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FURTHER STUDIES ON THE MODE-OF-ACTION OF THE ALPHA

HEMOLYSIN OF *STAPHYLOCOCCUS AUREUS*:

ROLE OF THE RECEPTOR IN HEMOLYSIS

by

John William Austin

A Thesis

submitted to the Faculty of Graduate Studies

through the Department of

Biology in Partial Fulfillment

of the requirements for the Degree

of Master of Science at

The University of Windsor

Windsor, Ontario, Canada

© 1984

This thesis is dedicated to my parents and

to the memory of the late Mrs. Elsie King.

ABSTRACT

Exposure of rabbit erythrocytes to staphylococcal α hemolysin had no effect on SO_4^{2-} efflux, but increased the rate of SO_4^{2-} influx two-fold during the prelytic lag phase. Since inhibition of anion exchange with DIDS did not inhibit hemolysis, the increased influx of SO_4^{2-} may have occurred through a DIDS insensitive pathway.

α hemolysin caused rabbit erythrocytes to expand during the prelytic lag phase. Rates of expansion were not affected by inhibition of anion exchange but were increased by furosemide, an inhibitor of passive cation transport in human erythrocytes.

Micromolar concentrations of parachloromercuribenzoate sulfonic acid, eosin-5-maleimide, and eosin-5-iodoacetamide increased the hemolytic sensitivity of rabbit erythrocytes to α hemolysin. N-ethylmaleimide was effective at millimolar concentrations. Eosin-5-maleimide and eosin-5-iodoacetamide and bound predominately to band 3. Trypsin and chymotrypsin digestion of band 3 demonstrated that eosin-5-maleimide bound to a fragment of band 3 with a molecular weight of approximately 20 000 daltons, which remained associated with the membrane after protease treatment. Specific labelling of band 3 with eosin-5-maleimide, followed by hemolysis of the labelled cells by α hemolysin, demonstrated the presence of a 50 000 dalton labelled polypeptide. This is presented as evidence that band 3 of hemolysin-lysed erythrocytes is hydrolysed by a protease.

Kinetic studies of hemolysis demonstrated the presence of two separate rates of hemolysis. This was made apparent by plotting the first derivative of the number of intact erythrocytes versus time after addition of α hemolysin to give a plot of the rate of lysis versus time. Von Krogh analysis, as well as other analyses suggest the first rate of lysis requires a single "hit," whereas the second rate requires more than one "hit."

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LIST OF ABBREVIATIONS

CNBr, cyanogen bromide; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonate;
DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); H_2DIDS, 4,4'-diisothiocyano-
1,2-diphenylethane-2,2'-disulfonate; imOsm, ideal milliosmolar; NAP-taurine,
N-(4-azido-2-nitophenyl)-2-aminoethylsulfonate; NEM, N-ethylmaleimide;
PBS, phosphate-buffered saline; PCMB, p-chloromercuribenzoate;
PCMBS, p-chloromercuribenzenesulfonate; SCB, sulfate chloride buffer;
SDS, sodium dodecyl sulfate; SITS, 4-acetamido-4'-isothiocyano-2,2'-stilbene
disulfonate.

INTRODUCTION

The receptor, on the rabbit erythrocyte, for the α hemolysin of
Staphylococcus aureus is a 95 000 dalton protein called "band 3" (Maharaj and
Fackrell, 1980). Band 3 of human erythrocytes is a transmembrane polypeptide
possessing several functions, including anion transport (Cabantchik and
Rothstein, 1974a; Ho and Guidotti, 1975), amino acid transport (Young *et al*,
1981), water transport (Brown *et al*, 1975), passive cation transport (Grinstein
and Rothstein, 1978), and attachment of the cytoskeleton to the lipid bilayer
(Bennett and Stenbuck, 1979b). After identification of the receptor, it was
natural to ask if α hemolysin disrupts any of these functions.

Disruption of normal cation permeability by α hemolysin is well
documented (Marucci, 1963a, b; Madoff *et al*, 1964; Cooper *et al*, 1964a, b;
Sengers, 1970; Cassidy and Harshman, 1976; M. Gorski unpublished data). The
effects of α hemolysin on anion and water transport by band 3 had not
been studied, perhaps because of the high permeability of the erythrocyte
membrane to anions and water in its natural state. To examine the possibility
that anion transport is altered by α hemolysin, SO_4^{2-} exchange was
measured. Equilibrium exchange of SO_4^{2-} is 1 / 10 000 as fast as Cl^-
exchange (Schnell *et al*, 1973, 1977). The relatively slow rate of SO_4^{2-}
exchange allowed the release or uptake of $^{35}SO_4^{2-}$ to be measured during
the prelytic lag phase, and during hemolysis.

A transport process may be inhibited or stimulated by several covalently
binding probes (for details see reviews by Cabantchik *et al*, 1978; Knauf,
1979). If the particular transport process is involved in hemolysis,
interference with transport will affect hemolysis, and these effects can be
detected by changes in the kinetics of hemolysis. The disulfonic stilbenes

inhibit anion exchange (Cabantchik and Rothstein, 1974a; Lepke *et al*, 1976), furosemide inhibits passive cation exchange (Wiley, 1977) and anion exchange (Brazy and Gunn, 1976), and *p*-chloromercuribenzenesulfonate inhibits water exchange (Naccache and Sha'afi, 1974; Sha'afi and Feinstein, 1976). The latter is a sulfhydryl specific reagent which also increases passive cation permeability (Sutherland *et al*, 1967). These are some of the reagents which can be used to disrupt functions of band 3, and by using these reagents the role of certain functions of band 3 in the hemolytic sequence can be studied.

Probes of band 3 can be used as specific markers for band 3, or for certain segments of band 3. Since the binding sites of many of the probes are known, the role of the various regions of band 3 in the mechanism of hemolysis can be examined.

A somewhat unrelated problem, in that it does not directly involve a function of band 3, was also addressed. Hemolysis may be the result of a single hemolysin molecule bound to the cell. This can be described as a single "hit." Or lysis may be caused by more than one "hit." Analysis of the kinetics of lysis suggested lysis occurrs by two modes-of-action. A single hemolysin molecule, or a single complex of hemolysin molecules caused an initial lytic phase. This was followed by a phase where more than one hit was required to lyse the cell.

The objective of this study was to determine which activities of band 3 are altered by α hemolysin, or are required for hemolysis, in the hope that this will elucidate the mode-of-action of this puzzling bacterial protein.

LITERATURE REVIEW

The gram positive bacterium, *Staphylococcus aureus*, a common inhabitant of the upper respiratory tract, produces several proteins that are correlated with the organism's pathogenicity (Lack and Wailling, 1954). Two epidermolytic toxins, exfoliatin A and exfoliatin B, are produced by *Staphylococcus aureus* and are the cause of scalded skin syndrome and Ritter's disease (Winternitz, 1898; Lyell *et al*, 1969; Melish and Glasgow, 1970). Enterotoxins A, B, C, D and E are frequent causes of food poisoning (for review see Bergdoll, 1976). Leukocidin (Panton-Valentine leukocidin) is produced in high yield by *Staphylococcus aureus* strain V8, a strain of human origin. Leukocidin consists of two polypeptides, of molecular weights 32 000 and 31 000 (Noda *et al*, 1980), which act synergistically to produce a channel in leukocyte membranes, permeable to cations (Woodin, 1970). Staphylokinase and staphylocoagulase (coagulase) are two enzymes which contribute to the pathogenicity of *S aureus*. The former is a 15 000 dalton protein (Fujimura *et al*, 1974; Kondo *et al* 1981), which activates the plasma enzyme plasminogen to plasmin (Robbins *et al*, 1967; Jackson *et al*, 1981), the major fibrinolytic enzyme in blood. Staphylocoagulase activates prothrombin, resulting in an enzyme termed "staphylothrombin". Staphylothrombin is able to convert the soluble plasma protein, fibrinogen, into insoluble fibrin.

Four hemolysins are produced by most strains of *Staphylococcus aureus*. The hemolysins are termed "α", "β", "γ" and "δ" hemolysin in the order of their discovery. All the hemolysins lyse erythrocytes and are cytotoxic to a variety of mammalian cells. The specificity and mode-of-action of each of the hemolysins is distinctive.

Staphylococcal α hemolysin possesses hemolytic, dermonecrotic and lethal activities (Bernheimer and Schwartz, 1963). Rabbit erythrocytes are much more susceptible to α hemolysin than erythrocytes of any other species yet tested. The mode-of-action of staphylococcal α hemolysin is not yet understood.

Sheep erythrocytes are especially sensitive to β hemolysin (Bigger, 1933; Glenny and Stevens, 1935). β hemolysin has been demonstrated by Doery et al (1963, 1965), and later by others (Maheswaran et al, 1967; Wiseman and Caird, 1967; Wadstrom and Mollby, 1971a, b) to be a Mg^{2+} dependent sphingomyelinase C. Not surprisingly, susceptibility of erythrocytes to β hemolysin is dependent on the sphingomyelin content of the membrane (Wiseman and Caird, 1967; Wadstrom and Mollby, 1971b; Bernheimer et al, 1974). This protein has a molecular weight of 30 000 daltons (Bernheimer et al, 1974; Wadstrom and Mollby, 1971b). Incubation of erythrocytes at $37^{\circ}C$ with low concentrations of β hemolysin will not lyse the cells. If these cells are cooled below $10^{\circ}C$, rapid lysis follows. This has been termed "hot-cold" lysis (Walbum, 1921; Bigger et al, 1927). Several hypotheses have been proposed to explain this phenomenon (Wiseman, 1970, Meduski and Hochstein, 1972, Bernheimer, 1974, Smyth et al, 1975, Low and Freer, 1977).

γ hemolysin is hemolytic toward human, rabbit, and sheep erythrocytes (Guyonnet and Plommet, 1970; Fackrell, 1973). Fackrell and Wiseman (1976a) purified γ hemolysin using ultrafiltration and gel filtration, followed by ammonium sulfate precipitation and extraction with NaCl. Fackrell (1973) determined the molecular weight of γ hemolysin, using gel filtration, to be 45 000 daltons. Using hydroxylapatite chromatography, Guyonnet and coworkers (1968, 1970) and Taylor and Bernheimer (1974) purified γ hemolysin to its two

components (I and II), which act synergistically. Components I and II have molecular weights of 29 000 and 26 000 respectively (Taylor and Bernheimer, 1974). These authors have shown γ hemolysin to be inhibited by several lipids

and sterols in which the 3–β–hydroxy group is esterified. γ hemolysin is also inhibited by cardiolipin, phosphatidyl inositol, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl serine, nervonic acid, stearic acid and palmitic acid, all at concentrations of 0.05–5μg/ml (Taylor and Bernheimer, 1974). No change in erythrocyte lipids has been observed after exposure to γ hemolysin. The mode–of–action of γ hemolysin remains unknown. Fackrell and Wiseman (1976b) demonstrated a release of nitrogen and acid–soluble phosphorous from human erythrocyte membranes upon the addition of γ hemolysin, suggesting that γ hemolysin possesses phospholipase activity.

δ hemolysin does not show the species specificity demonstrated by both α and β hemolysins (Marks and Vaughan, 1950; Kayser and

Raynaud, 1965). The amino acid sequence of δ hemolysin was recently reported by Fitton *et al* (1980). A molecular weight of 2 977 can be calculated from the sequence of the 26 amino acid long polypeptide. This value is in strong disagreement with several others reported. Molecular weight determinations of δ hemolysin range from 2 977 to greater than 200 000 (Hallander, 1963; Caird and Wiseman, 1970; Kantor *et al*, 1972). Kantor *et al* (1972) have suggested δ hemolysin consists of five subunits of 21 000 daltons each. Each subunit was said to be a tetramer of identical polypeptide chains. δ hemolysin from the Newman and E–delta strains of *S aureus* liberate organic phosphorus from erythrocytes in proportion to

their hemolytic sensitivity (Wiseman and Caird, 1968). An actual substrate, in the erythrocyte membrane has not been identified. Evidence has recently been presented that δ hemolysin causes hemolysis via high surfactant activity and hydrophobicity (Bernheimer, 1974) apparently causing hemolysis by its detergent-like action. Studies of the interaction of δ hemolysin with

phospholipid monolayers (Bhakoo *et al*, 1982) suggest δ hemolysin may aggregate in the plane of the membrane, resulting in transmembrane channels causing loss of selective ion permeability. Freer and Birkbeck (1982) suggested an α helical conformation for δ hemolysin resulting in a rod-shaped molecule with amphipathic properties, thereby allowing the molecule to orient itself across the bilayer.

Strains of coagulase positive *Staphylococcus aureus* produce several other extracellular products both *iv vivo* and *in vitro*. Strains "M 18" and "Smith 5R" produce as many as 25 to 30 extracellular proteins, while strain V8 produces four to ten proteins (Wadstrom *et al*, 1974).

OVERVIEW

α hemolysin is the most studied of the staphylococcal hemolysins. It plays an important role in the pathogenesis of staphylococcal infections, most likely by causing tissue damage near the focus of infection (for review see Arbuthnott, 1981)

Four "isotoxins" have been separated using various separatory techniques (Bernheimer and Schwartz, 1963; Six and Harshman, 1973a, b; Wadstrom, 1968; McNiven *et al*, 1972; Dalen, 1976). These components are conformers of

the same hemolysin (Wadstrom, 1978). This idea is supported by the observation that, although they possess charge differences, the isotoxins share similar biological properties (Wadstrom, 1978) .

Watanabe and Kato (1974, 1978) have isolated fragments of α hemolysin after digestion with trypsin and have suggested that different portions of the hemolysin are responsible for the cytolytic, dermonecrotic and lethal activities. The biological properties of α hemolysin include not only cytolysis, but also contraction and paralysis of smooth muscle (Thal and Egner, 1961) and cytopathic effects in cultured cells (Artenstein et al, 1963). α hemolysin is toxic to leukocytes (Gladstone, 1966) and platelets (Siegel and Cohen, 1964). The wide range of effects of α hemolysin have led McCartney and Arbuthnott (1978) to suggest this hemolysin be called a "membrane-damaging toxin" rather than "toxin" a term which suggests only the protein's lytic activity towards erythrocytes. Bernheimer (1974) has suggested the use of the term "cytolysin". For the sake of simplicity α hemolysin will be referred to as "hemolysin" throughout this thesis, as it is the hemolytic activity of this protein with which this thesis is about.

PHYSICAL AND CHEMICAL PROPERTIES

The α hemolysin monomer is a protein with a sedimentation coefficient of 3S (Bernheimer and Schwartz, 1963; Lominski et al, 1963; Arbuthnott et al, 1967; Six and Harshman, 1973b; Goode and Baldwin, 1973, 1974). Molecular weight determinations range from 26 000 to 45 000 daltons (Six and Harshman, 1973b;

Fackrell and Wiseman, 1976b; Watanabe and Kato, 1974; McNiven *et al*, 1972; Forlani *et al*, 1971; Bernheimer and Schwartz, 1963). The wide range of the reported molecular weights may be due to proteolytic degradation of the hemolysin by staphylococcal proteases. Dalen (1976) has suggested the existence of 27 500 and 39 000 dalton polypeptides. The 27 500 dalton "isotoxin" is hydrolysed to yield a 10 000 dalton fragment, this 10 000 dalton fragment then associates with the remaining 27 500 dalton fragment to give rise to the 39 000 dalton "isotoxin" (Dalen, 1976). This "self hydrolysis" to form the 10 000 dalton peptide is supported by the finding of Wiseman *et al* (1975) that the hemolysin itself has proteolytic activity.

The isoelectric point of α hemolysin is approximately 8.5 (Arbuthnott *et al*, 1967; McNiven *et al*, 1972; Fackrell and Wiseman, 1976b; Goode and Baldwin, 1974).

Monomers of α hemolysin associate to form 12S hexamers (Arbuthnott, 1970; Bernheimer, 1974) with a molecular weight of 240 000 to 330 000 daltons (Bernheimer, 1974).

MODE-OF-ACTION

Release of K^+ is the first detectable event after binding of α hemolysin to rabbit erythrocytes (Marucci 1963a, b; Madoff *et al* 1964; Cooper *et al*, 1964a, b). Cassidy and Harshman (1976) have demonstrated the prelytic release of ^{86}Rb and have shown this release to closely parallel the binding of ^{125}I hemolysin at $20^{o}C$ but could separate the two events at $0^{o}C$. Intracellular ^{86}Rb release occurred approximately 40 minutes after addition of α hemolysin at $0^{o}C$ and did not depend on the amount

hemolysin bound. The rate of ^{86}Rb release increased with increasing amounts of bound hemolysin, leading Cassidy and Harshman (1976) to suggest that, at 0°C, each hemolysin molecule bound to the erythrocyte establishes a focus of damage which, after a certain fixed time, allows release of intracellular ^{86}Rb and, with more hemolysin molecules bound, more ^{86}Rb is released. Cooper *et al* (1964b) demonstrated that lysis could be prevented by the addition of antibodies to α hemolysin. Further binding of hemolysin is prevented by the addition of antihemolysin but, once any hemolysin is bound, release of intracellular ^{86}Rb cannot be inhibited (Cassidy and Harshman, 1976).

α hemolysin is rapidly adsorbed to the erythrocyte membrane during a prelytic lag (Klainer *et al* 1964). The bound hemolysin reaches a maximum during the greatest rate of lysis, followed by a gradual release after lysis. Cassidy and Harshman (1973), using ^{125}I–α hemolysin, also found a partial release of bound hemolysin.

Mangalo and Raynaud (1959) measured hemolysis of a suspension of erythrocytes by α hemolysin using a turbidometric assay and found that the time required to lyse 50% of the suspension is directly related to the concentration of α hemolysin added to the suspension. Lominski and Arbuthnott (1962) determined that the rate of hemolysis also depends on the concentration of α hemolysin added to the suspension.

α hemolysin was first proposed to have enzymatic activity in the early 1930's (Forssman 1933, 1934a, b, c). Levine (1938, 1939) demonstrated that the hemolytic, dermonecrotic and lethal effects of α hemolysin could be adsorbed by concentrated suspensions of erythrocytes, leading him to suggest

that α hemolysin and its substrate reacted stoichiometrically, following the Freundlich adsorption isotherm and, therefore, the hemolysin was not an enzyme.

Wiseman and coworkers (1970, 1972, 1975) found a release of soluble nitrogen from ghosts exposed to α hemolysin. The nitrogen release continues with time and no acid soluble phosphorous is released. Proteolytic activity of α hemolysin is activated by trypsin and this activated hemolysin can hydrolyse tosyl arginine methyl ester (Wiseman *et al*, 1975). Wiseman (1975) suggests α hemolysin is activated by an erythrocyte protease, and this activated hemolysin reacts with membrane proteins leading to lysis. An attempt to demonstrate which membrane protein is the substrate of the hemolysin's proteolytic activity was made by Freer *et al* (1973). Polyacrylamide gels of erythrocyte ghosts exposed to α hemolysin did not detect hydrolysis of any of the major membrane proteins. Freer *et al* (1973) also could not inhibit hemolysis of erythrocytes by the protease inhibitor phenylmethane sulfonylfluoride, did not find a reduction of sedimentable protein, and found the freeze—etch patterns of α hemolysin—treated ghosts are not similar to those obtained using known proteases. Wiseman (1975) has suggested that the ghost preparation used by Freer *et al* (1973) did not contain the membrane protease responsible for activation of the hemolysin.

α hemolysin causes a release of CrO_4^{2-} and PO_4^{2-}, and glucose from artificial lipid spherules composed of lecithin, cholesterol and either dicetylphospate or stearyl amine (Weissman *et al* 1966). Freer *et al* (1968) observed 12S hexamers of α hemolysin on lipid spherules and erythrocyte ghosts. These treated ghosts were analyzed by polyacrylamide gel

electrophoresis and Freer *et al* (1973) claimed the protein band of
molecular weight of approximately 160 000 daltons to be a result of the
hexamers observed in the negatively stained ghosts after treatment with α
hemolysin. Bernheimer *et al* (1972) have also observed the ring
formations on rabbit, human, horse and guinea pig erythrocyte membranes,
platelet membranes, rat hepatocyte plasma membranes and lysosomal membranes
prepared from rabbit polymorphonuclear leukocytes. Ring structures are not
seen on membranes of various bacterial species. Buckelew and Colacicco (1971)
observed that α hemolysin penetrated lipids to differing degrees. Freeze-
fracture studies on platelets (Bernheimer *et al*, 1972) suggest that the
hexameric α hemolysin penetrates lipid and Freer *et al* (1973)
showed that the fracture plane of the membrane in freeze-fracture studies is
altered by exposure to α hemolysin.

It must be pointed out that the electron microscopy studies done by Freer
et al (1973), in which hexamers were observed in hemolysin-treated membranes
after negative staining, involved exposure of erythrocyte ghosts, rather than
intact erythrocytes, to α hemolysin. It has been shown by both Arbuthnott
and Billcliffe (1974) and Cassidy and Harshman (1973) that only about 5% of
added hemolysin binds to intact erythrocytes, whereas up to 60 to 70% binding is
obtained when osmotically prepared ghosts are used (Arbuthnott and Billcliffe,
1974). It is possible that the hemolysin binds non-specifically to the
ghosts, leading to ring formation.

Cassidy *et al* (1974) confirmed the release of markers from liposomes,
however they did not find the species specificity for lipids that exists for
erythrocytes. Several studies have revealed that α hemolysin does not exhibit a
specificity for any individual lipid.

The finding of Cassidy *et al* (1974), that liposomes prepared from human erythrocyte lipids are as sensitive to α hemolysin as those prepared from rabbit erythrocyte lipids, yet rabbit erythrocytes are 100 times more susceptible, suggests that more than lipid is involved in hemolysis. The studies with liposomes involved concentrations of α hemolysin (15 to 30 μg/ml) higher than that required to lyse rabbit erythrocytes (0.1 to 0.2 μg/ml) (Harshman, 1979). The lysis of the liposomes may be a result of non-specific binding to the lipid causing lysis through a different mechanism.

Although the lipid-binding hypothesis for the mode-of-action of the hemolysin seems suspect, Fussle *et al* (1981) and Bhakdi *et al* (1981) have demonstrated the ability of α hemolysin to self associate into hexamers in the presence of deoxycholate micelles, and these hexamers, when extracted, bound lipid and could be incorporated into artificial lecithin lipid vesicles. Fussle *et al* (1981) extracted α hemolysin hexamers formed on, or in, erythrocyte ghosts using Triton X-100 and reincorporate the hexamers into liposomes. On the basis of marker release studies Fussle *et al* (1981) have suggested that α hemolysin forms discrete transmembrane channels approximately three nanometers in diameter. Fussle *et al* (1981) account for the species sensitivity of whole erythrocytes by suggesting that human erythrocytes contain a protein, absent in rabbit erythrocytes, which somehow repairs the damage done by α hemolysin. Unfortunately, Fussle *et al* (1981) exposed their erythrocytes to concentrations (0.15 to 0.2 mg/ml) of α hemolysin, 1 000 times more than that required to cause hemolysis. Harshman (1979) has confirmed that rings are formed on the membranes of both human and rabbit erythrocytes at concentrations between 10 and 100 μg/ml . However

Harshman (1979) could not observe these same ring formations at lower
concentrations (0.1 to 0.2 μg/ml) which are still hemolytic to rabbit
erythrocytes.

Recent evidence has implicated a membrane protein as a specific receptor
for α hemolysin. Cassidy and Harshman (1976) found a close correlation
between binding of ^{125}I–α hemolysin to erythrocytes of various species and
hemolytic sensitivity of the erythrocytes. Rabbit erythrocytes bound 100 times
more α hemolysin than did human erythrocytes. Barei and Fackrell (1979)
found a correlation coefficient of 0.992 between receptor number and hemolytic
sensitivity and determined the number of receptors on rabbit erythrocytes to be
125 980. Cassidy and Harshman found 5 000 receptors per cell. The lower
number obtained by Cassidy and Harshman may be explained by a change in the
receptor on binding of the hemolysin. Barei and Fackrell used heat denatured
α hemolysin labeled with fluorescein, whereas Cassidy and Harshman used
^{125}I–α hemolysin which retained 10% of its hemolytic activity. This residual
activity may have caused release of the bound hemolysin, which is the case with
the active hemolysin (Klainer et al, 1964).

Using antigenic determinants as reference points, Lo and Fackrell (1979)
have shown that the hemolysin is oriented on the membrane in a specific manner
and possesses a membrane binding region. This is consistent with the idea of a
specific receptor for α hemolysin.

A finite number of high affinity binding sites and many low affinity
sites for α hemolysin are present on the rabbit erythrocyte (Barei and
Fackrell, 1979). Two types of binding exist, specific and non–specific, both
leading to hemolysis (Barei and Fackrell, 1979). The low affinity binding, at

high concentrations, may result in the appearance of the α hemolysin

hexamers observed by Freer *et al* (1968, 1973) and Bernheimer *et al.*

(1972).

Using polyacrylamide gel electrophoresis of sodium dodecyl sulfate

solubilized $^{125}I-\alpha$ hemolysin—treated rabbit ghosts, Cassidy and Harshman

(1979) located α hemolysin in protein bands 1.5×10^6, 1.1×10^6 and 1.6×10^5

daltons. They suggested that the 1.5×10^6 and 1.1×10^6 dalton bands

represented the α hemolysin hexamer—receptor complex and α hemolysin

dodecamer—receptor complex respectively. The 1.6×10^5 dalton band probably

represents the hemolysin—receptor complex in a ratio of one mole hemolysin :

one mole receptor.

Kato *et al* (1975) first suggested that a pronase sensitive flavine

mononucleotide binding glycoprotein was the receptor for α hemolysin.

Later, Kato and Naiki (1976) postulated that the receptor was a ganglioside.

Attempts to reproduce these results have been unsuccessful in ours and other

laboratories.

The receptor of α hemolysin on the rabbit erythrocyte membrane is

band 3 (Maharaj and Fackrell, 1981). Band 3 is a transmembrane protein of

molecular weight 90 000 to 100 000 daltons. Both concanavalin A and antibodies

to band 3 confer protection to rabbit erythrocytes. Purified band 3, when

preincubated with α hemolysin, causes the hemolysin to become non—hemolytic.

