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THE PREVENTION OF SICKLING HUMAN ERYTHROCYTES IN VITRO BY IMIDOESTER TREATMENT

Edwin Norris Bymun
(M. S. thesis)

June 1975

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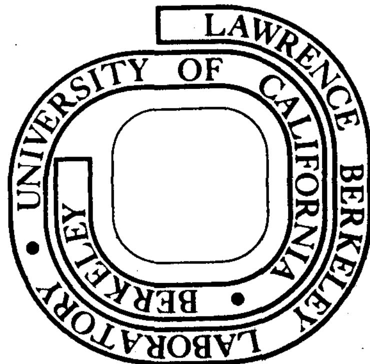

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Acknowledgment

I would like to acknowledge my appreciation for the expert advice given by Dr. Lester Packer, clinical samples given by Dr. Burt Lubin, and financial assistance given by the ERDA of the Lawrence Berkeley Laboratory in the preparation of this thesis.

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ABSTRACT

Previous studies have shown that sickling can be inhibited by increasing the hemoglobin-oxygen affinity. Evidence is presented here that brief treatment of human erythrocytes with a bifunctional cross-linking reagent dimethyl adipimidate at low concentrations prevents sickling, increases hemoglobin-oxygen affinity and preserves the physiological and and physical properties of treated cells.

Sickle erythrocytes treated with DMA washed and resuspended in original plasma showed the following properties under deoxygenated conditions: (a) the number of sickle forms was reduced from 80 to 4%; the higher viscosity of sickle cells was brought into the range for normal cells; (c) the K leak normally found under nitrogen was inhibited; (d) red cells indices were shifted, for example, mean corpuscular hemoglobin 92 to 97 μ^3; (e) osmotic fragility and auto hemolysis were decreased; (f) glucose utilization and ATP synthesis was retained; (g) the oxygen dissociation curve p50 value was shifted from 30 to 24 mm Hg. Normal cells treated under similar conditions retain normal properties.

Following these initial findings with dimethyl adipimidate three monofunctional and three other bifunctional imidoesters were also examined for their effect in inhibiting sickling <u>in vitro</u>. The short-chain bifunctional reagent, dimethyl malonimidate, is without effect but two other long-chain bifunctional compounds, dimethyl suberimidate and di-thio-bis-propionate, were quite effective. Two monofunctional reagents were found to be without effect, one of them being an impermeable reagent. However, a permeant monofunctional reagent, ethyl acetimidate, was found to be

almost as effective as the other long-chain bifunctional reagents in inhibiting sickling *in vitro*. In addition, cleavage of the di-thio compound does not reverse the antisickling effectiveness of this compound.

These results suggest that chemical modification of erythrocytes probably accounts for the effectiveness of these reagents in inhibiting the sickling of human erythrocytes.

Sickle cell anemia is a disease that affects the hemoglobin within the red blood cell. Specifically, the hemoglobin (Hb) S molecules polymerize within erythrocytes and form liquid crystals or gels.[1] It has long been known that the primary structural abnormality of Hb S that accounts for the aggregating properties of the deoxyhemoglobin is that valine instead of glutamic acid is the sixth amino acid in the beta polypeptide chains. The changes produced during deoxygenation have a profound effect on the membrane's functions. Indeed, an acquired membrane defect is believed to be responsible for the maintenance of the sickle shape in oxygenated irreversibly sickled cells[2], and even in reversible sickle cells temporary membrane defects can become accumulative and produce a permanent membrane change.[3] _In vitro_ studies have shown that the membrane defect is associated with alterations in cellular metabolism that apparently occur only if ATP is almost completely depleted while the cells are sickled. (ATP is responsible for preventing the accumulation of calcium ions and for providing the energy source for sodium potassium transport.[5,6,7] . ATP depletion, in turn, is consequent to deficiencies in those enzymes responsible for maintaining the level of ATP in the membrane.

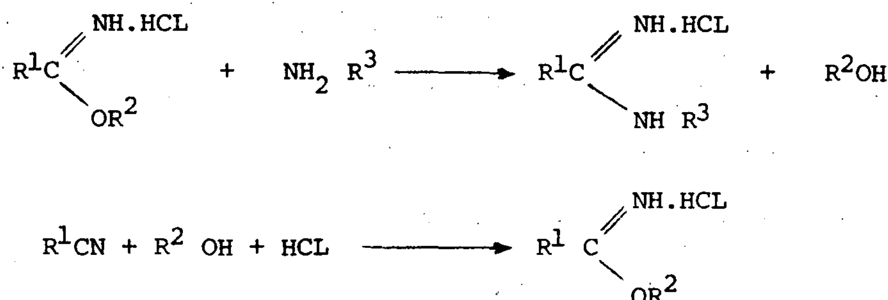
Membrane distortion during sickling has been shown to be associated with increased trans-membrane fluxes of potassium and sodium, a rapid initial calcium uptake, a decrease in ATP synthesis, a decrease in phospholipids and a decrease in osmotic lysis. These membrane defects can be prevented by _in vitro_ treatment with sodium cyanate[8]. Sodium Cyanate carbonylates, the N-terminal groups of free amino acids, specifically the valine in the beta chains, cause an increase in the hemoglobin oxygen affinity such that less oxygen is released from the cells. This

reagent was recently found to be useless in oral treatment of sickle cell anemia however, because of its many adverse side effects[9,10,11]. Urea also has been known to prevent sickling _in vivo_ and _in vitro_ by disrupting the hydrophobic bonds which form, when deoxygenated, hemoglobin S gels within the cell[12,13,14]. Unfortuantely, this method works only when the cells already have sickled; therefore, it has little or no therapeutic value in preventing sickle membrane changes[15,16,17].

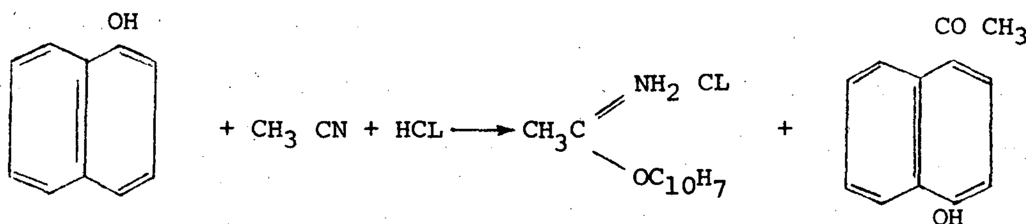
Packer and Pereira in 1972 showed that glutaldehydes prevented sickling by strenghthening or by inducing cross-linkage of the hemoglobin molecules; but further experiments indicated that they had deleterious effects on enzyme activity and potassium permeability of the membrane. Later work by Brooks, Vassar _et al_[18], indicated that treatment with milder aldehydes left intact the enzyme activity in the membrane with no apparent changes in potassium permeability. The electrokinetic studies of these investigators revealed that aldehyde treatment blocks the positive charge in the peripheral zone of the cells, thereby altering the permeability of the membrane in favor of the potassium leaking out of the cells during treatment. [19]

Recently, Bynum and Packer, reported that many commercially available bifunctional imidoesters would prevent sickling without posting any deleterious side effects upon the metabolic functions of its membrane. Many bifunctional reagents yield derivatives whose solubility in water is very limited due to the conversion of charge protein reactive groups to uncharged substituted groups. Imidoesters are reagents that when reacted with protein groups will not produce any charge different from that displayed on the new modified proteins.

The preparation and reactions of imidoesters were studied many years ago by Pinner, who prepared a variety of imidoesters from nitrilie by treatment with HCL and anhydrous alcohol[20,21]. His method:



remains the one generally employed in the preparation of this weak base. Another similar method for synthesizing imidoesters is that of Hoesch's. Essentially, the method consists of condensing a nitrile with a phenol or a phenolic ether in the presence of zinc chloride and anhydrous hydrogen chloride in a suitable solvent, the reaction proceeding via the imino chloride and then the ketimino hydrochloride to the ketone. In certain cases, however, imidate formation proceeds simultaneously[22].



The preparation of protein derivatives from imidoesters predicates the use of aqueous solutions and demands that the reaction be performed at low temperature and at pH values not too far removed from neutrality[23]. The reactions of imidoesters with amines have generally been performed in non-aqueous medium since imidoesters are subject to hydrolysis. The nature of the product (nitrile, ester, amide) depends on the structure of the imidoesters, the pH and the temperature of the reaction[24]. The tolerance of imidoesters to heat and hydrolysis depends on

the substituents imidoesters salts decompose to the corresponding amide and alkyl halide on pyrolysis by heating in inert solvent, but form esters and ammonium chloride in the presence of water[25]. The ammonium so produced reacts, in turn, with another molecule of imidoester to form an unsubstituted amidine[26].

The pH profiles of the rate of reaction of alpha and esplion amino groups with imidoesters are sufficiently different that preferential reaction of the alpha or the esplion amino groups can be achieved by adjusting the pH of the reacting solution. For example, at pH 8.5 and 37°C with equimolar concentration of alpha and esplion amino groups,the alpha amino groups react about four times faster than the esplion groups, whereas at pH 9.7, the esplion groups react seven times faster than the alpha amino groups. The reaction of a given imidoester in the presence of two amines of differing pH, therefore, can be controlled by a proper choice of the pH, permitting one or the other of the amino reactions to predominate[27].

Di-imidoesters have been useful for inter and intramolecular cross-linking of lysyl residues[28]. Because of the competing hydrolysis reaction, not every di-imidoester is in a position to actually form a cross-link: monofunctional substitution occurs at those sites where intramolecular cross links can form. Imidoesters also have been a very important tool in modifying RBC. Dutton, Adams and Singer reported in 1963 that sheep red blood cells treated with DEM for 24 hrs at 0°C and 280 mM still had the capacity to undergo immune hemolysis in the presence of quine pig complement and rabbit anti-sheep red cell antiserum after 86% of these free amino groups of BSA were blocked[29]. Niehaus in 1970 treated human erythrocyte membranes with DMA to determine the formation between soluble and insoluble protein molecules and found more insoluble proteins after prolonged treatment indicating that insoluble proteins were being cross-linked without any functional changes in the membrane[30]. Marinette used a combination of D.F.D.N.B and imidoesters to demonstrate crosslinking of phospholipids to proteins in erythrocyte membranes and

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showed that phospholipids could be crosslinked up to 70% without any noticeable metabolic defect[31]. Ji, proved in 1973 that sealoglycoproteins in human erythrocyte membrane were being crosslinked with low concentration of dimethyl adipimidate and with no significant change in membrane function[32].

I have found in my studies with imidoesters, that osmotic fragility increased, potassium leakage in sickle cells was reduced, sickling was inhibited and viscosity was decreased without any deleterious side effects on the functional characteristic of the cells.

These studies were performed by *in vitro* treatments only. The relative effectiveness of imidoesters in preventing sickling *in vivo* is unknown, but we have a very strong suspicion that the cell possesses the same potent inhibiting effects as *in vitro* studied cell without any tissue toxicity.

PURPOSE

The purpose of the research presented in this thesis is to study the effectiveness of imidoesters in preventing membrane changes that have been known to occur when erythrocytes undergo sickling.[33,34,35] By monitoring such changes as occur in association with sickling: increase in sodium and potassium transmembrane fluxes, decrease in ATP synthesis, in viscosity, and in osmotic fragility, on or within the membrane; with such techniques as low angle and 90° light scattering, nitrogen and gramicidin-induced potassium release, osmotic fragility, and TNBS reactions, the effectiveness of imidoesters treatment on functional characteristics of sickling erythrocytes, can be measured. The information gained from this study will delineate more precisely the role the membrane actually plays under sickling conditions and bring closer the possibility of designing effective therapy for the treatment of sickle cell anemia in humans.

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MATERIALS AND METHODS

Fresh heparinized blood samples were obtained from patients with sickle cell anemia at the Children's Hospital in Oakland, California. Fresh normal blood was obtained from myself, and expired normal blood was obtained from the Alameda Contra Costa County blood bank. The cells were washed with Krebs Henseleit buffer, pH 7.4, which contained 200 mg % glucose, and resuspended to a final hematocrit of 10-20%. To 4 ml of the red cell suspension was added 16 ml of 0.14M tris-hydrozymethyl methylamine propane sulfonic acid buffer, previously adjusted to pH 8.8 with 5N NaOH. The entire suspensions were then incubated with the various imidoesters. Control samples were prepared in an identical fashion except that sucrose was added in the same concentration as the imidoesters. Continuous mixing was maintained with a shaking water bath. The osmolarity of the incubated mixture was 280mosM. Following 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 were measured in one sample after the preparations were deoxygenated by equilibration with 3% O_2 for 1 hour in an II tonometer model #237. Following equilibration, aliquots were anaerobically transferred into 10% formaldehyde in saline for fixation. 500 cells were counted using the method of Butler[36]. Another sample was treated with sodium metabisulfate by adding one drop of a 2% solution to one drop of sickle blood and mixing on a 3 x 1 inch microscope slide. All the samples were sealed by placing a cover slip over the slides and adding vaseline around the edges. The number of fixed sickle cells were counted by one method described above.

Osmotic fragility of the treated and nontreated erythrocytes was tested by the method described by Parpart et al[37]. Hypotonic 10% buffered saline is made by adding NaCl 180g, $NaHPO_4$ 27.31g and $NaH_2PO_4.2H_2O$ 4.86g to a final volume of 2000ml of water. This solution is then buffered to pH 7.4 with 1N NaOH from which is derived the various NaCl concentrations gradient (0.9,0.75,0.70, etc). 0.05ml of a 40% Hct. of blood was added to 10 ml of each of the various concentrations. The tubes are allowed to stand at room temperature (20°C) for one hour, then remixed and centrifuged for 5 minutes at 2,000rpm. The amount of hemolysis in each tube was then compared with that in the 100% lysis tube (0.1% NaCl) by reading at 540 or 545 nM on the Beckman D.B spectrophotometer, Model 1400.

