SOME FUNCTIONAL PROPERTIES OF SUCCINYLATED BOVINE HEMOGLOBIN

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SUMMARY: Bovine hemoglobin was reacted with succinic anhydride at 20°C and a pH of 8.0 to 9.0 to form succinylated hemoglobin. The chemically modified hemoglobin was used to the maximum extent (100%) for the study on changes in functionality. The absorption spectrum of succinylated hemoglobin was close to that of cytochrome c both in the oxidized and reduced forms. Hemoglobin with succinylation became heat-stable at a neutral pH. No aggregation of the protein occurred by boiling at pH 7.0. Hemoglobin did not dissociate into subunits with succinylation. The precipitation of succinylated hemoglobin took place at pH 4.7 to 2.7. It seems that oxygen is no longer held by succinylated hemoglobin. Nitrosyl hemochrome of succinylated hemoglobin was formed in reaction with nitrite in the presence of ascorbic acid. Spray-dried powder of succinylated hemoglobin was prepared with no change in the properties mentioned above.

INTRODUCTION: Although blood from animals slaughtered in inspected slaughterhouses is a major potential protein resource, the utilization of animal blood appears to be limited in meat products (Wismer-Pedersen, 197[illegible] Slinde and Martens, 1982; Caldironi and Ockerman, 1982; Mielnik and Slinde 1983), animal food and fertilizers (Forrest et al., 1975; HPRD Committee, 1982). Blood contains about 18 % protein, and more than half of the protein (11 %) is hemoglobin (Sato, 1981).

Compared with the plasma protein, hemoglobin is troublesome, with black color and usually a characteristic odor. Trials of removing globin from heme moiety (Tybor et al., 1975; Sato et al., 1981) and of preparing hydrolyzed products (Drepper at al., 1981; Kodama et al., 1984; Nakamura et al., 1986) have been carried out in search of a suitable way to utilize hemoglobin for human foods.

One approach to the controlled alteration of protein functionality is the chemical modification of protein by N-acylation with succinic anhydride or acetic anhydride (Groninger, 1973). Many studies on N-acylation of various proteins (fish, soy, cotton-seed flour, myofibrillar proteins, sunflower and pea) revealed the improved functional characteristics of the proteins, such as solubility, foaming capacity, emulsification and water absorption (Groninger, 1973; Franzen and Kinsella, 1976; Childs and Park, 1976; Choi et al., 1981; Eisele and Brekke, 1981; Kabirullah and Wills, 1982; Johnson and Brekke, 1983). Klotz and Keresztes-Nagy (1963) used succinylation to demonstrate complete dissociation of hemerythrin, marine oxygen-carrying pigment, into subunits by the introduction of negative charge on the protein.

The purpose of this study is to chemically modify hemoglobin with succinic anhydride and to investigate resultant changes in functionality. A further objective is to prepare a dry product of succinylated hemoglobin which may find itself a material available for further study.

MATERIALS AND METHODS: Bovine hemoglobin was purchased from Sigma Chemical Co. (Type II) and Tokyo Kasei Kogyo Co. (crude one). Succinylation was carried out according to the procedure of Groninger (1973) with a

slight modification. Hemoglobin was reacted with succinic anhydride at 20°C and pH 8.0 to 9.0 over a period of 1 to 2 hr. The extent of succinylation of hemoglobin was estimated by the method of Kakade and Liener (1969). The molar absorption coefficient of trinitrophenyl-lysine, $1.46 \times 10 M^{-1}$, was used for calculation. The product in which all the lysines were succinylated was employed for the subsequent study after dialysis against distilled water at 4°C for 48 hr. Hemoglobin from Sigma Chemical Co. was used, if not stated specifically.

Spectrophotometric measurements were carried out with a Hitachi 220 spectrophotometer at room temperature (20°C). Thirty seconds were required to scan 60 nm.

Solubility of succinylated hemoglobin was determined at various pH values. The pH of the protein solution was adjusted with 2 M HCl or NaOH. 50 ml of 0.1 % protein solution was centrifuged at 1600 x g for 15 min. pH-solubility profile was obtained by measuring absorbance of the solution at 596.5 nm, because the magnitude of absorbance at 596.5 nm did not change with varying pH of succinylated hemoglobin solution (see text).

