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Dimethyl Adipimidate: A New Antisickling Agent

(sickle cell disease/erythrocytes/sickling)

BERTRAM H. LUBIN*, VICTOR PENA*, WILLIAM C. MENTZER†, EDWIN BYMUN‡, THOMAS B. BRADLEY§, AND LESTER PACKER‡

* Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center, Oakland 94609; † San Francisco General Hospital, San Francisco 94110; ‡ Energy and Environment Division, Lawrence Berkeley Laboratory and the Department of Physiology-Anatomy, University of California, Berkeley, Calif. 94720; and § V.A. Hospital, San Francisco, California 94121

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ABSTRACT **A new approach to the prevention of sickling *in vitro* by use of the bifunctional crosslinking reagent, dimethyl adipimidate, is described. Prior treatment of sickle erythrocytes with dimethyl adipimidate will inhibit sickling in completely deoxygenated erythrocytes. Treated erythrocytes do not demonstrate the potassium loss and viscosity increase that usually accompany sickling. The oxygen affinity of hemoglobin in these cells is increased independently from changes in the concentration of 2,3-diphosphoglycerate. The hemoglobin obtained from treated erythrocytes contains a high-molecular-weight component as well as additional positively charged components. The relative degree to which chemical modification and/or crosslinking is an essential part of the antisickling properties of the material is not known.**

Recently, several attempts to inhibit sickling by modification of the hemoglobin molecule have been successful *in vitro*. Nitrogen mustard has been reported to inhibit sickling by a direct effect on hemoglobin gelation (1). In contrast, cyanate appears to act primarily by modifying hemoglobin oxygen affinity (2). Preliminary clinical trials suggest that cyanate may be therapeutically beneficial in sickle cell disease (3), but potential side effects exist (4). In this report, we describe a new antisickling agent, dimethyl adipimidate. This material is a bifunctional crosslinking reagent that is known to link covalently the free amino groups in polypeptides (5). Brief treatment of erythrocytes at a concentration of 5 mM effectively inhibits sickling and appears to have few deleterious side effects *in vitro*.

METHODS

Fresh heparinized blood samples were obtained from patients with sickle cell anemia. The cells were washed with Krebs Henseleit buffer, pH 7.4, which contained 200 mg/100 ml of glucose, and resuspended to a final hematocrit of 20%. To 4 ml of the erythrocyte suspension was added 16 ml of 0.14 M Tris-hydroxymethyl methylamine propane sulfonic acid buffer that had been adjusted to pH 8.8 with 5 M NaOH. The entire suspension was then incubated for 10 min in a flask containing fresh, dry, powdered dimethyl adipimidate (from Pierce Chemical Co., P.O. Box 117, Rockford, Ill. 61105) at a final concentration of 5 mM. Control samples were prepared in an identical fashion except that sucrose (26 mM final concentration) was substituted for dimethyl adipimidate. The osmolarity of the incubation solution was 280 mosm. After incubation, the cells were washed with Krebs Henseleit buffer, resuspended in the original plasma at a final hematocrit of 40%, and equilibrated with room air.

The number of sickle forms was measured after the preparations were deoxygenated by equilibration with nitrogen for 1 hr in an IL tonometer model no. 237. After equilibration, aliquots were anaerobically transferred into 10% (v/v) formaldehyde in saline for fixation. Five hundred cells were counted by described criteria (6).

The viscosity of dimethyl adipimidate-treated and control cells was measured in a Wells Brookfield cone plate microviscometer model LVT, adapted to maintain controlled atmospheric gas tension (7). After adjustment of the hematocrit to 40 ± 1%, the blood was equilibrated for 1 hr with humidified 95% N_2 and 5% CO_2 in an IL tonometer model no. 237, then transferred in a closed system to the viscometer. The viscosity was measured at shear rates ranging from 4.5 to 90 sec^{-1} during continued exposure to nitrogen.