90° degree light scattering changes and potassium release from normal and sickle treated erythrocytes were measured simultaneously by adding fifty microlites of sample blood (Hct. 40%) to a dual curvet, containing 7.35 ml chloline chloride-T.E.S. buffer. Light-scattering changes were determined photometrically as described by Packer[38]. A potassium ion selective electrode (GKN33, electronic) was used to monitor the potassium release after the addition of 2.66ug of gramicidin D[39]. 1% triton-X-100 solution was used to induce lysizing of the erythrocytes. Continuous mixing was accomplished by magnetic stirring and the temperature (37°C) was held constant by a circulating water bath. Both responses were recorded on a Recti/Riter Texas Instrument recorder.

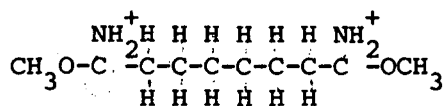
In vitro studies of erythrocyte metabolism and of net potassium loss were conducted by incubation at 37°C of thrice washed erythrocytes in Krebs Henseleit buffer with glucose (10mM) added in the presence of 10^{-4} quabain. The samples were continuously equilibrated with moistened gas mixtures consisting of nitrogen plus either 20%, 3.3% or 0% oxygen, and pH was maintained at 7.45 ± 0.05 by varying the CO_2 concentration utilizing a gasometric pH stat[39]. Supernatant of

potassium concentration was determined by flame photometry.

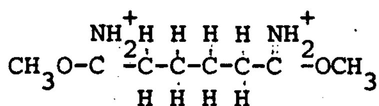
Low-angle light scattering and transmission were performed in a Brice Phoenix Universal light scattering photometer, series 2000, at 540nM[40]. 20 microliter of a 4% Hct. of treated and non-treated sickle and normal erythrocytes were added separately to a curvet that contain 5ml of buffered saline[41,42]. 0.36% $Na_2S_5O_4$ was added to the curvet to induce deoxygenation of the sickle cells[43]. Light scattering angles were set between 15-30 degrees and the percent transmission was set at 75 degrees. The cuvet was maintained at 37°C by a circulating water bath, and continuous mixing was maintained by a magnetic stir. The data were recorded on a Recti/Riter Texas Instrument recorder.

Trinitrobenzenesulfonate (TNBS) reaction was carried out by the method described by Habeeb[44]. This reaction measures free amino groups present after amidination. To 1ml of protein (0.2mg/ml) solution is added 1ml of 4% $NaHCO_3$, pH 8.5, and 1mL of 0.1% TNBS in water. The solution is held at 40°C for 2 hrs, then 1ml of 10% SDS and 0.5ml of 1N HCL are added, and the absorbance is read at 345nM[45]. The reading was taken on a Cary 14, Model #1115.

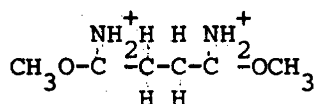
Pictures of sickle and normal blood were prepared by taking a 3x1 inch slide and adding 1 drop of 2% $Na_2S_5O_4$ solution to 1 drop of sickle blood, it was finalized by placing a cover slip over the mixture and sealing the edges by method previously described. The cells were then allowed to stand for 10 minutes so that the sickle shape could form. Normal blood was treated in the same manner except saline was added instead of $Na_2S_5O_4$. The slides were then placed on the stage of a phase contrast microscope camera attachment. Camera speed was set at 4sec. exposure and magnification at 900-x.



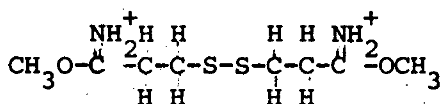
Dimethyl Suberimidate
(Dihydrochloride)



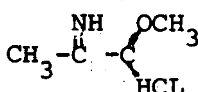
Dimethyl Adipimidate
(Dihydrochloride)



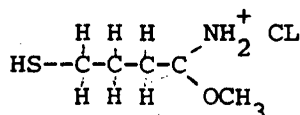
Dimethyl Malonimidate
(Dihydrochloride)



Dithiobis-Methyl Propricimidate



Ethyl Acetimidate



Methyl-4-Mercaptobutyrimidate
(Hydrochloride)

Imidoesters	Abbrv.	M.W.	#mg/20ml 1mM.	5mM.	10mM.
Ethyl Acetimidate	EA	123.6	2.4mg	12.3mg	24.6mg
Dimethyl Malonimidate (Dihydrochloride)	DMM	229g	4.5mg	22.9mg	44.8mg
Dimethyl Adipimidate (Dihydrochloride)	DMA	247.6g	4.9mg	24.7mg	49.4mg
Dimethyl Suberimidate (Dihydrochloride)	DMS	275g	5.5mg	27.5mg	55mg
Dithiobis-Methyl-proprioimidate	DMSSP	236g	4.7mg	23.6mg	47.2mg
Methyl-4-Mercaptobutyrimidate (hydrochloride)	MMB	169.7g	3.3mg	16.9mg	23.8mg

RESULTS

Osmotic Fragility

When cells are suspended in isotonic NaCl solution and diluted with water, the cells swell until they burst and liberate hemoglobin. This diffusion of hemoglobin from the cells continues until hemoglobin concentration is uniform in the resulting hemolysate. Hemolysis is an all-or-none effect as far as a single cell is concerned: i.e. lysis is either complete or no hemoglobin whatsoever is lost.[46]

Cells of varying ages are not all hemolysed at the same dilution, and their differing place at end when fixed can be obtained by plotting the percentage hemolysis against the concentration of external NaCl, a typical sigmoid curve is obtained showing that the cells in the population have different resistances to osmotic hemolysis.[47]

In order to determine the extent of the reaction of imidoesters on erythrocytes membranes, and to map out the ideal conditions for the therapeutic treatment of erythrocytes, it is important to assess the fragility of the cells. Osmotic fragility is arbitrarily taken as the reciprocal of the dilution of the isotonic medium required to produce a given degree of hemolysis. It will clearly depend upon the osmotically active substances in the cells, on ph, O_2 tension, temperature, and, in the present work, in the amount of cross-linkages present.

The amount of reaction between imidoesters and erythrocytes seems to depend on six major variables. The first being ph, the second the amount of alpha and epsilon amino groups available to react with imidoesters, the third with the specific modification of each imidoester on the hemoglobin and membrane, fourth,

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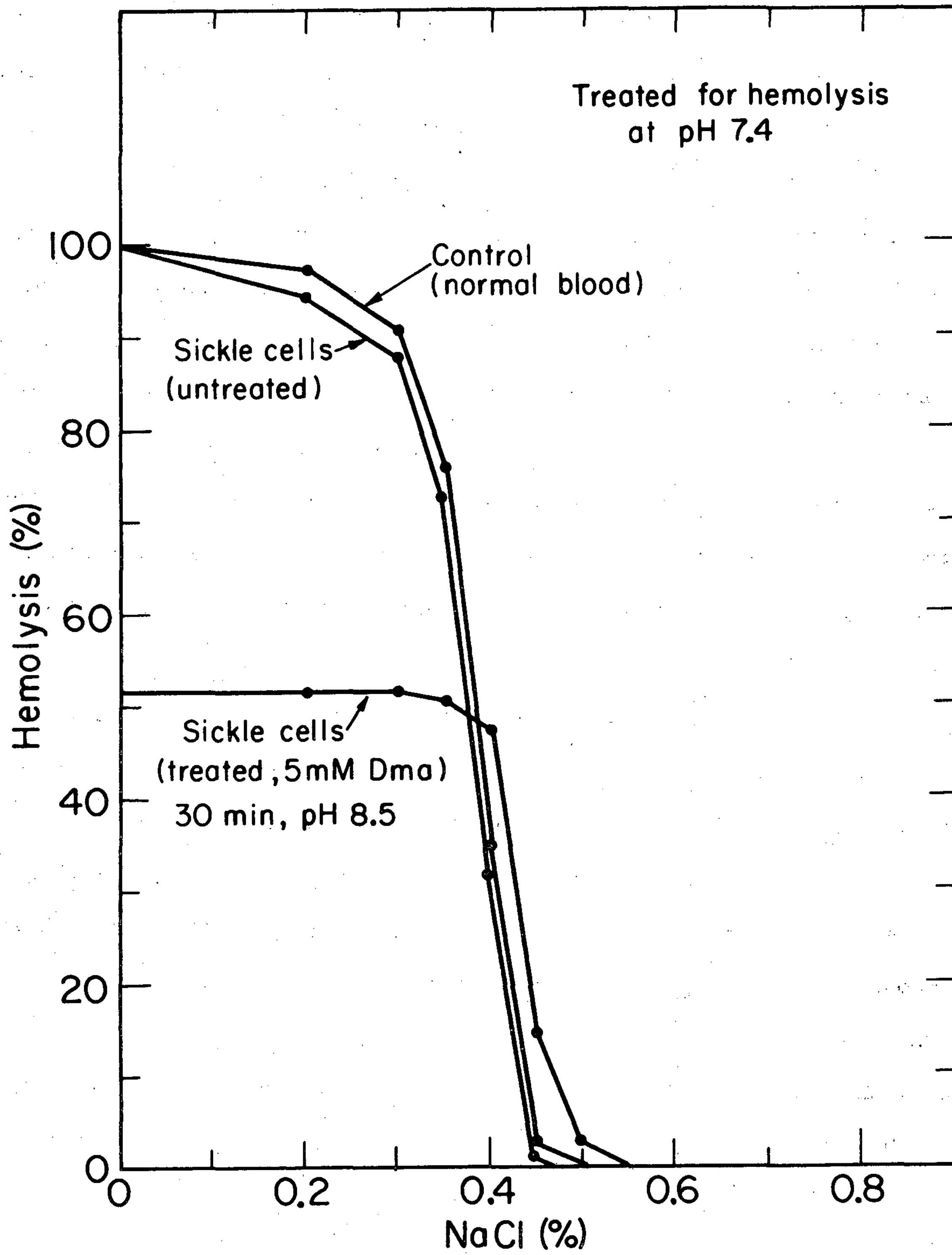

FIGURE I

temperature, fifth, time length of the reaction, and sicth, the imidoesters concentration. A visual summary of each variable can be seen in Figs. 1-8.

Fig. 1 shows the difference in osmotic fragility between sickle and normal erythrocytes, with and without treatment of imidoesters. We see that untreated sickle cells are more resistant to hemolysis than normal cells at room temperature. This resistance can be explained by the fact that sickle cell membranes form more denatured hemoglobin from the continuous hemoglobin breakages than do normal erythrocytes.[48] Denatured hemoglobin, bound to the membrane surface, makes it more rigid and resistant. Such damage to the membrane from this process shortens the circulatory survival of the red cells and thus contributes to the anemia associated with sickle cell disease. Fixed sickle cells behave no differently from normal cells after treatment with imidoesters. The amount of aminidation that takes place between various pH treated cells can be seen in Figure 2. Using equimolar concentrations of dimethyl adipimidate (DMA) significant differences were noted in pH values. At lower pH, a large amount of decomposition took place between water molecules and imidoester molecules.[49] Imidoesters in water undergo hydrolysis to the corresponding oxygen ester, and base-catalyzed decomposition to the corresponding nitrile; the uncatalyzed decomposition of the neutral molecule occurs much more slowly.[50] The hydrolysis reaction competes with the erythrocytes for the available DMA, therefore, at pH 6.0, the DMA is used up before the erythrocytes have a chance to react with it. A higher pH's (7.4, 8.5, 10) progressively more reaction occurs with the erythrocytes because hydrolysis isllessened.

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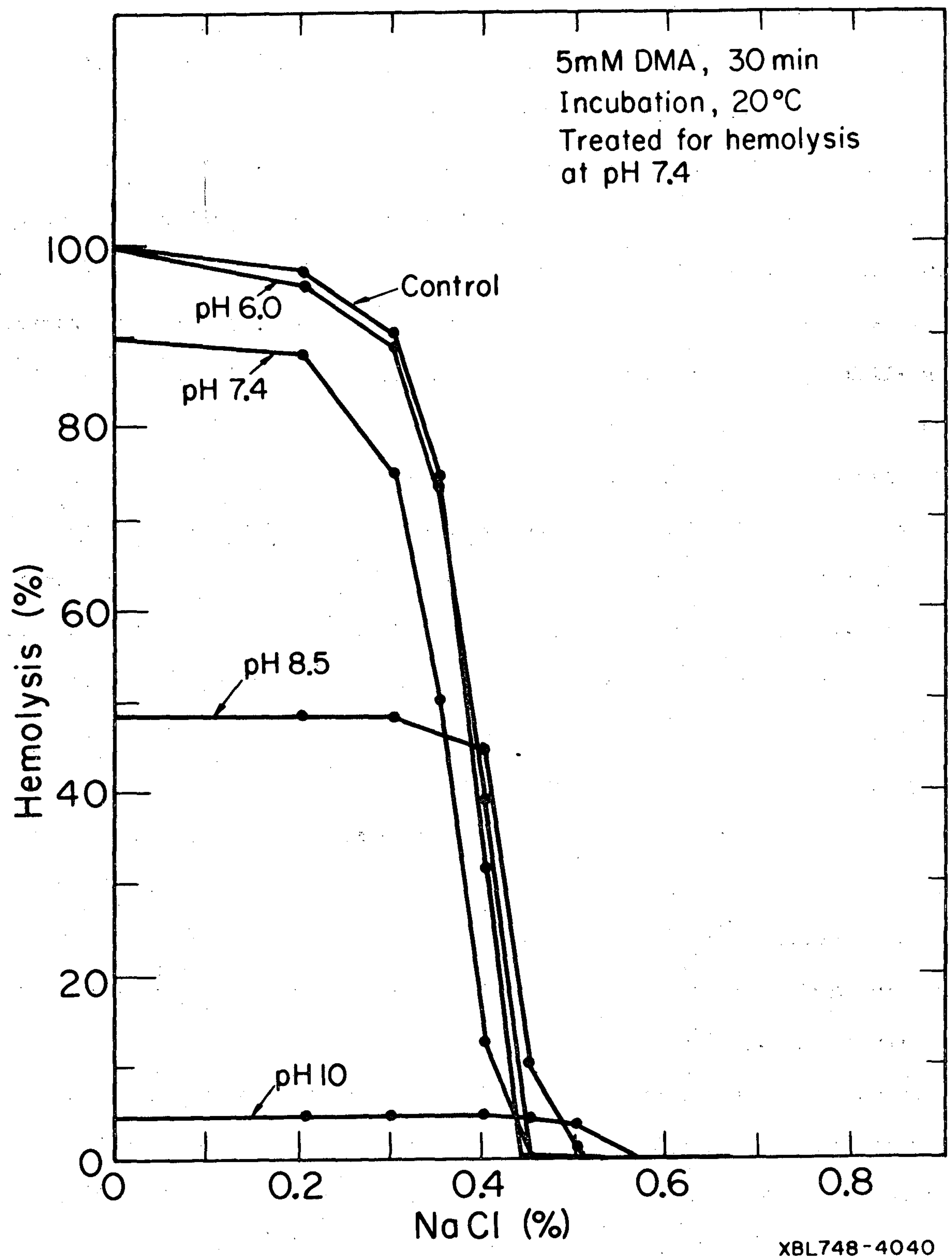

FIGURE 2

In Fig. 3, we see that varying the temperature from 20° to 0°C reduces the amount of osmotic hemolysis by a factor of five, therefore, temperature plays an important role in fixation. In Figs. 4-5, we see the difference in varying the concentration. Increased concentration always increases the rate of the reaction between the erythrocyte and imidoester until cross-linking reached a maximum. Fig. 6 shows the difference in time length of the reaction with erythrocytes. We see that, with an increase in time, there will also be an increase in cross-linking. This increase will continue until all of the imidoester is used up.

