A Modification of Red Blood Cells by Isocyanates[1]

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Modified red cells that have a reinforced membrane and hemoglobin which is difficult to denature are prepared by treating red cells with some isocyanates. These modified cells have the same shape as normal red cells, but they are not lysed by osmotic pressure or by the actions of any reagents except for some ionic surface-active agents. No agglutination was observed when they were mixed with the blood of foreign animals or with blood of different blood groups. The hemoglobin within the cell gave a reversible oxygenation curve and had a high oxygen affinity. Toluene-2,4-diisocyanate was found to be the most suitable reagent among the many isocyanates examined. A suspension reaction method which consists of the addition of an isocyanate emulsion to a red-cell suspension at 5°C is developed for the modification process.

The present authors have studied the preparation of artificial red blood cells by using microencapsulation techniques. A hemoglobin solution was microencapsulated with synthetic polymers, such as polystyrene, a silicone derivative, poly-γ-benzyl-L-glutamate, and dextrane stearate, into sphere particles five to ten microns in diameter.[3-5] The hemoglobin contained in these microcapsules was found to be preserved in a normal state, and it gave an oxygenation curve identical with that of normal hemoglobin. The oxygen permeability of the hemoglobin-containing silicone microcapsules was comparable with that of natural red cells. They are free from problems of agglutination and hemolysis, and can be preserved over a long period. It is expected that the hemoglobins obtained from many mammalias can be used as artificial red cells when subjected to microencapsulation. The present authors are now studying the metabolism of the polymers used as capsulating materials and the rheology of the suspension containing such rigid particles.

This paper will describe a method of preparations and the properties of the modified red cells that have a reinforced membrane and normal hemoglobin. It is known that the shape of red cells can be fixed by treating them with some protein precipitants, such as aldehydes,[6,7] tannic acid, or hydrogen peroxide.[8] However, these reagents are soluble in water, so that they can penetrate into the cell to denature hemoglobin. These fixed cells have been used for studies of the morphology or the surface properties of red cells. In this modification reaction, water-immiscible isocyanates are used in the suspension reaction method that consists of the addition of an isocyanate emulsion to a red cell suspension. The modified red cells thus obtained show some specific behavior concerning hemolysis and agglutination. They are considered to be suitable as samples for a biological, physiological, or rheological study of erythrocytes. The present authors are now studying their use in the medical field as a kind of artificial red cell in a way similar to the hemoglobin-containing microcapsules.[9,10] This modification technique can be employed generally in studying the nature of cell membranes other than that of the erythrocytes.

Experimental

Materials. Toluene-2,4-diisocyanate (TDI), from the Tokyo Kasei Co., was purified by distillation (146°C/38 mmHg). Phenyl and ethyl isocyanate, phenyl and ethyl isothiocyanate, hexamethylene diisocyanate (Tokyo Kasei Co.) and diphenylmethane diisocyanate (Hodogaya Chemicals) were obtained commercially.

Polyoxyethylene hydrogenated caster oil ether (Nikko Chemicals), Tween 20, sodium dodecylbenzenesulfonate (Kao-Atlas Co.), saponin, lecithin from eggs (Merck Co.), and cetyl, dodecyl and ethyl-pyridinium salt (Tokyo Kasei Co.) were obtained commercially. The other surface-active agents were synthesized and purified in our laboratories.

Reagent-grade chemicals were used in all the experiments.

Modification of Red Cells by TDI. The suspension reaction method developed by the authors was used to modify red cells suitably. Mammalian red cells were collected by centrifugation and washed three times with isotonic saline solution. In 200 m*l* of pH 7.2 phosphate-buffered isotonic saline, we suspended 10 g of the washed red-cell slurry at 5°C. The TDI emulsion was prepared by emulsifying 0.15 m*l* of the reagent in 30 m*l* of the saline, by means of an ultrasonic emulsifier, into microdroplets of about one micron in diameter. This TDI emulsion was quickly stirred into the red-cell suspension at 5°C. The modified cells were recovered after 5 min by centrifugal separation at 1500 rpm for 10 min and then washed with the saline twice.

The same method was employed in the cases of the other isocyanates, with varying amounts of the reagents and with varying reaction times.

