THE UNIVERSITY OF MANITOBA

STUDIES WITH GAMMA HEMOLYSIN OF *Staphylococcus aureus*

by

HUGH BRYSON FACKRELL

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ABSTRACT

Highly purified gamma hemolysin of *Staphylococcus aureus* was obtained from the Smith 5R strain by ultrafiltration, gel filtration on Sephadex G-75, followed by ammonium sulphate precipitation and extraction with NaCl. The hemolysin was shown to be a protein which was homogeneous when subjected to isoelectric focusing, disc gel electrophoresis and immunodiffusion. One peak of 2.6 S was observed in the analytical ultracentrifuge and methionine was identified as the only N-terminal amino acid. Gamma lysin was distinguished from the alpha, beta and delta hemolysin immunologically and by its physical-chemical properties. Investigation of the biological properties of gamma lysin showed that it attacked human and rabbit platelets, human leucocytes and C-6 cells in tissue culture. Rabbit, sheep and human erythrocytes were also lysed. Intravenous injection with 50 µg of gamma lysin killed guinea pigs but had no effect on mice, nor was it dermonecrotic for rabbits or guinea pigs. Kinetic analysis of the hemolytic reaction suggested that the gamma lysin was an enzyme. Furthermore, hemolysis was competively inhibited by phospholipids of erythrocyte membranes although the phospholipids were not degraded.

A new method of purification of the alpha hemolysin has also been devised. This method, which utilizes the

first step of Wittler and Pillemer (J. Biol. Chem., *174*:23, 1948) further consists of gel filtration on Sephadex G-75, ammonium sulphate fractionation and ion exchange chromatography on carboxymethyl cellulose. The alpha lysin was shown to be homogeneous by the same criteria applied to gamma lysin.

ACKNOWLEDGEMENTS

Although a thesis is the presentation of one individual's research, the results could not be obtained without the willing cooperation of the entire Department. The author especially wishes to thank Dr. J.C. Wilt for the use of the facilities of the Department and his personal encouragement. The guidance of Dr. G.M. Wiseman was not only patient but enthusiastic. Finally, the author gratefully acknowledges the technical assistance provided by J.D. Caird.

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INTRODUCTION

Since the review of Elek in 1959 (55), knowledge of the hemolysins of *Staphylococcus aureus* has expanded rapidly. In spite of the proliferation of literature, considerable discrepancies are apparent in the individual properties of the hemolysins. While a great deal of work has been published with regard to properties of the alpha, beta and delta lysins, only recently have investigators turned their attention to the gamma lysin. This lysin, discovered by Smith and Price in 1938 (177), was largely ignored until the work of Guyonnet *et. al* (85,86,158), mainly because of the view taken by Elek (55) that it was identical with the delta lysin.

As the result of the work of Guyonnet's group, based on the gamma lysin produced by the Smith 5R strain of *S. aureus*, it is now possible to separate the gamma lysin as a distinct entity apart from the other hemolysins. However, very little is known about its nature and mode of action and how these compare with what is known of the alpha, beta and delta lysins. It is with these questions that the present investigation is concerned.

LITERATURE REVIEW

ALPHA HEMOLYSIN

Burnet (27,28) first suggested that several of the properties now associated with alpha hemolysin were multiple manifestations of a single toxic protein. Many of the individual properties had previously been reported as separate factors. Both De Christmas (45) and Leber (124) realized that staphylococcal products caused inflammation and, in 1890, Breiger and Fraenkel (22) noted that staphylococcal culture fluids, lethal for guinea pigs and rabbits, caused formation of sterile pus and dermonecrosis at the injection site. Van de Velde (184) reported that a leucocyte damaging factor called "leukocidin", also lysed rabbit erythrocytes. Although these earlier observations were confirmed in the following years (118, 153,166,167), it was Burnet's "unitarian hypothesis" that gave insight into the distinctive character of alpha hemolysin. Since then, numerous attempts to purify alpha hemolysin have met with varying degrees of success (Table I) and the use of relatively homogeneous preparations of the hemolysin has firmly established its characteristics.

Biological Effects

Hemolysis

Unfortunately, there exists no internationally accepted definition of a unit of alpha hemolysin since abandonment of the circular definition; one hemolytic unit

is the amount neutralized by one unit of anti hemolysin. Hemolysis of rabbit erythrocytes is the most common, convenient and sensitive index of alpha hemolysin, because a few nanograms will cause lysis (10,81,127). Erythrocytes of human, sheep, horse and monkey are notably resistant (7,9,41) with the red cells of other animal species occupying positions of intermediate sensitivity (7,9,26, 50,63,68,147,162,199). When erythrocytes from different rabbits are titrated against a single batch of alpha hemolysin, the titres may vary several fold (10,41,182). This individual animal variation may be greater than species variation (7) making generalizations concerning sensitivity between erythrocytes of different species difficult. The variation also makes standardization of alpha lysin in terms of hemolytic activity unfeasible. Bernheimer and Schwartz (10) made all assays comparable by titrating a standard hemolysin preparation with each unknown and correcting the results according to deviation from the fixed value of the standard. The necessary assumptions that alpha lysin can be maintained in a stable condition for long periods and that all crude alpha lysin preparations are the same have yet to be proved. An adequate definition of a unit of alpha hemolysin can be derived only from a precise understanding of the mechanism of action at the molecular level.

Toxicity

Intravenous or intraperitoneal injections of alpha hemolysin are lethal for all species of experimental animals that were tested; mice, rabbits, chickens, frogs, cats, dogs, rats and horses (12,50,112,171,211). The LD_{50} ranges from 2 µg/kg for rabbits to 40 µg/kg for mice to 400 µg/kg for chickens (2). Mice are generally used for convenience, economy and because smaller absolute amounts are required. Large doses of alpha hemolysin kill in seconds but smaller amounts take days (2,55). Initially, the animal appears to be normal, then in rapid sequence it becomes unsteady, paralysis of the hind legs develops, the respiration becomes irregular, incoordinate movements occur, the pupils dilate and the animal dies after violent convulsions. In rapid death, post mortem macroscopic lesions are absent, but petechial hemorrhages are found on the serosal surfaces of the internal organs, particularly the kidneys, and most organs show congestion (55).

Subcutaneous injection of microgram amounts of alpha hemolysin causes dermonecrosis. The minimal dermonecrotic dose (MDD) varies from 0.01-2.4 µg (10,97,114,127). This wide range may be accounted for by the variability in rabbits tested and the use of a poor statistic. Finally, Goshi *et al.* (81) reported that subcutaneous injection into man caused only induration and erythema, illustrating the difficulty in defining the minimal criteria for a

dermonecrotic lesion.

Elek (55) and other investigators (2,76) postulated that dermonecrosis was the result of the direct action of alpha hemolysin on skin tissue, because alpha lysin is cytotoxic for a large number of cell types beside erythrocytes. Alternatively, Thal and Egner (181) believed that dermonecrosis was the result of the vascular effects caused by the selective action of alpha hemolysin upon the smooth muscle of blood vessels. It is now clearly established that alpha lysin causes contraction and subsequent paralysis of smooth muscle (23,24,25,181,196,197,198,213). The contraction effected by alpha lysin can be prevented by the addition of anti-alpha hemolysin (213). Wiegerhausen (196,197,198) considered that the alpha hemolysin acted directly by causing the release of pharmacological substances, but Brown and Quilliam (25) were unable to detect the release of such substances from guinea pig ileum segments after exposure to alpha lysin. Resolution of the question, how does alpha lysin cause dermonecrosis, will have to await a more complete understanding of the mode of action of this lysin.

Gengou (74,75) first attributed the ability of staphylococci to destroy platelets to alpha hemolysin and this conclusion has since been confirmed (12,103,138,172). Alpha lysin causes a rapid reduction of absorbance and loss of contents into the suspending medium (12,138).

Electron microscopy revealed the loss of organelle contents and irregularities in the membrane (138). Bernheimer and Schwartz (12) reported that other hemolysins affected platelets but non-hemolytic toxins did not.

Bernheimer and co-workers had suggested that protoplasts of L forms of Gram-positive bacteria were lysed by alpha hemolysin and that spheroplasts of Gram-negative organisms were resistant (8,13,14) but it now appears that lysis was due to contaminating amounts of delta hemolysin(15).

Rabbit polymorphonuclear leucocytes are destroyed by alpha lysin (76,212) but human polymorphs are resistant (7,76,120,121). Goshi *et al.* (81) reported that alpha hemolysin destroyed human leucocytes, but the observation should be confirmed.

Physical-Chemical Properties

Alpha hemolysin is a protein which can be destroyed by proteolytic enzymes (10,43,82,126). As expected, alpha hemolysin exhibits an ultraviolet absorption spectrum characteristic of proteins (10,43,114). However, the preparation of Goshi *et al.*(81) contained 6-9% carbohydrate. Kumar *et al.* (120,121) from preliminary studies assumed the presence of carbohydrate and the purified material was shown to contain carbohydrate by Kitamura *et al.* (114). In contrast, purer preparations indicate that carbohydrate represents less than 1% of the weight of

alpha lysin (10,43,59).

The results of the amino acid analysis reported by Bernheimer and Schwartz (10) and Coulter (43) are in reasonable agreement except that Bernheimer reported five times the amount of methionine than the latter. Since both preparations were acid-hydrolysed, cystine or cysteic acid and tryptophan were not detected. Therefore the presence of disulfide bridges or free sulphhydryl groups cannot be excluded. Coulter (43) detected arginine and histidine as N-terminal amino acids but in a later report, Wiseman and Caird (208) detected only histidine.

Current research suggests that alpha hemolysin exists in varying degrees of molecular association. Highly purified preparations of alpha lysin have a sedimentation coefficient of 3.0S (3,10,41,43,127) (see Table I). An additional smaller peak representing 10-15% of the protein, with a sedimentation coefficient of 12-16S has been reported (3,10,127). Three pieces of evidence suggest that the 12S molecule is an aggregate of the 3S alpha lysin: (i) 12S alpha hemolysin can be disaggregated by urea to yield biologically active alpha hemolysin, (ii) removal of the urea allowed spontaneous reformation of the 12S component, and (iii) in Ouchterlony double diffusion tests, 12S preparations exhibited a partial line of identity with alpha lysin (3). Larger insoluble precipitates of alpha hemolysin will form if preparations

TABLE 1

COMPARISON OF PURIFICATION PROCEDURES OF ALPHA HEMOLYSIN

REFERENCE	PURIFICATION PROCEDURE	HEMOLYSIN			PURITY		
		SPECIFIC ACTIVITY	PURIFICATION (fold)	RECOVERY %	PRECIPITATION LINES	ELECTROPHORESIS BANDS	ULTRA-CENTRIFUGE
29	Acetic acid ppte.	66,000#	18#	85	*	*	*
20	TCA ppte.	*	75#	75	*	*	*
210	Methanol ppte pH ppte. acetate extraction	1,200	200	75	*	*	*
183	Metaphosphoric acid ppte phosphate ppte. methanol ppte. ethyl-cellusolve.	660,000	1,850	100	2	*	*
30	Ammonium sulphate ppte. ethanol fraction-ation cellusolve frac-tionation	2,400#	120#	*	2	*	*
164	Dialysis CM-	18,200	*	*	*	*	*

TABLE 1 (CONTINUED)

REFERENCE	PURIFICATION PROCEDURE	HEMOLYSIN SPECIFIC ACTIVITY	HEMOLYSIN PURIFICATION (fold)	HEMOLYSIN RECOVERY %	PURITY PRECIPITATION LINES	PURITY ELECTROPHORESIS BANDS	PURITY ULTRA-CENTRIFUGE
120,121	Curtain electro-phoresis	*	*	*	*	*	1.4S
81	TCA Methanol ppte DEAE Hydroxylapatite	80,000	2,000	*	1	1	*
97	Dialysis Zn++-ethanol Ethanol Hydroxylapatite	34,300	250	65#	*	*	*
127	Methanol ppte. Seph-G-75 DEAE Seph Methanol ppte. DEAE Seph Methanol ppte.	119,000	440#	1.5#	1	*	3.1
10	$(NH_4)_2SO_4$ ppte. Curtain electro-phoresis $(NH_4)_2SO_4$ ppte.	19,000	1,200	40	2	2	3S,12S
165	Zn++ Ethanol EDTA CM-cellulose Zone electro-phoresis	4,000	194	*	*	*	*

Continued.....

TABLE 1 (CONTINUED)

REFERENCE	PURIFICATION PROCEDURE	HEMOLYSIN			PURITY		
		SPECIFIC ACTIVITY	PURIFICATION (fold)	RECOVERY %	PRECIPITATION LINES	ELECTROPHORESIS BANDS	ULTRA-CENTRIFUGE
114	Methanol ppte. DEAE-Seph CM-Seph	8,000	200-350	10-20	1	2	One peak
43	Acid-methanol ppte. Seph-G-100. Electrophoresis-G-100	10,000	*	25	1	*	2.8
40,41	$(NH_4)_2SO_2$ ppte. Curtain electrophoresis DEAE-cellulose CM-cellulose	1,200,000	*	*	1	*	2.8
3	Bernheimer & Schwartz Heat 1 min. 60°C Urea 8M Dialysis	20,400	3,100#	13#	1	*	1.9S,12S,3S
188	Roto-evaporation Dialysis PO_4= Dialysis-glysine Iso-electric focusing Seph-G-75	50,000-100,000	200-400	50-60	*	*	*

* - Not reported.
# - Calculated from reported data.

are left standing at $0^{o}C$ (3,43). This precipitate, although non-toxic and non-hemolytic, can elicit an anti-alpha hemolysin response in rabbits (3,43), and only a single line is observed in gel diffusion against crude and purified alpha lysin. There is some evidence that the alpha lysin may exist as a molecule of 1.4-1.9S dimensions (2,120,121). These varying degrees of molecular association are reflected in the different values for the molecular weight of alpha hemolysin, which range from 8,000 to 50,000 daltons (10,43,87,121).

The 12S component of alpha hemolysin has a characteristic morphology when examined by negative staining in the electron microscope (2,70,161). Each aggregate presents the appearance of a ring composed of 6±1 subunits arrayed in a circle, the diameter of which is about 8.7 and the centre hole is 2.0 nm. In the absence of imposed orientating forces, these rings exhibit hexagonal close packing (2,70).

Electrophoretic studies indicate that alpha lysin has a charge heterogeneity. Butler (30) reported a single component with an isoelectric point (pI) of 6.4. Later Kitamuro *et al.* (114) discovered at least two components with pI values of about 6.8. Using curtain electrophoresis, Bernheimer and Schwartz (10) isolated four biologically active forms of alpha hemolysin. Wadstrom (188), who employed the technique of isoelectric focusing,

also suggested that four biologically active forms of alpha lysin occurred, the major form at pI 8.5 and others at pI 9.2, 7.0 and 6.0-6.5. McNiven *et al.* (146) obtained similar results. Researchers now believe that alpha lysin exists in several polymeric forms in rapid equilibrium, the whole of which shifts slowly to inactivation and higher molecular weight. The charge heterogeneity exhibited by isoelectric focusing could be another manifestation of polymeric forms in rapid equilibrium rather than the existence of "isotoxins" as suggested by Wadstrom and Mollby (189).

Mode of Action

Studies of the cytotoxicity of alpha hemolysin suggest a direct action against cell membranes. Immunofluorescent techniques have detected the hemolysin on the erythrocyte surface (106,115). Scanning electron microscopy (116) and freeze etching studies (71) have demonstrated the formation of primary lesions on the erythrocyte membrane after incubation with alpha lysin. Transmission electron microscopy has revealed that alpha hemolysin forms regular arrays on erythrocyte surfaces (2,70,71,161).

Artificial phospholipid membranes form spherules or liposomes that have been used as a model system to study the mechanism of action of alpha lysin. Since spherules trap ions within the membrane, the release of trapped ions

is an index of the toxicity against the artificial membrane. Weissmann *et al.* (195), when studying the effect of alpha hemolysin on spherules of varying lipid composition, noted such a release of marker molecules. This release was prevented by prior incubation with anti-alpha hemolysin. The spherulytic action of alpha lysin was independent of the net surface charges since spherules containing dicetyl phosphate were as sensitive as those containing stearyl amine, which suggested a hydrophobic interaction. Experiments with lipid monolayers showed changes in the surface pressure and surface potential consistent with a penetration of the monolayer due to a hydrophobic interaction with the lysin. The spherulytic effect or release of trapped ions was independent of cholesterol in the artificial membrane which seemed to indicate that the hemolysin acted upon the phospholipid components (195). In a later study, Arbuthnott *et al.* (4) concluded that the interaction of alpha hemolysin and biological membranes probably depended upon the location of various lipids in the membrane and their distribution in relation to one another, rather than the presence of a single component.

As stated previously, alpha hemolysin can be induced to form regular arrays of ringed structures on susceptible erythrocyte membranes. Pretreatment of the membranes with phospholipase C abolishes the formation of the regular arrays of alpha lysin but similar pretreatment with other

hydrolytic enzymes has no effect (4,161). The phospholipase C is probably destroying the hydrophobic receptor sites as opposed to competing for any type of substrate with the alpha lysin, because alpha hemolysin has no phospholipase activity (43,208). The antagonism of staphylococcal beta lysin, which is a phospholipase C, towards alpha hemolysin (55) may be because of the destruction of postulated receptor sites.

As noted by Freer *et al.* (71) the key question with regard to the mode of action of alpha hemolysin is whether the demonstrated surface activity is sufficient to account for its biological activity, or whether an additional enzymatic mechanism is also involved. This concept that staphylococcal alpha hemolysin is an enzyme has received consideration from several quarters. Forssman (64-67) first postulated that the action of alpha hemolysin was enzymatic because adsorption of the hemolysin to the erythrocytes of rabbit and sheep was weak, irregular and easily reversible. Van Heyningen (185) noted that both alpha hemolysin and enzymes were capable of acting in extremely low concentrations. Hemolysis of rabbit erythrocytes by alpha hemolysin exhibits a sigmoid curve characterized by a pre-lytic lag, a period of rapid lysis, and a slower rate of lysis (2,39,40,126,130,143,144). If the maximum slope of the rate of hemolysis is plotted against lysin concentration, the result is a straight line, indicative

of an enzymatic reaction (126,143,144). Lominski and Arbuthnott (126) thought that the decreasing rate of lysis in the terminal portion of the hemolytic curve could reflect that the erythrocytes were more resistant to alpha lysin. Later Marucci (143) demonstrated that no difference existed in the susceptibility of erythrocytes remaining after 85% of the population was lysed when compared with the original suspension. Cumulatively a detailed analysis of hemolysis suggested that alpha lysin may be an enzyme but no indication has been given as to the necessary substrate.

Recently, a series of experiments by Wiseman and Caird (208,209) has demonstrated the enzymatic action of alpha lysin. They believe that alpha hemolysin is secreted by *Staphylococcus aureus* as an inactive protease zymogen which is activated by proteolytic enzymes, either from erythrocyte membranes or insoluble trypsin (Wiseman, unpublished data). After activation, alpha lysin has proteolytic activity in addition to hemolytic activity. Both the proteolytic and hemolytic activities are equally neutralized by a monospecific anti-alpha hemolysin. The inactive alpha lysin contains histidine as the N-terminal amino acid; activated hemolysin contains isoleucine or leucine as the N-terminus (Wiseman, unpublished data). Earlier, Bernheimer and Schwartz (12) considered that their alpha-2 preparation consisted of an inactive

hemolysin capable of activation although they did not know the mechanism of action of the activated hemolysin.

Freer *et al.* (71) on the other hand could obtain no evidence for the involvement of a proteolytic mechanism in the mode of action of alpha lysin. They reported that alpha hemolysin caused no changes in erythrocyte ghost protein as would be expected if the alpha hemolysin was indeed activated. However, these workers did not prove that the erythrocyte ghosts possessed biologically active protease necessary for the activation of the alpha lysin. It is quite possible that the method of obtaining "ghosts" is also critical as illustrated by Dodge *et al.* (47).

BETA HEMOLYSIN

The property that drew attention to beta lysin was the classical "hot-cold" hemolytic phenomenon. Hemolysis which is either incomplete or absent at 37^{o}C, becomes readily evident by subsequent cooling to 4^{o}C. This phenomenon, originally attributed to alpha hemolysin (18), was eventually associated with serologically distinct beta lysin (63,79). Reinterpretation of earlier data suggests that several previous researchers were in fact dealing with the beta hemolysin (17,18,152,155,192-194).

Biological Effects

Hemolysis

Apparently the "hot-cold" hemolytic phenomenon is only one expression of a more general effect. The primary result of incubation of either alpha or delta lysins with erythrocytes is direct lysis of the red cells but the primary result of incubation with beta lysin is an increased fragility of the cells in the absence or near absence of hemolysis. Lysis of sensitized erythrocytes by beta lysin can be effected at 37^{o}C by rapid changes in pH or by decreasing the concentration of salt (159,201). Smith and Price (176) noted that glycerols and broths enhanced beta lysin activity and staphylococcal lipase, non-hemolytic by itself, lyses sensitized erythrocytes (36). Strains of Lancefield Group B streptococci produce an agent that lyses sheep erythrocytes exposed to beta lysin (38). In all the above examples, sheep erythrocytes are insensitive unless there is a prior exposure to beta lysin. Because beta hemolysin reacts with a substrate only indirectly related to the integrity of the erythrocyte membrane, Wiseman (201) termed beta lysin a "secondary hemolysin".