The last and most important feature that was detected through the use of osmotic fragility is the amount of osmotic hemolysis displayed between the various imidoesters. It has been illustrated in Figs. 7 and 8 that imidoesters with a chain length similar to DMS, DMA and DMSSp turn out to be the better cross-linker than imidoesters with a structure similar to DMM and MMb. The difference that is displayed between these compounds can be associated with the lack of specific structure that is needed for a specific attachment, The amount of monofunctional linkage that will take place, and the amount of decomposition that will occur between the specific imidoester and the water molecules.

Monofunctional imidoesters generally do not prevent hypotonic hemolysis because n-n-disubstitation does not occur between proteins. This can be seen in Fig. 8 with respect to ethyl acetimidate.

Although osmotic lysis can be used to determine changes in the ability of the erythrocyte membrane to respond to osmotic shock, it does not, by itself, yield information about the functional characteristics of the intact cell. To investigate functional stability of the erythrocyte after treatment with imidoesters, we simultaneously recorded volume changes photometrically and the cell's ability to retain

0 0 0 0 4 4 0 4 8 0 2

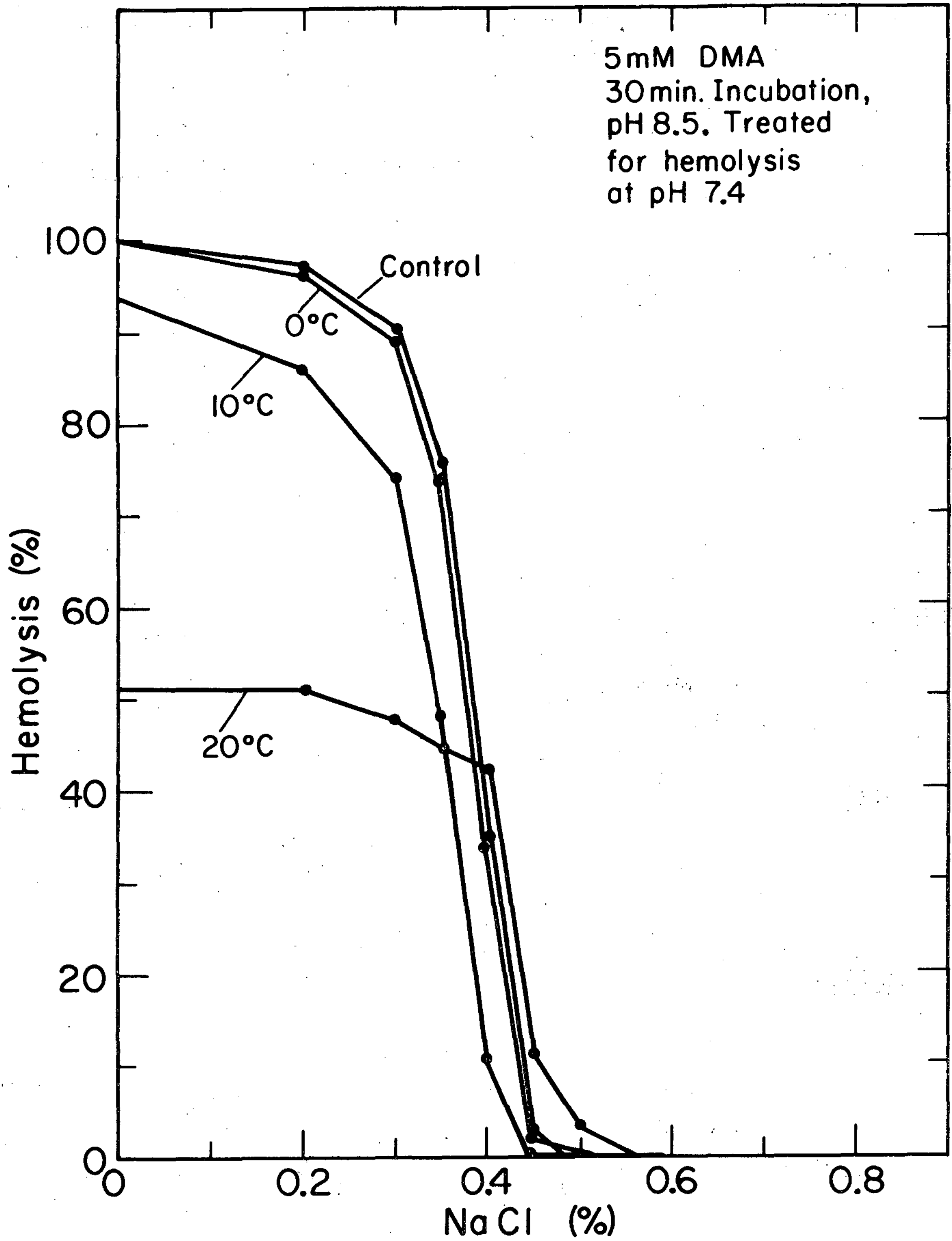

FIGURE 3

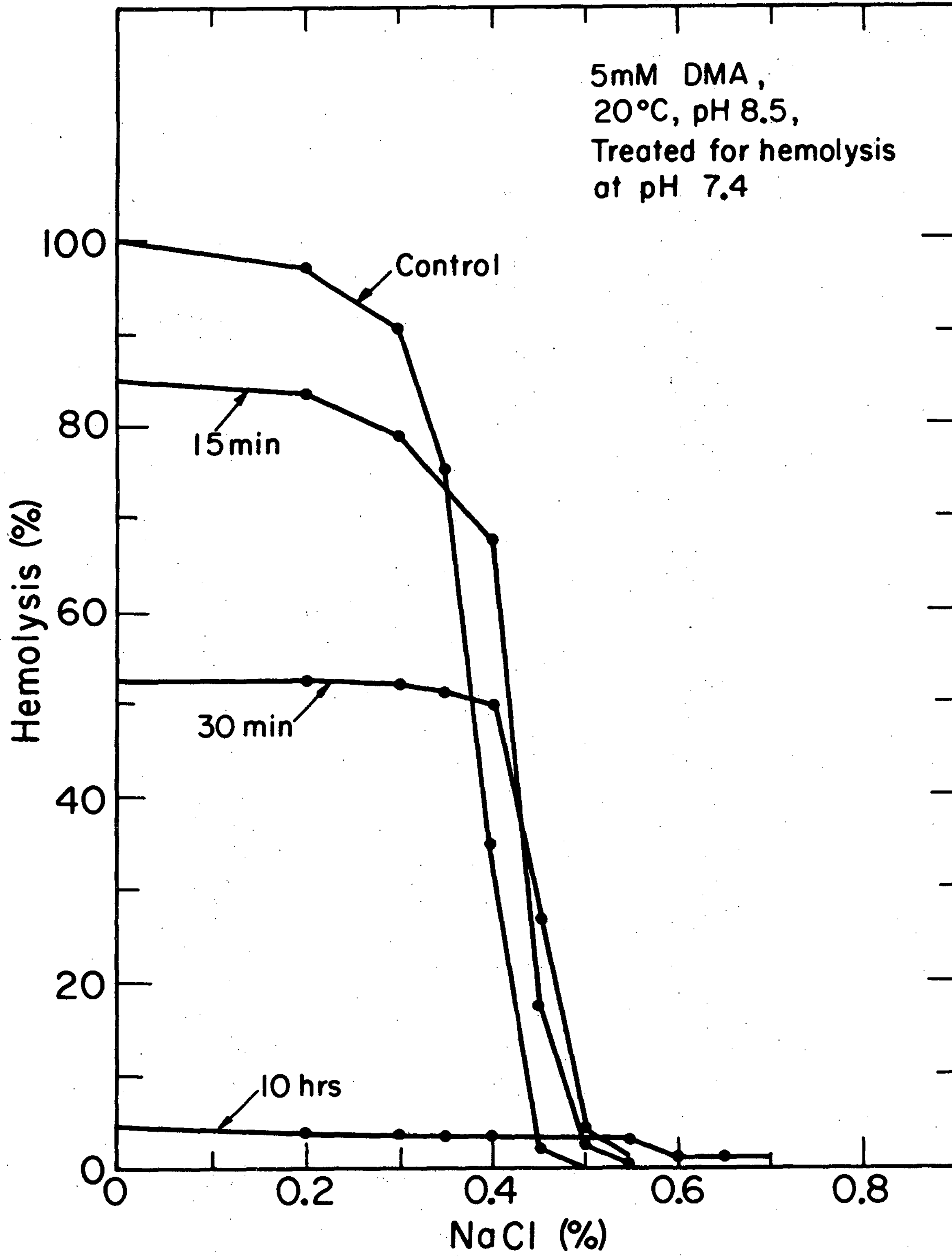

FIGURE 4

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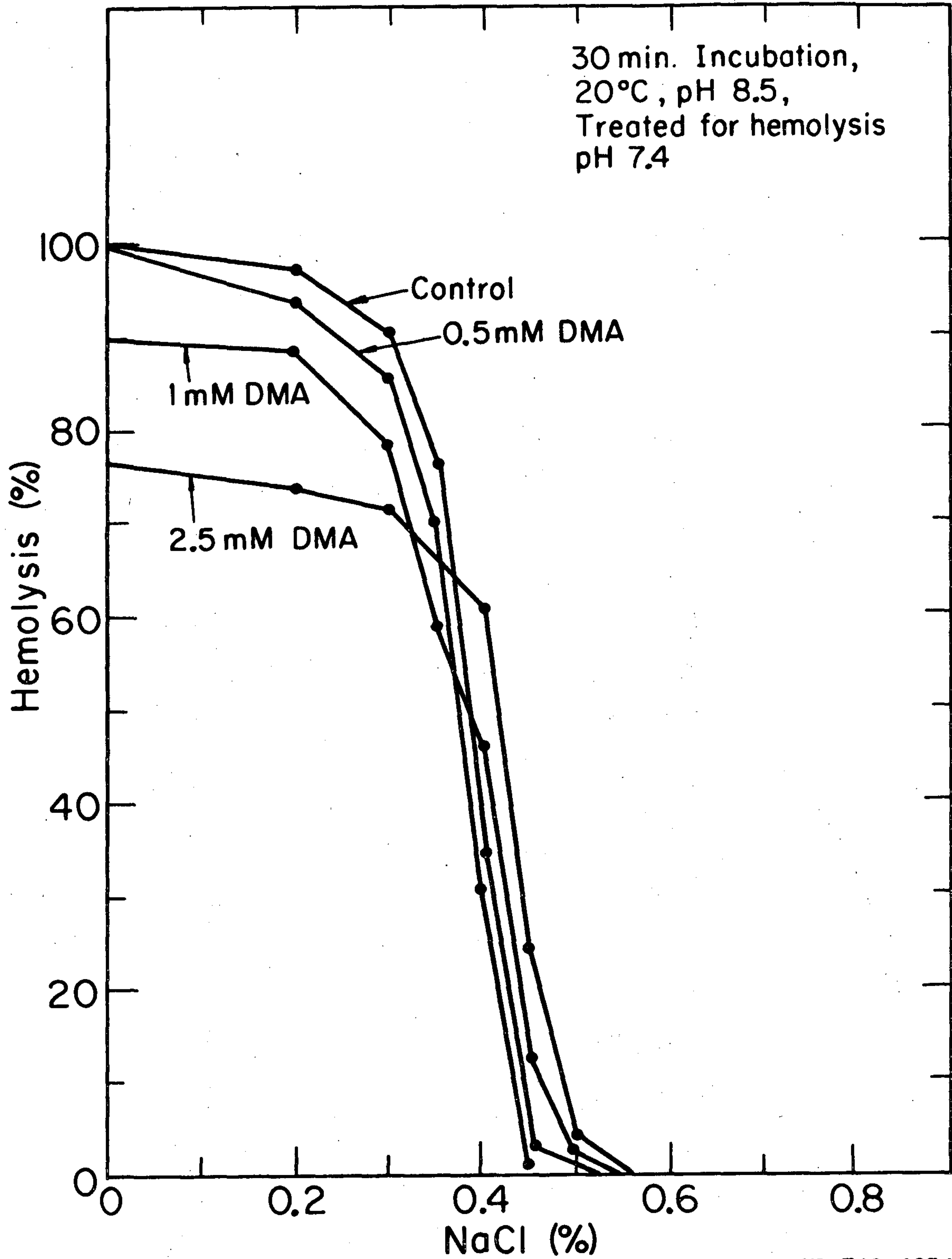