BAND 3 PHYSICAL AND CHEMICAL PROPERTIES

Band 3 is an integral glycoprotein. It can be extracted from the membrane only by dissolution of the lipid (Juliano, 1973; Steck, 1974; Yu *et al*, 1973). Band 3 is more hydrophobic than most soluble proteins, containing 37.5% hydrophobic residues (Steck *et al*, 1978) and is held into the lipid bilayer by strong hydrophobic interactions. Estimates of the carbohydrate content of band 3 vary from three to 15% by weight (Ho and Guidotti, 1975; Yu and Steck, 1975a; Furthmayr *et al*, 1976; Jenkins and Tanner, 1977b; Fukuda *et al*, 1978). Galactose and N–acetylglucosamine are the predominant sugars with mannose, fucose and sialic acid present in lower amounts. The carbohydrate of band 3 is exposed at the outer surface of the membrane (Steck and Dawson, 1974; Yu and Steck, 1975a; Jenkins and Tanner, 1977b; Fukuda *et al*, 1978; Drickamer, 1978).

Band 3 was named by Fairbanks *et al* (1970) for its position in sodium dodecyl sulfate gel electrophoretograms as the third major band from the origin corresponding to a molecular weight of about 95 000 daltons. Gel filtration suggests the actual weight of band 3 to be closer to 80 000 daltons (Ho and Guidotti, 1975; Clarke, 1975). The reason for the higher estimate of molecular weight of band 3 by sodium dodecyl sulfate electrophoresis may be its large content of carbohydrate.

HETEROGENEITY OF BAND 3

Although band 3 appears homogeneous by polyacrylamide gel electrophoresis, it actually consists of several proteins. In electrophoretograms a major peak containing 90% of the protein is apparent at the leading edge of the band followed by a diffuse trail which contains gradually less protein at the higher weights. The minor components differ in protease susceptibility (Carraway,

1975; Cabantchik and Rothstein, 1974b; Knauf *et al*, 1974; Reichstein and Blostein, 1975; Jenkins and Tanner, 1977a,b) and can be separated by other electrophoretic (Anselstetter and Horstmann, 1975; Ballou and Smithies, 1977; Conrad and Penniston, 1976) techniques or immunoelectrophoretic (Bhakdi *et al*, 1976; Golovtchenko-Matsumoto and Osawa, 1980) techniques. Heterogeneity is observed when band 3 is exposed to proteases when in the intact cell (Carraway, 1975; Cabantchik and Rothstein, 1974b; Jenkins and Tanner, 1977b; Steck *et al*, 1971; Reichstein and Blostein, 1975). Most of band 3 is hydrolysed to polypeptides of 35 000 and 65 000 daltons in pronase treated cells, however three pronase resistant bands remain in the band 3 region (Cabantchik *et al*, 1978).

It has been reported that band 3 contains a phosphorylated intermediate of the Na,K-ATPase (Williams, 1972; Avruch and Fairbanks, 1972; Knauf *et al*, 1974; Bjerrum and Bog-Hansen, 1976) and acetylcholine esterase activity has been detected in band 3 (Bellhorn *et al*, 1970). Brown *et al* (1975) found a protein responsible for water transport migrates in the band 3 region of electrophoretograms.

Band 3 demonstrates variable behaviour towards binding of lectins (Findlay, 1974; Adair and Kornfeld, 1975), and towards endogenous protein kinases (Roses, 1976).

No heterogeneity of the major component is found in sites of proteolytic cleavage (Steck *et al*, 1976) or in the binding of anion exchange inhibitors (Findlay, 1974) suggesting the polypeptide portion of the major component of band 3 is homogeneous. The major component of band 3 contains only a single C-terminal amino acid (Drickamer, 1976) and the cytoplasmic fragment of band 3 derived from trypsin hydrolysis contains only a single N-terminal amino acid

(Jenkins and Tanner, 1977b). Heterogeneity of the major component of band 3 is attributed to the varying carbohydrate composition (Yu and Steck, 1975a; Findlay, 1974; Adair and Kornfeld, 1974, Jenkins and Tanner, 1977b; Drickamer, 1978) of the protein.

ARRANGEMENT OF BAND 3 IN THE MEMBRANE

Band 3 is cleaved at the cytoplasmic face of the membrane by mild trypsin or chymotrypsin treatment yielding a soluble 40 000 dalton fragment, not associated with the bilayer (fragment A), and a membrane bound 55 000 dalton fragment. Recently, Appell and Low (1981) have found that fragment A is released from the membrane as a highly elongated, noncovalent dimer of molecular weight 90 000 daltons. Fragment A is not required for anion transport (Lepke and Passow, 1976; Grinstein et al, 1978; Ross and McConnell, 1978; England and Steck, 1978), and functions mostly as a link between the bilayer and the cytoskeleton or cytoplasmic proteins. Fragment A contains the binding sites for several cytoplasmic proteins: aldolase (Yu and Steck, 1975b; Murthy et al, 1981; Strapazon and Steck, 1977), glyceraldehyde–3– phosphate dehydrogenase (Yu and Steck, 1975b), phosphofructokinase (Richards et al, 1979) and hemoglobin (Karadsheh and Uyeda, 1977; Salhany and Shaklai, 1979; Salhany et al, 1980; Salhany and Gaines, 1981; Cassoly and Salhany, 1983). The cytoplasmic portion of band 3 also binds ankyrin (band 2.1) (Bennett and Stenbuck, 1979; Branton et al, 1981) which couples band 3 to the cytoskeleton (Bennett and Stenbuck, 1979, 1980). The cytoplasmic fragment A contains the reactive sulfhydryl groups responsible for crosslinking of band 3 by Cu^{2+}/o–phenanthroline (Steck et al, 1976; Rao and Reithmeier, 1979; Reithmeier and Rao, 1979). The Nα–terminal 201 residues of human

erythrocyte band 3 have recently been sequenced by Kaul *et al* (1983). These authors suggest a polyanionic 23-residue Nα-terminal segment binds aldolase (Murthy *et al*, 1981), glyceraldehyde-3-phosphate dehydrogenase (Tsai *et al*, 1982), and hemoglobin (Kaul and Kohler, 1983).

The 17 000 dalton membrane spanning portion of band 3 is separated from the rest of band 3 by exposure of leaky ghosts to low concentrations of chymotrypsin (Steck *et al*, 1976). Exposure of leaky ghosts to higher concentrations of chymotrypsin results in further cleavage to a 15 000 dalton polypeptide (DuPre and Rothstein, 1981; Ramjeesingh *et al*, 1980a; Ramjeesingh and Rothstein, 1982). A 4 000 dalton CNBr fragment contains the binding site for DIDS (Ramjeesingh et al, 1980b). DIDS covalently binds to an ε amino group of lysine located 9 000 daltons from the C-terminus of fragment B (Ramjeesingh, 1981). A single cysteine residue is located 5 000 daltons from the C-terminus of fragment B (Rothstein and Ramjeesingh, 1982).

The 35 000 dalton fragment resulting from exposure of intact cells to chymotrypsin contains the single carbohydrate attachment site. This fragment contains two cytoplasmic sulfhydryl groups (Rao, 1979; Ramjeesingh *et al*, 1981a). Since the carbohydrate is located on the outside of the membrane, and the sulfhydryl groups are cytoplasmic, this fragment must cross the membrane. Cleavage of band 3 to give the 60 000 dalton and 35 000 dalton fragments does not inhibit anion transport (Cabantchik and Rothstein, 1974b), and the two fragments remain associated in the membrane (Grinstein *et al*, 1978). However, cleavage of the 35 000 dalton fragment by papain or chymotrypsin results in inhibiton of anion exchange (Jennings and Passow, 1979; DuPre and Rothstein, 1981). Phenylglyoxal, an inhibitor of anion transport, which is both impermeant and covalent, binds to the 35 000 dalton fragment of band 3 (Wieth

et al, 1982). It appears that both the 17 000 dalton and the 35 000 dalton

fragments are required for anion transport. These two segments of band 3 can

be crosslinked with H_2DIDS at alkaline pH (Jennings and Passow, 1979) and

comigrate during centrifugation after extraction in Triton X–100 (Reithmeier,

1979), suggesting that they exist in close proximity in the membrane.

ROLE OF BAND 3 IN ANION TRANSPORT

The $Cl^--HCO_3^-$ exchange system of the red blood cell is required for CO_2

transport from tissues to lungs. This exchange of anions represents the rate

limiting step in CO_2 transport to the lungs. Gaseous CO_2 diffuses into the red

cell, where it combines with H_2O to produce H_2CO_3. H_2CO_3 is then converted to

HCO_3^- and a H^+ the latter is taken up by hemoglobin which acts as a H^+ sink.

The HCO_3^- produced is then transported out of the cell into the plasma in

exchange for the inward transport of Cl^- (Harris and Pressman, 1967). The

bulk of the CO_2 produced by metabolism is transported to the lungs as plasma

HCO_3^-.

Since the main function of the red cell is removal of CO_2 from the

tissues and the time of passage of a red cell through a capillary is less than

one second, the HCO_3^-/Cl^- exchange protein must be present in many copies

per cell. Band 3, the anion transporter of the red cell membrane, is present

in approximately 10^6 copies per cell (Cousin and Motais, 1979; Knauf

et al, 1978; Funder *et al*, 1978; Halestrap, 1976; Ship *et al*, 1977;

Lepke *et al*, 1976; Zaki *et al*, 1975). The half–time for Cl^- exchange is

about 50ms at 37^oC (Brahm, 1977), corresponding to a Cl^- permeability

of about 2.3 X 10^{-3}cm/sec. Cl^- exchange is much more rapid than the passive cation fluxes (2 X 10^{-10}cm/sec at 37^0C) (Sachs, 1977). Thus the red cell membrane exerts an anion / cation selectivity of about seven orders of magnitude.

Anion transport across the red cell membrane is mediated by band 3 (Cabantchik and Rothstein, 1972, 1974a; Zaki *et al*, 1975; Ho and Guidotti, 1975; Ship *et al*, 1977). This has been determined by the use of membrane probes which inhibit anion transport, the most useful probes being the disulfonic stilbenes 2,2'–diisothiocyano–4,4'–distilbene disulfonate (DIDS) and 4–acetamido–4,4'–isothiocyano–2,2'–distilbene disulfonate (SITS). When red cells are exposed to radioactively labelled inhibitors of anion transport, followed by electrophoresis of the membranes, most of the label is found in band 3. Reconstitution of band 3 into liposomes confers anion transport ability upon the liposomes (Rothstein *et al*, 1975, 1976; Feinstein *et al*, 1977; Ross and McConnell, 1977, 1978; Cabantchik, 1982; Darmon *et al*, 1983). Wolosin *et al* (1977) used negative purification to produce vesicles from red cell membranes containing only band 3 and demonstrated the capability of these vesicles to transport anions.

Cl^- transport is a saturable, electroneutral, one for one exchange. The mechanism of transport is still not understood at the molecular level. Transport is thought to follow the "ping pong" model (Gunn and Frohlich, 1978), in which positively charged anion binding sites alternate between exposure to inside and outside compartments, but can only shift from one position to another when occupied by an anion. The unidirectional translocation of an anion (ie. one half cycle of the 1 : 1 exchange) is triggered upon binding of an anion to a transport "site" (Jennings, 1982).

Transport occurs via a fixed protein pathway. A small segment of peptide undergoes a small rearrangement from topologically out to topologically in. The turnover of Cl⁻ is high (2 X 10⁵ sec⁻¹), therefore the change in conformation must be small, and over a small distance (Rothstein and Ramjeesingh, 1982).

Anions to be transported appear to combine with a substrate site which is half saturated with Cl⁻ at 65mM (Gunn *et al*, 1973; Brazy and Gunn, 1976; Dalmark, 1976). A second site of anion binding is saturated at Cl⁻ concentrations of 335mM (Dalmark, 1976). This site has been termed the modifier site. The modifier site has a high affinity for organic anions, such as N−(4−azido−2−nitrophenyl)−2−aminoethylsulfonate (NAP−taurine) (Cabantchik *et al*, 1978; Knauf, 1979). The modifier site appears to be functionally linked to the transport site, as binding of anions to this site causes non−competetive inhibition of transport. Binding of the impermeant inhibitor, NAP−taurine, to the modifier site indicates that this site is exposed to the outside surface of the membrane (Knauf *et al*, 1978).

FIG1

FIG2

Figure 1

Figure and legend reproduced without permission from Rothstein and
Ramjeesingh (1982). A proposed arrangement of band 3 in the bilayer.
C_t and N_t, C-terminus and N-terminus; CHO, site of carbohydrate
attachement; SH, locations of sulfhydral groups; DIDS, binding site for DIDS;
Pa_1 and Pa_2 are papain cleavage sites; C_1, C_2,
C_3, C_4, and C_5 are chymotrypsin cleavage sites.

N,

42

SH SH SH

C_2

C_3

15

DIDS

17

SH

C_1, Pa1

7

C_4, Pa2

C_5

35

8

SH

SH SH

CHO

C_1
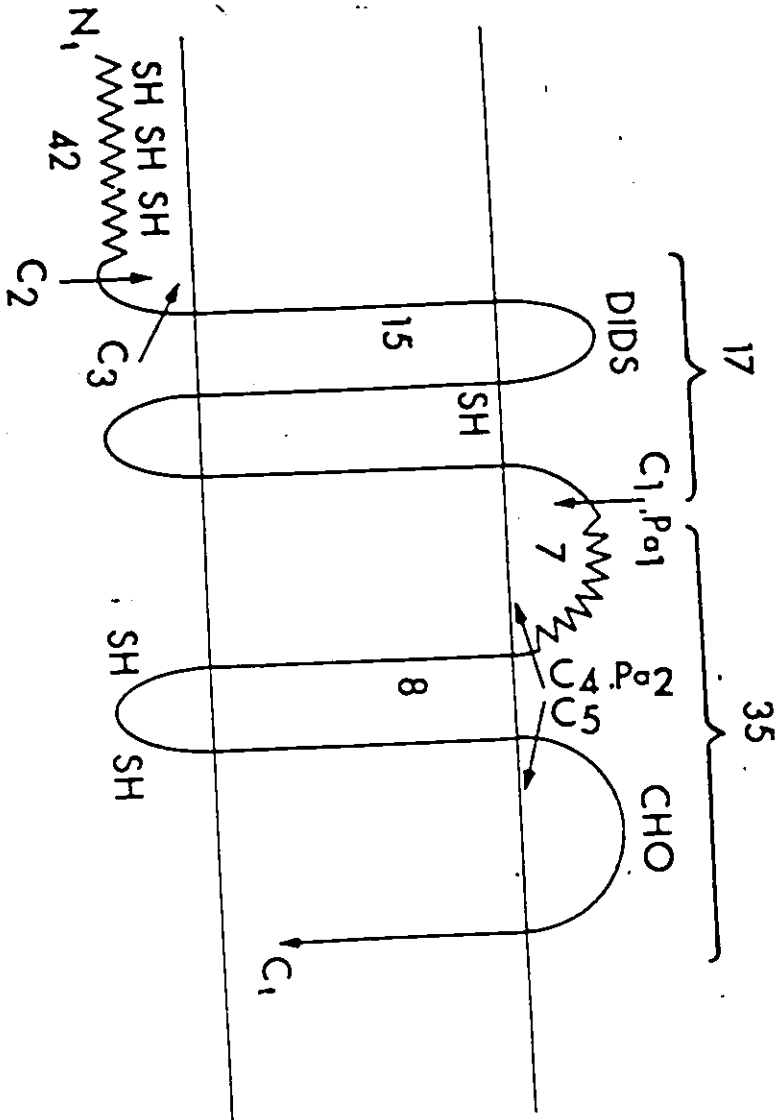
Figure 2

Figure and legend reproduced without permission from Rothstein and
Ramjeesingh (1982). A functional model for anion transport. (a) A kinetic
model with T representing the transport site and M the modifier site;
(b) the model accomodated to band 3 structure, with the dotted line
representing a diffusion barrier, the gate the transport site, and the square
the modifier site.

(a)

OUTSIDE

BILAYER

INSIDE

A⁺ ──→ T⁺ ⇌ ∿∿∿ ⇌ T⁺ ──→ A⁺

AT ──→ AT ──→ AT

AM ──→ AM ──→ A⁺

(b)

OUTSIDE

BILAYER

INSIDE

INTRINSIC SEGMENTS OF BAND 3
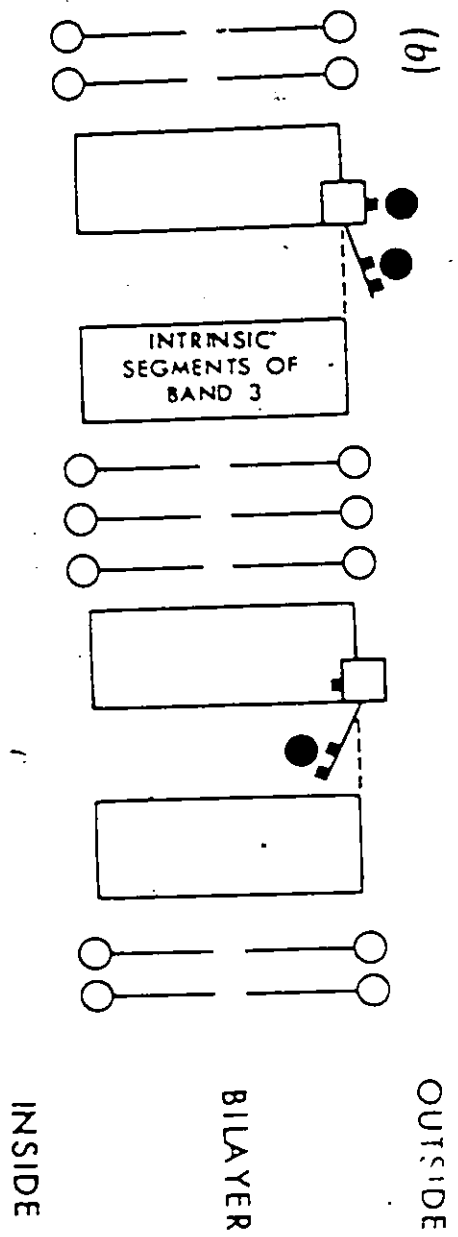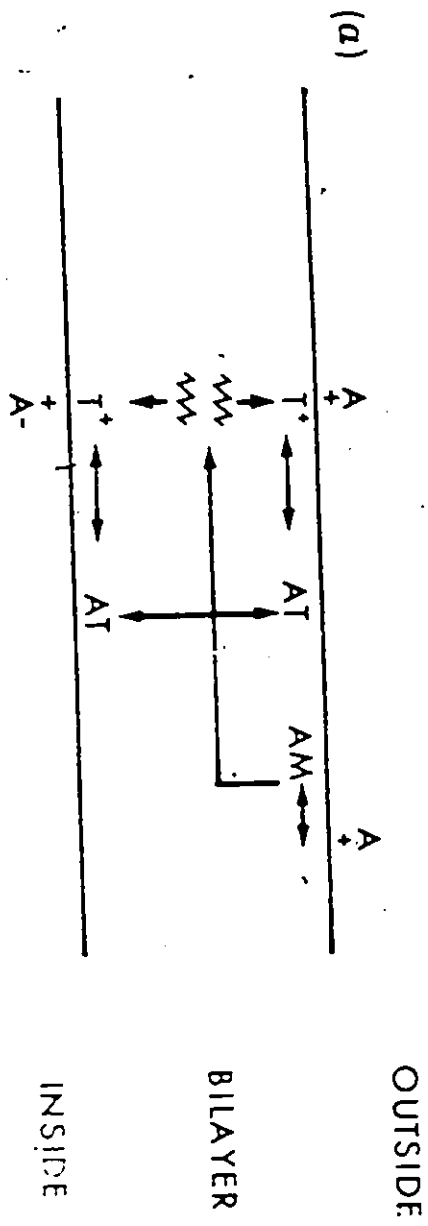
MATERIALS AND METHODS

Cultures

Cultures of *Staphylococcus aureus* strain Wood 46 (Fackrell, 1973) used in this study met the requirements set by Baird–Parker (1972) to be classified as *Staphylococcus. aureus*. Phage–typing of the strain was done at the Canadian Communicable Disease Centre in Ottawa. Lyophilized cultures were reconstituted and grown on rabbit blood agar plates, incubated at 37^0C for 36 hours in an atmosphere of 10% CO_2 and 90% air. Colonies showing the largest zones of hemolysis were subcultured on rabbit blood agar plates, and used to produce α hemolysin.

Preparation of α hemolysin.

Staphylococcus aureus strain Wood 46 was inoculated into Dolman–Wilson medium (Dolman and Wilson, 1940) and incubated at 37^0C with shaking in an atmosphere of 10% CO_2 and 90% air for 36 hours. The supernatants of the cultures were harvested by centrifugation at 8000 x *g* for 15 minutes and frozen immediately. Culture supernatants were used as the source of α hemolysin. All hemolytic activity of these supernatants could be neutralized by monospecific anti–hemolysin.

Heat inactivation of α hemolysin.

Staphylococcal α hemolysin was heated to 60^0C at pH 4 for five minutes (Dalen, 1976).

Reagents and buffers.

Tritiated dihydro-2,2'-diisothiocyano-4,4'-distilbene disulfonic

acid ([$^3H_2$]DIDS) (200mCi/mmole) and dihydro-2,2'-diisothiocyano-4,4'-distilbene

disulfonate (H$_2$DIDS) were gifts from M. Ramjeesingh and A. Rothstein, The

Hospital for Sick Children, Toronto, Canada. Na$_2$^{35}SO$_4$ (2 mCi/ml),

N-[ethyl-2-^{3}H]-ethylmaleimide (2.2 Ci/mmole) and Bray's solution were from

New England Nuclear, Boston Mass. 4,4'-diisothiocyano-2,2'-stilbene disulfonic

acid (DIDS), 4-acetamido-4'-isothiocyano-2,2'-stilbene disulfonate (SITS),

4-(2-hydroxyethyl)-1-piperazineethane sulfonate (HEPES), p-chloro-

mercuribenzene sulfonate (PCMBS), N-ethylmaleimide, iodoacetamide and 1,1'-

azobis(N,N-dimethylformamide) (diamide), and bovine serum albumin were obtained

from Sigma Chemical Co., St. Louis, MO. Eosin-5-maleimide and eosin-5-

iodoacetamide were obtained from Molecular Probes, Junction City, OR.

Electrophoresis reagents were from Sigma. NCS tissue solubilizer was obtained

from Amersham, Oakville, Ontario.

The sulfate-chloride buffer, used for all SO$_4^{2-}$ exchange experiments,

consisted of 130mM NaCl, 5mM KCl, 10mM Na$_2$SO$_4$, 5mM HEPES, and 5mM

D-glucose pH to 7.4 with 0.5N NaOH (Rakitzis *et al*, 1978). Phosphate-

buffered saline consisted of 145 mM NaCl and 10 mM sodium phosphate buffer

(pH 7.2). The milliosmolar buffer (20 ideal milliosmolar) was identical to the

buffer described by Dodge *et al* (1963). 20imOsm buffer was prepared as

follows: 0.155M NaH$_2$PO$_4$ was mixed with 0.103M Na$_2$PO$_4$ to make 310 imOsm

buffer pH 7.4. This buffer was then diluted to make 20 imOsm buffer pH 7.4.

Preparation of erythrocytes and erythrocyte membranes.

Blood from New Zealand white rabbits was collected in sterile Alsever's solution (Carpenter, 1965). The erythrocytes were collected by centrifugation at 2000 x g for 5 minutes, and the plasma and buffy coat were removed by aspiration. Erythrocytes were washed four times in six volumes of PBS before use. Erythrocyte membranes were prepared by the method of Dodge *et al* (1963).

Hemolytic titration of α hemolysin.

The 50% endpoint method of Lo and Fackrell (1979) was performed in Microtitre equipment. The reciprocal of the dilution of hemolysin that gave 50% hemolysis was defined as one hemolytic unit.

Kinetic hemolysis assay.

Two-hundred microlitres of a 2% suspension of rabbit erythrocytes was added to 3ml of the required dilution of α hemolysin in a spectrophotometer cuvette. The lysis of the suspension of erythrocytes was measured by the decrease in absorbance of the suspension at 650nm. Readings were taken from a Perkin-Elmer Coleman 124 spectrophotometer and recorded by an Apple II+ computer (Glasgow and Fackrell, 1984). A BASIC program, called KINETICS, calculated several parameters including, the length of the prelytic lag phase, the time to 50% hemolysis, the time to completion, the average and maximum rates of hemolysis, and the percentage of unlysed cells at completion.

Kinetic expansion assay

Two—hundred microlitres of a 2% suspension of rabbit erythrocytes was added to 3ml of the required dilution of α hemolysin in a spectrofluorometer cuvette. The rate of swelling of the erythrocytes measured by the decrease in light scattered by the suspension at 650nm. Readings were taken from a G. K. Turner Model 430 spectrofluorometer and recorded by an Apple II+ computer (Glasgow and Fackrell, 1984). A BASIC program, called KINETICS, calculated several parameters including, the length of the pre—expansion lag phase, the time to 50% expansion, the time to completion, and the average and maximum rates of expansion.

Gel electrophoresis.

Twenty microlitres of a five times concentrated sample buffer (Laemmli, 1970) was mixed with 100μL of packed membranes and boiled for 4 minutes. A 100μL sample was applied to each 8% acrylamide discontinuous tube gel (Laemmli, 1970) and electrophoresed at 2.5 ma per gel. To detect $[^3H_2]$DIDS or N—[ethyl—2—^{3}H]—maleimide, the gels were sliced into 2mm thick discs. Each slice was swelled in 90% NCS tissue solubilizer and $[^3H_2]$DIDS or N—[ethyl—2—^{3}H]—maleimide was detected by liquid scintillation counting in a Beckman LS—3150P liquid scintillation counter. The gels of eosin—5—maleimide labelled proteins were fixed in a solution of 45% methanol and 9% acetic acid and photographed under long wave ultraviolet light with an O2 (orange) filter.