Studies *in vitro* of erythrocyte metabolism and of net K^+ loss were conducted by incubation at 37° of thrice-washed erythrocytes in Krebs Henseleit buffer with added glucose (10 mM) in the presence of 0.1 mM ouabain. The samples were continuously equilibrated with moistened gas mixtures consisting of nitrogen plus 20%, 3.3%, or 0% oxygen, and pH was maintained at 7.45 ± 0.05 by varying the CO_2 concentration with a gasometric pH stat (8). Supernatant K^+ concentration was determined by flame photometry. 2,3-Diphosphoglycerate (9) concentrations in erythrocytes were determined in perchloric acid extracts.

The oxygen affinity of whole blood, expressed as p50 (that oxygen tension at which hemoglobin is half-saturated), was determined by measurement of hemoglobin oxygen saturation and pO_2 after equilibration of the samples with gas mixtures containing 2.2%, 2.8%, 3.2%, and 3.9% oxygen plus 5% CO_2 and the remainder nitrogen. The p50 was calculated by a best fit analysis of these data and corrected to a pH of 7.4 (10).

Hemolysates and globin were prepared by standard methods. Gel filtration in Sephadex G-100 was performed by the method of Andrews (11). The column was calibrated with aldolase (molecular weight 158,000), ovalbumin (molecular weight 45,000), and chymotrypsinogen A (molecular weight 25,000). Hemoglobin electrophoresis was performed on cellulose acetate strips at pH 8.65 in a Beckman microphor system. Barbital buffer, 25 mM, pH 8.0, was used for electrophoretic separation of globin chains and the cellulose acetate strips were soaked in the same buffer with 8 M urea and 5 mM 2-mercaptoethanol. Spectra were recorded with a Cary 14 recording spectrophotometer.

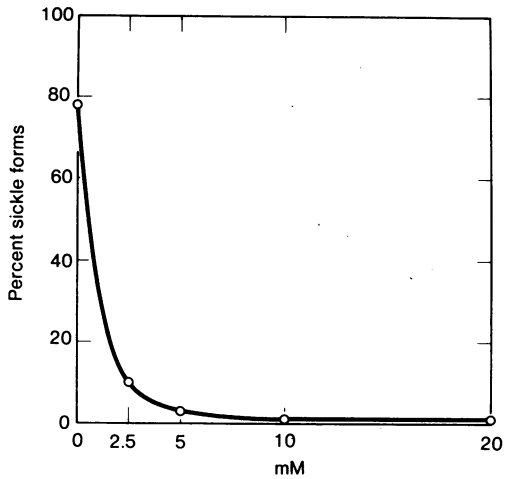


FIG. 1. Influence of dimethyl adipimidate on sickling. After a 10-min treatment with various concentrations of dimethyl adipimidate, the cells were equilibrated with nitrogen for 1 hr and fixed in formalin. The percent sickled forms was determined by counting at least 500 cells (6).

RESULTS

The influence of dimethyl adipimidate on the number of sickle forms after deoxygenation is shown in Fig. 1. The percentage of sickled cells was reduced from 80% in untreated cells to 4% by 5 mM dimethyl adipimidate and to 0% by 10 mM dimethyl adipimidate. The pH remained stable at 7.4 during these incubations. In all samples, measurements of hemoglobin saturation were less than 5% at the end of these incubations.

The inhibition of sickling by dimethyl adipimidate was further evaluated by measurement of net K^+ loss from sickle cells incubated under hypoxic conditions. As previously shown, a marked increase in net K^+ loss accompanies sickling (12). Determination of net K^+ loss is, therefore, a quantitative measure of the amount of sickling (13, 14). Ouabain was added

