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# Kinetics of hemolysis of normal and abnormal red blood cells in glycerol-containing media

Alexandra Sauer [1], Tamar Kurzion [1], Dan Meyerstein [1] and Naomi Meyerstein [2]

[1] *Department of Chemistry, Ben-Gurion University of the Negev, Beer Sheva (Israel) and* [2] *Dr. J. Kaufmann Laboratory, Unit of Physiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva (Israel)*

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**The kinetics of hemolysis of erythrocytes in glycerol-containing media was studied spectrophotometrically. The hemolytic process starts by a rapid process, obeying a first order rate law, which is followed by a slow change in absorbance. The kinetics of hemolysis may be described by (a) the maximum absorption, $E_{max}$, due to cellular expansion, (b) the rate constant, $k$, of the fast process and (c) the final absorption at its end, $E_{inf}$ and the ratio $E_{inf}/E_{max}$. At pH 6.85 in normal human cells, $k = 0.72$ min$^{-1}$ while in hereditary spherocytosis cells, $k = 1.06$ min$^{-1}$, iron deficiency $k = 0.52$ and $\beta$-thalassemia minor $k = 0.36$ min$^{-1}$. The percentages of $E_{inf}/E_{max}$ were 35.3 in control cells, while they were 9.8, 50.0 and 88.3 in spherocytosis, iron deficiency and thalassemia, respectively. Thus these kinetic parameters may help to distinguish and understand the above mentioned erythocyte disorders. At physiological pH (7.4–7.2), no hemolysis was detected in the medium used. When the pH decreased, hemolysis occurred, its rate increasing gradually until pH 6.3. On further acidosis, the hemolytic rate slowed down again. Addition of DIDS to the whole blood prior to the test inhibits hemolysis. Similar effect of DIDS was noted in washed cells; this effect was partially reversed by albumin. These results suggest that a process involving band 3 affects the rate and degree of glycerol-induced hemolysis of normal red blood cells.**

## Introduction

Hemolysis of erythrocytes in glycerol media may be measured easily by observing the reduction in turbidity of the cell suspension [1]. Gottfried and Robertson introduced the glycerol lysis time (GLT) as a rapid screening test for various erythrocyte disorders [2]. The time for 50% hemolysis has been used for comparing different pathological cells. This test is quite sensitive, mainly for identifying decreased glycerol lysis time (in spherocytosis) but also for increased lysis time (iron deficiency and thalassemia). In order to augment sensitivity, Zanella et al. [3] introduced the Acidified Glycerol lysis test (AGLT), which is very sensitive in the diagnosis of spherocytosis but cannot distinguish between healthy subjects and patients with normal or increased lysis times, since they all have $AGLT_{50}$ values of more than 1800 s [3,4]. Our previous studies [5,6] yielded faster $AGLT_{50}$ times; the source of this discrepancy has not been clarified. In both $GLT_{50}$ and $AGLT_{50}$ the samples are characterized by the time needed for the absorption to reach half its initial value (at $\lambda = 626$ nm). This value describes a combination of two components: the degree of lysis and its rate. Thus it seemed conceivable that by measuring each of these properties separately, additional differentiation would be obtained. As results at pH 7.4 differ from those at pH 6.85, we studied correlation of hemolysis rates with pH. Because of the similarity between the observed pH dependence with that of anion transport in red cells, we investigated the effect of preincubation of DIDS, a known inhibitor of anion transport, on glycerol hemolysis rates. The results indeed support the hypothesis that band 3, the anion transport protein, is involved in the glycerol lysis processes.

Correspondence: N. Meyerstein, Dr. J. Kaufmann Laboratory, Unit of Physiology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel.

## Materials and Methods

### (a) Collection of samples

Blood samples were collected from the following four

groups in accordance with the principles of the Helsinki Declaration:

(1) Six healthy volunteers with normal hematological values.

(2) Four adult volunteers who were considered to be iron deficient: they had microcytic, hypochromic cells (mean cell volume (MCV) was lower than 78 fl, mean corpuscular hemoglobin concentration (MCHC) lower than 32 g/dl). Hemoglobin levels were below the accepted normal values for males and females of their age groups. Serum iron levels were low, iron binding capacity was high and percent transferrin saturation was low, as expected in iron deficiency anemia (IDA). Thalassemia and other causes for anemia were ruled out.

