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CRM's of the alpha toxin of Staphyloccoccus aureus.

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CRM'S OF THE ALPHA TOXIN OF *Staphyloccoccus aureus*

by

© URSULA P. MACHOWSKI-SYROWIK

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This thesis is dedicated to my parents.

ABSTRACT

32 non-hemolytic mutants of Staphylococcus aureus Wood 46 strain, were isolated following treatment with ultraviolet light. These non-hemolytic mutants were characterized biochemically, and immunologically. As a result of the immunological characterization, the mutants were classified based on their ability to react with antibodies to alpha toxin immunoadsorbent beads.

One mutant, P1, was studied in greater detail because of its peculiar nature. P1 was found to be temperature-sensitive in that it produced a hemolytic toxin when grown at 24°C, and a non-hemolytic form of the toxin when grown at 37°C. P1 could bind onto rabbit erythrocyte membranes, compete with alpha toxin for receptors and protect the red cells from lysis by alpha toxin. This mutant's CRM was isolated and purified by affinity chromotography, after which antibodies were raised against P1CRM 37°C. The P1CRM 37°C had a molecular weight of 44,000 daltons.

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LITERATURE REVIEW

Introduction

Staphylococcus aureus is a microbe mainly associated with skin, and mucous membranes of warmblooded animals. This microbe secretes several potential lethal exotoxins, the most potent of which is alpha toxin. Alpha toxin is antigenic, causing disruption of normal physiological processes in both man and other animals. Alpha toxin has a wide range of manifestations. Not only is alpha toxin a hemolysin affecting rabbit erythrocytes, but it is also dermonecrotic, neurotoxic and cytolytic to a wide variety of cells (4). Despite a large amount of research in the last 70 years, the exact mode of action, the structure and function, and the antigenic determinants of alpha toxin remain unknown.

Experimenters with other protein antigens such as the diphtheria toxin and hemoglobin (Hb) have used mutants to increase their understanding of the structure, activity and function of these proteins. As a result, the structure and antigenic determinants of diphtheria toxin and hemoglobin have been characterized immunologically. Since work with mutants has proven successful with diphtheria toxin and hemoglobin, it was thought that this approach could be used to determine the unknown characteristics of alpha toxin.

The objective of this investigation was to isolate and partially characterize non-hemolytic mutants of alpha toxin. Subsequently, emphasis was placed on one mutant, P1, because of its peculiar temperature-sensitive characteristic.

Production and Purification

In the production of Staphylococcus aureus, most workers have used Staphylococcus aureus, Wood 46 stain, which is coagulase positive and which is noted for high alpha toxin production. The best medium for alpha toxin production is Dolman-Wilson medium (31). The toxin is formed during the log phase of growth and proceeds until late stationary phase (81).

The alpha toxin has been purified by a number of investigators by a variety of isolation procedures (78). Two frequently used methods are: (a), selectively eluting the alpha toxin from controlled pore glass beads (23) and (b), heating the toxin and reactivating it with 8 M urea (28).

Physical and Chemical Characteristics

Alpha toxin is the subject of several relatively recent reviews (4, 10, 40, 72, 78). Improvements in methodology have permitted a better understanding of the biological effects of alpha toxin, as well as its physical and chemical characteristics.

Purified alpha toxin of S. aureus is a protein (4) having an estimated molecular weight of between 10^4 to 4.5×10^4 daltons (78). A molecular weight of 36,000 was determined by SDS-gel electrophoresis (58), 22,000 by equilibrium centrifugation (78), and 44,000 by ultracentrifugation (12, 34).

Amino acid analysis has shown a variation in the N-terminal part of the toxin. Histidine, arginine, glycine and alanine variously have been detected as N-terminal amino acids and this variation is attributed to proteolytic enzymes present in crude

toxin preparation (78).

The sedimentation coefficient of alpha toxin is 3.0S. The toxin can also exist as 12S to 16S form as described by Bernheimer and Schwartz (12) and Lominski et al (45). The 3S form aggregates into the 12S form when heated to 60°C. The 12S aggregate has a uniform appearance when examined by negative staining in the electron microscope. It consists of small rings, having an outside diameter of approximately 9 nm, which contain 6 subunits arranged hexagonally (6). The 12S form is non-hemolytic and is biologically inactive (78). Treatment with 8 M urea causes the 12S form to become reactivated to the 3S hemolytic form, which is indistinguishable from the 3S native form (5).

Alpha toxin occurs in multiple forms as shown by sucrose density gradient centrifugation (78). The toxin separates into four electrophoretically distinct variations that are immunologically and biologically identical (12).

The molecular heterogeneity of alpha toxin has also been revealed by its four isoelectric points. The major component of the four electrophoretic variants has an isoelectric point of 8.5 which accounts for 80% of the total hemolytic activity (6). The three minor components, which accounts for 20% of the hemolytic activity, have isoelectric points of 6.3, 7.2, and 9.1.

Mode of Action

The mode of action of the alpha toxin at the molecular level is not fully understood. Most investigators agree that the cell membrane is the site of action for alpha toxin.

Discussions have centered mainly on the question of whether alpha toxin has an enzymatic or a non-enzymatic mode of action.

The kinetic studies of Marucci (53) and Madoff et al (48) suggest that two stages are required for alpha toxin to lyse rabbit erythrocytes. First, a pre-lytic stage, possibly enzymatic in nature, in which the leakage of K^+ ions occurs (24, 25) and second, an actual lytic stage in which hemoglobin is released from the erythrocytes. The binding of ^{125}I-labeled alpha toxin and the subsequent leakage of intracellular K^+ is independent of lysis suggesting that membrane damage is a separate sequential step (22). The second stage is blocked by anti-alpha toxin (43, 76) or alpha toxoid (9).

The non-enzymatic theory of the mode of action, involves the surface activity of the toxin, which penetrates and disorganizes the hydrophobic regions of the cell membrane. Weissman et al (76) showed the effects of alpha toxin on phospholipid liposomes. Alpha toxin causes the rapid release of cations, anions, and other marker molecules from the liposome. This reaction was inhibited by prior incubation of the alpha toxin with anti-alpha toxin. These observations suggested that alpha toxin acted on the phospholipid component of spherules, and that this interaction involved hydrophobic regions of the toxin (4).

Freer et al (35) carried out comparative studies of morphological changes caused by alpha toxin in artificial and natural membranes. Using purified toxin, they showed that liposome disruption resulted in the formation of ring-like structures. A detailed study of the ring formations suggested that alpha toxin

is polymerized on the surface of the lipid membranes to form aggregates resembling 12S toxin aggregates (7). Buckelew and Colacicco (15) observed that alpha toxin penetrated aqueous monolayers of different lipids, and that the interaction between toxin and lipid membrane was hydrophobic.

Arbuthnott et al (7) showed that polymerization resulted, when alpha toxin was exposed to diglycerides, lecithin, cholesterol, or lysolecithin. Based on these findings, it was suggested that surface active properties of alpha toxin were responsible for the destructive effects (7).

Liposomes suffer from a major limitation in that they cannot be compared to natural membranes. For example, Cassidy and Harshman (55) showed that liposomes prepared from rabbit erythrocyte lipids, or human erythrocyte lipids were equally susceptible to alpha toxin, even though the rabbit erythrocyte is 100 times more sensitive than the human erythrocyte.

Recently, McCartney and Arbuthnott (55), Cassidy and Harshman (22), and Barei and Fackrell (9) have suggested that the surface active properties of alpha toxin are not the sole factors involved in hemolysis.

There is current evidence which suggests that alpha toxin binds to receptors located on rabbit erythrocyte membrane. Cassidy and Harshman (22) used ^{125}I-labeled alpha toxin to estimate that intact rabbit erythrocytes contain approximately 5×10^3 binding sites per cell. They also speculated that the receptor may be a glycoprotein or lipoprotein.

More recently, in this laboratory, Maharaj and Fackrell (49)

have isolated, and characterized the receptor for alpha toxin. The receptor is a transmembrane glycoprotein which is the band 3 of the rabbit erythrocyte membrane, with a molecular weight of 90,000 daltons. The band 3 is an anion channel (50).

Lo and Fackrell (43) found that alpha toxin was specifically oriented on rabbit erythrocytes, and that a membrane-binding region existed on the toxin. Thus, they were able to separate two antibody populations; an antibinding antibody (AB-Ab) and a hemagglutinating antibody (IHA-Ab). Neutralization tests showed that the two populations are directed to different determinants on the alpha toxin.

Barei, and Fackrell (9) found that alpha toxin binds to receptor sites present on erythrocytes using fluorescein-labeled alpha toxoid. They also demonstrated that there is a correlation between the number of specific receptors and the hemolytic sensitivity of various animal species.

Wiseman (78) and his co-workers (79, 80, 81) have postulated that alpha toxin is activated by membrane-bound proteases, which convert the alpha toxin from an inactive to an active protease, and becomes capable of hydrolyzing "structural" membrane polypeptides (79).

Alpha toxin was shown to be proteolytic when activated by trypsin and could hydrolyze tosyl arginine methylester (81). Dalen (27) has reported that extracellular proteolytic activity modified the alpha toxin in such a way as to change the molecular weight of the toxin thus suggesting autodigestion.

Apparently, binding of the alpha toxin molecule to a receptor is required for lysis of rabbit erythrocytes, but there is a

question as to the exact mode of action of lysis after binding has occurred.

Genetics

In 1961, Blair and Carr (13) noted that alpha toxin may be under bacteriophage control. However, Altenberg (1, 2) could not find any evidence to support this hypothesis, although Corynebacterium diphtheriae, and group A streptococci toxins are regulated by bacteriophage. Once the bacteriophage is lost, so too is the ability to produce that toxin.

It is known that lysogenic staphylococcal strains are quite common, and that most Staphylococcus aureus strains carry temperate bacteriophage (37). It has been demonstrated that there is lysogenic conversion associated with beta-toxin(30) (40). We do not know if such an association occurs with alpha toxin.

Genetic experiments were done by McClatchy and Rosenblum in 1966 (56), who investigated alpha toxin mutants obtained by ultraviolet irradiation and studied their biological properties. McClatchy and Rosenblum showed genetic recombination between Staphylococcus aureus non-hemolytic mutants by transductional analysis (57). The result of the two-point reciprocal transduction analysis placed the mutants into two genetic groups. Group 1 mutants were structural gene mutants for alpha toxin. All members of this group were fibrinolysin-positive and cross reactive material (CRM) positive (57). Group 2 mutants were pleiotropic mutants which lacked both the alpha-toxin producing gene, and fibrinolysin-producing gene (57). The pleiotropic effect of alpha toxin production and fibrinolysin production indicated that a regulatory

gene existed at either the transduction or translation level (57). Therefore, these investigators suggested that there are at least two genetic loci involved in alpha toxin production (57).

Chromosomal mapping in Staphylococcus aureus, has been done by Altenbern (1, 2) and more recently by Brown and Pattee (14). In 1971, Altenbern presented a partial genomic map of Staphylococcus aureus (2,3) based on a method of inducing synchronous chromosomal replication by treatment with phenethanol (3).

Nearly all DNA-mediated transformation in Staphylococcus aureus has been done in the phage group 3 recipient strain 8325 (51), and there are three genetic linkage groups for the chromosome of strain 8325 (14). Brown and Pattee (14) found that the chromosomal determinant for alpha toxin production is between purine biosynthesis and isoleucine-valine biosynthesis in linkage group three.

Studies have shown that staphylococcal cells belonging to the same species display unexpected property variations (71). One source of variation in Staphylococcus aureus is the presence of extrachromosomal genetic elements or plasmids (71). Plasmids are probably always found in all staphylococcal cells. The first plasmids to be studied in S. aureus, were those strains carrying genes resistant to penicillin. In 1963, Novick postulated that the penicillinase genes were carried by plasmids (61). Current evidence indicates that many different types of plasmids are involved in resistance to drugs and inorganic ions.

Preliminary work by Witte (82) points to the fact that alpha

toxin can also be controlled by a plasmid. For example, the alpha toxin marker is lost at a high spontaneous rate in a number of clinical strains, which is characteristic of plasmids. Also, the alpha toxin marker is lost after grown in the presence of SDS.

MATERIALS AND METHODS

BIOLOGICAL METHODS

Cultures

All cultures were presented by lyophilization. Mutants from Staphylococcus aureus, strain Wood 46 and strain Wood 46 (16) were subcultured once a month in Dolman-Wilson medium (31), and T-soy agar slants (Difco). The subcultured organisms were incubated at 37°C, under 10% CO_2, and air for 36 hours, and stored at 4°C. All strains were examined periodically for hemolysis on rabbit blood agar plates, and by Gram stain.

Production of Crude Mutant Material

Mutants were grown for 36 hours under 10% CO_2, at 37°C in Dolman-Wilson medium (31). The method of Wiseman et al (81) was used. Cultures were centrifuged at 6,000 x g for 20 minutes. Supernatants were kept, and either sodium azide or pentachlorophenol was added as a preservative. Supernatants were then frozen at -20°C until use.

Media

The following Difco media were prepared in distilled water, Trypticase-soy (T-soy) broth, Trypticase-soy agar, nutrient broth, nutrient agar, Nutrient Gelatin media, Coagulase Plasma, Deoxyribonuclease Test Agar (DNase), Egg Yolk agar base, Mannitol Salt Agar, Mannitol broth, and Phosphatase agar.

Antibiotic Sensitivity

Difco antibiotic sensitivity disks were used to test for the sensitivity, or resistance of the mutant cultures to the

following antibiotics: Bacitracin (10 units), Polymycin B (100 units), Chlorotetracycline (30 µg), Penicillin (5 units), Dehydrostreptomycin (10 µg), Erythromycin (5 µg), Tetracycline (30 µg), and Chloramphenicol (30 µg). The tests were performed according to the Kirby-Bauer Method (18).

Erythrocytes

New Zealand White rabbits maintained in this department were bled for erythrocytes. Citrated sheep blood was supplied by Woodlyn Laboratories, Guelph, Ontario. Outdated human blood, type A Rh+, was obtained from the Red Cross.

The rabbit blood was defribinated with glass beads. The erythrocytes were centrifuged at 600 x g, for 20 minutes, and washed three times in Phosphate Buffer Saline (PBS) (0.01 M, pH 7.2, 0.85% NaCl). The packed red cells were resuspended to a final concentration of 2% (V/V).

Hemolytic Titration

The method of Lo and Fackrell (43) was used, except the volume was increased proportionally. Alpha toxin (100 µl) was diluted two-fold in PBS in Micotitre® plates, (Cool Lab, Products) having a U-shaped well. One hundred µl of a 2% (V/V) suspension of rabbit erythrocytes was added. The mixture was incubated at 24°C for one hour, and then centrifuged to remove unlysed cells. After centrifugation, the supernatant was diluted 1:3 in PBS, and the absorbance was read spectophotometrically at 541 nm, (1-cm light path). The hemoglobin content of the supernatants thus measured was translated into percent hemolysis by comparison to

standards which were prepared from a 2% suspension of cells lysed with distilled water.

Kinetic Hemolytic Test

The amount of hemolysis plotted against time was graphically recorded by a Sargent Recorder, using a Colman 124 double beam spectrophotometer. A mixture of rabbit erythrocytes in PBS, was adjusted to a final absorbance of 1.6 to 1.8 at 650 nm. After the addition of alpha toxin, the rate of hemolysis was traced. This rate of lysis, expressed as $\Delta A/min$, is proportional to either the amount of toxin or available receptor sites.

IMMUNOLOGICAL TESTS

Immunodiffusion

Immunodiffusion in Noble Agar (Difco) was performed by the method of Ouchterlony (63). Gel diffusion was carried out on a microscope slide, with a 3mm thick layer of 1% Noble Agar in PBS. A 3mm center well was cut from the gel with four peripheral antigen wells of equal size, spaced 5mm from the well. Immunodiffusion slides were incubated at 4°C in a humidity chamber for several weeks.

Antisera

Rabbit anti-alpha toxin previously prepared in the laboratory was purified by ammonium sulphate precipitation to obtain the IgG fraction (19). To obtain antibodies to PlCRM, a seven month old New Zealand White rabbit was injected with purified PlCRM 37°C.

A suspension of 0.8 ml of P1CRM (1.22 mg/ml) was mixed with a 0.8 ml of Freund's complete adjuvant (Difco), and 0.8 ml of the mixture was injected intramuscularly into each of the hind legs of the rabbit.

One month later, the rabbit was reinjected with the same dose and after a waiting period of an additional month, the rabbit was bled for antibodies.