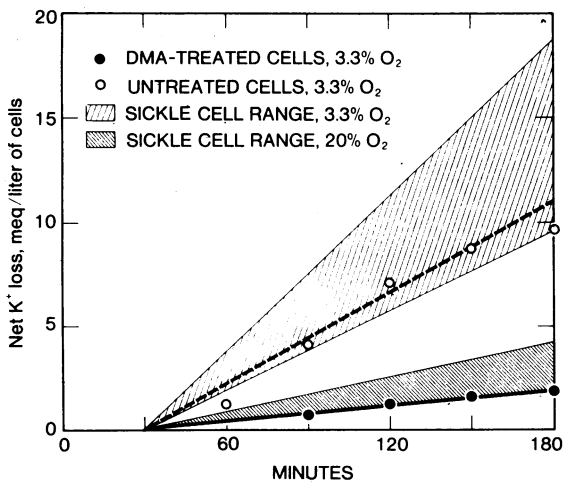


FIG. 2. The affect of dimethyl adipimidate on net K^+ loss from hypoxic sickle cells. The range of net K^+ loss from sickle cells after equilibration with either 3.3% or 20% O_2 is indicated by the shaded areas. A representative experiment in which sickle cells were treated with 5 mM dimethyl adipimidate (DMA) and then equilibrated at 3.3% O_2 is shown by the solid circles. Net K^+ loss from the same cells not treated with dimethyl adipimidate is shown by the open circles. Ouabain (0.1 mM) was present in all incubations.

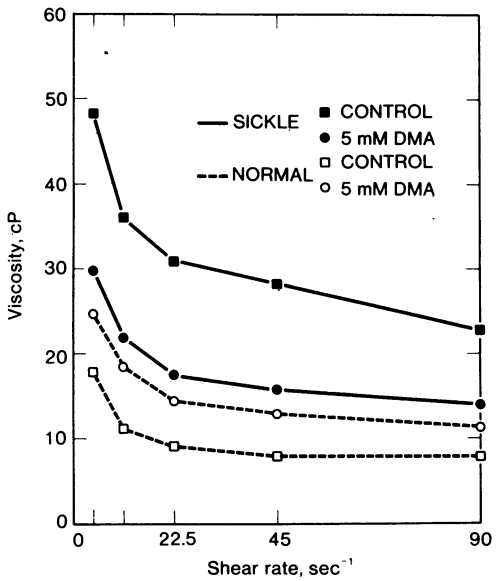


FIG. 3. Viscosity of dimethyl adipimidate-treated erythrocytes. After treatment with either 5 mM dimethyl adipimidate or 26 mM sucrose, the whole blood was equilibrated with nitrogen for 1 hr, and the viscosity was measured in a cone plate viscometer at various shear rates. The hematocrit of all samples was 40 ± 1%.

to the incubated cells in order to inhibit the ATP-mediated cation pump and thus magnify the K^+ loss induced by sickling. When sickling was absent, a small loss of K^+ nevertheless occurred, due to the presence of ouabain. K^+ loss was greatly accentuated by hypoxia, reflecting active sickling of erythrocytes. Dimethyl adipimidate (5 mM) completely eliminated the net K^+ loss induced by hypoxia in sickle cells. A representative experiment is shown in Fig. 2. In this experiment, 3.3% oxygen was chosen to induce a physiological level of hypoxia. Treated sickle cells incubated at 3.3% O_2 show net K^+ loss within the usual range of 20% O_2 incubations where sickling is not observed. These results were not due to prior depletion of intracellular K^+ by dimethyl adipimidate since intracellular cation concentrations were unaffected by dimethyl adipimidate (data not shown).

The whole blood viscosity of deoxygenated sickle cells was reduced at all shear rates by treatment with dimethyl adipimidate. (Compare *closed circles* with *closed squares* in the representative experiment shown in Fig. 3.) In contrast, the viscosity of deoxygenated normal blood, which is less than that of sickle blood at similar shear rates, was slightly increased after dimethyl adipimidate treatment. (Compare *open circles* and *squares*.) However, it should be noted these slight changes in normal cells are still within our normal limits for this determination.