(3) Four hereditary spherocytosis (HS) patients were studied: their diagnosis was confirmed by typical blood smears findings, osmotic fragility and AGLT studies [4]. Three of them had mild, compensated anemia and one was a post-splenectomy, non-anemic patient with very high glycerol permeability, as expected [2].

(4) Three patients were mildly anemic, $\beta$-thalassemia minor patients. Their diagnosis was confirmed by hemoglobin electrophoresis studies and low glycerol permeability. None of the subjects had ever been transfused.

*(b) Reagents*

(1) Isotonic phosphate-buffered saline (PBS), prepared by adding 9 vol. of 0.15 M NaCl to 1 vol. of 0.10 M phosphate buffer [3]. The phosphate buffer was prepared from sodium phosphate, adjusted to the desired pH by HCl or NaOH.

(2) 0.3 M glycerol. 27.62 g glycerol, reagent grade, was added to 300 ml PBS and brought to a final volume of 1.0 litre with distilled water [3].

(3) DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) was purchased from Sigma Chemical, Israel and used in PBS (pH 7.4).

(4) Bovine serum albumin was obtained from Sigma, Israel.

*(c) Glycerol kinetics*

Blood samples were taken into heparin or EDTA test tubes and examined immediately, or stored for less than 3 days in the refrigerator in CPDA-Media. Glycerol lysis was induced according to the method of Zanella et al. by mixing suspensions of whole blood in a phosphate-buffered saline (PBS) solution with a glycerol/PBS solution at pH 6.85 [3]. 0.5 ml of the suspension of whole blood in the PBS solution was mixed with 1 ml glycerol/PBS solution in a two-compartment mixing cell immediately prior to a run. The cell was washed several times with distilled water and finally with PBS before each experiment. The rate of hemolysis was followed by measuring the fall in turbidity of the reaction mixture with a HP-Diode Array Spectrophotometer HP 8452. Several samples were analyzed in parallel, using a Cary 17 or a Bausch and Lomb Spectronic 2000 spectrophotometer. The reference cell contained 0.5 ml of glycerol/PBS mixed with 1 ml of the PBS solution. The kinetic data were evaluated from the absorption changes at 626 nm. The best standard procedure for evaluating the data was found to be a first-order fit for the time interval of 40–500 s after mixing. Hemolysis was confirmed and followed up by measuring the appearance of free hemoglobin at 415 nm.

*Preincubation with DIDS*

DIDS was dissolved in PBS (pH 7.4); whole blood and washed cells were preincubated with DIDS in final concentration 10–1000 $\mu$M. Glycerol lysis rates were performed after 1 hour preincubation. Some of the samples were also incubated with DIDS as washed cells. The effect of incubation with 1% albumin was also performed, to study the reversibility of the DIDS effect.

All experiments were performed at room temperature (23° C).

## Results

Preliminary experiments showed that unstirred solutions can be studied for about 30 min without precipitation affecting the measurements, as verified by comparing stirred and unstirred solutions. As the fall in turbidity (i.e., the decrease of light scattering) was followed in regular UV-Vis spectrophotometers, measuring the absorbance changes of the solutions, the results varied with the type of spectrophotometer (i.e., the slit width) used: initial absorptions differed considerably (1.2–0.2), as did the measured $AGLT_{50}$ values which ranged between 50 to 300 s for a normal blood sample. It was thus necessary to calibrate the testing procedure according to the spectrophotometer used. (All the tabulated data are derived from measurements with the HP Diode Array spectrophotometer with a comparative narrow

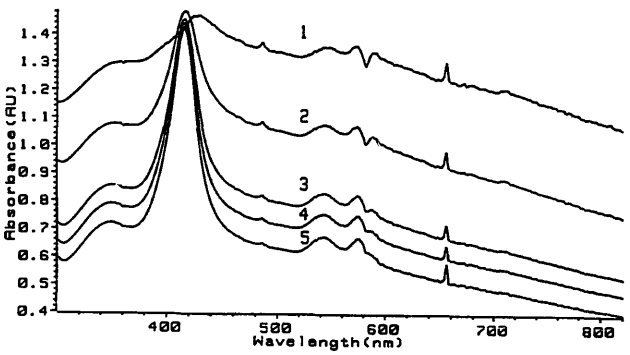


Fig. 1. Spectral changes after mixing control red cells with glycerol-PBS (pH 6.85). Curves 1, 2, 3, 4, 5 recorded after 5, 65, 125, 185 and 545 s, respectively. The peaks observed at 486, 582 and 656 nm, are due to instrument artifacts.