Beta lysin is inactivated by dialysis (82,101,163). Similarily, addition of EDTA or citrate to the lysin causes loss of hemolytic activity (163,200,201). The activity of the hemolysin is restored and enhanced by the addition of Mg++ (34,83,90,101,163,200,201). This enhancing effect

can be achieved by several other cations, notably Mn++ (101, 163,200,201), Co++ (163,200,201), and Fe++ (90,163). Although Robinson *et al.* (163) reported that Ni++ stimulated beta lysin, the research has not been confirmed. Unexpectedly, Ca++ did not enhance hemolytic activity (90,101,163,200,201), and depending upon experimental conditions, Zn++ had either no effect (163) or was inhibitory (200,201).

The red cells of sheep, goat and ox are most susceptible to beta hemolysin whereas erythrocytes of the horse, mouse, rat and rabbit are comparatively resistant to lysis (26,63,90,147,170,177,200,201). Other mammalian species of red cells occupy an intermediate position between the extremes. Some workers report that human red cells are resistant to beta lysin (37,90,199); others suggest that human erythrocytes are susceptible although to a lesser extent than sheep cells (170,200,201). Cristie and North (37), who believed that neither alpha nor beta lysin is capable of lysing human red cells, noted that the combination would destroy human erythrocytes. Elek and Levy (56, 58) on the other hand, report an antagonism between alpha and beta lysins on sheep blood agar. Beta and delta lysins have a synergistic effect (55,56,58,142,199). Therefore the effect of beta lysin on human erythrocytes should be interpreted cautiously since there may be small amounts of other hemolysins present.

Toxicity in Animals

The toxic effect of beta hemolysin on whole animals is uncertain. Subcutaneous injection of beta hemolysin into rabbits causes mild erythema but no dermonecrosis (29,79,83, 133,134,200,201). Intravenous injections of crude beta lysin are lethal for rabbits (29,62,79,176), but the effect of purified beta hemolysin is in doubt. Some authors report negative results (200,201) while others claim that doses of 40-160 µg of beta hemolysin are lethal (82,83). Similarly, Heydrick and Chesbro (93) reported that beta lysin in the presence of Mg++ was lethal for guinea pigs and mice but Wiseman (200) was unable to confirm their observations. Expression of lethality as an LD_{50} dose in terms of both hemolytic titre and protein content would resolve the problem since discrepancies may be due to the amount of active lysin injected. The lethal effect of crude beta lysin can be attributed to several factors, such as the presence of small amounts of alpha lysin, or the synergistic effect of beta and delta hemolysin. In early reports, the lethal effect of beta lysin may be due entirely to the presence of delta hemolysin.

Cytotoxicity

The toxicity of beta lysin for various mammalian cells in tissue culture has been reported extensively (34,78,89,102,117,203). However, Gladstone and Yoshida (78)

and Hallander and Bengtsson (89) were unable to demonstrate toxicity of beta hemolysin for several cell lines; HeLa, L, HL, FL, HEp^{-2}, chick fibroblast and human, bovine and monkey kidney cells. Beta hemolysin does not disrupt lysosomes of rabbit leucocytes or rabbit liver cells (11) but it does lyse rabbit blood platelets (12). The presence of beta lysin causes KB and monkey kidney cells to detach from glass (117,203). Generally, susceptible cells are characterized by swelling, disintegration and progressive vacuolation. Wiseman (203) reported an increased uptake of trypan blue and an absence of acid production by mammalian cells incubated with beta lysin. Jeljasewicz *et al.* (102) reported that histochemical studies revealed that beta lysin decreased the number of KB cells that produced acid phosphatase but had no effect on alkaline phosphatase or 5'-nucleosidase activity. They also showed increases in the number of cells containing lipids as detected by Sudan B. Beta lysin has a leucocidal effect upon guinea pig macrophages (34) and Wiseman (203) reported that affected cells can no longer reduce phenolindo-2,6-dichlorophenol to a colourless product, which suggests that beta lysin interferes with respiration.

Physical-Chemical Properties

Little is known about the physical-chemical properties of beta lysin. It appears to be a simple protein with a

sedimentation coefficient of 1.8S (83) (Table 2). Chesbro and Kucic (35) determined that the molecular weight of the beta hemolysin was about 11,000 daltons but the "Donita" strain was unusual as its beta lysin had a molecular weight of 59,000 daltons. Wadstrom and Mollby (189-191) and Maheswaran and Lindorfer (136) reported that the isoelectric point of beta lysin was 9.5, whereas Chesbro *et al.* (34) established a pI of 8.6-8.9.

Mode of Action

Beta hemolysin, a Mg++-dependent sphingomyelinase, catalyzes the following reaction (48,49):

$$\text{Sphingomyelin} + \text{water} \xrightarrow[\text{Mg++}]{\text{beta lysin}} \text{N-acylsphingosine} + \text{phosphorylcholine}$$

Others have shown that purified sphingomyelin is degraded to phosphorylcholine in the presence of highly purified beta lysin (132,134,205,206), and the sphingomyelinase does not appear to be a contaminant of beta lysin as the two activities cannot be separated. Doery *et al.* (48) demonstrated that sphingomyelin was no longer present in phospholipid extracts of erythrocytes that had been pre-treated with beta hemolysin. Then Wiseman and Caird (206) correlated the relative sensitivity of erythrocytes from various species with sphingomyelin concentration in the

REFERENCE	PURIFICATION PROCEDURE	HEMOLYSIN			PURITY		
		SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)	RECOVERY %	PRECIPITATION LINES	ELECTROPHORESIS BANDS	ULTRA-CENTRIFUGE
200,201	Hydroxylapatite Dialysis	1.17×10^6	29#	17	2	*	*
97	Zn++ Ethanol Zn-Hg++ Hydroxylapatite	22,500	250	50-60	*	*	*
91	Acetone fractionation DEAE	6.55×10^6#	256	100	1	*	*
190	CM-Sephadex Isoelectric focusing gel filtration	3.3x10"	40,000	15,000	1	1	*
34	Pervaporation Cellulose phosphate	60,000#	150		2	2	1 peak
133	$(NH_4)_2SO_4$ ppte Seph^{2}G-100 CM or DEAE cellulose	68,200	245	*	1	1	*
83	$(NH_4)_2SO_4$ Seph^{2}G-100 CM-cellulose Density gradient electrophoresis	5.24×10^6	180,800	100	1	8	1.8S

-Not reported.
-Calculated from reported data.

erythrocytes. Maheswaran and Lindorfer (136) confirmed and extended the correlation by using protein-free erythrocyte "ghosts". Beta hemolytic and sphingomyelinase activity are both activated by Co++ and Mg++ (49,134) and, as expected, EDTA inhibits the release of organic phosphorus by this enzyme. In addition, thiol-reacting agents such as p-chloromercuribenzoate and iodoacetate inhibit both hemolytic (34) and sphingomyelinase activity.

Unfortunately, little is known about the *in vivo* mode of action of beta hemolysin. Injection of crude beta lysin increased blood sugar levels in rabbits (42) and intravenous injection of rabbits or cats produced biphasic changes in blood pressure followed by a decline to zero (6). Respiration is stimulated just before death (6,82). Phospholipases are known to inhibit electron transport in mammalian cells (54) and beta lysin has been shown to inhibit the ability of leucocytes to reduce dichlorophenol-indophenol (204). Fritsche (73) could not show that sphingomyelinase affected the cells of staphylococci and concluded that beta lysin acted as a poison in host cells.

DELTA HEMOLYSIN

Williams and Harper (199) discovered a fourth hemolysin while studying staphylococcal hemolysins on sheep blood agar. The activity of delta hemolysin was not neutralized by the presence of anti-alpha lysin and/or anti-beta lysin. This fourth hemolysin was differentiated from the gamma lysin of Smith and Price (177) on the basis of its hemolytic spectrum. These initial observations were confirmed by Marks and Vaughan (142) who also showed that delta hemolysin acted synergistically with beta lysin on sheep erythrocytes. Marks (141) was able to demonstrate, by using several antisera, that the alpha-2 lysin of Minett (147) and Morgan and Graydon (150) was identical to delta lysin. Similarly, he showed that delta was distinct from alpha, beta and gamma lysins.

Biological Effects

Hemolysis

Delta hemolysin possesses a wider hemolytic spectrum than alpha and beta lysin, being lytic for erythrocytes of rabbit, sheep, man, guinea pig, monkey and horse (119, 142,199,204). When Wiseman (204) compared the sensitivities of various erythrocytes to beta and delta lysins, he reported that guinea pig and horse red cells

were sensitive to delta lysin but resistant to beta hemolysin. As pointed out in the review (204), there has never been any clear agreement among investigators regarding the hemolytic spectra of the staphylococcal lysins; a fact mediated by (A) variability of erythrocyte sensitivity to hemolysins in a single animal, (B) differences in hemolysin concentration and techniques of titration used in various laboratories, and (C) use of impure preparations.

Unlike beta lysin, metal cations fail to enhance the hemolytic activity of delta lysin (100,104,119,204) and neither EDTA nor citrate inhibits hemolysis. It is now well established that most proteins can inhibit or reduce the hemolytic activity of delta lysin (78,100,104,119,207). Gladstone and Yoshida (78) measured inhibition of delta lysin by different serum proteins and found that 0.1% concentrations of Cohn fractions I,III,IV and VI inhibited delta hemolysin completely. By contrast, five times this concentration of normal gamma globulin, fraction II, inhibits only 55%. These observations were confirmed by Wiseman and Caird (207). Normal human or rabbit sera also inhibited expression of delta lysin (78,104,119,207). The normal serum inhibitor of delta hemolysin has not been identified but the discovery of similar concentrations of inhibitor in many human sera (52,137) and its presence in fetal calf serum (53) does not suggest naturally occurring antibodies. Microgram quantities of phospholipids inhibit

delta lysin (107,108,119) and Donahue (52) and Kapral (108) have suggested that the neutralizing activity of normal serum is due to lipid(s). Kantor *et al.* (105) believe that delta hemolysin can combine with the alpha-lipoproteins. Some workers (77,207) reported that cholesterol was inhibitory but others failed to confirm this observation (78, 119).

Toxicity in Animals

Data regarding the toxicity of delta hemolysin for laboratory animals are scant. The use of impure preparations had suggested that delta lysin was dermonecrotic for rabbits (76,142) and Kreger *et al.* (119) reported that both his preparation and that of Kapral *et al.* (109) were dermonecrotic for rabbits and guinea pigs if milligram quantities were used. By comparison, a few micrograms of alpha lysin caused greater dermonecrosis.

McLeod (145) found that intravenous injection of a heated crude culture fluid into rabbits was lethal. Although delta lysin is known to be relatively heat stabl- (100,105,119,142), this lethal effect for rabbits should be re-examined with purified preparations.

Wadstrom and Mollby (189) claim that delta hemolysin has an LD_{50} of 125 mg for mice. From the data reported by Kreger *et al.* (119), their preparation of delta lysin has an LD_{50} of 2.0±0.1 mg for mice and 7.17± 0.12 mg for guinea pigs. These values seem unusually

high since alpha hemolysin has an LD_{50} of half a microgram for mice. Comparison of the LD_{50} doses suggests possible contamination of delta lysin with alpha lysin. Neutralization of either dermonecrosis or lethality of delta lysin by anti-alpha lysin was not shown. Gladstone (76) observed that intravenous injection of delta lysin into mice had no visible effect.

Cytotoxicity

Even less information is available regarding the toxicity of delta lysin for individual cell types. Jackson and Little (98,99), working with crude preparations of delta lysin freed of alpha hemolysin, found that it was lytic for human leucocytes. The leucolytic action of delta lysin ran parallel with its hemolytic action on human and horse red cells and like hemolysis, was inhibited by cholesterol (77). Gladstone and Yoshida (78) reported that leucocytes of a number of animals were susceptible to delta lysin but their preparation was contaminated with beta lysin and ribonuclease. This preparation released aldolase and B-glucuronidase from HeLa cells, indicating a toxic action on cell membranes and lysosomes. However, other workers using a similar preparation were unable to demonstrate release of B-glucuronidase or acid phosphatase from lysosomes of rabbit liver.

TABLE 3

COMPARISON OF PURIFICATION PROCEDURES OF DELTA LYSIN

REFERENCE	PURIFICATION PROCEDURE	HEMOLYSIN			PURITY		
		SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)	RECOVERY %	PRECIPITATION LINES	ELECTROPHORESIS BANDS	ULTRA-CENTRIFUGE
142	Ethanol extraction Ether extraction Acetone precipitate	*	*	25-50	*	*	*
99,100	pH precipitate Ethanol extraction	3,200	8	38	*	*	*
214	Calcium phosphate chromatography TEAE cellulose Crystallize	400	22	16	2	2	6.1S
111	Sephadex G-200 pH 4.5 Na_2SO_4 precipitate crystallize	600 (HU/ml)	*	*	3	*	5.5S
110	Calcium phosphate Citric acid Sephadex G-100	100 (HU/ml)	*	2.5	*	*	*
88	Sephadex G-100 polyacrylamide gel electrophoresis	90	11	21	2	*	*
207	pH precipitate Hydroxylapatite DEAE-cellulose	*	30	*	2	*	*

Continued.....

TABLE 3 CONTINUED

REFERENCE	PURIFICATION PROCEDURE	HEMOLYSIN			PURITY		
		SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)	RECOVERY %	PRECIPITATION LINES	ELECTROPHORESIS BANDS	ULTRA-CENTRIFUGE
148	CM-cellulose Isoelectric focusing	*	*	*	*	*	*
105	Ammonium sulphate precipitate Membrane ultra-filtration DEAE cellulose	*	*	26	>1	*	*
135	Isoelectric focusing DEAE cellulose Sephadex G-200 CM cellulose Sephadex G-150	*	*	*	1	1	8.7S
31	pH precipitate Ammonium sulphate DEAE cellulose	11,600	32	16	1	1	2.8S,9.8S
119	Hydroxylapatite	200	15	20-30	*	2	4.9S to 11.9S

*-Not reported.

Physical Chemical Properties

Considerable information is now emerging about the physical-chemical nature of delta lysin. Crude delta lysin is thermostable (87,88,100,142,145) but whether purified delta lysin is thermostable or not seems to depend upon the purification techniques employed (31,104, 105,214). Initially it was believed that delta lysin may have been a lipid because it was soluble in absolute alcohol (142,214) (Table 3). Workers now agree that purified delta lysin is a protein (31,105,119,214) (Table 3). The high percentage of hydrophobic amino acids may explain the solvent behaviour of the crude lysin (105,119). The N-terminal amino acid is proline (31).

Available data suggest that multiple molecular forms of the hemolysin can occur. The lysin is non-dialysable (87,99,142,204) and is excluded from a column of Sephadex G-200 with the void volume (31,88,89,105) indicating a high molecular weight compound. Several workers have reported molecular heterogeneity as indicated by wide elution patterns of hemolytic activity on columns, after ultracentrifugation or by electrophoresis (88,94, 105,119). There has been a corresponding variance in the sedimentation coefficient of delta lysin. Values range from 2.8S to 11.9S (see Table 3). The reported value for the molecular weight has ranged from 5,100 daltons (105) to greater than 200,000 daltons (31,87,88,105,119,204).

This molecular heterogeneity seems also to be reflected in the isoelectric points of delta lysin. Kreger *et al.* (119) reported the presence of acidic and basic delta hemolysins having pI values of 5.0 and 9.5, respectively. Kantor *et al.* (105) separated purified delta hemolysin into three active hemolytic peaks with pI values of 4.65, 6.7 and 7.0. However, Mollby and Wadstrom (148) obtained a single pI of 9.6 for delta lysin. The reported pI of 9.5 for gamma lysin could easily lead to confusion between delta and gamma lysin (149).

Mode of Action

There is now some evidence to suggest that delta hemolysin is an enzyme. The rate of hemolysis of a 1% suspension of erythrocytes was proportional to the delta hemolysin concentration (100). Kapral (107,108) reported that the hemolytic activity of delta lysin was neutralized or inhibited by phospholipids and that the hemolysin appeared to be absorbed to the phospholipids from which it could be recovered by removal of the phosphate radical by non-hemolytic *Bacillus cereus* phospholipase. Earlier Magnusson *et al.* (131) observed that a crude delta lysin preparation of *S. aureus* contained a phospholipase which hydrolysed phosphatidylinositol and lysophosphatidylinositol. They demonstrated that this phospholipase C was distinct from the sphingomyelinase activity of beta lysin.

The highly purified delta lysin of Wiseman and Caird released acid-soluble phosphorus from phosphatidylinositol (31,207) and sphingomyelin was not hydrolysed by delta lysin in contrast with its susceptibility to beta lysin. The release of acid-soluble phosphorus was linear with respect to time (204) and when delta lysin concentration was plotted against the reaction velocity with phosphatidylinositol as a substrate, a straight line was obtained (207). The activation energy, determined from an Arrehenius plot was 18,750 cal, within the range of 1,000-25,000 cal observed for most enzymes (174). The reaction velocity of delta lysin was directly proportional to the temperature from 20-56^{o}C (204).

Several researchers have established that *S. aureus* produces a phospholipase that degrades phosphatidylinositol (31,48,131,207). Wiseman and Caird (31,207) believe that this phospholipase and the delta lysin are identical because they demonstrated a correlation between sensitivity of various erythrocytes to delta lysin and the phosphorus released from these erythrocytes by incubation with the lysin.

GAMMA HEMOLYSIN

Morgan and Graydon (150) reported the existence of two antigenically distinct lysins that attacked rabbit erythrocytes, which they termed "alpha$_1$" and "alpha$_2$".

In 1938, Smith and Price (177) reported the presence of a "gamma" lysin that they thought was the same as "alpha$_2$" lysin. Their conclusion was based on the observation that all sera with a high anti-alpha$_2$ titre also had a high anti-gamma lysin titre. However, the titres were never identical. Subsequently, Marks (141) confirmed the existence of gamma lysin but at the same time demonstrated immunologically that "alpha$_2$" and delta lysins were identical. Hemolysin plate tests with blood agar were unable to differentiate delta and gamma lysin (57,58) and in his monograph, Elek (55) concluded that only three hemolysins existed; alpha, beta and delta. Several pieces of information refute this conclusion. Smith and Price (177) noted that normal sera from a variety of animals possessed low anti-gamma hemolysin titres. Delta lysin is inhibited by serum proteins but Marks (141) was unable to correlate neutralization of gamma hemolysin with inhibition of delta lysin by the same serum. Delta lysin is heat stable whereas gamma lysin is heat labile (85,86,96, 149,158,189). Gamma lysin is characteristically inhibited by agar (85,86,96,189), and for this reason it cannot be detected on hemolysin plate tests.

Jackson (96) reported that mild reducing agents such as cysteine and ascorbic acid inhibited gamma lysin but Guyonnet and Plommet (86) disagreed. However, the technique of the latter workers would not detect reversible

inhibition since the reducing agents were not incorporated into the buffer used for titrations.

A wide range of erythrocytes are lysed by gamma lysin. Rabbit, sheep and human erythrocytes are all sensitive but horse red cells are resistant (86,141,149, 158,177,189).

Smith and Price (177) claimed that crude gamma lysin was slightly dermonecrotic for guinea pigs and lethal when injected intravenously into rabbits. Intraperitoneal injection of low doses did not affect guinea pigs and mice survived injection of 100 hemolytic units of the hemolysin. However, since only crude culture filtrates adsorbed with anti beta lysin were used in these experiments, the significance of the results is doubtful.

Smith (175) suggests that R-variants of *S. aureus* can be characterized by a loss of alpha lysin production without loss of gamma lysin. While the idea is of practical importance, whether or not the observation is correct is in doubt, as only ten strains were studied. However, one of the variants, Smith 5R, produces mainly gamma lysin and little or no alpha lysin and is now used as a prototype strain for the production of gamma lysin in several laboratories.

Mollby and Wadstrom (149) separated culture filtrates of *S. aureus* strain Smith 5R into two hemolytic fractions by isoelectric focusing. The pI = 8.5 peak was

alpha hemolysin and they concluded that the second hemolytic fraction which had a pI of 9.5 was gamma lysin after comparison of the hemolytic spectrum with those of alpha, beta and delta lysins. This second fraction was inhibited by agar but not by agarose.

Plommet and Bouillanne (158) described rather exacting conditions for the production of gamma lysin in broth, in which aeration was critical. With the Smith 5R strain, titres of 250 HU/ml with human erythrocytes were obtained after 16 h incubation in 20% carbon dioxide and 80% oxygen. On the other hand, Mollby and Wadstrom (149) obtained maximum yields of 40 HU/ml after 24 h incubation in air with no special conditions.

Guyonnet *et al.* (85,86) purified gamma lysin 30-fold with 60-65% recovery by the use of hydroxyapatite columns. Two slightly hemolytic protein peaks called "gamma-I" and "gamma-II" were obtained. Gamma-I was heat sensitive, inhibited by agar and lysed rabbit, sheep and human erythrocytes but not horse red cells. This fraction was contaminated by at least three other proteins, one of which was beta lysin. Gamma-I and gamma-II acted synergistically on human, rabbit and sheep erythrocytes and gamma-II was poorly immunogenic. Two precipitin lines were obtained when gamma-II was diffused in gel against antisera (86). These authors suggest that gamma lysin is a complex of gamma-I and gamma-II, but an alternative

explanation is that gamma-I is contaminated with beta lysin and that gamma-II contains delta lysin, since the synergism between beta and delta lysins has been well established. Mollby and Wadstrom (149,189) were unable to confirm the work of Guyonnet and co-workers. It should be emphasized that none of the data obtained for gamma lysin has been confirmed with the use of homogeneous material.

