ANTI-SICKLING NATURE OF DIMETHYL ADIPIMIDATE

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Dimethyl adipimidate (DMA), a bifunctional cross-linking agent, is known to be a powerful anti-sickling agent in vitro. The hemoglobin isolated from DMA-treated erythrocytes is heterogeneous with respect to charge and size as shown by starch gel electrophoresis and sedimentation velocity studies; some hemoglobin tetramers being cross-linked together resulting in high molecular weight species. Measurement of transverse water proton relaxation times of DMA-treated sickle erythrocytes shows that while some aggregation may take place within deoxygenated erythrocytes, it is not of sufficient extent to cause the cells to change shape. Therefore, it is suggested that the anti-sickling effect of DMA is due to its ability to react with hemoglobin S inside sickle erythrocytes and inhibit the aggregation process.

Erythrocytes from individuals with sickle cell disease show a marked decrease in their ability to sickle in vitro following treatment with the bifunctional cross-linking reagent dimethyl adipimidate (1,2,3). Dimethyl adipimidate (DMA) is known to covalently link free amino groups in polypeptide chains, and has been shown to cross-link human erythrocyte membranes (4,5). When erythrocyte membranes are extensively cross-linked they retain potassium gradients (5), and potassium loss is one of the primary events in sickling (6,7). In addition to membrane effects, it has been shown that the oxygen affinity of DMA-treated erythrocytes is shifted to substantially lower oxygen tensions and that upon gel filtration of hemolysates from such erythrocytes high molecular weight hemoglobin components are found (1,2,3).

The technique of measuring transverse water proton relaxation times has been found to be useful in monitoring the aggregation of deoxyhemoglobin S inside intact erythrocytes (8,9). In the present study, use has been made of this technique to investigate the anti-sickling nature of dimethyl adipimidate.

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MATERIALS AND METHODS

Samples of blood collected in sodium citrate were obtained from the Hematology Service of the Department of Internal Medicine, The University of Texas Health Science Center at Dallas. Hemoglobin from normal (A/A) and sickle cell disease (S/S) individuals was identified by polyacrylamide disc gel electrophoresis following lysis of a small sample of the erythrocytes.

The spin-echo measurements were carried out on packed erythrocyte samples (1 min at 1000 x g) at 24.3 MHz using a Nuclear Magnetic Resonance Specialties PS-60AW pulsed nuclear magnetic resonance (NMR) spectrometer. The temperature of the sample was maintained constant at the temperatures indicated ($\pm$ 0.5°C). The transverse relaxation time (T_2) was determined from the echo envelope of a series of 180° pulses as described by Carr and Purcell (10). Deoxygenation of erythrocyte suspensions was accomplished by using 95% N_2-5% CO_2 (water saturated) as the gas phase and gently shaking for 90 minutes. The deoxygenated samples were transferred into standard 5 mm NMR tubes and sealed prior to packing the erythrocytes. All manipulations were carried out inside a glove bag filled with the same N_2-CO_2 gas mixture. The percentage of deoxyhemoglobin in all samples was determined using a CO-Oximeter model 182 from Instrumentation Lab, Inc. The amount of methemoglobin in each sample was measured according to Tönz (11). The extent of erythrocyte sickling was determined by preparing a smear microscopy slide in an atmosphere of the same gas mixture described above.

Reaction of erythrocytes with dimethyl adipimidate dihydrochloride (Pierce Chemical Co.) was carried out in the following fashion. After removal of the serum, the cells were washed with isotonic Krebs-Henseleit buffer, pH 7.4 (12). Aliquots of erythrocyte suspensions were incubated at 37°C with DMA in Tris-hydroxymethyl methylaminopropane sulfonic acid buffer, pH 8.8 and 280 milliosmolar. The final hematocrit was 4-5% and the final DMA concentration was 5 mM. Sucrose was substituted for DMA in controls. After incubation for periods ranging between 15 minutes and one hour, the cells were washed with Krebs-Henseleit buffer 3 times to remove excess DMA and to lower the pH back to 7.4.

This incubation procedure is similar to that reported by Lubin, et al. (3).

For studies with the isolated hemoglobin from DMA-treated erythrocytes, the cells were lysed with water (3 times volume of packed cells) and the stroma removed by centrifugation. Starch gel electrophoresis was carried out as described by Yamaoka, et al. (13). The heme concentration of all samples was determined by measuring the absorbance at 419 nm after reduction with sodium dithionite and bubbling with carbon monoxide (ε = 191 mM^{-1} cm^{-1}) (14). Sedimentation velocity measurements were carried out at 42,040 rpm on samples containing 6 mg protein/ml in 0.05 M phosphate buffer, pH 7.0, using double sector cells in a Spinco Model E analytical ultracentrifuge (15). The solubility of the hemoglobin isolated from treated and untreated erythrocytes was measured in 1.96 M phosphate buffer, pH 7.0 (16). The minimum gelation concentration of samples of isolated deoxyhemoglobin S from treated and untreated erythrocytes was measured using dry 95% N_2-5% CO_2 (17). The heat stability of samples of isolated hemoglobin from treated and untreated erythrocytes was measured according to Huehns, et al. (18) at 70°C, 75°C and 80°C.