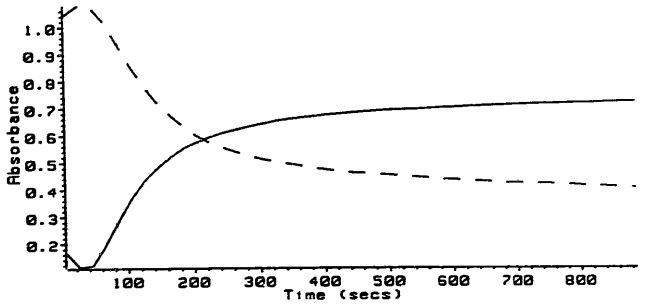


Fig. 2. Rate of hemolysis of normal red cells, recorded at 626 nm (— — —). Formation of free hemoglobin measured at 415 nm after correction for turbidity.

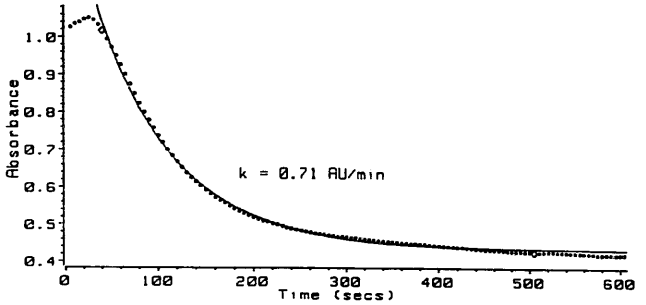


Fig. 3. Absorbance at 626 of normal red cells in glycerol-PBS (pH 6.85), recorded at 626 nm (·····). Theoretical first order rate with $k = 0.71\ \text{min}^{-1}$ is presented by the unbroken line (———).

slit width). Fig. 1 shows the spectral changes during the hemolysis of a normal blood sample. As the turbidity decreased, due to the lysis of the cells, the resolution of the peaks at 415 and 550 nm resulting from free hemoglobin, was observed (Fig. 1). The rate of hemolysis was usually observed at 626 nm (as suggested by Gottfried and Robertson [2]), as at this wave length there is no interference by formation of free hemoglobin. As Fig. 2 shows, this rate was identical to the rate of formation of the absorption at 415 nm, after correction of the latter value for the turbidity. The hemolysis measured at 626 nm, was characterized by an increase in absorption during the first 20–30 s, followed by a more or less rapid decay between 40 and 500 s, which was well described by a first order rate law, followed in turn by a slow decay. A typical glycerol lysis for a normal blood sample and its fit to a first order rate law are shown in Fig. 3. As the $AGLT_{50}$ does not adequately describe the kinetics, especially in the cases of high residual absorption, we characterized the results by three factors: (1) the maximum absorption ($E_{max}$, which is slightly higher than the initial absorption due to an expansion of the cells before the lysis); (2) the rate constant $k$ derived from the first-order fit of the decrease of the absorption between 40 and 500 s and (3) the final absorption according to the fit ($E_{inf}$) and its percentage of $E_{max}$.

The results of glycerol lysis kinetics of cells of healthy subjects and various patients are presented in Table I and Fig. 4. As before, $AGLT_{50}$ values may differentiate between normal cells and those known to have different rates of hemolysis, i.e., fast hemolysis (hereditary spherocytosis) on the one hand, and slow rates (IDA and thalassemia) on the other hand. Hemolytic rate is again more rapid (i.e., short $AGLT_{50}$) than reported by Zanella probably due to the use of a different spectrophtometer (see above). The differences between IDA and thalassemia are obvious but individual measurements may vary. $k$ values may help to differentiate between normal control and IDA and thalassemia ($P < 0.01$). $E_{max}$ is quite similar in all cells, but calculation of the percentage of $E_{inf}$ may enable a distinction to be made ($P < 0.001$).