A column (2.6 x 70 cm) for gel chromatography was packed with Toyopearl HW-50S purchased from Toyo Soda Kogyo Co. An elution buffer contained 0.2 M NaCl and 25 mM phosphate at pH 5.70. 1 ml of protein solution was loaded onto the gel, and eluted at a flow rate of 40 ml/hr at 10°C. 5.5 ml of the effluent was collected a tube and measured absorbance at 280 nm. The proteins used for gel chromatography were purchased from Sigma Chemical Co.; hemoglobin (Bovine Type II), myoglobin (Horse heart Type III) and cytochrome c (Horse heart Type III). The identification of the heme proteins in the effluent was determined by measuring absorption spectrum with or without adding solid sodium dithionite.

The reaction of succinylated hemoglobin with nitrite was examined in a medium containing 0.1 % succinylated hemoglobin, 0 to 50 mM sodium nitrite, 0 to 50 mM sodium ascorbate and 50 mM phosphate at pHs 5.20 to 6.95. The reaction mixture was incubated at room temperature for 24 hr. The reaction product was extracted with 75 % acetone, in which solution heme pigments both from native and denatured nitrosomyoglobins were equally extracted (Sakata and Nagata, 1983). The acetone extraction was carried out in a dark room at 0°C for 10 min, and the extract was filtrated through Toyo filter paper No. 6, then followed by spectrophotometric measurement.

Hemoglobin from Tokyo Kasei Co. was used for the preparation of a spray-dried product. At a ratio of hemoglobin to succinic anhydride (2 to 1), succinylated hemoglobin was prepared. The pH value of the succinylated hemoglobin solution (4 liter of 3.5 % protein solution) was lowered to 4.5 with 2 M HCl to form the precipitate. The precipitate was centrifuged in a basket centrifuger (Type SYK-3800-15A, Sanyo Rikagaku-Kikai Seisakusho, Tokyo, Japan) through a cotton flannel. The precipitate was suspended in 2 liter of distilled water and filtrated through Toyo filter paper No. 2. Succinylated hemoglobin washed was resolved in 2 liter of distilled water by adjusting pH to 7.0 with 2 M NaOH. Spray drying was conducted with a spray dryer (Model OC-16, Ohkawara Kakohki Co., Tokyo, Japan) in the following condition: air inlet temperature, 120°C; air outlet temperature, 98°C; atomizer, 16000 rpm; flow rate, 1 l/hr.

RESULTS AND DISCUSSION: The spectrum patterns of 100% succinylated hemoglobin solution are shown in Figure 1 both in the oxidized and reduced

forms. The spectra did not change by the addition of ferricyanide (Left panel). It had peaks at 410 and 529 nm and a shoulder at 562 nm. This spectrum was almost the same as that of cytochrome c in the oxidized form (Izumi et al., 1982). On the other hand, the spectra of succinylated hemoglobin solution revealed peaks at 532 and 560 nm by the addition of dithionite. It had also a strong resemblance to the spectrum of cytochrome c in the reduced form, although the peaks shifted about 10 nm to a longer wavelength. The same spectrum changes were obtained with 98.7 % succinylation of hemoglobin from Tokyo Kasei Kogyo Co. Succinylation did not affect the redoxing ability of hemoglobin.