MATERIALS AND METHODS

BIOLOGICAL METHODS

Cultures

Characteristics of the strains of *Staphylococcus aureus* used in this study are given in Table 4. The phage susceptibility patterns were determined at the Canadian Communicable Disease Centre in Ottawa. Immunodiffusion of crude hemolysin dilutions against constant amounts of specific antisera gave an estimate of the relative amount of hemolysin produced by each strain. The strains were coagulase-positive and fulfilled the criteria for *S. aureus* given by Breed *et al.* (21). All strains were preserved by lyophilization and by subculturing once a month on Brain-Heart Infusion agar (Difco). Those that were subcultured were incubated at 37°C for 24 h and stored at 4°C. The strains were examined periodically for purity and types of hemolysin produced.

Production of Crude Hemolysins

The semi-synthetic medium of Dolman and Wilson (51) was used for the production of alpha hemolysin. Five ml of a 16 h broth culture of *S. aureus* strain Wood 46 were inoculated into 500 ml of sterile broth in a one litre erlenmeyer flask. The cells were grown for 36 h in 10% carbon dioxide at 37°C on a New Brunswick reciprocating incubator shaker (model R25) at 100 rpm after which the cultures were centrifuged at 10,000 x g for 15 minutes.

TABLE 4

CHARACTERISTICS OF THE STRAINS OF *S. aureus* EMPLOYED IN THIS RESEARCH

STRAIN	PHAGE TYPE	HEMOLYSIN PRODUCTION ALPHA	BETA	DELTA	GAMMA	LITERATURE REFERENCES
Wood 46	Non-typable	4+*	2+	1+	2+	83
Smith 5R	6/47/53/54/85	±	1+	1+	4+	85,86,158,175
E-delta	3C/55/71	2+	1+	4+	0	203,204
R-1	Non-typable	1+	4+	2+	1+	200-202
MB-534	47/53	3+	4+	1+	0	83
Newman	47/54/75/77/84/85	2+	3+	3+	1+	31,203,204

*0 - none detected
1+ - slight
2+ - moderate
3+ - high
4+ - very high

Delta hemolysin was isolated from strain Newman. The hemolysin that gave a 4+ value was purified from all the other strains.

In this manner 7½ litres of crude supernatant hemolysin could be obtained easily at one time.

The technique of Birch-Hirschfeld (19) was utilized for the production of crude gamma lysin by *S. aureus* strain Smith 5R. Difco agar (1.5% w/v) was added to 500 ml amounts of Dolman-Wilson broth and the autoclaved mixture was poured into sterile stainless steel trays with an area of 1,800 cm^2. After cooling, the solid agar was overlaid with cellophane (Dennison, Drummondville, Que. #57001) which had been sterilized by immersion in water and exposure to steam for 1 hour. The cellophane surface was then inoculated with the organisms. The inoculum was prepared from slants of 16 h cultures which were flooded with 5 ml of sterile phosphate buffered saline and the cells suspended by agitation. This suspension was distributed over the surface of the cellophane with a glass spreader and the trays were covered before being placed in "perspex" boxes. The boxes were flushed with 10% carbon dioxide in air at a rate of 20 l/min for 10 minutes before being incubated at 37°C for 24 hours. At the end of the period, the trays were removed and the cellophane was flooded with 50 ml of phosphate buffered saline. The cells were suspended with a bent glass rod and centrifuged at 10,000 x g for 15 minutes. The supernatant which contained crude gamma lysin was retained. Petri dishes were used instead of steel trays when several variables

were examined at one time, and consequently all volumes were reduced proportionally.

Beta and delta lysin were produced in the same way as gamma lysin except that different strains were used (Table 4).

Purification of the Hemolysins

Gamma and alpha hemolysin were purified as described in the Results section (see pp. 79-100). Beta hemolysin was purified by a modification of the method of Wiseman and Caird (206) in which the hemolysin was passed through Sephadex G-75 equilibrated with the buffer of Hallander (87). The active material was pooled when removed from the column, dialysed against distilled water for 48 h, centrifuged and lyophilized. The delta hemolysin was purified according to the method of Caird and Wiseman (31). Both the beta and delta lysin were homogeneous by the criteria used in this thesis.

Erythrocytes

Human blood (type O, Rh+) was obtained from the Red Cross in Winnipeg. Sheep erythrocytes were supplied by the National Biological Laboratory, Winnipeg, and rabbit red cells were obtained by bleeding animals maintained in this Department. Erythrocytes of other species were obtained from departmental animals or from the National Biological Laboratory except for the monkey erythrocytes

which were purchased from Connaught Laboratories (Toronto). All erythrocytes, except those of man, were stored in an equal volume of Alsever's solution (33). Human blood was stored in acid-citrate dextrose solution as supplied by the Red Cross. Prior to use, the red cells were washed three times in phosphate buffered saline and resuspended to a 2% concentration in buffer.

Hemolysin Titration

Hemolysins were titrated by a modification of the method of Weissmann (201). Serial two-fold dilutions of the lysin were made in 1 ml of phosphate buffered saline in tubes measuring approximately 1 x 8.5 cm. An equal volume of a suspension of 2% erythrocytes was added to each tube to give a final volume of 2 ml and the mixture was incubated at 37^{o}C for 1 h. After 16 h in the cold, the erythrocytes had settled and any hemolysis was readily apparent. The series of tubes which contained the 50% visual end point was centrifuged at 2,000 x g and 1 ml of supernatant fluid was diluted 1:3 with distilled water. The absorbance of this fluid was read at 541 nm in a spectrophotometer and plotted against the $\log_{10}$ dilution of the hemolysin. The 50% end point was obtained by comparison with data taken from a standard curve prepared by lysis of the erythrocytes with distilled water. One hemolytic unit (HU) was defined as the amount of hemolysin which would cause 50% hemolysis under the

conditions just described.

Alpha hemolysin was titrated against rabbit red blood cells while beta lysin was assayed with sheep erythrocytes. Gamma and delta lysins were titrated against human red cells. When necessary, delta hemolysin could be distinguished from gamma hemolysin by incorporation of 1% normal (non-immune) human sera into the buffer to inhibit delta hemolysin. Since the hemolytic effect of gamma and delta lysins was additive, any residual hemolysis was attributed to gamma lysin.

The activity of beta hemolysin was enhanced by the presence of 0.001 *M* $MgSO_4$, and the salt was incorporated into the buffers used in these titrations.

Kinetics of Hemolysis of Erythrocytes by Gamma Lysin

Some experiments required that the rate of hemolysis be measured. Washed erythrocytes were resuspended in buffered saline so that a 1:2 dilution would have an absorbance of 1.8 at 650 nm. After an equal volume of gamma hemolysin in buffered saline was added to the erythrocyte suspension, the absorbance at 650 nm was monitored continuously in a cuvette with a 1 cm light path. The slope of the plot of absorbance decrease vs time, expressed as absorbance change/min, reflected the amount of hemolysin present.

Growth of S. aureus

The growth of *S. aureus* was determined spectrophotometrically. Cell cultures were diluted 1:20 with phosphate buffered saline and the absorbance at 650 nm was taken as an index of the cell mass. Viable counts were performed by the standard pour plate method with Dolman-Wilson agar and the plates were incubated for 24 h at 37^{o}C.

Tissue Culture

Cells of the C-6 line, which are a clonal subline derived from the NC-37 cell line of human lymphoblast-like cells (95) were supplied by Dr. A. Wallbank of this Department. These cells were grown in stationary culture at 37^{o}C in McCoy's 5A medium (Grand Island Biological, New York) supplemented with 10% fetal calf serum, until the concentration reached 1 x 10^{7} cells/ml. The cells were harvested, washed once with the same medium but with 2.5% added fetal calf serum, and resuspended to the original volume in this medium.

Antisera

Antibodies were prepared in two month old New Zealand white rabbits purchased from the Canadian Research Animal Farm, Bradford, Ontario. Two injection schedules were used, one for crude hemolysins and one for purified lysins. Rabbits were injected subcutaneously with doses

of crude hemolysin increasing from 0.10 to 1.00 ml at two week intervals for a period of three months. A solution of purified hemolysin (2 mg/ml) in buffered saline was injected subcutaneously every three days for three weeks and after a two week rest the schedule was repeated. The first three injections consisted of 0.10, 0.25 and 0.50 ml of hemolysin respectively, followed by 1.00 ml thereafter. The purified alpha hemolysin was converted to a toxoid by heating (60^{o}C, 30 min). The animals were bled from the heart, the serum was separated from the blood and stored at -20^{o}C. The longer injection schedule for the crude hemolysin was required because all the hemolysins contained a small amount of alpha hemolysin which was dermonecrotic and the animals were allowed to recover from each injection.

Before use, antisera were twice precipitated with ammonium sulphate and fractionated with diethylaminoethyl cellulose (32). All purified gamma globulin samples gave a single precipitin line in immunoelectrophoresis when diffused against goat anti-rabbit sera.

Immunological Tests

Immunodiffusion, immunoelectrophoresis, and quantitative precipitin tests were performed according to the methods of Campbell *et al.* (32). Blood agar was used in some immunodiffusion tests and was prepared by

addition of 1.0 ml of packed washed erythrocytes to 100 ml of a 1% (w/v) solution of agarose in phosphate buffered saline. Antigen concentrations were always 10 mg/ml.

PHYSICAL AND CHEMICAL METHODS

Buffers

All buffers were prepared according to Gomori (80). Phosphate buffer, 0.01 *M*, pH 7.0, was supplemented with 0.85% NaCl.

Protein Assays

Protein in column eluates was expressed in terms of absorbance at 280 nm in a quartz cuvette with a 1 cm light path. The amount of protein in crude culture filtrates was measured by Bailey's modification (5) of the technique of Lowry *et al.* (128) with bovine serum albumin as a standard. Since more accurate evaluations were required for the purification techniques and for the purified hemolysins, the micro-kjeldahl technique described by Markham (140) was used to assay nitrogen and the value was multiplied by 6.25 which gave an estimate of the protein content of the sample.

Phosphorus Assays

Acid-soluble phosphorus was determined by the method of Fiske and Subbarow (61) as described by Leloir

and Cardini (125), and total phosphorus was measured as suggested by the latter.

Thin Layer Chromatography

Identification of N-Terminal Amino Acids

The technique of Fraenkal-Conrat *et al*. (69) was applied to the determination of the N-terminal amino acids of the hemolysins, except that the dinitrophenyl amino acids were chromatographed on Silica gel G thin layer chromatographic plates prepared by the method of Stahl (178). Both water-soluble and ether-soluble extracts were chromatographed by ascending chromatography in several solvent systems: n-propanol/ammonium hydroxide (7:3), chloroform/benzyl alcohol/acetic acid (70:30:3), chloroform/methanol/acetic acid (95:5:1), benzene/pyridine/acetic acid (80:20:2) and chloroform/t-amyl alcohol/acetic acid (70:30:3) (v/v) (156).

Identification of Phospholipids

Phospholipids were identified by ascending chromatography on Silica gel G plates. The plates were developed in the solvent of Marinetti (139) and the spots were visualized by spraying the plates with rhodamine 6G or ammonium molybdate (1).

Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was performed as described by Davis (44) and Ornstein (154).

A 7.5% gel, alkaline system with separation at pH 9.3 was used. After extrusion, gels were stained with 1% Amidoschwartz in 7% acetic acid for 1 h and then electrolytically destained. The absorbance of the gels was measured with a Chromoscan densitometer (Joyce Loebl, Gateshead, England).

Ultracentrifugation

Solutions of the hemolysins were subjected to sedimentation velocity analysis in a Spinco Model E analytical ultracentrifuge with a Spinco AN-D analytical rotor operating at 60,000 rpm at 20°C. The diffusion coefficients at 20°C were determined with a synthetic boundary cell in an AN-D rotor that was operated at 4,000 rpm for the gamma lysin and 6,000 rpm for the alpha lysin. In all cases, photographs of the Schlieren patterns were taken at 8 minute intervals.

Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of purified staphylococcal hemolysins were determined with lyophilized preparations which were reconstituted to 1 mg/ml in phosphate buffered saline.

Amino Acid Analysis

The percent composition of the different amino acids in the staphylococcal hemolysins was determined with an Autotechnicon autoanalyzer after the proteins

were hydrolysed in 6 *N* HCl under vacuum for 24 h at 105°C. The analyses were performed by Dr. Labella of the Department of Pharmacology and by Dr. Fritz-Stevens of the Department of Biochemistry.

Isoelectric Focusing

The isoelectric focusing technique of Vesterberg *et al.* (187) was employed and a 110 ml LKB 8101 column (LKB Produckter, Stockholm) was used with ampholytes in the pH range 3-10. The pH value of each fraction (2 ml) was measured immediately after elution from the column. After dialysis against phosphate buffered saline, the absorbance at 280 nm and the hemolytic activity were measured.

Gel Filtration

Sephadex gels and columns (K50 x 100 cm) were supplied by Pharmacia of Canada Ltd., Montreal. The procedures for preparation of gels and packing of columns were those recommended by the manufacturer (157). The columns were fitted with flow adapters and the samples were separated on the column by upward elution.

STATISTICAL METHODS

Statistical analyses were performed with the standard statistical programs supplied by the Health Sciences Centre Computer Department for the CDC 1800 computer. The statistical equations were derived from

the text by Steel and Torrey (179), except for the probit analysis of the LD_{50} dose which was developed from that of Finney (60). Dr. Stevens of the Department of Physiology, University of Manitoba, gave guidance in the statistical analyses.

RESULTS

CHAPTER 1

PRODUCTION OF GAMMA HEMOLYSIN

Attempts to produce gamma hemolysin by the method of Guyonnet *et al.* (85) were unsuccessful and, therefore, the conditions required for production of gamma lysin were examined in detail. Cell growth and hemolysin activity were measured in these experiments. The medium of Dolman and Wilson (51), Gladstone (77) and Stolp and Petzold (180), as well as Brain-heart Infusion Broth (Difco) all supported the growth of *S. aureus* in liquid culture but consistently gave low hemolysin titres (Table 5). Growth of *S. aureus* strain Smith 5R on sloppy agar (0.6% w/v) or solid agar (1.5% w/v) and extraction of the hemolysin by freezing and thawing the media also resulted in low recoveries (Table 5).

Comparison of Solid Media Overlain with Cellophane

The technique of Birch-Hirschfield (19) was used in which solid media were overlain with sterile cellophane followed by inoculation of the cellophane surface. In this manner, the ability of ten different solid media to support growth and hemolysin production was examined after 24 h incubation at 37^{o}C in an atmosphere of 10% carbon dioxide (Table 6). Regression analysis of these results in Table 6 revealed a positive correlation between

TABLE 5

GROWTH AND HEMOLYSIN PRODUCTION ON LIQUID, SEMI-SOLID AND SOLID MEDIA

MEDIUM	GROWTH (O.D. 650 nm)	HEMOLYSIN TITRE (HU/ml)
Liquid Medium		
Dolman-Wilson	0.51	78
Gladstone's	0.11	0
Stolp and Petzold's	0.24	13
Brain Heart Infusion	0.48	23
Semi-Solid Medium*		
Dolman-Wilson	0.55	0
Gladstone's	0.35	0
Stolp and Petzold's	0.30	0
Brain Heart Infusion	0.45	0
Solid Medium*		
Dolman-Wilson	0.63	0
Gladstone's	0.59	0
Stolp and Petzold's	0.52	0
Brain Heart Infusion	0.40	0

*Media subjected to freezing and thawing.

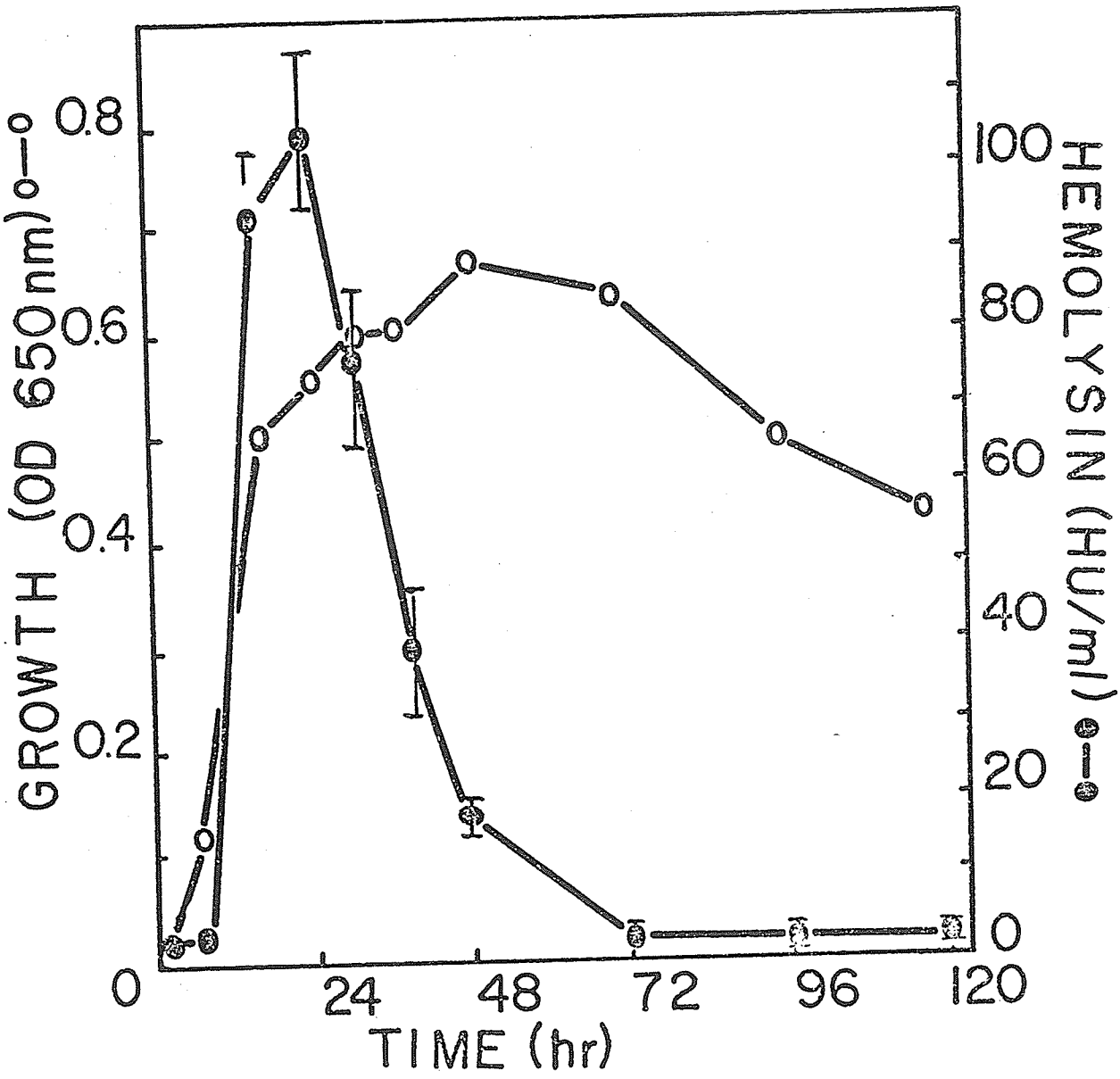
TABLE 6

GAMMA HEMOLYSIN PRODUCTION ON DIFFERENT MEDIA

MEDIUM	GROWTH (O.D. 650 nm)	HEMOLYSIN TITRE (HU/ml±S$\bar{x}$)
Dolman-Wilson	0.810	453±21
Gladstone's	0.850	441±27
Brain-Heart Infusion	0.650	263±14
Veal Infusion	0.710	205±20
Liver Infusion	0.790	152±10
Stolp & Petzold	0.516	136±9
Blood Agar	0.610	86±6
Trypticase Soy	0.492	30±2
Nutrient Agar	0.320	15±1
Casamino Acids	0.116	6±1

FIGURE 1

Effect of incubation time on the production of gamma hemolysin by S. aureus strain Smith 5R


GROWTH (OD 650nm)
0.8
0.6
0.4
0.2
0
HEMOLYSIN (HU/ml)
100
80
60
40
20
0
24
48
72
96
120
TIME (hr)

hemolysin production and growth. Cells of *S. aureus* grown on Dolman-Wilson's medium or Gladstone's medium gave the highest titre crude hemolysin but Dolman-Wilson's medium was selected for routine use because it was easier to prepare.

Effect of Incubation Time on Production

In this experiment, cells were grown for various intervals so that the time for best recovery of the hemolysin could be determined. The hemolysin samples were frozen at -20°C until completion of the experiment and then titrated against the same sample of blood. Gamma hemolysin produced on Dolman-Wilson agar reached a maximum after 24 h incubation, at the end of the logarithmic phase of growth (Fig. 1). Maximum growth was not reached until 48 h but by this time only 15% of the total hemolytic activity demonstrable at 24 h was still present.