Indirect Hemagglutination Test

The Indirect Hemagglutination Test (IHA) was performed as described by Lo and Fackrell (43). Briefly, a serial, two-fold dilution of 50 µl of serum was done in PBS. To each well, 50 µl of a 1% suspension of immunoadsorbent was added. The IHA titre was the reciprocal of the last dilution that gave hemagglutination after 1.5 hours at 4°C..

Preparation of Immunoaffinity Agarose Beads

The method described by Cuatrecasas and Anfinsen (26) was used in the preparation of immunoadsorbent beads, which included binding the anti-alpha toxin to the cyanogen bromide activated agarose beads. The immunoadsorbent was used to bind either alpha toxin or alpha toxin mutants' CRM so that they could be isolated.

Sepharose 4B (40 grams) was added to an equal amount of distilled water. Ten grams of finely powdered cyanogen bromide (CNBr) was added to the agarose suspension. While stirring the suspension, 1N NaOH was then added to raise the pH to 11. The agarose-CNBr solution was transfered rapidly to a Buchner funnel, and washed under vacuum with a large volume of cold sodium acetate

buffer (0.2M, pH 4.5). The agarose-CNBr beads were then removed from the filter paper, and the anti-alpha toxin (IgG) or anti-binding antibody (AB-Ab) was added to the mixture. The resulting mixture was stirred gently overnight at 4°C in sodium acetate buffer. These agarose beads were used as immunoadsorbents in the IgG Bead Test, the AB-Ab Bead Test, and in the immunoaffinity procedure for the purification of P1CRM 37°C.

Preparation of Immunoadsorbent with Purified P1CRM

An immunoadsorbent was prepared by the method of Lo and Fackrell (43). The immunoadsorbent was rabbit erythrocytes coated with purified P1CRM that were then washed, covalently linked with glutaraldehyde, and washed again with lysine-HCl. Rabbit erythrocytes were washed three times in PBS, and were adjusted to 10% suspension in purified P1CRM (1 mg/ml). The combination was stirred at room temperature for 1 hour. The unbound P1CRM was removed by washing the cells three times with PBS.

The coated erythrocytes were adjusted to a 2% suspension in 0.1% (V/V) glutaraldehyde in PBS. The mixture was agitated at room temperature for 1 hour after which the cells were washed three times in PBS and brought to a 20% (V/V) suspension in 0.1M lysine-HCl in PBS to neutralize excess glutaraldehyde. After incubation at room temperature for one hour the immunoadsorbent was washed 3 times in PBS, and stored at 4°C as a 10% (V/V) suspension in PBS.

PHYSICAL AND CHEMICAL METHODS

Buffers

Phosphate buffered saline (PBS) 0.01M, pH 7.2, with NaCl (0.85%, W/V) was prepared with glass distilled water. All other buffers were prepared by methods described by Gomori (36).

Protein Assays

The amount of protein was determined by the Lowry method (47), using bovine serum albumin as a standard. The presence and concentration of protein from column eluates was expressed in terms of absorbance at 280 nm in a quartz cuvette (1-cm light path).

Molecular Weight Determination

The molecular weight of purified PlCRM 37°C was determined by the method of Laemmli (41) SDS-gel electrophoresis in 9% polyacrylamide. The relative mobility was calculated for several protein standards such as, Bovine serum albumin (66,000; Sigma), alpha amylase (48,000; Sigma), egg albumin (45,000; Sigma), carboxypeptidase A (34,000; Sigma), trypsin (23,000; Sigma), B-lactalbumin (18,480; Sigma), and lysozyme (14,300; Sigma). A plot of the logarithm of the above molecular weights versus their relative mobilities was obtained.

Polyacrylamide Disc Gel Electrophoresis (PAGE)

Polyacrylamide disc gel electrophoresis (29) was used to determine if purified PlCRM was homogenous. To form an electric

field gradient, two acrylamide gels differing in concentration, in ionic strength, and in pH were cast in the same tube. A small pore gel (7% acrylamide), polymerized with ammonium persulphate, was at the lower end of the tube, where the separation took place. Above the small pore gel a shorter "spacer" gel was cast. The spacer gel (3% acrylamide) had a larger pore size, and was polymerized with riboflavin as a catalyst. PICRM (500 µl of 1.22 mg/ml) was mixed with 10 µl of 05% bromophenol blue in PBS, and about 0.1 mg. of sucrose. This mixture (50 µl to 200 µl) was placed on top of the "spacer gel". The electrophoretic buffer comprises TRIS-Glycine at pH 8.3. Coomassie Brilliant Blue R 250 (Sigma) was used for staining and a mixture of 7.5% glacial acetic acid and 5% methanol was used for destaining and storage.

RESULTS

CHAPTER 1

ISOLATION OF NON-HEMOLYTIC MUTANTS OF Staphylococcus aureus WOOD 46 STRAIN

Several non-hemolitic mutants of S. aureus were isolated and characterized.

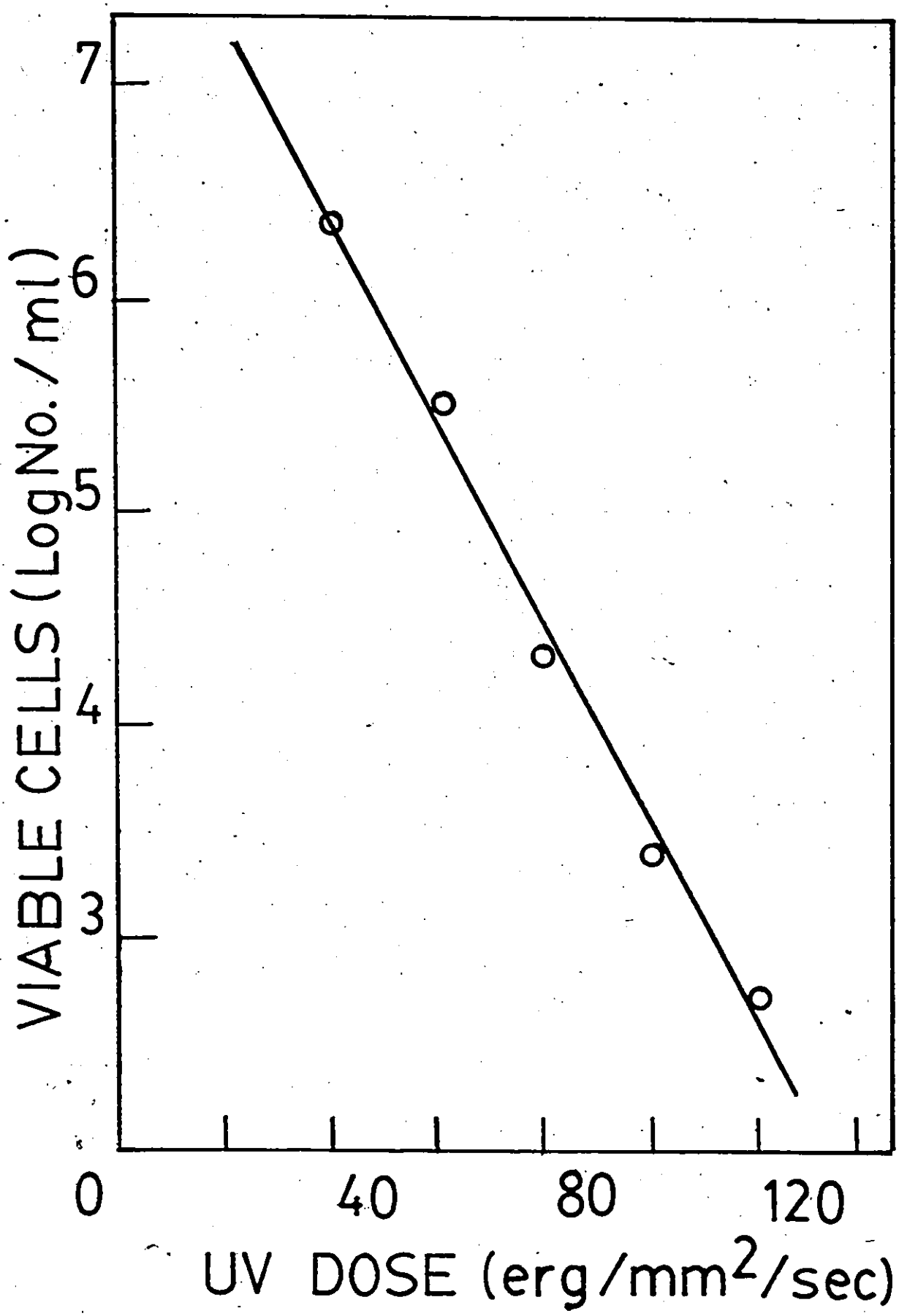
Alpha toxin from Staphylococcus aureus, is normally a hemolysin which also causes dermonecrosis. To obtain non-hemolytic mutants from S. aureus, strain Wood 46, the organism was grown for 36 hours in 1000 ml of semi-synthetic Dolman-Wilson medium (31). The cells were grown in 10% CO_2 and 90% air, in an incubator shaker at 37°C. The above cultures were centrifuged (6,000 x g, 20 min.), and the bacterial pellet was washed three times in PBS. The pellet was resuspended in PBS to obtain 1×10^7 cells /ml. Figure 1 shows a typical survival curve. The number of viable cells decreased from 9×10^8 to 1.2×10^4 after exposure to 80 erg/mm^2/second, a greater than 99% kill rate. Therefore, washed cultures were routinely exposured to 80 erg/mm^2/second irradiation before plating to increase the mutation rate.
To identify possible mutants, 0.5 ml aliquots of these irradiated samples were plated on rabbit blood agar plates. Those colonies which were non-hemolytic were picked and re-streaked on rabbit blood agar plates to confirm their non-hemolytic activity.

Using this differential screening process, 36 non-hemolytic mutants were isolated in our laboratory from several hundred-thousand colonies. Each mutant collected was then tested for cell morphology and Gram stain. All were found to be Gram positive cocci, arranged in characteristic irregular clusters. To determine

FIGURE 1

Survival Curve

This figure shows the logarithm of the number of viable cells per ml after exposure to varying doses of ultraviolet light. Before exposure the number of viable cells per ml was 9×10^8.


VIABLE CELLS (Log No. / ml)
7
6
5
4
3
0
40
80
120
UV DOSE (erg/mm2/sec)

whether these ultraviolet mutants were stable, they were subcultured several times on Dolman-Wilson agar plates. Four mutants could not be subcultured over a period of time due to poor growth and therefore, were discarded.

It is known that staphylococcal cells probably carry one or more plasmids at any instant in time (33). Most plasmids are lost spontaneously from the bacterial cell at a high rate (33). The loss occurs when plasmid replication fails to keep up with the growth of the cell. Therefore, to determine whether the alpha toxin gene is on a plasmid or on a chromosome of Wood 46 strain, the spontaneous rate of loss of the marker was measured. Witte (82) has suggested that alpha toxin was coded on the plasmids of some strains. More recently the alpha toxin gene was found to be on a chromosome (14). We attempted to re-confirm this point.

S. aureus, Wood 46 strain, was grown at 37°C, under 10% CO_2 for 48 hours. The culture was centrifuged (6,000 x g, 20 min.), and the bacterial pellet was washed and diluted to give approximately one hundred colonies per petri dish. Samples were removed at T=1 hour, and T=2 hours, plated onto rabbit blood agar plates, and incubated until colonies were visible. At T=1 hour and T=2 hours, the number of non-hemolytic mutant colonies was counted, and this number was divided by the number of generations. The spontaneous rate of loss of the marker was found to be 1×10^{-8} mutations / cell / generation.

To determine the back mutation rate of the non-hemolytic mutant, P1, was centrifuged, diluted, and plated on rabbit blood agar plates.

The colonies which were hemolytic were counted. The back mutation rate was 1 x 10^{-8} mutations/ cell/ generation. Since both the back mutation rate, and the loss of the hemolytic marker were much lower than the maximum of 2 x 10^{-4} for plasmids, the alpha toxin gene is probably chromosomal.

By means of genetic mapping, the chromosomal location of the alpha toxin gene in S. aureus was sought. Very little is known about the genetics of S. aureus, except for the preliminary genetic mapping done by Altenbern (1, 2, 3) and Brown and Pattee (14). Using genetic transformation, Brown and Pattee (14) placed an alpha toxin marker between purine-110, and isoleucine-129 markers on the chromosome.

The chromosomal mapping method of Altenbern (3) was used to detect the position (replication time) for the alpha toxin gene. A gene will normally double its DNA when the DNA replicating fork passes it. If exposed to ultraviolet light, the mutation rate should also double. Altenbern's method (3) relies on the fact that there exists an initiation point on the chromosome for replication to occur. In the presence of phenyl ethyl alcohol (PEA or phenethanol) replication will only continue through to termination and reinitiation will not occur.

Briefly, 10 ml of an 18 hour culture of the mutant, P1, which was grown in T-soy broth, was added to 100 ml of T-soy broth (not inoculated). This culture was incubated for three hours at 37°C in a shaker. A DNA inhibitor, 0.4% phenyl ethyl alcohol, was then added to the culture. The mixture was then incubated for two hours at 30°C without shaking. At this point, all of the cells of the culture had finished chromosomal replication and were unable to

replicate any further. Afterwards, 40 ml of the culture was centrifuged, and the cells were resuspended in 100 ml of T-soy broth prewarmed at 30°C. At this time, the cells were fee from the PEA inhibition, were synchronized and, once again, chromosomal replication continued. The bacteria were then incubated at 30°C without shaking.

Five samples were removed at 5 minute intervals, and each sample was removed, centrifuged, and resuspended in 5 ml of PBS. Each sample was then irradiated for 80 seconds with ultraviolet light. The cells were then diluted and plated on rabbit blood agar plates to determine if an increase in the number of mutations displaying hemolytic activity had occurred. At T=0 and T-5 min., there were 10 hemolytic colonies per 100,000 colonies. At both T=10 and T=20 min., there were 19 colonies per 100,000 colonies. The number of mutants induced in the ultraviolet exposed cells was constant for the initial time period. This was followed by a sharp increase by a factor of approximately 2. Therefore, the duplication of the alpha toxin gene occurred during the first 10 minute time period of the 60 minute time period required for chromosomal replication.

The Altenbern method has limited value for the accurate mapping of the alpha toxin gene because a differential rather than a selective test had to be used.

The mutants of S. aureus were examined for their ability to produce other extracellular products in the hope that a simple correlation with alpha toxin production might exist. Such a correlation would allow the development of a selective rather than a differential test. There are a number of examples in which the

loss of one product is simultaneously associated with the loss of other products. For example, nuclease mutants also lose their free coagulase and their beta-hemolytic activity (62). In one study, methicillin-resistant strains showed genetic correlation of methicillin resistance, enterotoxin B and penicillinase (32). Winkler et al (77) have shown genetic correlation between the loss of beta-hemolysis and the gain of fibrinolysin production. Even more pertinent, McClatchy and Rosenblum (56) have identified a group of non-hemolytic alpha toxin mutants that had simultaneously lost their ability to produce fibrinolysin but were similar to their parent strain in the production of lipase, deoxyribonuclease, and fermentation of mannitol. These mutants possibly represent regulatory gene mutations.

The following biochemical tests were performed on each mutant. DNase, phosphatase, free coagulase, lipase, gelatinase, and fibrinolysin. Also, each mutant was checked for "hot-cold" lysis, that is, testing for the presence of beta-hemolysin. The results of the above test are summarized in Table 1. From these data, there does not appear to be any correlation between the loss of any extracellular products and the loss of hemolytic activity in the mutants. These data disagree with the data obtained by McClatchy and Rosenblum (57).