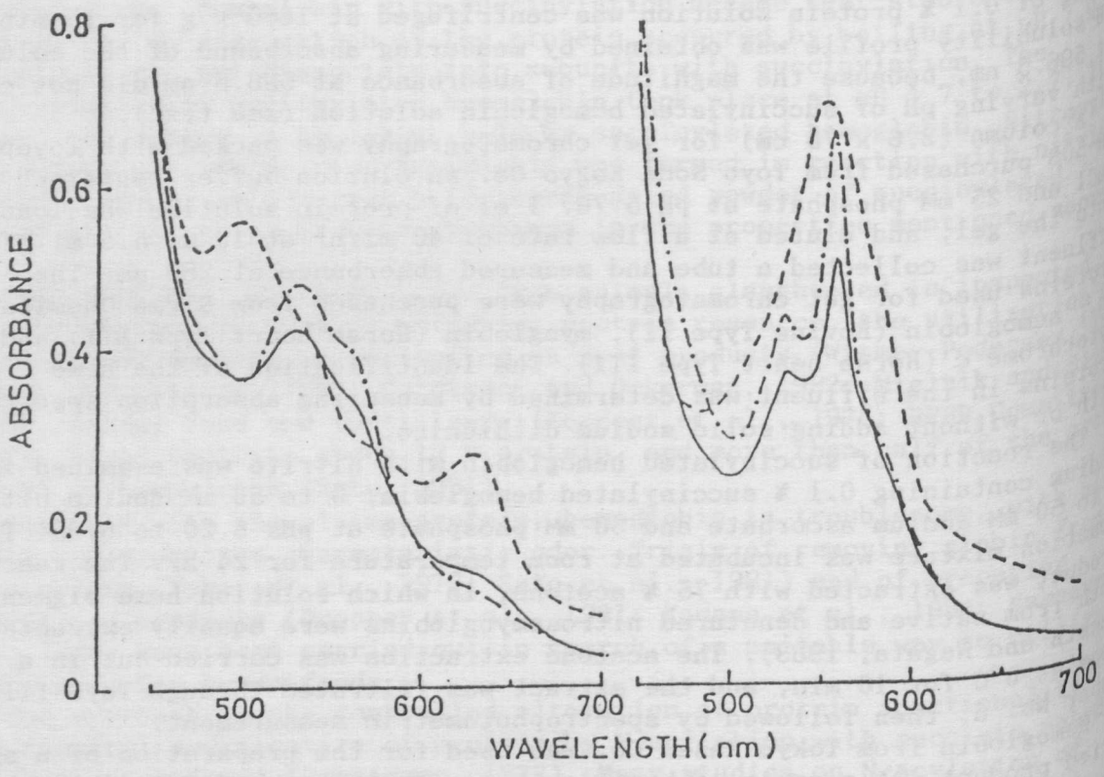

Figure 1.-Absorption spectra of hemoglobin and succinylated hemoglobin. The medium contained 50 mM phosphate and 0.1 % hemoglobin or succinylated hemoglobin. Left panel- scanning was conducted after the addition of ferricyanide to hemoglobin solution, and before and after the addition of ferricyanide to succinylated hemoglobin solution. Right panel- scanning was conducted after the addition of dithionite. – – –: Hemoglobin at pH 5.85, ———:Succinylated hemoglobin at pH 5.85, —•—: Succinylated hemoglobin at pH 6.95.

With the increasing extent of succinylation of hemoglobin (Table 1), the spectrum changes followed was that the absorbance at 529 and 562 nm increased, while the absorbance at 504 nm decreased. The ratio of the absorbance at 529 to 504 nm at pH 5.85 was 0.818, 0.833, 0.845, 0.928 and 1.127 with succinylation of 0, 23.5, 34.9, 74.3 and 100 %, respectively.

Table 1.-Extent of modification of succinylated hemoglobin

g Anhydride/ g Protein		Reactive ε -amino groups	
		μ mole/ g protein	% modified
SA	0.0	629.45 (563.70)	0.0
	0.3	481.51 (22.23)	23.5 (96.1)
	0.5	409.59 (5.14)	34.9 (99.1)
	1.0	161.64 (7.36)	74.3 (98.7)
	1.4	0.0	100.0

SA: succinic anhydride
Parentheses: values obtained from hemoglobin from Tokyo Kasei Kogyo Co.

The absorbance of succinylated hemoglobin in either the oxidized or reduced form decreased with decreasing pH of the solution. The magnitude of absorbance at 690 to 700 nm of 0.1 % succinylated hemoglobin solution was examined at pHs 5.20, 5.68, 6.85 and 7.93. It was found that the absorbance (0.182) at 596.5 nm did not change with varying pH values. The pH-solubility profile of succinylated hemoglobin was determined by measuring the absorbance at 596.5 nm at various pH values (Figure 2). Succinylated hemoglobin precipitated at pH 2.7 to 4.7, and at 4.7 to 5.0 its solution became turbid.