Measurements of Properties. Hemolysis. Surface-active agents were dissolved in water to make a 1% solution, and the hydrogen-ion concentration was adjusted to neutral by adding dilute hydrochloric acid or a sodium hydroxide solution. In a test tube we placed 5 m*l* of this solution and then added several drops of the modified red cells. The suspension was

1) A part of this study was prestented at the 22nd Annual Meeting of the Chemical Society of Japan, Osaka, 1969, by M. Kitajima and A. Kondo.

2) Department of Surgery, Faculty of Medicine, the University of Tokyo, Hongo, Bunkyo-ku, Tokyo.

3) T. Toyoda, *Nihon Gekagaku Zasshi*, **67**, 36 (1966).

4) A. Kondo and S. Miyano, Japan. 529014 (1968).

5) M. Kitajima, S. Miyano, and A. Kondo, *Kogyo Kagaku Zasshi*, **72**, 493 (1969).

6) M. Moskowitz and S. Carb, *Nature*, **180**, 1049 (1957).

7) D. H. Heard and G. V. F. Seaman, *Biochem. Biophys. Acta*, **53**, 366 (1961).

8) H. Funaki, *Jap. J. Physiol.*, **7**, 153 (1957).

9) W. Sekiguchi, M. Kitajima, and A. Kondo, *Nihon Yuketsu Gakkai Zasshi*, **16**, 149 (1969).

10) W. Sekiguchi, M. Kitajima, and A. Kondo, Abstracts, the 12th Congress of the International Society of Blood Transfusion, Moscow, Aug. 1969, p. 512.

stirred for 5 min, and then observed under a microscope in order to see whether the cells were lysed, swelled, or unchanged.

Spectral Measurements. The visible absorption spectra for suspensions of the modified cells were taken with a Hitachi EPS-3T spectrophotometer, according to the opal glass method. A small amount of sodium hydrosulfite was added to deoxygenate hemoglobin. The infrared spectra were taken with a Hitachi Perkin-Elmer 125 spectrophotometer.

Oxygenation Curve. The modified cells were suspended in a solution phosphate-buffered at pH 6.5, and then put in a modified Wyman-type tonometer. The air inside the tonometer was replaced by nitrogen purified with Fieser's solution and saturated potassium chloride solutions. After the hemoglobin had been completely deoxygenated, a measured amount of air was introduced into the tonometer with a syringe. It was shaken for 20 min to equilibrate the hemoglobin with the gas, and then the sample was submitted to absorption spectrum measurements. The percentage of oxygenation, Y, of the hemoglobin was calculated from the spectra according to the following equation:

$$Y=\{[(E_a-E_b)-(D_a-D_b)]/[(O_a-O_b)-(D_a-D_b)]\}\times 100$$

where the terms O, D, and E represent the absorbance at the completely oxygenated state, the completely deoxygenated state, and the state between the two, respectively. The suffixes a and b represent the wavelength at which the absorption was measured: a, 578: b, 562.5 nm.

Results and Discussion

Modification Reaction. Various isocyanates were examined for use in modifying red cells, but most of them were found to be unsuitable. Monoisocyanates such as phenyl and ethyl isocyanate did not reinforce the membrane. The same was true with phenyl and ethyl isothiocyanate. Diisocyanates such as hexamethylene and diphenylmethane diisocyanate reinforced the membrane under some conditions, but they required too long a time to complete the modification, as their reactivity was not high enough,[11] and the hemoglobin within the cell was denatured. It was effective to add some catalysts, such as ferric chloride or sodium phenate, to accelerate the reaction, but they were undesirable because of their deleterious action on hemoglobin.

TDI was found to be the most suitable reagent. It gave modified cells that had a sufficiently reinforced membrane and hemoglobin which was hardly denatured at all. The reactivity of TDI is very high, so that the modification reaction is completed in a few minutes. The excess TDI is exhausted by reactions with water molecules and becomes inert solid particles in this suspension reaction. When the isocyanate is used as an organic solution, the shape of the cells changes into a sphere and a denaturation of the hemoglobin takes place. Actions based on the low surface tension of the solvent or on the extraction of lipids from the membrane must be the reason. Thus, both the high reactivity and the reaction method serve to prevent the reagent from penetrating into the cell to denature hemoglobin.