Labelling of rabbit erythrocyte membranes with [³H₂]DIDS.

Rabbit erythrocytes were labelled with [³H₂]DIDS using the method of Ship *et al* (1977). Briefly, rabbit erythrocytes were suspended at 40% hematocrit in PBS containing 10 μM H₂DIDS containing [³H₂]DIDS at 37°C for 15 minutes. The erythrocytes were then washed once in HEPES-saline buffer (150 mM NaCl, 10 mM HEPES, pH 7.4 with 2 N NaOH). This buffer was supplemented with 0.5% BSA to remove [³H₂]DIDS which may have bound non-specifically to the membrane. This wash was followed with two washes in PBS.

Incubation of rabbit erythrocytes with sulfhydryl-specific probes.

Two-fold serial dilutions of each of the probes were performed in PBS. An equal volume of a 4% suspension of rabbit erythrocytes in PBS was added to each dilution. The final hematocrit of the suspensions was 2%. All incubations lasted one hour at room temperature. Cells labelled with eosin-5-maleimide or eosin-5-iodoacetamide were incubated in the dark.

Labelling of intact erythrocytes and erythrocyte membranes with N-[ethyl-2-³H]-maleimide.

Rabbit erythrocytes and rabbit erythrocyte membranes were labelled by reacting a 25% suspension of cells or membranes in PBS pH 7.4 with N-[ethyl-2-³H]-maleimide at room temperature for one hour (Rao, 1979).

Labelling of intact erythrocytes with eosin–5–maleimide.

The labelling procedure was the same as that used by Nigg and Cherry (1979). Two milligrams of eosin–5–maleimide, dissolved in 4 ml of PBS was added to 10 ml of packed erythrocytes. After incubation in the dark for 60 minutes, the cells were washed three times in 40 volumes of PBS.

Labelling of erythrocyte membranes with eosin–5–maleimide.

A 2.5 ml volume of packed rabbit erythrocytes was lysed in 37.5 ml of 20 imOsm buffer pH 7.4 (Dodge *et al*, 1963) and washed six times in 50 ml of the same buffer. The ghosts were incubated in the dark for 90 minutes in 1 ml of 20 imOsm buffer containing 0.5 mg of eosin–5–maleimide and then washed six times with 50 ml of 20 imOsm buffer.

Chymotrypsin treatment of eosin–5–maleimide labelled erythrocytes.

Ten ml of a 50% suspension of erythrocytes, labelled with eosin–5–maleimide, were incubated overnight in PBS containing 100 μg/ml of chymotrypsin. The suspension was centrifuged at 5 000 x g for 5 minutes and both cells and supernatant retained. The cells were washed three times in 50 volumes of phosphate–buffered saline, and lysed in 75 ml of 20 imOsm buffer. The ghosts were washed with 20 imOsm buffer until they were the characteristic light pink colour of eosin–labelled ghosts.

Chymotrypsin and trypsin treatment of eosin–5–maleimide labelled ghosts.

A 5 ml volume of labelled, packed erythrocytes was lysed in 75 ml of 20 imOsm buffer and washed in the same buffer until they were light pink. Two

ml of these ghosts was shaken for one hour at room temperature with 2 ml of 20 imOsm buffer containing 200 μg/ml of either trypsin or chymotrypsin. The suspension was centrifuged at 5 000 x g for five minutes and the supernatant and cells were retained.

Sulfate efflux determinations.

The method used was essentially that of Rakitzis et al (1978). All steps were carried out at 37°C unless stated otherwise. Fresh rabbit blood was collected from the ear of a New Zealand white rabbit into sulfate chloride buffer supplemented with 1mM ethylenediaminetetraacetic acid (EDTA). This blood was centrifuged at 2000 x g for 5 minutes and the plasma and buffy coat were removed. The erythrocytes were washed three times with five volumes of sulfate–chloride buffer. The washed cells were resuspended to 6% hematocrit in sulfate–chloride buffer and equilibrated at 37°C for two hours with gentle shaking. The equilibrated cells were centrifuged at 2000 x g for 5 minutes and the supernatant was discarded. The cells were resuspended at 16% hematocrit in sulfate–chloride buffer containing aqueous $Na_2^{35}SO_4$ (1μL per 1ml qf packed cells) and loaded in this medium for 90 minutes with gentle shaking. The loaded cells were centrifuged at 2000 x g for 5 minutes and the supernatant was removed by aspiration. The cells were resuspended in sulfate–chloride buffer to 10% hematocrit. A volume of the 10% suspension of cells was pipetted into sulfate–chloride buffer containing the desired concentration of α hemolysin or inhibitor, to give a final hematocrit in the flux medium of 0.5%. A 0.5ml aliquot of the cell suspension was removed at various times and pipetted into 0.5ml of ice cold sulfate–chloride buffer. This sample was immediately centrifuged at 10 000 x g for 3 minutes at 4°C and the supernatant

removed and saved. Portions of the supernatants were retained for hemoglobin measurements (absorbance at 541 nm) and the remainder of the supernatants, after precipitation of the protein with 2.5% (w/v) trichloroacetic acid, was added to 10 ml of Bray's solution and counted in a Beckman LS-3150P liquid scintillation counter to determine $^{35}SO_4^{2-}$ content.

Sulfate influx determinations.

The method of Young and Ellory (1982) was used for SO_4^{2-} influx determinations. One-hundred μL of sulfate-chloride buffer containing 0.5μCi of $^{35}SO_4^{2-}$ and the desired concentration of α hemolysin was added to several 1ml centrifuge tubes (one tube for each sample) and warmed to 37^{0}C. 100μL of a 10% suspension of prewarmed rabbit erythrocytes in sulfate-chloride buffer were added to each tube so that the final suspension contained 5% erythrocytes. The time of addition of the cells to the buffer containing $^{35}SO_4^{2-}$ was considered time zero. At each sampling time, 1000μL of ice-cold sulfate-chloride buffer containing 1mM SITS, a noncovalent inhibitor of anion transport, was added to one of the tubes to stop the influx. Samples were centrifuged at 15 000 x g for 20 seconds at 4^{0}C in a microcentrifuge and the supernatants were removed and saved for determination of hemoglobin. The cells were washed twice in ice-cold sulfate-chloride buffer containing 1mM SITS. Packed cells were lysed by resuspension in 500μL of 1% (v/v) Triton X-100 in sulfate-chloride buffer and the lysate was deproteinized with 2.5% (w/v) trichloroacetic acid. Samples were then centrifuged at 15 000 x g for ten minutes. Two-hundred μL of the deproteinized lysates were added to 10mL of Bray's solution for liquid scintillation counting.

RESULTS

Chapter 1

DISRUPTION OF SULFATE TRANSPORT BY α HEMOLYSIN

I. Binding of $[^3H_2]$DIDS to the rabbit erythrocyte membrane.

Exposure of intact rabbit erythrocytes to $[^3H_2]$DIDS resulted in the labelling of two polypeptides. A major peak of radioactivity appeared at 90 000 daltons, corresponding to band 3 in Coomassie Blue stained SDS electrophoresis gels (figure 3). A much smaller peak appeared at approximately 40 000 daltons. The small peak contained less than 5% of the $[^3H_2]$DIDS.

II. Effect of DIDS and SITS on SO_4^{2-} exchange.

The effects of DIDS and SITS on SO_4^{2-} efflux in human erythrocytes are shown in figure 4. The covalently binding inhibitor, DIDS, inhibited the rate of exchange 98.5% at a concentration of 195μM. SITS inhibited the rate of exchange 82.6% at the same concentration (figure 4).

III. Effect of heat inactivated hemolysin on SO_4^{2-} release.

Heat inactivated α hemolysin can bind to erythrocytes but is not hemolytic (Maharaj and Fackrell, 1980). The binding of heat-inactivated α hemolysin to rabbit erythrocytes does not have a pronounced effect on the rate of exchange (figure 4). The rate of exchange with inactivated hemolysin bound to the membrane was only 12% less than that of the untreated cells.

IV. Effect of α hemolysin on SO_4^{2-} release.

Exposure of rabbit erythrocytes to α hemolysin increased the rate of $^{35}SO_4^{2-}$ release over that of the untreated cells (figure 5). The release of SO_4^{2-} preceeded the release of hemoglobin. This increased rate of SO_4^{2-} release in the presence of α hemolysin was assumed to be the result of both anion transport and hemolysis. Therefore, to measure only the SO_4^{2-} released that was a result of hemolysis, cells were loaded with $^{35}SO_4^{2-}$, exposed to DIDS to inhibit anion exchange, and then exposed to hemolysin. The data presented in figure 6 show that the SO_4^{2-} release, from DIDS labelled cells exposed to the hemolysin, began slowly during the prelytic lag phase and increased once hemolysis began. This observation suggests the SO_4^{2-} released during the prelytic phase was via the DIDS sensitive pathway, and was not a result of membrane damage due to the hemolysin. If the rate of SO_4^{2-} release in DIDS treated cells exposed to the hemolysin is subtracted from the rate of release from the cells not labelled with DIDS but exposed to the hemolysin, the rate of release due to transport during lysis can be obtained. When this was done little difference in the rates was observed. The rate of exchange from red cells exposed to α hemolysin and DIDS was 6.66 attomoles/cell per min whereas, the exchange rate from hemolysin treated cells was 29.3 attomoles/cell per min. The difference in the rates was 23.6 attomoles/cell per min. This difference reflects the rate of exchange through band 3 in hemolysin treated cells, and was comparable to the exchange rate of 20.7 attomoles/cell per min for untreated red cells.

V. Effect of α hemolysin on SO_4^{2-} efflux during hemolysis.

To confirm that the rate of efflux in cells about to lyse was identical to that of cells not exposed to α hemolysin, the net efflux was measured. To do this, the percentage of the initial SO_4^{2-} remaining in the cells (ie. $P - P_t / P$) was expressed as a fraction of the per cent cells unlysed and plotted versus time (figure 7). The initial $^{35}SO_4^{2-}$ content of the intact cells decreased as it did in the cells not exposed to hemolysin. However, in the later stages of hemolysis, the SO_4^{2-} content of the intact cells increased, suggesting that SO_4^{2-} accumulated in the cells.

VI. Effect of α hemolysin on influx of SO_4^{2-}.

SO_4^{2-} entered rabbit erythrocytes exposed to α hemolysin at a rate double that of the unexposed cells (figure 8). This entry occurred during the prelytic lag period and stopped once hemolysis began.

Figure 3

SDS polyacrylamide gel electrophoretogram and labelling profile of rabbit
erythrocyte membrane proteins of erythrocytes labelled with 10μM
[^{3}H$_2$]DIDS.
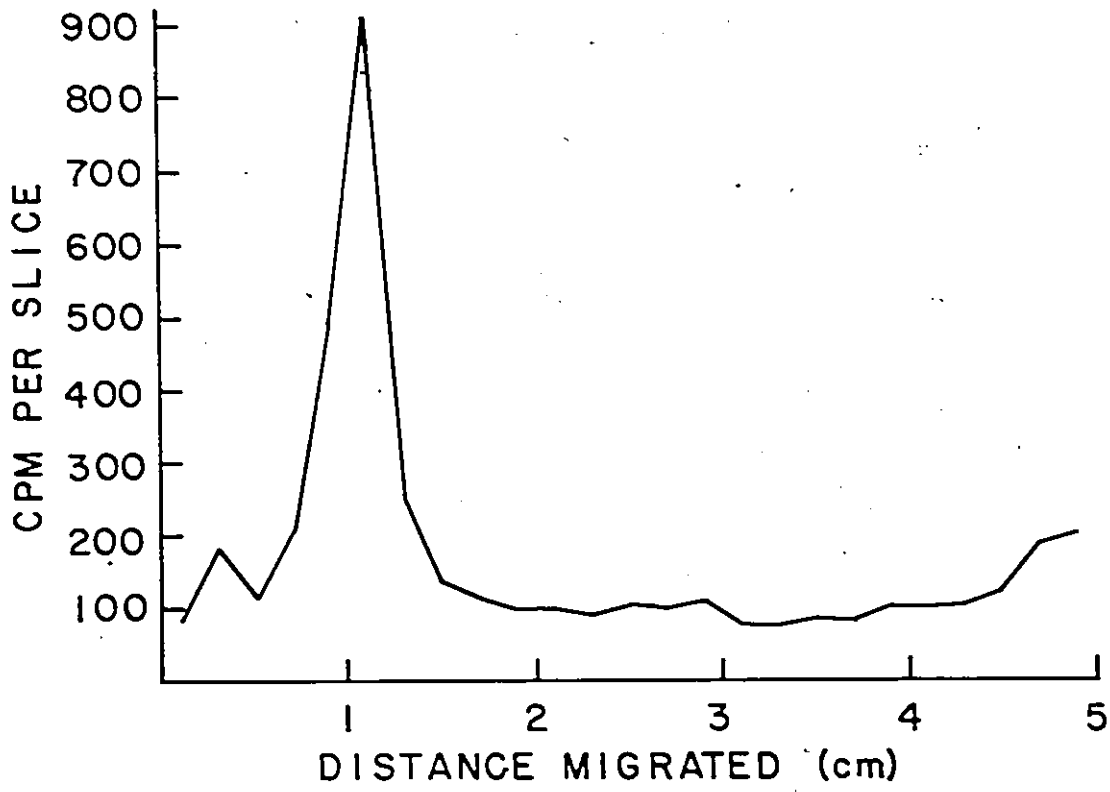
Figure 4

Sulfate efflux from rabbit erythrocytes. ▲ control cells,

○ cells exposed to 195μM DIDS, ◑ 195μM SITS, △ heat inactivated

α hemolysin (equivalent to 2 H.U. active hemolysin).
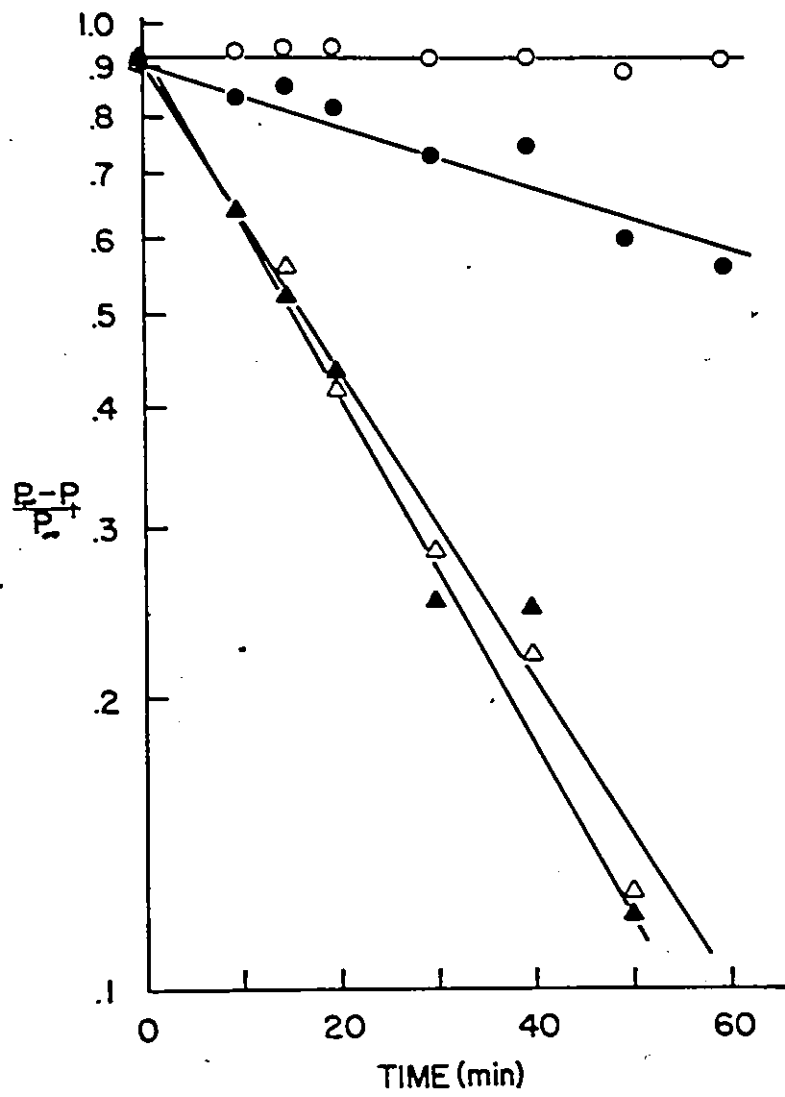
Figure 5

Release of SO_4^{2-} and hemoglobin from toxin–treated and untreated cells. Toxin–treated cells were exposed to 2 H.U. of toxin in the flux medium. O SO_4^{2-} release toxin–treated cells, △ hemoglobin release toxin–treated cells, ● SO_4^{2-} release untreated cells, ▲ hemoglobin release untreated cells.
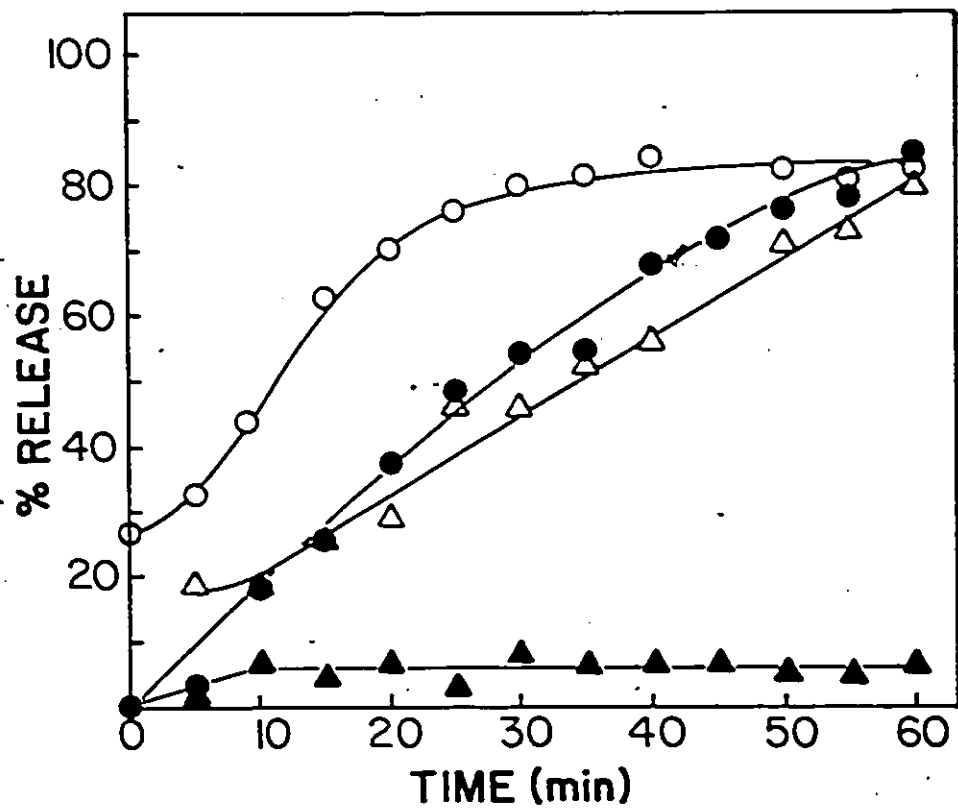
Figure 6.

Loss of SO_4^{2-} from cells exposed to α hemolysin in media with DIDS $\triangle$, and without DIDS $\bigcirc$. In both cases, the flux medium contained 2 H.U. of α hemolysin.
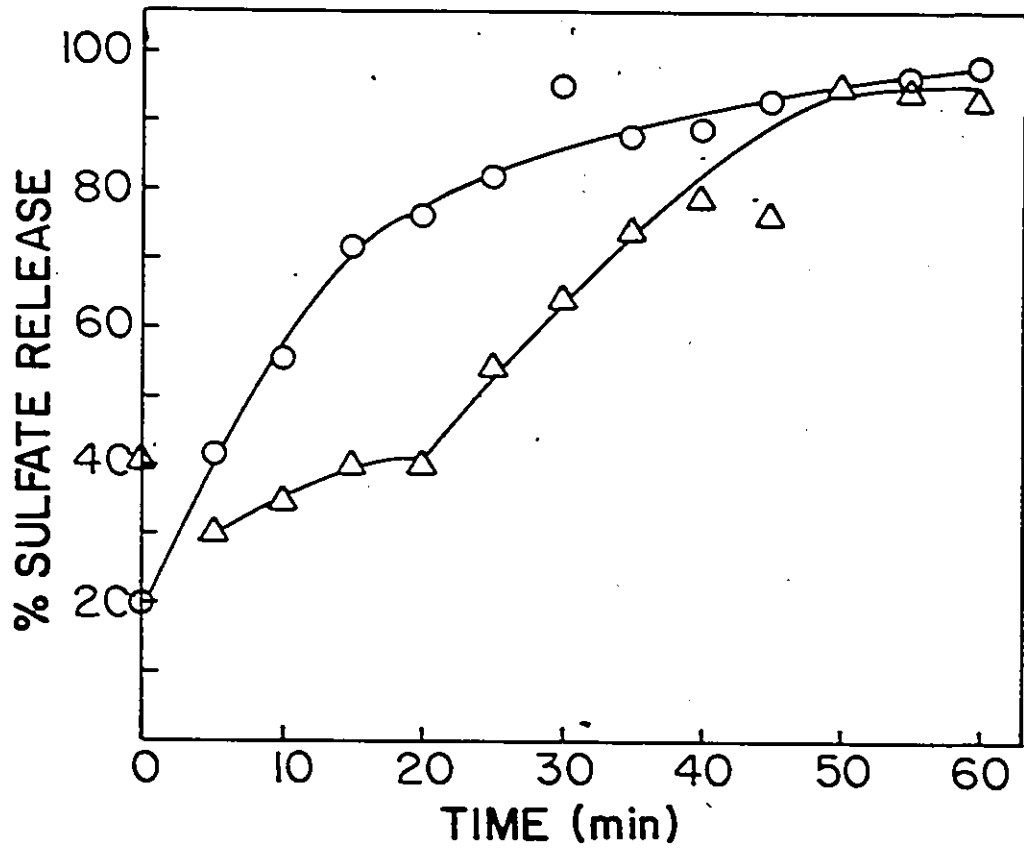
Figure 7.

Residual SO_4^{2-} in intact erythroctyes upon exposure to α
hemolysin. Data taken from figure 3. The residual SO_4^{2-} per cell $\bigcirc$
was calculated by the formula:

$$(P_\infty - P_t) / (100 - \%lysis) \; P_\infty$$

Values for % lysis $\triangle$ were obtained from hemolglobin measurements.
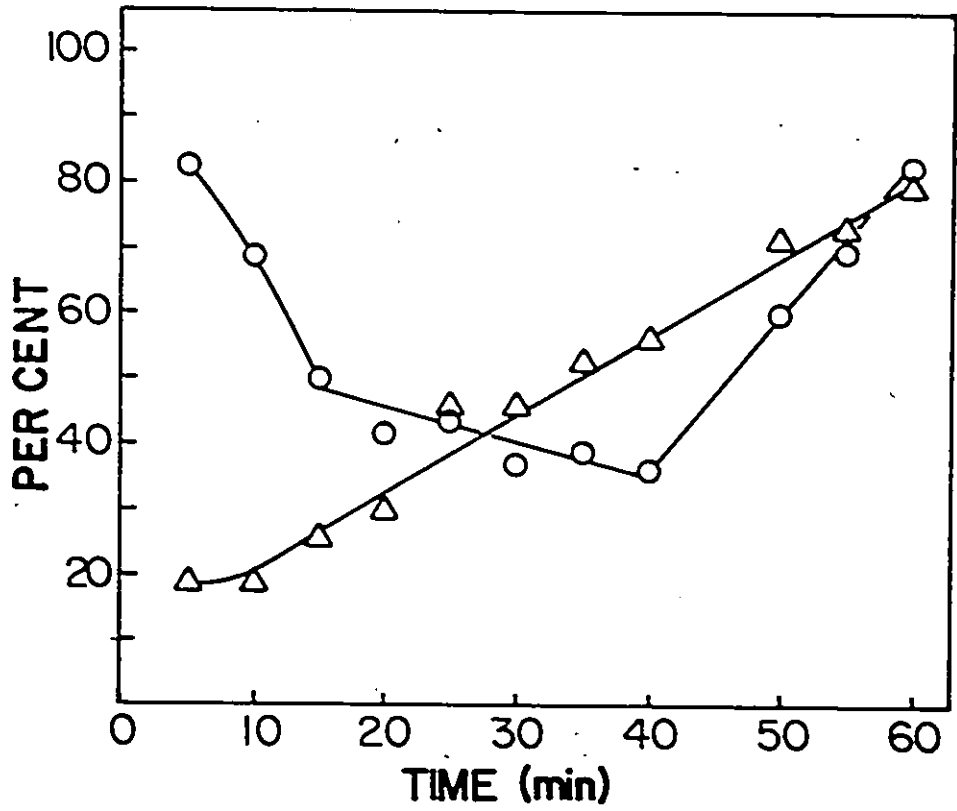
Figure 8.