McClatchy and Rosenblum postulated a regulatory gene that controlled both alpha toxin and fibrinylysin production (57). This conclusion was based in part upon the observation that 5 out of 7 mutants that were fibrinolysin positive were also CRM positive. Therefore, we sought to confirm whether there was a correlation between the loss of fibrinolysin production, and the loss of alpha

TABLE I

BIOCHEMICAL PROPERTIES OF ALPHA TOXIN MUTANTS OF *Staphylococcus aureus*

\+ positive test

\- negative test

Mutant No.	MANNITOL	DNAse	PHOSPHATASE	COAGULASE	CATALASE	B-HEMOLYSIN	LIPASE	GELATINASE	FIBRINOLYSIN
1	-	-	-	-	+	-	+	-	-
2	+	+	+	+	+	+	+	+	+
3	+	-	-	-	+	-	-	-	-
4	-	-	+	-	+	-	-	-	-
5	+	-	-	-	+	-	-	+	-
6	+	-	-	-	+	-	-	-	-
7	+	+	+	+	+	-	+	+	+
9	-	-	-	-	+	-	-	-	-
10	-	-	-	-	+	-	-	-	-
11	-	-	-	-	+	-	-	-	-
12	-	-	+	-	+	-	-	+	-
13	-	-	+	-	+	-	-	-	-
14	+	-	-	-	+	-	-	-	-
15	-	-	+	-	+	-	-	-	-
16	-	+	+	-	+	-	-	-	-
17	+	+	+	+	+	+	+	+	+
18	+	-	-	-	+	-	-	-	-
19	-	-	+	-	+	-	-	-	-
20	+	-	-	-	+	-	-	-	-
22	+	-	-	-	+	-	-	-	-
23	+	-	-	-	+	-	-	-	-
24	-	-	+	-	+	-	-	-	-
25	+	+	+	+	+	-	+	+	+
26	-	-	+	-	+	-	-	-	-
27	-	-	+	-	+	-	-	-	-
28	-	-	-	-	+	-	-	-	-
31	-	-	-	-	+	-	-	-	-
32	-	-	+	-	+	-	-	-	-
33	-	-	+	-	+	-	-	-	-
34	-	-	+	-	+	-	-	-	-
35	-	-	+	-	+	-	-	-	-
P1	+	+	+	+	+	-	+	+	+
W46	+	+	+	+	+	+	+	+	+

toxin production using more qualitative tests. We found 5 out of 25 mutants that were fibrinolysin positive and CRM positive, and 20 out of 25 mutants that were fibrinolysin negative and CRM positive. However, McClatchy and Rosenblum found only 2 out of 7 mutants that were fibrinolysin negative and CRM positive. Thus, while a regulatory gene for alpha toxin may exist, the pleiotropic nature of its regulation is in doubt.

Since there was no correlation of alpha toxin loss with the extracellular products, a determination of the antibiotic resistance of the mutants was sought, using common antibiotics. Since antibiotic resistance can be located on chromosome or on a plasmid, antibiotic resistance can be linked with other antibiotic markers. If a correlation with an antibiotic marker did exist, a very easy selection system could be used, for example, the non-hemolytic mutants could be screened on rabbit blood agar plates supplemented with an antibiotic. Difco antibiotics sensitivity disks were used to compare the mutants' sensitivity or resistance to several widely used antibiotics with the sensitivity of S. aureus, Wood 46 strain. (Table II). There is no apparent correlation with the loss of hemolysis and the ability of the mutants to become sensitive or resistant to any of the tested antibiotics. Therefore, a simple selection mechanism could not be developed and further attention was focused on the characterization of the mutants already collected.

Each mutant toxin preparation was tested to determine its potential lethal activity in mice. It is known that alpha toxin is fatal to mice within 24 hours, but when maintained at an elevated temperature (60°C for 1/2 hour) the alpha toxin (toxoid) preparation is not. Thirty-seven experiments were performed in each of which

TABLE II

SENSITIVITY OF ALPHA TOXIN MUTANTS OF Staphylococcus aureus TO VARIOUS ANTIBIOTICS

R = resistant

S = sensitive

I = intermediate

Mutant No.	Bacitracin 10 units	Polymycin B 100 units	Chlorotetra - cycline 30 µg	Penicillin G 5 units	Dihydrostrepto- mycin 10 µg	Erythromycin 5 µg	Tetracycline 30 µg	Chloramphenicol 30 µg
1	S	I	S	S	S	S	S	S
2	S	R	S	S	S	S	S	S
3	S	S	S	S	S	S	S	S
4	S	R	S	S	S	S	S	S
5	S	S	S	S	S	S	S	S
6	S	S	S	S	S	S	S	S
7	S	I	S	S	S	S	S	S
9	S	S	R	S	S	S	R	S
10	S	S	R	S	S	S	R	S
11	S	S	S	S	S	S	S	S
12	S	I	S	S	S	S	S	S
13	S	S	S	S	S	S	S	S
14	S	S	S	S	S	S	S	S
15	S	I	S	S	S	S	S	S
16	S	S	S	S	S	S	S	S
17	S	I	S	S	S	S	S	S
18	S	S	S	S	S	S	S	S
19	S	S	S	S	S	S	S	S
20	S	S	S	S	S	S	S	S
22	S	S	R	S	S	S	R	S
23	S	S	S	S	S	S	S	S
24	S	S	S	S	S	S	S	S
25	S	R	S	S	S	S	S	S
26	S	S	S	S	S	S	S	S
27	S	I	S	S	S	I	S	S
28	S	I	R	S	S	S	R	S
31	I	S	S	S	S	S	S	S
32	S	I	S	S	S	S	S	S
33	S	I	S	S	S	S	S	S
34	S	I	S	S	S	S	S	S
35	S	I	S	S	S	S	S	S
P1	S	R	S	S	S	S	S	S
W46	S	R	S	S	S	S	S	S

either 10 or 12 C3H or C57 mice were injected intraperitoneally with 1.0 ml of each freshly prepared mutant toxin. Control animals were injected with 1.0 ml Dolman-Wilson medium, alpha toxin, or alpha toxoid. All of the animals injected with alpha toxin died; all the others survived. Apparently, the loss of hemolytic activity is accompanied by a loss of lethal activity.

When one mutant, P1, was inadvertently left out at room temperature for 2 hours, zones of lysis were observed around all its colonies. As a result, a temperature shift experiment was performed on all the mutants. Each mutant was grown on rabbit blood agar plates at 37°C and 24°C. After colonies became visible, each mutant that was grown at 37°C, was incubated at 24°C, for 2 hours, and each mutant that was grown at 24°C was incubated at 37°C for two hours. Control cultures were left at the original incubation temperatures. Colonies were examines for zones of lysis. None of the mutants were hemolytic as a result of a shift in the temperature with the exception of P1. P1 was hemolytic when grown at 24°C but not when grown at 37°C. This phenomenon was later studied in greater detail as will be described in Chapter 3.

CHAPTER 2

IMMUNOLOGICAL CHARACTERIZATION OF ALPHA TOXIN MUTANTS OF Staphylococcus aureus WOOD 46 STRAIN

After biochemical characterization, the mutants were studied immunologically. Immunodiffusion was a suitable technique because this technique is useful for the detection of small amounts of immunochemically active proteins.

Immunodiffusion tests in agar gel slides were performed with anti-alpha toxin to determine which mutant produced a cross-reacting material (CRM). Clarified culture fluid of each mutant was diffused against antibodies to purified alpha toxin. It was found that the following mutants were positive for CRM and gave lines of identity with alpha toxin: 1, 2, 7, 9, 14, 17, 25, 32, 34, 35, and P1. The tests were negative for all of the other mutants. Figure 2 shows immunodiffusion patterns for each positive CRM mutant of this test.

In order to further characterize the mutants immunologically, immunoadsorption was then employed. This method has been used to purify antigens and toxins (38) and to prepare beads coated with anti-insulin (26). The antibodies used in this experiment were anti-alpha toxin (IgG) and anti-binding antibody (AB-Ab). There are two species of antibodies to alpha toxin. The indirect hemaglutinating antibody or IHA-Ab and the anti-binding antibody or AB-Ab (43).

The IHA-Ab was removed from a previously prepared immune serum by absorption with toxoid-coated erythrocytes, thus leaving the AB-Ab present in the serum. A 5 mg/ml solution of each of the antibodies, anti-alpha toxin (IgG) and AB-Ab was mixed with 40

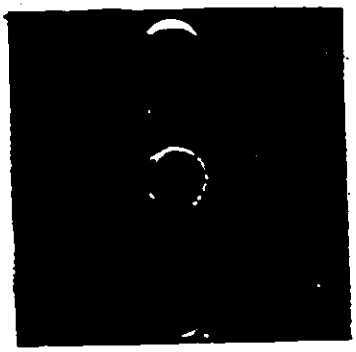
FIGURE 2

Immunodiffusion patterns

The supernatants of the culture fluid of the mutants are diffused against anti-alpha toxin. Only these supernatants that gave a precipitin line are shown. The center well contained undiluted anti-alpha toxin. The top, left, and bottom wells contained the supernatants from centrifuged culture fluids. The right hand well contained the supernatant of the centrifuged culture fluids of strain Wood 46. For Pl see Figure 12a.

1

2



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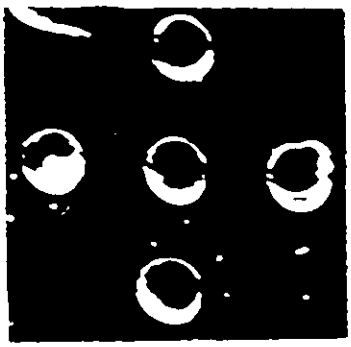

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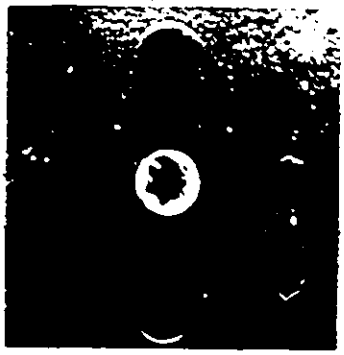

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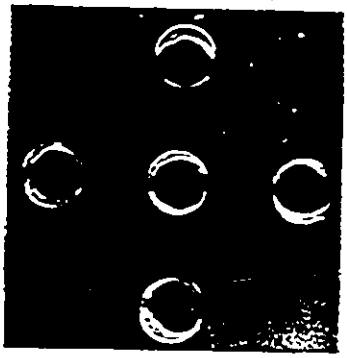

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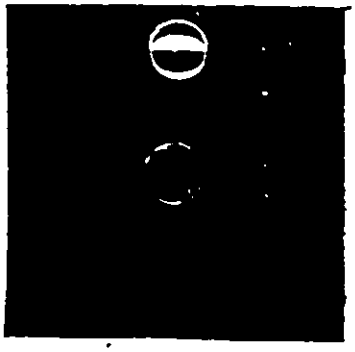
25

32



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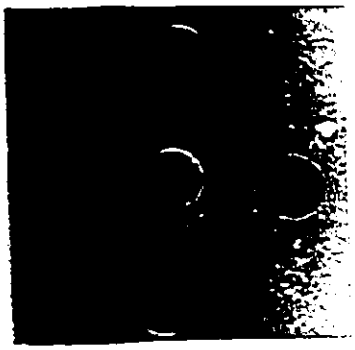

grams of activated CNBr beads, and the CRM material was then bound to these antibodies. Aliquots of the coupled beads (0.1 ml) were mixed with a 0.2 ml solution of each of the 32 mutants, and were combined with controls comprising alpha toxoid, PBS, and Dolman-Wilson medium. The coupled beads were mixed with each of the samples and each of the controls at room temperature and afterwards were incubated overnight at 4°C. Resulting supernatants were removed from the samples and controls, and the coupled beads were washed three times in PBS and allowed to settle to the bottom of the test tubes. This wash removed any excess CRM added to the samples.

Alpha toxin (0.1 ml) was then added to the samples and the controls and these mixtures were then incubated for 1.5 hours at room temperature. Each supernatant was titrated to determine residual hemolytic activity. (See hemolytic titrations in methods and materials for procedure). The hemolytic activity was measured in HU_{50}, and the data was normalized.

The results of the previously described immunoadsorbent IgG bead test are shown in Table III. The alpha toxoid had a titer of 175 HU_{50}. This value represents the upper limits of the HU_{50}, and was set at 100% after normalization. The PBS and Dolman-Wilson medium control had a titer of 47 HU_{50}. This value represents the lower limit of the HU_{50} and was set at 0% after normalization. The higher the value, the better the possibility that the CRM tested was bound to the activated agarose beads. Any CRM which had a normalized value above 50% was characterized as positive (i.e. possessing an anti-binding and/or IHA determinant. Any

TABLE III

IMMUNOADSORBENT IgG BEAD TEST

MUTANT NO.	HU_{50}	NORMALIZED
1	104	44
2	104	44
3	84	29
4	128	63
5	104	44
6	97	39
7	128	63
9	84	29
10	97	39
11	104	44
12	111	50
13	119	56
14	169	95
15	119	56
16	104	44
17	111	50
18	79	25
19	84	29
20	119	56
22	194	114
23	147	78
24	90	34
25	128	63
26	169	95
27	97	39
28	104	44
31	157	86
32	157	86
33	111	50
34	147	78
35	157	86
P1 37 C	147	78
toxoid	175	100
toxin titer	495	-
PBS and DW MEDIUM	47	0

CRM which had a normalized value below 50% was characterized as negative (i.e. the alpha toxin molecules were bound to the activated agarose beads instead of the CRM). According to this test, 18 strains were positive, and all other strains had both determinants missing or were unavailable.

The results of the immunoadsorbent AB-Ab bead test are shown in Table IV. The alpha toxin control had a titer of 725 HU_{50}. This value represents the upper limit of the HU_{50}, and was normalized at 100%. The PBS and Dolman-Wilson controls had a titer of 157.6 HU_{50}. This value was normalized at 0%. Any mutant which had a normalized value of 40% or greater was characterized as being positive (i.e. the mutant CRM had an AB-Ab determinant present). Any mutant CRM which had a normalized value below 40% was characterized as being negative (i.e. the alpha toxin not the mutant CRM was bound to the AB-Ab bead complex). If a value of 50% had been chosen as the cut-off point, mutant 17 and mutant 34 which are shown to be weakly positive, would have been characterized as negative. However, this was not done because mutant 34, which had already been isolated in this laboratory, gave a line of identity with anti-alpha toxin in an immunodiffusion test (Fackrell, unpublished).

Based on the immunoadsorbent AB-Ab bead test, 8 mutants were characterized as being positive. The remainder of the mutants were characterized as being negative. The immunoadsorbent IgG bead test and immunoadsorbent AB-Ab bead test were then compared to determine which mutants were IHA+, AB-, and which were IHA-, AB+. For example, mutant 25 was positive for the immunoadsorbent IgG bead test. Therefore, mutant 25 had either an AB-Ab determinant

TABLE IV

IMMUNOADSORBENT AB-Ab BEAD TEST

MUTANT NO.	HU_{50}	NORMALIZED
1	550	69
2	208	9
3	169	2
4	256	17
5	295	24
6	169	2
7	111	0
9	338	32
10	223	12
11	195	6
12	223	12
13	147	0
14	208	9
15	201	8
16	315	28
17	388	41
18	445	51
19	231	13
20	157	0
22	223	12
23	588	76
24	194	6
25	223	12
26	256	17
27	256	17
28	137	0
31	137	0
32	512	62
33	223	12
34	388	41
35	588	76
P1 37 C	630	83
toxoid	548	70
toxin titer (beads)	725	100
PBS and DW MEDIUM	157	0

and/or an IHA-Ab determinant present. Since mutant 25 was negative for the immunoadsorbent AB-Ab bead test, it was assumed that it only had an IHA-Ab determinant. As expected, 6 out of 8 mutants characterized as being positive in the immunoadsorbent AB-Ab bead test were also positive for the immunoadsorbent IgG bead test, the remaining two mutants were characterized as being negative in the immunoadsorbent IgG bead test. This may have occurred due to experimental error, or because the IgG preparation contained more IHA-Ab than AB-Ab. In any event, mutants 1 and 18 remained in doubt, and consequently, have been grouped into class 5, the uncertainty class. In each case, if the mutant did not fit with the immunoadsorbent IgG bead test, it was placed in class 5.

An IHA-Ab consumption test was performed which involved incubation of CRM with IHA positive antisera. The IHA titer was then compared both before and after absorption.

IHA positive antiserum (50 µl) was mixed with a 0.5 ml solution of crude CRM and was incubated at room temperature for 1.5 hours. A two-fold dilution series was made with 50 µl of the above mixture in 50 µl of PBS and 50 µl of lysine-glutaraldehyde coated erythrocytes (LGCE) was then added to each sample. After incubation for 90 minutes at 4°C, hemagglutination titers were read. Antisera mixed with PBS, Dolman-Wilson medium and toxoid were the controls used. The results are shown in Table V.

In the presence of an IHA determinant, the IHA-Ab binds the mutant CRM. Thus, in the presence of LGCE there was a decreased hemagglutination titer compared to the controls. If a hemagglutination titer has a value of less than 10, this indicated that the

TABLE V

IHA-Ab CONSUMPTION TEST

MUTANT NO.	IHA TITER
1	20
2	80
3	10
4	10
5	80
6	40
7	10
9	160
10	160
11	160
12	20
13	20
14	10
15	10
16	10
17	80
18	10
19	160
20	20
22	80
23	80
24	80
25	10
26	10
27	10
28	20
31	80
32	10
33	80
34	10
35	10
P1CRM 37 C	10
toxoid	10
PBS	160

mutant consumed the IHA-Ab and, therefore, has an IHA determinant. If the titer had a value greater than 10, this indicated that the IHA determinant was missing or unavailable. A titer of 10 was chosen to ensure that the CRM was negative.