FIGURE 5

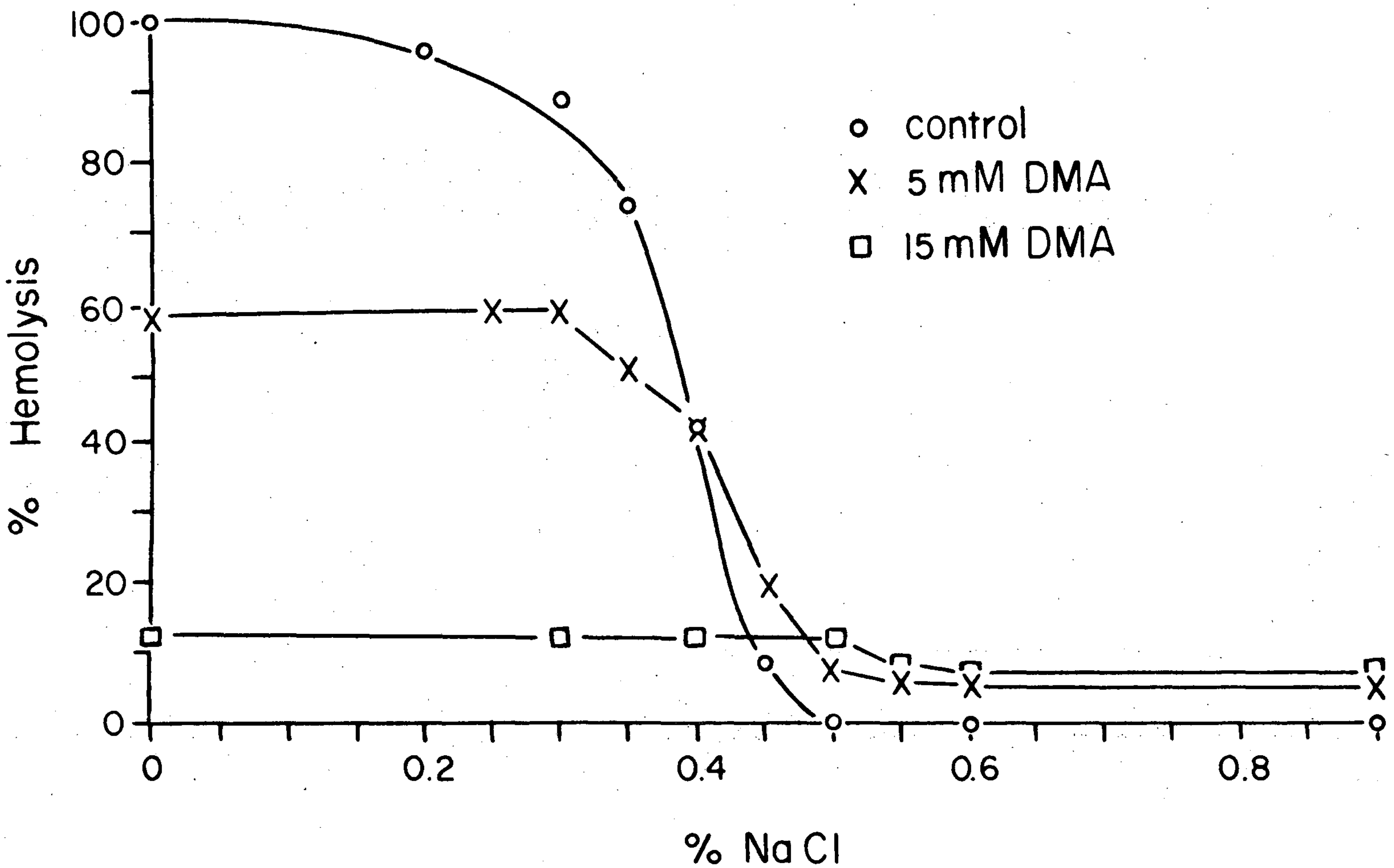

FIGURE 6

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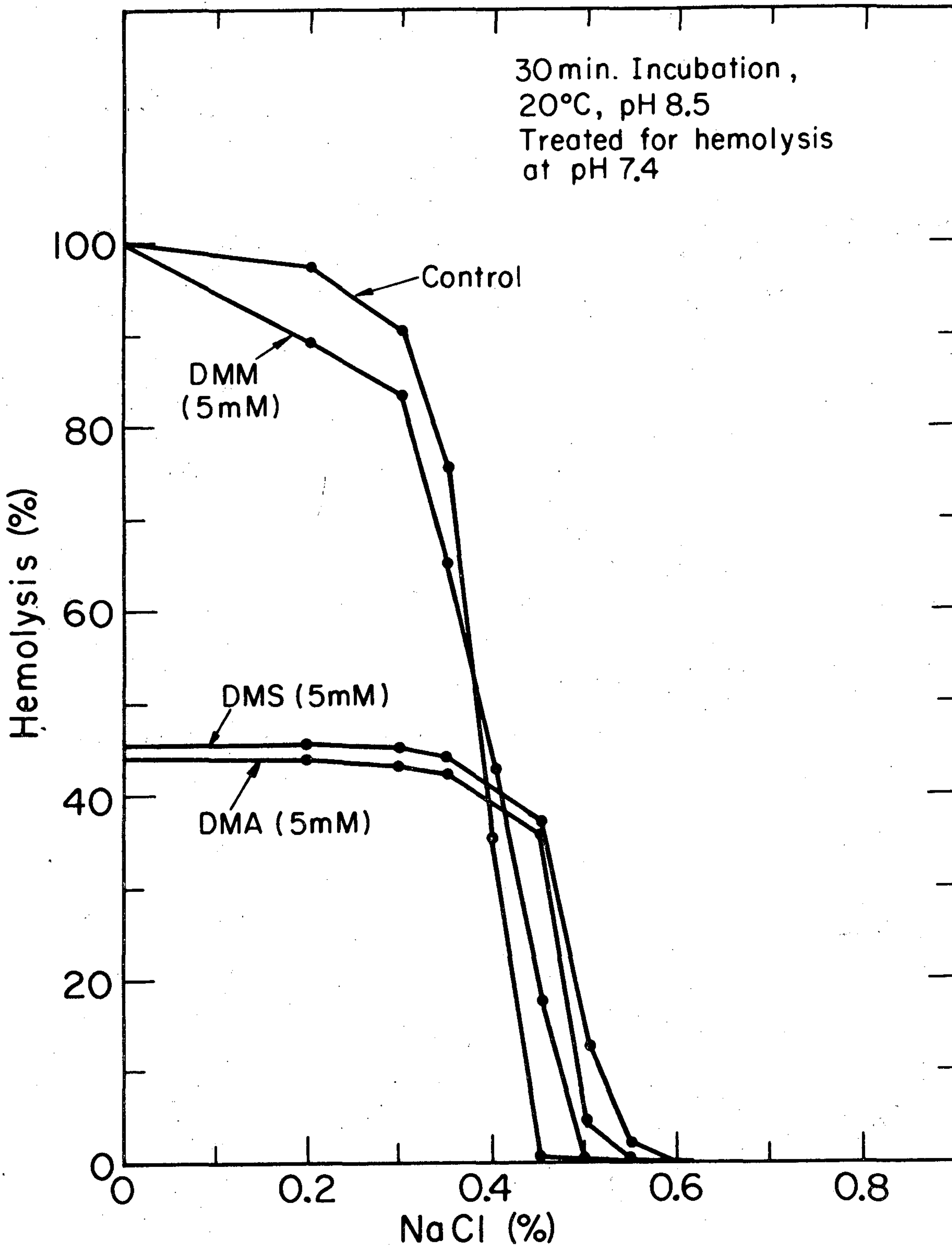


FIGURE 7

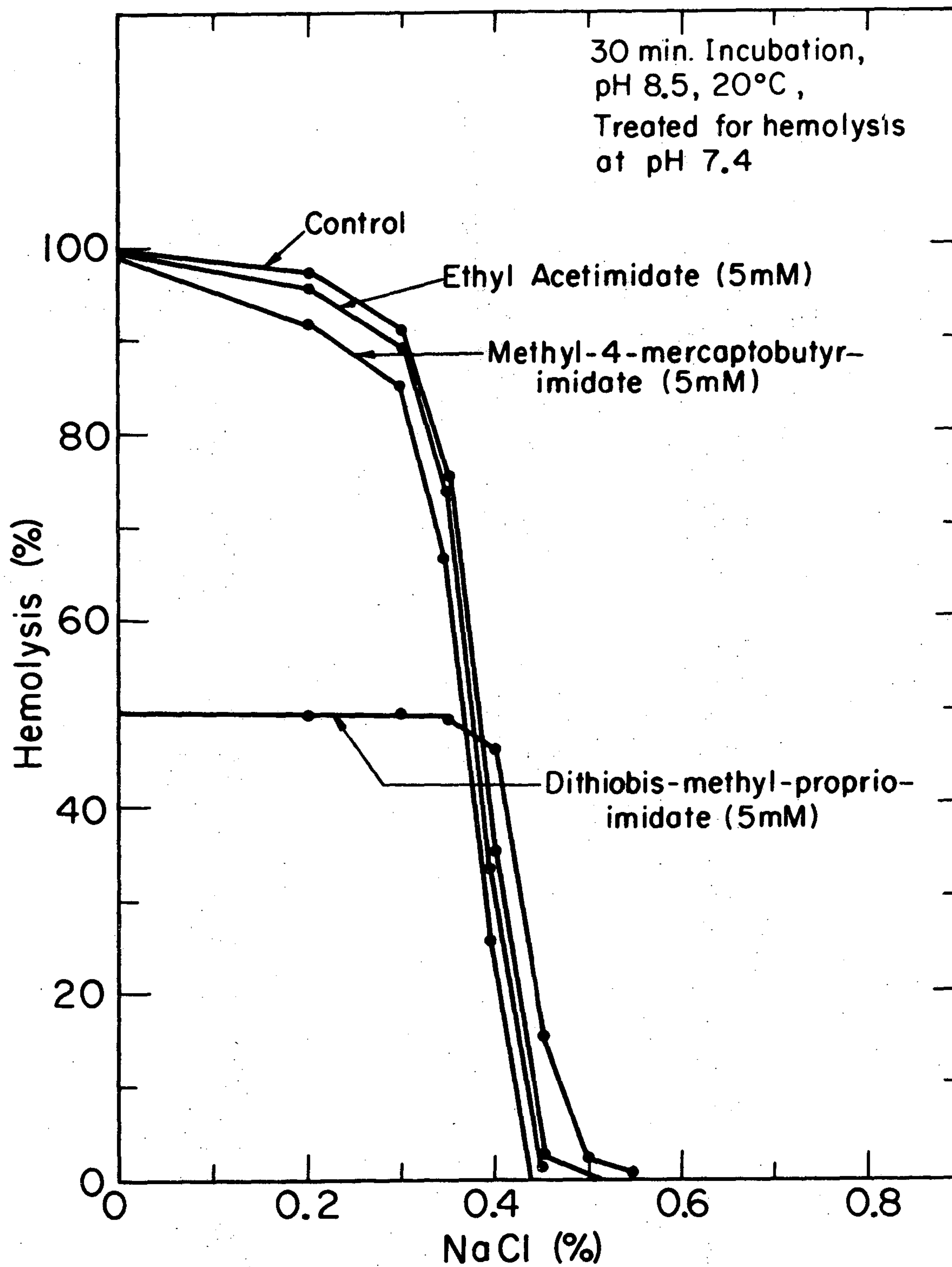
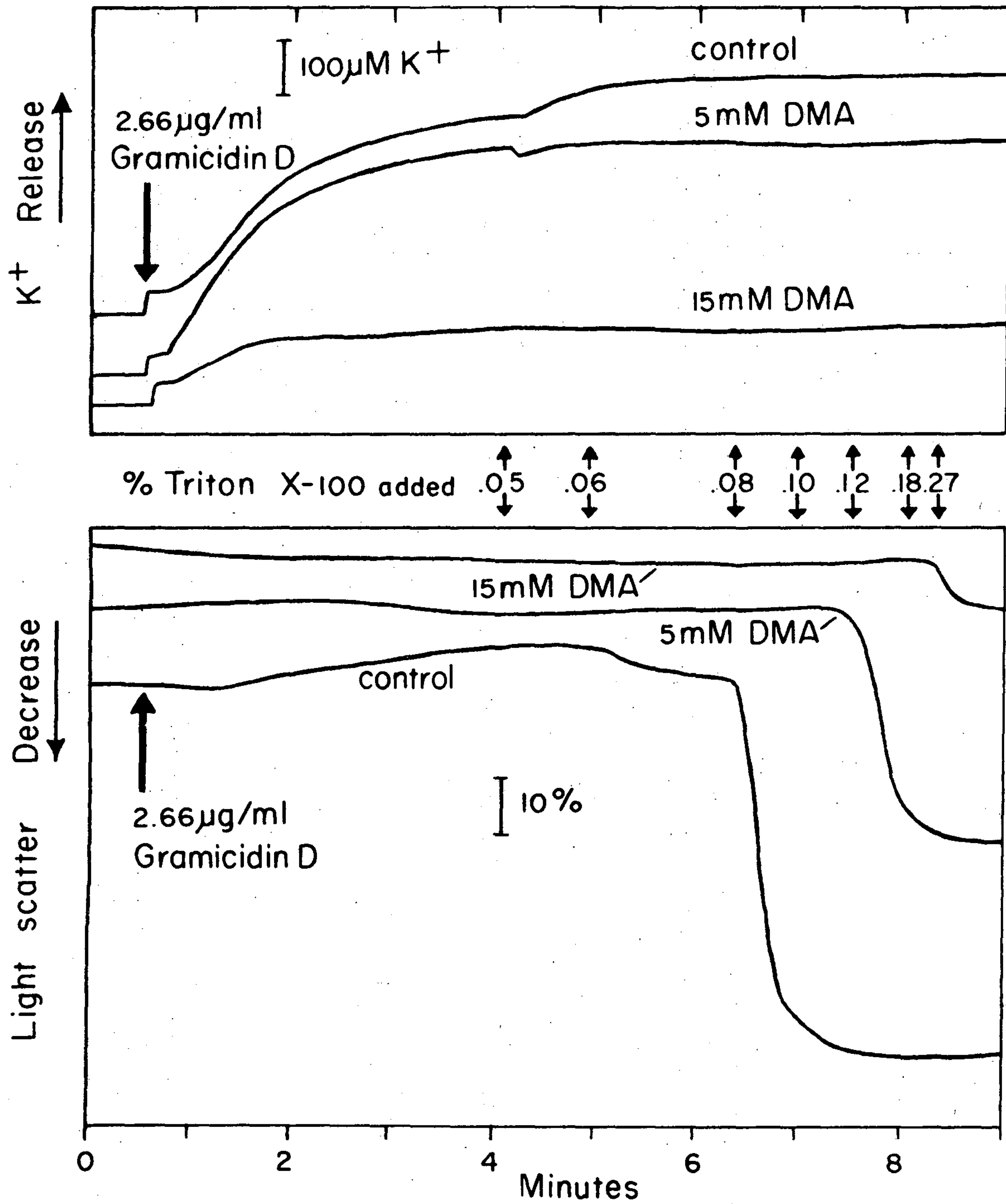


FIGURE 8

energy dependent potassium gradients. A typical experiment is shown in Fig. 9. Using the same preparation as for osmotic lysis (Fig. 5) control cells were treated with gramicidin D and demonstrated a loss of approximately 90%of their internal potassium, accompanied by slight shrinkage. Subsequent treatment with triton-x-100 released the remaining 10% of their potassium and, at 0.08% triton-x-100, these cells undergo lysis, as measured by the large and rapid decrease in light scattering. Cells treated with 5 mM DMA show a very similar response with respect to potassium, and in fact,release the identical amount of potassium during this process. Very different behavior is observed with respect to light scattering, however, in DMA-treated cells. At. 0.08% triton-x-100 treated cells do not undergo lysis and, in fact, lysis does not occur until the concentration of triton-x-100 is increased to 0.12%. At this concentration a 40% decrease in light scattering occurs in contrast to control cells which show a 60% decrease in light scattering after triton treatment. Cells treated with 15mM DMA still retain approximately 35% of their potassium,are almost unresponsive to triton-x-100, and show only a 10% decrease in light scattering after treatment with 0.27% triton-x-100.