Influx of SO_4^{2-} into toxin-treated and untreated erythrocytes. $\bigcirc$ SO_4^{2-} influx of untreated cells. $\triangle$ SO_4^{2-} influx of toxin-treated cells. $\bullet$ hemoglobin release of untreated cells. $\blacktriangle$ hemoglobin release of toxin-treated cells.
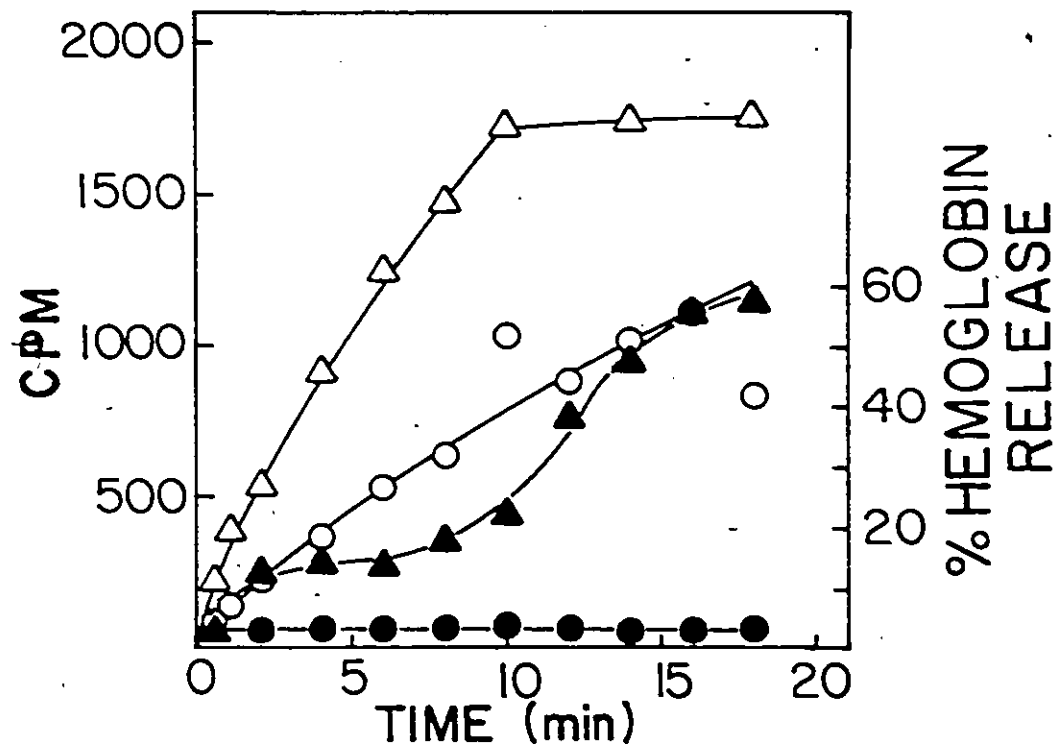
Chapter 2

EXPANSION OF RABBIT ERYTHROCYTES DURING

THE PRELYTIC LAG PHASE

I. Measurement of $HCO_3^-Cl^-$ exchange.

Addition of 20 μL of 0.2 M sodium succinate (pH 6.0) to 3ml of a

0.13% suspension of rabbit erythrocytes in 150 mM NaCl caused a decrease in

light scattered by the suspension. The decrease in scattered light was

inhibited by addition of micromolar concentrations of SITS, demonstrating the

involvement of anion transport in the swelling process. To eliminate the

possibility that the decrease in scattered light was a result of some type of

non—osmotic hemolysis, the pH of the suspension of swollen cells was raised by

the addition of 25 μL of 1M NaOH. Raising the pH of the suspension

immediately increased the amount of scattered light, due to shrinkage of the

erythrocytes. If the decrease in scattered light was a result of hemolysis,

rather than expansion, increasing the pH of the suspension would not cause

shrinkage of the erythrocytes, and the light scatter would not increase.

Addition of NaOH to a suspension of erythrocytes caused an increase in

scattered light, which indicated shrinkage of the cells (figure 9).

Preincubation of erythrocytes with SITS prevented shrinkage (figure 10).

Addition of SITS after NaOH stopped shrinkage immediately (figure 11).

II. Expansion caused by α hemolysin.

Addition of α hemolysin to a 0.13% suspension of rabbit erythrocytes
in 150 mM NaCl or PBS caused the erythrocytes to expand, as demonstrated by
the decrease in scattered light (figure 12). Kinetic assays of expansion
recorded by an Apple II+ computer show the presence of a brief lag phase
before expansion. This was unlike the expansion caused by HCO_3^-, which
occurred immediately on acidification of the suspending medium. The
expansion occurred before the first cells began to lyse, the swelling of the
cells then passed into hemolysis. The rate of decrease in scattered light did
not change when hemolysis began, making it impossible to distinguish when
swelling stopped and lysis began. To overcome this problem, hemolytic kinetic
assays were run in parallel with the expansion kinetic assays. Heat denatured
α hemolysin (toxoid) did not cause the erythrocytes to expand. Prior
exposure of rabbit erythrocytes to SITS did not affect swelling caused by
α hemolysin (figure 13).

III. Effect of furosemide on expansion.

Labelling of rabbit erythrocytes with furosemide, an inhibitor of passive
cation fluxes, increased the rate of swelling of erythrocytes caused by
α hemolysin. Time to 50% swelling decreased linearly when plotted
against the logarithm of the furosemide concentration from furosemide
concentrations of $78 \mu M$ to 5 mM (figure 14).

Figure 9.

Shrinkage of rabbit erythrocytes caused by addition of NaOH, to give a final concentration of 3.3 μM. Erythrocytes were suspended in unbuffered media (150 mM NaCl).
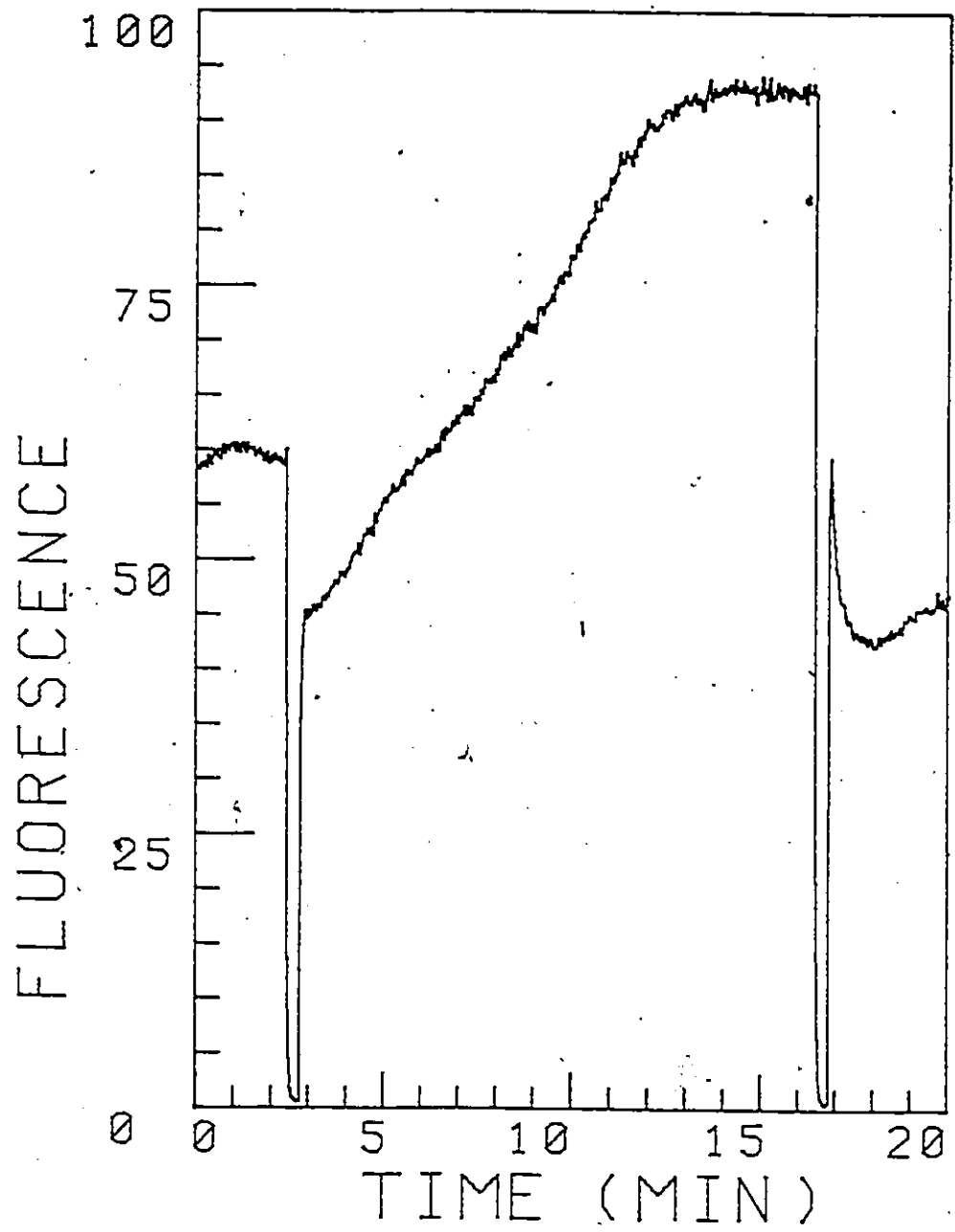
Figure 10.

Inhibition of shrinkage of rabbit erythrocytes by SITS before addition of NaOH. Erythrocytes were preincubated in 150 mM NaCl with 300 μM SITS for one minute. NaOH was then added to give a final NaOH concentration of 3.3 μM.
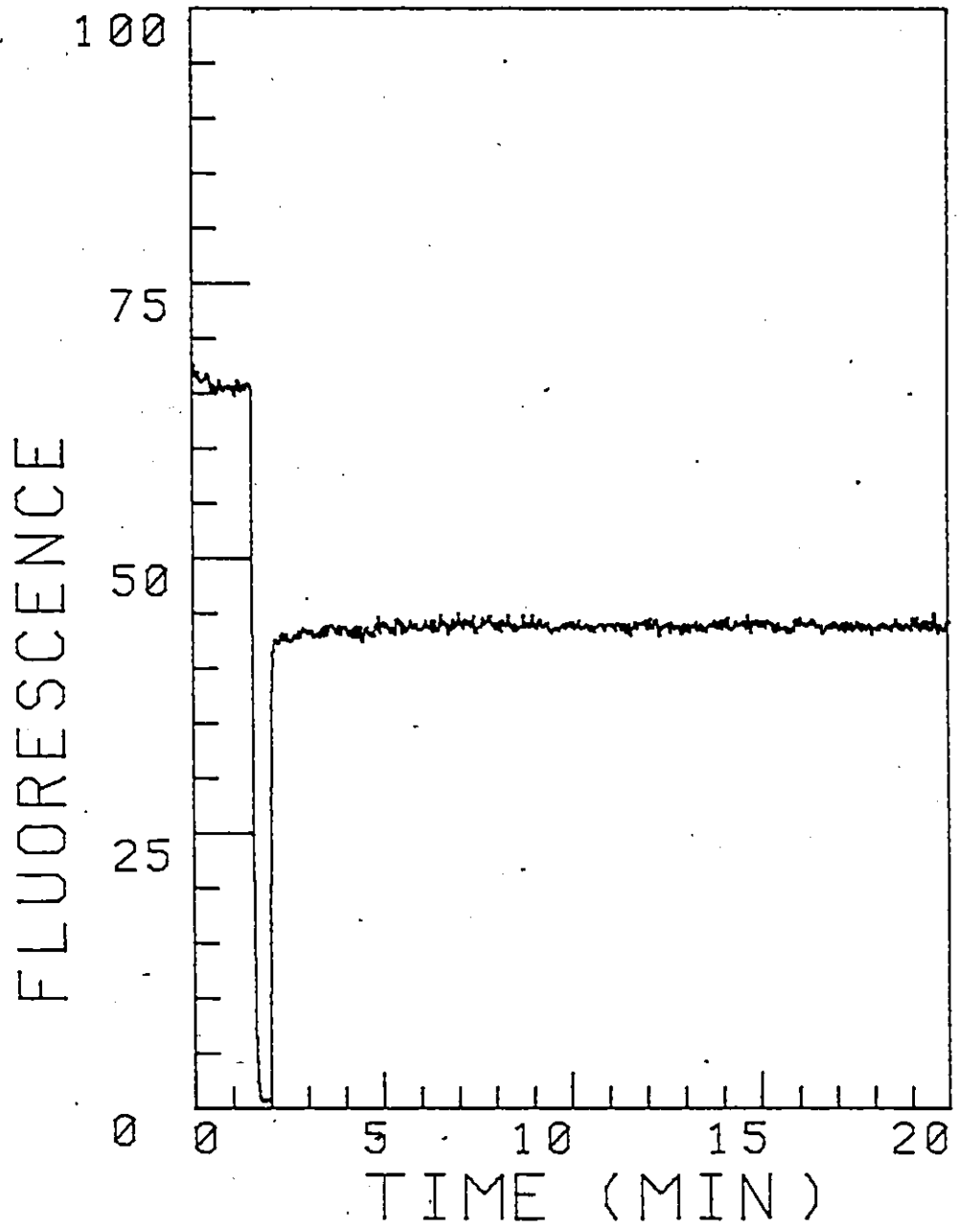
Figure 11.

Inhibition of shrinkage by SITS after addition of NaOH. Same as Figure 10, except SITS added after NaOH.
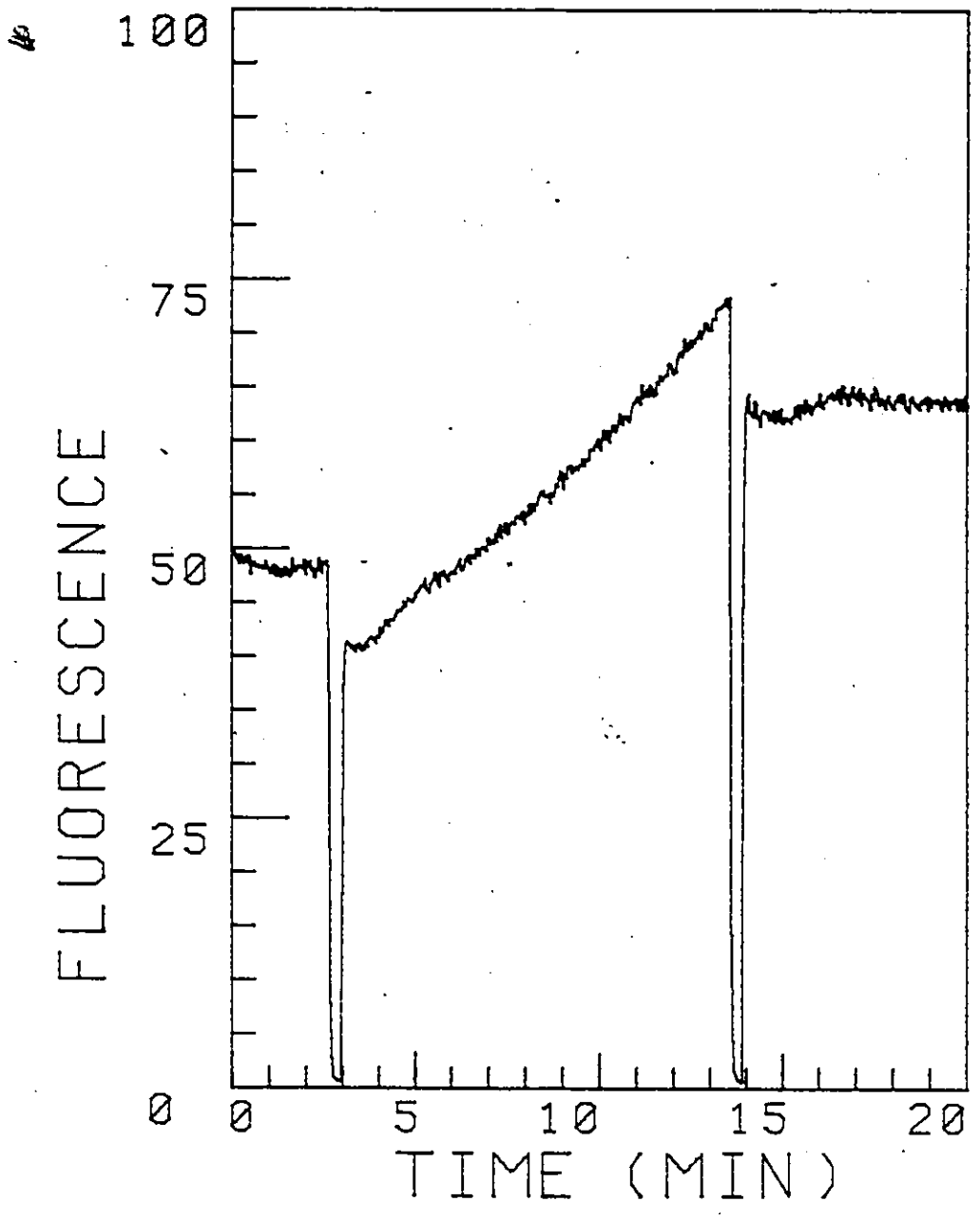
Figure 12.

Expansion of rabbit erythrocytes caused by α hemolysin.
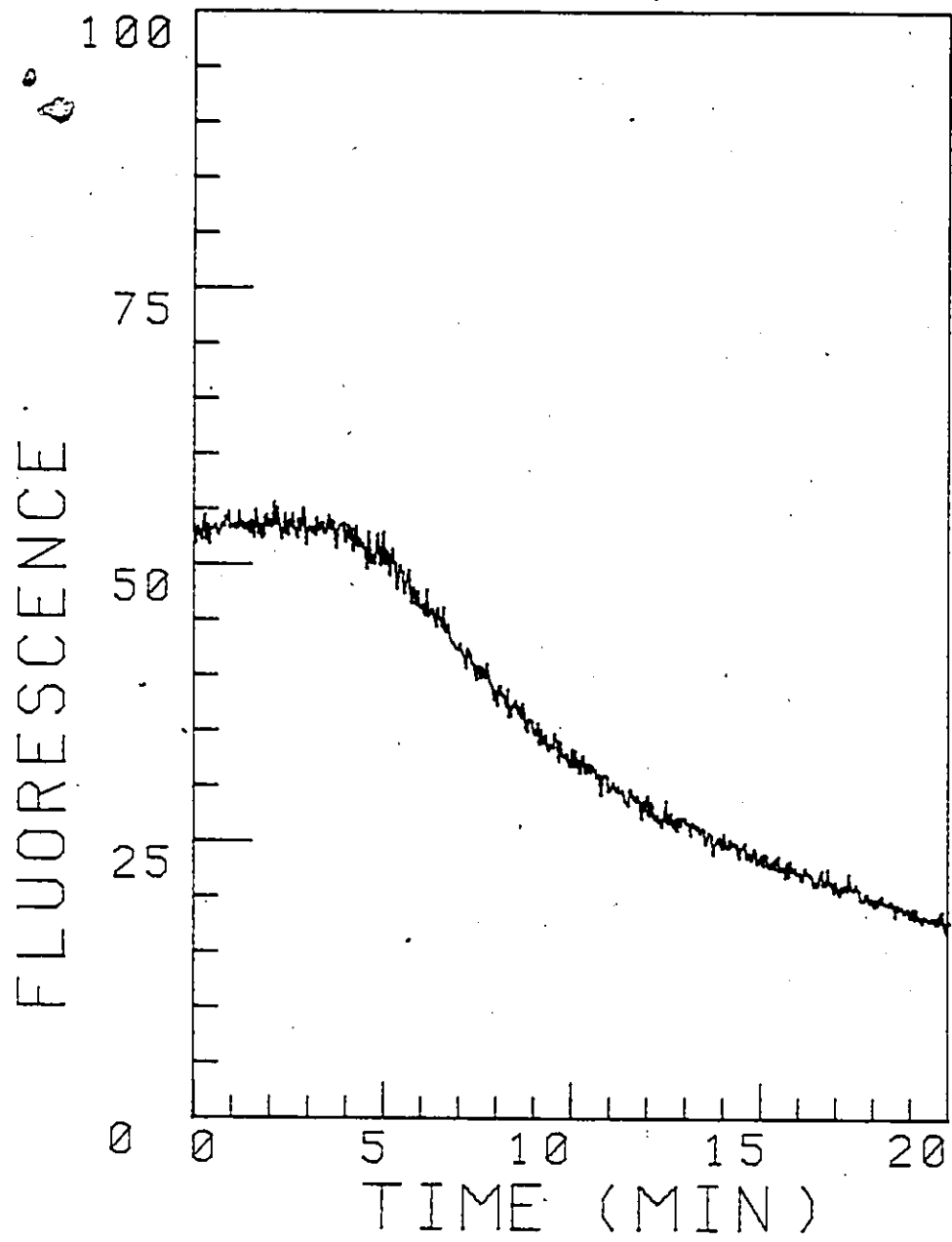
Figure 13.

Expansion of rabbit erythrocytes caused by α hemolysin in buffer containing SITS.
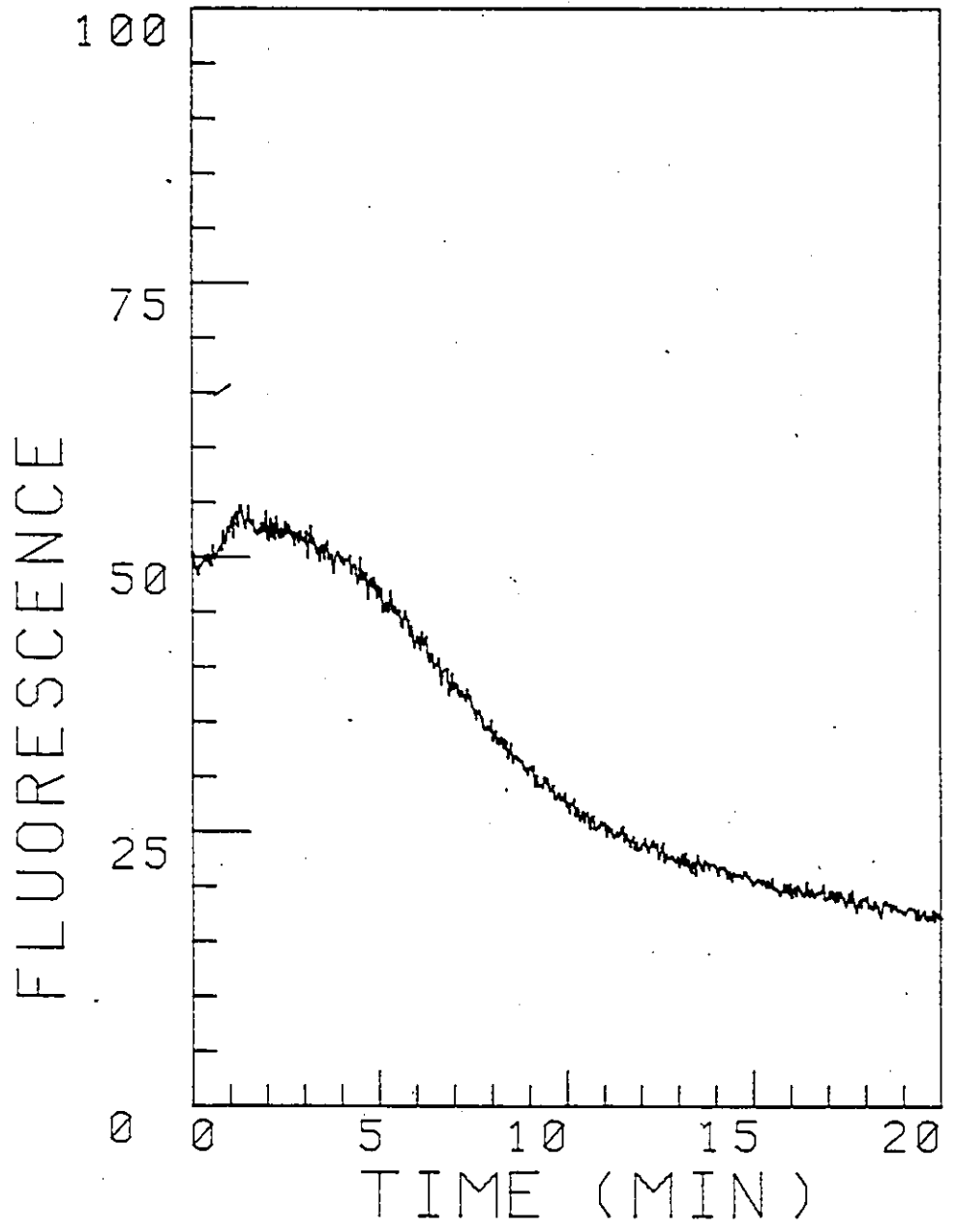
Figure 14.

