (PP Hb) solution was assayed for several parameters. Haemoglobin was measured by Cyan-methaemoglobin method[5] and methaemoglobin was determined by the method of Malloy and Evellyn[6]. The electrolytes, sodium and potassium, were assayed by flame photometry and oncotic pressure using a colloid osmometer (Wescor 4400). The pH was measured in a digital pH meter. Cellulose acetate electrophoresis was done using Cosmos electrophoretic apparatus and the absorbancy curves between 650 and 350 nm examined using a spectrophotometer (Hitachi 220). The ability of the solution to carry oxygen reversibly was determined by controlled oxygenation and

deoxygenation made possible by a manometric apparatus designed by our Institute and measuring oxygen pressure by using a blood gas analyser (Radiometer, Copenhagen). The solution was tested for the presence of blood groups. The effect of this solution on platelet aggregation was assessed[7] and the effect on coagulation was studied.

RESULTS AND DISCUSSION

The earlier attempts to use haemoglobin solution as a blood substitute were limited by problems such as renal damage and rapid removal from the circulation[8,9]. To overcome this, Chang prepared the first artificial red blood cells by microencapsulating haemoglobin solution[10,11] and also by cross-linking haemoglobin using glutaraldehyde[12]. Although they could carry oxygen, the affinity for oxygen was high. These major drawbacks of high oxygen affinity and low intravascular retention time were overcome by pyridoxalation and polymerization. During the procedure haemoglobin becomes diluted, but can be concentrated to the desired extent. Removal of all stromal particles facilitates the maintenance of normal oncotic activity even at higher concentrations of haemoglobin.

All the essential parameters like haemoglobin concentration, pH, electrolyte concentration and oncotic activity were similar to those of the whole blood. The final solution was devoid of any blood group substances. Figure 1 shows the absorbancy curves between 350 and 650 nm of unmodified (A) and pyridoxalated polymerized haemoglobin (B). The spectra are identical and represent the spectrophotometric curve of oxyhaemoglobin with absorption maxima at 414, 540 and 576 nm. The present solution was able to carry oxygen and give off oxygen as evidenced by PO_2 measurements in a blood gas analyser at frequent intervals of oxygenation and deoxygenation at a constant rate. As far as the electrophoretic pattern was concerned, the unmodified haemoglobin solution showed minor peaks associated with traces of haemoglobin A_2 and carbonic anhydrase. The pyridoxalated haemoglobin solution exhibited a single peak as can be seen from the densitometric scan (figure 2). Broadening of the peak may be either due to the modification caused by polyethylene glycol[13] used for adjusting concentration or due to the presence of both modified and unmodified solution in different ratios after modification which can be resolved by chromatography. Table 1 shows no significant difference between the

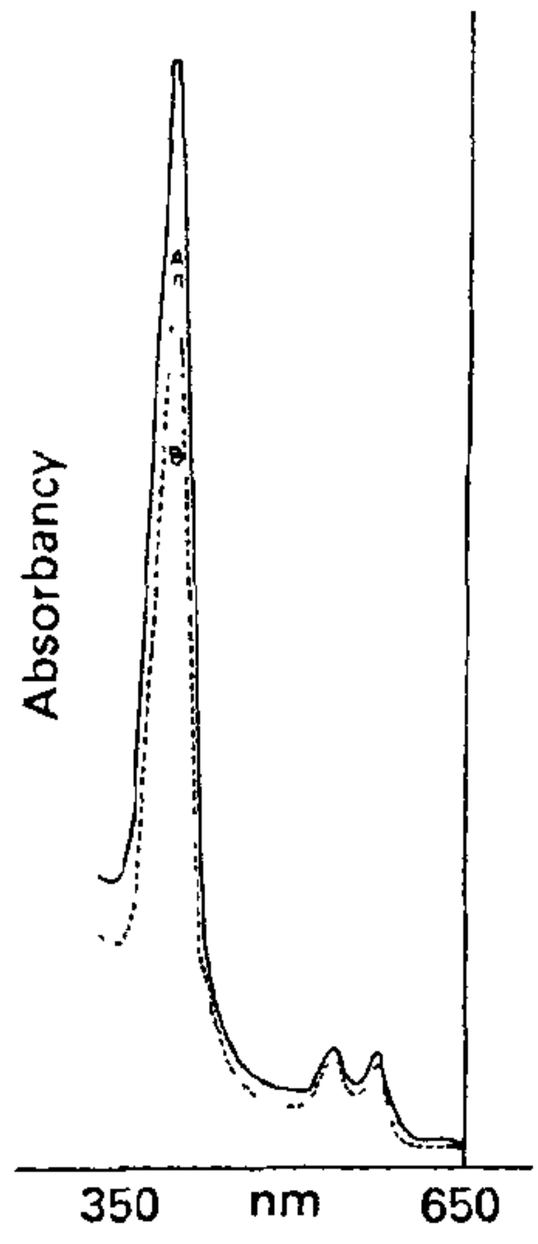

Figure 1. Absorbancy curves of unmodified (A) and modified (B) haemoglobin.

haemoglobin solution and albumin control in the coagulation screening test. Haemoglobin solution neither induced platelet aggregation nor inhibited platelet aggregation to added ADP.