The amount of TDI to be used was determined by examining the degree of the reinforcement and that of the denaturation of hemoglobin. The optimum amount of TDI to modify 10 g of the red cell slurry properly was found to be 0.15 m*l*. When the amount was insufficient, the cells became more sensitive to hemolytic action than intact cells. A large excess of TDI caused serious denaturation. The use of small amount of surface-active agents served to keep the isocyanate emulsion stable. Polyoxyethylene hydrogenated caster oil ether, which is known to be inert to blood, was added to the suspension in an amount of 0.05%.

Shape. The shape of the modified cells was biconcave discoide, as is shown in Fig. 1. It cannot be distinguished from that of normal cells. These cells were neither ruptured nor deformed when suspended in a hypotonic or hypertonic salt solution.

It was assumed that the cells were fixed hard as rigid particles by the modification. However, viscometric measurements over a shear rate of 2 to 100 sec^{-1} showed that the flow behavior of the modified cell suspension was non-Newtonian, although to a lesser extent than with the intact red-cell suspension.[12] The result for the red cells hardened by formaldehyde was found to be Newtonian. These facts suggest that the membrane of the modified red cells has some flexibility.

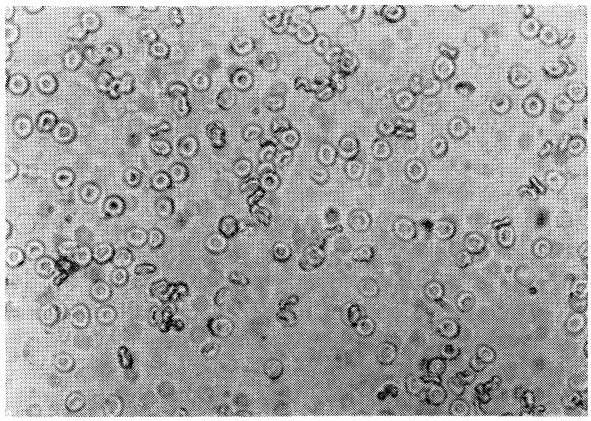

Fig. 1. Micro photograph of the modified human red cells suspended in distilled water.

Hemolysis. The modified cells showed some specific characteristics concerning hemolysis. They were not lysed by the action of osmotic pressure. They were recovered by centrifugation after being suspended in distilled water without releasing free hemoglobin into the medium. Neither lysis nor swelling was observed when they were suspended in a 50% aqueous solution of ethyl alcohol, acetone, or glycerine. No change in shape was observed in 2N sulfuric acid, but they swelled very much in 0.1N sodium hydroxide. Nonionic surface-active agents, such as Tween 20, polyoxyethylene hydrogenated caster oil ether, and saponin, were found not to affect the lysis of the modified cells. The same result was obtained with lecithin from eggs.

On the contrary, some of the ionic surface-active agents caused a lysis or swelling of the cells. The

11) W. Cooper, R. W. Pearson, and S. Drake, *Ind. Chemist*, **36**, 121 (1960).

12) M. Kaibara and E. Fukada, Institute of Physical and Chemical Research, Saitama, personal communication, 1969.

Table 1. Effect of ionic surface-active agents on the hemolysis of the red cells modified by TDI[a)]

Anion	Result[b)]	Cation	Result[b)]
$C_{12}H_{25}SO_4Na$	H	$C_{12}H_{25}NH_2HCl$	H
$C_{12}H_{25}PhSO_3Na$[c)]	H	$C_{12}H_{25}PyCl$[c)]	H
$C_{11}H_{23}COONa$[d)]	S	$C_{16}H_{33}PyCl$[c)]	H
$C_{12}H_{25}PO_4Na_2$	U	$C_{12}H_{25}N(CH_3)_3Cl$	S
$C_{16}H_{33}O(CH_2)_3SO_3Na$	U	C_2H_5PyBr[c)]	U
C_4H_9COONa	U	$C_{17}H_{35}(CONHC_2H_4)_2NH_2$[d)]	U

a) Concentration of the detergents: 1%
b) H: hemolysed, S: swelled, U: unchanged.
c) Ph: phenylene, Py: pyridinium
d) A concentration below 1% was used because of the low solubility.

results are shown in Table 1.

There were clear differences among the hemolysed, the swelled, and the unchanged state. A hemolytically-active agent like dodecylpyridinium chloride caused lysis instantaneously, and a clear hemoglobin solution was obtained. Sodium laurate caused a swelling into a sphere and the hemoglobin within the cell was released into the medium, but a turbidity remained in the solution. No change in shape nor any release of hemoglobin was observed in the case of ethylpyridinium bromide. The hydrogen-ion concentration of the solution affected the hemolytic behavior sensitively. The modified cells were apt to lyse easily in a medium with a high pH value.