The PBS control had a titer with a value of 160. In this IHA-Ab consumption test 15 mutants were characterized as being positive, all of the other mutants were characterized as being negative, or the IHA determinant was considered to be missing or unavailable.

The immunoadsorbent IgG bead test was compared with the IHA-Ab consumption test. As expected, 11 out of 15 mutants were characterized as being positive in the IHA-Ab consumption test, were also positive for the immunoadsorbent IgG bead test. Mutants 3, 16, 18 and 27 which were characterized as being positive for the IHA-Ab consumption test, were negative for the immunoadsorbent IgG bead test. As was done previously with mutants 1 and 18 these mutants were placed into class 5, the uncertainty class, because they did not fit with the immunoadsorbent IgG bead test.

Erythrocytes were coated with CRM from each of the mutants, washed with PBS and then exposed to IHA-Ab. A high IHA titer indicated the presence of both the IHA determinant and a binding site. A low IHA titer indicated that the IHA determinant was missing or unavailable.

Mutant CRMS (10 ml) were incubated with 0.2 ml of packed rabbit erythrocytes. Samples were mixed, incubated for 2 hours at room temperature, centrifuged, and any resulting supernatants were discarded. The coated erythrocytes were washed three times

in PBS and brought up a final volume of 10 ml (2% V/V suspension). A serial two-fold dilution of anti-alpha toxin was made in PBS, and an equal volume of CRM coated erythrocyte solution was added to each mixture so that a final volume of 100 µl was obtained. After an hour incubation, the hemagglutination titers were recorded to determine whether the IHA-Ab was bound to the CRM. An agglutination titer of 8 or greater was considered as being positive for the presence of an IHA determinant. The toxoid control had a titer of 8. The results in the Table VI show that 12 out of the 32 mutants were considered negative, or non-reactive for the IHA determinant.

The immunoadsorbent IgG bead test was compared with the IHA test. As expected, 11 out of 12 mutants characterized as being positive in the IHA-Ab test were also positive for the immunoadsorbent IgG bead test. Mutant 27 was not considered as positive for the immunoadsorbent IgG bead test but was considered as positive for the IHA-Ab test. Therefore, mutant 27 was also placed into class 5, the uncertainty class.

As a result of the five previous tests the mutants of S. aureus were placed into five tentative classes. The first class included mutants which were IHA positive, and not reactive to AB-Ab. The second class included mutants which were IHA negative and did react with AB-Ab. The third class of mutants lacked both antigenic determinants, while the fourth class of mutants had both sets of antigenic determinants. The nature of some mutants was uncertain and they were placed into the fifth class pending further characterization. A summary of all these five tests is shown in Table VII. Table VIII summarizes which class each mutant was placed in.

TABLE VI

MUTANT-COATED ERYTHROCYTES IHA-AB TEST

MUTANT NO.	IHA-Ab TITER
1	4
2	0
3	8
4	0
5	4
6	4
7	8
9	0
10	0
11	4
12	8
13	8
14	8
15	0
16	0
17	0
18	4
19	2
20	2
22	0
23	0
24	2
25	8
26	16
27	8
28	0
31	4
32	8
33	4
34	8
35	8
P1CRM 37 C	16
Toxoid	8
PBS	0

TABLE VII

SUMMARY TABLE OF ALL IMMUNOLOGICAL TESTS PERFORMED ON THE MUTANTS

Mutant Number	IgG Bead Test	AB-Ab Bead Test	Immuno-diffusion Test	IHA-Ab Consump-tion Test	Mutant-coated IHA-Ab Test
1	-	+	+	-	-
2	-	-	+	-	-
3	-	-	-	+	+
4	+	-	-	+	-
5	-	-	-	-	-
6	-	-	-	-	-
7	+	-	+	+	+
9	-	-	+	-	-
10	-	-	-	-	-
11	-	-	-	-	-
12	+	-	-	+	+
13	+	-	-	-	+
14	+	-	+	+	+
15	+	-	-	+	-
16	-	-	-	+	-
17	+	+	+	-	-
18	-	+	-	+	-
19	-	-	-	-	-
20	+	-	-	-	-
22	+	-	-	-	-
23	+	+	-	-	-
24	-	-	-	-	-
25	+	-	+	+	+
26	+	-	-	+	+
27	-	-	-	+	+
28	-	-	-	-	-
31	+	-	-	-	-
32	+	+	+	+	+
33	-	-	-	-	-
34	+	+	+	+	+
35	+	+	+	+	+
P1	+	+	+	+	+

TABLE VIII

MUTANT CLASSES

CLASS	IHA-Ab	AB-Ab	MUTANT NO.
1	+	-	4, 7, 12, 14, 15, 25, 26,
2	-	+	17, 23
3	-	-	5, 6, 10, 11, 19, 24, 33
4	+	+	32, 34, 35, P1
5	?	?	1, 2, 3, 9, 13, 16, 18 20, 22, 27, 28, 31

CHAPTER 3

CHARACTERIZATION OF THE MUTANT P1

This chapter considers in detail the mutant P1, which was found to be particularly interesting since it was temperature-sensitive. As previously mentioned, P1 was non-hemolytic when grown at 37°C on rabbit blood agar plates (Figure 3), but was hemolytic when grown at 24°C on these plates (Figure 4). Conversely, S. aureus Wood 46 is hemolytic at both temperatures.

After P1 was grown on rabbit blood agar plates at 37°C the temperature was changed to 24°C, after which zones of lysis were observed around the colonies (Figure 5a, 5b).

Initially, it was thought that P1 multiplied slower than S. aureus Wood 46 and hence produced less alpha toxin. However, a comparative growth curve showed that P1 and S. aureus Wood 46 grew equally well (Figure 6). For this growth curve experiment, the initial cultures of P1 and S. aureus Wood 46 were adjusted to the same Klett reading. A one ml solution of both P1 and S. aureus Wood 46 were inoculated separately into 1000 ml of Dolman-Wilson medium. One flask of each culture was incubated at 37°C and 24°C. Cultures were incubated under 10% CO_2 and air in an incubator shaker. At 8 hour intervals, 1 ml samples were removed and a plate count performed in triplicate. Figure 6 shows no apparent difference in the growth rate between P1 and S. aureus Wood 46 when compared at either 24°C and 37°C. A lag phase of 8 to 10 hours was observed when the cultures were incubated at 24°C, but not at 37°C.

The amount of hemolytic activity of P1CRM at 24°C was

FIGURE 3

P1 grown on a rabbit blood agar plate at 37°C to show colonial morphology and non-hemolytic colonies.

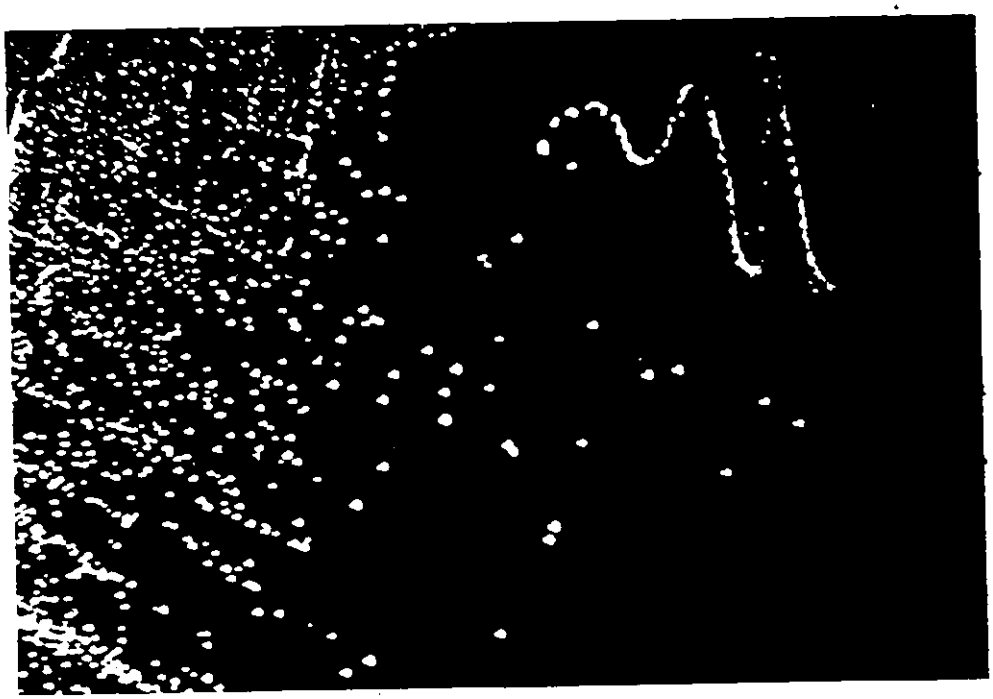

FIGURE 4

P1 grown on a rabbit blood agar plate at 24°C to show zones of lysis around the colonies.

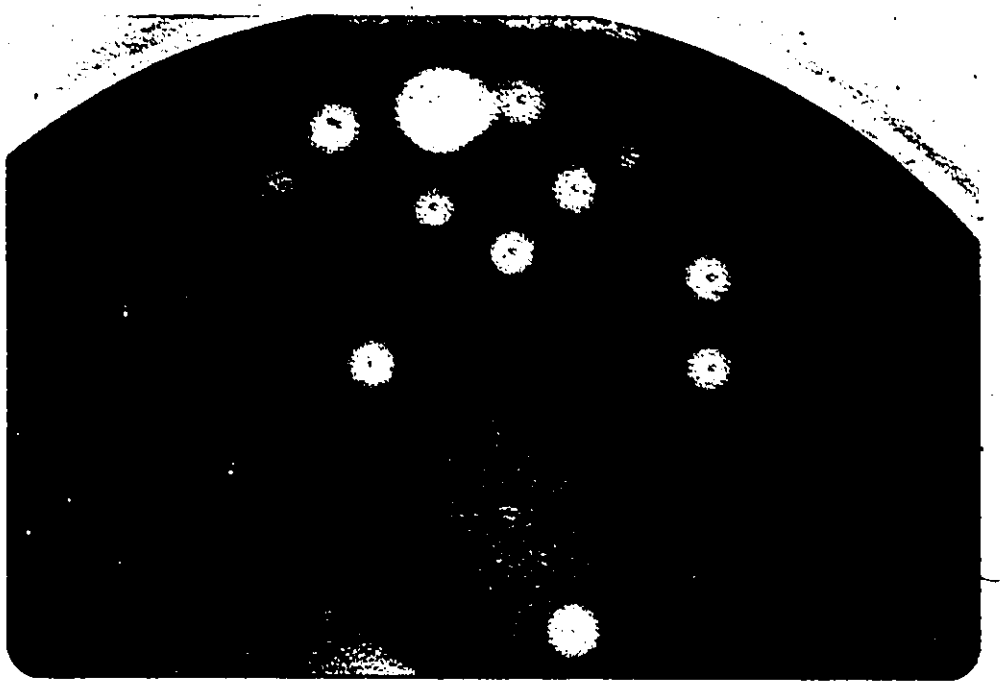

FIGURE 5

Effects of change in temperature on production of hemolysis by mutant P1. P1 was grown on a rabbit blood agar plate at 37°C until colonies were visible (Figure 5a). No hemolysis was observed. The plate was then incubated at 24°C. Zones of lysis were observed within 2 to 3 hours after the temperature shift (Figure 5b).

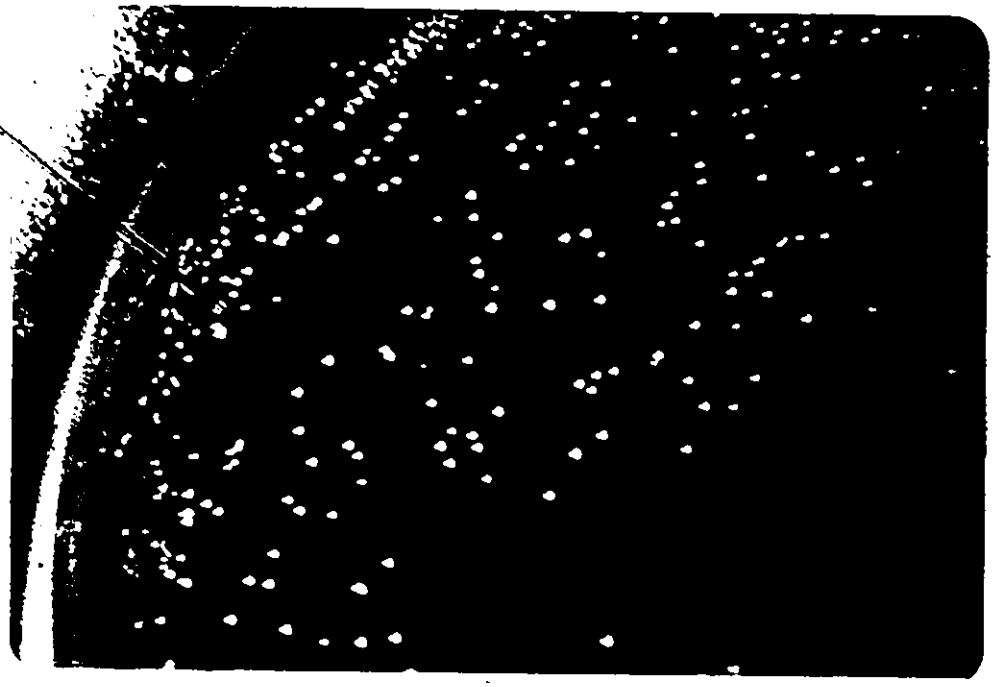

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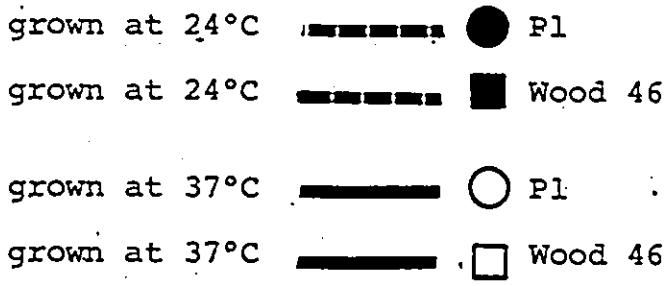
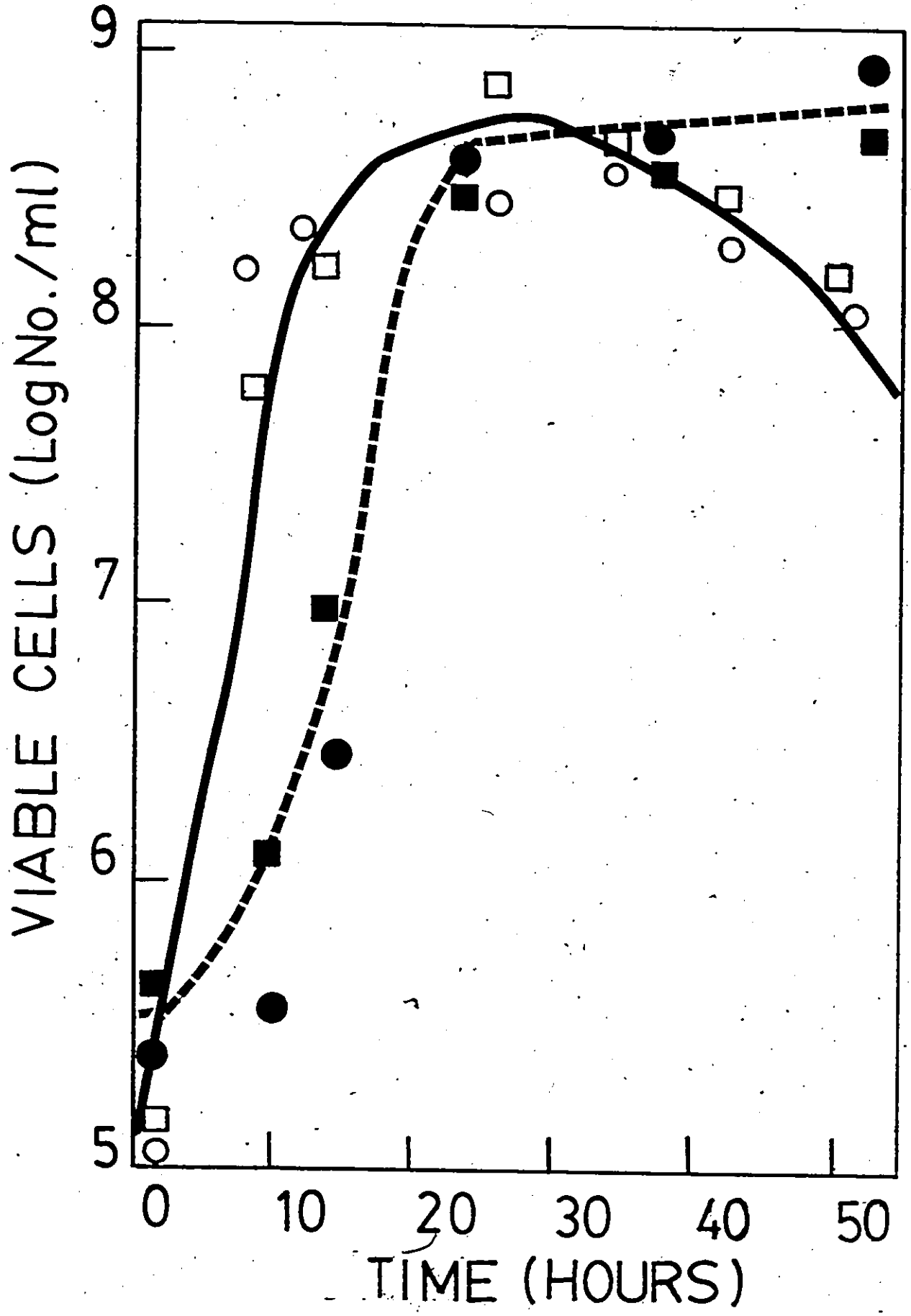
FIGURE 6

Comparison of the growth curves for S. aureus strains P1 and Wood 46.