Effect of furosemide on the time required for erythrocytes exposed to α hemolysin to reach 50% of their final expansion volume.
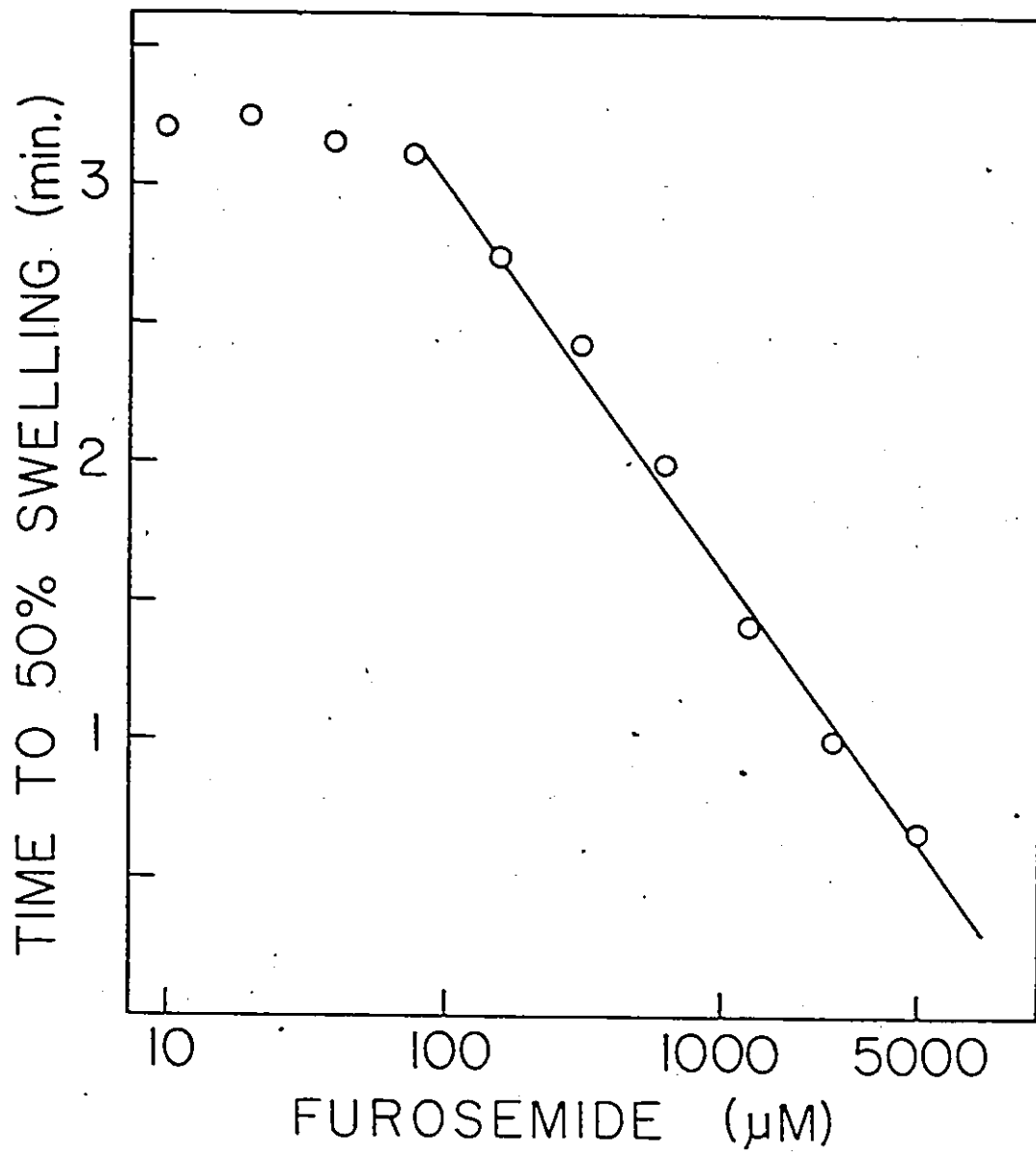
CHAPTER 3

STIMULATION OF HEMOLYSIS BY SULFHYDRAL REAGENTS

I. Effect of maleimide derivatives on hemolytic kinetics.

Cytoplasmic hemoglobin and reduced glutathione act as a sink for permeant sulfhydryl reagents. To ensure saturation of the membrane sulfhydryls, concentrations of NEM greater than the concentration of total cellular sulfhydryls must be used. This was done to overcome the binding of the permeant reagent to the cytoplasmic sulfhydryls. The amount of NEM bound to rabbit erythrocyte membrane proteins at each concentration of NEM used to label intact cells and membranes is shown in figure 15. Similar curves were obtained by plotting both the time to 50% lysis, or total cells lysed, versus concentration of NEM (figure 16). At concentrations less than 7mM, NEM reduced both the number of cells remaining intact after hemolysis and the time to 50% lysis. At concentrations greater than 7mM, this reduction continued, but not as markedly as it did at lower concentrations. The effect of NEM upon the rate of hemolysis is shown in figure 17. Eosin–5–maleimide and eosin–5–iodoacetamide each shortened the prelytic lag (figure 18) and decreased the time to 50% lysis (figure 19). Neither probe had an effect on the time to 50% lysis at concentrations below 5μM. NEM was hemolytic to the cells alone, at concentrations greater than 20 mM for 12 hour incubation periods. Neither eosin–5–maleimide or eosin–5–iodoacetamide were hemolytic themselves, at the concentrations used for the kinetic studies (to 2 mM).

II. Effect of PCMBS on hemolytic kinetics.

Preincubation of rabbit erythrocytes with PCMBS affected all the kinetic parameters. The prelytic lag, time to 50% lysis and the fraction of cells remaining intact were decreased, while the rate of lysis was increased. A stimulatory effect on the rate of lysis was observed at concentrations of PCMBS less than $20\mu M$, (figure 20). A maximum rate, 15 times faster than the untreated cells, was reached at $20\mu M$ PCMBS. At concentrations of PCMBS greater than $20\mu M$ the rate of lysis decreased with further increases in PCMBS concentration, approaching a rate approximately 5 times that of untreated cells. The time to 50% lysis decreased as the PCMBS concentration was increased to $20\mu M$, and increased at higher concentrations (figure 21). PCMBS alone did not cause hemolysis at concentrations below $100\mu M$ (figure 22).

The effect of exposure of erythrocytes to differing concentrations of PCMBS for various times, followed by exposure to α hemolysin is demonstrated in figure 23. At concentrations below $20\mu M$ the stimulatory effect of PCMBS increased with both concentration of PCMBS and incubation time. After incubation in PCMBS for 30 minutes, the stimulatory effect increased the greatest at $20\mu M$, reaching a rate about 15 times that of the untreated cells. Longer incubations at this concentration did not result in further increases in the rate of lysis. At PCMBS concentrations greater than $20\mu M$, the maximum rate of lysis obtained was approximately 9 times higher than the rate of lysis for untreated cells.

III. Effect of diamide and iodoacetamide on hemolytic kinetics.

Diamide is a permeant reagent which oxidatively crosslinks the proteins of the human erythrocyte cytoskeleton (Haest *et al*, 1977; Fischer *et al*, 1978; Kursantin-Mills and Lessin, 1981). Iodoacetamide is a sulfhydryl specific reagent with no ionic charge at pH 7. Concentrations of diamide and iodoacetamide ranging from 0 to 20mM had no effect on the rate of lysis of erythrocytes by α hemolysin.

IV. Binding of N-[ethyl-2-^{3}H]-maleimide to intact erythrocytes and erythrocyte membranes.

Incubation of either intact erythrocytes, or isolated membranes with N-[ethyl-2-^{3}H]-maleimide labelled spectrin (bands 1 and 2), band 3, and several lower molecular weight polypeptides as resolved by SDS electrophoresis (figures 24 and 25). When intact erythrocytes were incubated with N-[ethyl-2-^{3}H]-maleimide, the label bound to proteins known to be exposed only to the cytoplasmic side of the membrane, indicating that N-[ethyl-2-^{3}H]-maleimide crossed the membrane.

V. Binding of eosin-5-maleimide to intact erythrocytes.

Eosin fluorescence was quantitated in the electrophoresis gels by densitometer scans of photographic negatives (figure 26). Approximately 84% of the eosin fluorescence co-migrated with band 3 in the coomassie-blue stained gels. Four other proteins were labelled with eosin-5-maleimide and show faintly in the photograph (figure 26). The large fluorescent band at the bottom of the gel is free eosin-5-maleimide (Nigg and Cherry, 1979).

VI. Distribution of bound eosin–5–maleimide after proteolysis of membrane proteins.

Intact erythrocytes exposed to 200 μg/ml chymotrypsin did not release a fluorescently labelled polypeptide (figure 27B), but did possess fluorescent polypeptides of molecular weights 95 000 daltons and 60 000 daltons (figure 27A). Exposure of ghosts derived from eosin–5–maleimide labelled erythrocytes to chymotrypsin resulted in the appearance of three low molecular weight polypeptides (less than 20 000 daltons) labelled with eosin–5–maleimide (figure 27C). No fluorescent polypeptide was released from chymotrypsin treated ghosts (figure 27D). Exposure of ghosts derived from eosin–5–maleimide labelled erythrocytes to trypsin also did not release a labelled polypeptide (figure 27F), but resulted in the appearance of three labelled polypeptides of molecular weights less than 20 000 daltons (figure 27E).

VII. Binding of eosin–5–maleimide to erythrocyte membranes.

Several polypeptides were labelled when ghosts were exposed to eosin–5–maleimide (figure 28). This indicates that eosin–5–maleimide can bind to the sulfhydryl groups of the proteins on the cytoplasmic side of the membrane, if it is allowed to cross the membrane.

VIII. Use of eosin–5–maleimide to detect a proteolytic fragment of band 3 in membranes of rabbit erythrocytes lysed by α hemolysin:

Exposure of rabbit erythrocytes, labelled with eosin–5–maleimide, to α hemolysin caused the appearance of an additional labelled polypeptide (figure 29). This polypeptide could not be detected in coomassie blue stained electrophoresis gels of α hemolysin treated erythrocytes. Selective labelling

of band 3 increased the contrast between the proteolytic fragment and the additional membrane polypeptides. The new polypeptide appears as a broad fluorescent band with a molecular weight between 50 000 and 60 000 daltons.
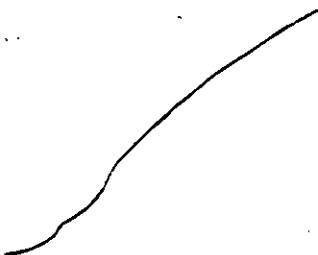
Figure 15.

Binding of [³H]NEM to membranes of intact rabbit erythrocytes ▲ and to isolated rabbit erythrocyte membranes △ .
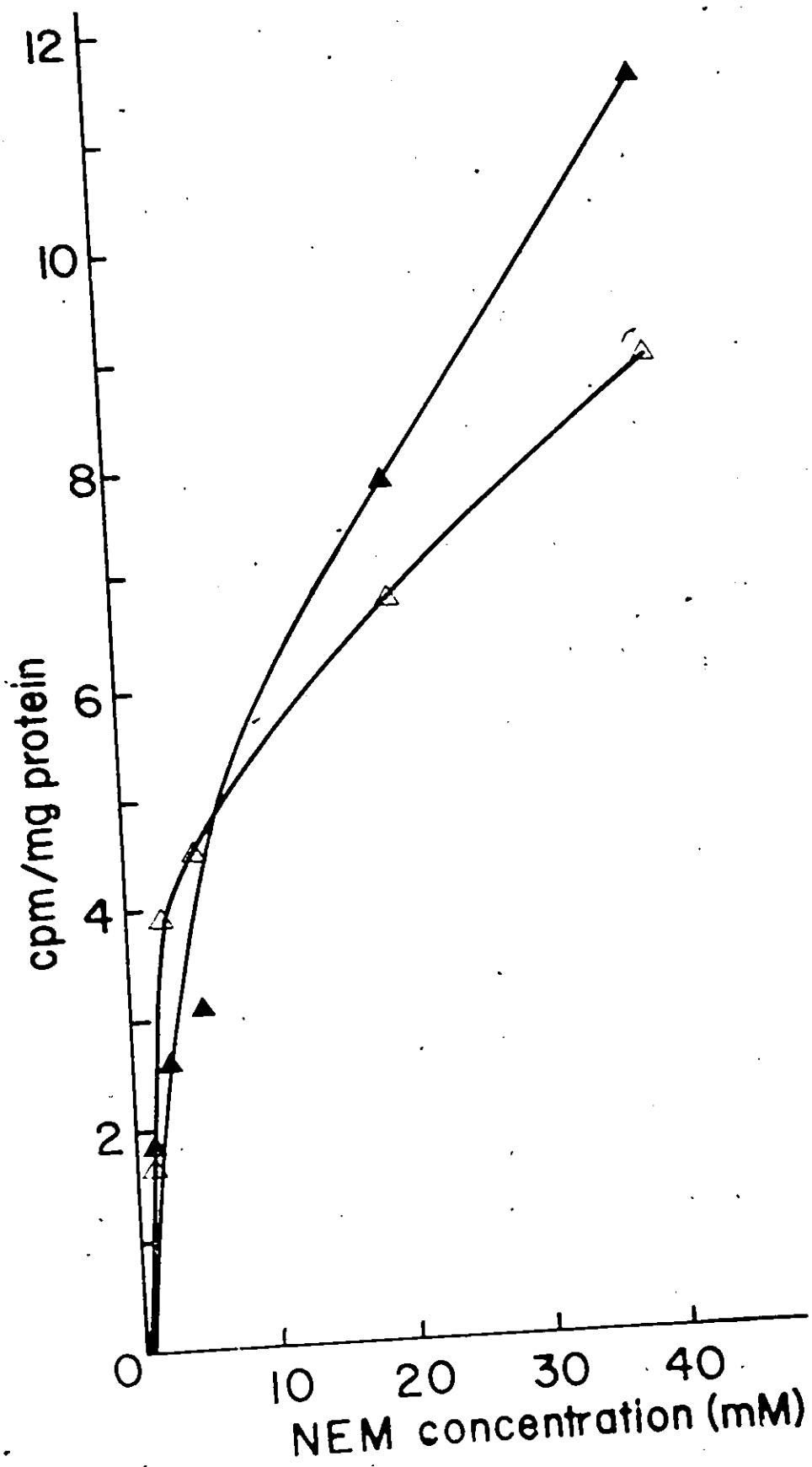
Figure 16.

Effect of N–ethylmaleimide upon lysis of rabbit erythrocytes by α
hemolysin. Erythrocytes were incubated with various concentrations of N–
ethylmaleimide for one hour at room temperature. The cells were then exposed
to α hemolysin. ▲ total cells lysed (baseline) ▨ time to 50% lysis.
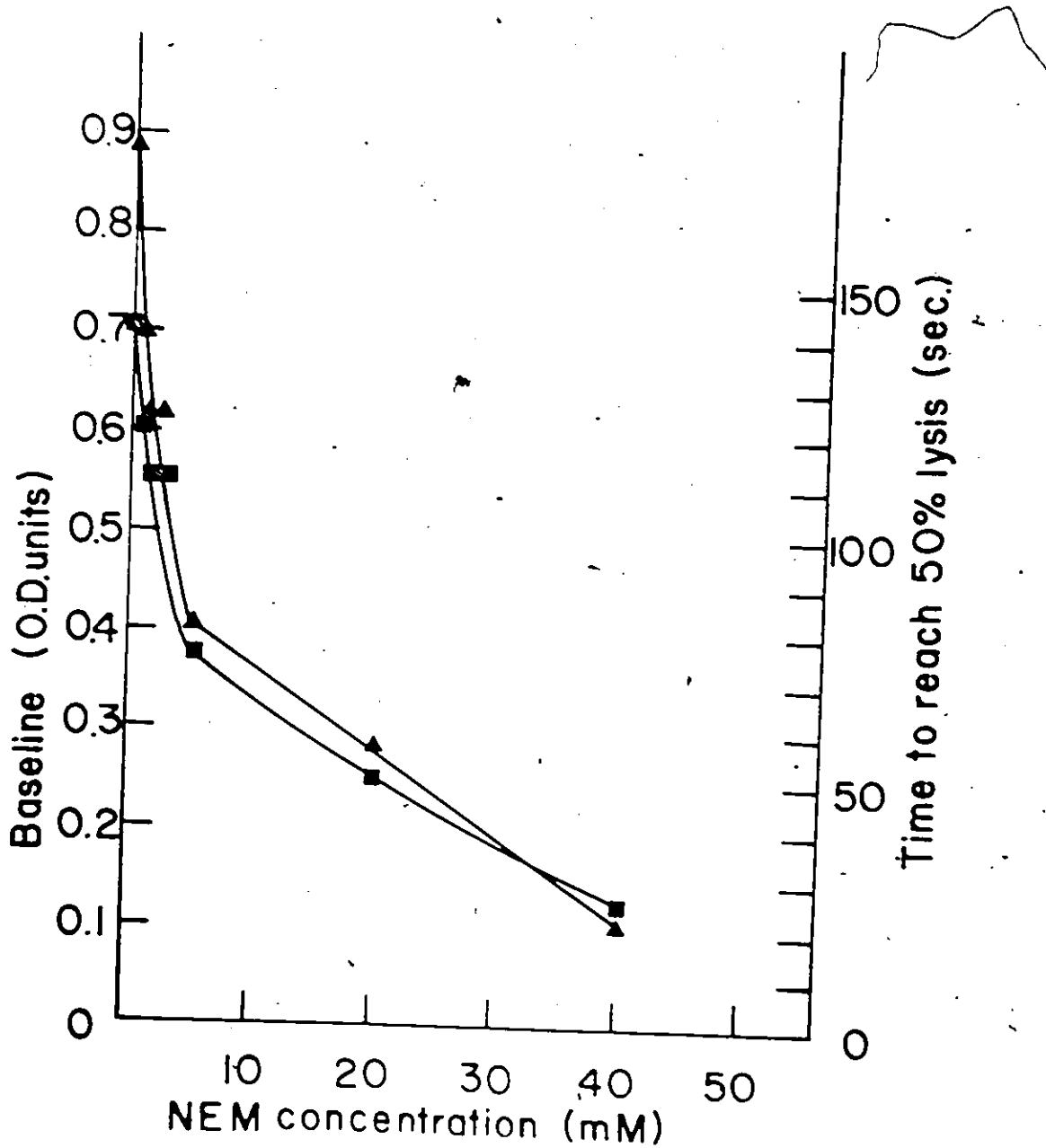
Figure 17.

Effect of N—ethylmaleimide on the rate of lysis of rabbit erythrocytes by α hemolysin.
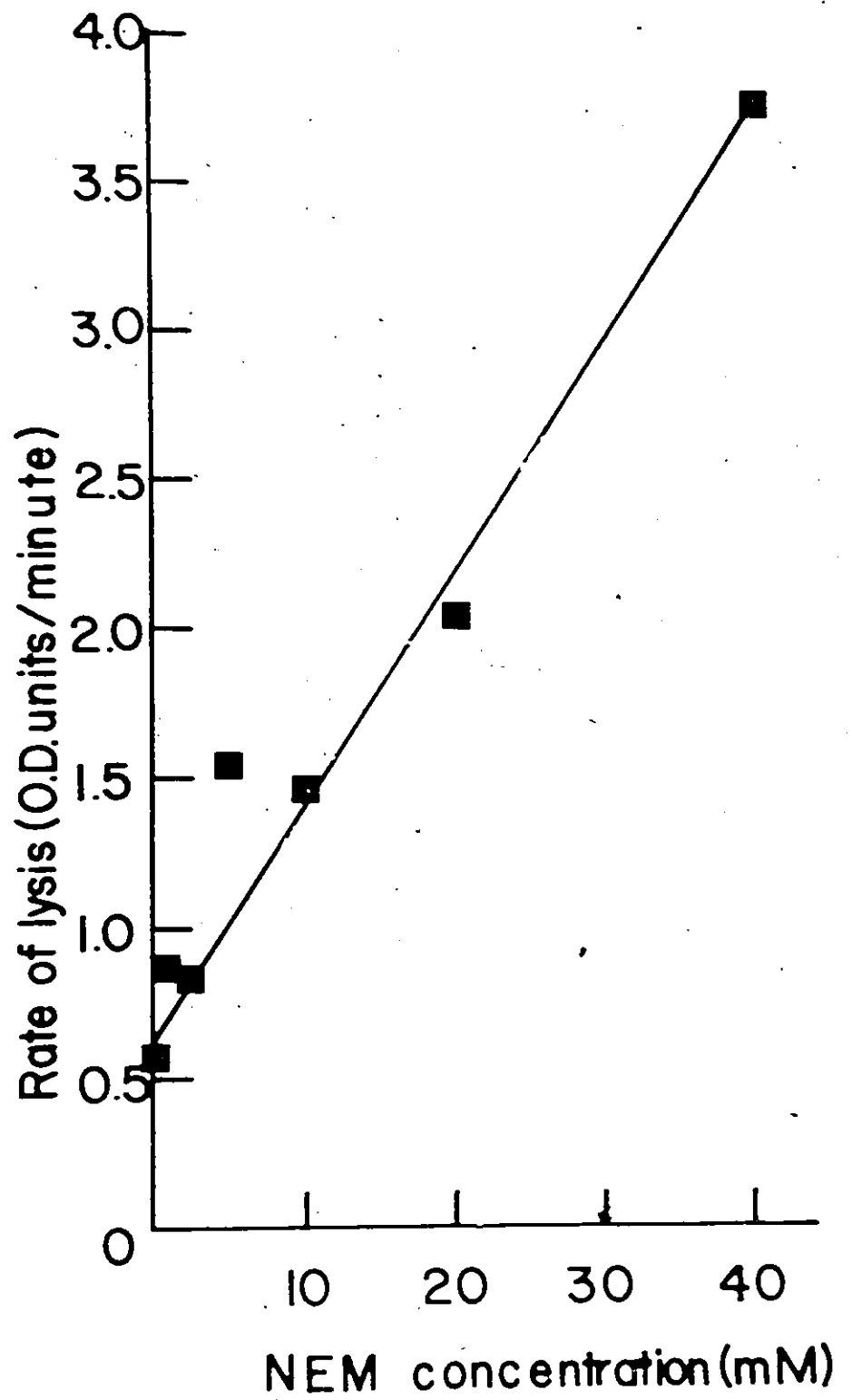
Figure 18.

Effect of eosin-5-maleimide $\bigcirc$ and eosin-5-iodoacetamide $\triangle$ on the length of the prelytic lag phase.
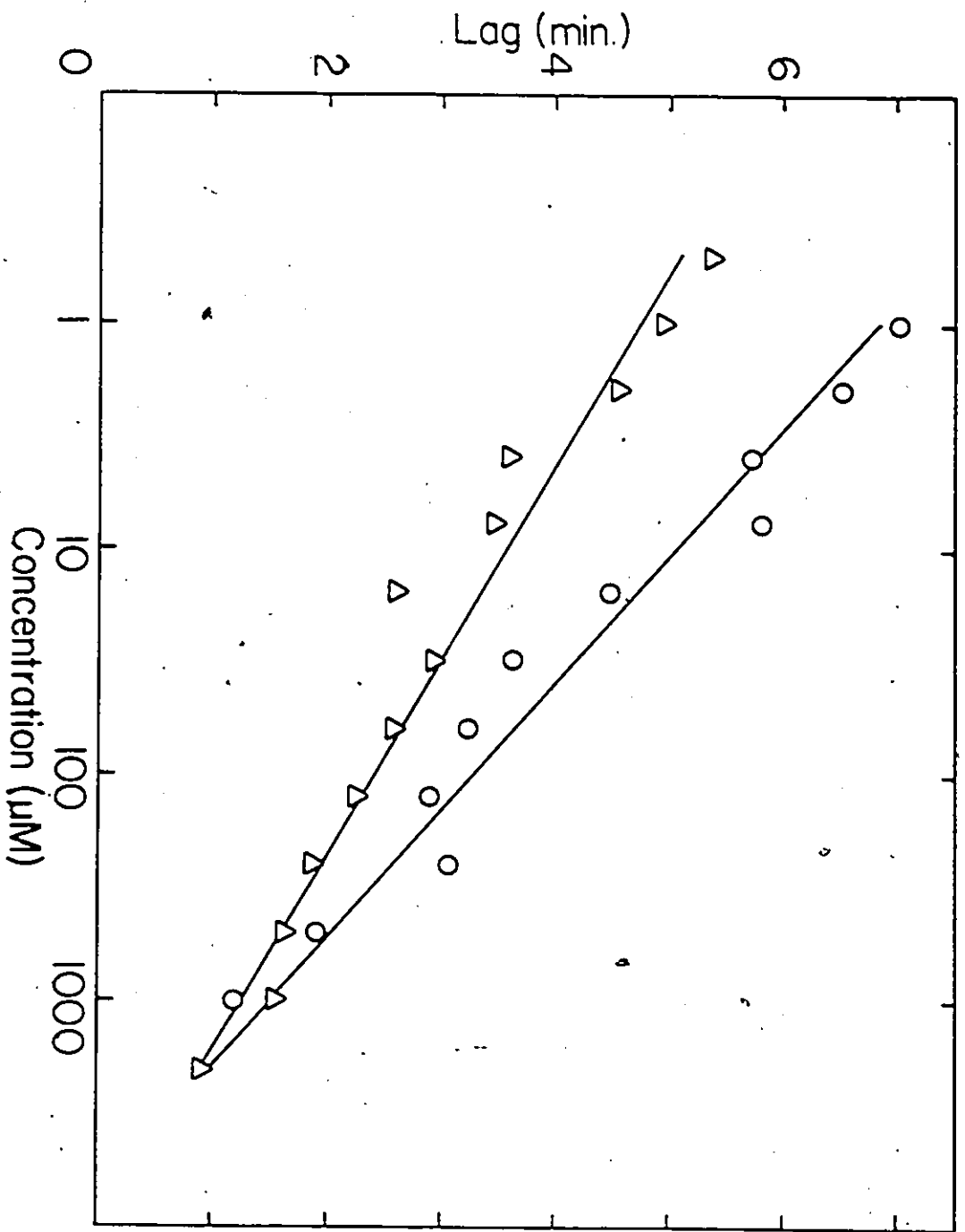
Figure 19

Effect of eosin–5–maleimide ◯ and eosin–5–iodoacetamide △ on the time required to reach 50% hemolysis.
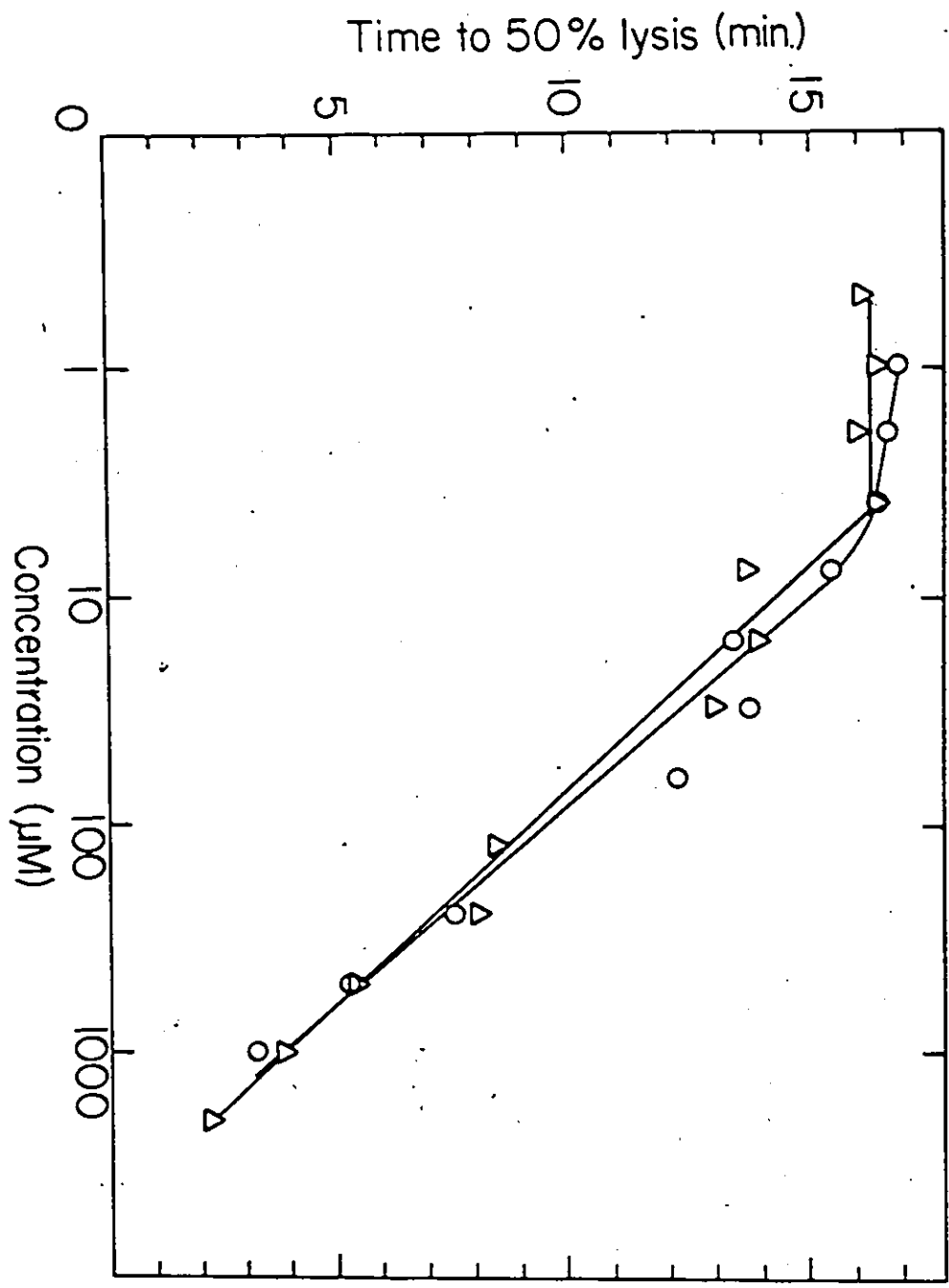
Figure 20.