The rapid appearance of gamma hemolysin during the logrithmic phase of growth suggested that it may have been an extracellular product (160). In a time-course experiment similar to that shown in Fig. 1, cells of *S. aureus* were pelleted by centrifugation and washed twice in phosphate buffered saline. Both the supernatant and the washed cells were incubated with Lysostaphin (1 mg/ml) (Mead-Johnson Co.) for 15 minutes at 37°C and the fluid was then assayed for gamma hemolysin and protease. The

solutions were incubated with Azocoll (Calbiochem) (5 mg/ml) for 15 minutes at 37°C and the absorbance at 520 nm at the end of this time was used as an index of the amount of protease present. Although *S. aureus* is known to produce extracellular proteases, the fact that strain Smith 5R does not, allowed the presence of a protease to be used as an indicator of autolysis. Intracellular hemolysin that was present in the washed pellet was detected after 8 h (Fig. 2) and reached a maximum at 16 h, whereas the extracellular hemolysin in the supernatant reached a peak at 24 h with a curve similar to that shown in Fig. 1. Although protease was present intracellularly, it did not appear in the supernatant until 36 h had elapsed, which suggests that the cell membrane was intact when gamma lysin was released into the medium.

pH

Cells of *S. aureus* grew well over a pH range of 5.0-8.5 (Fig. 3) but the gamma hemolysin was produced optimally at pH 7.0. The observed reduction in activity above and below pH 7.0 was not the result of instability of the hemolysin as the pH of the solution was adjusted to 7.0 immediately after recovery of the crude hemolysin. The buffered salts used in this experiment had no intrinsic effect upon the hemolysin stability; that is, they neither enhanced nor inhibited the hemolysin's activity. Hemolysin

FIGURE 2

Production of intracellular and extracellular hemolysin

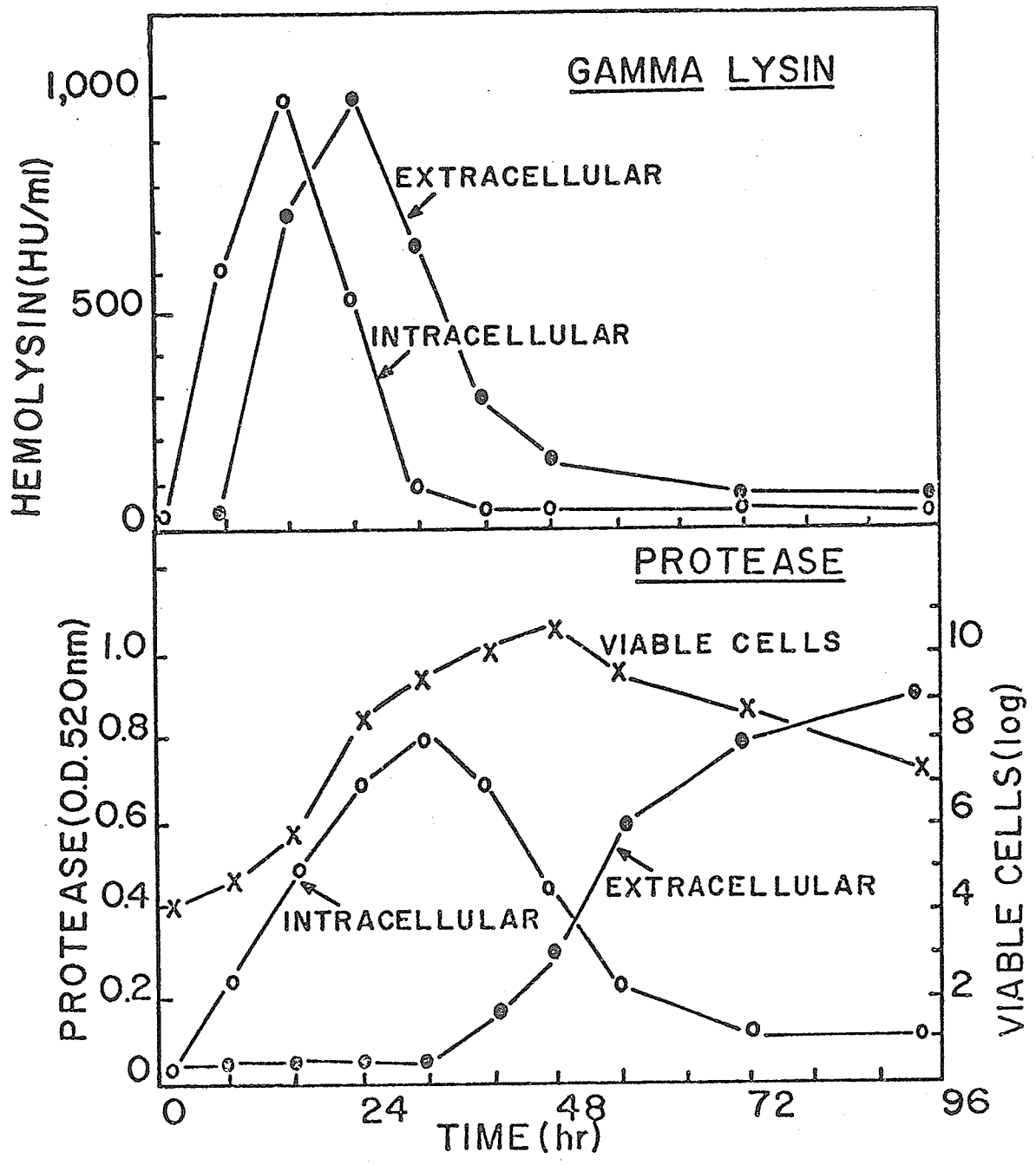
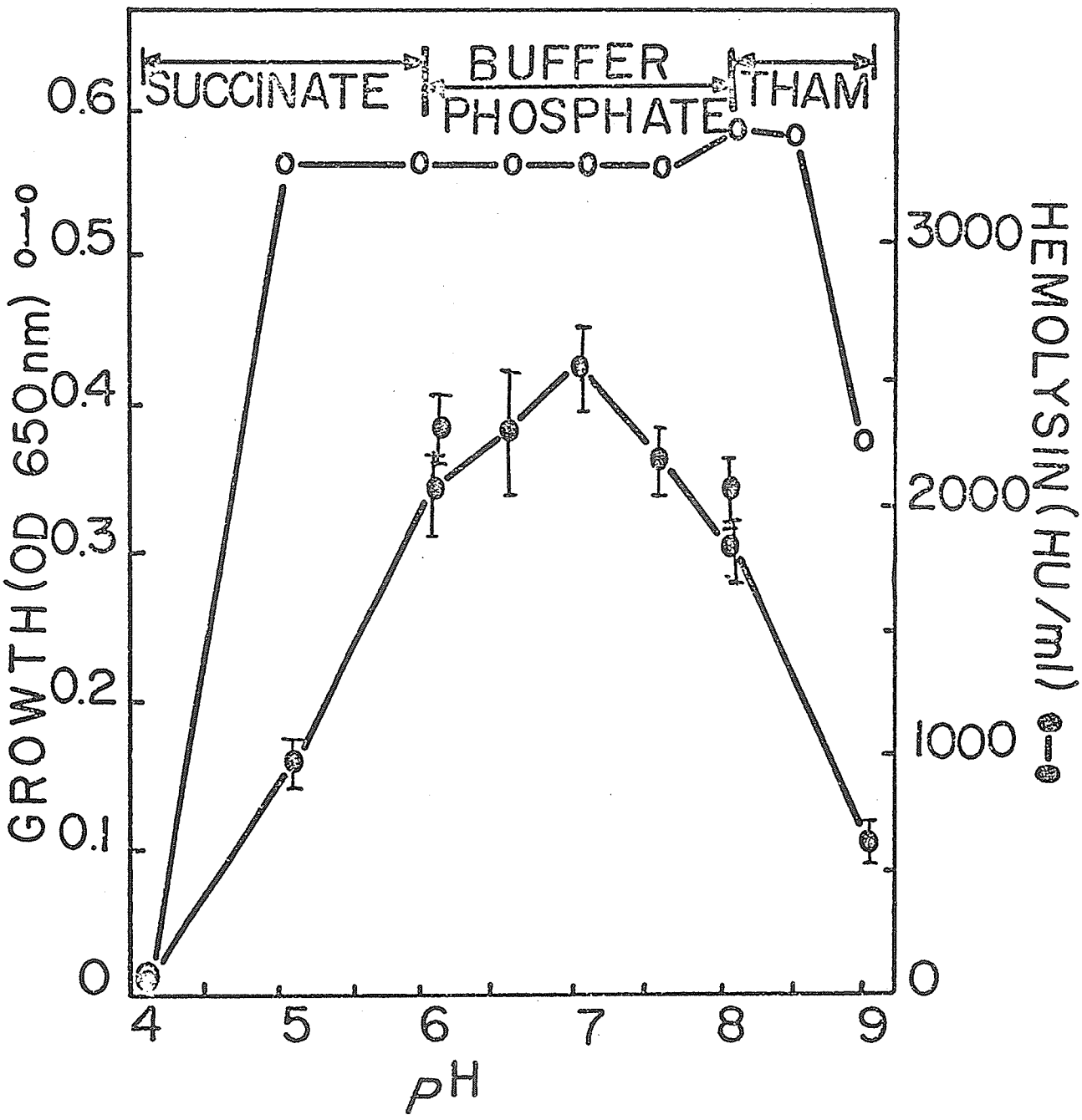

GAMMA LYSIN
1,000
500
0
HEMOLYSIN(HU/ml)
EXTRACELLULAR
INTRACELLULAR
PROTEASE
1.0
0.8
0.6
0.4
0.2
0
PROTEASE(O.D.520nm)
VIABLE CELLS
10
8
6
4
2
VIABLE CELLS(log)
INTRACELLULAR
EXTRACELLULAR
0
24
48
72
96
TIME(hr)

FIGURE 3

Production of gamma hemolysin at various pH values


BUFFER
SUCCINATE
PHOSPHATE
THAM
0.6
0.5
0.4
0.3
0.2
0.1
0
GROWTH (OD 650nm)
3000
2000
1000
0
HEMOLYSIN (HU/ml)
4
5
6
7
8
9
pH

dialysed against succinate and phosphate or Tris-HCl and phosphate buffers at the same pH gave similar levels of activity when titrated.

In another experiment, crude hemolysin was dialysed against buffers with a range of pH's from 4.0-9.5 but retained all of its activity even after storage for 4-6 weeks at 4°C. However, crude hemolysin was rapidly destroyed by exposure to pH values below 3.5 or above 10.0 (data not shown).

Temperature

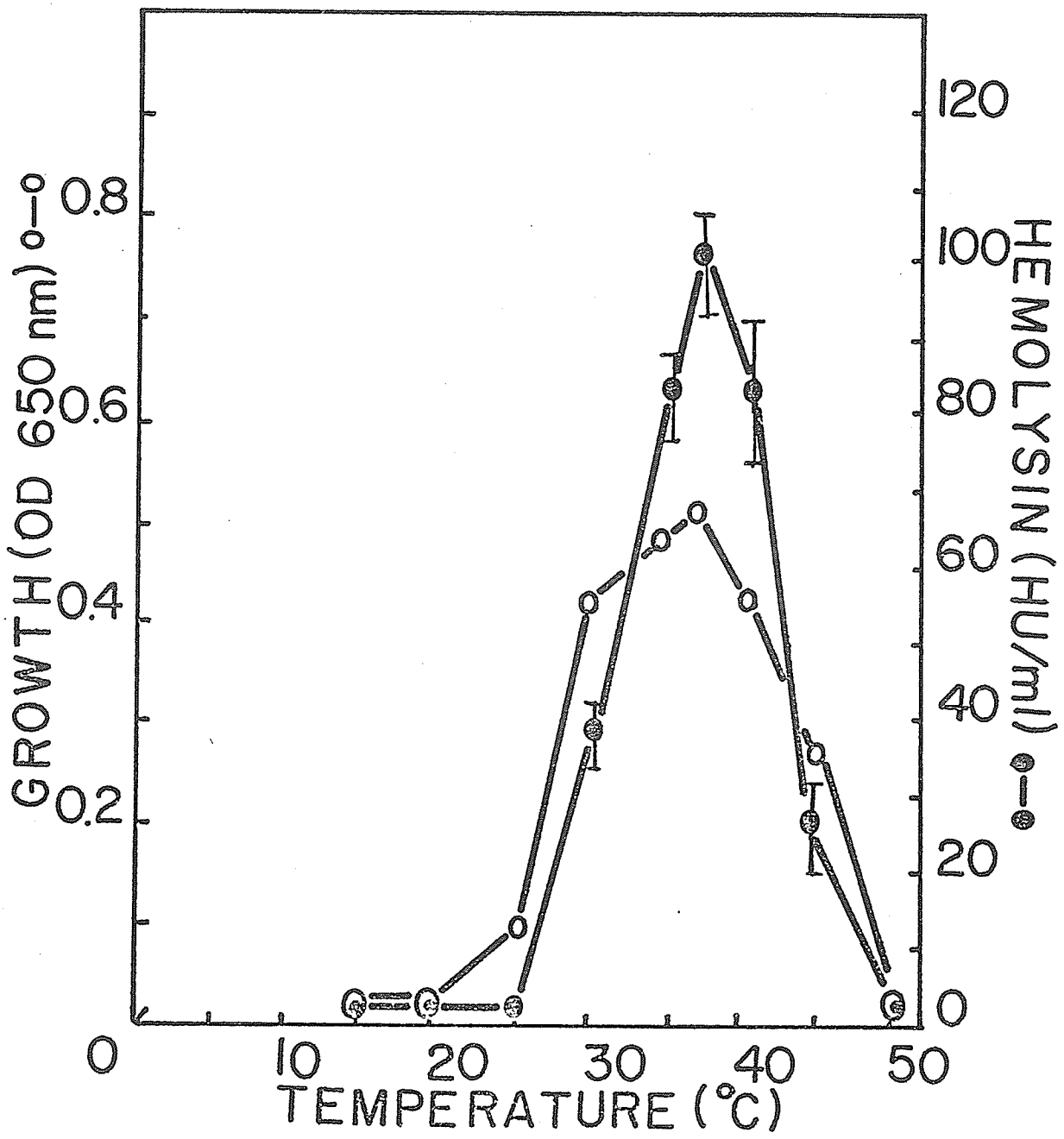
Temperature influenced both growth and hemolysin production (Fig. 4). Optimal hemolysin production and growth both occurred at 37°C but a shift of 3°C from this optimum resulted in a significant decrease in the amount of hemolysin in the supernatant. No hemolysin was detected at 25°C or 50°C although growth still occurred at these temperatures.

Carbon Dioxide

In this experiment, inoculated petri dishes placed in Torbol anaerobic jars were flushed with a mixture of carbon dioxide and air then sealed and incubated at 37°C for 24 h. The amount of carbon dioxide in the mixture, determined by relative flow rates of carbon dioxide and compressed air, was measured at lower concentrations with a carbon dioxide analyser. The production of gamma hemolysin

FIGURE 4

Production of gamma hemolysin at different temperatures


GROWTH (OD 650 nm)
0
0.2
0.4
0.6
0.8
HEMOLYSIN (HU/ml)
0
20
40
60
80
100
120
0
10
20
30
40
50
TEMPERATURE (°C)

exhibited a marked dependence upon the carbon dioxide concentration (Fig. 5) with optimal yields of the hemolysin being produced in an atmosphere of 10% carbon dioxide. Growth decreased at higher concentrations of the gas but an increase of growth was observed repeatedly at 90% concentrations. Maximum growth occurred in air but maximum amounts of hemolysin were formed in 10% carbon dioxide.

The possibility was considered that the carbon dioxide was dissolved and reduced the amount of hemolysin formed because the pH of the medium would then be lower. However, in a separate experiment, pH remained constant over the range of carbon dioxide concentrations tested (Data not shown).

Carbon Source

The ammonium lactate in the medium of Dolman and Wilson was replaced by a variety of different carbon sources to determine the carbohydrate requirements for production of gamma lysin. When 1% concentrations of the carbohydrates were used, ammonium lactate could be replaced only by sodium lactate and beta glycerophosphate (Table 7). Although many of the carbon sources supported the growth of *S. aureus* Smith 5R, only these three permitted the production of hemolysin. Similar results were obtained when 0.1% concentrations were used.

FIGURE 5

Production of gamma hemolysin in different atmospheres of carbon dioxide and air.

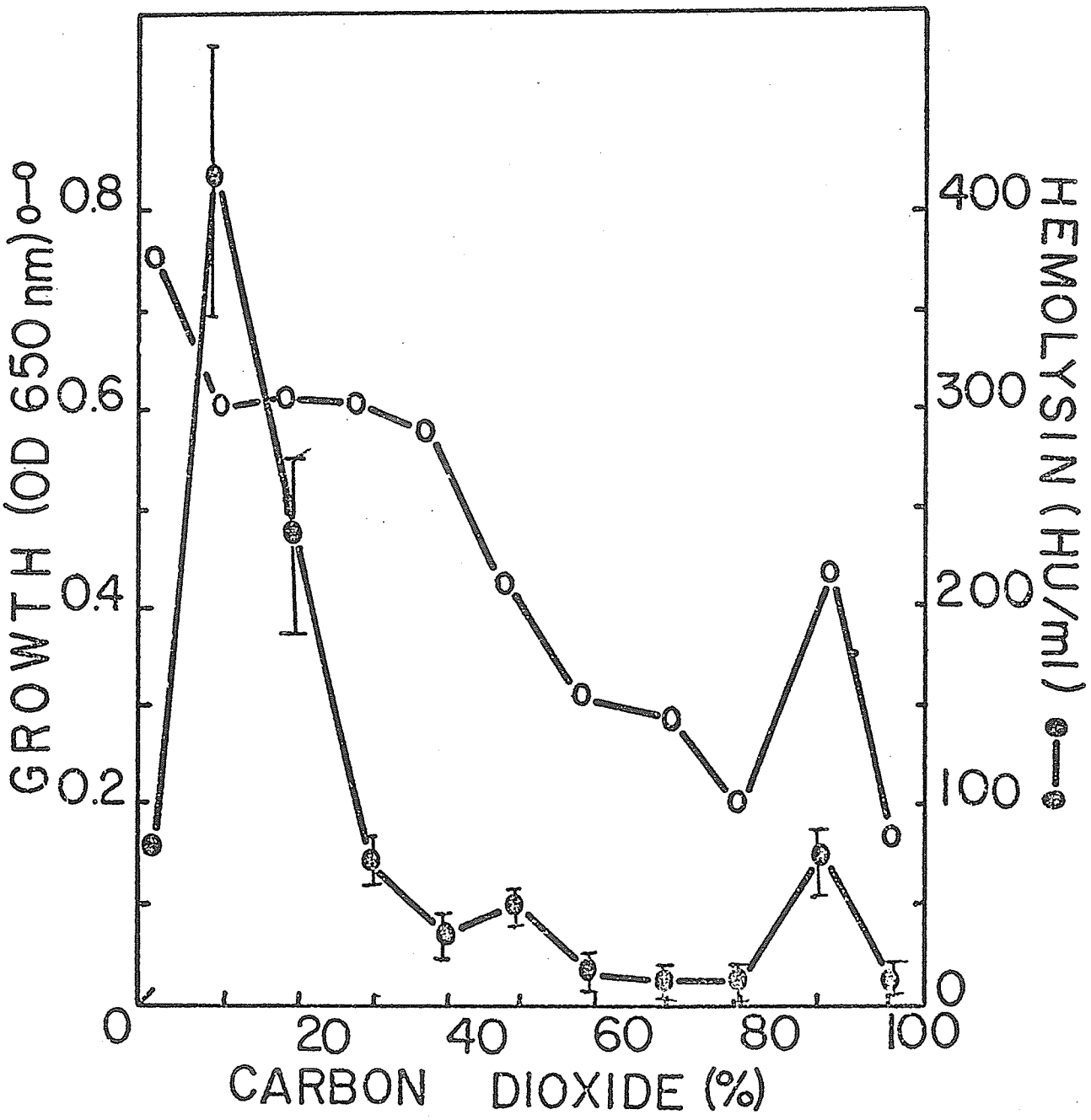

GROWTH (OD 650 nm)
0.8
0.6
0.4
0.2
0
HEMOLYSIN (HU/ml)
400
300
200
100
0
0
20
40
60
80
100
CARBON DIOXIDE (%)

TABLE 7

GAMMA HEMOLYSIN PRODUCTION ON DIFFERENT CARBON SOURCES

CARBON SOURCE (1% conc.)	GROWTH (O.D. 650 nm)	HEMOLYSIN (HU/ml±$S\bar{x}$)
Lactose	0.720	0
Maltose	0.496	0
Fructose	0.316	0
Sucrose	0.560	0
Glucose	0.292	0
Galactose	0.520	0
Raffinose	0.302	0
Arabinose	0.276	0
Melibose	0.292	0
Cellibose	0.314	0
Levulose	0.364	0
Xylose	0.300	0
Mannitol	0.466	0
Sorbitol	0.600	0
Dextran	0.610	0
Starch	0.326	0
Glycerol	0.680	0
Ammonium Lactate	0.680	517±29
Sodium Lactate	0.480	471±23
Glycerophosphate	0.260	487±24
None	0.260	0

CHAPTER 2

ASSAY OF GAMMA HEMOLYSIN

SECTION A: OPTIMAL CONDITIONS FOR ASSAY

The research involving gamma hemolysin was directly dependent upon the assay of its biological activity. A large number of methods for determining hemolytic activity were available for the other staphylococcal hemolysins but the factors that could affect hemolysis by gamma lysin were unknown. Consequently, optimal conditions for the hemolysin assay were investigated with crude hemolysin.