Plot of the logarithm of viable cells against time for P1 and Wood 46 when grown at 24°C and 37°C.




VIABLE CELLS (Log No./ml)
9
8
7
6
5
0
10
20
30
40
50
TIME (HOURS)

compared with that of S. aureus Wood 46. Both P1 and S. aureus Wood 46 were incubated for 3 days at 24°C and 37°C. At various time intervals, aliquots were removed, centrifuged and the resulting supernatants titrated for hemolytic activity. All samples were titrated in triplicate at 37°C (Figure 7a, 7b). S. aureus Wood 46 released alpha toxin at the same rate whether grown at 24°C or at 37°C. Its titer was not affected by the temperature of incubation of the assay. P1 produced a hemolytic toxin at 24°C but not at 37°C, and its rate of production at 24°C was less than that of S. aureus Wood 46. Immunodiffusions were set up for all of the samples with anti-alpha toxin. The resulting supernatants were removed every 8 hours during bacterial growth and tested for CRM. Precipitin lines were detected at 8 hours of bacterial growth for both P1 and S. aureus Wood 46, whether grown at 24°C or at 37°C.

This experiment also suggested that P1CRM, when grown at 24°C, was lytic when assayed at both temperatures. However, the apparent decrease in hemolytic activity suggested that the P1CRM was more labile than S. aureus Wood 46. Therefore, a more sensitive comparison of heat lability was performed.

Supernatants from cultures of P1 and S. aureus Wood 46 were grown at 24°C and samples were later incubated at various temperatures. Aliquots were removed from each of the samples tested after 30 minutes, 60 minutes, and 90 minutes of incubation. Fifty microliters of each sample were serially diluted in 50 µl of PBS in Microtitre plates® and assayed at 24°C for 1.5 hours. Figure 8 shows that P1CRM and alpha toxin have similar heat denaturation

FIGURE 7

Comparisons of the hemolytic activity produced by S. aureus strains P1 and Wood 46 when grown at 24°C and 37°C.

Growth temperature 24°C	●	P1
Growth temperature 24°C	■	Wood 46
Growth temperature 37°C	○	P1
Growth Temperature 37°C	□	Wood 46

Fig. 7(a) - Hemolytic activity was titrated at 24°C.

Fig. 7(b) - Hemolytic activity was titrated at 37°C.

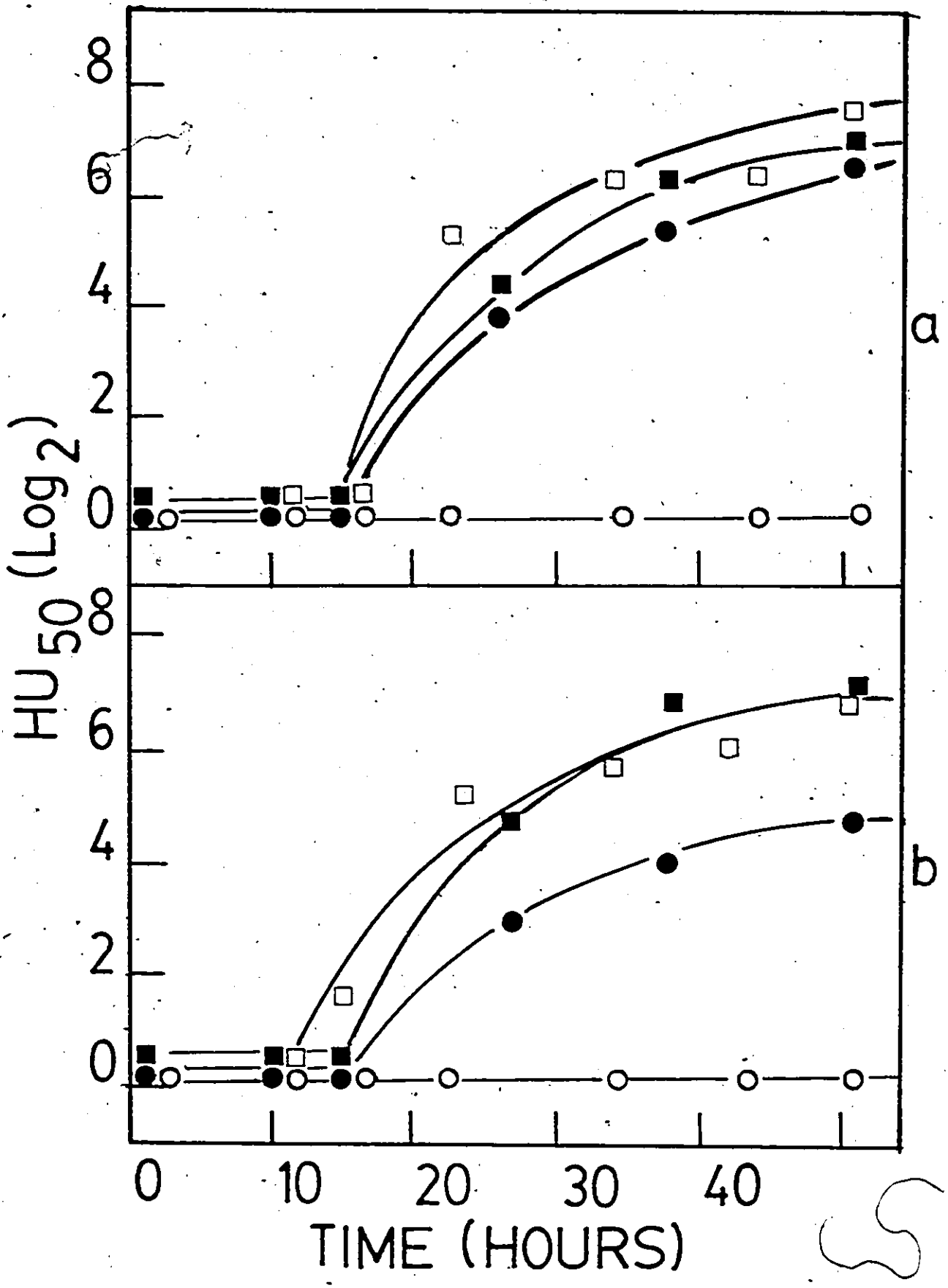
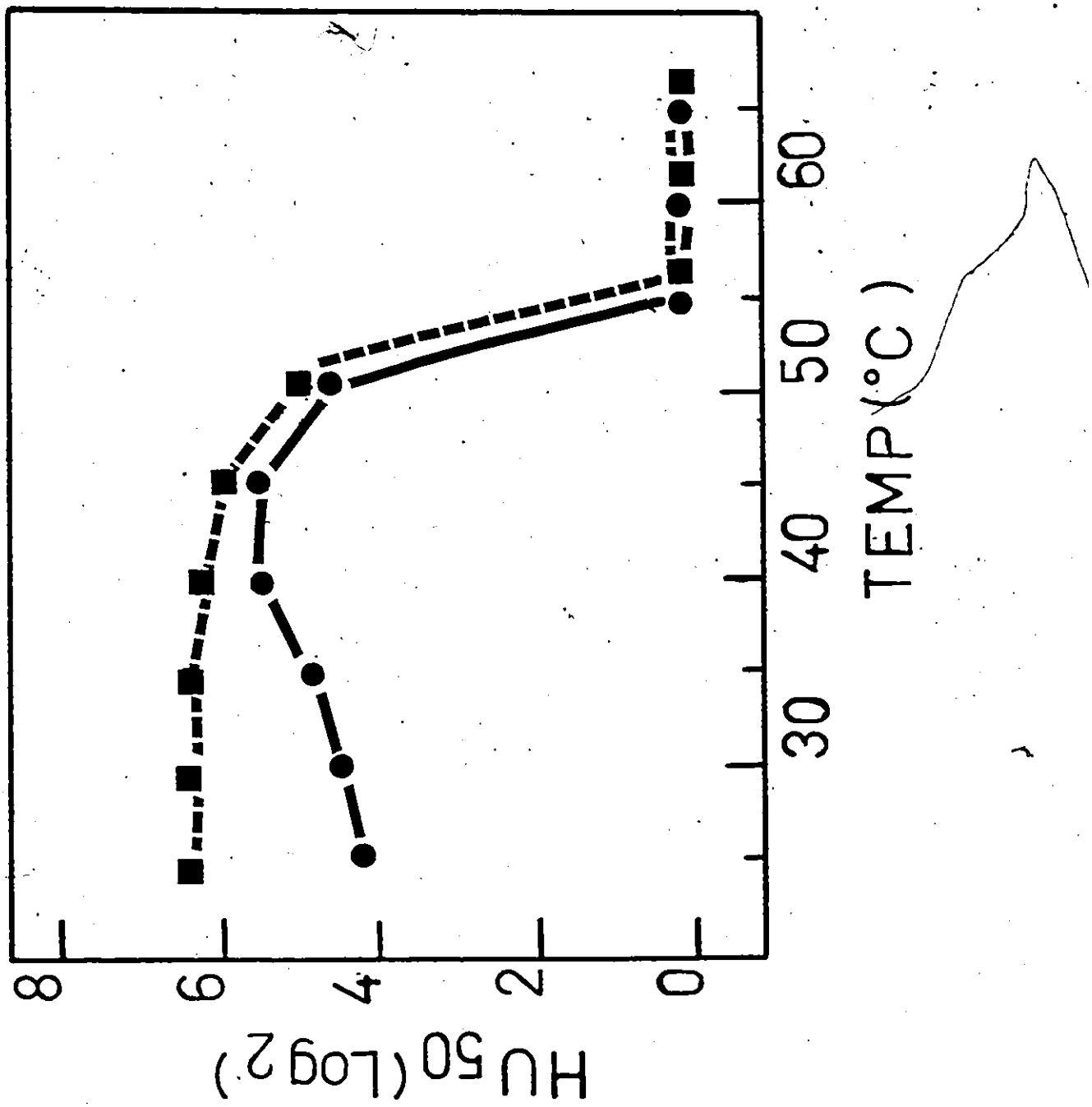

8
6
4
2
0
a
8
6
4
2
0
b
HU_{50} (Log_2)
0
10
20
30
40
TIME (HOURS)

FIGURE 8

Comparison of the thermodenaturation profiles for PlCRM and alpha toxin.

Supernatants from Pl and Wood 46 cultures were incubated at various temperatures. Aliquots were removed from each sample at 30, 60 and 90 minutes of incubation. Samples were diluted and titrated for hemolytic activity at 24°C. The same profile was obtained for Pl and Wood 46 whether the samples were incubated for 30, 60 or 90 minutes.


HU50 (Log 2)
8
6
4
2
0
30
40
50
60
TEMP(°C)

profiles at the upper temperatures between 40°C and 60°C. This same profile was obtained whether the samples were incubated for 30, 60 or 90 minutes. PlCRM was less stable at the lower temperatures (24°C) than alpha toxin. PlCRM appeared to be more heat stable at 37°C than at 24°C. These results suggest that at 37°C, Pl does not lose its hemolytic activity because of immediate denaturation of its PlCRM.

It is known that alpha toxin spontaneously polymerizes and forms an insoluble non-hemolytic precipitate, and that this process can be enhanced by maintaining alpha toxin at 60°C for 1/2 hour. Hemolytic activity can be restored to this precipitate by treatment with 8 M urea (28).

An attempt was made to renature the PlCRM using this method. Briefly, samples of PlCRM 24°C and PlCRM 37°C were maintained at 60°C for 12 hours to precipitate the proteins. The precipitates were resuspended in 7 ml of 8 M urea. Each sample was then incubated at 24°C and 37°C for one hour and then dialyzed overnight against PBS to remove the urea at the same temperatures. All of the samples were then titrated at 24°C and 37°C. PlCRM 24°C and alpha toxin became non-hemolytic when maintained at 60°C for several hours but became hemolytic after treatment with the urea. (Table IX). PlCRM 37°C, originally non-hemolytic, remained non-hemolytic after heating, and was unable to become hemolytic after treatment with the urea at either 24°C and 37°C. Therefore, PlCRM 37°C and PlCRM 24°C are not the same proteins. However, PlCRM 24°C is similar to alpha toxin in that it can be reactivated to a hemolytic state.

TABLE IX

UREA REACTIVATION TEST

Titrated at 24°C	P1CRM 24°C $HU_{50}(log_2)$	P1CRM 37°C $HU_{50}(log_2)$	ALPHA TOXIN $HU_{50}(log_2)$
No treatment	3.8	0	7.2
Heat treatment	0	0	0
Urea and dialyzed at 24°C	4.8	0	6.3
Urea and dialyzed at 37°C	4.6	0	6.3
Titrated at 37°C			
No treatment	3.8	0	7.2
Heat treatment	0	0	0
Urea and dialyzed at 24°C	4.6	0	6.2
Urea and dialyzed at 37°C	4.2	0	7.8

In order to study the physical, biological, and immunological characteristics of P1CRM 37°C more closely, it was first purified.

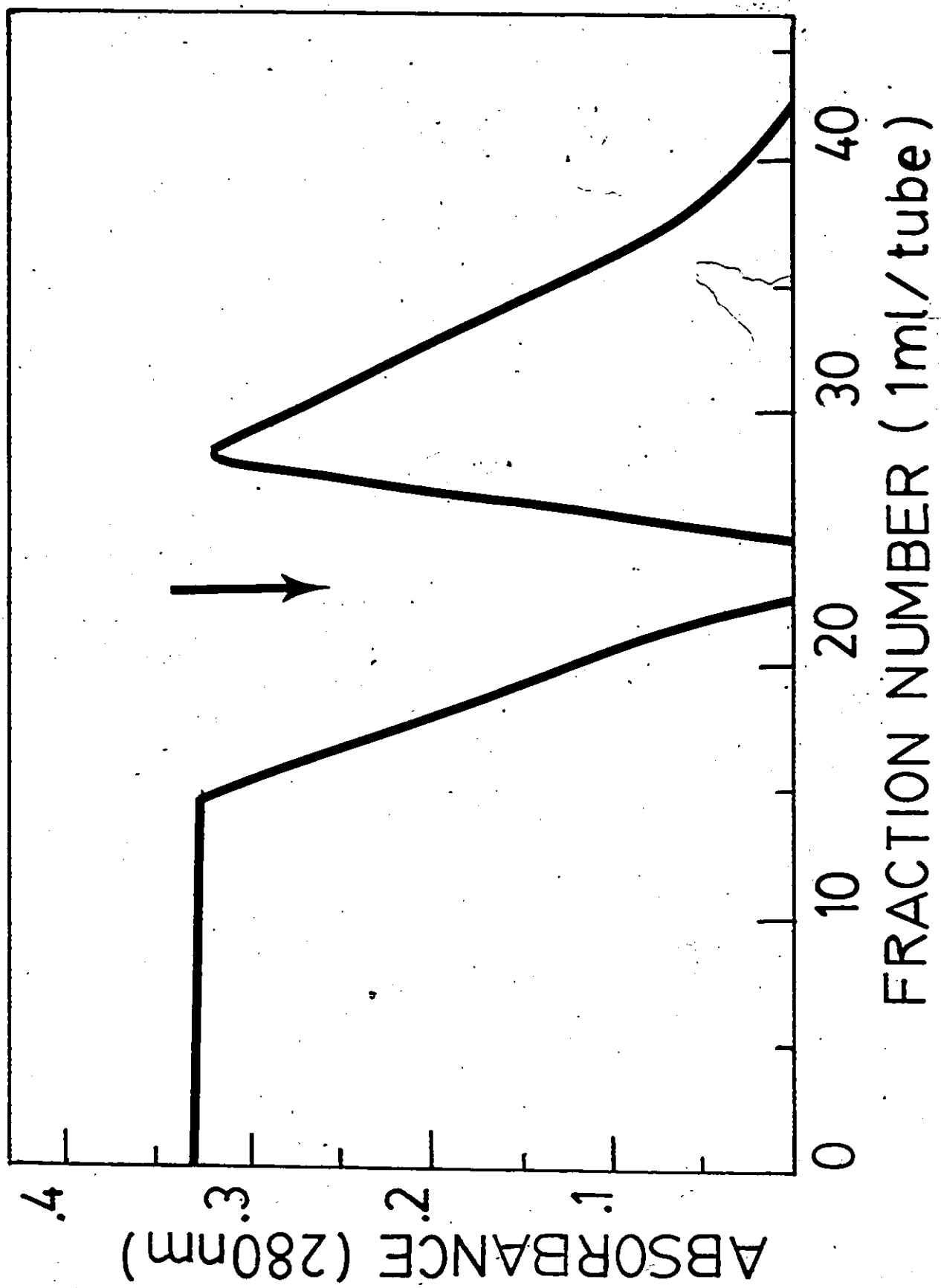
There are a number of methods available to purify alpha toxin. The method of Wiseman (81), and ammonium sulphate precipitation was employed to purify P1CRM 37°C. Since P1CRM 37°C was not hemolytic no quantitative purification factor could be determined. The method of Dalen (28) was used to purify both P1CRM 37°C and P1CRM 24°C. The purity of P1CRM 37°C could not be tested. The purity of P1CRM 24°C could be tested and the titer increased only four-fold.