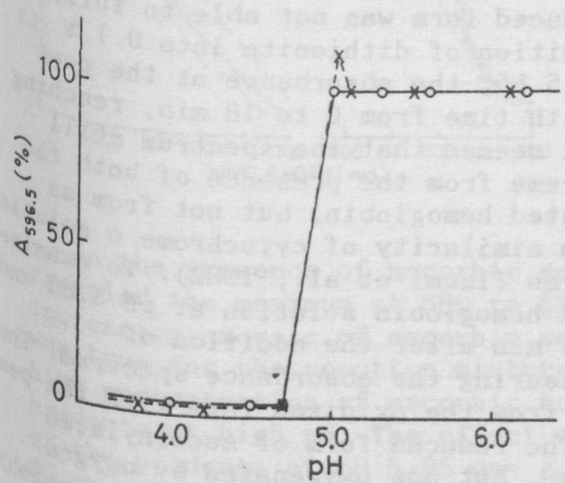

Figure 2.-Solubility profile of succinylated hemoglobin in water. o: hemoglobin from Sigma Chemical Co. and with 100 % succinylation, x: hemoglobin from Tokyo Kasei Kogyo CO. and 98.7% succinylation.

The possibility of dissociation of hemoglobin into subunits with succinylation was examined by gel chromatography (Figure 3). The elution profile showed that the proteins were eluted in the order of succinylated hemoglobin with peak fractions 34 and 35, hemoglobin (Fraction 36), myoglobin (Fraction 38) and cytochrome c (Fraction 40). When succinylated hemoglobin was loaded alone onto the gel, it was eluted with peaks at fractions 34 and 35. Hemoglobin did not dissociate into subunits with succinylation, rather, an increase in molecular weight of hemoglobin was indicated by the elution profile. About 4400 dalton can be added with succinylation to one mole of hemoglobin having 44 moles of lysines (Price and Schweigert, 1971).

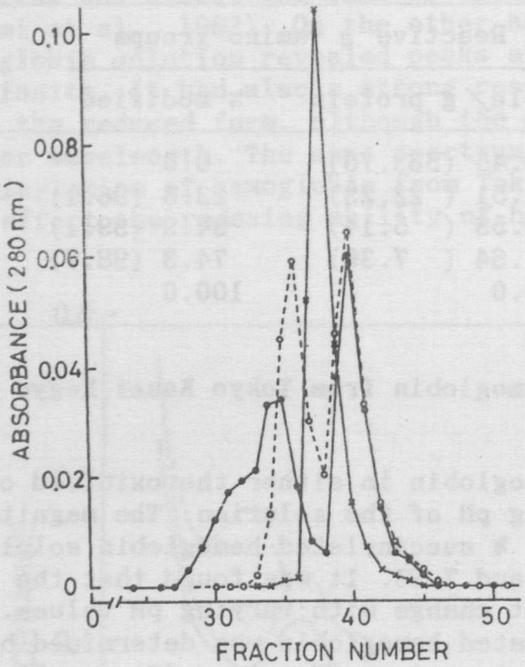

Figure 3.-Elution profile of hemoglobin, myoglobin and cytochrome c. 1 ml of the protein solution was chromatographed on Toyopearl HW-50S. o: solution containing 0.1 % hemoglobin and 0.1 % cytochrome c loaded, ●: solution of 0.1 % succinylated hemoglobin and 0.1 % cytochrome c, x: solution of 0.1 % myoglobin.

As shown in Figure 1, succinylated hemoglobin in the oxidized form was reduced by dithionite. However, the reduced form was not able to fully maintain its form in air. After the addition of dithionite into 0.1 % succinylated hemoglobin solution at pH 5.85, the absorbance at the peak 560 nm decreased from 0.565 to 0.435 with time from 0 to 18 min, reaching a plateau at the 40 min measurement. It seemed that the spectrum still having the peak 560 nm at the plateau came from the presence of both the reduced and oxidized forms of succinylated hemoglobin, but not from an oxygenated one, because of the spectrum similarity of cytochrome c solution containing the reduced and oxidized forms (Izumi et al., 1982). To examine this point, 10 ml of 0.1 % succinylated hemoglobin solution at pH 5.85 and 6.95 was bubbled with oxygen gas for 15 min after the addition of dithionite, immediately followed by measuring the absorbance spectrum. The spectrum obtained was nothing but that from the oxidized form with the peak at 529 nm and the shoulder at 562 nm. The reduced form of succinylated hemoglobin appears to be easily oxidized, but not oxygenated by oxygen.