Fig. 10 shows the effects of light scattering and potassium release after treatment with DMM and DMS. DMS with an 8 carbon chain length and DMM with 4 carbon chain length, at equimolar concentrations under the same optical conditions, showed a great deal of difference in 90^0 light scattering, but no apparent difference in potassium release. In both DMM and DMS treated cells at 2.66 ug/ml of gramicidin D caused 90% of the cell's potassium to be released. In control and DMM treated cells, the addition of triton-x-100 caused a 60% decrease in light scattering in contrast to DMS-treated cells where no changes were observed. At 0.12% light scattering decreased 42%. These data indicate that DMS is just as effective as



XBL 755-1376

FIGURE 9

0 0 0 0 4 4 0 4 8 0 6

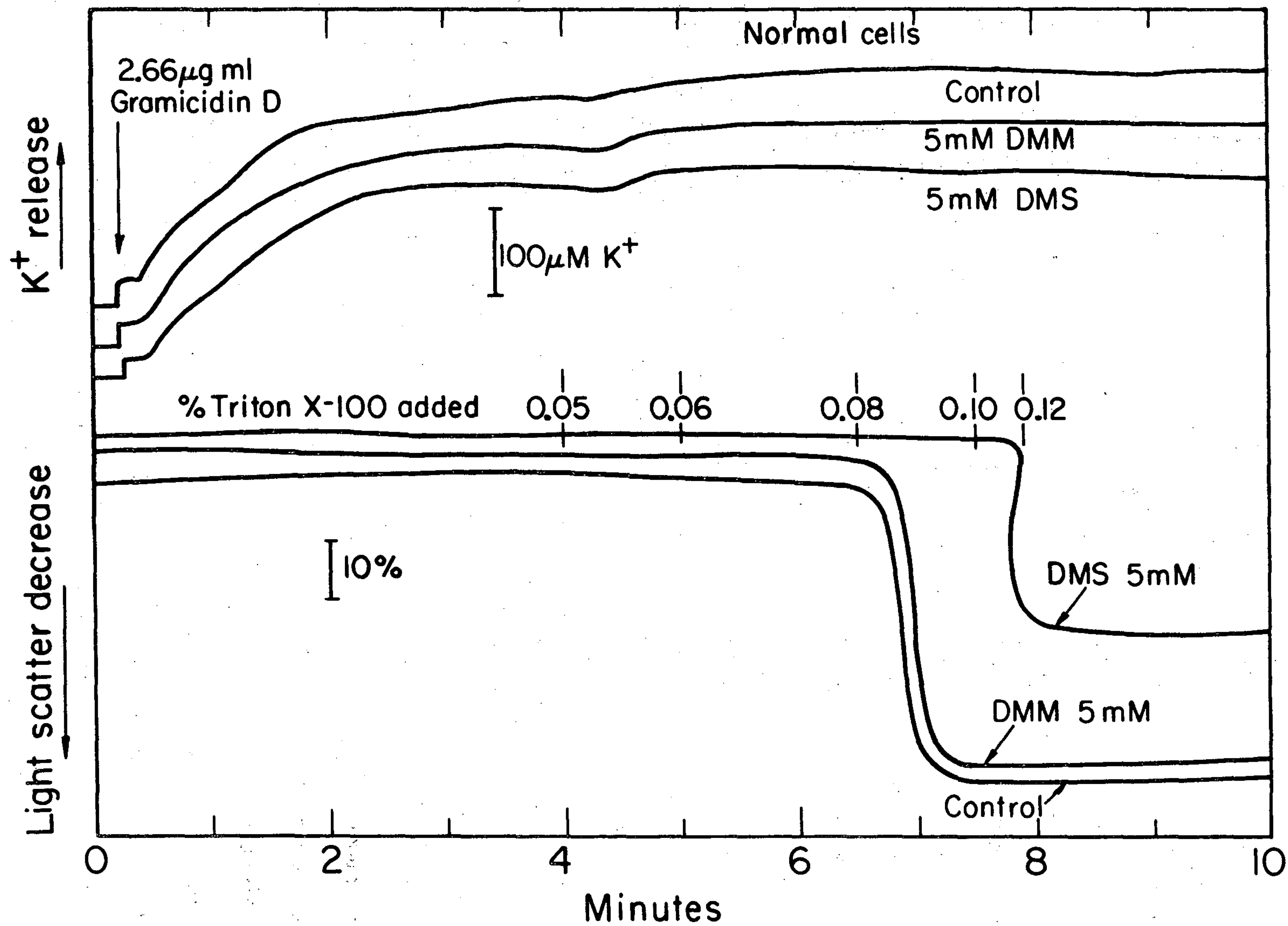


FIGURE 10

DMA in preventing light scattering changes.

A comparison of normal and untreated sickle cells shows differences both in potassium release and light scattering. As indicated in Fig. 11, potassium release rate from sickle cells was twice that of normal cells. This difference can be explained by the fact that polymerized hemoglobin molecule particle under sickling conditions poke holes in the membrane.[50] These holes under appropriate stimuli, act as channels for sodium and potassium transport.[51] Light scattering changes in sickle cells correspond to those observed in osmotic fragility studies in that the sickle cells are slightly more resistant to triton-x-100 induced hemolysis than are normal cells.

Thus far we have looked at the effects of imidoesters on normal and sickle erythrocytes with respect to osmotic fragility, 90° light scattering and potassium release. Although good indicators of metabolic and functional characteristics, these parameters tell us very little about the kinetics of the morphological changes characteristic of cell sickling. Low-angle light scattering studies performed by Rabinowitz in 1973 indicate that as sickle-prone cells begin to sickle, incident light is first scattered away from and then toward the incident beams. This observation suggested that low-angle light scattering techniques could be used to evaluate the effects of imidoesters in preventing the typical morphological changes of deoxygenated sickle cells. Optical density changes have been employed in similar fashion to follow the swelling process in mitochondrial particles,in the aggregation of platelets, and in several other studies involving presumed particles whose diameters are several times larger than the wavelength of incident light. The major contribution to scattering by particle is the forward direction. For a single particle, or dilute solution of monodispere particles, the Fraunhofer diffraction

0 0 0 0 4 4 0 4 8 0 7

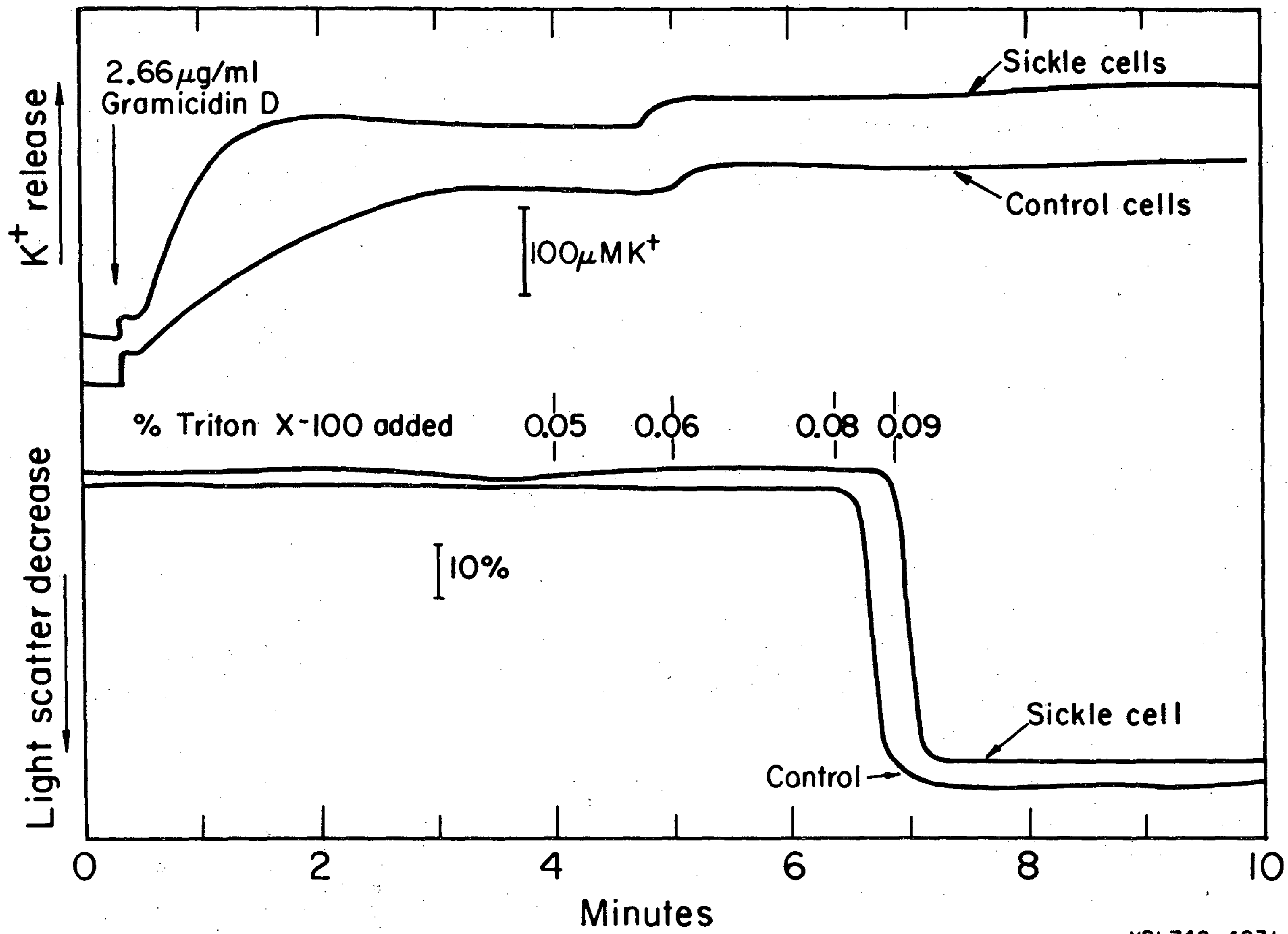
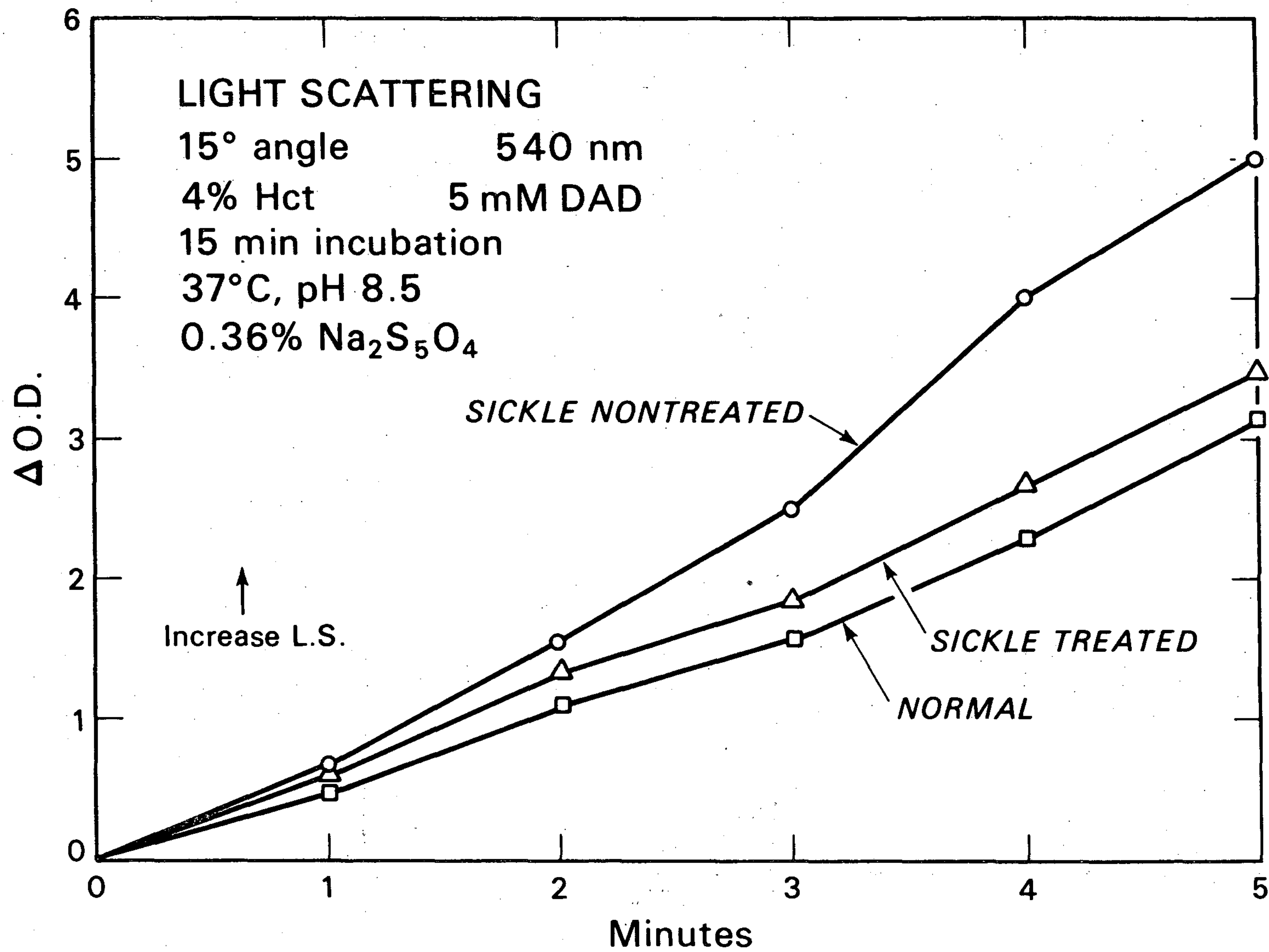