Hemolysin Concentration

Washed human erythrocytes were resuspended to a final concentration of 1% in phosphate buffered saline containing various amounts of gamma hemolysin and the absorbance at 650 nm was observed continuously so that the rates of lysis of the erythrocytes could be determined (Fig. 6). At concentrations of 4-10 HU/ml, the reaction was completed within 9 minutes. After a short pre-lytic lag, the hemolysis curve became linear with respect to time and was then followed by a "tailing off" of the rate. The slopes of the time-absorbance curves in Fig. 6 were taken as the velocity of the hemolytic reaction and were plotted as a function of the hemolysin concentration (Fig. 7). The velocity of the lysis was directly proportional

FIGURE 6

Hemolysis of human erythrocytes by gamma lysin

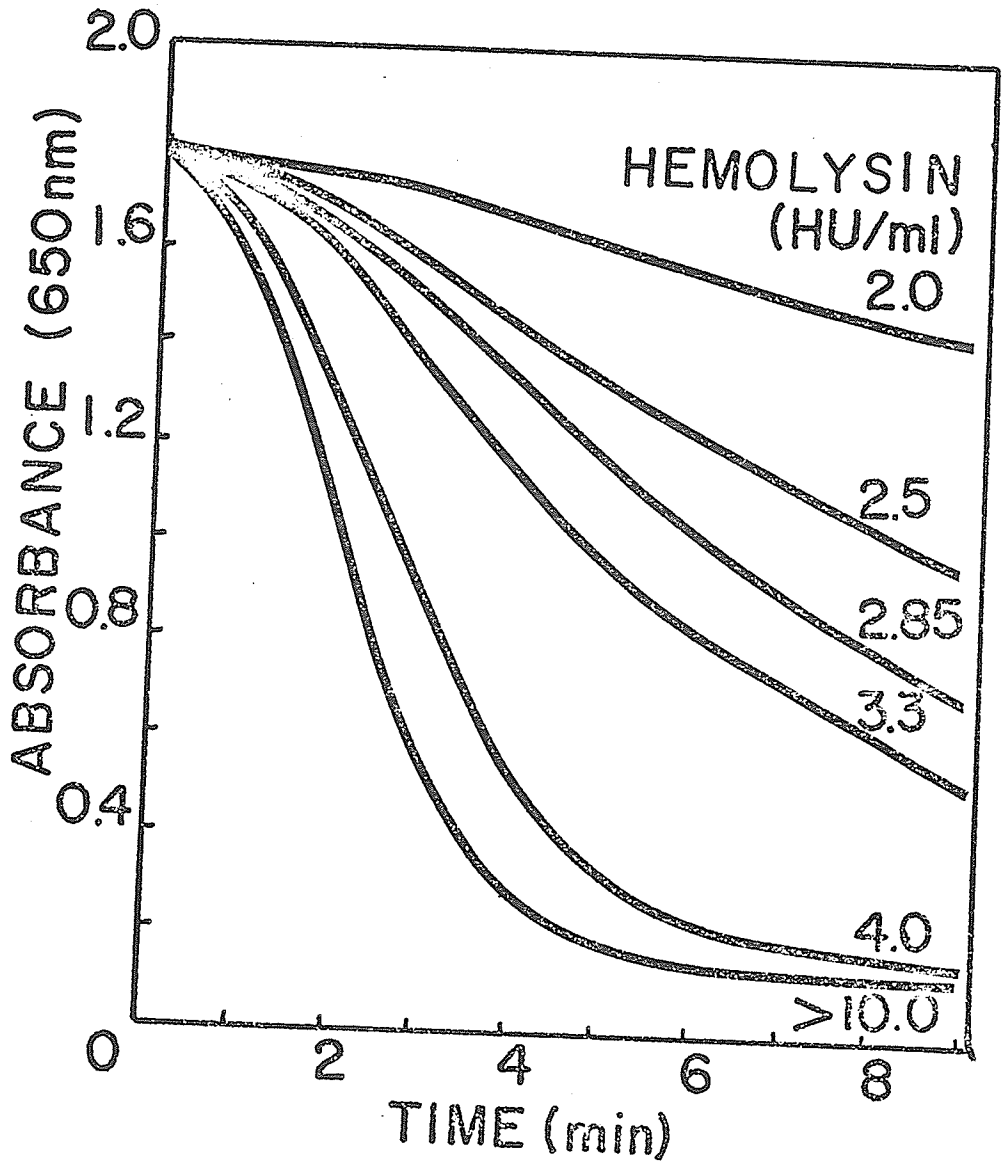
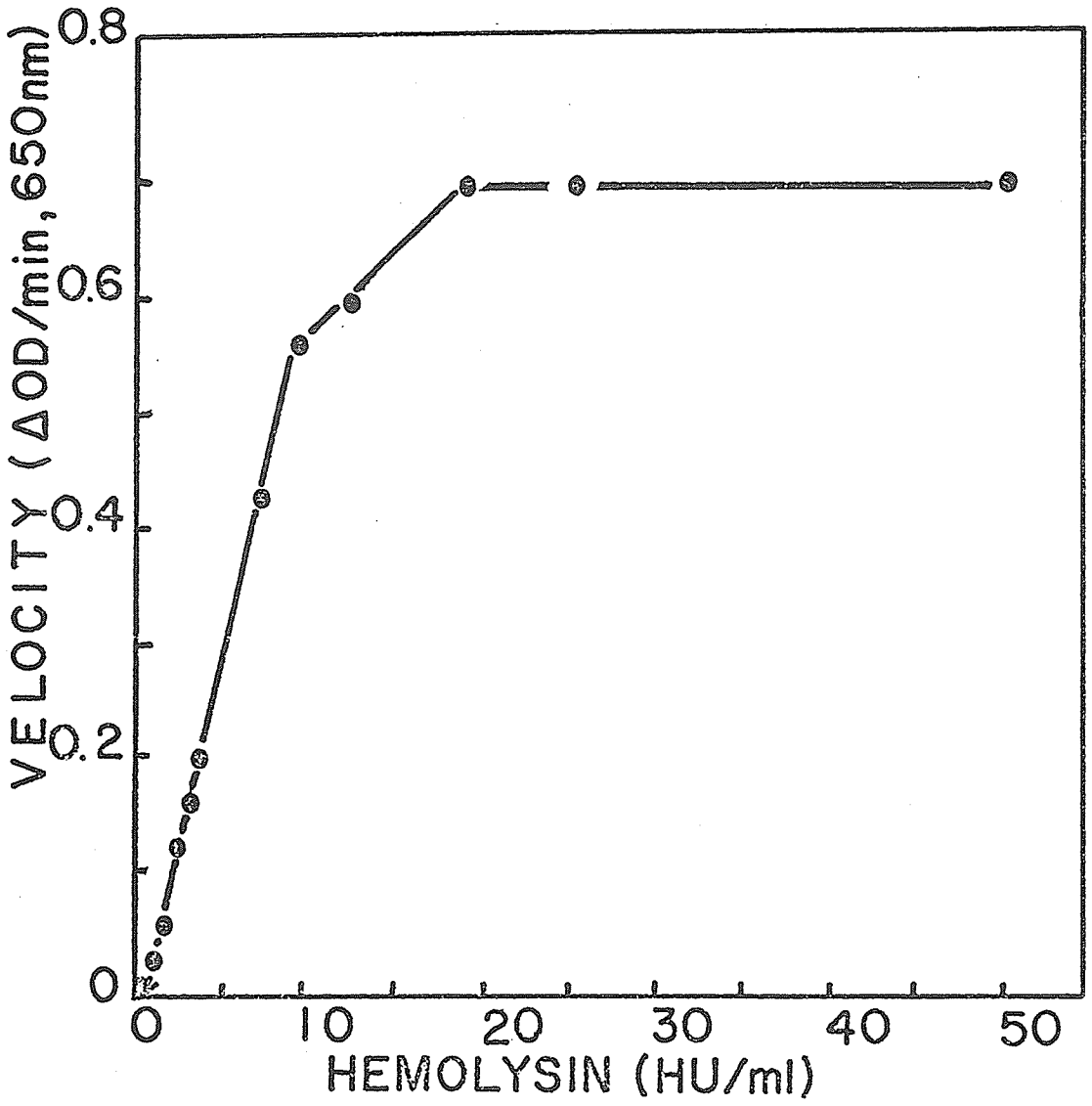

2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE (650nm)
2
4
6
8
TIME (min)
HEMOLYSIN
(HU/ml)
2.0
2.5
2.85
3.3
4.0
>10.0

FIGURE 7

Velocity of hemolysis of human erythrocytes by different concentrations of gamma hemolysin


VELOCITY (ΔOD/min, 650nm)
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
HEMOLYSIN (HU/ml)

to the concentration of hemolysin up to 10 HU/ml and reached a maximum at 25 HU/ml. The maximum probably reflects a limit of the detection system rather than a true limit of the rate of lysis.

Erythrocyte Concentration

The erythrocyte concentration was plotted against the reaction velocity of gamma lysin for rabbit, human and sheep red cells as shown in Fig. 8. As illustrated in the insert of Fig. 8, the ratio of rabbit to human titres is about 2.5 while the ratio of sheep to either rabbit or human titrations is less. These ratios are reflected in the ratios of the slopes of the Lineweaver-Burk plot, which one would expect. It should also be pointed out that the kinetics of the hemolysis of the three erythrocyte species in the presence of crude gamma lysin are first order reactions and that there is a common Y-intercept.

Temperature

The temperature at which erythrocytes are incubated affected the rate of lysis of red cells by gamma lysin as shown in Fig. 9. The initial velocity of lysis increased to a maximum at 37-50^{o}C and then decreased rapidly. This sensitivity to heat, indicated by the decline in the initial velocity above 50^{o}C, has been reported by others (85,86,158) but the effect was not measured quantitatively. In addition,

FIGURE 8

Lineweaver-Burk plot of rate of hemolysis vs substrate concentration for rabbit, sheep and human erythrocytes

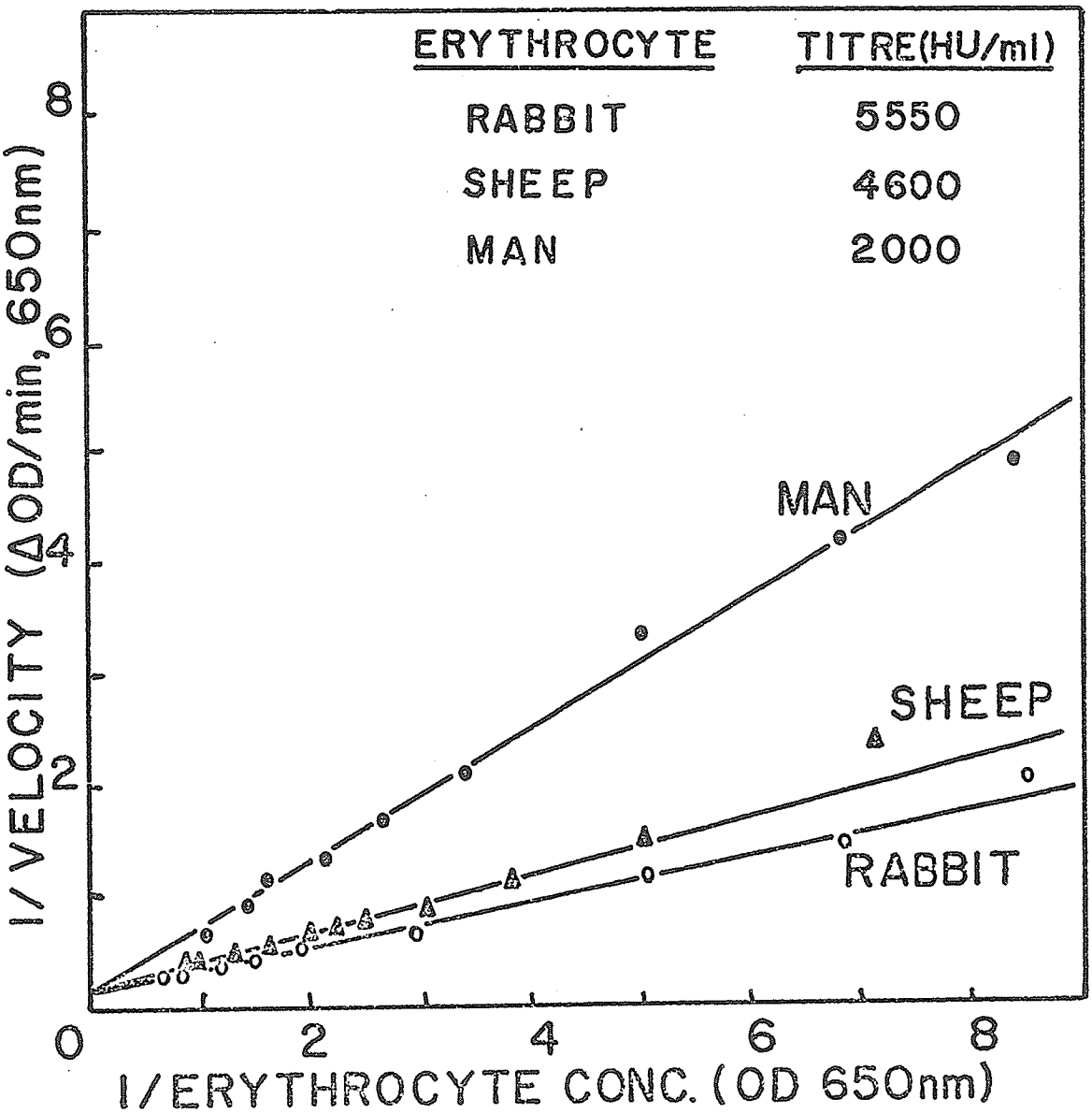
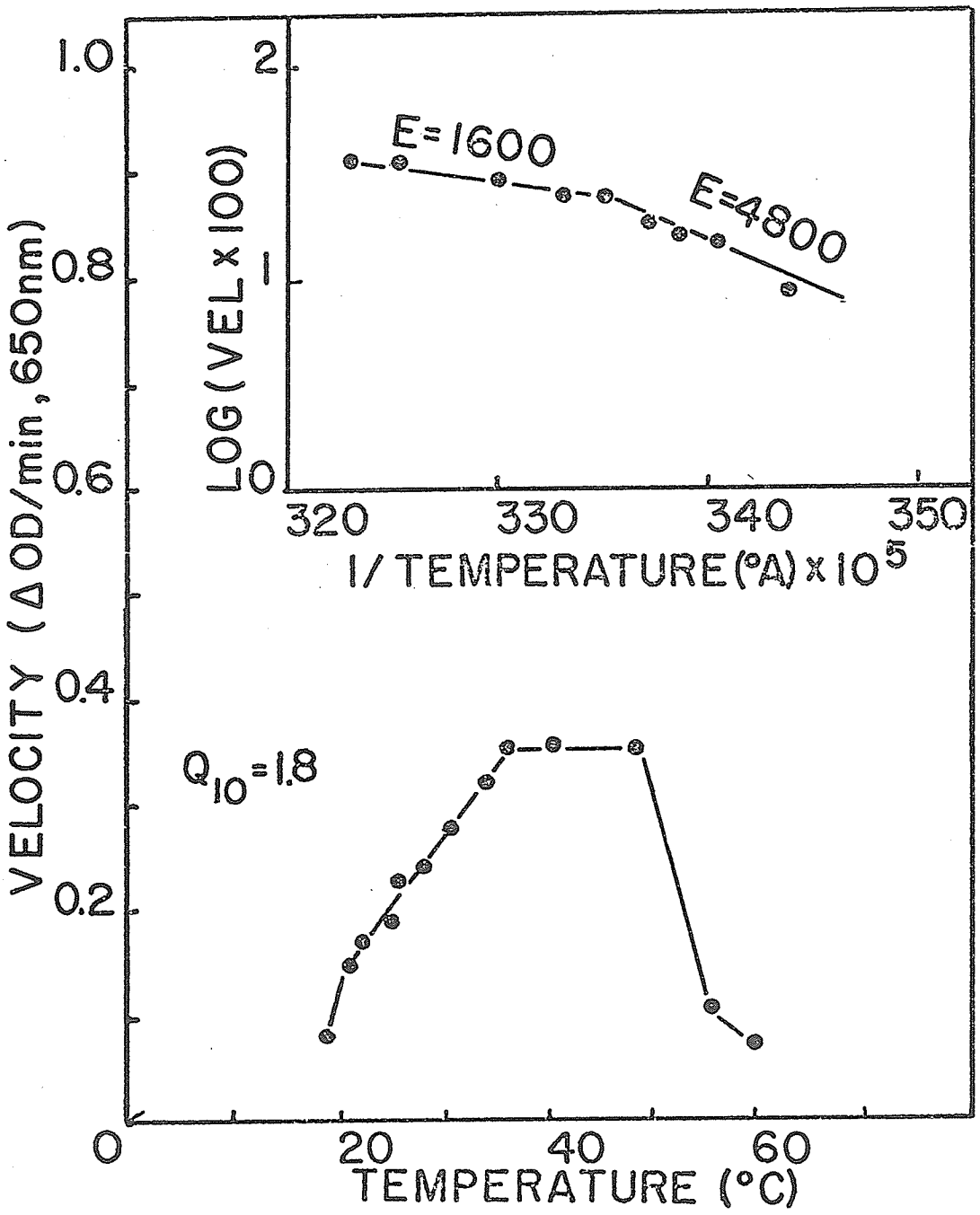

ERYTHROCYTE
TITRE(HU/ml)
RABBIT
5550
SHEEP
4600
MAN
2000
MAN
SHEEP
RABBIT
1/VELOCITY (ΔOD/min, 650nm)
1/ERYTHROCYTE CONC. (OD 650nm)
0
2
4
6
8

FIGURE 9

Rate of hemolysis of human erythrocytes at different temperatures by gamma lysin at a final concentration of 20 HU/ml.


VELOCITY (Δ OD/min, 650nm)
1.0
0.8
0.6
0.4
0.2
0
20
40
60
TEMPERATURE (°C)
Q_{10}=1.8
LOG (VEL x 100)
2
1
0
320
330
340
350
1/ TEMPERATURE (°A) x 10^5
E=1600
E=4800

these velocity measurements allowed determination of the Q_{10} of the reaction, which for this experiment was 1.8. An Arrhenius plot of the logarithm of the velocity against the reciprocal of the absolute temperature is shown in the insert. The two slopes that were obtained from this plot gave activation energies (E) of 4,800 and 1,600 cal. for the hemolytic reaction.

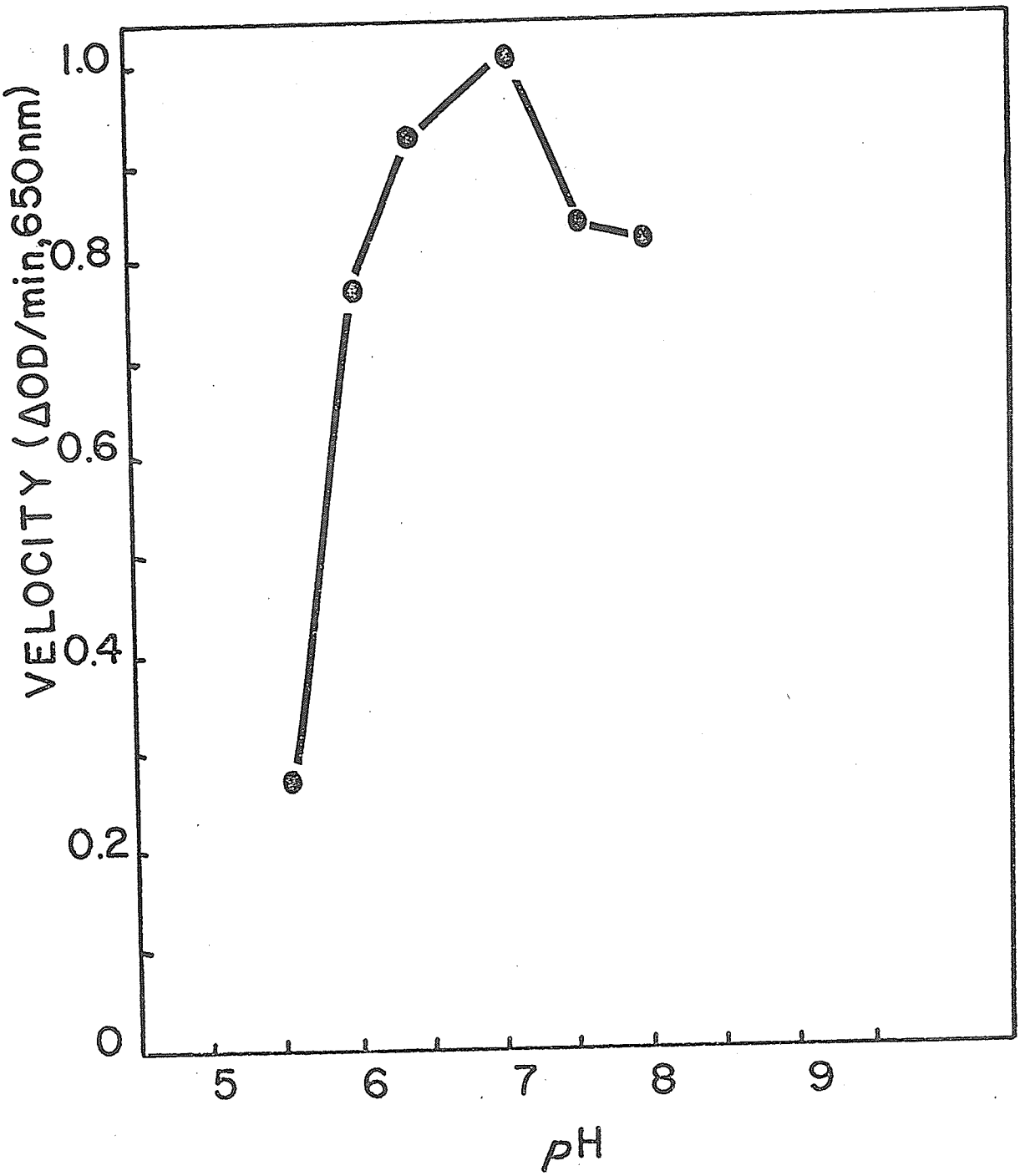
pH

The effect of pH on the rate of hemolysis of human erythrocytes also was measured by velocity experiments (Fig. 10). The maximum velocity of lysis occurred at pH 7.0 with a rapid decrease in the rate on either side of neutrality. This decrease in velocity at other than neutral pH values was not the result of denaturation of the gamma hemolysin because constant titres could be maintained with crude hemolysin when stored for one month over a pH range of 4.0-10.0.