Stimulatory effect of PCMBS acid upon the rate of lysis of rabbit
erythrocytes exposed to α hemolysin.
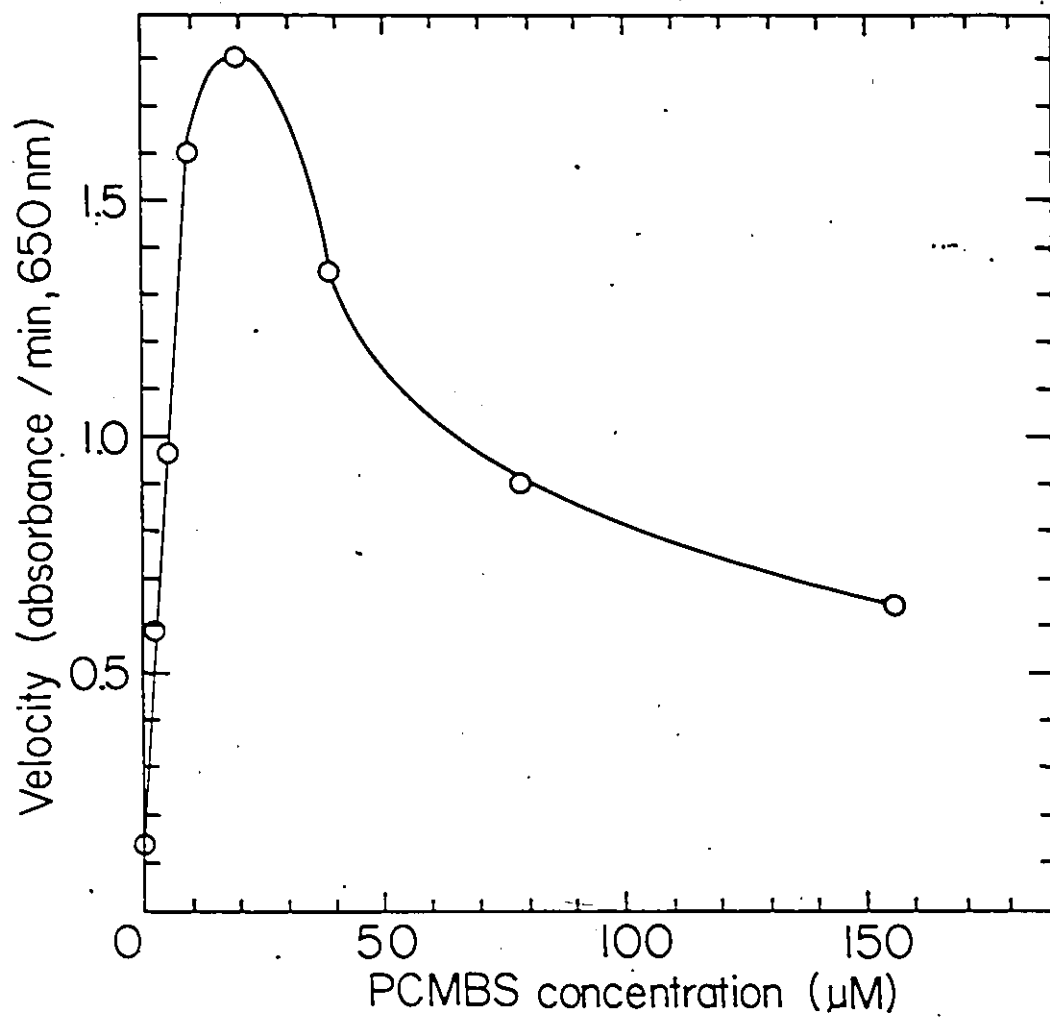
Figure 21

Effect of PCMBS upon the time required to reach 50% hemolysis.
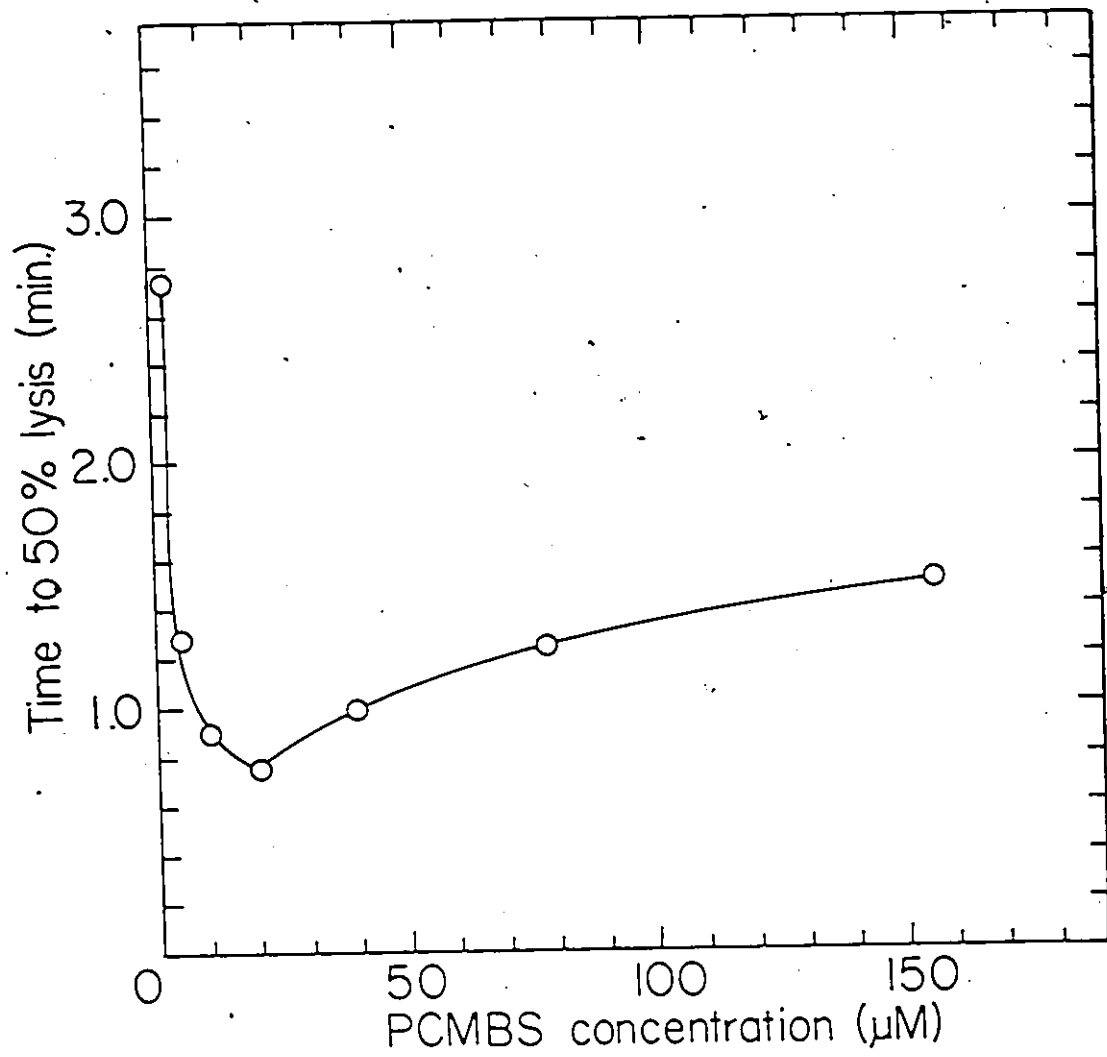
Figure 22.

Hemolysis of rabbit erythrocytes caused by PCMBS alone.
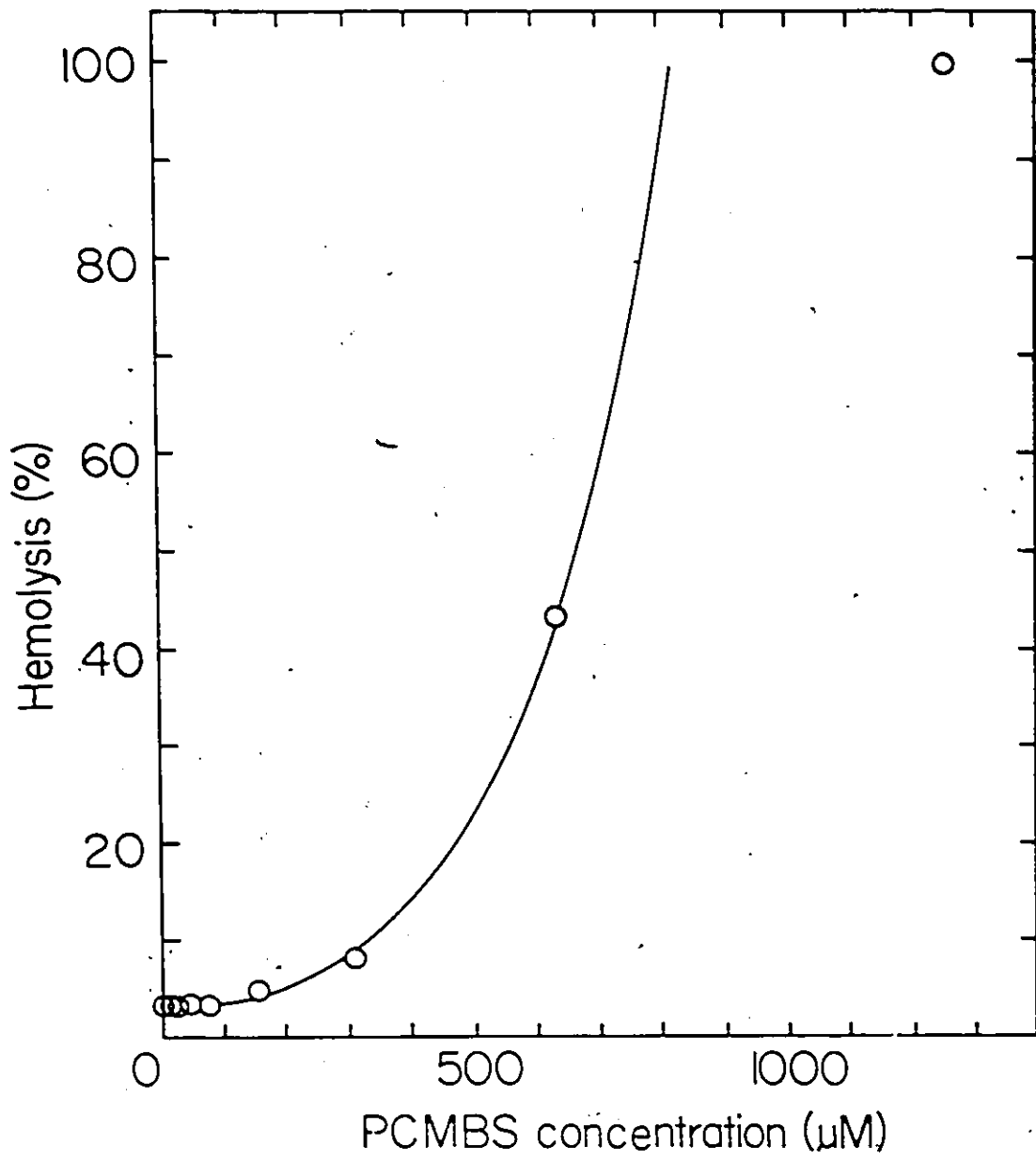
Figure 23.

Effect of length of exposure of rabbit erythrocytes to PCMBS upon rate of
lysis. Erythrocytes were incubated at room temperature in PBS containing
various concentrations of PCMBS. At the required time interval, these
erythrocytes were exposed to α hemolysin and the rate of lysis of the
suspension was recorded.

● 2.4 μM   PCMBS
■ 5 μM   PCMBS
▲ 20 μM   PCMBS
○ 39 μM   PCMBS
□ 78 μM   PCMBS
△ 156 μM PCMBS
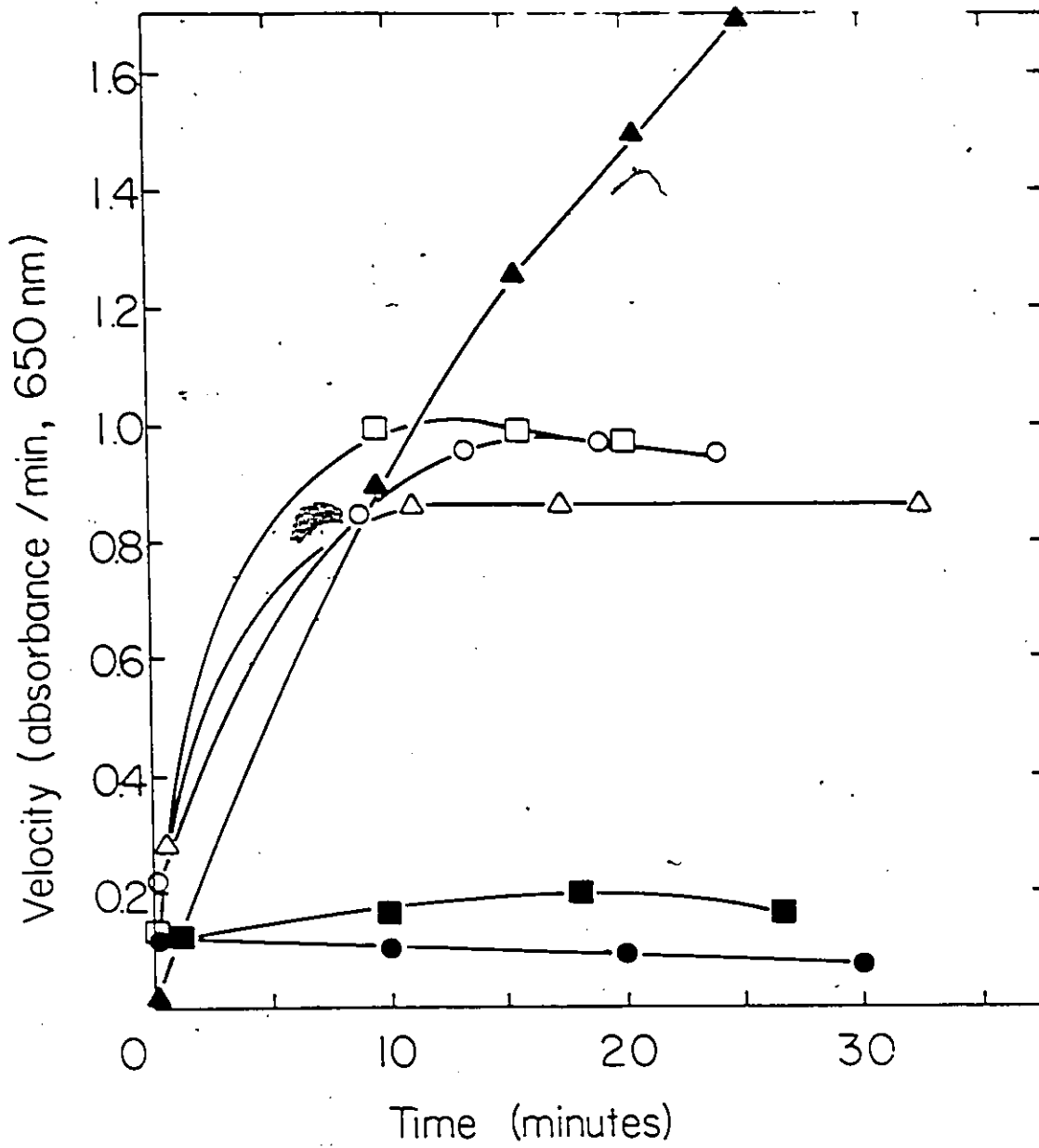
Figure 24.

Polyacrylamide gel electrophoretogram of N–[ethyl–2–^{3}H]–maleimide labelled rabbit erythrocyte membranes proteins. Intact erythrocytes were exposed to 40mM .
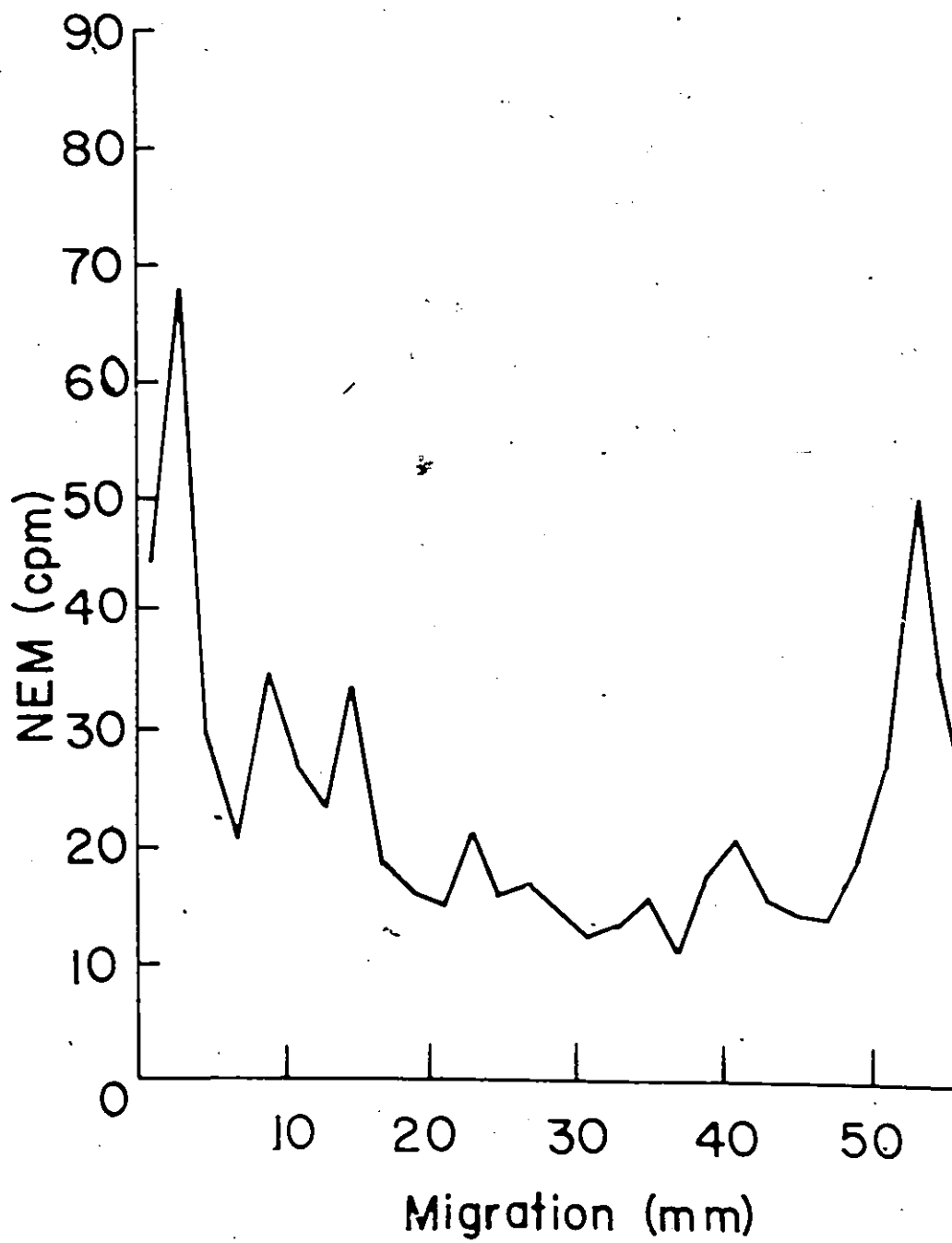
Figure 25

Polyacrylamide gel electrophoretogram of N–[ethyl–2–^{3}H]–maleimide

labelled rabbit erythrocyte membrane proteins. Isolated erythrocyte membranes

were exposed to 40mM N–[ethyl–2–^{3}H]–maleimide
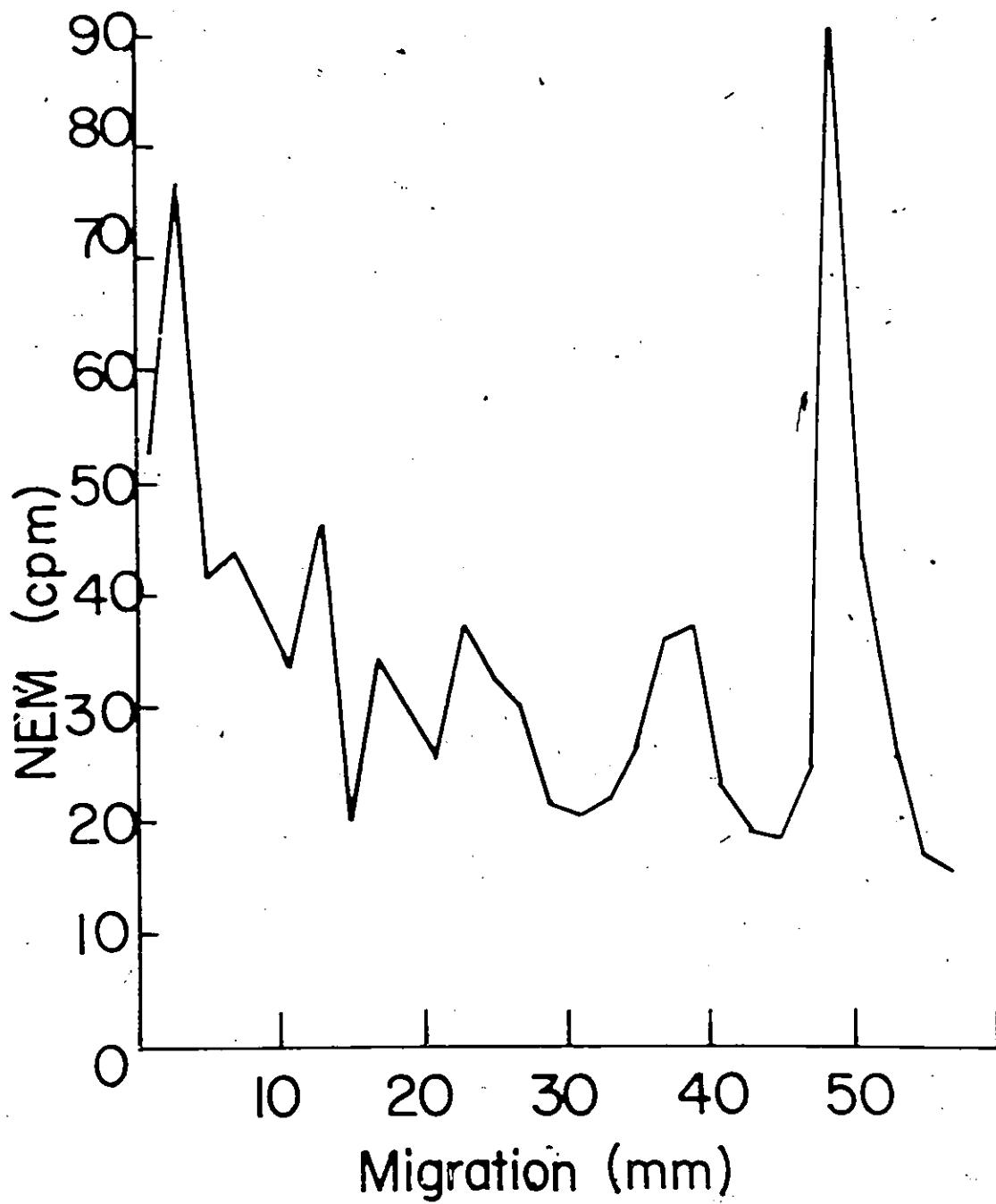
Figure 26.