FIGURE 11

approximation will yield the diameter of the particle from the scattered intensity as a function of angle.[52] For the measurement of erythrocyte size and shape, more significant information would be added if biochemical and physiological parameters could be simulated. Fig. 13 shows low angle light scattering set at a 15° angle in a medium of KHB, buffered at ph 7.4 with 0.36% sodium metabisulfate solution used to induce deoxygenation in sickle cells. As illustrated, the sickled non-treated cells produced more light scattering changes than imidoester-treated cells or control cells. This difference may indicate that the hemoglobin in the non-treated cells are polymerizing within the cell causing gellation and density changes. These same differences between non-treated sickle cells and control and treated cells occurs in light transmission, which, in the former, increases after the addition of sodium metabisulfate and then decreases, whereas no change occurs in the latter.

The <u>in vitro</u> study of potassium release in sickle membranes incubated with nitrogen has shown clear metabolic defects in the sickled membrane. During sickling a net potassium release occurs.[53] Determination of net potassium loss is, therefore, a quantitative measure of the amount of sickling.[54,55] In these studies Quabain was added to the incubated cells in order to inhibit the ATP mediated cation pump and, thus, magnify the potassium loss induced by sickling. When sickling is absent, a small loss of potassium occurred, due to the presence of quabain. Potassium loss is modified by hypoxia reflecting active sickling of erythrocytes. 5mM of DMA or DMS completely eliminated the net potassium loss induced by hypoxia in sickle cells: treated sickle cells incubated at 3.3% shows net potassium loss within the usual range of 20% O_2 incubations where sickling is not observed. These data indicate that the net potassium release related to sickling can be prevented through imidoester treatment.



XBL 749-4926

FIGURE 12

0 0 0 0 4 4 0 4 8 0 8

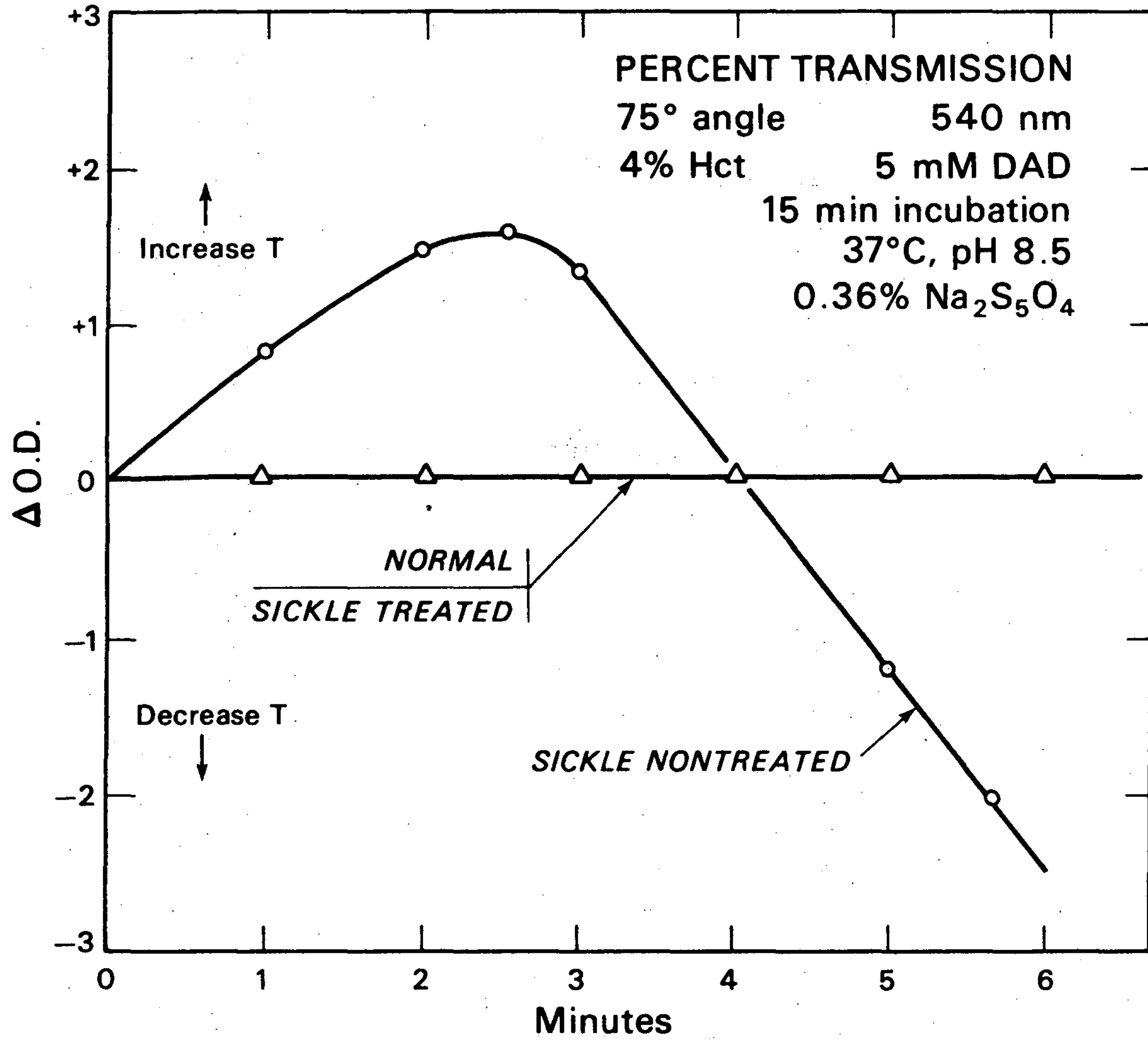


FIGURE 13

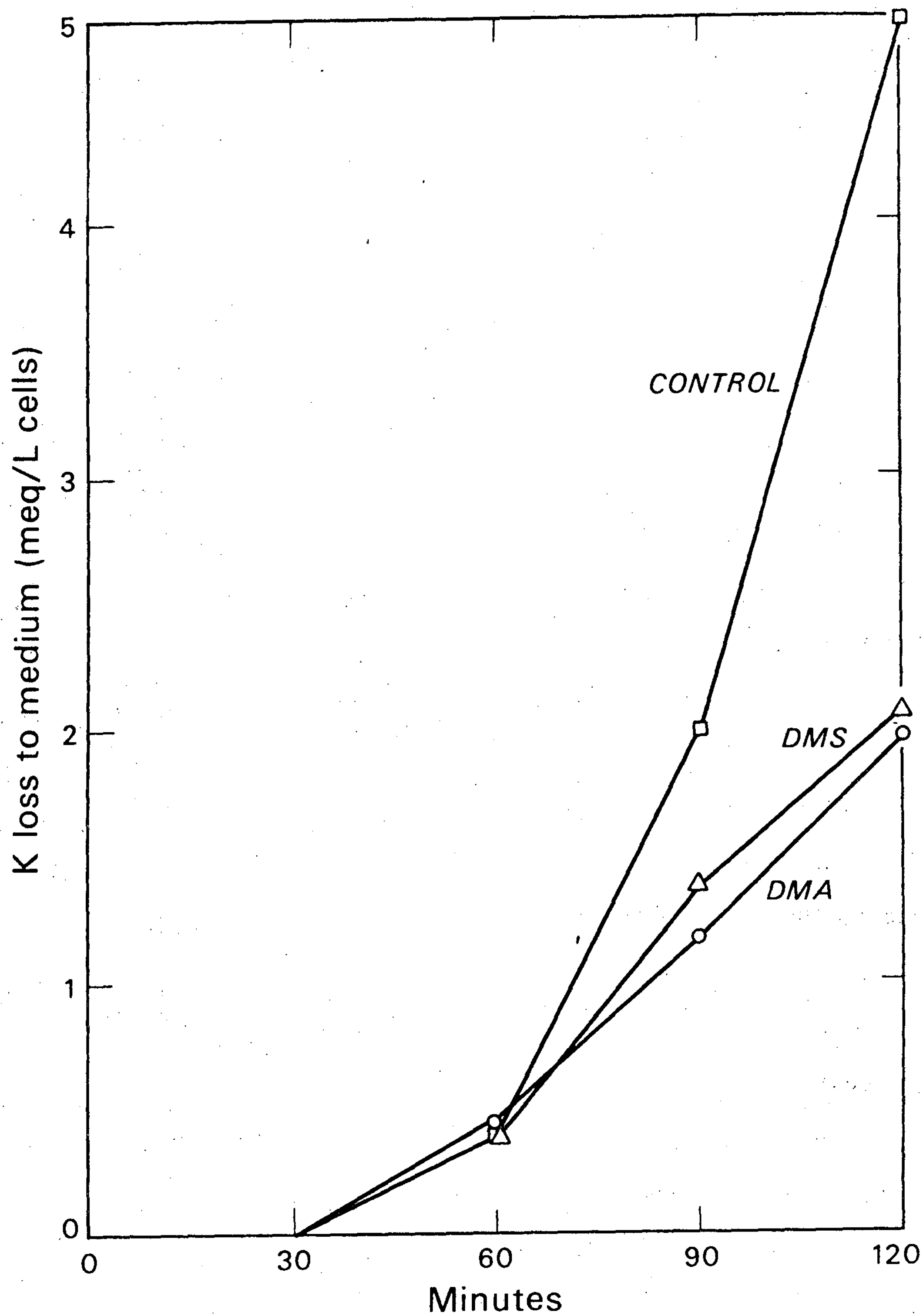


FIGURE 14

The amount of amidation of normal cells was expressed by the TNBS reaction. In Fig. 15, DMA, DMS, DMSSP and EA were compared at various concentrations on the percentages free amino acids groups present after cross-linkages. At 5mM concentration, there were 80% bound amino groups in DMSSP treated cells compared to 65% at DMS, 52% in DMA and 50% in EA; at 1 mM concentration, there were 40% free amino groups bound in DMS, DMSSP, and EA, compared to 25% in DMA. Taking these same imidoesters and looking at their effectiveness in inhibiting sickling, we see in Fig. 16 that at 1mM concentration inhibition with DMA is 80% DMSSP, 77% and EA, 80%. From this data we can correctly say that a fewer cross-linkages and the highest degree of inhibition of sickling is achieved with DMA. As previously discussed under conditions of constant temperature, ph and concentration, the amount of free amino groups bound depends solely on the structure of the specific imidoesters, the amount of decomposition and the spacing of the amino groups available for corss-linkages.

The influence of imidoesters on the percentage of sickle forms following incubation with sodium metabisulfate and 95% nitrogen, respectively, is shown in Figs. 17, 18. The cells incubated in 92% nitrogen shows a decrease from 80% in control sickle cells to 15% in 1mM treated with DMA, DMS and EA and 20% in the case of SMSSP; DMM and MMB had little effect on cell sickling. Fig. 20 shows the same effects when cells are incubated with sodium metabisulfate instead of 92% nitrogen. At .25mM however, imidoesters does not not protect against sickling, but at .50mM this protection increases from 42-48% in the three imidoesters tested. DMA, DMS, DMSSP and EA have proven to be the most effective reagents known today in terms of preventing sickling without destroying the metabolic functions of the cell's membrane. DMA and MMB proved to be not so effective at this concentration, but at higher concetration 100mM-800mM, sickling was prevented.

Fig. 19 indicates the events that take place in a sickle cell from the moment of deoxygenation to the loss of Hbs to the surrounding medium. Fig. 20 is a diagram showing how bifunctional imidates cross-link to membrane or protein molecules.