The *in vitro* characteristics of the modified and unmodified haemoglobin were essentially the same

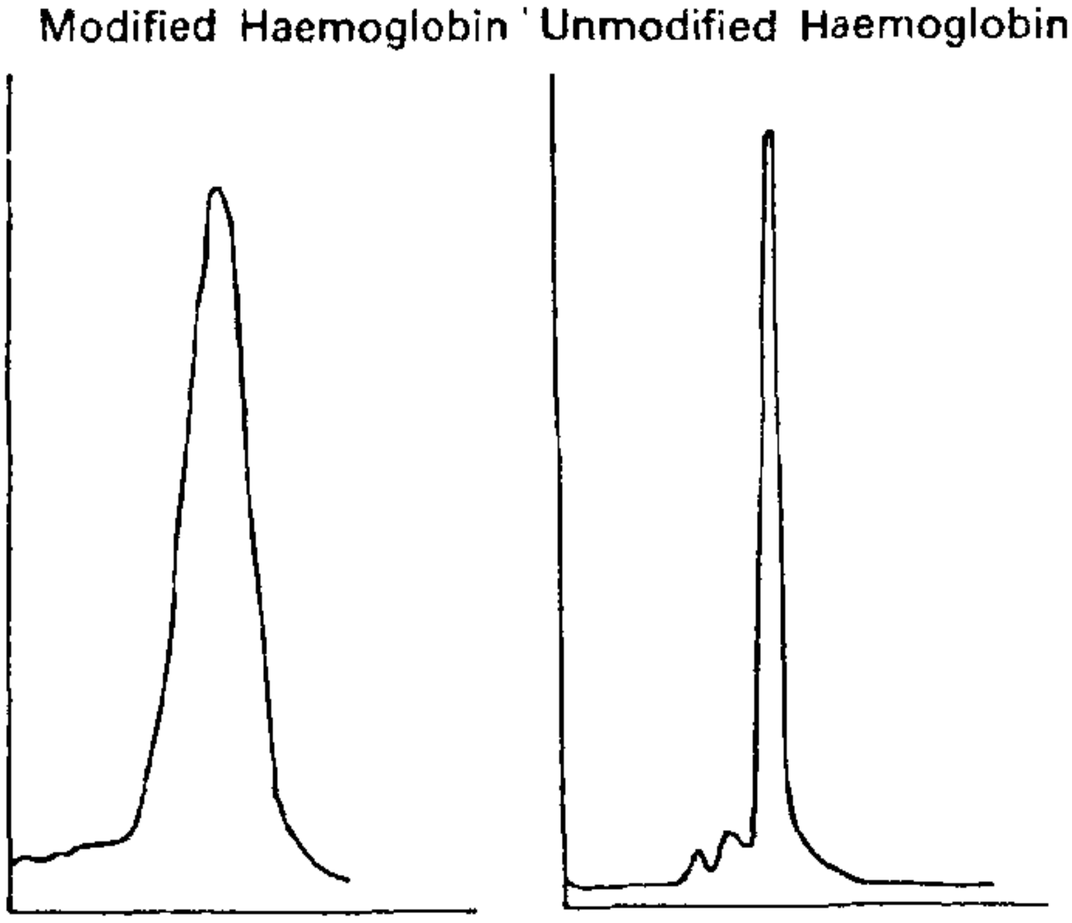

Figure 2. Densitometric scan of cellulose acetate electrophoresis of modified and unmodified haemoglobin.

Table 1 *Effect of PPHb on the coagulation system*

	Pro-thrombin time (sec)		Partial thrombo plastin time (sec)		Thrombin time (sec)	
	0 min	30 min	0 min	30 min	0 min	30 min
Plasma	14		45		15	
Plasma:Albumin 3:1	14	14	44	34	14	14
Plasma:PPHb 3:1	17	17	46	38	18	15
Plasma:Albumin 1:1	15	19	44	38	16	17
Plasma:PPHb 1:1	18	19	42	37	17	17
Plasma:Albumin 1:3	17	19	49	53	13	14
Plasma:PPHb 1:3	24	25	–	43	15	17

in their composition except for the methaemoglobin content which was higher in the modified haemoglobin solution[14,15]. The special structure of haemoglobin molecule, made up of four subunits with different oxygen affinities depending upon the degree of oxygenation, gives the oxygen dissociation curve, a nonlinear, sigmoidal shape and in the presence of the effector 2,3 DPG, ensures a substantially better oxygen exchange in the physiologic range. Pyridoxal 5 phosphate can function as an effector, which can take over the role of intraerythrocyte 2,3 diphosphoglycerate, with the added advantage of being more firmly bound to the haemoglobin by a covalent bond[16,17]. The problem f rapid elimination' of haemoglobin solution was solved by intermolecular cross-linking of haemoglobin[3,18,19].

Animal studies are in progress to assess the intravascular retention time and other *in vivo* characteristics of the modified haemoglobin solution in comparison with the unmodified solution. In view of the improved *in vitro* characteristics of this solution, as well as the achievement of normal blood electrolyte content, oncotic activity and no adverse effects on coagulation and aggregation, an increased efficacy and tolerance can be expected *in vivo*.

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