The experiment of oxygen bubbling was applied to succinylated hemoglobin solution incubated with 50 mM ascorbic acid at pH 5.20, 5.85 and 6.95 for 24 hr. No particular change in the spectrum was observed, except for that from the oxidized form. In addition, no significant change in the spectrum took place in the presence of 50 mM ascorbic acid; the spectrum was basically that from the oxidized form. In the case of the solution in which each solution was bubbled with nitrogen gas for 1 hr before mixing up the reaction medium (0.1 % succinylated hemoglobin, 50 mM ascorbic acid and 50 mM phosphate at pH 5.20, 5.85 and 6.95), no significant change in the spectrum from the oxidized form to the reduced form occurred. Ascorbic acid did not act as a strong reductant to succinylated hemoglobin.

The reaction of succinylated hemoglobin with nitrite in the presence or absence of ascorbic acid was investigated at pHs 5.20, 5.85 and 6.95. In the absence of ascorbic acid (Figure 4, Left panel), the spectra of the reaction products lost the peak at 529 nm and the shoulder at 562 nm with increasing amounts of nitrite, and showed a hyperbolic curve with a slight shoulder at 600 nm. Concomitantly, the color of the reaction mixture became green from brownish red. This change was dependent upon pH value of the reaction mixture, being easier at low concentration of nitrite with decreasing pH. The spectrum pattern of the products formed at 10 mM nitrite and pH 5.20 was almost the same that formed at 50 mM nitrite and pH 5.85, revealing a less discernible shoulder at 600 nm. On the other hand, no significant change in the spectrum of succinylated hemoglobin occurred at 50 mM nitrite and pH 6.95. About 2 % of a slight decrease in absorbance at 500 to 600 nm was observed.

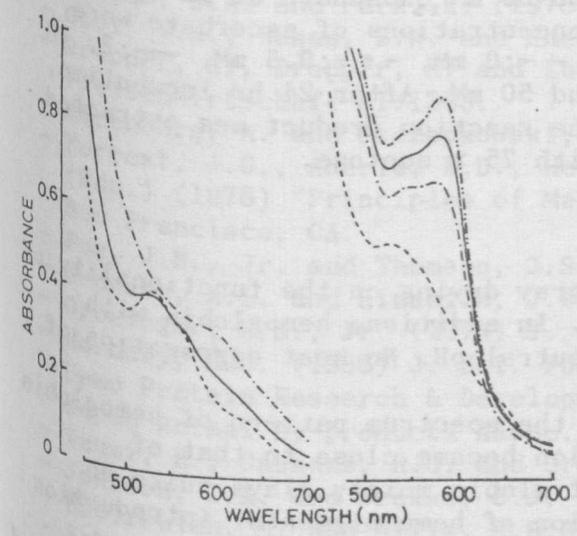

Figure 4.-Absorption spectrum of the product formed from reaction of succinylated hemoglobin with nitrite in the presence or absence of ascorbic acid. Left panel: Reaction mixture containing 0.1 % succinylated hemoglobin, sodium nitrite (- - -;3 mM, ——;10 mM, -•-;50 mM) and 50 mM phosphate at pH 5.85 was incubated for 24 hr. Right panel: Reaction mixture containing 0.1% succinylated hemoglobin, 10 mM sodium nitrite, varying amounts of sodium ascorbate and 50 mM phosphate at pH 5.85 was incubated for 24 hr. Concentration of ascorbate were - - -;0.5 mM, -•-; 2 mM, ——;10 mM, -••-;50 mM.

In the presence of ascorbic acid (Figure 4, Right panel), the spectra revealed the maximum at 580 to 583 nm and minimum at 524 to 525 nm with increasing amounts of ascorbic acid. This pattern change in the spectrum was true for the reaction mixtures at pHs 5.20 and 6.95. However, high concentration of ascorbic acid was needed to form the reaction products at high pH; The effect of 2 mM ascorbic acid on the formation of the products at pH 5.85 was equal to or a little stronger than that of 50 mM ascorbic acid at pH 6.95 in the presence of 10 mM nitrite. The two spectra from the reaction mixtures containing 10 and 50 mM ascorbic acid (Figure 4, Right panel) did not change by the addition of dithionite, so that the reaction products were in the reduced form. This spectrum pattern was different from either that of nitrosyl myoglobin (Fox and Thomson, 1963; Sakata and Nagata, 1983) or that of ferrocytochrome c nitrosyl compound II (Ehrenberg and Szczepkowski, 1960; Izumi et al., 1982).