*pH effects*

To study the effect of pH on lysis times, we exposed normal cells to different pH values, ranging from physiological pH, pH 7.4, to pH 5.7, at constant osmolality. No hemolysis was detected at pH 7.4. When the pH was gradually reduced to 6.0, hemolysis became more rapid and complete (Fig. 5). However, when the pH was further reduced to 5.7, hemolysis became slower, and was not complete (Fig. 5).

TABLE I

*Glycerol kinetics of red cells (at pH 6.85)*

Absorbance measured at 626 nm. Data are given as averages ± S.D. Number of subjects is given in parenthesis. All differences are significant, $P < 0.01$ or $< 0.001$, except for $AGLT_{50}$ between iron deficiency and thalassemia.

| Diagnosis | $AGLT_{50}$ (s) | $k$ ($\text{min}^{-1}$) | $E_{max}$ | $E_{inf}$ | $E_{inf}/E_{max}$ (%) |
|---|---|---|---|---|---|
| Control | | | | | |
| (6) | 162 ± 52 | 0.72±0.12 | 1.13 ±0.10 | 0.393±0.11 | 35.33±6.59 |
| Hereditary spherocytosis | | | | | |
| (4) | 53.7± 8.2 | 1.06±0.13 | 0.89 ±0.09 | 0.091±0.036 | 9.82±2.16 |
| Iron deficiency | | | | | |
| (4) | 476 ±113 | 0.52±0.02 | 0.737±0.048 | 0.417±0.068 | 50.0 ±5.34 |
| β-Thalassemia minor | | | | | |
| (3) | > 600 | 0.36±0.05 | 0.847±0.025 | 0.747±0.040 | 88.33±5.31 |

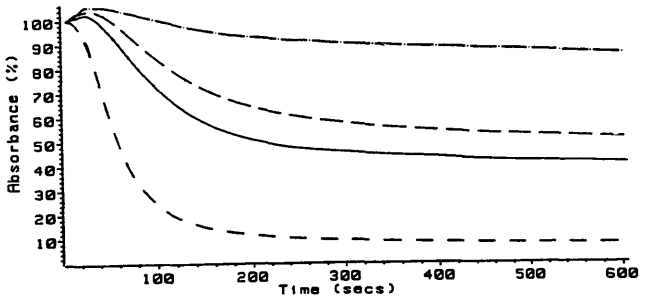


Fig. 4. Glycerol lysis kinetics, of normal and pathological cells, at 626 nm (pH 6.85). Normal cells (———), Hereditary Spherocytosis cells (- - - - -), iron deficiency (— — —) and thalassemia cells (·—·—·).

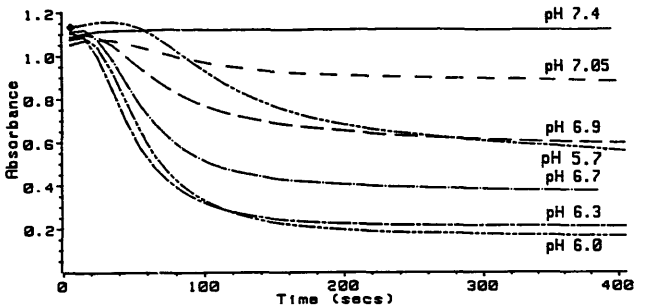


Fig. 5. Glycerol lysis rates of normal cells, at different pH values. ($k$ = 0.01, 0.76, 0.71, 1.51, 1.50, 1.29, 0.55 $min^{-1}$; 100($E_{inf}/E_{max}$ = 99, 82, 53, 47, 19, 16, 46 at pH 7.4, 7.04, 6.85, 6.70, 6.30, 6.00 and 5.70, respectively).

*Effect of DIDS*

Whole blood, whole blood diluted 1 : 1 with PBS and washed cells were incubated with DIDS for 60 min. The rate of lysis was then determined. The results are summarized in Table II and a representative result is shown in Fig. 6. The results indicate that the rate of glycerol-induced lysis is slowed down considerably by the washing procedure itself: $AGLT_{50}$ exceeds 300 s and the ratio of $E_{inf}$ to $E_{max}$ is increased from 35% to over 60%. Addition of albumin to the washed cells partially reverses the effect of washing, as reported before by Janas et al. [7]. Preincubation with 1 mM DIDS inhibits glycerol-induced hemolysis; 100 $\mu$M and 10 $\mu$M have smaller inhibitory effect, as expected. The addition of albumin to DIDS treated samples also increases the