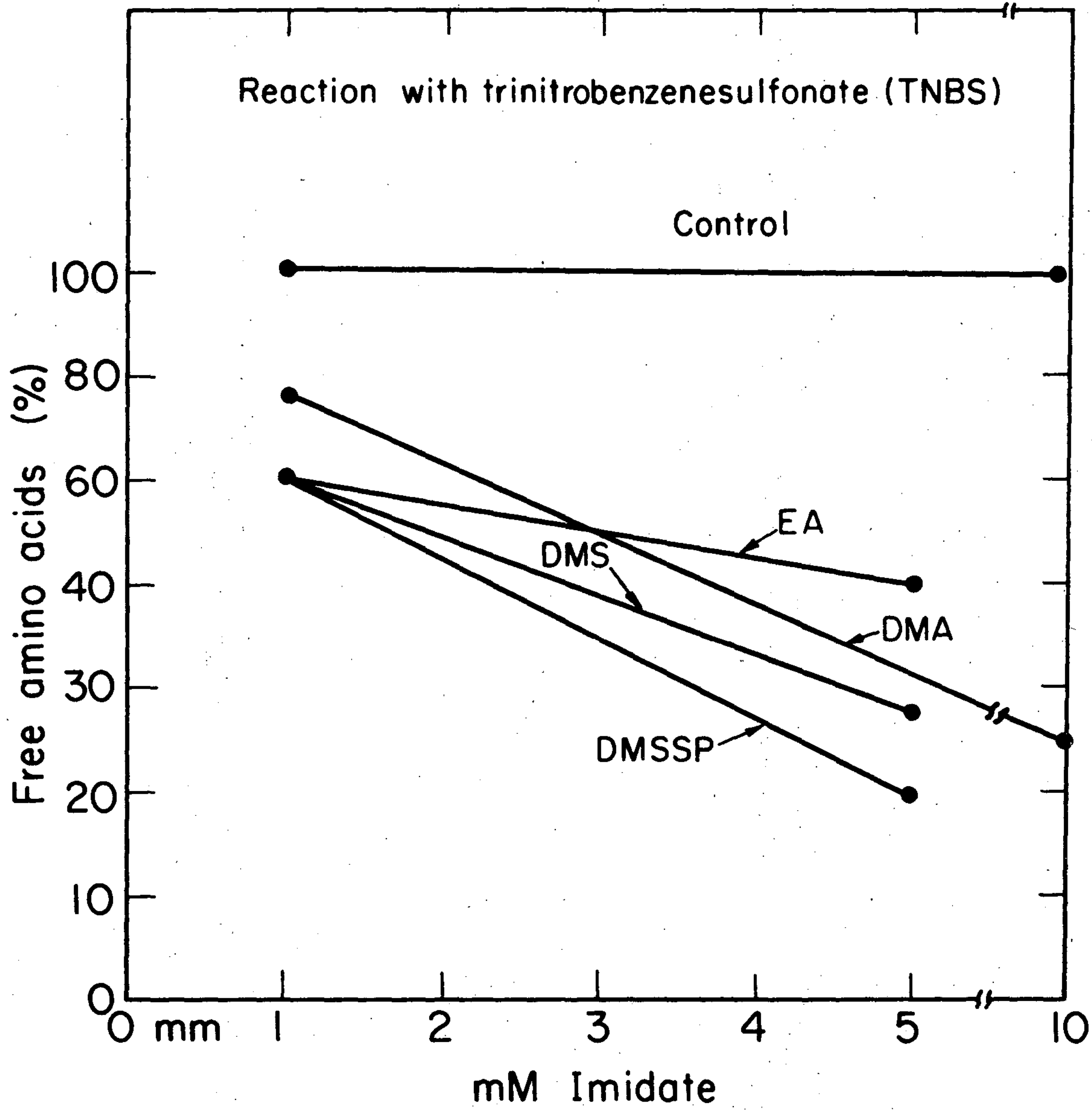


FIGURE 15

EVALUATION OF THE RELATIVE EFFECTIVENESS OF VARIOUS IMIDOESTERS AS ANTI-SICKLING AGENTS

	% inhibition of sickling	% amino groups bound	Index of effectiveness as an anti-sickling agent
CONTROL	0	0	---
DMA	80	25	3.2
DMS	80	40	2.0
DMSSP	77	40	1.9
EA	80	40	2.0

1 mm imidoester, pH 8.5, 1 hr 20 C, test at pH 8.5

XBL 748-1410

FIGURE 16

0 0 0 0 4 4 0 4 8 1 1

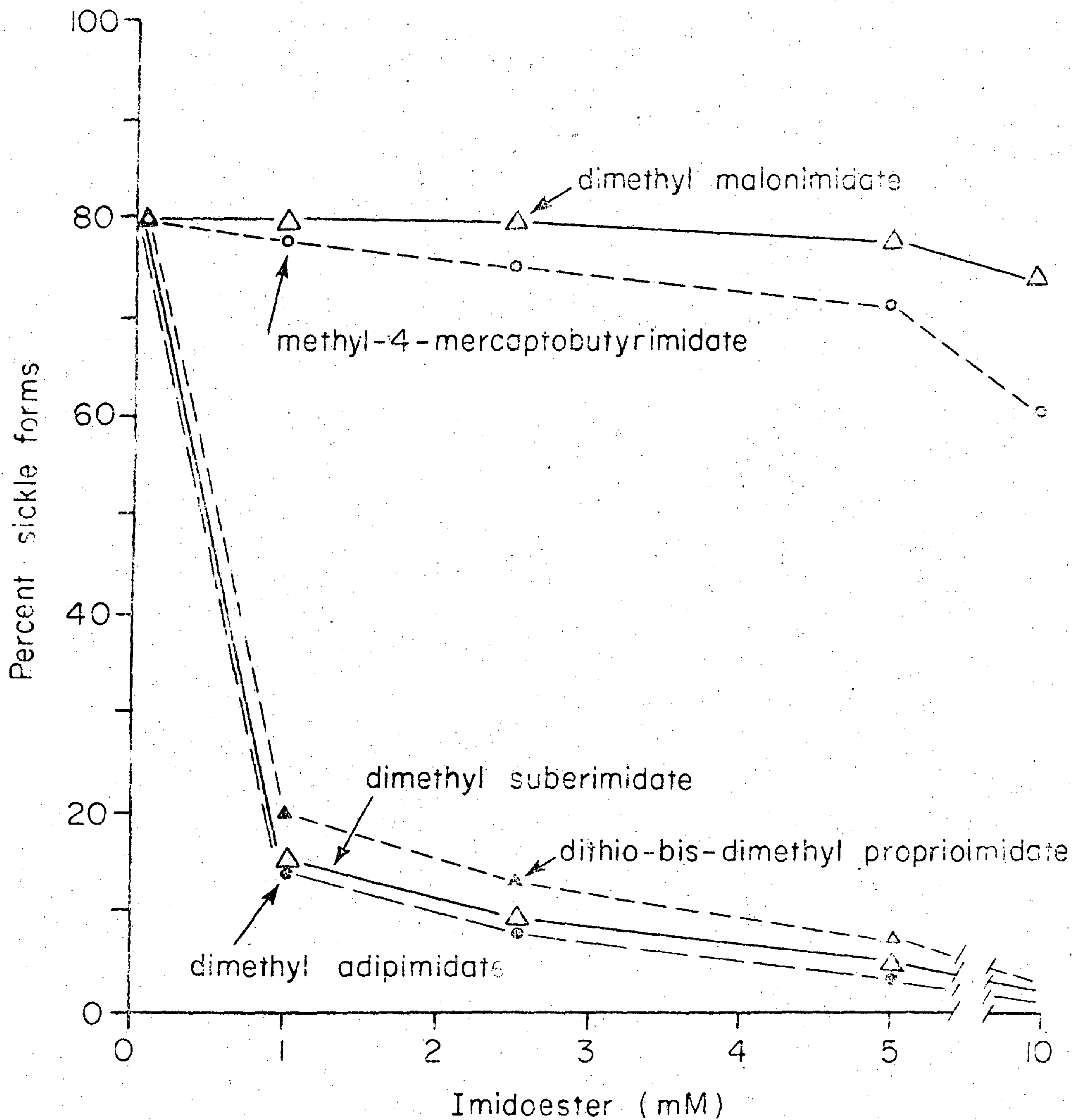


FIGURE 17

0 0 0 0 4 4 0 4 8 1 2

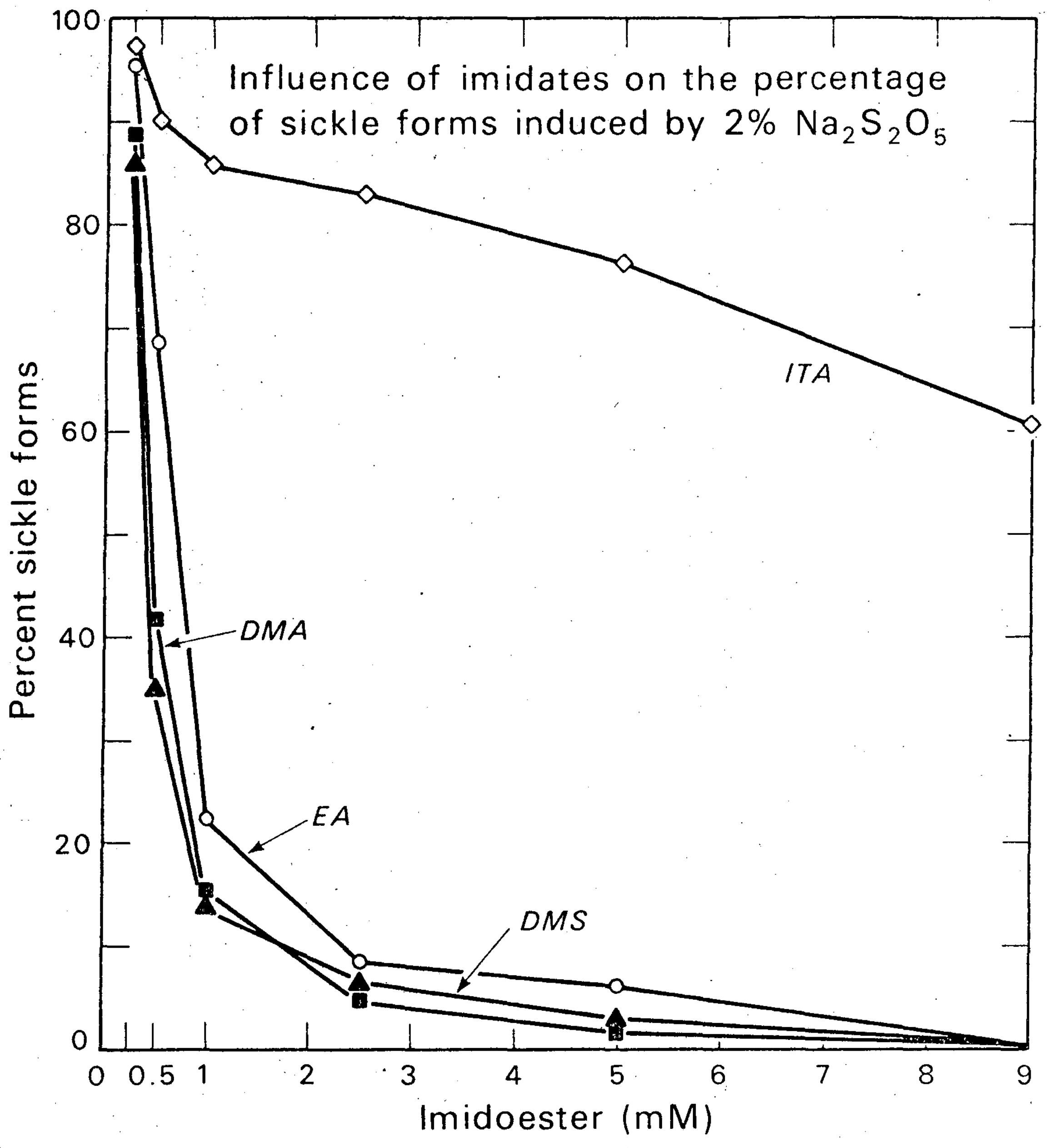
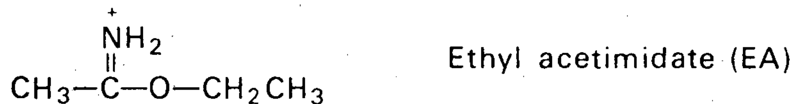


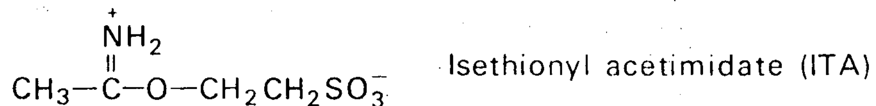
FIGURE 18

IMIDOESTERS

MONOFUNCTIONAL

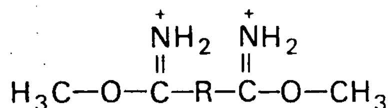


Ethyl acetimidate (EA)



Isethionyl acetimidate (ITA)

BIFUNCTIONAL



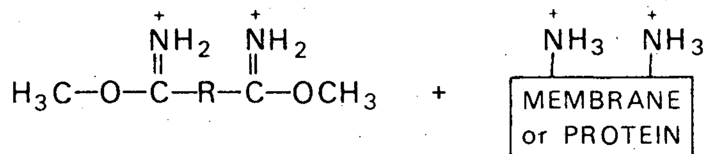
R = $-CH_2-$ Dimethylmalonimidate (DMM)

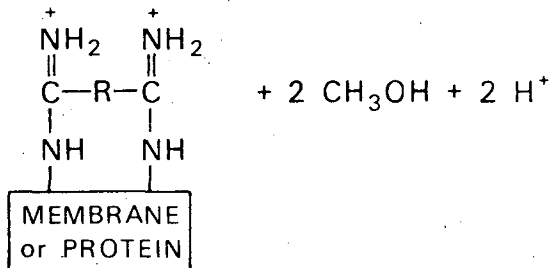
R = $-(CH_2)_4-$ Dimethyladipimidate (DMA)

R = $-(CH_2)_6-$ Dimethylsuberimidate (DMS)

R = $-(CH_2)_2-S-S-(CH_2)_2$ Dithio-bis-Dimethylproprioimidate (DMSSP)