Although different pH values cause changes in cell volume and hence the number of cells required to give an absorbance of 650 nm, the numbers present at various pH levels were constant when measured with a Coulter Counter. Therefore, the decrease in velocity was not due to variations in the numbers of erythrocytes (data not shown).

FIGURE 10

Rate of hemolysis of human erythrocytes at different pH values by gamma lysin at a final concentration of 20 HU/ml.


VELOCITY (ΔOD/min,650nm)
1.0
0.8
0.6
0.4
0.2
0
5
6
7
8
9
pH

Ion Requirements

Titration and velocity experiments showed that the activity of gamma lysin was inhibited by ethylene-diaminetetraacetic acid (EDTA). Hemolysis was entirely inhibited by EDTA at a concentration of 100×10^{-4} *M* (Fig. 11). Dialysis of the gamma hemolysin-EDTA complex against phosphate buffered saline restored activity completely (Table 8). The combined results of Fig. 11 and Table 8 suggested that gamma hemolysin required an ion that was present in trace amounts. However, titration and velocity experiments (data not shown) with the following ions gave no enhancement of lysis when these ions were tested at concentrations of 1×10^{-8} *M* to 0.1 *M*: magnesium, calcium, iron, aluminum, zinc, manganese, nickel, cobalt, ammonia and chloride. A requirement for sodium was demonstrated and at concentrations of less than 7.5×10^{-3} *M* NaCl, no lytic activity was observed (Fig. 12). The isotonicity of the solutions was maintained with either sucrose or Tris buffer in which gamma hemolysin was inactive if sodium was absent. The gamma hemolysin was also active if sodium was replaced with potassium.

SECTION B: RELIABILITY OF THE ASSAY

The specificity, sensitivity and reproducibility or variation in the hemolysin assay are the three factors that contribute to an evaluation of the reliability of the

FIGURE 11

Inhibition by EDTA of the rate of lysis of human erythrocytes in the presence of gamma lysin

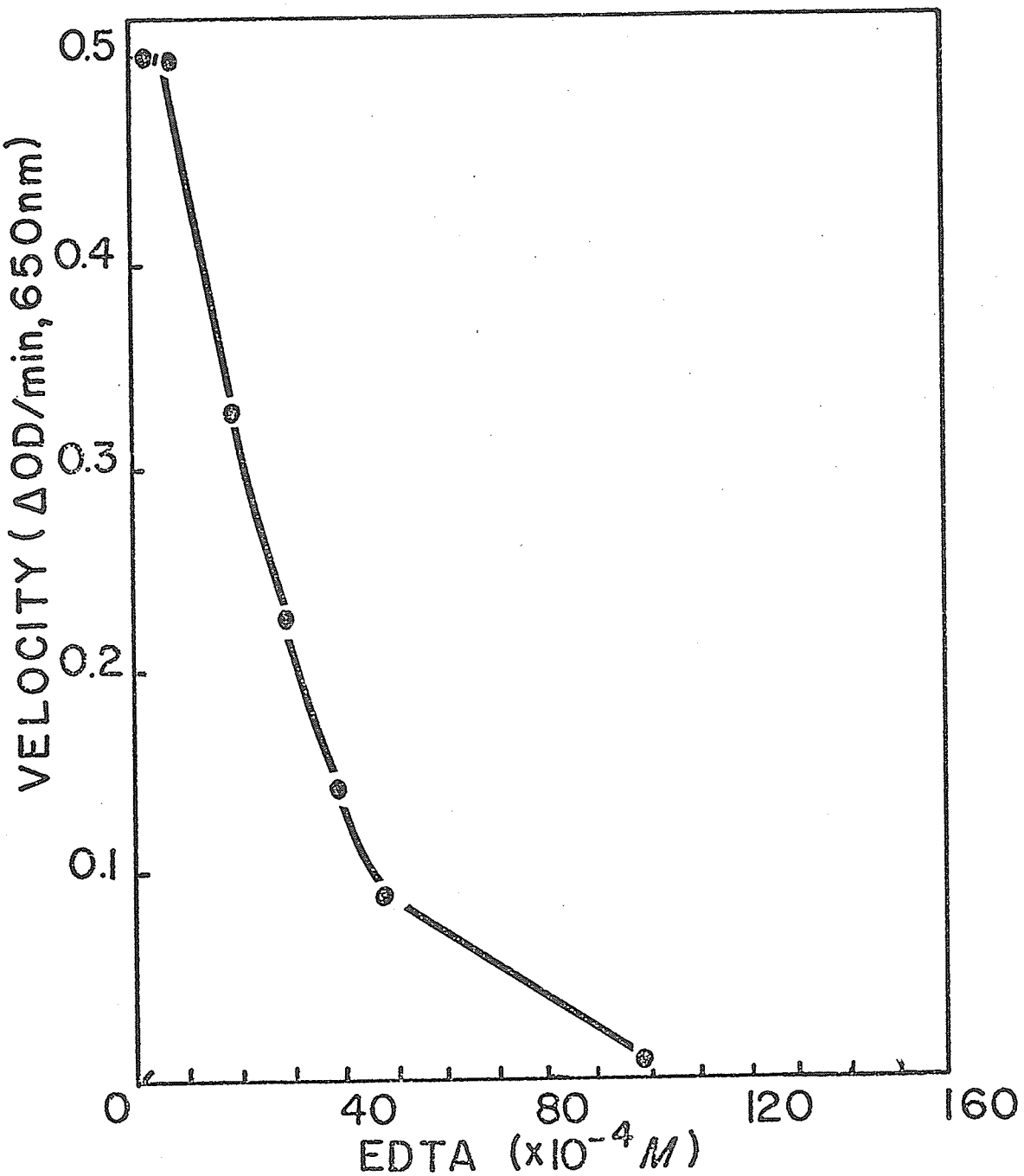

0.5
0.4
0.3
0.2
0.1
VELOCITY (ΔOD/min, 650nm)
0
40
80
120
160
EDTA (x10⁻⁴ M)

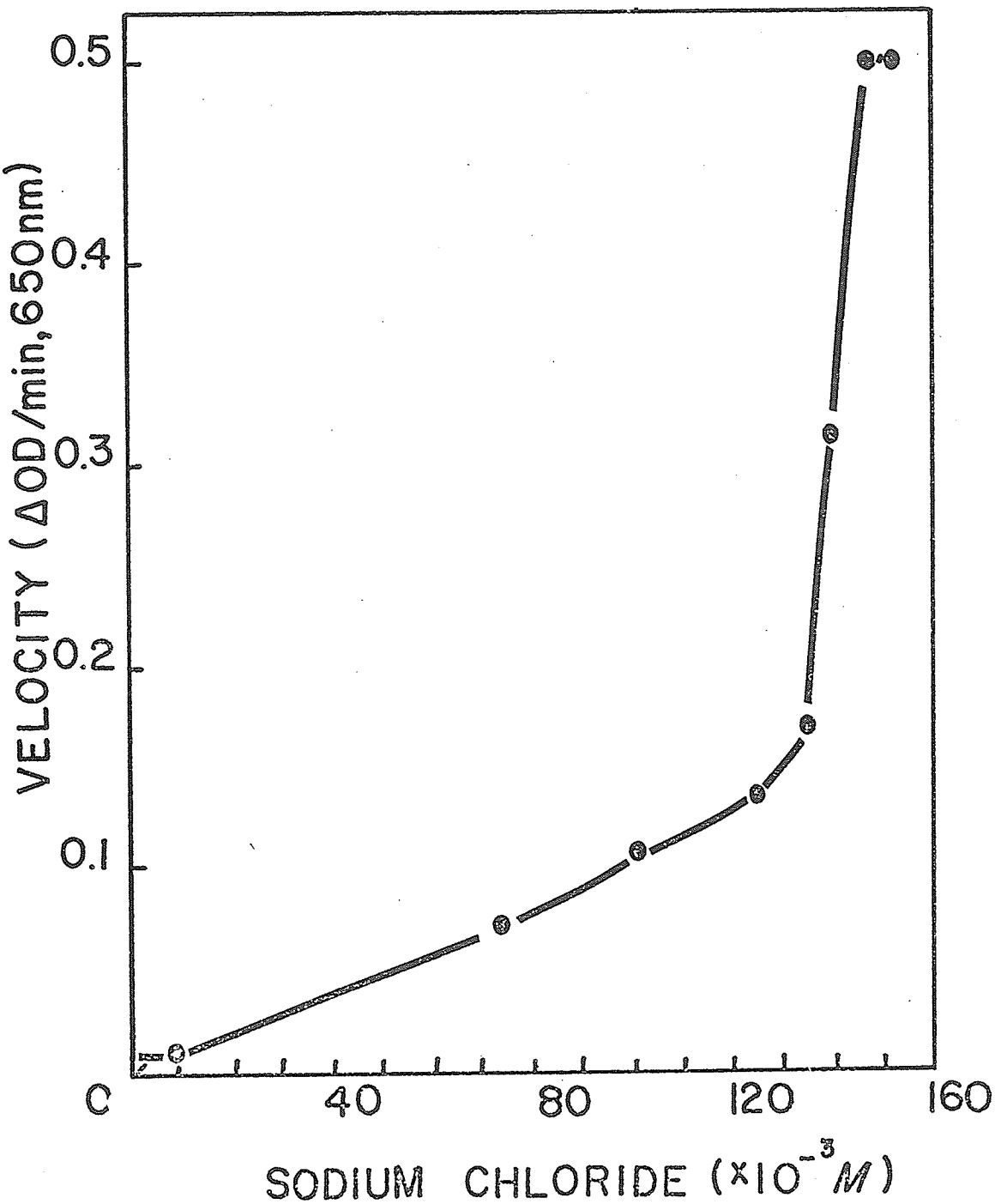
TABLE 8

INHIBITION OF GAMMA HEMOLYSIN BY EDTA

TREATMENT	EDTA (*M*)	TOTAL HEMOLYSIN (HU)
Untreated gamma lysin	-	11,800
Gamma lysin + EDTA	$1x10^{-2}$	0
	$1x10^{-3}$	0
	$1x10^{-4}$	6,500
	$1x10^{-5}$	7.300
	$1x10^{-6}$	11,200
	$1x10^{-7}$	11,800
Gamma lysin + EDTA + dialysis	$1x10^{-2}$	11,900
	$1x10^{-3}$	12,100
	$1x10^{-4}$	11,600
	$1x10^{-5}$	11,750
	$1x10^{-6}$	11,300
	$1x10^{-7}$	11,500

FIGURE 12

Rate of lysis of human erythrocytes by gamma lysin with increasing concentrations of NaCl


0.5
0.4
0.3
0.2
0.1
0
VELOCITY (ΔOD/min, 650nm)
40
80
120
160
SODIUM CHLORIDE (×10⁻³M)

test. Although the hemolysin titration has a degree of inherent non-specificity as shown in Table 20, nevertheless, it can still be useful if the test is sensitive and reproducible. The hemolysin titration is non-specific because human erythrocytes can be destroyed by many agents other than gamma hemolysin.

Reproducibility

The variability that occurred between different hemolysin preparations and blood from different individuals was analysed by titrating each hemolysin preparation sixteen times against each blood sample. The results shown in Table 9 are presented as the arithmetic mean and the standard error of the mean, expressed as HU/ml. After Bartlett's test had established a homogeneity of the variances of the different sets of titrations, a two-way analysis of variance of the arithmetic means suggested that there was a significant difference between blood samples, but that the hemolysin preparations were the same ($p = 0.05$). However, the more sensitive multi-factorial analysis of variance demonstrated that there was a significant difference between hemolysin samples and therefore, Duncan's New Multiple Range Test was employed to identify these differences where they occurred. This test compares each mean with every other mean and, assuming the variances are equal, determines whether the

TABLE 9

HEMOLYSIN	BLOOD SAMPLE 1	2	3	4	5
A	759±36*	1431±67	550±42	1997±62	2476±64
B	896±25	1399±54	679±33	2041±93	2183±71
C	999±51	1752±36	715±31	2499±67	2457±68
D	1180±54	1644±56	730±17	3086±80	2207±76

DUNCAN'S NEW MULTIPLE RANGE TEST .01 protection level

A3 B3 C3 D3 A1 B1 C1 D1 B2 A2 D2 C2 A4 B4 B5 C5 D5 A5 C4 D4

* HU/ml ± $S_{\bar{x}}$

means are significantly different from one another. These results are presented in Table 9 at a protection level of 0.01. The results mean that titrations can only be compared within an experiment, but not between experiments because of the variability of blood samples and hemolysin preparations.

Over a two-year period, more than two thousand hemolysin titrations were performed with each titration expressed as the arithmetic mean ± the standard error. If the standard error is expressed as a percent of the mean and all the percentages averaged, the value is 5%. This high degree of precision is affected by the potency of the material tested. The assay is most precise if the titre is between 1,000-2,000 HU/ml when the average standard error is 4.75% of the mean compared to 7% for titres above 2,000 HU/ml. By comparison, many researchers use a visual titration, where a one-tube difference is not considered significant in a doubling dilution series.

Sensitivity

The sensitivity of the hemolysin assay was measured by titrating hemolysin samples prepared by a harmonic dilution series. The series was prepared by taking 0.9, 0.8, 0.7 ... 0.1 ml portions of a sample which contained 0.5 HU/ml and making the volume up to 1.0 ml with phosphate buffered saline. After erythrocytes were added and

the tubes incubated at 37°C, the absorbance was measured at 541 nm. It was determined that the hemolysin test will detect as little as 0.1 HU/ml or less than a nanogram of purified material (data not shown).

In summary, the assay is precise and sensitive but it lacks specificity.

CHAPTER 3

PURIFICATION OF GAMMA HEMOLYSIN

Ultrafiltration

Crude gamma lysin was concentrated initially so that it could be handled more easily. Ultrafiltration was selected because a crude estimate of the molecular weight could be obtained and if the value was greater than 10,000 daltons, purification as well as concentration would result. A series of Amicon membranes (Amicon Co., Mass.); UM-10, PM-30, XM-50 and XM-100 which theoretically partitioned at 10,000, 30,000, 50,000 and 100,000 daltons, respectively, was tested for its ability to concentrate and purify crude gamma lysin. Ten ml of hemolysin were concentrated to one ml, the concentrated material diluted to 10 ml and the procedure repeated three times to ensure that all the hemolysin capable of passing through the membrane was removed. Both the retained material and the eluted fraction were assayed for gamma hemolysin and protein. In the results shown in Table 10, gamma hemolysin was completely retained by the first three membranes but passed through an XM-100 membrane. Approximately a three-fold increase in specific activity was observed with the XM-50 membrane and the hemolysin could be concentrated 10-20 fold. Consequently, this membrane is

TABLE 10

ULTRAFILTRATION of GAMMA HEMOLYSIN

MEMBRANE		TOTAL HEMOLYSIN (HU)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)
NONE		125,000	90	1,400	1.0
UM-10	retained	125,000	64	2,000	1.4
	passed	0	15	—	—
PM-30	retained	125,000	62	2,000	1.4
	passed	0	14	—	—
XM-50	retained	125,000	31	4,000	2.9
	passed	0	56	—	—
XM-100	retained	35,000	17	2,000	1.4
	passed	90,000	66	1,400	1.0

now used as the first step in the purification of gamma lysin.

In a similar series of experiments, alpha and beta hemolysin passed through an XM-100 membrane but were retained by an XM-50 membrane while delta hemolysin was only retained by the XM-100 membrane (data not shown). This points to the unreliability of the manufacturer's stated exclusion limits.

Gel Filtration

Sephadex gel filtration was used as the second step in the purification procedure. The Sephadex gel that would give the best purification was determined empirically. Gamma lysin which first had been concentrated with the XM-50 membrane was fractionated on Sephadex columns which contained G-50, G-75, G-100 or G-200. Columns of Sephadex G-50 only gave a further two-fold purification whereas those with Sephadex G-75, G-100 or G-200 all gave a four or five-fold purification (Table 11). As the recovery in each case was 100%, a Sephadex G-75 column was selected for subsequent use because it had a faster flow rate than Sephadex G-100 or G-200 yet was equally effective.

An elution profile of gamma lysin on the G-75 column is shown in Fig. 13. The gamma lysin, with a relative elution volume (V_e/V_o) of 1.45 was eluted

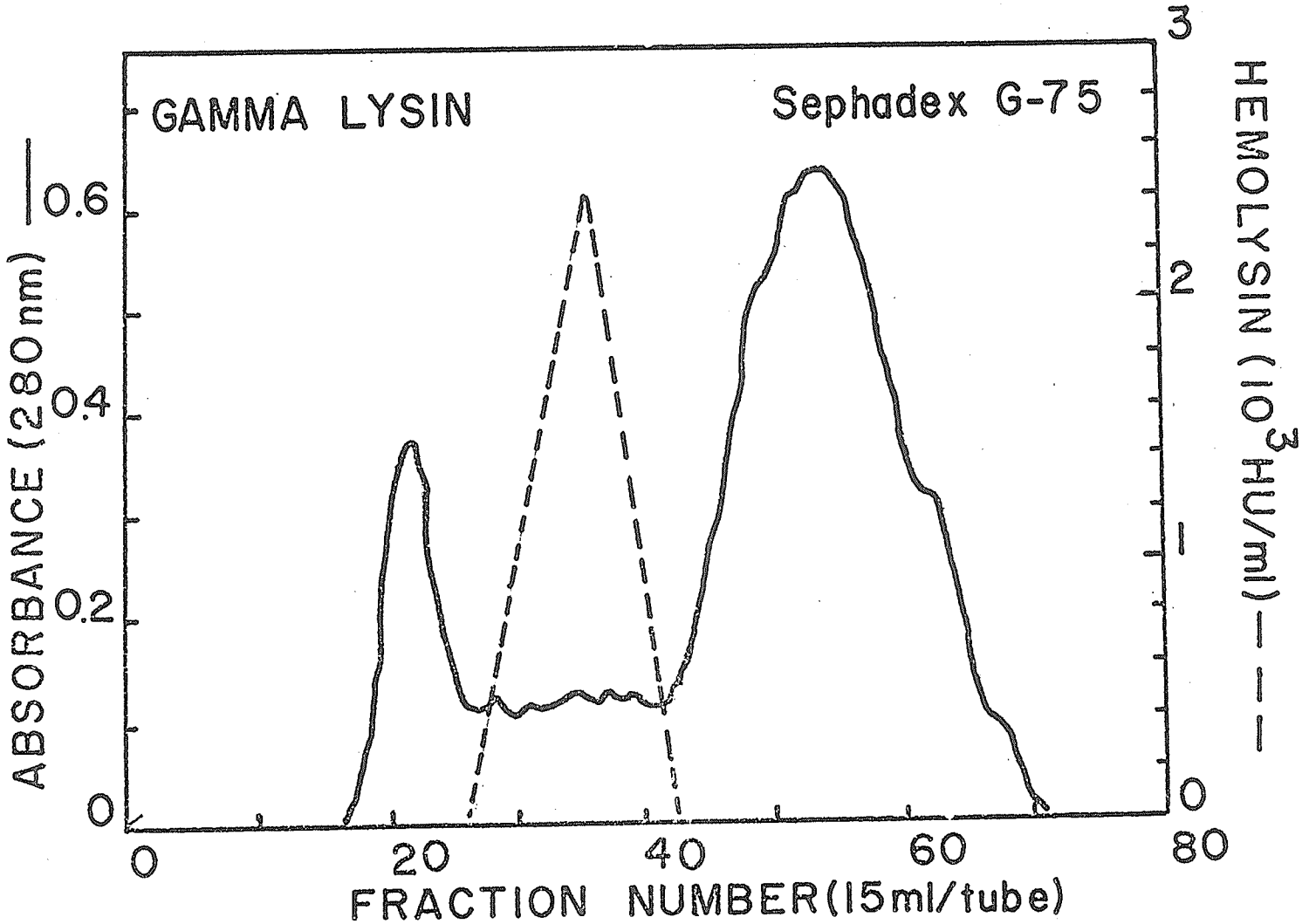
TABLE 11

PURIFICATION OF GAMMA LYSIN BY USE OF DIFFERENT TYPES OF SEPHADEX GELS

CONDITION	TOTAL HEMOLYSIN (HU)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)
Crude lysin	20,000	29	690	1.0
Ultrafiltration (XM-50 retained)	20,000	9.6	2,000	3.0
G-50	20,000	4.65	4,300	6.2
G-50	20,000	3.9	5,100	7.4
G-75	20,000	2.0	10,000	14.5
G-75	20,000	2.6	7,700	11.1
G-100	20,000	2.9	6,900	10.0
G-100	20,000	2.2	9,000	13.2
G-200	20,000	2.9	6,900	10.0
G-200	20,000	2.0	10,000	14.5

FIGURE 13

Elution profile of gamma lysin on Sephadex G-75


GAMMA LYSIN
Sephadex G-75
ABSORBANCE (280nm)
0.6
0.4
0.2
0
HEMOLYSIN (10^3HU/ml)
3
2
1
0
0
20
40
60
80
FRACTION NUMBER (15ml/tube)

between two major peaks of protein.

