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# HEMOGLOBIN-INULIN CONJUGATE AS AN OXYGEN CARRYING BLOOD SUBSTITUTE

Keiji Iwasaki, Katsumi Ajisaka and Yuji Iwashita

Ajinomoto Co., Inc., Central Research Laboratories
1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi, 210 Japan

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Pyridoxylated hemoglobin was modified with the activated ester of inulin. From the oxygen equilibrium curve, this conjugate was estimated to have about fivefold oxygen carrying capacity compared with a free hemoglobin. The half disappearance time of this conjugate in the circulation of a rat was 21 hours in contrast to 3 hours of a free hemoglobin. The oncotic pressure and the viscosity can be adjusted nearly equal to those of whole blood. This conjugate can be concluded to have sufficient properties for use as a blood substitute.

Many researchers have tried to use a hemoglobin solution as a blood substitute (1,2). But this approach has several limitations. When the hemoglobin solution is infused into a blood vessel, it is rapidly excreted through glomeruli to urine. And the oxygen affinity is so high that it can not deliver enough oxygen to tissues. In order to overcome these limitations, modified hemoglobins have been studied by several groups (3-6). But the modified hemoglobin with sufficient properties for clinical use has not been prepared. As one of the approaches to artificial blood, the authors modified hemoglobin with inulin, polyfructan. In this communication we would like to report the characteristics of the pyridoxylated hemoglobin-inulin conjugate as a potential candidate for a blood substitute.

## Materials and Method

A stroma free hemoglobin solution was prepared from outdated human blood by the method of Savitzky et al (7), and the hemoglobin was pyridoxylated by the procedure of Benesch et al (8). Inulin was activated as follows. First, inulin was succinylated by reacting with succinic anhydride in N,N-dimethylformamide at

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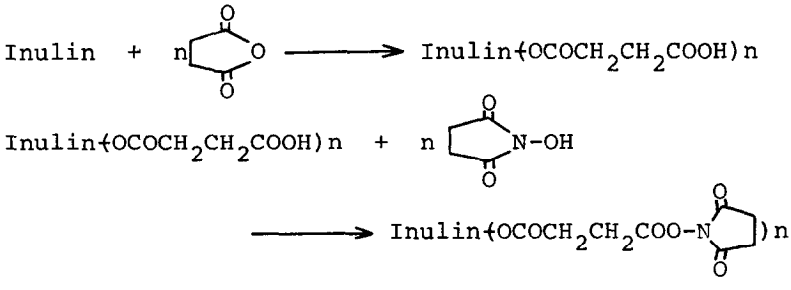
100°C for 2 hours. And then the succinylated inulin was allowed to react with N-hydroxysuccinimide by the help of dicyclohexylcarbodiimide in N,N-dimethylformamide at room temperature overnight. The pyridoxylated hemoglobin was allowed to react with the activated inulin in 0.1M tris buffer (PH=7.0) at 4°C for one hour. The reaction mixture was analyzed with a JASCO Trirotor HPLC apparatus equipped with a TSK G3000 SW column (7.6 x 600 mm). The modified hemoglobin solution was purified with an Amicon PM 30 membrane filter until the unreacted inulin and other low molecular weight compounds were detected no more. The oxygen equilibrium curves were measured by the method of Imai (9). Molecular weight was determined with a low angle laser light scattering photometer LS-8 (Toyo Soda Co., Ltd.) Viscosity was measured with an Uberode type viscometer and oncotic pressure was measured with 4100 colloid osmometer (Wescor Inc.). The half disappearance time in the circulation was determined as follows; male Spraque Dawley rats (200-300g) were anesthetized by ether. They were exchange-transfused to 72-75 percent blood replacement with a hemoglobin-inulin solution through a polyethylene catheter inserted in a tail vein. The rate of the transfusion was 0.2-0.25 ml/min.. Phlebotomy was carried out through a polyethylene catheter inserted in a tail artery. Aliquots (0.5 ml) of the blood were collected at 1,2,4,8,12 and 24 hours after the exchange-transfusion and the concentration of the infused hemoglobin-inulin conjugate in the plasma was determined by the cyanomet-hemoglobin method after centrifugation. The half disappearance time was obtained from semilogarithmic plots of the concentration against the time after the exchange-transfusion.