RESULTS AND DISCUSSION

The starch gel electrophoresis patterns of hemoglobin samples isolated from DMA-treated and untreated erythrocytes are shown in Fig. 1. It should be noted that in all DMA-treated samples there is a trailing of protein and some hemoglobin is seen to remain at the origin. This pattern suggests a very heterogeneous reaction of DMA with hemoglobin including cross-linked hemoglobin species having molecular weights higher than 64,000 daltons, a view which is confirmed by the ultracentrifugation pattern shown in Fig. 2. The hemoglobin from DMA-treated samples shows a schlieren pattern which is skewed toward the bottom of the cell indicating a heterogeneous mixture of higher molecular weight forms. In addition, the electrophoretic pattern in Fig. 1 shows that identical patterns are found when either oxy or deoxy erythrocytes are incubated with DMA. However, since the DMA incubation is carried out at pH 8.8, the deoxy erythrocytes will not be sickled and the intermolecular con-

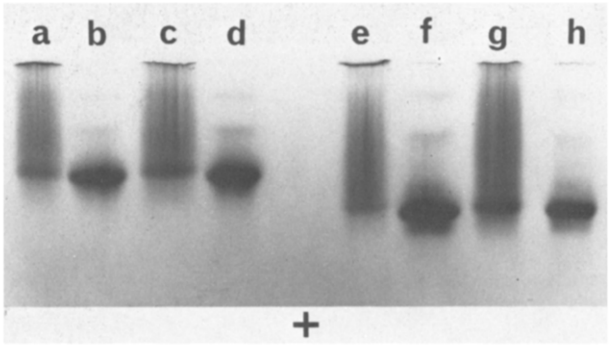

Fig. 1. Starch gel electrophoresis pattern of hemoglobin isolated from DMA-treated erythrocytes. Samples a through d are from sickle cell disease erythrocytes while samples e through h are from normal erythrocytes. Samples a and e are from oxy erythrocytes following DMA treatment while samples b and f are the respective controls. Samples c and g are from deoxy erythrocytes following DMA treatment while samples d and h are the respective controls. All DMA incubations were carried out for 15 minutes. Electrophoresis was carried out at room temperature for 90 minutes at pH 8.6, and the gel was stained with amido black and destained with methanol:acetic acid:water (13). The anode is at the bottom of the picture.

tacts between deoxyhemoglobin S molecules will be greatly decreased (19,20). Therefore, the similarity in electrophoretic patterns is not surprising and qualitatively there appears to be no difference in the extent of DMA reaction between oxy and deoxy hemoglobin S.

The solubility in 1.96 M phosphate buffer, pH 7.0, of hemoglobin S and A isolated from DMA-treated erythrocytes was less than 0.01 mg/ml in either the oxy or deoxy samples. This result suggests that essentially all the hemoglobin isolated from DMA-treated erythrocytes is reacted with DMA although only a portion of these reacted molecules are linked together as seen in Fig. 2. Thus, modification of free amino groups of the hemoglobin molecule by DMA results in loss of solubility in high phosphate concentration. The minimum gelation concentration of hemoglobin S solutions from DMA-treated erythrocytes was found to be about 350 mg/ml while that from untreated erythrocytes was about 250 mg/ml.

The electrophoresis and ultracentrifugation studies indicate that treatment of erythrocytes with DMA leads to a heterogeneous mixture of hemoglobin derivatives including some molecules which are cross-linked together to yield