Although further investigations are required to establish the mechanism of the hemolysis of the modified cells, some tendencies can be seen in the table. There is some relationship between the structure of the ionic groups and the hemolytic effect. The orders are considered to be as follows: $-SO_4Na$ and $-SO_3Na$ > $-COONa$ > $-PO_4Na_2$ for the anionic series and $-NH_2HCl$ and $-PyCl$ > $-N(CH_3)_3Cl$ for the cationic series. Hydrophobic groups are also considered to greatly affect the hemolytic action. Surface-active agents that have too short or too long alkyl chains cannot cause lysis. An alkyl chain that has ten to fifteen carbons is considered to be most effective.

These facts are in fair agreement with the results obtained for intact red cells. It has been reported that the order of the hemolytic activity of ionic surface-active agents according to the polar groups is as follows:[13)]

$$-SO_4Na > -SO_3Na > -NH_2HCl > -COONa > -N(CH_3)_3I$$

Kondo and Tomizawa studied the hemolysis by cationic surface-active agents and proposed a mechanism.[14)] They stated that lysis was caused by the adsorption of the detergent molecules onto the cell surface in terms of ionic attraction at first step, followed by the solubilization of lipids in the membrane in terms of hydrophobic interaction. They considered that the bulkiness of the hydrophobic group of reagents was an important factor in effective adsorption.

13) B. A. Pethica and J. H. Schulman, *Biochem. J.*, **53**, 177 (1953).
14) T. Kondo and M. Tomizawa, *J. Pharm. Sci.*, **58**, 255, 1378 (1969).

It is possible to consider that the mechanism of the hemolysis of the modified cells is the same as that for intact cells. This means that the isocyanate molecules do not affect the sites where the ionic surface-active agents attack to lyse. On the other hand, the sites where nonionic agents attack are assumed to be reinforced by the modification reaction.

Agglutination. The modified cells were found to be mixed with the blood of foreign animals or with that of different blood groups. Neither agglutination nor lysis was observed in the mixture. Figure 2 shows microphotographs of the modified human red cells of the A, B, O, and AB blood groups, suspended in plasma of the A blood group. Not much difference can be seen among them. It was also observed that the modified bovine red cells were mixed with the whole blood of a rabbit, homogeneously. No lysis nor agglutination was caused.

These facts suggest that red cells are inhibited or lose the power to agglutinate by the modification reaction. It has been reported that red cells lose their agglutinability towards appropriate antisera when they are treated with a formaldehyde solution.[5)] It is expected that some information on the mechanism of the agglutination or other surface activities of red cells can be gotten by studying the properties of the modified cells further.

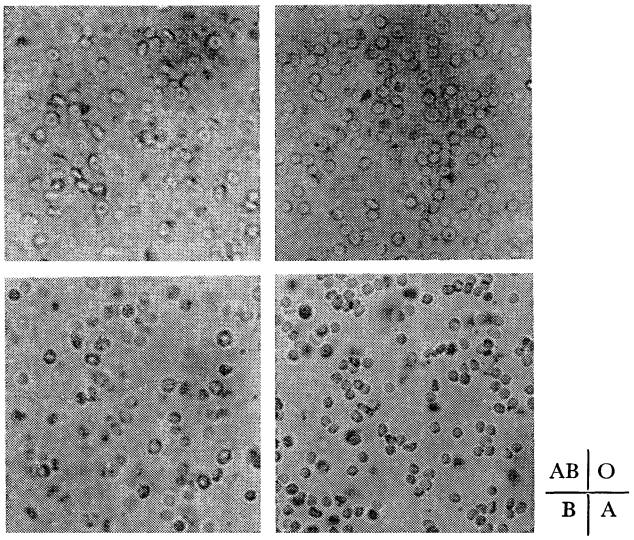

Fig. 2. Micro photographs of the modified red cells of the A, B, O, and AB blood group, suspended in plasma of the A blood group.