Polyacrylamide gel electrophoretograms of the membrane proteins of
rabbit erythrocytes exposed to eosin–5–maleimide. Band 3 is visible as
the bright band approximately one–quarter of the way down the gel. The
additional bands are marked by arrows. The densitometer scan of the
photographic negative of the gel is shown next to the gel.
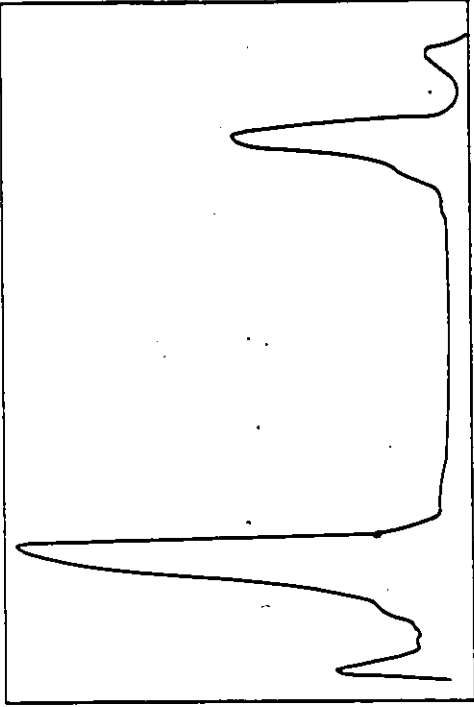
Fluorescence intensity

Migration

Figure 27.

Polyacrylamide gel electrophoresis patterns after degradation of labelled proteins with trypsin and chymotrypsin. A: chymotrypsin digestion of intact erythrocytes (pellet); B: chymotrypsin digestion of intact erythrocytes (supernatant); C: chymotrypsin digestion of ghosts (pellet); D: chymotrypsin digestion of ghosts (supernatant); E: trypsin digestion of ghosts (pellet); F: trypsin digestion of ghosts (supernatant).
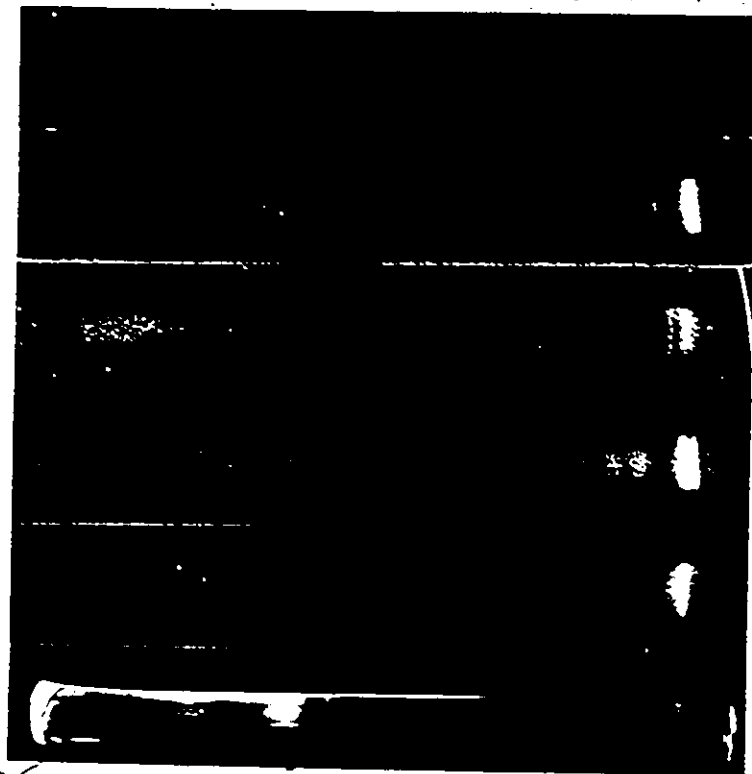
A B C D E F

Figure 28.

Polyacrylamide gel electrophoretograms of rabbit erythrocyte ghosts labelled with eosin–5–maleimide. The label appears in several polypeptides. The predominance of the label at the top of the gel suggests the formation of complexes upon the addition of eosin–5–maleimide.

Figure 29.

Polyacrylamide gel electrophoretogram of hydrolysed band 3. Intact erythrocytes were labelled with eosin-5-maleimide, then lysed by α hemolysin. The membranes were isolated, solubilised, and electrophoresed. Polypeptide bands containing eosin-5-maleimide were detected by fluorescence.
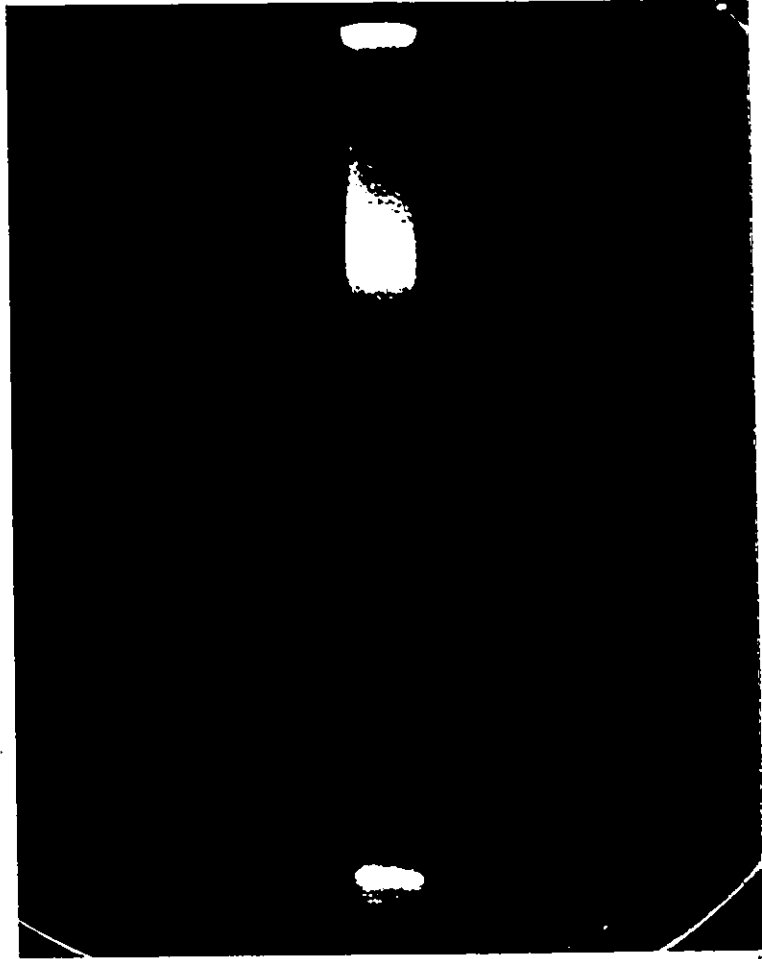
CHAPTER 4

CALCULATION OF THE NUMBER OF "HITS"
REQUIRED TO LYSE ONE ERYTHROCYTE

A typical kinetic curve of intact cells versus time, can be separated into

two rates of lysis. This separation was made more apparent when the first

derivative of the rate of lysis was plotted versus time (figure 30). The

presence of two rates of hemolysis suggests either one complicated mode-of-

action of α hemolysin, or two more simple mechanisms, whose rates are first

order with respect to the hemolysin concentration. If the latter is assumed,

the number of "hits", by monomers or oligomers of α hemolysin, required

to lyse one rabbit erythrocyte can be calculated. Detailed theoretical

analyses of the number of hits needed to lyse a cell were developed by Mayer

(1961a, b) and Inoue *et al* (1976).

The first rate of lysis began when the prelytic lag ended, and eventually

decreased, causing a brief period during which lysis stopped, or slowed. The

rate of lysis then increased, and this rate continued until all the erythrocytes

were lysed, in conditions of excess hemolysin. Hemolysis reached completion

without all cells being lysed when the hemolysin concentration was the limiting

factor.

If the logarithm of the proportion of the cells lysed (y) was taken

when the first rate ended, and before the second rate began, and was plotted

against the logarithm of the amount of α hemolysin added to the cells, a

straight line was obtained (figure 31). The slope of this line was one, that is

the fraction of cells lysed is directly proportional to the amount of α

hemolysin added. Therefore one hit was required to lyse one erythrocyte .

If the logarithm of the non–lysed erythrocytes $(1-y)$, after the first rate, was plotted versus the amount of α hemolysin, a single hit survival curve was obtained (figure 32), with a y–intercept of 0.982. If the logarithm of the non–lysed erythrocytes, after the second rate, was plotted versus the amount of α hemolysin, a typical multi–hit survival curve was obtained. Extrapolation of the linear part of the curve to the ordinate gave a y–intercept of 11.3. This shows that one hit was required for the first rate, and approximately 11 hits were required for the second rate.

When a von Krogh plot (von Krogh, 1916; Kabat, 1961) was used to analyze the same data, slopes of 0.912 and 0.204 were obtained (figure 33). These slopes represent the reciprocal of the number of hits required to lyse one erythrocyte ($1/n$ in the von Krogh equation). The respective values for n for the first and second rates were 1.10 and 4.90. Again, one hit is suggested for the first rate, and more than one hit for the second rate.

When the logarithm of the amount of α hemolysin was plotted against log log $1/(1-y)$ for the first rate, the slope of the line was one (figure 34). This was similar to the plot of logarithm α hemolysin versus the logarithm of the proportion of cells lysed, and also suggests one hit was required to lyse one erythrocyte.

Figure 30.

The upper graph represents a typical kinetic curve obtained from
monitoring the absorbance of a suspension of erythrocytes exposed to α
hemolysin. The lower graph is a plot of the first derivative values of each of
the data points of the upper graph versus time. When this is done a plot of
rate of lysis versus time is obtained.

HEMOLYTIC KINETICS PROGRAM

DEGREE OF SMOOTHING OF RAW DATA = 17

DEGREE OF SMOOTHING OF 1-ST DERIVATIVE = 5
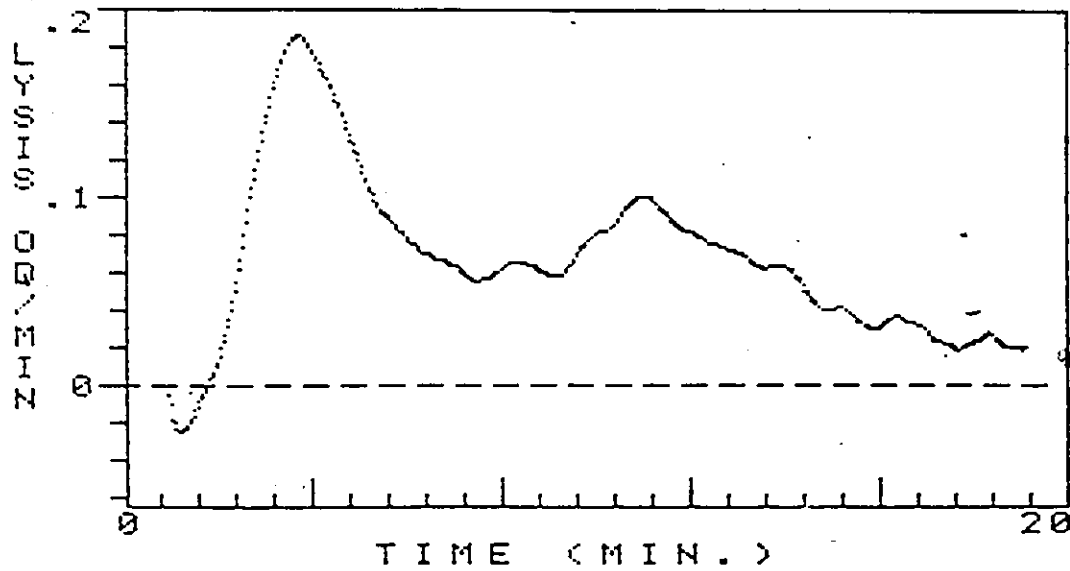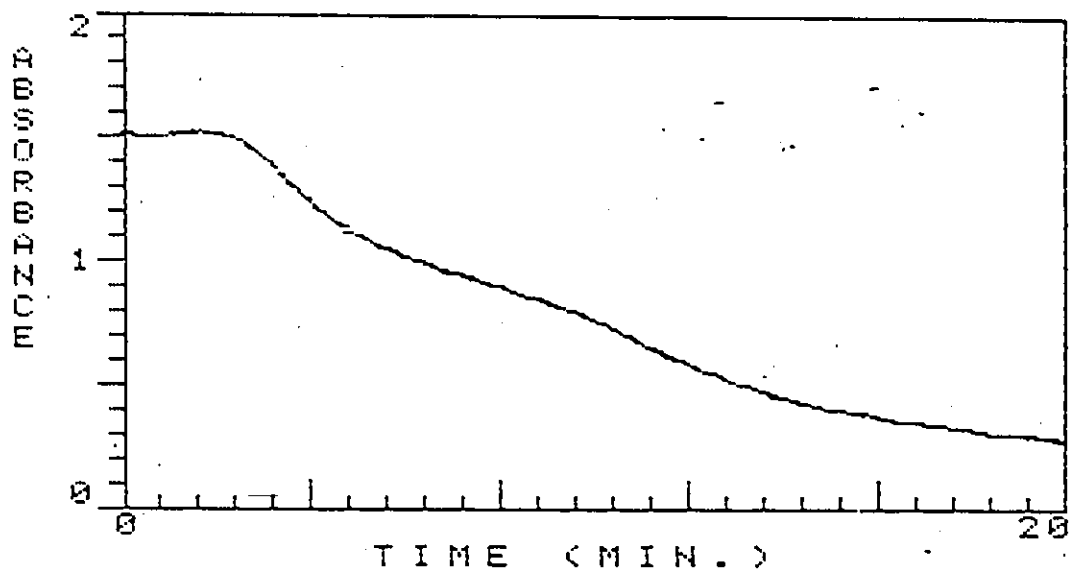
Figure 31.

Fraction of lysed cells plotted against volumes of α hemolysin added.
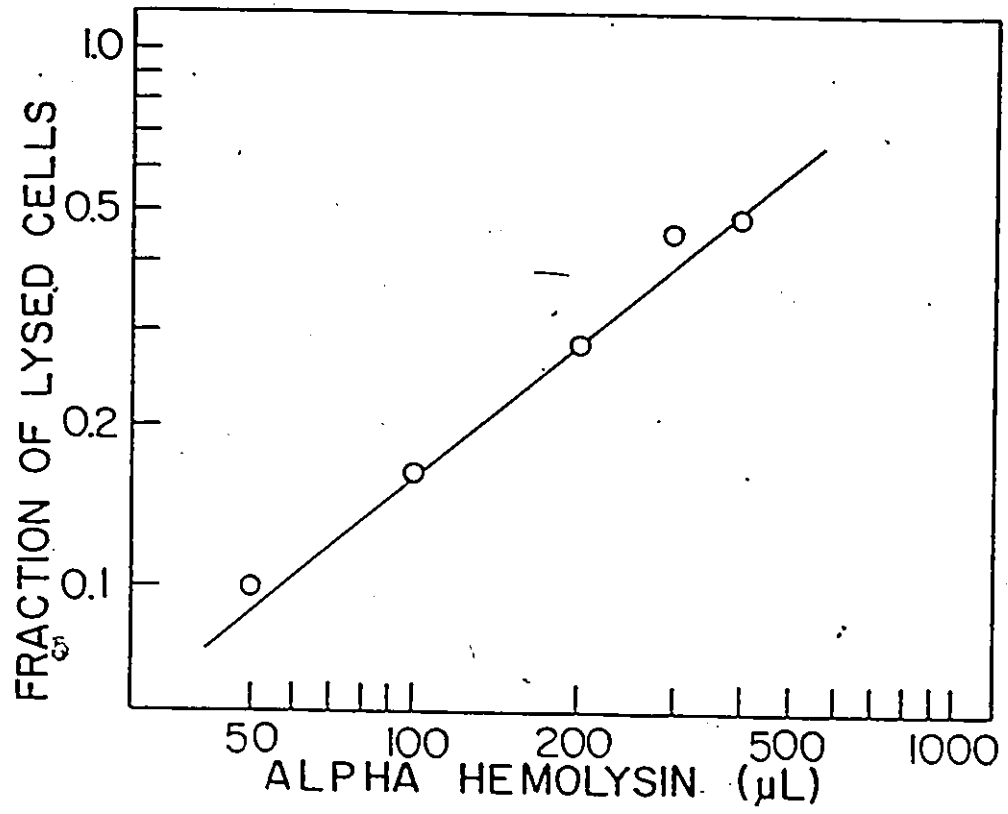
Figure 32.

Fraction of intact cells plotted against volumes of α hemolysin
added.

ALPHA HEMOLYSIN (μL)

100   200   300   400

FRACTION OF INTACT CELLS

1.0   0.5   0.2
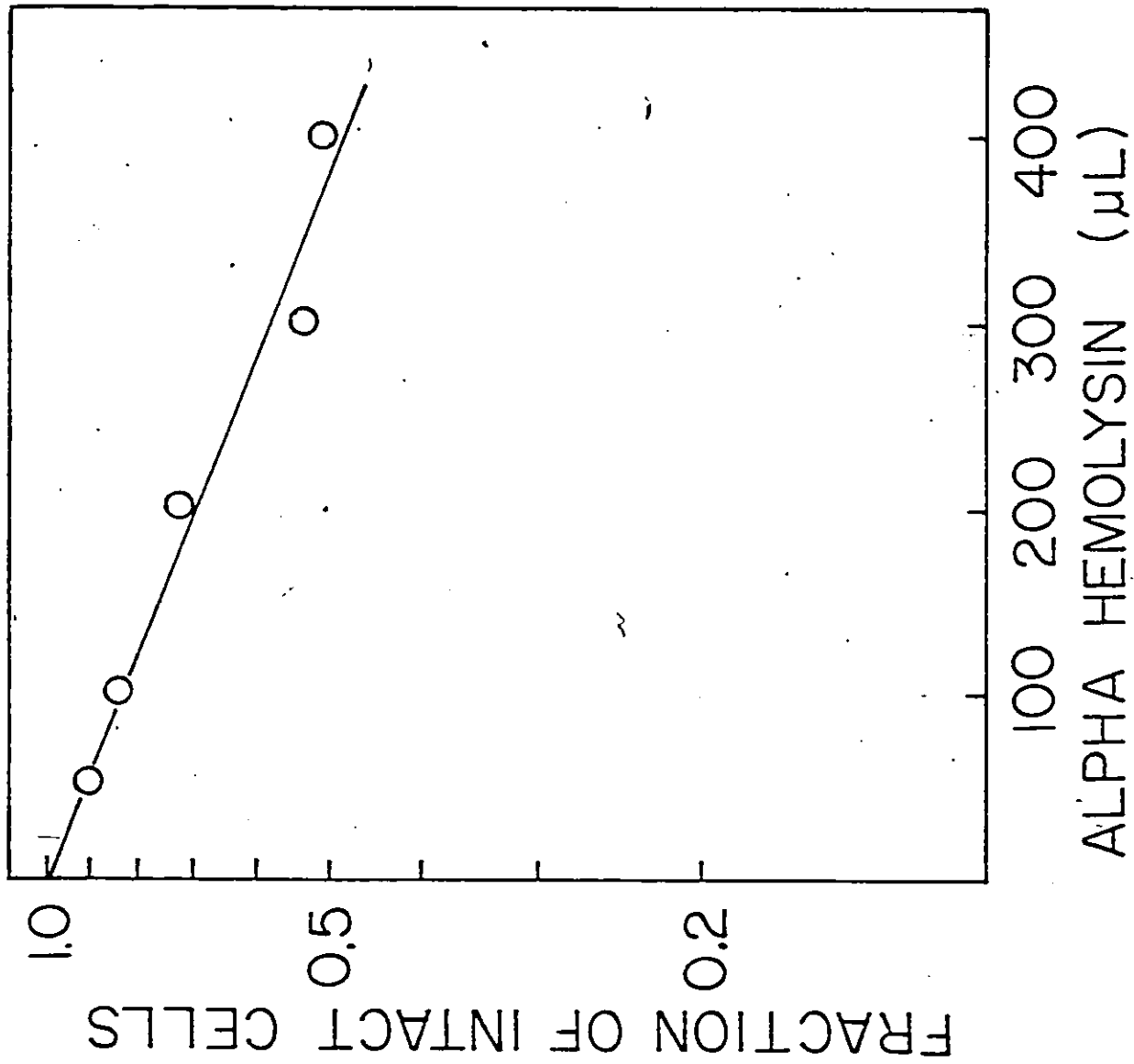
Figure 33.

Volume of α hemolysin versus (fraction of lysed cells / 1– fraction
of lysed cells)
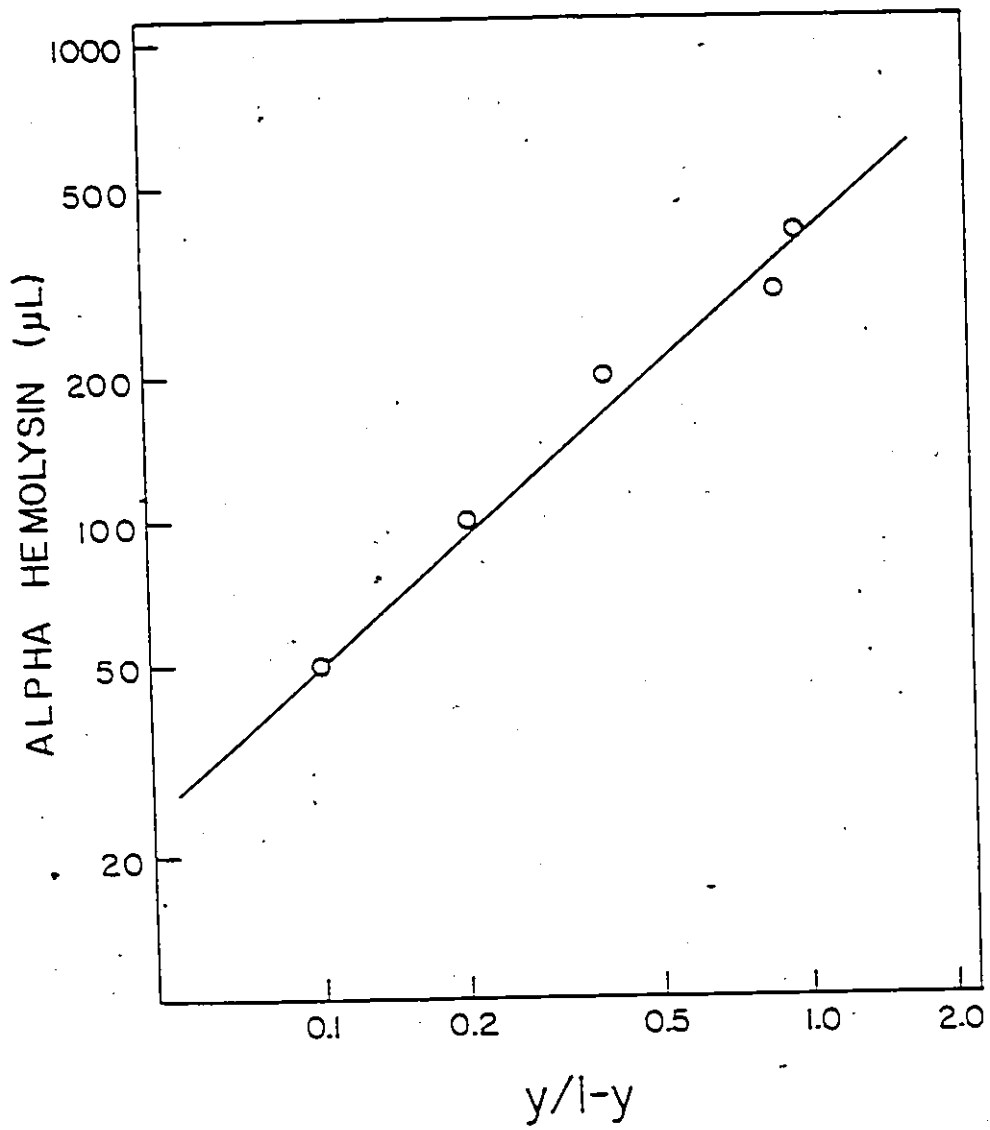
Figure 34.

log (fraction of lysed cells / 1- fraction of lysed cells) versus volume of α hemolysin added.
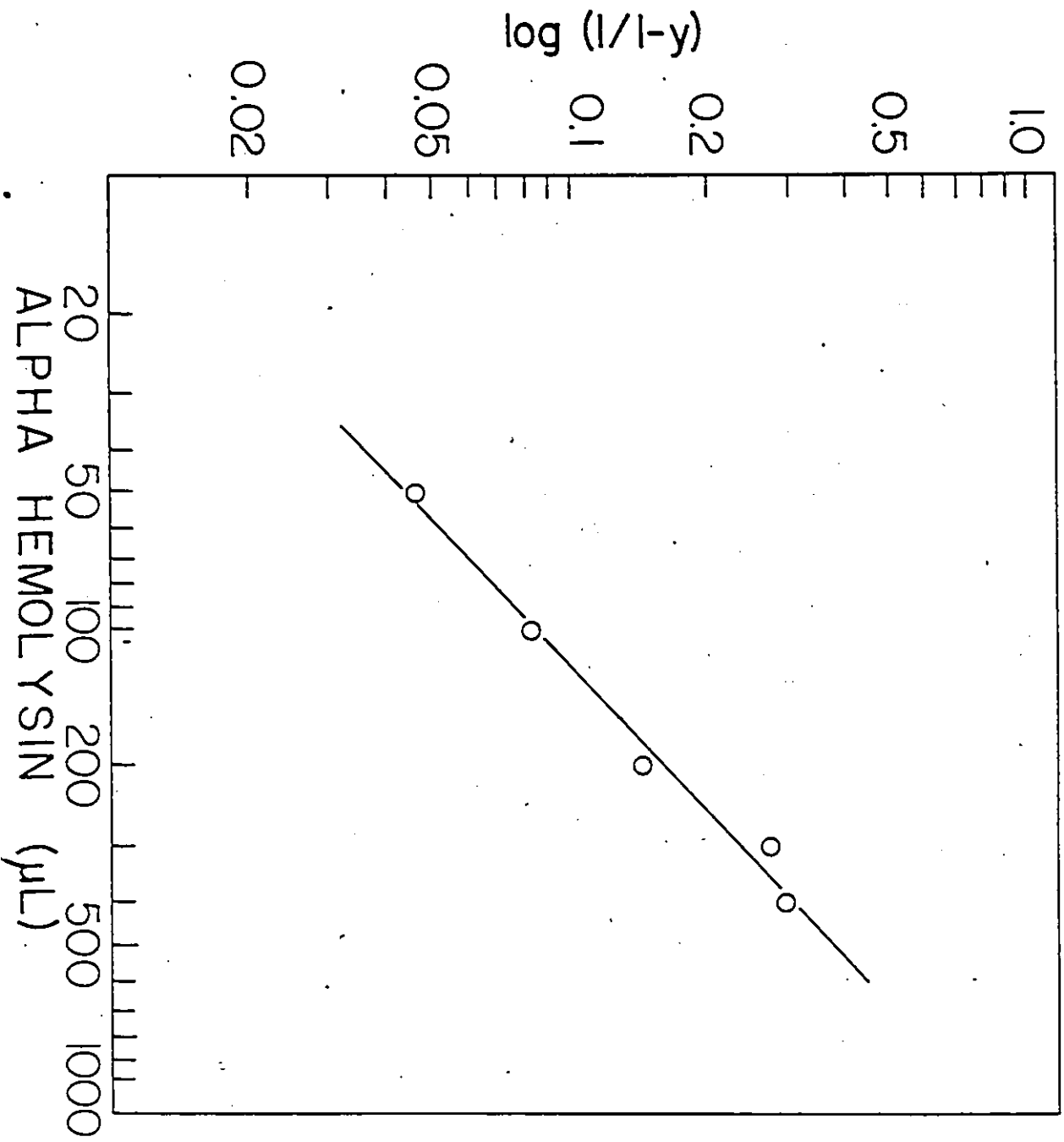
DISCUSSION

The rate of SO_4^{2-} exchange in rabbit erythrocytes, and its

inhibition by DIDS and SITS suggests similar mechanisms of transport in

rabbit and human erythrocytes. $[^3H_2]$DIDS bound predominantly to a 90 000

dalton polypeptide, corresponding to rabbit erythrocyte band 3 in Coomassie

Blue stained SDS PAGE gels. This suggests band 3 is the anion transport

protein of rabbit erythrocytes. Binding of $[^3H_2]$DIDS to rabbit erythrocyte

band 3 and a 40 000 to 50 000 dalton polypeptide identified as a lactate

transport protein has been previously demonstrated (Jennings and Adams–Lackey,

1982).