## Results and Discussion

In order to bind inulin to hemoglobin, some of the primary hydroxy groups of inulin were esterified with succinic anhydride, and then the carboxylic groups attached to inulin were activated by a reagent used for peptide synthesis such as N-hydroxysuccinimide as shown below.

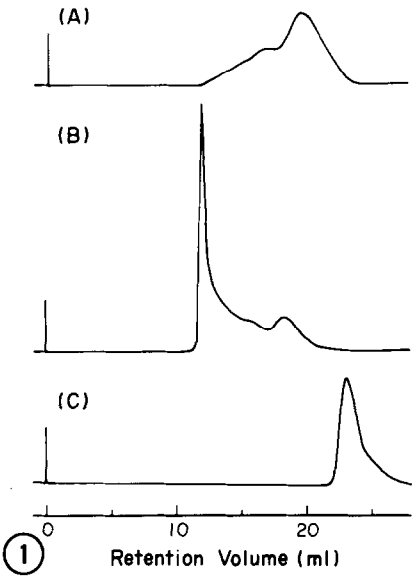



The number of activated groups in one inulin molecule depends on the molar ratio of succinic anhydride to inulin in the reaction mixture. Molecular weight of the hemoglobin-inulin conjugate depends not only on the molar ratio of the activated

inulin to hemoglobin but also on the number of the activated groups in one inulin molecule.

Figure 1 shows the high pressure liquid chromatograms of hemoglobin-inulin conjugates. In each case tenfold excess moles of the activated inulin were added to a hemoglobin solution. The result indicated that when the inulin which had a small number of activated groups was used, the reaction product was the low molecular weight hemoglobin-inulin conjugate (Figure 1 A). On the other hand, when the inulin which had a large number of activated groups was used, the reaction product was the high molecular weight conjugate (Figure 1 B). For example, the molecular weight of the main peak in Figure 1 A was estimated to be 82,000 by the laser light scattering method, and that of the shoulder peak in this chromatogram was 170,000. The molecular weight corresponding to the void peak in Figure 1 B was estimated to be more than 300,000. The high molecular weight hemoglobin-inulin conjugate in Figure 1 B must have highly crosslinked structure. The product shown in Figure 1 A is assumed to be non-crosslinking structure, and it was used in the following experiments.

The oxygen affinity of modified hemoglobins are usually higher than that of free hemoglobin. In order to decrease the oxygen affinity of hemoglobin-inulin conjugate, the authors combined hemoglobin with pyridoxal 5'-phosphate before the modification with inulin, because the oxygen affinity of the hemoglobin can be decreased by combining with pyridoxal 5'-phosphate (8). Figure 2 shows the oxygen equilibrium curve of the pyridoxylated hemoglobin-inulin conjugate. The $P_{50}$ value, the oxygen partial pressure necessary to produce 50 percent saturation of hemoglobin, of the conjugate (17.3 mmHg at 37°C PH=7.40) is higher than that of free hemoglobin (14.3 mmHg),



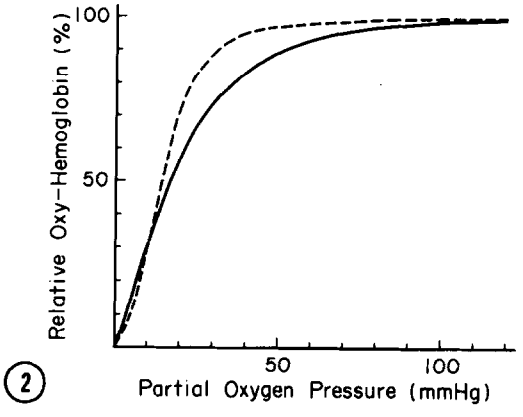




Figure 1 High pressure liquid chromatograms of the pyridoxylated hemoglobin-inulin conjugates (A,B) and free hemoglobin (C) TSK G3000 SW column was used with 0.1M phosphate buffer (PH=7.0) at 1.0 ml/min..