The oxygen affinity of sickle and normal cells was consistently and significantly increased after treatment with 5 mM dimethyl adipimidate (Fig. 4). The mean p50 value for whole blood for six patients with sickle cell anemia was decreased from 33.0 ± 1.7 mm Hg to 24.1 ± 0.56 mm Hg. In five normal controls, the p50 value was decreased from 28.7 ± 1.4 mm Hg to 23 ± 1.4 mm Hg. Erythrocyte 2,3-diphosphoglycerate levels in sickle cells treated with dimethyl adipimidate were slightly lower (5.69 + 0.5 mM/liter of cells) than in sickle cells not treated with dimethyl adipimidate (5.90 +

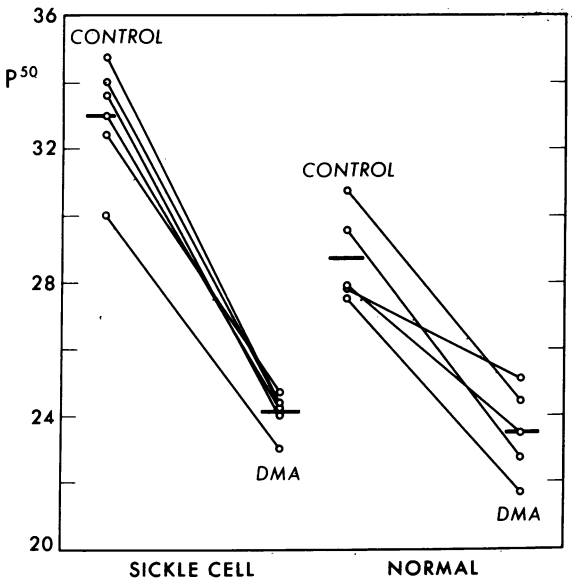


FIG. 4. The influence of dimethyl adipimidate on the oxygen affinity of hemoglobin. The oxygen affinity of hemoglobin in sickle cells and normal cells was measured before and after treatment with 5 mM dimethyl adipimidate. The mean for each group is indicated by the horizontal bars. 2,3-Diphosphoglycerate levels did not change significantly (see *text*).

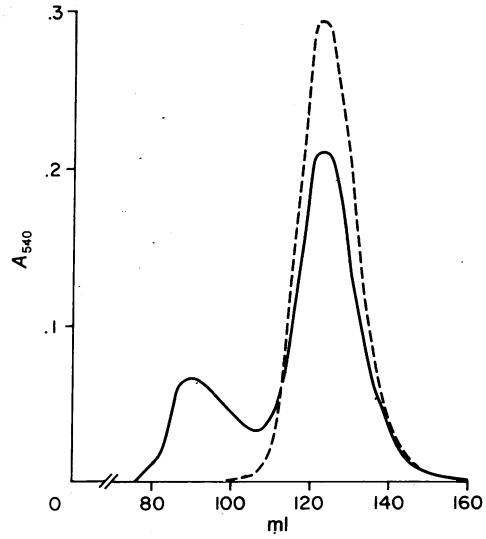


FIG. 5. Gel filtration in Sephadex G-100. The column was 2 × 65 cm. The buffer was 50 mM Tris·HCl, pH 7.5, with 0.1 M KCl. Three milliliters of hemoglobin, 0.13 mM (heme basis), were applied to the column. Solid curve, unreacted hemoglobin; dashed curve, 5 mM dimethyl adipimidate.

0.23 mM/liter of cells). Changes of similar magnitude were produced in control erythrocytes. In neither sickle nor control erythrocytes were the differences in erythrocyte 2,3-diphosphoglycerate large enough to account for the changes observed in hemoglobin oxygen affinity (15).