Heat–inactivated α hemolysin (toxoid) retains its ability to bind to

its receptor although it is no longer hemolytic (Barei and Fackrell, 1979;

Maharaj and Fackrell, 1980; Lo and Fackrell, 1979; Lo et al, 1982). When rabbit

erythrocytes were exposed to heat–inactivated α hemolysin, the rate of

SO_4^{2-} exchange only decreased by 12%. The binding of heat–inactivated

hemolysin may exert a minimal effect on anion transport. Heat denaturation of

native α hemolysin seems to cause a loss of all the protein's functions

except its ability to bind to the receptor. α hemolysin apparently possesses

a heat–labile "active site" and a heat–stable binding site. The inability of

toxoid to influence the flux of SO_4^{2-} supports Cassidy and Harshman's

(1976) observation that binding is separate from subsequent events leading to

lysis of the erythrocyte.

Release of SO_4^{2-} by rabbit erythrocytes exposed to α hemolysin

was a result of both transport (ie. equilibrium exchange) and hemolysis. Since

release occurs by two mechanisms, the rate of release due to hemolysis was

subtracted from the combined rates to obtain the rate due to transport during

hemolysis. When this was done, no change in the transport rate was seen by addition of α hemolysin. The release of SO_4^{2-} during the prelytic lag phase was inhibited by DIDS, suggesting that the prelytic release of SO_4^{2-} was via the DIDS sensitive component of anion exchange.

Efflux of SO_4^{2-} is not changed by α hemolysin, therefore, the increased influx results in an accumulation of SO_4^{2-} in the cell. This accumulation occurred during the prelytic lag phase and stopped once hemolysis began. This is similar to the influx of Na^+ into human erythrocytes on exposure to α hemolysin (Sengers, 1970). It is tempting to think that accumulation of anions leads to expansion of the cell resulting in osmotic lysis. However, the causal relationship, in the case of α hemolysin remains to be demonstrated.

Anion transport in human erythrocytes is accomplished by a one-for-one exchange (Gunn and Frohlich, 1978). An increase in rates of influx, without an accompanying increase in efflux rate, is an unusual result. Influx of SO_4^{2-} was measured in a medium containing high activity of $^{35}SO_4^{2-}$ as compared to the medium in which efflux was measured. Resealing of the lysed erythrocytes could possibly trap $^{35}SO_4^{2-}$ in the cells, resulting in an apparent increase in the net influx. This situation could not occur in the efflux experiments, as the released $^{35}SO_4^{2-}$ was diluted 95.5%. Also, the increased rate of influx occurred during the prelytic lag phase, before hemolysis of the cells and, before any possible resealing could occur. Finally, attempts to reseal ghosts derived from erythrocytes lysed by α hemolysin, or with hypotonic buffer, using the method of Steck (1974b) were unsuccessful. Fussle *et al* (1981) were also unable to reseal rabbit erythrocyte ghosts, but were able to reseal ghosts prepared from human and

sheep erythrocyte membranes. This inability to reseal rabbit erythrocyte ghosts may represent a fundamental difference in the structures of rabbit erythrocyte membranes from membranes of other species.

Whether the accumulation of SO_4^{2-} before lysis occurred via band 3, or whether it occurred via a "pore" in the membrane, has not been determined. DIDS did not inhibit hemolysis and may not have inhibited the accumulation of SO_4^{2-}. The lack of effect of DIDS suggests the usual anion exchange mechanism is not involved in the accumulation of anions. It is possible that the net accumulation of SO_4^{2-} occurred through a DIDS insensitive component of the net anion flux since 20 to 30% of the net chloride flux is not inhibited by DIDS (Knauf et al, 1977). However, the SO_4^{2-} transporting form of the carrier is not involved in net anion flux (Knauf et al, 1983). It remains possible that binding of α hemolysin to band 3 results in a conformational change in band 3 causing a radical change in either the exchange flux or net flux of anions. Alterations in kinetic characteristics of erythrocyte transport proteins, by binding other proteins, is not without precedent. Sachs et al (1974) demonstrated an increase in active K^+ influx in goat red blood cells, induced by binding of antibody to low K^+ goat erythrocytes. The antibody reduced the inhibition of the Na, K−ATPase produced by K^+.

Both the 35 000 dalton and 17 000 dalton segments of band 3 are required for transport. Binding of α hemolysin to either segment may cause a small conformational change, which may result in an alteration of the kinetics of anion transport. The change in conformation may be a small change, as the fast turnover of Cl^- (2×10^5 sec^{-1}) indicates the change in conformation to transport an anion involves a small perturbation over a small distance.

Binding of DIDS changes the structure of band 3 at locations other than the DIDS binding site, suggesting a functional assembly of band 3. DIDS affects interactions of band 3 with surrounding annular lipids (Snow *et al*, 1978), locations of tryptophan residues in relation to the bilayer (Kleinfeld *et al*, 1982), and binding of hemoglobin to the cytoplasmic segment of band 3 (Salhany *et al*, 1980). Therefore, it is possible binding of α hemolysin, to a site removed from the actual anion–binding site, can cause a conformational change in band 3, resulting in alteration of normal transport kinetics.

The release of CrO_4^{2-} and HPO_4^{2-}, as well as glucose, from artificial lipid spherules exposed to α hemolysin has been demonstrated (Weissman *et al*, 1966). These observations led them to suggest that α hemolysin interacted with the phospholipid of the erythrocyte through its inner hydrophobic amino acids. Several groups have suggested α hemolysin imbeds itself into the phospholipid of the erythrocyte forming an ion channel leading to osmotic lysis (Freer *et al*, 1968, 1973; Bernheimer *et al*, 1972; Bhakdi *et al*, 1981). Although Cassidy and coworkers (1974) confirmed the release of markers from liposomes, they found no correlation between the lipid composition of the erythrocytes and their sensitivity to α hemolysin.

The finding of Cassidy *et al* (1974), that liposomes prepared from human erythrocyte lipids are disrupted by the same concentration of α hemolysin as those prepared from rabbit erythrocyte lipids, even though rabbit erythrocytes are 100 times more susceptible, suggests that more than lipid is involved in hemolysis. The studies with liposomes involved concentrations of α hemolysin more than 100 times those required to lyse rabbit erythrocytes (0.1 to 0.2μg/ml) (Harshman, 1979). The lysis of liposomes may be a result

of non–specific binding to the lipid causing lysis through a different mechanism.

Cassidy and Harshman (1976) found a correlation between binding of $^{125}I–\alpha$ hemolysin to erythrocytes of various species and the hemolytic sensitivity of the erythrocytes. Barei and Fackrell (1979) found a correlation between receptor number and hemolytic sensitivity. They determined the receptor number to be 125 980 per rabbit erythrocyte and postulated these to be high affinity binding sites. A much larger number of low affinity sites were suggested on the basis of Scatchard plots. Two binding mechanisms were proposed, specific and non–specific, both mechanisms leading to hemolysis.

Band 3 may act simply as a receptor for α hemolysin. After binding to band 3, the hemolysin may insert itself into the membrane and act either as a nonspecific or specific pore, or as a carrier. Using concentrations of α hemolysin greater than the hemolytic concentration for rabbit erythrocytes, Bhakdi *et al* (1981) and Fussle *et al* (1981) have demonstrated the appearance of hexamers of α hemolysin on both erythrocyte membranes and deoxycholate detergent micelles. They have proposed that these hexamers form a cylinder with a central aqueous pore which disrupts the permeability barrier of the cell. Hexamers of α hemolysin have also been observed in other laboratories (Freer *et al*, 1968, 1973; Bernheimer *et al*, 1972) including this laboratory. In this laboratory, hexamers could not be detected at low, but lytic, concentrations of one to two hemolytic units. Two possible explanations for this inability to detect the hexamers are possible. Perhaps only a few hexamers are required to cause hemolysis of a cell or, two modes–of–action exist. The latter possibility may involve hexamer formation at high hemolysin concentrations and disruption of the ion transport function of band 3 at low

concentrations.

The presence of two rates of hemolysis, combined with the values obtained for the number of "hits" required to lyse an erythrocyte support the suggestion that two modes-of-action exist. The first rate required only one "hit" per cell and was the rate stimulated by sulfhydryl reagents. This mode-of-action probably involves high affinity, specific binding of α hemolyin to band 3, and causes the increased influx of SO_4^{2-} accompanied by expansion of the cell. The second rate is probably a result of low affinity, nonspecific binding of α hemolysin to the phospholipid, resulting in the appearance of hexamers. This results in nonspecific disruption of the permeability barrier to small molecules and ions. The value for the number of "hits" for the second rate suggests six (or possibly more) hemolysin molecules bind separately to the cell. The molecules must then associate, by moving laterally in the bilayer, to form the pore. A similar mechanism has been postulated for streptolysin O (Kanbayashi *et al* 1972).

Fussle *et al* (1981) demonstrated the release of marker molecules of diameter less than 30A from resealed human erythrocyte membranes exposed 30 μg/ml of α hemolysin. The release of marker molecules is through hexamers of α hemolysin embedded in the membrane. Alteration of SO_4^{2-} exchange, in rabbit erythrocytes, by one or two hemolytic units (0.1 μg/ml) of α hemolysin illustrates a different action of the hemolysin. It is unlikely that a pore which allows flow of molecules 30A in diameter would allow SO_4^{2-} to enter, but not exit, the rabbit erythrocyte. The activity of α hemolysin at low concentrations is probably one of disruption of the usual mechanism of

anion exchange on binding to band 3.

Barer and Ross (1952) found that addition of BSA to a suspension of earthworm erythrocytes caused them to reverse their contrast, from phase bright to phase dense, when viewed by phase contrast microscopy. The erythrocytes, with their high content of hemoglobin, appeared bright in isotonic media. Addition of BSA to the suspending medium caused an increase in the refractive index of the medium. When the refractive index of the suspending medium equalled that of the cytoplasm, the cells became invisible. The difference in the refractive indices of the erythrocyte cytoplasm and the suspending medium that makes the erythrocytes scatter light, as detected by the phase contrast microscope.

Changes in the concentration of erythrocyte hemoglobin result in changes in the refractive index of the erythrocyte. As erythrocytes shrink, the hemolglobin becomes more concentrated, resulting in an increase in the refractive index of the cell. As erythrocytes expand, the hemoglobin is diluted and the refractive index of the erythrocyte approaches that of the suspending medium.

Measurement of cell volume by detection of light scattered at 90° to the incident rays was first used by Orskov (1934) and Parpart (1935). The technique is used mainly to measure the rates of penetration of various substances across the erythrocyte membrane. Cl^-/HCO_3^- exchange can be measured by changes in scattered light. Uptake of H^+ and its buffering by hemoglobin, results in the efflux of HCO_3^- and the corresponding influx of Cl^- (Jacobs and Stewart, 1941). The uptake of Cl^- increases the number of osmotically active particles in the cytoplasm, causing the erythrocyte to take up H_2O, which results in

swelling.

Light scatter was chosen to measure expansion of erythrocytes for several reasons. Using this method, it is possible to determine small volume changes in cells without disturbing them as hematocrit determination would. The hematocrit method cannot be used to determine volume changes caused by lytic concentrations of hemolysin, since the cells would hemolyse before the hematocrit determination. Increased fragility caused by sublytic concentrations of α hemolyin would make hematocrit determinations difficult, possibly causing the cells to hemolyse during the centrifugation. Hendry (1954) criticized the hematocrit method for the measurement of the volume of erythrocytes in hypotonic systems. He has shown that swollen erythrocytes are much more likely to be compressed by centrifugal forces than cells of whole blood. The conditions of the expansion assay are identical to those used in the kinetic hemolysis assay, allowing direct comparisons to be made between the two assays. Measurement of light scattered by a suspension of erythrocytes has the added advantage of being a continuous measurement, amenable to computer analysis.

The cause of expansion of rabbit erythrocytes, after binding α hemolysin, has not been determined. Heat inactivation of α hemolysin destroyed the ability of the hemolysin to cause expansion. This suggests expansion may be a necessary precusor to hemolysis. Several authors have demonstrated K^+ release during the prelytic lag (Marucci, 1963a,b; Madoff, 1964; Cooper et al, 1964a,b). Heat inactivated hemolysin does not cause K^+ release (M. Gorski, unpublished data). Future experiments should be done to determine if pre–lytic expansion is correlated with pre–lytic K^+ release. If these two phenomena are correlated, measurement of cell

expansion may be used as an indicator to study the early events in the mode-of-action of the hemolysin.

A short lag phase preceeded expansion of the erythrocytes. This pre-expansion lag was shortened by increasing the hemolysin concentration. Binding of the hemolysin to band 3, and the subsequent "alteration" of band 3 probably occur during the lag. The rate of expansion was increased by addition of more hemolysin. Inhibition of $Na^+ + K^+$ cotransport by furosemide, decreased the time to 50% expansion, implicating involvement of passive cation movements in the hemolytic mechanism of α hemolysin. The lack of effect of SITS on prelytic expansion further supports the idea that the polysulfonic acid-sensitive anion channel is not involved.

To completely characterize the role of membrane sulfhydryl groups, it is necessary to investigate their reactions with sulfhydryl reagents of various types. PCMBS forms a mercaptide with protein sulfhydryl groups, and is considered to be highly specific for sulfhydryls. The stimulation of α hemolysin mediated lysis of rabbit erythrocytes by PCMBS occurred at concentrations less than $20 \mu M$. The high specificity of PCMBS for sulfhydryls, combined with its effectiveness at micromolar concentrations, strongly implicates the involvement of a sulfhydryl in the stimulatory effect.

Maleimide derivatives are considered relatively specific at concentrations below 1mM. NEM was used in high concentrations to overcome the binding of this permeant reagent to cytoplasmic sulfhydryls. Since eosin-5-maleimide is impermeant, we were able to use concentrations of this reagent well below 1mM, thereby increasing the specificity of eosin-5-maleimide for sulfhydryls.

Eosin–5–iodoacetamide and iodoacetamide are relatively specific for sulfhydryls but often bind to other nucleophilic groups. As with eosin–5–maleimide, low concentrations of this reagent produced marked effects on the rate of hemolysis, suggesting involvement of a sulfhydryl. In addition to the effectiveness of these sulfhydryl reagents at low concentrations, several amino specific reagents had no effect on hemolysis of rabbit erythrocytes by α hemolysin. Therefore, although no direct proof is available, the involvement of a sulfhydryl in the stimulatory effect is likely.

PCMBS is a slowly permeant molecule (Knauf and Rothstein, 1971). At low concentrations, and for short labelling periods, it may be used to selectively label external sulfhydryls. Stimulation of lysis is observed when cells are incubated with PCMBS for short periods or at low concentrations, further incubation or at higher concentrations, decreased the initial stimulatory effect. This suggests the stimulatory effect is caused by binding to a sulfhydryl located in an accessible region of the membrane, perhaps exposed to the outside of the membrane, or in a hydrophilic channel. This population of sulfhydryls may be similar to the sulfhydryls in human erythrocytes involved in inhibition of water transport. These sulfhydryls react within the first ten minutes of exposure to PCMBS (Naccache and Sha'afi, 1974).

A second, less accessible, population of sulfhydryls may be responsible for the decrease in the initial stimulation of lysis. In intact cells, reactivity of sulfhydryls is more dependent on the location of sulfhydryls in the membrane, than the chemical reactivity of a particular sulfhydryl for the reagent. Membrane functions are disturbed by sulfhydryl reagents in sequence from the periphery toward the inside of the cell (Rothstein, 1970). Therefore, the second population is probably deeper into the membrane than the more

reactive sulfhydryls. This would explain why PCMBS acid, a permeant reagent, first stimulates, then inhibits hemolysis. Eosin-5-maleimide, an impermeant reagent, can bind only to the external sulfhydryl and, therefore exerts only a stimulatory effect on hemolysis.

α hemolysin and PCMBS appear to interact synergistically. When rabbit erythrocytes were first treated with a sub-lytic concentration of α hemolysin, then exposed to sub-lytic concentrations of PCMBS, the erythrocytes lysed at a rate much greater than if the effects of the two agents were additive. The kinetics of this lysis were identical to the kinetics of lysis resulting from exposure to hemolysin alone. The stimulatory effect of PCMBS on α hemolysin-mediated lysis is independent of the order of addition of these two reagents.

Uptake of PCMBS can be separated into two components. A rapid binding of PCMBS to 1.2×10^6 sites per cell is independent of temperature and can be inhibited by PCMB (Rothstein, 1982). The slow component is not temperature dependent, does not compete with PCMB, and is not saturable (Rothstein, 1982). The slow component of PCMBS uptake is inhibited by SITS, and therefore occurs via the anion transport channel (Knauf and Rothstein, 1971).

Human erythrocytes exposed to PCMBS demonstrate an increased permeability to cations (Sutherland et al, 1967). The sulfhydryl involved in control of cation permeability in human erythrocytes is located in the 17 000 dalton transmembrane portion of band 3 (Grinstein and Rothstein, 1978). These authors have shown the sulfhydryl to be more accessible from the inside of the membrane. Release of K^+ occurs only after prolonged exposure of the erythrocytes to PCMBS (Sutherland et al, 1967). The stimulation of hemolysis was observed immediately after exposure of the erythrocytes to

PCMBS and reached a maximum after only ten minutes exposure. The accessibility of the sulfhydryl of the transmembrane portion of band 3 may be much greater in the rabbit erythrocyte, compared to that of the human erythrocyte. It is intriguing to consider that both PCMBS and α hemolysin cause a release of K^+ from human and rabbit erythrocytes respectively, and both lead to hemolysis.

N-[ethyl-2-^{3}H]-maleimide and eosin-5-maleimide were used to identify the membrane protein involved in the stimulatory effect. Jacob and Jandl (1962) demonstrated the entry of the lipid soluble reagent, NEM into erythrocytes by its reaction with intracellular reduced glutathione. NEM labelled all the major proteins of the rabbit erythrocyte membrane. Although preincubation of rabbit erythrocytes with NEM increased their hemolytic sensitivity to α hemolysin, NEM was not used as a probe for the protein involved in the stimulatory effect because of its lack of specificity.

Concentrations of eosin-5-maleimide or eosin-5-iodoacetamide ranging from 5 to 1 000μM decreased both lag and the time required to reach 50% hemolysis. When rabbit erythrocytes were exposed to eosin-5-maleimide or eosin-5-iodoacetamide, most of the probe was bound to band 3. When ghosts were exposed to the same concentration of eosin-5-maleimide, several additional polypeptides were labelled. Although potentially available for binding eosin-5-maleimide, the sulfhydryls on the cytoplasmic side of the membrane did not bind the probe when intact cells were used, indicating that eosin-5-maleimide is impermeant.

An exofacial sulfhydryl, located on a 65 000 to 70 000 dalton polypeptide on the human erythrocyte, is required for glucose transport (Batt *et al*, 1976). Eosin-5-maleimide labelled a rabbit erythrocyte membrane polypeptide in this

region of electrophoresis gels to a small extent. Less than 2% of the

eosin–5–maleimide migrated in this region, suggesting the existence of a few

copies of this polypeptide in rabbit erythrocytes or a low affinity of eosin–5–

maleimide for the sulfhydryl in this polypeptide. While it is possible that the

sulfhydryl involved in the stimulatory effect is in a protein other than band 3,

this is unlikely considering that, not only is band 3 the receptor for α

hemolysin, but at least 85% of the eosin–5–maleimide added to the red cells

bound to band 3.

Labelling of rabbit erythrocytes with eosin–5–maleimide, followed by

chymotrypsin–treatment of these cells, resulted in the appearance of a labelled

membrane–bound polypeptide, with a molecular weight of 60 000 daltons. Exposure

of ghosts to chymotrypsin, resulted in further hydrolysis of band 3 to give a

membrane–bound 20 000 dalton fragment which contained the label. No labelled

polypeptide was released from the membrane during incubation with chymotrypsin.

Exposure of ghosts to trypsin also resulted in the appearance of the label in a

20 000 dalton polypeptide which remains associated with the membrane. Again, no

soluble labelled polypeptide was released during treatment of the ghosts with

trypsin. The reactive sulfhydryl is probably in the 20 000 dalton

transmembrane portion of band 3.

Diamide is a permeant sulfhydryl–oxidizing reagent that has been used to

determine nearest–neighbour relationships of the proteins of human erythrocyte

membranes by crosslinking the proteins through their sulfhydryl groups (Haest

et al, 1977; Fischer *et al*, 1978; Kursantin–Mills and Lessin, 1981). Exposure

of rabbit erythrocytes to concentrations of diamide up to 20mM had no effect on

lysis of the cells by α hemolysin. This suggests that the cytoskeleton

(spectrin, actin and band 4.1) can be crosslinked, without affecting the

kinetics of hemolysis.

The lack of effect of iodoacetamide on lysis of rabbit erythrocytes by α hemolysin is not surprising. Iodoacetamide does not inhibit glucose transport in human erythrocytes (Smith and Ellman, 1973), or affect Rh antigen activity (Green, 1967), functions which are known to be disrupted by other sulfhydryl reagents. A similar reagent, iodoacetate, does not inhibit glucose transport (LeFevre, 1948), and has no effect on passive cation permeability (Passow, 1964) of human erythrocytes.

Comparisons between the rabbit and human erythrocyte membranes must be made cautiously. Comparatively little is known about the rabbit erythrocyte membrane, and in this study we have assumed the structure of the rabbit erythrocyte membrane to be similar to that of humans. Rabbit erythrocyte band 3 has been demonstrated to be a receptor for staphylococcal α hemolysin, the involvement of a sulfhydryl of band 3 suggests an active role for band 3 in the mode-of-lysis of erythrocytes by α hemolysin. Direct involvement between the sulfhydryl and the hemolysin is unlikely. The synergistic nature of the stimulation of hemolysis suggests that PCMBS acts at a site different from that of α hemolysin. The sites, however, may be located on the same molecule. The sulfhydryl is likely to be involved in maintenance of either the conformation of band 3, or in maintenance of a permeability barrier, either of which may be disrupted upon binding of the reagents to the sulfhydryl, thereby increasing the sensitivity of the erythrocyte to lysis by staphylococcal α hemolysin.

SUMMARY

1. Band 3 was identified as the anion transport protein in rabbit erythrocytes.

2. α hemolysin caused an increased influx of SO_4^{2-} into the rabbit erythrocyte, but did not change the rate of efflux of SO_4^{2-}. This increased influx occurred after binding of the hemolysin and before the onset of hemolysis.

3. Heat inactivated α hemolysin had no effect upon equilibrium exchange of SO_4^{2-}.

4. Inhibition of anion transport with DIDS had no effect upon hemolysis or release of SO_4^{2-} during the prelytic lag phase.

5. α hemolysin caused rabbit erythrocytes to swell prior to hemolysis, the rate of swelling was increased by micromolar concentrations of furosemide. Inhibition of anion exchange with SITS did not affect the prelytic swelling. Heat inactivated hemolysin did not cause erythrocytes to swell.

6. Several sulfhydral-specific agents stimulated the hemolytic activity of α hemolysin. Two of these reagents, eosin-5-maleimide and eosin-5-iodoacetamide bound to a 15 000 dalton, membrane-bound chymotryptic fragment of band 3. The binding site of these probes was shown to be exposed to the outside surface of the membrane.

7. Graphic analysis of total cells lysed versus hemolysin concentration, suggested hemolysis occurs by two modes-of-action. The initial hemolysis required binding of a single hemolysin molecule to an erythrocyte. A second mechanism required binding of more than one hemolysin molecule.

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