Figure 2 Oxygen equilibrium curves of the pyridoxylated hemoglobin-inulin conjugate (——) and free hemoglobin (----) in 0.1M phosphate buffer (PH=7.40) at 37°C

but it is still lower than that of whole blood. In many cases oxygen delivering capacity is discussed based on $P_{50}$ value. But we should like to suggest that the important factor is the difference between the oxyhemoglobin content in the vein and the artery. The amount of oxygen corresponding to the difference between oxyhemoglobin content in the vein and that in the artery will be supplied to tissues. The partial oxygen pressure in the vein is 40 mmHg and that in the artery is 100 mmHg under normal conditions. From Figure 2, in the case of free hemoglobin about 100 percent of hemoglobin is oxyganated at 100 mmHg oxygen partial pressure, and 95 percent is oxygenated at 40 mmHg oxygen partial pressure. It means that the only 5 percent of the free hemoglobin delivers oxygen to tissues. On the other hand in the case of the pyridoxylated hemoglobin-inulin conjugate, 98 percent of hemoglobin is oxygenated at 100 mmHg oxygen partial pressure

and 83 percent of hemoglobin is oxygenated at 40 mmHg. Therefore 15 percent of the pyridoxylated hemoglobin-inulin conjugate works to deliver oxygen to tissues. Although the difference of $P_{50}$ values between free hemoglobin and this conjugate is rather small, this conjugate can off-load threefold excess amount of oxygen. And this conjugate has another advantage. Usually many researchers used the solution of 6 percent hemoglobin to keep the oncotic pressure equal to that of whole blood, but the concentration of the present preparation was able to be increased up to 10 hemoglobin percent preserving the oncotic pressure and the viscosity, 24.3 mmHg and 1.89 cst., respectively, as shown in Table 1. In total the oxygen off-loaded to tissues by this conjugate can be said to be fivefold excess volume of oxygen compared with hemoglobin.

The disappearance of the hemoglobin and the modified hemoglobin in the circulation is shown in Figure 3. When the hemoglobin-inulin conjugate solution was used to exchange-transfuse 72-75 percent of the total blood volume of rats, the half disappearance time of the conjugate is 21 hours in contrast to 3 hours of the free hemoglobin. This value is long enough for using this hemoglobin-inulin conjugate as a blood substitute. Hemoglobinuria was scarcely observed in the case of

Table 1 Characteristcs of the pyridoxylated hemoglobin-inulin conjugate 1)

| | |
|---|---|
| Concentration (percent) | 10.0 |
| Oncotic Pressure (mmHg) | 24.3 |
| Viscosity (cst.) | 1.89 |
| $P_{50}$ (mmHg) | 17.3 |
| Half Disappearance Time (hrs.) | 21 |

1) Oncotic pressure and viscosity were measured at 25°C and 37°C, respectively, and $P_{50}$ was measured at 37°C in 0.1M phosphate buffer (PH=7.40).

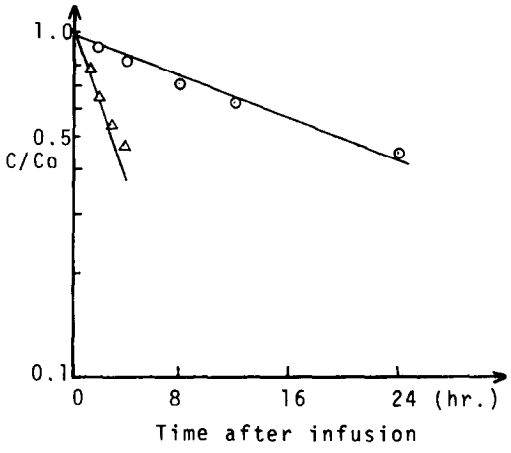



Figure 3 The disappearance of the hemoglobin-inulin conjugate ( o ) and free hemoglobin ( △ ) in the circulation

the hemoglobin-inulin conjugate, whereas the severe hemoglobinuria was observed in the case of free hemoglobin. The present results show that the pyridoxylated hemoglobin-inulin conjugate has sufficient properties for use as an oxygen carrying blood substitute.

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