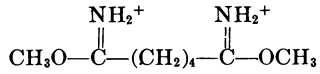
Gel filtration of hemolysates from erythrocytes that had been treated with dimethyl adipimidate revealed the presence of a high-molecular-weight component that was proportional to the concentration of dimethyl adipimidate in the reactive mixture (Fig. 5). It constituted 25–30% of the hemoglobin at 5 mM dimethyl adipimidate, 15–20% at 2.5 mM dimethyl adipimidate, and 8–10% at 1.25 mM dimethyl adipimidate. Similar proportions of this component were observed with normal and sickle cell anemia erythrocytes. The mean molecular weight of this component was in excess of 150,000. Although it was eluted as a symmetrical peak, the portion with the lowest elution volume exhibited spontaneous precipitation. The chromatography on Sephadex G-100 of individual fractions of this peak failed to reveal further evidence of heterogeneity with respect to molecular size. The remainder of the hemoglobin of reacted erythrocytes had elution characteristics similar to those of unreacted hemoglobin. Both components were in the oxygenated state and showed no evidence by spectral criteria for the loss of heme groups (16).

Electrophoresis of the reacted hemolysates (5 mM dimethyl adipimidate) revealed a 5% component with an additional positive change. This component was limited to the normal peak after gel filtration. Electrophoresis of globin prepared after isolation of this component by DE-52 chromatography revealed both normal and positively changed α and β chains with predominance of the normal α and altered β chains.

Preliminary studies with sickle erythrocytes have revealed that 5 mM dimethyl adipimidate does not substantially alter glucose consumption, lactate production, or glycolytic erythrocyte enzyme activity. Erythrocyte indices were altered slightly by dimethyl adipimidate. A 5% increase in the mean corpuscular volume and a 5% decrease in the mean corpuscular hemoglobin concentration occurred in both normal and sickle cells, while the mean corpuscular hemoglobin remained unchanged.

DISCUSSION

In this paper, we present evidence that indicates that an 8-carbon bifunctional crosslinking reagent, dimethyl adipimidate,



is a potent inhibitor of sickling. Like cyanate, another inhibitor of sickling, dimethyl adipimidate alters hemoglobin oxygen affinity. Unlike cyanate, dimethyl adipimidate inhibits sickling even under conditions of complete deoxygenation, indicating that factors other than altered oxygen affinity must also be involved.

The molecular basis for the inhibition of sickling by dimethyl adipimidate is not known. The binding of dimethyl adipimidate to hemoglobin may directly prevent the polymerization of deoxygenated sickle hemoglobin. Although the influence of dimethyl adipimidate on the oxygen affinity of the erythrocytes may partially explain its effects under hypoxic conditions, this mechanism alone cannot account for its antisickling properties in the absence of oxygen. In addition, a direct effect on the membrane or membrane–hemoglobin interaction which stabilizes the cell in the discoid configuration and prevents cation alterations might be considered.

Previous studies have indicated that bifunctional crosslinking reagents may alter the structure of proteins without affecting their functional properties. For example, when ribonuclease is treated with dimethyl adipimidate, enzymatic function is slightly altered despite intrachain crosslinking between the epsilon-amino groups of lysine residues (17). In human erythrocytes, Neihaus and Wold (5) have demonstrated that concentrations of dimethyl adipimidate as low as

0.08 mM covalently crosslink lysine residues in both the membrane and hemoglobin. This crosslinking had no adverse effect on glucose consumption by intact cells and inhibited the release of oxygen from hemoglobin (5). Krinsky, Bymun, and Packer have demonstrated that human erythrocytes treated with dimethyl adipimidate are resistant to osmotic lysis but maintain their responsiveness to gramicidin-induced K^+ permeability (18). This observation suggests that a physiologic stabilization of the membrane may have been induced by this agent. These data support the possibility that a combined effect on both the membrane and hemoglobin may account for the inhibition of sickling by dimethyl adipimidate.

It is premature to speculate on the possible clinical role for dimethyl adipimidate in the treatment of sickle cell disease. However, both the marked inhibition of sickling and the apparent lack of major effects on erythrocyte metabolism, suggest that this agent might be used without seriously disturbing normal erythrocyte physiology. Any therapeutic application must await delineation of the mechanism whereby sickling is inhibited and a thorough evaluation of possible toxicity.

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