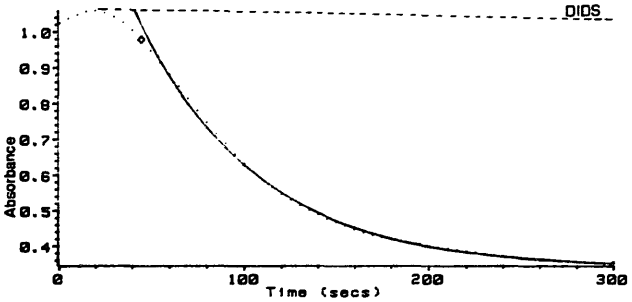


Fig. 6. Effect of preincubation of whole blood with 1 mM DIDS on the rate of hemolysis. Absorbance at 626 nm of normal cells. Lower curve: measured hemolysis of normal, untreated cells (·····). Theoretical first order rate is represented by unbroken line (———). Upper curve: DIDS-treated blood (- - - - -).

degree of lysis but the effect is considerably smaller than in the nontreated samples. These results clearly point out that DIDS slows down the rate of the glycerol lysis process in all these systems.

## Discussion

The rate of hemolysis of erythrocytes in glycerol media may be studied spectrophotometrically by recording the decreasing turbidity of the cells at 626 nm. The rate of hemolysis is determined by several factors, as suggested by Wessels [8]: (a) The ratio surface-to-volume: spherocytes, with low surface-to-volume ratio, hemolyse rapidly, while cells with abundant surface (i.e., target cells) hemolyse slowly. (b) It has been claimed that membrane composition, mainly lipid composition, is a major determinant of glycerol permeability [9] but this theory has been disproved in human diseases and in various species [9,10]. (c) Loss of osmotic material from the cells during the experiments.

The fall in the turbidity of cellular suspension corresponds to the increase in free hemoglobin in the medium, as can be seen in Fig. 2. The effect of. pH on hemolytic rate in glycerol media was studied in normal and pathological cells. An unexpected finding was that when this medium, containing 0.3 M glycerol and 50 mM sodium chloride, was buffered to pH 7.4, no hemolysis was

TABLE II

*Glycerol-induced hemolysis of normal red cells after different treatments*

Data given as averages ± S.D.

| | $E_{inf}/E_{max}$ (%) | | | | |
|---|---|---|---|---|---|
| | whole blood | | washed cells | | |
| | untreated | 1 mM DIDS | untreated | 1 mM DIDS | 100 $\mu$M DIDS |
| No albumin | 35.3 ± 6.6 | 99 ± 1 | 65 ± 3.9 *** | 98 ± 1 *** | 93.7 ± 5.2 *** |
| With albumin | - | - | 54.7 ± 9.1 | 93.2 ± 3 | 86.5 ± 4.5 |

*** Difference between whole blood to washed cells, as well as the DIDS effects are highly significant. Effect of albumin on washed cells is significant only at 1 mM DIDS ($P < 0.05$).

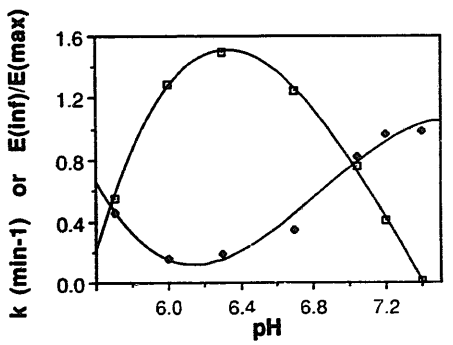


Fig. 7. pH dependence of the rate and degree of hemolysis. □——□, $k(min^{-1})$; ◆——◆, $E_{inf}/E_{max}$.

observed. When the pH was gradually reduced to pH 6.0, hemolysis was apparent, with increasing hemolytic rate, until pH reached 6.0. With a further reduction in pH to 5.7, the hemolytic rate became slower.