Purification of P1CRM 37°C was best achieved by using the method of immunoaffinity chromatography. Briefly, Sepharose 4B was activated with CNBr, and then anti-alpha toxin (IgG) was added so that it was covalently linked to the beads as described previously. A 10 ml column was constructed and the matrix was washed extensively with PBS. Excess crude P1CRM 37°C was added to saturate the column. An overnight incubation at 4°C was done to insure maximum adsorption. The column was then also extensively washed with PBS to remove the unadsorbed protein. When the absorbence of the eluted wash had a reading of 0.0 at 280 nm, absorbed P1CRM 37°C was then removed by elution with a glycine-HCl buffer (0.2 M, pH 2.0). Fractions were collected in 1 ml volumes, neutralized with NaOH 2 M and read at 280 nm. The elution profile for P1CRM 37°C is shown in Figure 9. The P1CRM 37°C was eluted in the second protein peak. The second peak fractions were pooled and the amount of protein recorded was 0.69 mg/ml. An immunodiffusion test was performed and the second peak fractions gave

FIGURE 9

Immunoaffinity chromatography of S. aureus, strain P1CRM 37°C.

Arrow indicates when glycine - HCl 0.2M, pH 2.0 was added. Fractions 26 to 40 which contained P1CRM 37°C were pooled and concentrated by lyophilization.


ABSORBANCE (280nm)
.4
.3
.2
.1
0
10
20
30
40
FRACTION NUMBER (1ml/tube)

faint lines of identity with anti-alpha toxin. These columns were reused three to four times and gave relatively good yields for the first three runs.

When 100 µl of P1CRM 37°C (1.22 mg/ml) was subjected to polyacrylamide gel electrophoresis (PAGE), a single protein band was observed suggesting that the P1CRM 37°C was not contaminated. The Rf (0.66) of P1CRM 37°C is similar in that of alpha toxin (i.e. Rf (0.60)) (Figure 10).

The molecular weight of purified P1CRM 37°C was determined by the SDS-polyacrylamide gel electrophoresis test using the method of Laemmli (41). The molecular weight can be determined because there is a linear relationship between the log of the molecular weight and the relative mobility of the standard proteins. The results of a typical experiment performed are shown in Figure 11. The molecular weight of P1CRM 37°C was determined to be 44,000 daltons.

In the SDS-polyacrylamide gel electrophoresis test a single protein band appeared. An immunodiffusion test was performed and P1CRM 37°C gave a single line of identity with anti-alpha toxin (Figure 12a). When purified P1CRM 37°C was injected intramuscular into a rabbit, the resulting antibody gave a line of identity with crude P1. Therefore, P1CRM 37°C elicited a single antibody (Figure 12b).

In summary, P1CRM 37°C was found to be homogenous because single protein bands appeared in both the PAGE and SDS-polyacrylamide gel electrophoresis tests. Purified P1 CRM 37°C elicited a single antibody and formed a single precipitin line when crude P1 was diffused against the antibody.

FIGURE 10

Densitometric tracing of disc gel electrophoresis of purified P1CRM 37°C. The arrow denotes the origin.

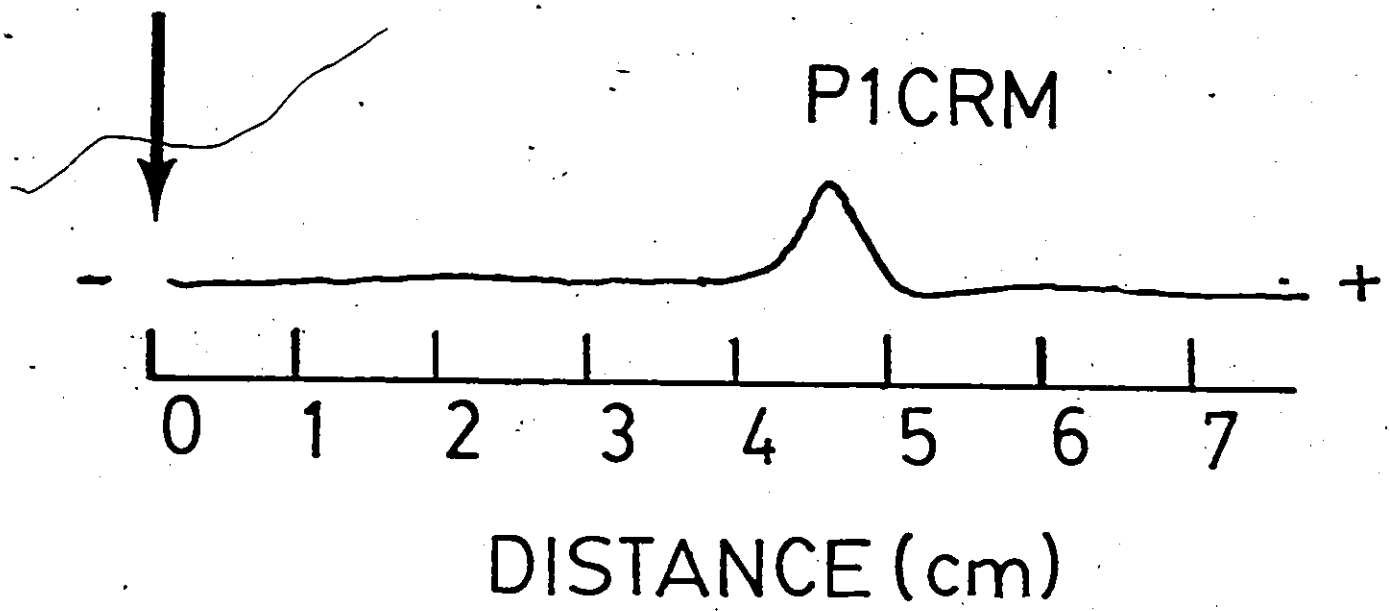

P1CRM
-
+
0
1
2
3
4
5
6
7
DISTANCE (cm)

FIGURE 11

Determination of the molecular weight of PlCRM 37°C.

Semi-log plot of molecular weight verse distance of migration (Rf). A line of best fit has been drawn through the data points.

- ● a Bovine serum albumin
- ● b alpha amylase
- ● c egg albumin
- ● d carboxypeptidase A
- ● e trypsin
- ● f beta-lactalbumin
- ● g lysozyme
- ○ PlCRM 37°C

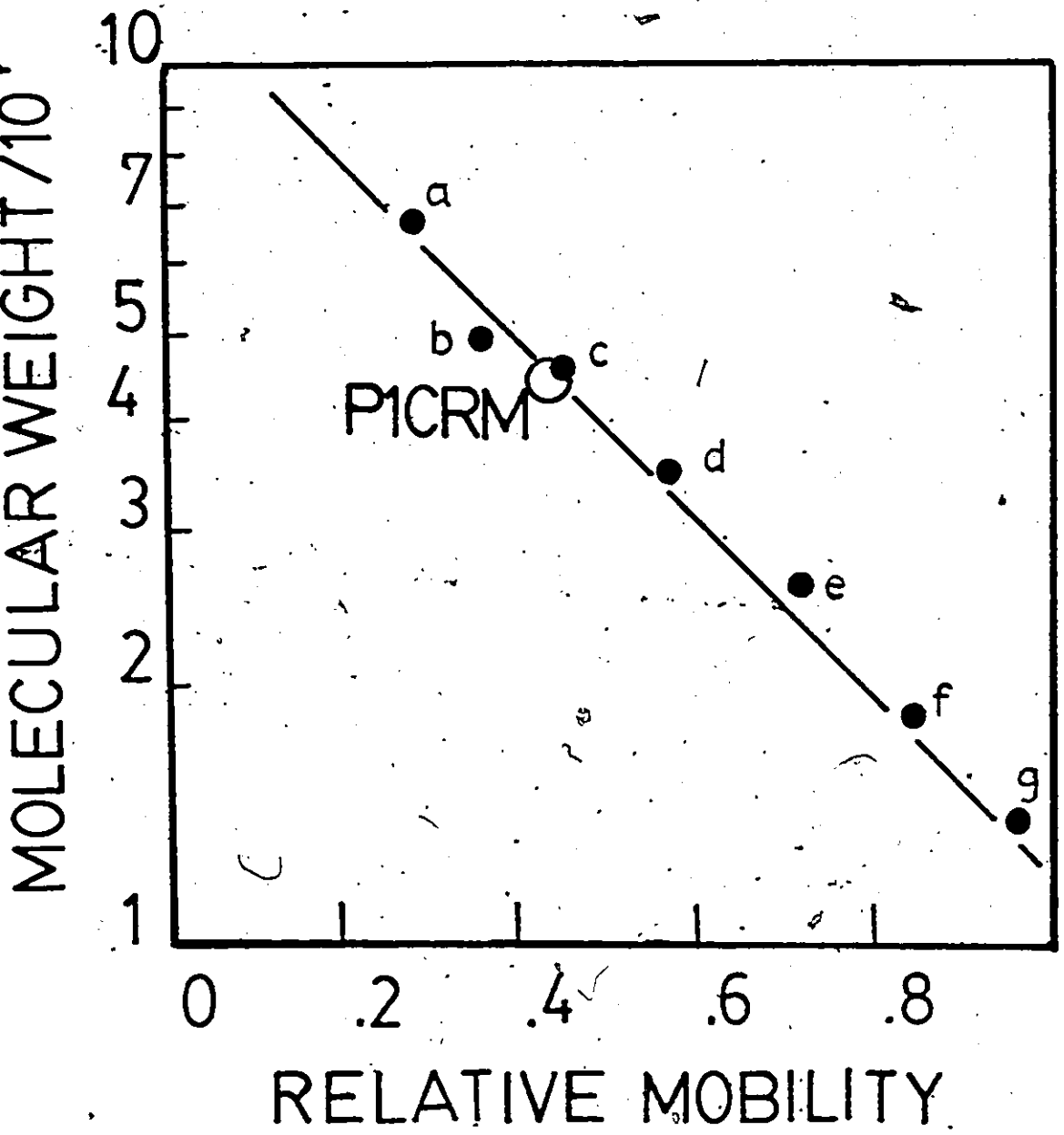

MOLECULAR WEIGHT /10^4
10
7
5
4
3
2
1
a
b
c
P1CRM
d
e
f
g
0
.2
.4
.6
.8
RELATIVE MOBILITY

FIGURE 12

Immunodiffusion patterns of purified P1CRM 37°C

Figure 12a

Diffusion to antibodies to alpha toxin

Center well: anti-purified alpha toxin

top well: purified P1CRM 37°C

left well: purified P1CRM 37°C

Right well: phosphate buffered saline

bottom well: unpurified alpha toxin

Figure 12b

Diffusion to antibodies to purified P1CRM 37°C

center well: anti-purified P1CRM 37°C

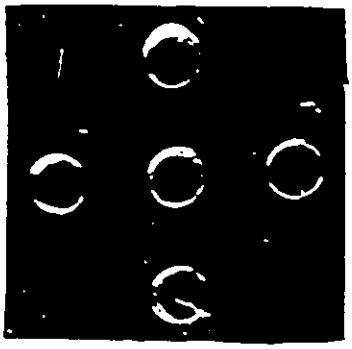
top well: unpurified P1CRM 37°C

left well: unpurified P1CRM 37°C

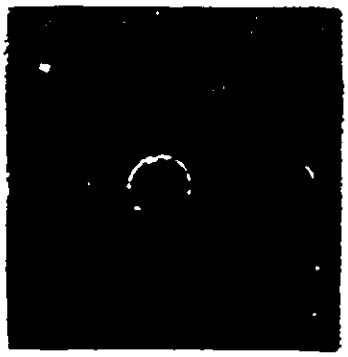
bottom well: unpurified P1CRM 37°C

right well: unpurified alpha toxin

a



b



The original observation that led to the selection of P1 for study occurred when P1 grown on rabbit blood agar plates was flooded with alpha toxin. The erythrocytes surrounding the P1 colonies were protected from lysis. This protection could have been provided by P1CRM 37°C and, therefore, a series of experiments were done to prove this. The protective nature of P1CRM 37°C was demonstrated in a number of kinetic assays. Protection assays were first performed with crude P1CRM 37°C and later with purified P1CRM 37°C. The results described in this thesis are presented for purified P1CRM 37°C.

A small amount of alpha toxin was mixed with a suspension of rabbit erythrocytes in PBS and incubated at 24°C. A turbidity decrease was measured at 650 nm which reflected hemolysis of the red cells. A typical S-shaped curve is shown in Figure 13, showing a characteristic pre-lytic lag phase followed by a period of rapid lysis and a tailing-off period.

Purified P1CRM 37°C was added to the system containing suspension of rabbit erythrocytes in PBS, and having an absorbance of 0.8 at 650 nm. This system was challenged with alpha toxin and when P1CRM 37°C was added, less lysis was detected thereby indicating protection. Because P1CRM 37°C alone is non-hemolytic, as the concentration of P1CRM 37°C increases so did the amount of protection. The results also indicated that the amount of protection was dependent upon the amount of P1CRM 37°C available in the system. Therefore, P1CRM 37°C and the alpha toxin molecules may compete with each other for binding sites present on the rabbit cell membrane. This supposition was subsequently confirmed in a later experiment.

FIGURE 13

Inhibition of alpha toxin by PlCRM 37°C.