Properties of Hemoglobin. Figure 3 shows the visible absorption spectra of the red cells modified by TDI. The spectra for both the oxygenated state and the deoxygenated state are identical with that for normal blood, except that the peak intensity at 430 nm of the deoxygenated is slightly low. Figure 4 shows the oxygenation curves of the modified cells and intact cells. It is known that the hemoglobin within the modified cell retains the property to combine and to release molecular oxygen reversible in relation to the partial pressure of oxygen. It may be seen in the figure that hemoglobin within the modified cell has a higher oxygen affinity than normal hemoglobin, and that the

slope of the curve over the 10 to 90% oxygenated region is less steep. The n value, as calculated by using Hill's equation,[15] is 1.7. This is an intermediate value between 2.8 for normal hemoglobin and 1.0 for myoglobin. These changes in the oxygenation curve are considered to correspond to the decrease in the cooperation of hemoglobins or in the heme-heme interaction.

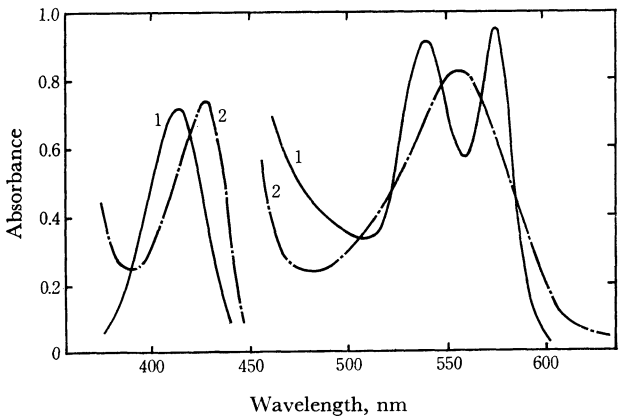

Fig. 3. Absorption spectra of the modified red cells.
(1) oxygenated; (2) deoxygenated with sodium hydrosulfite.
(The normal red cells give almost same absorption spectra as this figure.)

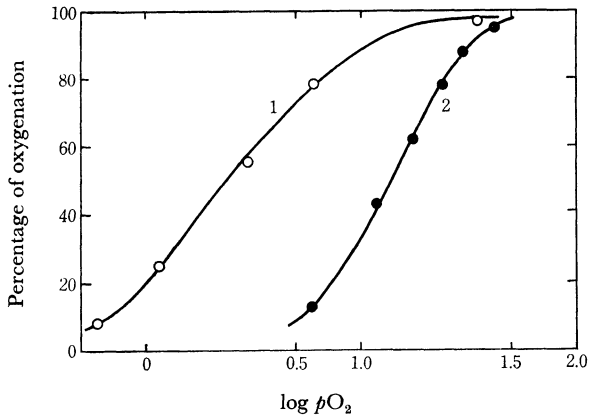

Fig. 4. Oxygenation curves of hemoglobin at pH 6.5. (1) the modified cells; (2) intact cells.

It is known that the absence of organic phosphates, such as D-2,3-diphosphoglycerate, or adenosine triphosphate, makes the oxygen affinity of hemoglobin higher.[16] It is considered that hemoglobins are not affected by the modification reaction, but some steric change or dissociation of the molecules may take place. On the other hand, it is also possible that the permeability of the cell membrane increases and that the organic phosphates within the cell come out, resulting in the high oxygen affinity. Further studies are necessary to ascertain the reasons and to find a way to protect hemoglobin from this change.

Reaction Sites. The facts described hitherto give us an idea of the modification reaction, that TDI reacts rapidly and selectively with specific components of the cell membrane to reinforce it, while the hemoglobin within the cell is kept unreacted. Figures 5 and 6 show the IR spectra of stroma and hemoglobin. The spectral samples of the modified cells were prepared through a process of lysis with sodium dodecylbenzenesulfonate, centrifugal separation of the stroma from the hemoglobin solution, and drying. Some differences can be seen between the spectrum of stroma separated from the modified cells and that for the intact cells. A sharp absorption band at 2250 cm^{-1} due to the stretching vibration of the isocyanate group, and additional bands at 805 and at 865 cm^{-1} corresponding to the out-of-plane deformation vibration of hydrogen on the 1,2,4-tri-substituted aromatic ring, appear in the spectrum for the modified stroma. On the other hand, the spectrum of the hemoglobin recovered from the modified cells is almost identical with that for the intact cells. This indicates that the hemoglobin is kept unreacted.