First Ammonium Sulphate Fractionation

Ammonium sulphate fractionation was used as a third step in the purification of gamma hemolysin. Pooled active fractions from a Sephadex G-75 column were divided into several parts and increased amounts of ammonium sulphate were added to each. The precipitate, after being allowed to aggregate overnight at $4^{o}C$ was centrifuged, resuspended, dialysed against phosphate buffered saline and then titrated for hemolysin and protein content. Gamma hemolysin passed through Sephadex G-75 had a specific activity of 2,162 HU/mg (Table 12). About 95% of the hemolysin was recovered in the precipitate of the 60% ammonium sulphate saturation fraction and the specific activity had increased to 8,431 HU/mg. Higher concentrations of ammonium sulphate only caused precipitation of contaminating proteins. Subsequent experiments at slightly different concentrations of ammonium sulphate demonstrated that no gamma hemolysin was precipitated by 25% ammonium sulphate. As a third step in the purification of gamma hemolysin, 25% ammonium sulphate was added to the hemolysin that was eluted from a Sephadex G-75 and this precipitate was discarded. More ammonium sulphate was added to give a final concentration of 60% and the second precipitate with the active gamma hemolysin was

TABLE 12

AMMONIUM SULPHATE FRACTIONATION OF GAMMA HEMOLYSIN

AMMONIUM SULPHATE (% SATURATION)	PRECIPITATE			
	TOTAL HEMOLYSIN (HU)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)
0	0	0.110	-	-
0-15	0	0.205	-	-
0-30	2,200	0.325	6,769	3.1
0-45	3,500	0.480	7,291	3.4
0-60	4,300	0.510	8,431	3.9
0-75	4,400	1.100	4,000	1.9
0-90	4,300	1.790	2,402	1.1
0-100	4,500	2.010	2,238	1.0
Control, G-75	4,800	2.220	2,162	1.0

collected for further purification.

Dialysis and Salt Extraction

Gamma hemolysin fractionated by ammonium sulphate could be dissolved by dialysis against phosphate buffered saline. However, subsequent dialysis of the solution against distilled water caused formation of a white precipitate and loss of hemolytic activity. If dissolved hemolysin, which contained protein, phosphate and pentose, was treated with ribonuclease and then dialysed against distilled water the amount of precipitate was much smaller and contained only protein. This suggested that gamma hemolysin was co-precipitated with nucleic acids. Since nucleic acids are irreversibly denatured by dialysis against distilled water, an attempt was made to dissolve selectively the precipitated gamma hemolysin with NaCl.

After fractionation with ammonium sulphate, gamma hemolysin was dialysed against distilled water for three days and various concentrations of NaCl in 0.01 *M* phosphate buffer were added to suspensions of the precipitate. The treated suspensions then were centrifuged to separate the insoluble pellets from the supernatants and the pellets were washed with 2.0 *M* NaCl which dissolved any residual material. Finally, both the pellet and the supernatant were assayed for hemolysin and protein. The results, which are summarized in Table 13 show that all the hemolytic

TABLE 13

DIALYSIS AND SALT EXTRACTION OF GAMMA HEMOLYSIN

NaCl (*M*)	SUPERNATANT		PRECIPITATE	
	HEMOLYSIN (HU/ml)	PROTEIN (mg/ml)	HEMOLYSIN (HU/ml)	PROTEIN (mg/ml)
0.01	320	0.130	880	0.225
0.03	470	0.135	795	0.230
0.06	520	0.140	630	0.218
0.10	640	0.150	510	0.205
0.30	970	0.154	275	0.200
0.50	1060	0.160	145	0.190
0.60	1240	0.174	70	0.180
1.00	1300	0.220	0	0.140
2.00	1300	0.230	0	0.125
CONTROLS				
Distilled water	0	0.045	1300	0.305
Phosphate buffer, .01 *M*	270	0.104	990	0.245

activity is present in the precipitate when it is suspended in distilled water, but that the addition of phosphate buffer causes some of the hemolytic activity to appear in the supernatant. As progressively more NaCl is added, more hemolysin is dissolved and less activity remains in the precipitate. Although all of the hemolytic activity is recovered by the addition of 1.0 *M* NaCl, 45% of the protein is still insoluble. This NaCl-extracted hemolysin contained protein but no phosphate or pentose.

Second Ammonium Sulphate Fractionation

Gamma hemolysin was unstable after extraction with NaCl, all activity being lost after 24 h storage at $4^{o}C$. If activity were to be retained, the hemolysin had to be stored as a precipitate in ammonium sulphate. As a final step in the purification procedure, the hemolysin was fractionated a second time with ammonium sulphate. The precipitate which occurred after addition of 30% ammonium sulphate was discarded and the hemolysin collected by further addition of the salt to 60% saturation. The active material was washed once and stored in 100% saturated ammonium sulphate solution.

Effectiveness of the Purification Procedure

The flow diagram in Fig. 14 summarizes the procedure used to purify gamma hemolysin. Table 14 summarizes the purification data. Crude gamma hemolysin was concen-

FIGURE 14

Flow diagram of the procedure for the purification of gamma hemolysin

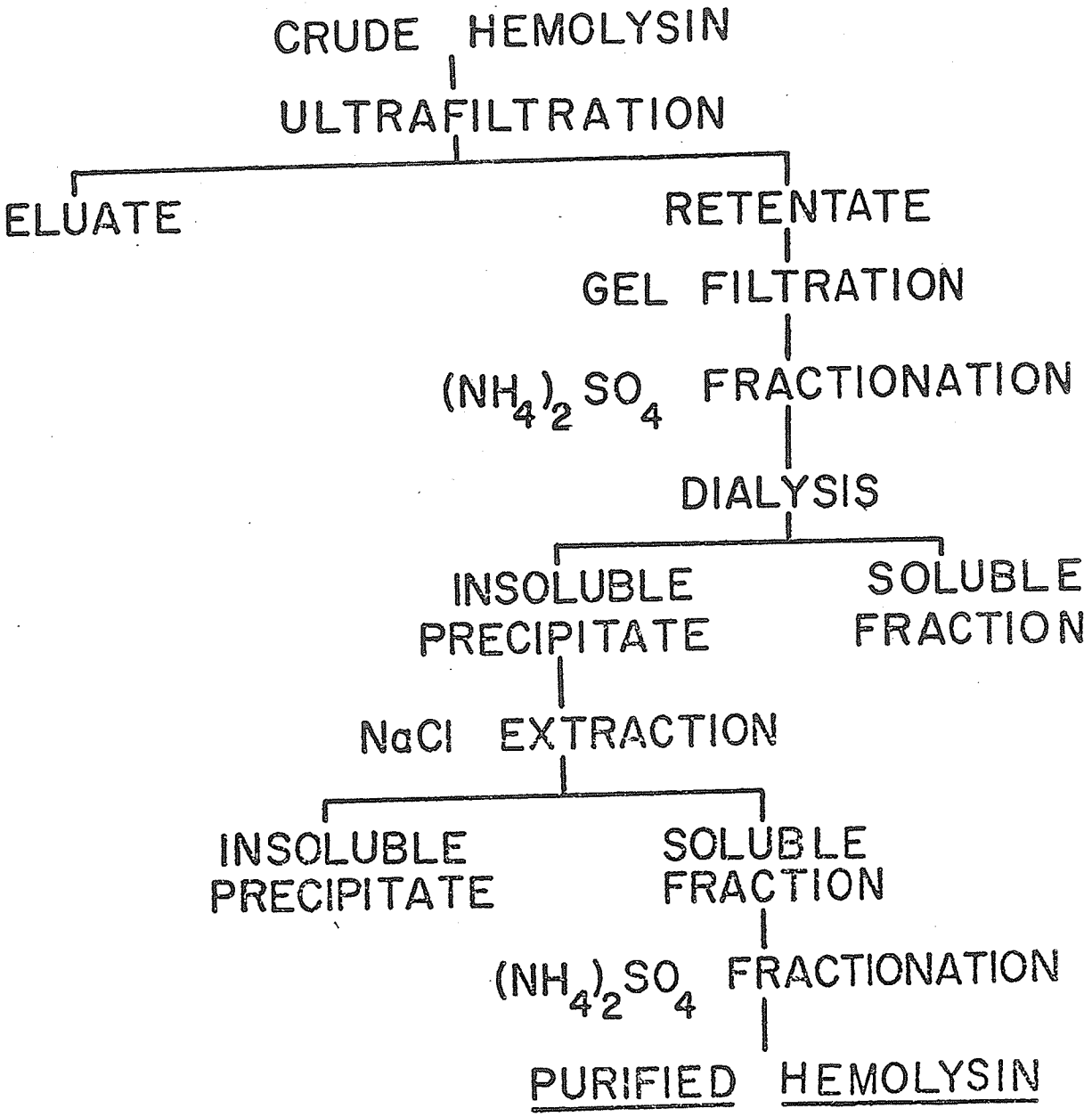

CRUDE HEMOLYSIN
ULTRAFILTRATION
ELUATE
RETENTATE
GEL FILTRATION
$(NH_4)_2SO_4$ FRACTIONATION
DIALYSIS
INSOLUBLE PRECIPITATE
SOLUBLE FRACTION
NaCl EXTRACTION
INSOLUBLE PRECIPITATE
SOLUBLE FRACTION
$(NH_4)_2SO_4$ FRACTIONATION
PURIFIED HEMOLYSIN

TABLE 14

PURIFICATION OF GAMMA HEMOLYSIN: SUMMARY OF DATA

PURIFICATION STEP	TOTAL HEMOLYSIN (HU)	TOTAL PROTEIN (mg)	RECOVERY %	SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)
Crude Lysin	151,900	3,844.0	100	40	1.0
Ultrafiltration	148,750	1,214.0	98	123	3.1
Gel Filtration	128,000	198.9	84	643	16.3
$(NH_4)_2SO_4$ Fractionation	107,250	41.7	70	2,572	64.0
Dialysis	115,500	15.8	76	7,333	185.6
NaCl Extraction	115,500	9.8	76	11,737	297.0
$(NH_4)_2SO_4$ Fractionation	112,450	1.1	74	108,125	2,736.0

trated and purified three-fold by an Amicon XM-50 membrane with complete recovery of the hemolytic activity. After passage through a Sephadex G-75 column, 84% of the hemolytic activity was recovered with a 16-fold increase in the specific activity. Ammonium sulphate fractionation further concentrated and purified the gamma hemolysin four-fold. The precipitated hemolysin was then dialysed against distilled water and the hemolytic activity extracted from the insoluble residue by treatment with 1.0 *M* NaCl. Finally the hemolysin was stabilized and concentrated by a second ammonium sulphate fractionation. With this step, gamma hemolysin has been purified 2,700 fold with 74% recovery of hemolysin. The specific activity of the purified hemolysin was 10^5 HU/mg.

CHAPTER 4

PURIFICATION OF ALPHA HEMOLYSIN

Although there are many recent reports of techniques for purifying alpha hemolysin, the techniques required equipment that was unavailable and therefore a new method was devised.

Methanol Precipitation

The earlier technique of methanol precipitation as described by Wittler and Pillmer (210) was re-examined. After the pH of the crude alpha hemolysin was first adjusted to 4.0 with concentrated HCl, the hemolysin was cooled to 4^{o}C and various amounts of cold methanol, -20^{o}C, were added to the crude hemolysin. The mixture was allowed to sit in the cold for 2 h before the precipitate was collected by centrifugation and dialysed against phosphate buffered saline. Table 15 shows that over 50% of the alpha hemolysin was precipitated with 35% methanol and that higher concentrations of methanol only decreased the amount of recoverable alpha lysin. As a first step, crude alpha hemolysin at pH 4.0 was mixed in the cold with methanol to a final concentration of 35% and the precipitate dialysed against phosphate buffered saline.

TABLE 15

METHANOL PRECIPITATION OF CRUDE ALPHA HEMOLYSIN

METHANOL CONCENTRATION (%)	TOTAL HEMOLYSIN (HU)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (HU/ml)	RECOVERY (%)
0	1,142,980	300	3,810	100.0
5	8,036	9.63	835	0.7
10	10,475	11.77	890	0.9
15	20,896	18.85	1,108	1.8
20	65,722	20.90	3,144	5.8
25	319,980	23.6	13,558	28.0
30	540,094	28.2	19,152	47.3
35	642,970	32.0	20.092	56.3
40	420,994	31.1	13,558	36.8
45	295,766	42.0	7,042	25.9
50	262,887	42.1	6,245	23.0
60	266,651	43.5	6,127	23.3

Ammonium Sulphate Fractionation

Alpha hemolysin partially purified by methanol precipitation, was divided into several tubes and increased amounts of ammonium sulphate were added. The precipitate was treated in the manner described for the gamma lysin and then assayed for alpha hemolysin and protein content. About 70% of the alpha hemolysin was precipitated with 60% ammonium sulphate with a two-fold increase in specific activity (Table 16) but no significant amounts of hemolysin were precipitated with 40% ammonium sulphate. Therefore methanol-precipitated alpha lysin was further purified by collecting the hemolysin which precipitated in the range of 40-60% ammonium sulphate.

Gel Filtration

The same Sephadex G-75 column that was used for fractionation of gamma lysin was used to further purify the alpha hemolysin. The elution profile for alpha lysin, shown in Fig. 15, is similar to that for gamma lysin and the relative elution volume was also 1.45. All the alpha hemolysin was recovered from this step.

Ion Exchange

A second ammonium sulphate fractionation was performed; primarily to concentrate and stabilize the alpha hemolysin that was eluted from the Sephadex G-75 column. The precipitate was dialysed against distilled water,

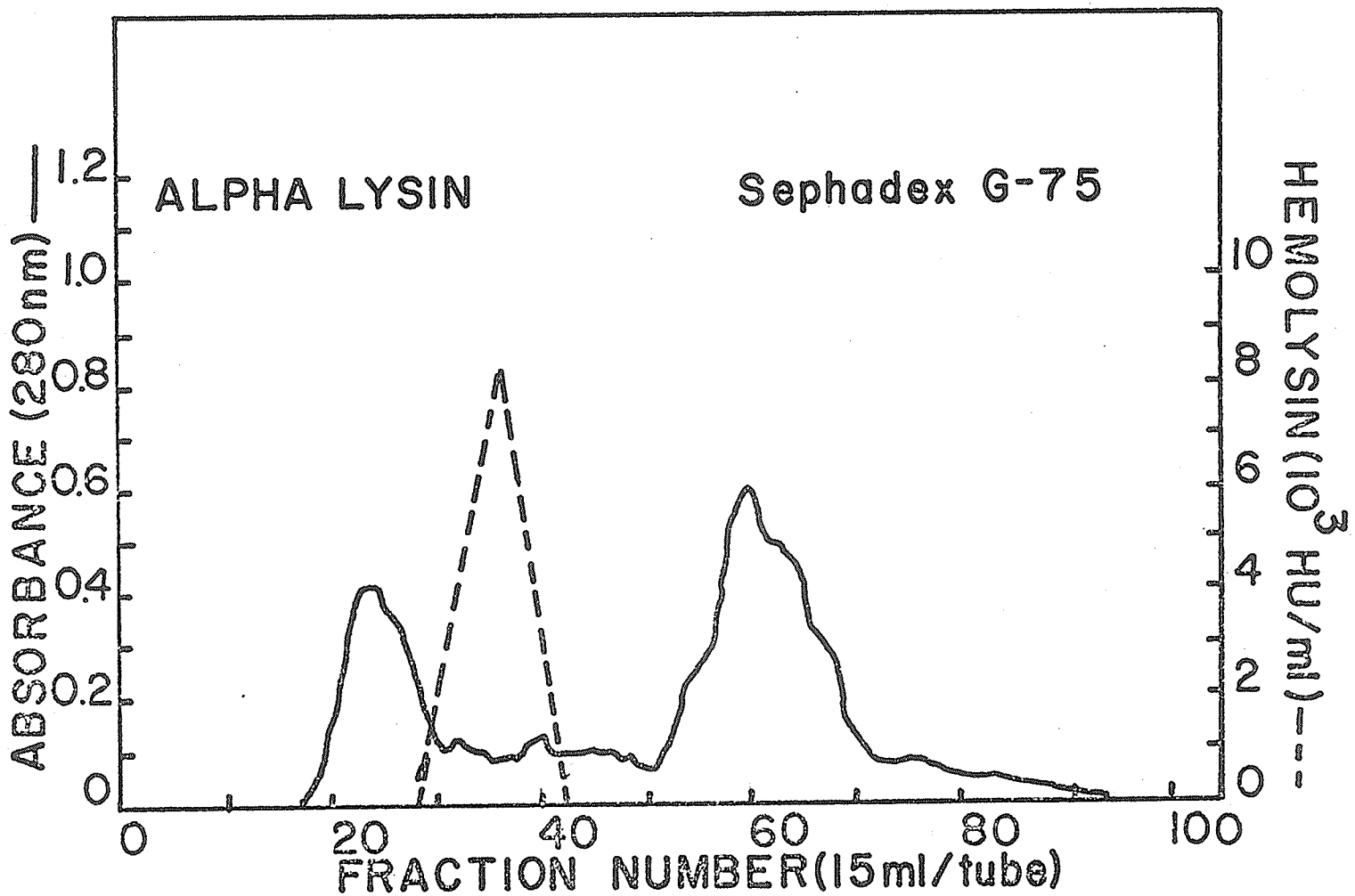
TABLE 16

AMMONIUM SULPHATE FRACTIONATION OF ALPHA HEMOLYSIN

AMMONIUM SULPHATE (% SATURATION)	PRECIPITATE TOTAL HEMOLYSIN (HU)	PRECIPITATE TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)
0	0	0.1	-	-
0-15	0	2.4	-	-
0-30	4,100	3.2	1,281	0.6
0-40	12,500	4.7	2,659	1.2
0-50	21,600	5.2	4,153	1.9
0-60	27,350	5.6	4,884	2.3
0-75	28,650	11.2	2,558	1.2
0-90	28,600	12.3	2,325	1.1
0-100	31,400	13.1	2,396	1.1
Methanol ppte (control)	42,200	19.5	2,164	1.0

FIGURE 15

Purification of alpha hemolysin by gel filtration on Sephadex G-75


ALPHA LYSIN
Sephadex G-75
ABSORBANCE (280nm)
HEMOLYSIN (10^3 HU/ml)
FRACTION NUMBER(15ml/tube)
0
0.2
0.4
0.6
0.8
1.0
1.2
0
2
4
6
8
10
0
20
40
60
80
100

centrifuged to remove any residue and then dialysed against 0.056 *M* phosphate buffer, pH 6.0. This material was then applied to a carboxymethylcellulose column equilibrated with the same buffer and eluted with the stepwise gradient procedure described by Robinson *et al.* (164). The alpha hemolysin was eluted in the third peak (Fig. 16) as shown previously by Gow (82). Essentially all the alpha hemolysin was recovered by this step and the purified product was stored under saturated ammonium sulphate at 4°C.

Effectiveness of Procedure for Purification of Alpha Hemolysin

The flow diagram in Fig. 17 summarizes the procedure used to purify alpha hemolysin. Table 17 summarizes the purification data. Crude hemolysin was purified 4.4-fold with methanol and another 2-fold with ammonium sulphate. Sephadex G-75 gel filtration increased the specific activity from 4,200 HU/mg to 24,200 HU/mg and the second ammonium sulphate fractionation and ion exchange chromatography gave a product with a specific activity of 125,000 HU/mg. The hemolysin was purified 300-fold with 40% recovery.