Spectrophotometric tracing of hemolysis of rabbit erythrocyte suspensions after addition of 50 µl of alpha toxin and challenged with various amounts of PlCRM 37°C. The reaction mixture contained 100 µl of erythrocytes, 50 ul of alpha toxin and 150 µl of PlCRM 37°C dissolved in PB5. The final concentrations of PlCRM 37°C were as follows:

a = 14.64 µg/ml

b = 12.81 µg/ml

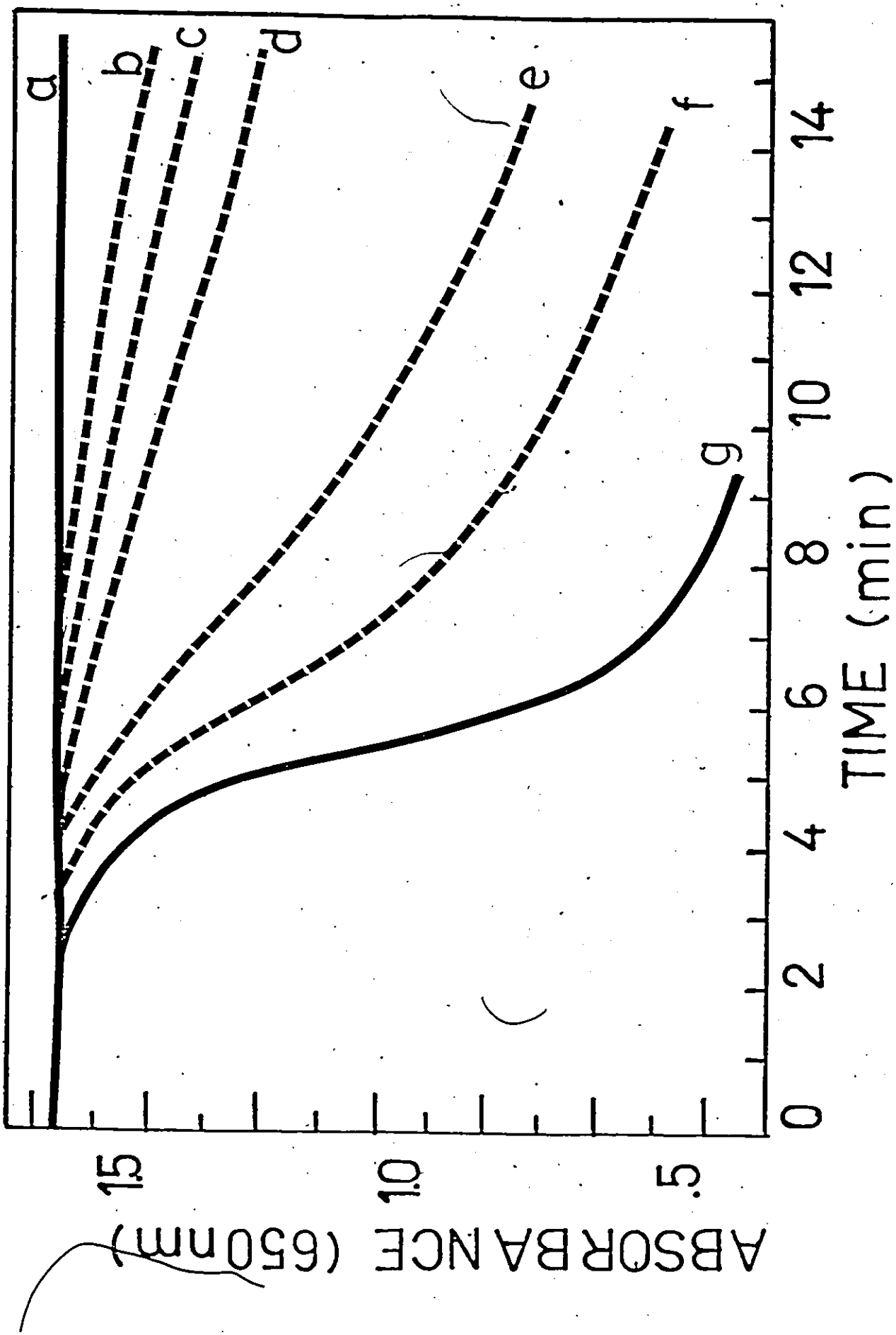
c = 10.98 µg/ml

d = 9.15 µg/ml

e = 7.32 µg/ml

f = 3.66 µg/ml

The solid line (line g) represents lysis due to 50 µl of alpha toxin alone.


ABSORBANCE (650nm)
15
10
5
0
2
4
6
8
10
12
14
TIME (min)
a
b
c
d
e
f
g

Previously, this laboratory had shown that alpha toxoid protected red cells in a similar manner (9). However, this protection lasted only for the pre-lytic lag phase. Consequently, similar time experiments were performed with purified PlCRM 37°C.

In a series of kinetic hemolytic tests, a constant amount of PlCRM 37°C was added at different times (0 minutes, 3.5 minutes. 5 minutes, and 6 minutes) to a constant amount of erythrocyte-toxin mixture (Figure 14). The PlCRM 37°C gave complete protection only if added during the initial lag phase of lysis at time equal to zero. When PlCRM 37°C was added during the exponential phase of lysis, the erythrocytes had less than 100% protection. Curves for each different time were traced, and are shown in Figure 14. A series of similar experiments were performed, and it was found that PlCRM 37°C mimics alpha toxoid.

It was found that the further addition of 80 µl of PlCRM 37°C at 5 minutes did not give any additional protection (Figure 15). Results showed that a two-fold increase in the amount of PlCRM 37°C only resulted in a slight increase in protection. This suggested that alpha toxin had already been bound to the red blood cells causing considerable damage to the membrane and therefore prevented further protection by PlCRM 37°C.

PlCRM 37°C was then used to coat rabbit erythrocytes. The excess PlCRM 37°C was removed by washing the coated erythrocytes (CE) with PBS. When later challenged with alpha toxin, the CE was resistant to hemolysis. This is shown in Figure 16. In summary, PlCRM 37°C protected like alpha toxoid and competed with alpha toxin for receptor sites present on rabbit erythrocytes.

FIGURE 14

Protective effects of PlCRM 37°C when added at various times during hemolysis by alpha toxin. The reaction mixture (final volume 300 µl) contained 100 µl of erythrocytes, 50 µl of alpha toxin and 150 µl of PlCRM 37°C dissolved in PBS. The solid line represents hemolysis due to 50 µl of alpha toxin alone. Broken lines represent the effect of adding PlCRM 37°C at different times during the course of hemolysis. Final concentration of PlCRM 37°C was 14.64 µg/ml.

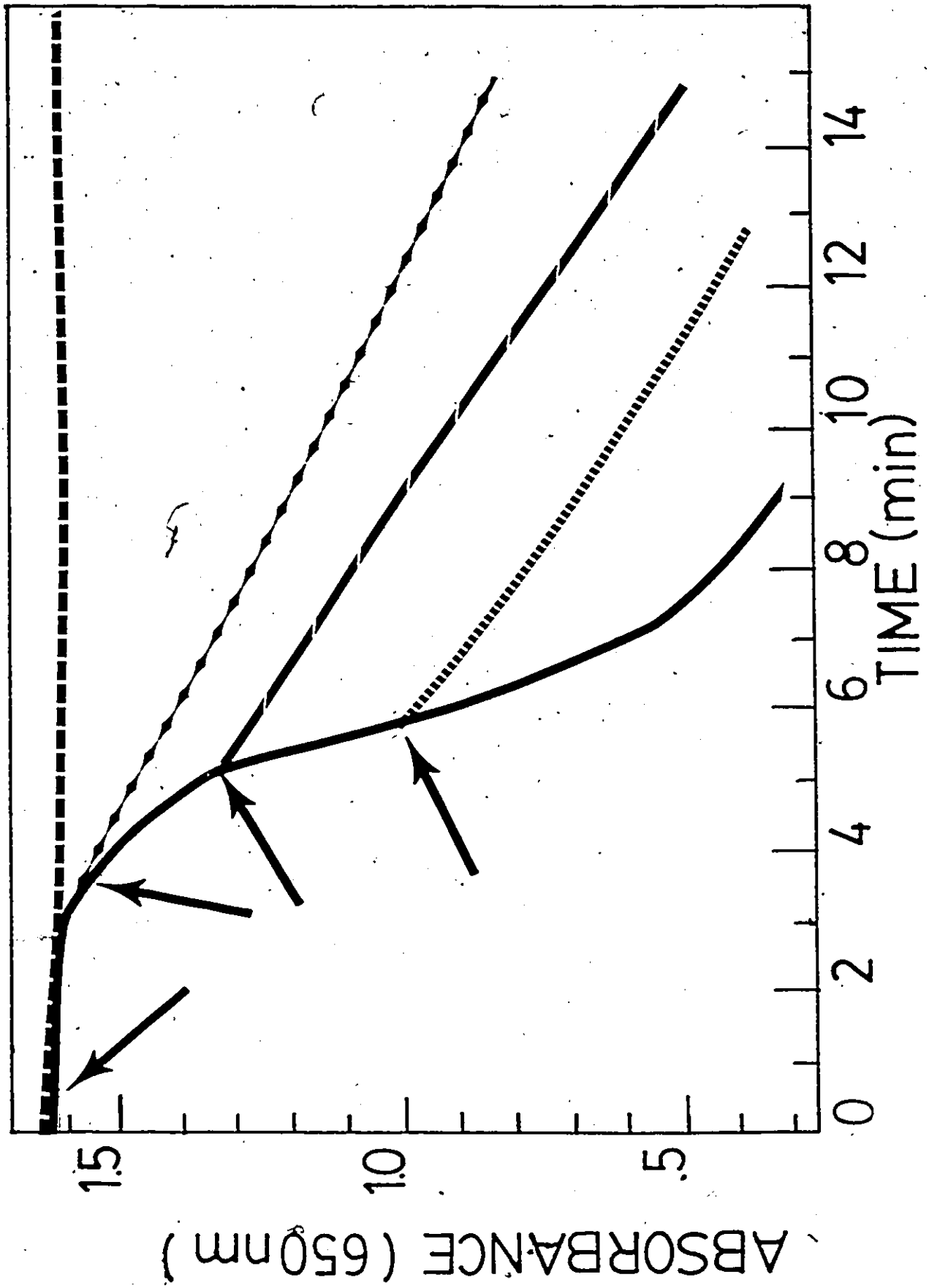

ABSORBANCE (650nm)
15
10
5
0
2
4
6
8
10
12
14
TIME (min)

FIGURE 15

Protective effects of various concentrations of P1CRM 37°C when added at the five minute time period during hemolysis by alpha toxin. The reaction mixture contained 100 µl of erythrocytes, 50 µl of alpha toxin, and 150 µl of P1CRM 37°C dissolved in PBS. The solid line represents hemolysis due to 50 µl of alpha toxin alone. The line (◆——◆——◆) represents the effect of adding 14.64 µg/ml of P1CRM 37°C at 5 minutes, and the line (▪▪▪▪▪▪▪▪▪▪▪▪) represents the effect of adding 30 µg/ml to P1CRM 37°C at 5 minutes. Rate of hemolysis was unaltered when double the amount of P1CRM 37°C was added at 5 minutes.

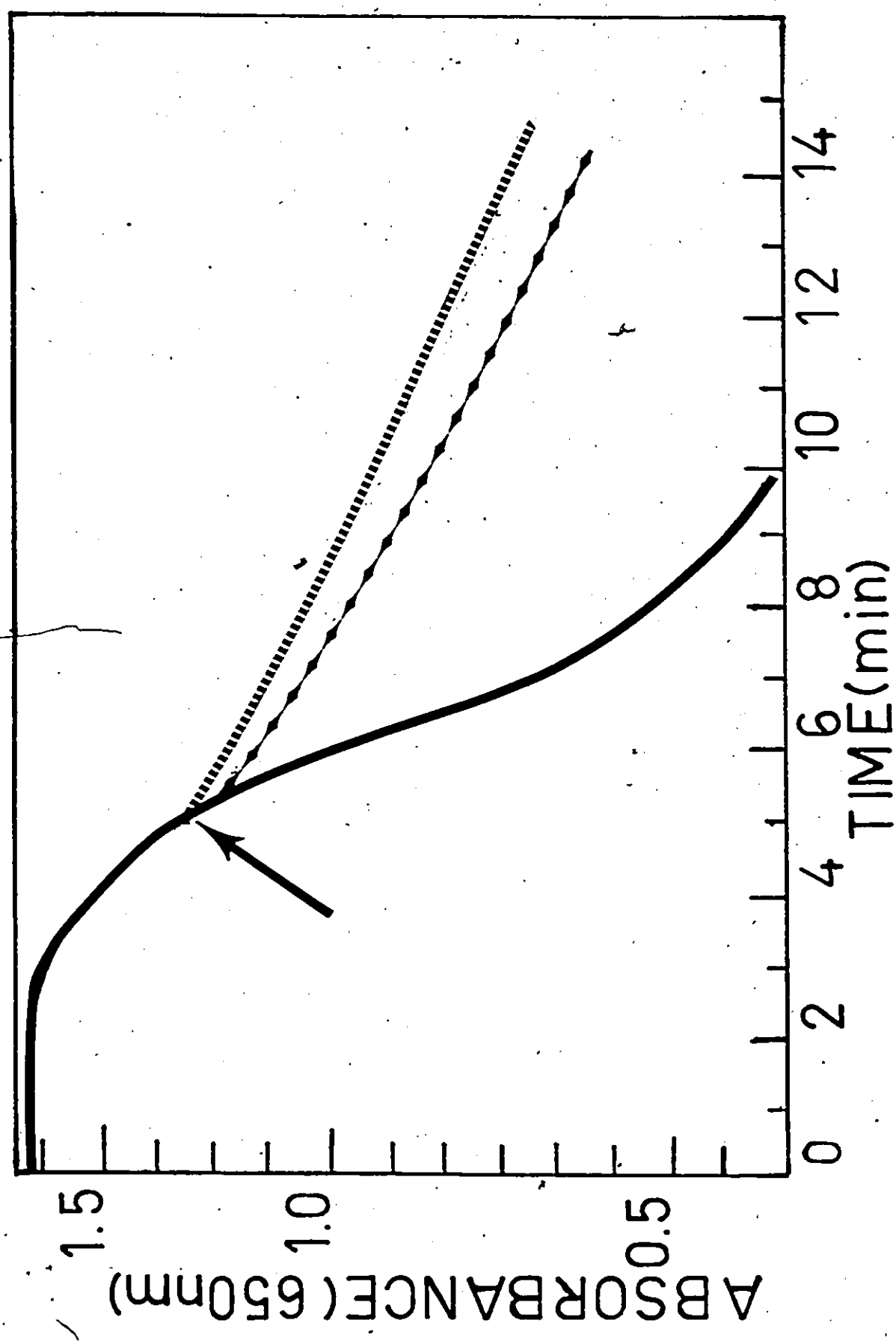
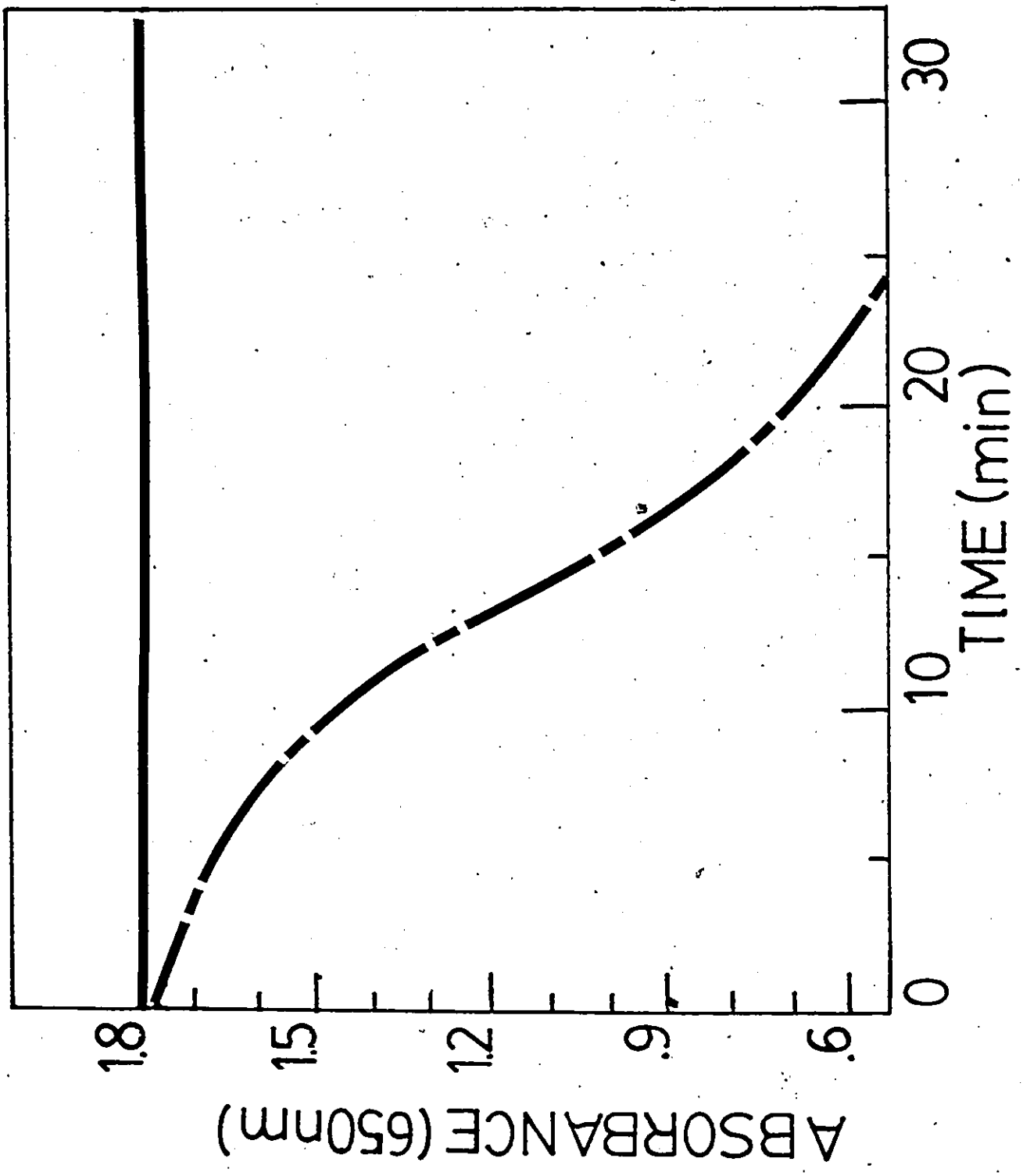

ABSORBANCE(650nm)
1.5
1.0
0.5
0
2
4
6
8
10
12
14
TIME(min)

FIGURE 16

Protective effect of PlCRM 37°C - coated erythrocytes. The broken line represents lysis of a mixture of 50 µl of alpha toxin, 100 µl of erythrocytes, and 150 µl of PBS. When the erythrocytes were exposed to PlCRM 37°C and washed with PBS prior to challenge with alpha toxin, no lysis was observed. (Solid line).