It is of interest to note that the pH effect observed in this study, at least at the range 7.4 to 6.0, does not correspond with the pH effect on glycerol permeability demonstrated by Carlsen and Wieth [11]. The latter have studied the kinetics of glycerol transport in human red blood cells by determining the rate of tracer efflux from radioactively labelled cells [$^{14}$C]glycerol) in 1% cells, in isotonic media. They found that glycerol is transported by two mechanisms: (a) by facilitated diffusion, with permeability depending on glycerol concentration, inhibited by $Cu^{2+}$ and by $H^+$, and (b) by a unspecific pathway, possibly by diffusion of individual molecules, independent of pH. The glycerol permeability has been demonstrated to be independent of pH, above pH 6.5. This inhibitory effect is competitive and is abolished when the glycerol concentration is high (approaching 2 M). Thus, the present results indicate that the pH effect on the rate and degree of hemolysis is not due to glycerol permeability, but to another phenomenon.

It is of interest to note that when either the rate of hemolysis or the ratio of $E_{inf}/E_{max}$ is plotted versus pH (Fig. 7) the curves indicate maximal effect at pH 6.25 ± 0.15. This pH dependence resembles the pH dependence of anion transport through the erythrocyte membrane [12,13].

It is therefore tempting to suggest that the rate and degree of hemolysis are due to a process involving band 3 which has been shown to mediate anion transport [12,13]. In order to check this hypothesis we added DIDS to normal blood at pH 6.85. We found that indeed, practically no hemolysis occurs in the AGLT test after DIDS preincubation. This result supports the hypothesis that the rate and degree of hemolysis are caused by a process involving band 3. This conclusion is corroborated by the effect of albumin on the lysis process as albumin reverses DIDS inhibition [7].

Several studies have shown marked differences in non-electrolyte permeability of liposomes with varied lipid composition [9]. In other studies these correlations were not so apparent [1]. Later, when studies were performed in several high permeability animal species, no distinct correlation between lipid composition and glycerol permeability was established [8]. However, in low permeability species, glycerol permeability decreased with an increase in chain length and a decrease in double bonds in the hydrocarbon chains [8].

When glycerol-induced hemolysis was measured in different human disorders, it was found that changes in membrane lipids affect the rate of hemolysis [2]. These changes were not specific, however, and any increase in lipids such as cholesterol of phosphatidylcholine delayed the hemolysis, indicating that the major factor was the membrane surface area: any lipid addition leading to an increase in membrane surface area, thereby increasing critical hemolytic volume, decreasess the hemolytic rate.

Therefore, it is reasonable to use the rate of glycerol-induced hemolysis for diagnosing disorders with increased surface-to-volume ratios, such as iron deficiency and thalassemia, as suggested by Gottfried and Robertson for GLT [2]. (However, as mentioned above, both $GLT_{50}$ and $AGLT_{50}$ could not always distinguish between iron deficiency and thalassemia).

The results of this study show, that instead of using a single parameter, (i.e., $AGLT_{50}$), two independent parameters should be used as criteria for diagnostic implications. The two parameters, $k$ and $E_{inf}/E_{max}$, describe two physical different phenomena, which were mixed in the use of $AGLT_{50}$. It has been demonstrated that most of the hemolysis, at least during the first 500 s, occurs according to a first order rate law and may therefore be described by a simple rate constant. This constant, $k$, gives more information about the hemolytic process than the time till the absorbance decreases to 50%, which sometimes is not reached at all. In addition, the final absorption value $E_{inf}$, as calculated at the latter stage of the hemolysis, also reflects the pathological state of the erythrocyte. These two parameters correlate well and very significantly with the type of erythrocyte disorder.

*Conclusion*

In addition to the determination of $AGLT_{50}$ values, it seems that the kinetic data above mentioned, such as $k$ values and the ratio of $E_{inf}$ to $E_{max}$, describe the hemolytic processes more aptly. These data may differentiate between the normal cell or spherocytic cell on the one hand, and the small, cell with an over-abundance of membrane, such as the red cells in iron deficiency anemia and thalassemia, on the other. Therefore we suggest that in addition to $AGLT_{50}$, these two new parameters should be used. The results of this study

suggest that the glycerol- induced lysis process occurrs via band 3. This conclusion is in accord with the suggestion that small non-electroctrolyte molecules penetrate the red cell membrane via band 3 [14,15]. The effect of albumin and DIDS (Table II and Fig. 6) clearly support this mechanism Thus, it is concluded that the glycerol-induced lysis indeed is mediated by a process involving band 3.

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