In order to evaluate the formation of nitrosyl hemochrome, the reaction mixture containing 0.1 % succinylated hemoglobin, 10 mM nitrite and varying amounts of ascorbic acid at pH 5.85 was extracted with acetone just like the procedure applied to nitrosyl myoglobin (Hornsey, 1956; Sakata and Nagata, 1983)(Figure 5). An equal amount of nitrosyl hemochrome was

extracted from the reaction mixtures containing 2, 10 and 50 mM ascorbic acid, although the spectra of the reaction mixtures were different from one another (Figure 4, Right panel). In the absence of ascorbic acid, some products were extracted with 75% acetone solution. The extract bore green, and at low pH more green product was extracted.

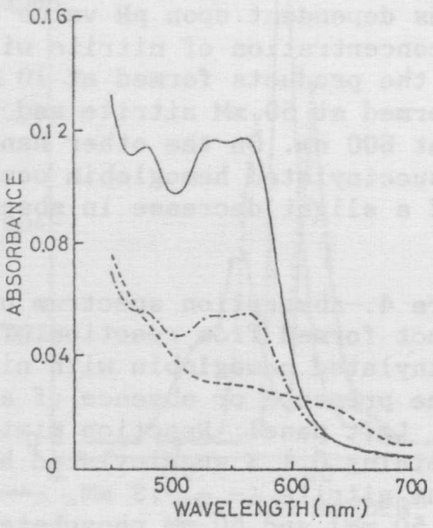

Figure 5.-Absorption spectra of 75 % acetone extracts from the reaction mixtures. The reaction mixture containing 0.1 % succinylated hemoglobin, 10 mM sodium nitrite, varying amounts of sodium ascorbate and 50 mM phosphate at pH 5.85. Concentrations of ascorbate were - - -;0 mM, -•-;0.5 mM, ——;2, 10 and 50 mM. After 24 hr incubation, the reaction product was extracted with 75 % acetone.

There was no significant effect of spray drying on the functionality of succinylated hemoglobin mentioned above. In addition, hemoglobin with succinylation became heat-stable at a neutral pH. No heat aggregation of the protein occurred.

It is an interesting phenomenon that the spectrum pattern of hemoglobin with an increasing extent of succinylation became close to that of cytochrome c (Figure 1), indicating that globin moiety plays quite an important role in the color representation of heme pigments. Introduction of a negative charge on proteins with succinylation can shift an isoelectric point to lower pH and increase protein solubility. Succinylated hemoglobin also gained high solubility at pH above 5.0 including the isoelectric zone of hemoglobin (Figure 2). The pH-solubility profile of succinylated hemoglobin was the same as that of succinylated globin from which heme moiety has been already removed (Miyaguchi et al., 1989). Nakamura et al. (1984) acetylated globin isolated from bovine hemoglobin and showed an improved emulsifying activity of acetylated globin at the pH region where globin was fully solubilized with acetylation. The authors also tried acetylation of hemoglobin, and it was found that the spectrum of acetylated hemoglobin was close to that of cytochrome c as shown in the case of succinylated hemoglobin.

There is an interesting comparison between succinylations of hemoglobin and hemerythrin which is devoiding heme groups. Hemerythrin dissociated into subunits and lost its oxygen binding ability, but did not change in its spectrum with succinylation (Klotz and Keresztes-Nagy, 1963). Hemoglobin with succinylation still remained in its tetramer form (Figure 3). It seems that the loss of oxygen binding ability of hemoglobin was incurred with succinylation.

The reaction of succinylated hemoglobin with nitrite seemingly proceeded as well as that of hemoglobin in the presence of ascorbic acid. Nitrosyl

hemochrome with form of ferrous iron was formed, but the color representation was modified by succinylated globin moiety or by reaction of succinylated globin with nitrite which may occur.

CONCLUSIONS: The spectrum of hemoglobin with succinylation became close to that of cytochrome c. Heat-stability of hemoglobin at a neutral pH was acquired with succinylation. Hemoglobin did not dissociate into subunits with succinylation. It seems that oxygen is no longer held by succinylated hemoglobin. Nitrosyl hemochrome of succinylated hemoglobin was formed in reaction with nitrite in the presence of ascorbic acid. Spray-dried powder of succinylated hemoglobin was prepared with no change in its functionality.

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