ABSORBANCE (650nm)
18
15
12
9
6
0
10
20
30
TIME (min)

DISCUSSION

This thesis describes the isolation, and partial characterization of 32 non-hemolytic mutants of Staphylococcus aureus, following ultraviolet irradiation. It has been shown that the mutants with defective alpha toxin can be collected by a differential screening method, and can be categorized immunologically into four classes with respect to their antigenic determinants and their ability to cross react with anti-alpha toxin. These mutants should prove to be useful tools in order to study the mechanism of action and antigenicity of alpha toxin.

As previously stated, mutants have proven to be invaluable tools in the study of other proteins such as hemoglobin and diphtheria toxin. Mutations affecting hemoglobin have had a major impact on molecular biology, medicine, and genetics. For example, the alpha and beta chains of hemoglobin are controlled by two distinct genes so that a single mutation will affect either the alpha chain or the beta chain, but not both; and the antigenic determinants of hemoglobin are well characterized using immunochemical techniques (69). Mutations affecting hemoglobins have provided important information about the relationship between the structure and the antigenicity of normal hemoglobin. Particularly noteworthy is the work of Morris Reichlin (68, 69, 70). Reichlin used quantitative complement fixation reactions with rabbit anti-hemoglobin sera to distinguish altered hemoglobin from normal hemoglobin. His studies showed that certain single amino acid substitutions in the hemoglobin protein can alter the antigenic determinant of hemoglobin (70). While the complement fixation test may be

possible for other systems, it did not work for alpha toxin (44). This is because both alpha toxin and the toxin-antibody complex bind onto erythrocyte membranes (44).

Another example in which work with mutants had proven to be invaluable was in the study of diphtheria toxin (11, 64). A series of mutated forms of diphtheria toxin were isolated, purified, and characterized immunologically. The use of these mutants increased the understanding of the mode of action, the biosythesis, the regulation, and the biochemistry of the toxin molecule, as well as the mechanism by which diphtheria toxin crosses cell membranes.

Production of diphtheria toxin depends on both the phage and the host (64), unlike alpha toxin. The structural gene for diphtheria toxin is on the phage chromosome. Nevertheless, the mutants of diphtheria toxin produce cross-reactive material (CRM) which react variably with diphtheria antitoxin (64). As a result of this variable reaction characteristic, the mutants have been placed into two different classes: class (a) mutants which are non-toxic proteins and which show lines of partial identity with diphtheria toxin when tested by immunodiffusion (11); and class (b) mutants which are non-toxic proteins and which show lines of identity with diphtheria toxin when similarly tested.

Class (a) mutants are the result of either frameshift or nonsense mutations within the structural gene which leads to premature chain termination, whereas class (b) mutants appear to be the result of missense mutations within the structural gene (11).

Many class (a) mutants have been found to be as enzymatically

active as fragment A of the wild type diphtheria toxin, and have had an ADP-ribosylation activity equivalent of about 50% of that of fragment A. All of these mutants lacked a portion of fragment B, thus preventing binding to the receptors on the cell membrane specific for the diphtheria toxin (75).

Most class (b) mutants have a normal B fragment which competes with diphtheria toxin for receptor sites. Class (b) mutants have either a reduced to no enzymatic activity (75). Mutations in fragment A alter the NAD:EF-2 ADPR-transferase activity; whereas, mutations in fragment B alter the binding characteristics of the mutants toward eukaryotic cell membranes (11).

The isolation and characterization of class (a) and class (b) CRMs of diphtheria toxin provided evidence that fragment A of the toxin catalyzes the NAD:EF-2 ADPR-transferase reaction.

McClatchy and Rosenblum (56, 57) isolated 20 non-hemolytic mutants of S. aureus, which they tested for lethal and dermonecrotic activity and for the presence of CRM with anti-alpha toxin. They grouped the mutants according to their ability to cross-react with anti-alpha toxin by using the immunodiffusion test.

A qualitative method such as immunodiffusion has a limited value, since it fails to detect changes in single amino acids when the quantitative methods reveal large changes. Also, immunodiffusion does not recognize CRM which possess a single antigenic determinant. For example, seven CRMs of hemoglobin are easily distinguishable from normal hemoglobin by the complement fixation test using three different rabbit antisera. The immunodiffusion test can distinguish only two of the seven CRMs of hemoglobin

from normal hemoglobin using two of the antisera, and only three of the seven CRMs of hemoglobin using all three antisera (69, 70).

Because of the above-mentioned deficiencies of the immunodiffusion test, a more sensitive method was developed in order to classify the CRMs of alpha toxin. The method was based on immunoadsorption with anti-alpha toxin antibodies, since there are two distinct populations of alpha toxin antibodies; the IHA-Ab and the AB-Ab (43). The CRMs were characterized based on the presence or absence of antigenic determinants to these populations. Although there are two classes of antibodies for anti-streptolysin-O (67), classification of mutants with respect to antigenic determinants had not been done before. The mutants were classified as having both determinants, only IHA, only AB, or neither IHA or AB. Undoubtedly, the mutant classes discussed in the results will have to be re-evaluated as more is learned about the mutants. The CRM of mutants 32, 34, and 35 have already been isolated by others in this laboratory and confirmed to possess both the IHA and AB determinants.

The development of these classes of mutants can provide the foundation for the possible identification and isolation of the regulatory protein for alpha toxin and for the further study of the mechanism of action.

Studies have indicated that alpha toxin is oriented on rabbit erythrocyte membranes (43) and that there are two binding mechanisms for alpha toxin: a low and a high affinity binding mechanism (9). The CRMs can be tested to determine whether or not they are capable of binding onto receptors of the rabbit erythrocyte. This

can be done with fluorescein isothiocyanate (FITC)-labeled CRM as was performed by Barei and Fackrell to its wild type toxin (9). High and low affinity binding can thus be measured with fluorescein-label CRMS.

A reliable test that could be used to determine susceptibility of rabbit erythrocyte membranes to CRMs is to demonstrate the leakage of K+ ions from intact cells after their exposure to all of the mutants CRMs. The binding between alpha toxin and erythrocytes is characterized by rapid release of K+ ions from cells early in the pre-lytic period (22, 48). The CRMs can be tested to see if K+ ions are released when they are added to erythrocytes either alone, or in competition with alpha toxin. The information from these leakage experiments could provide some insight into the kinetics of the interactions between the CRMs and the membrane. These results could be correlated with the previously mentioned receptor studies.

According to Wiseman et al (81) alpha toxin is activated by membrane proteases to become proteolytic. One could test whether any of the mutants are naturally proteolytic and whether they can be activated. Of particular interest would be the class 1 mutants which are IHA positive and AB negative.

One possible reason why the CRMs of alpha toxin have a variety of reactions to the antibody beads tests can be due in part to the variety of potential mutations caused by ultraviolet light. Ultraviolet light is known to cause deletions and amino acid substitutions in DNA. Such mutations can occur either in the structural gene or regulatory gene for alpha toxin.

If the mutation is the result of an amino acid substitution in the antigenic determinant, it can be determined by peptide mapping in which the exact amino acid substitution can be identified. If the mutation is the result of a deletion it can be determined by comparing the molecular weight of each CRM with that of the alpha toxin.

Any conformational change in the CRM would result in steric blockage of the antigenic determinant. To determine whether the change in the CRM is the result of a conformational change, the CRM can be compared to the alpha toxin by ultracentrifugation. If the diffusion coefficient and molecular weight of the CRM are known, the frictional ratio can be calculated and compared to the alpha toxin. If the frictional ratio deviates from that of alpha toxin, then one can assume that a conformational change has occurred.

Since protein synthesis starts at the amino terminal end of the polypeptide, the mutants of class 1 and class 2 could provide evidence as to whether the IHA or the AB determinant is made first. If the IHA and AB portions are in the sequence IHA-AB, there may be either a substitution or deletion in the AB portions but merely a substitution in the IHA portion. If the IHA and AB portions are in the sequence AB-IHA there may be either a substitution or a deletion in the IHA portion, but merely a substitution in the AB portion. The mutants of classes 1 and 2 can be isolated by the immunoaffinity, and thereafter tested to see if deletions occur. One could expect decreases in molecular weight in only that class of mutants which has an antigenic determinant closest

to the carboxyl terminus.

Mutants which are able to synthesize CRM which react in turn with both antigenic determinants, probably have only minor lesions in the structural gene for alpha toxin. These mutants could be useful in study, the mode of action for alpha toxin through the use of binding and activation studies previously mentioned.

It may be possible to use CRMs to isolate the regulatory protein for alpha toxin by using the immunoadsorbent bead test. The immunoadsorbent technique could be used to re-evaluate mutants of class 3 which are negative for the IHA an AB determinants. The class 3 mutants may have a mutation in the regulatory gene for alpha toxin which can prevent the expression of the structural gene for alpha toxin. There is probably a number of regulatory gene mutants among the mutants of this class 3.

The bead technique could also be used to confirm whether alpha toxin has two genetic loci involved in alpha toxin production.

Class 1 mutants have a genotype of R+, tox+, and a phenotype of R+, $tox^{IHA+,\ AB-}$ given that "R" is the regulatory gene, and "tox" is the structural gene for alpha toxin. Class 2 mutants have a genotype of R+, tox+, and a phenotype of R+, $tox^{IHA-,\ AB+}$. Class 3 mutants can have a genotype of R-, $tox^{IHA+,\ AB+}$ or R+, $tox^{IHA-,AB-}$ and a phenotype of R+, $tox^{IHA-,AB-}$, or R- $tox^{IHA+,AB+}$.

By combining the cytoplasmic protein machinery of a regulatory mutant of class 3, phenotype of R-, $tox^{IHA+,AB+}$, with a structural gene mutant of class 1, phenotype R+, $tox^{IHA+,AB-}$, wild type alpha toxin could be formed. To the combined cytoplasms, ^{14}-C amino acid precursors, ATP and GTP, could be added to obtain an <u>in vitro</u>

protein synthesis in the combined systems (54, 60). Production of ^{14}-C labeled alpha toxin could then be monitored by the use of anti-alpha toxin beads bound to radioactive alpha toxin. If two different mutants had lesions on the same gene, then no radioactive alpha toxin could be made. The results would give evidence as to whether there are two genes responsible for alpha toxin synthesis. This information could lead to the eventual isolation of a regulatory protein which then could be used to increase alpha toxin production.

Although little is known about the structural gene and regulatory gene for alpha toxin, the mutants could be useful in genetic and metabolic studies.

As previously mentioned, one mutant, P1, was found to be particularly interesting in that it was discovered to be a functional temperature-sensitive mutant. This mutant was able to produce a hemolytic toxin at 24°C but not at 37°C. Yet, P1 produced CRM at both temperatures. This was the first discovery of a functional temperature-sensitive mutant for any of the staphylacoccal toxins and, in fact, for any toxin.

There are several examples of functional temperature-sensitive enzymes in the literature. Functional temperature-sensitive enzymes have been demonstrated in bacterial systems (46, 59, 73) and in somatic cells (39, 74). For example, Escherichia coli contains a temperature-sensitive valyl-tRNA synthetase (59) which was the first temperature-sensitive sythetase mutant to be identified. As a wild type, this mutant failed to grow at 40°C but grows quite well at 30°C. Growth of the mutant stopped abruptly when the temperature

was suddenly raised from 30°C to 40°C. This abrupt halt in growth suggested that the valyl-tRNA synthetase is a non-functional temperature sensitive enzyme and not that a failure occurred at the restricted temperature.

In somatic cell genetics, a cell mutant of Chinese hamster ovary (CHO) has a structural alteration in the leucyl-tRNA synthetase enzyme whose activity is temperature sensitive. The CHO mutant was unable to use leucine for charging the tNRA (74).

In most studies, enzymatic assays of the synthetase enzyme were done at two temperatures, a restrictive temperature and a lethal temperature. However, most investigators failed to determine whether a non-functional enzyme was produced at the lethal temperature by CRM, or whether the enzyme was produced at all.

P1CRM 37°C has been isolated and purified by immunoaffinity chromatography, and the homogeneity was shown by single bands in PAGE and SDS-polyacrylamide gel electrophoresis. Purified P1CRM 37°C gave a single line of identity with antibodies to purified alpha toxin in immunodiffusion. When injected into a rabbit, P1CRM 37°C elicited a single antibody as shown by diffusion against unpurified P1CRM 37°C. P1CRM 37°C reacts with the IHA and AB determinants of the alpha toxin antibody. The molecular weight of P1CRM 37°C was determined to be 44,000 daltons, which was also the molecular weight for alpha toxin (34). This indicates that the mutation was not the result of a deletion or addition. It is clear that P1CRM 37°C and alpha toxin have many similarities, and the only detectable difference is that P1 when grown at 37°C is a non-hemolytic, while the wild type is hemolytic at that temperature.

The change that accounts for this difference, however, is not detected by PAGE, SDS-polyacrylamide gel electrophoresis (molecular weight determination), immunodiffusion or the antibody bead tests.

The change in P1 may be the result of a point mutation in which the lesion is very small. There may be a change in the DNA which may have no effect at the lower temperature (24°C), but which may alter the structure and function of the protein at the slightly higher temperature (37°C). The structural gene in P1 may be misread at 37°C, thus giving a non-hemolytic toxin. The P1CRM at 24°C may be equivalent to the wild type alpha toxin in which the DNA is read normally. In support of this hypothesis, P1 and S. aureus Wood 46 grew equally well at both of these temperatures, indicating that nothing was wrong with their protein synthesizing machinery. Also, P1 and S. aureus Wood 46 have similar heat denaturation profiles. When an attempt was made to denature P1CRM 37°C using urea so that the original conformation could be restored, P1CRM 37°C remained in its non-hemolytic form, while P1CRM 24°C was reactivated to its hemolytic form. Thus, the difference in P1 is probably not the result of a conformational change, but rather the result of a point mutation.

P1 may be used in many different ways. P1 can be used as a tool in studying the mechanism of action of the alpha toxin, using its unique temperature-sensitive nature. The interest in our laboratory, has focused on the mode of action of alpha toxin, mainly as to the possible enzymatic nature of alpha toxin. By using peptide maps, one can compare P1CRM 37°C with alpha toxin and determine where any changes have occurred. Any changes in P1

can show which amino acids are involved for the hemolytic activity of alpha toxin, assuming no conformational change.

PI can serve as a specific probe in the study of receptors, since alpha toxin is specific in its interaction with rabbit erythrocyte receptors.

P1 can be used in the practical production of vaccine. Presently, Staphylococcal Toxoid (42) is a peptic digestion of alpha toxin which is being used as a vaccine in medicine for the prevention and treatment of staphylococcal infections. Since P1 is already non-hemolytic, has both antigenic determinants for anti-alpha toxin, and is similar to the wild type alpha toxin, it may be the ideal vaccine.

SUMMARY

1. Staphylococcus aureus, strain Wood 46, was exposed to ultraviolet irradiation, after which 32 non-hemolytic mutants were isolated.

2. These mutants were characterized biochemically and with respect to antibiotic sensitivity, but no simple correlation was observed between loss of hemolytic sensitivity and any of the characteristics tested.

3. The mutants were placed in one of four classes, based on their reactions with either anti-alpha toxin or its subpopulation.

4. P1, one of the 32 non-hemolytic mutants was a temperature-sensitive mutant. The permissive temperature was 24°C and the non-permissive temperature was 30°C to 37°C.

5. The cross reactive material from P1 cultures, incubated at 37°C was isolated by affinity chromatography and characterized.

6. This protein, which has a molecular weight of 44,000 daltons does not seem to be an alternative conformation of the hemolytic protein produced at 24°C, since urea experiments failed to activate the cross reactive material.

7. The cross reactive material from P1 reacts with all the subpolution of anti-alpha toxin.

8. The cross reactive material from P1 binds onto the receptors sites of rabbit erythrocytes membranes and protects them from lysis by alpha toxin.

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