AMINIDATION WITH BIFUNCTIONAL REAGENTS

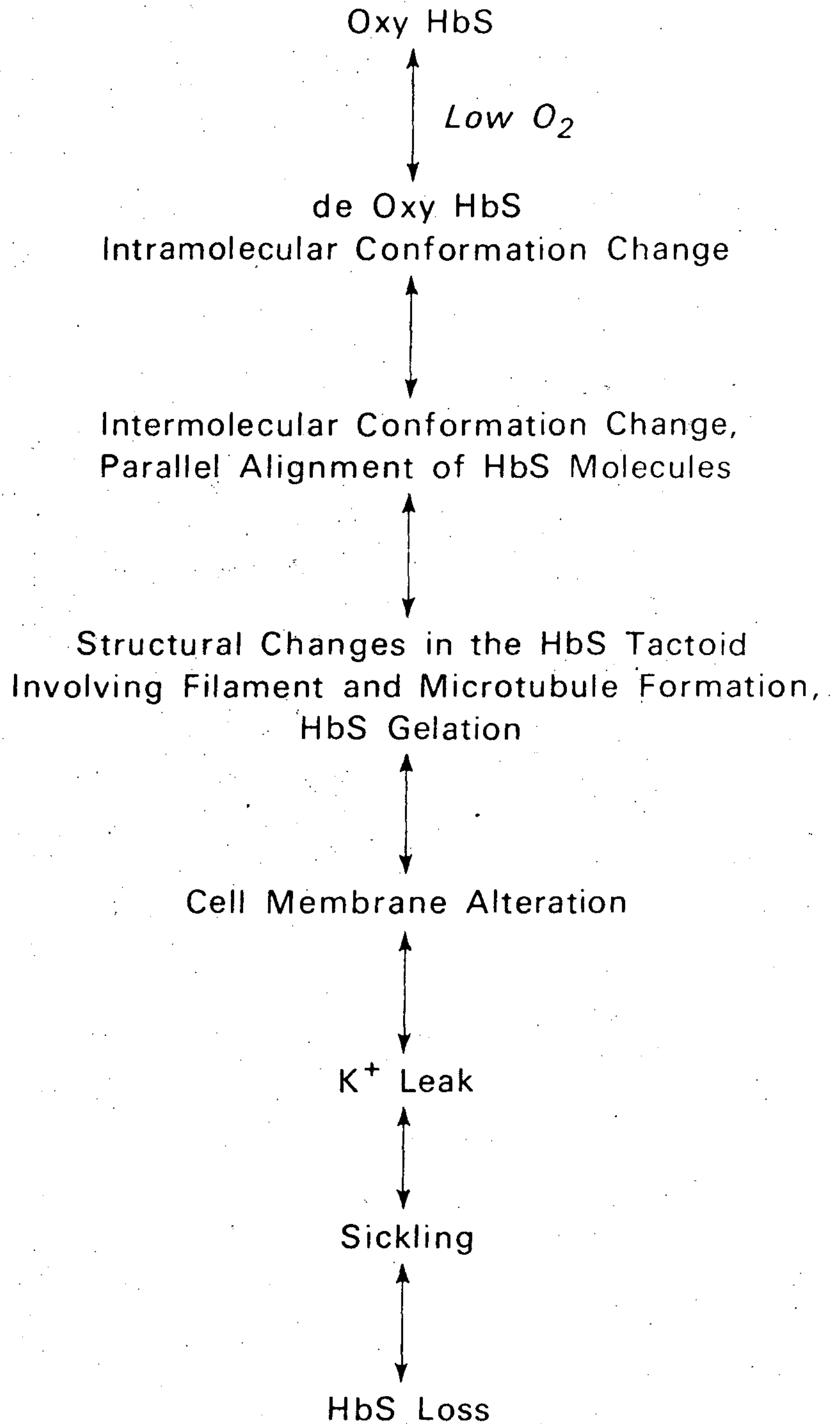




XBL 751-4642

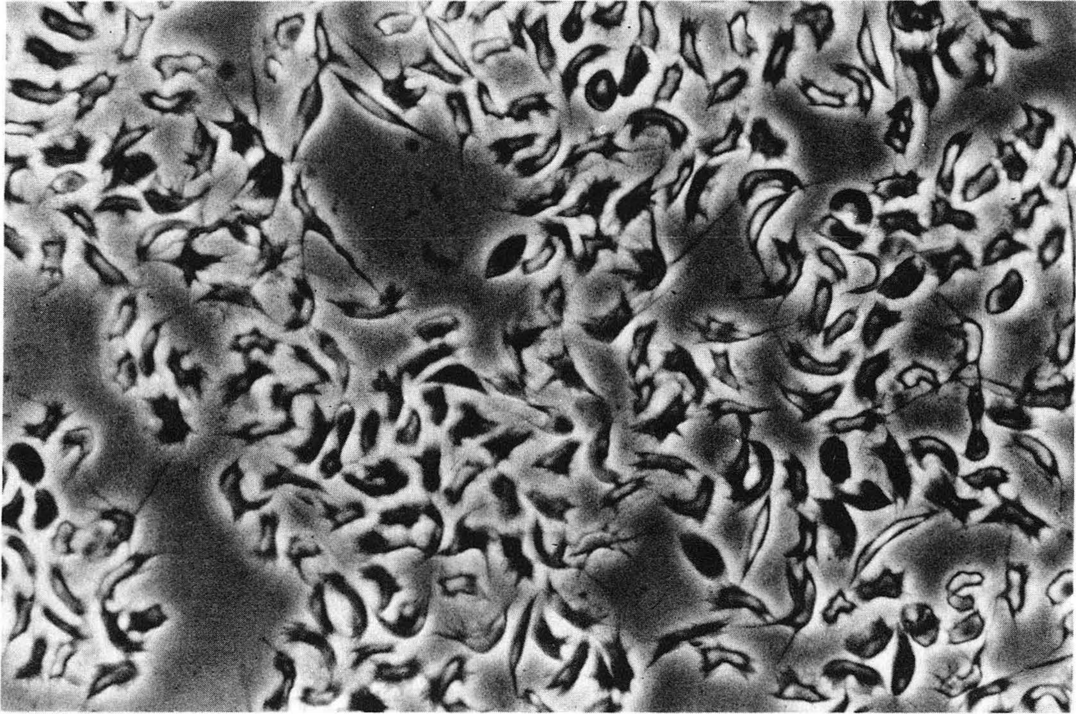
FIGURE 19

0 0 0 0 4 4 0 4 8 1 3



XBL 751-4643

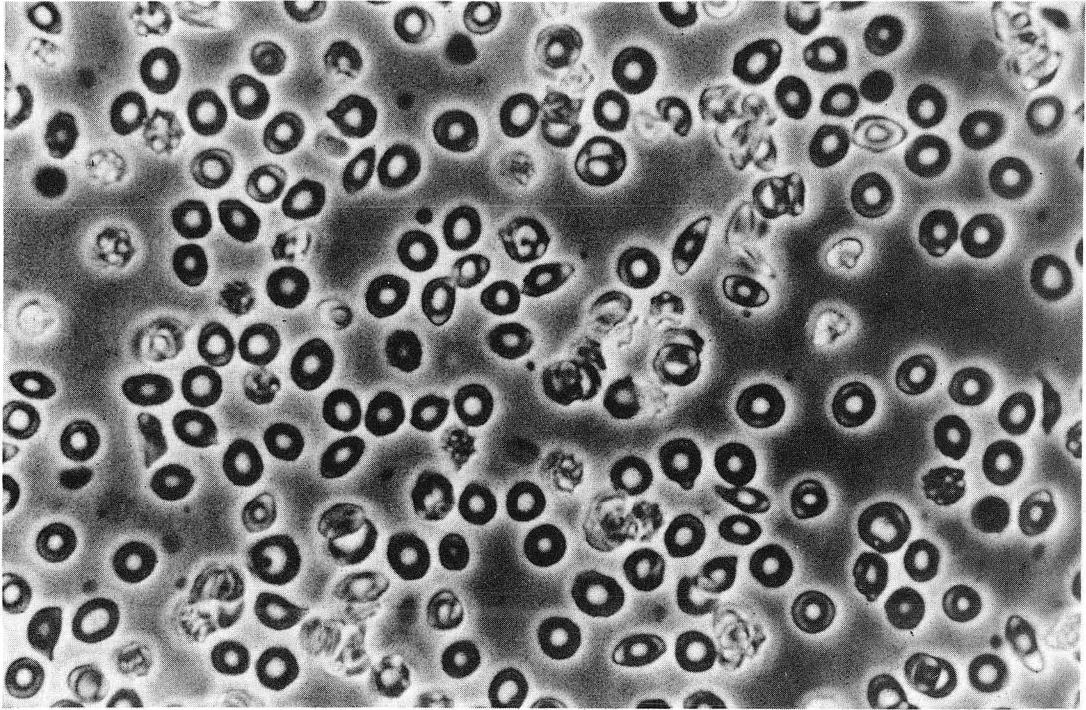
FIGURE 20



CBB 749-6002

FIGURE 21

0 0 0 0 4 4 0 4 8 1 4



CBB 749-6003

FIGURE 22

DISCUSSION

The optical density changes which have been displayed in sickle cells at low angle light scattering, were taken near an isosbestic wavelength to minimize absorption contribution to O.D. changes. The analysis of changes in optical density of sickle cells consequent to low-angle light scattering appears to be a good method for evaluating the effects of imidoester treatment on sickling.

For smaller particles, an unambiguous determination of size and shape, known refractive index, can be determined from light scattering pattern.[56,57,58] For large particles, where diffraction effects dominate, the scattering and refractive index contribution are not significant and the Fraunhofer approximation to Lorenz (Mie) theory is better used for complete analysis of the results.[59,60] The morphological kinetics of sickle cells, described by Ponder, suggests that light scattering techniques will be a valuable aid in elucidating the sequence of events that occurs in sickling.

The reaction of imidoesters with erythrocytes was found to be controlled by ph, temperature, time and concentration, whereas amidination seems to be controlled by the specific structure of each imidoester and the spacing of the available amino or protein groups.

Many studies performed on the effects of imidoester modification of proteins, seem to indicate that bifunctional cross-linking reagents alter the structure of proteins without affecting their functional properties. The metabolic functions of they erythrocyte membrane did not change during mild imidoester treatment, even though the membrane protein became cross-linked up to 70% (e.g. potassium transport, enzymatic activity, and oxygen transport).[61] In this regard the data on the

0 0 0 0 4 4 0 4 8 1 5

physical properties of certain amidinated proteins reported by Wofsy and Singer[62] would indicate that extensive amidination caused only minor, if any, conformational changes in the proteins.

Sickle cells treated with DMA and DMS, seem to prevent In Vitro potassium leakage during hypoxia incubation with quabian. This finding suggests that potassium leakage is directly controlled by sickling, even though there appear to be extra channels in the membrane.[63]

The use of hypoxia-induced potassium loss from sickle cells is based on Tosteson's observations of increased potassium flux in deoxygenated sickle cells.[64] If quabian is added to the system to inhibit active cation transport, the acceleration of potassium flux is reflected as potassium loss from the cells to the medium. The rate of potassium loss is linear and inversely related to the oxygen tension. The magnitude of the loss varies somewhat in different runs, but the rate at any oxygen tension is directly proportional to the concentration of S hemoglobin in the cells and known inhibitors of sickling such as increased ph or carbon monoxide decrease the rate in a predictable manner.[65] Therefore, measurement of the rate of the potassium loss provides a quantifiable method for the assessment of sickling, and possible beneficial effects of drugs such as imidoesters can be more precisely evaluated.

Data from osmotic fragility tests, and TNBS reactions show that both hemoglobin and membrane proteins are being cross-linked (N-N disubstituted) by bifunctional imidoesters, and that monosubstitution (N-substitution) is formed with the monfunctional imidoesters. These observations, and the finding that Ethyl Acetimidate (monofunctional reagent) effectively prevents sickling suggests that bifunctional imidoesters are not the deciding factor in preventing of sickling.

In other sickling studies, Gillette, showed that potassium cyanate reacts specifically with the amino Terminal valine residue to prevent sickling.[66] His theory is that cyanate prevents the intramolecular salt bridge formation in the deoxy conformation, thus increasing oxygen affinity, like cyanate bifunctional imidoesters increase oxygen affinity, but to a lesser extent. The P_{50} in DMA-treated cells decreases from 35mm to 24mm[67] and with cyanate, the decrease is from 35mm to 13mm[68], indicating that hemoglobin treated with cyanate holds on to the oxygen molecule more tightly than do DMA-treated cells. Unlike cyanate, however, imidoesters inhibit sickling under conditions of complete deoxygenation, indicating that factors other than altered oxygen affinity are involved. On the basis of the foregoing, it seems reasonable to hypothesize that the mechanism behind the prevention of sickling by imidoesters is the ability to stabilize the previously unstable deoxy hemoglobin molecule.[69] Most investigators would agree that rodlet formation during deoxygenation requires at least two interacting regions, opposite each other for nearly spherical molecules. Since we are dealing with only one kind of molecule, these regions are self-complementary.[70] Residue beta (6) is normally glutamic acid. It has a charged side chain that sticks outward.[71] In sickle cell hemoglobin, the glutamyl residue is replaced by valine.[72] Charache has speculated, and it seems probable, that the uncharged side chain of valine beta (6) also sticks outward and can make contact with other molecules. Since the side chain is uncharged, we might suppose that a hydrophobic bond is formed during sickling.[73] Normal red cells do not sickle because the net energy of interaction, i.e. the sum of attractive and repulsive forces between molecules - comes out negative.[74] Valine at beta (6) tips the balance (to a net attrative) and rodlets form, but only in the deoxy conformation. As the beta chains swing outward during deoxygenation, two self-complementary sites fall into alignment and molecules stack one upon another like building

0 0 J 0 4 4 0 4 8 1 6

blocks. Within the red cell, the resulting rodlets are packed so tightly that they cannot rotate freely, and gellation and membrane distortion occurs. TNBS data seem to indicate that imidoester treatment removes or reduces self-conplementarity action within sickle hemoglobin to stabilize the deoxy form. The data also indicates that only a small percentage of the total free amino acids groups need amidination to prevent sickling. For example, 25% bound free amino acids groups will prevent 80% sickling.

Even though the hypotheses discussed here seem to suggest that hemoglobin amidination alone is responsible for the prevention of sickling, the possibility remains that inhibition of sickling may result from a combined effect on both the membrane and hemoglobin.

I have presented evidence that imidoesters are the most potent antisickling agent known to man today. Possible clinical use of this reagent, therefore, looks very promising. However, the marked inhibition of sickling and the apparent lack of major effects on erythrocytes metabolism, suggest that these reagents might be used without seriously disturbing normal red cell physiology.

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