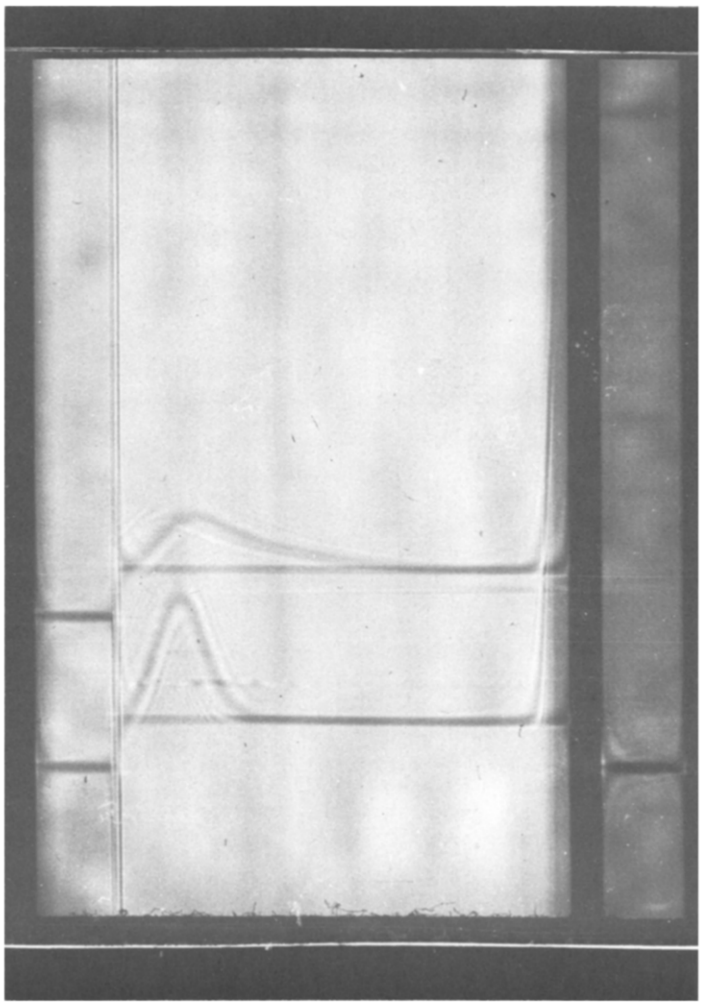

Fig. 2. Sedimentation velocity pattern obtained with hemoglobin S from DMA-treated erythrocytes. The pattern on top was obtained with isolated hemoglobin S from sickle cell disease erythrocytes following DMA treatment for 15 minutes while the pattern on the bottom was obtained with hemoglobin S from untreated erythrocytes. Both samples contained 6 mg hemoglobin/ml in 0.05 M phosphate buffer, pH 7.0. The speed of the rotor was 42,040 rev/min and the temperature was maintained at 20°C. The above picture was taken 50 min. after attaining maximum speed utilizing the normal optical system of a Spinco Model E analytical ultracentrifuge with red light supplied by a Baird-Atomic Filter having a transmission maximum of 691 nm (15).

higher molecular weight species. The effect of DMA on the hemoglobin inside red cells was also examined by measurement of transverse water proton relaxation times (T_2). In Table I are shown the observed T_2 values at 37°C from both DMA-treated and untreated, oxy and deoxy, (A/A) and (S/S) erythrocytes. As

TABLE I

Effect of DMA Treatment of (S/S) and (A/A) Erythrocytes on the Observed Transverse Water Proton Relaxation Times

SAMPLE	DMA	T_2 (sec)
(S/S) Erythrocytes		
oxy	-	0.141 ± 0.044
	+	0.057 ± 0.014
deoxy	-	0.038 ± 0.006
	+	0.048 ± 0.008
(A/A) Erythrocytes		
oxy	-	0.207 ± 0.046
	+	0.067 ± 0.038
deoxy	-	0.177 ± 0.030
	+	0.067 ± 0.039

The spin echo measurements were at 24.3 MHz at 37°C. The reaction with DMA (5 mM) and subsequent deoxygenation are described in the text. The untreated deoxy (S/S) samples contained greater than 90% sickled cells, while the DMA-treated deoxy (S/S) samples contained less than 10% sickled cells. The deoxygenated samples all contained more than 93% deoxyhemoglobin. The T_2 values are the mean value and one standard deviation observed with five different (S/S) and (A/A) erythrocyte samples.

previously reported, deoxygenation of (S/S) samples results in a shortening of the observed T_2 value which is interpreted as being due to an increase in the mean correlation time for the small fraction of water having a correlation time equal to or greater than that for the rotational motion of a hemoglobin molecule [irrotationally bound water] (9). It is assumed that aggregation of deoxyhemoglobin S causes the increase in the correlation time of this water fraction and the shortened T_2 values. In Table I it can be seen that treatment of oxy (S/S), oxy (A/A) and deoxy (A/A) erythrocytes with DMA results in a drastically shortened observed T_2 value. This suggests that DMA cross-links the hemoglobin inside the erythrocytes thereby increasing the mean molecular weight and also the mean rotational correlation time for the irrotationally bound water. Deoxygenation of the DMA-treated (S/S) erythrocytes results in no significant decrease in the observed T_2 value. If some aggregation of deoxyhemoglobin S does occur in

these samples it is clearly not sufficient to cause a change in cell shape. No change is observed in the concentration of methemoglobin between untreated and DMA-treated erythrocytes and the heat stability of the hemoglobin isolated from cells reacted with DMA is not altered.

The conclusion drawn from this study is that while DMA can react with both the erythrocyte membrane and the intracellular hemoglobin, the anti-sickling effect is due to inhibition of deoxyhemoglobin S aggregation. The limitation on deoxyhemoglobin S aggregation may result from DMA interaction at specific sites on the hemoglobin molecule or from the cross-linking together of hemoglobin tetramers thereby decreasing the concentration of deoxyhemoglobin S molecules which can interact to form an aggregate. In either case, it is important to recognize that DMA has a different anti-sickling effect than cyanate in that it inhibits sickling even under conditions of greater than 90% deoxygenation. Both compounds result in a shift in the hemoglobin oxygenation curve to the left, and this has been proposed as the mechanism by which cyanate functions as an anti-sickling agent (21). However, cyanate-treated erythrocytes will sickle when completely deoxygenated while DMA-treated erythrocytes will not. The anti-sickling effect of DMA is the result of a structural modification of the hemoglobin molecule rather than being due to the shift in the hemoglobin S oxygenation curve. We, therefore, propose that the in vivo effect of this compound with respect to toxicity should be examined.

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