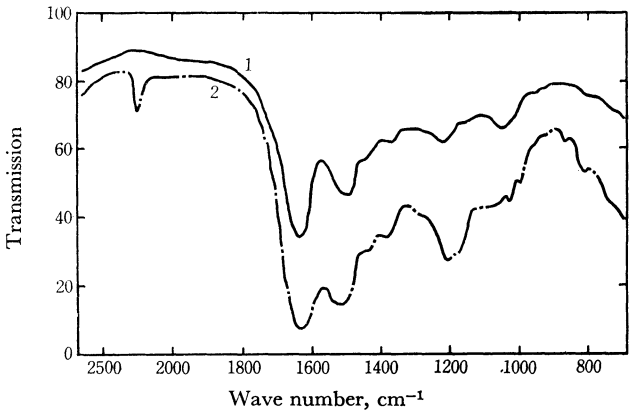

Fig. 5. IR spectra of stroma separated from (1) intact cells and (2) the modified cells.

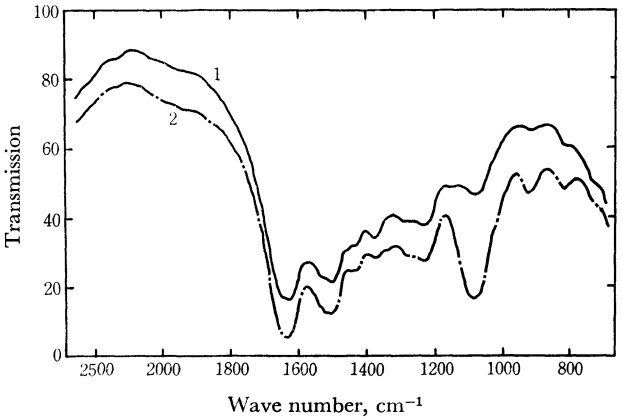

Fig. 6. IR spectra of hemoglobin separated from (1) intact cells and (2) the modified cells.

It is known that isocyanates react with compounds containing active hydrogen to give addition products. The order of reactivity on the bases of functional groups has been reported to be as follows:[17]

15) $Y=Kp^n/(1+Kp^n)$ where Y represents the oxygenated rate at partial pressure of oxygen being p mmHg, and K is the dissociation constant of oxygenated hemoglobin.

16) R. Benesch and R. E. Benesch, *Nature*, **221**, 618 (1969).

17) J. M. Buist and H. Dudgeon Ed., "Advances in Polyurethane Technology," J. Wiley and Sons Inc., New York (1968), p. 8.

Aliphatic NH_2>aromatic NH_2>primary OH>water>secondary OH>tertiary OH>phenolic OH>COOH and RNHCONHR'>RCONHR'>RNHCOOR'

As the modification reaction proceeds in an aqueous suspension, the reaction sites must have more isocyanate-reactive groups than water such as amino and primary hydroxy group. Other functional groups, carboxy, carbamoyl, imino, mercapto, formyl, *etc.*, may be kept unreacted. Diisocyanates are known as a kind of bridging reagents for enzymes and proteins.[18] It has been reported that the lysine residue was attacked exclusively in the treatment of pancreatic ribonuclease with hexamethylene diisocyanates.[19]

It has also been reported that comparatively large amounts of lysine and arginine residues are in the red-cell membrane as components of the protein.[20] Other components which have reactive groups are the cephalin and plasmalogens in the lipid layer and the serine residue in the protein. The isocyanate molecules must react with these components and reinforce the cell membrane through inter- or intra-molecular cross-linkings. It is reasonable to consider that ionic interaction between the polarized isocyanate group and polar group on the cell surface, or hydrophobic interaction between the hydrophobic group of the reagent and lipids in the membrane, gives some additional selectivity to the reaction.

One of the reactants must be the blood-group factor itself or one that is closely related to the agglutination phenomena. The isocyanate molecules may combine directly with it or mask the active site sterically, so that agglutination is inhibited. It is left for further studies now in progress to ascertain the exact mechanism of the modification reaction.

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18) S. Petersen, *Angew. Chem.*, **59**, 266 (1947).
19) H. Ozawa, *J. Biochem.*, **62**, 419 (1967).
20) H. Behrendt, "Chemistry of Erythrocytes," C. C. Thomas, Springfield, I 11 (1957), p. 6.