FIGURE 16

Purification of alpha hemolysin by ion exchange chromatography on carboxymethyl cellulose

The arrows denote the addition of the different buffer

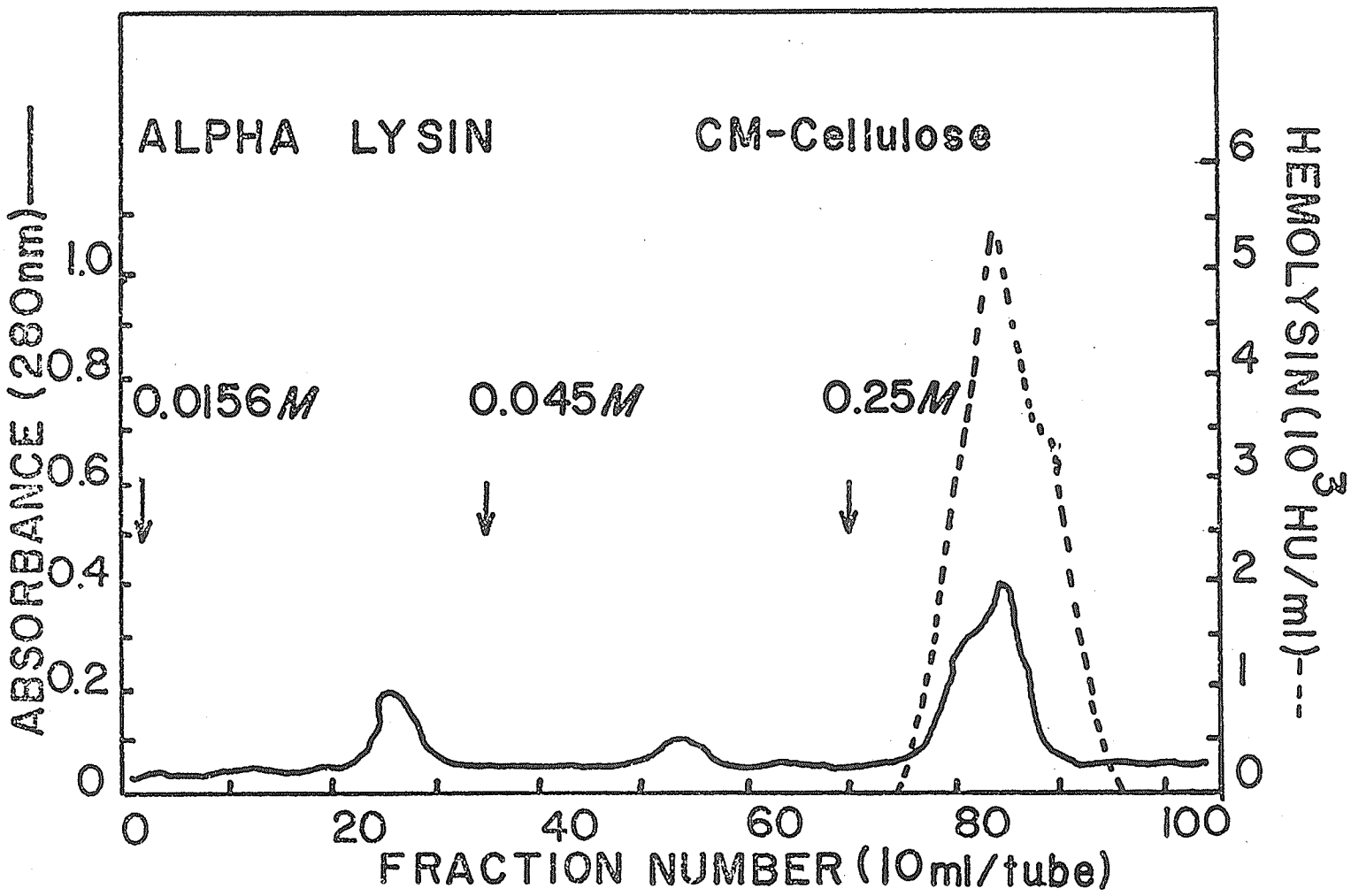

ALPHA LYSIN
CM-Cellulose
0.0156M
0.045M
0.25M
ABSORBANCE (280nm)
HEMOLYSIN (10^3 HU/ml)
FRACTION NUMBER (10ml/tube)
0
0.2
0.4
0.6
0.8
1.0
1
2
3
4
5
6
0
20
40
60
80
100

FIGURE 17

Flow diagram of the procedure for the purification of alpha hemolysin

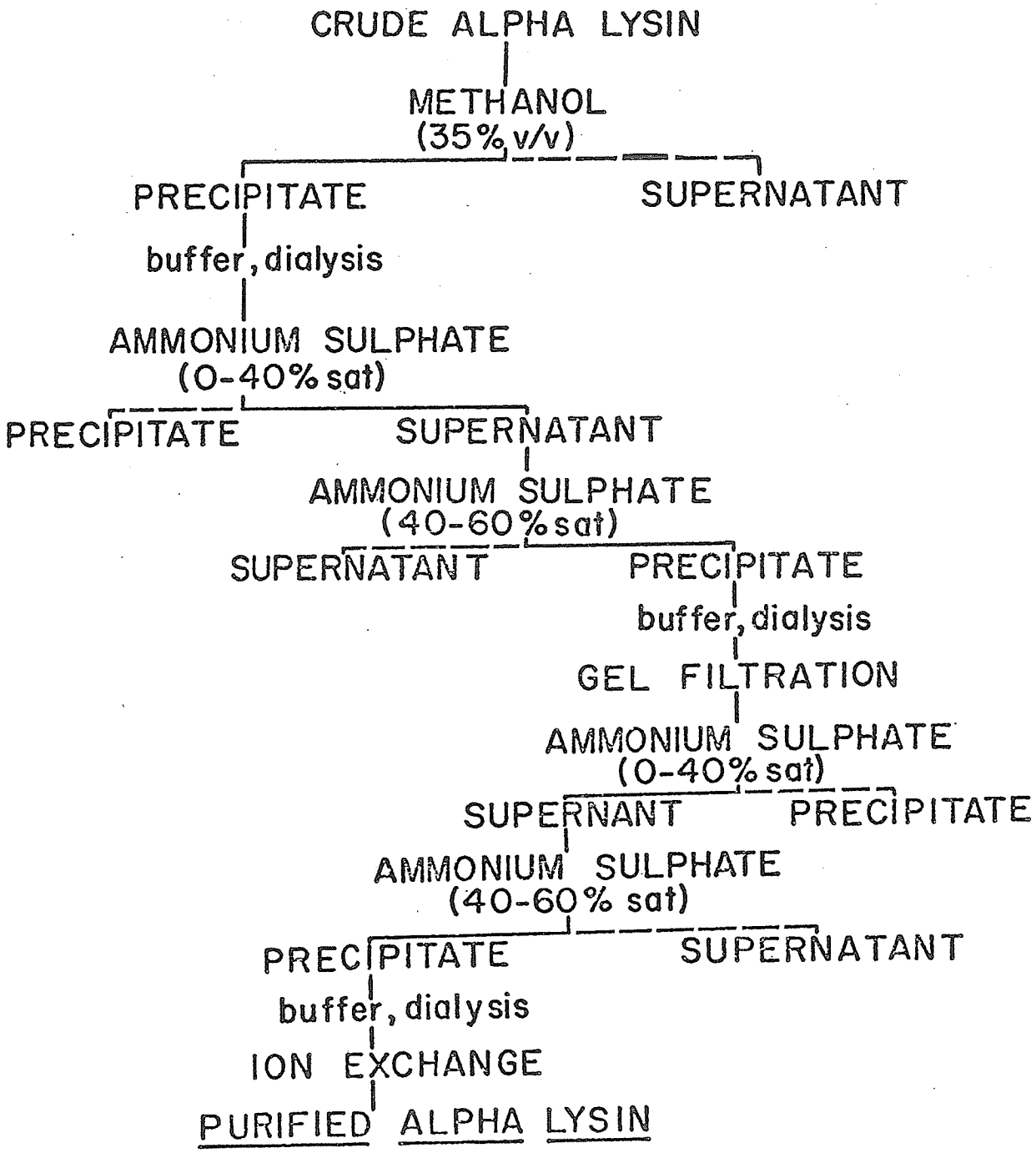

CRUDE ALPHA LYSIN
METHANOL
(35% v/v)
PRECIPITATE
SUPERNATANT
buffer, dialysis
AMMONIUM SULPHATE
(0-40% sat)
PRECIPITATE
SUPERNATANT
AMMONIUM SULPHATE
(40-60% sat)
SUPERNATANT
PRECIPITATE
buffer, dialysis
GEL FILTRATION
AMMONIUM SULPHATE
(0-40% sat)
SUPERNANT
PRECIPITATE
AMMONIUM SULPHATE
(40-60% sat)
PRECIPITATE
SUPERNATANT
buffer, dialysis
ION EXCHANGE
PURIFIED ALPHA LYSIN

TABLE 17

SUMMARY OF DATA FOR PURIFICATION OF ALPHA HEMOLYSIN

STEP	TOTAL HEMOLYSIN (HU)	RECOVERY (%)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (HU/mg)	PURIFICATION
Crude lysin	20,235,000	100	49,962	405	1
Methanol ppte	11,347,200	56	6,304	1,800	4
Ammonium sulphate precipitation	7,375,500	36	1,755	4,200	10
Sephadex G-75	8,118,000	40	335	24,200	60
Ammonium sulphate precipitation	8,200,000	41	185	44,200	109
CM cellulose	7,790,000	39	62.3	125,000	308

CHAPTER 5

A PHYSICAL-CHEMICAL COMPARISON OF GAMMA HEMOLYSIN WITH ALPHA, BETA AND DELTA LYSINS

Disc Gel Electrophoresis

When 100 µg of the purified hemolysins were applied to polyacrylamide columns, a single protein band was observed in each case (Fig. 18) which suggested that the hemolysins were free of contamination. The R_f (0.60) of gamma hemolysin cannot be distinguished from that of alpha lysin but is distinct from those of beta and delta hemolysins.

Analytical Ultracentrifugation

Results shown in Fig. 19 indicate that single peaks were obtained with purified alpha, beta and gamma hemolysins when they were examined by sedimentation velocity analysis. If purified alpha lysin was immediately analysed, the S_{20w} value was 1.4, but if the preparation was kept at $4^{o}C$ for 1 week, the S_{20w} was 3.0. The values for beta and gamma lysins were 1.8S and 2.6S, respectively. The photograph and S_{20w} value for delta lysin were supplied by Dr. Wiseman as already published (31). As shown in the figure, two peaks were observed with the purified delta hemolysin, the S_{20w} values of which are 2.8 and 9.8. These results support those obtained by disc gel electrophoresis to the effect that the alpha, beta and gamma lysins were homogeneous.

The diffusion coefficients for alpha and gamma lysin were calculated to be 7.3×10^{-7} cm^2/sec and 13.9×10^{-7}

FIGURE 18

Densitometer tracings of disc gel electrophoresis of the purified staphylococcal hemolysins. The arrow denotes the origin where the hemolysin was added.

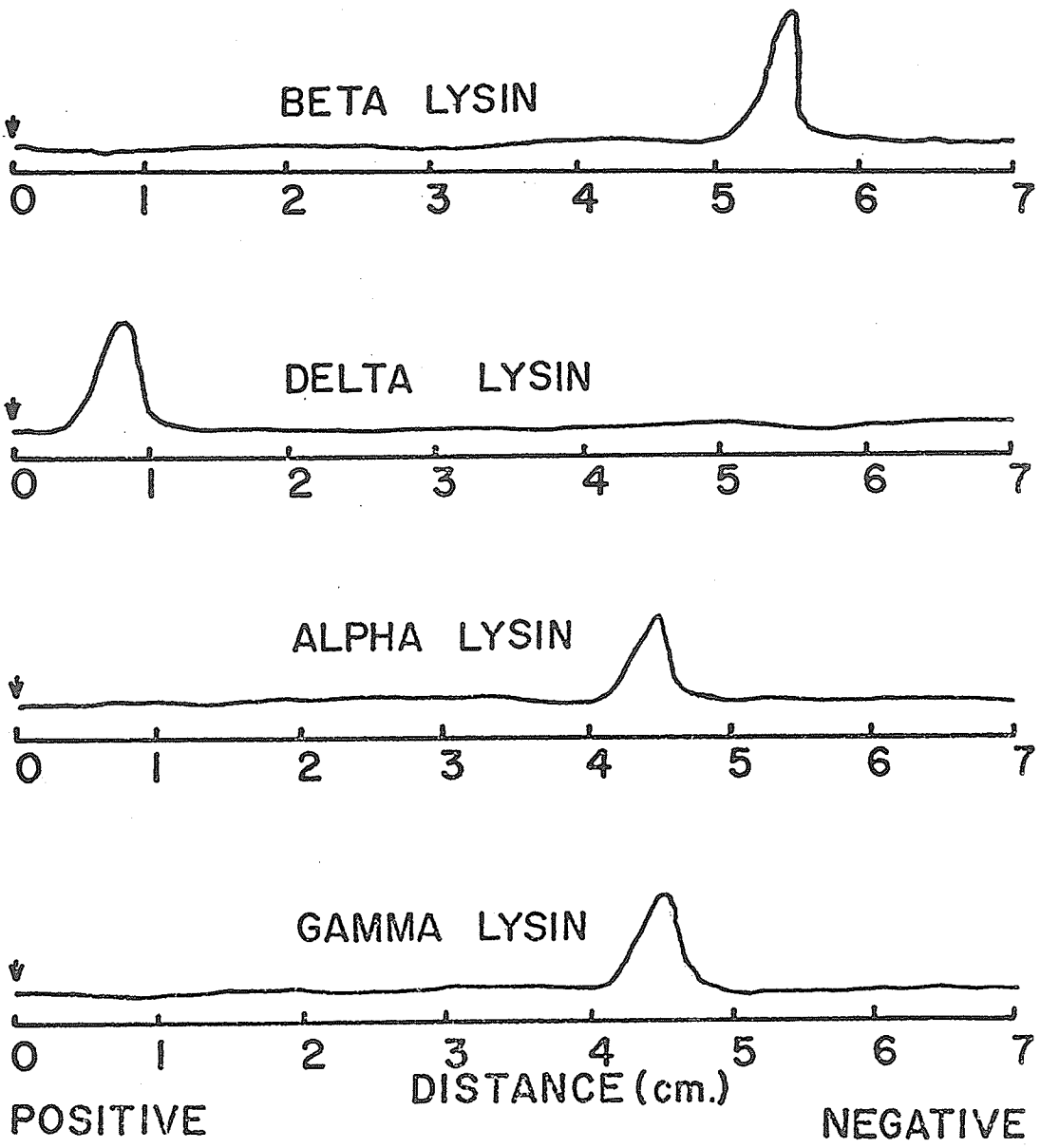

BETA LYSIN
0 1 2 3 4 5 6 7
DELTA LYSIN
0 1 2 3 4 5 6 7
ALPHA LYSIN
0 1 2 3 4 5 6 7
GAMMA LYSIN
0 1 2 3 4 5 6 7
DISTANCE (cm.)
POSITIVE
NEGATIVE

FIGURE 19

Sedimentation Velocity Analysis: The Fig. shows representative photographs of the Schlieren patterns of the four hemolysins. The hemolysin concentrations for these photographs were 6.3 mg/ml in all cases and the photographs were taken 24 min after the maximum speed of 60,000 RPM was reached. The photograph of the delta lysin is the same as published by Caird and Wiseman (31).

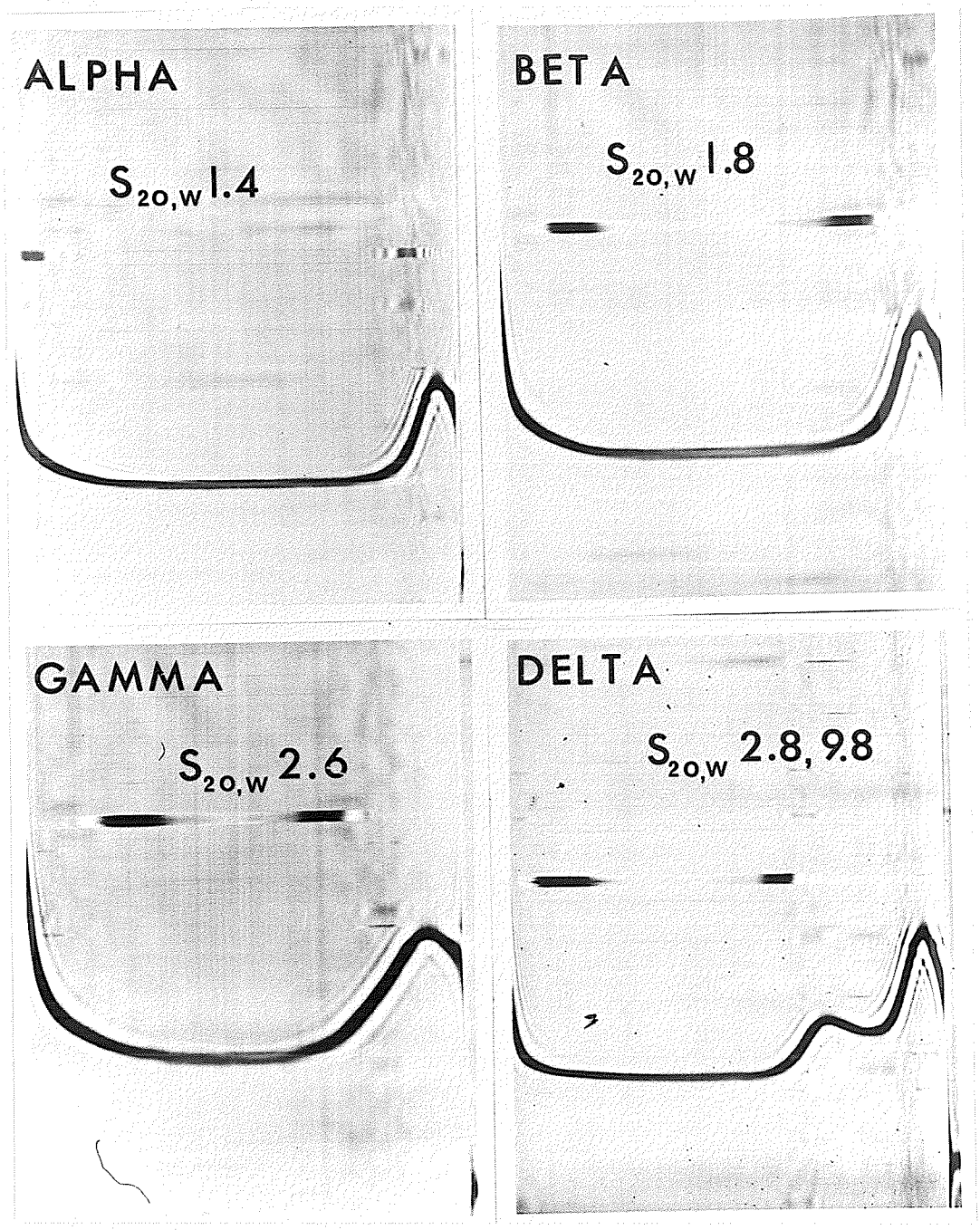
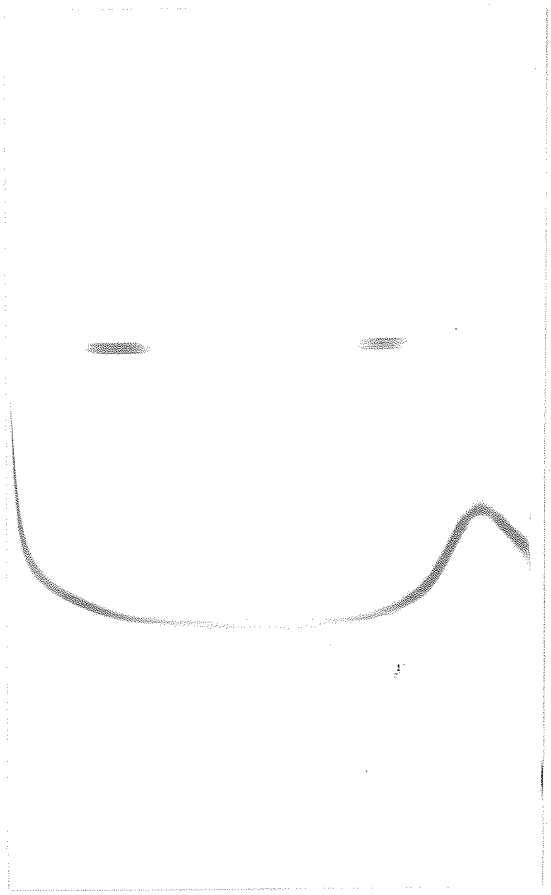

ALPHA
$S_{20,w}$ 1.4
BETA
$S_{20,w}$ 1.8
GAMMA
$S_{20,w}$ 2.6
DELTA
$S_{20,w}$ 2.8, 9.8

FIGURE 19

Photographs of the Schlieren patterns of the purified lysins (cont)

The 3.0 S molecule of the alpha lysin



cm^2/sec, respectively.

Ultraviolet Absorption Spectra

The ultraviolet absorption spectra of the four purified hemolysins were determined with lyophilized preparations that were reconstituted to 1 mg/ml in phosphate buffered saline. The results, presented in Fig. 20, show that gamma hemolysin has an absorption maximum at 277 nm with the minimum at 250 nm. By comparison, alpha and beta lysins have absorption maxima at 275 nm and the minima are at 250 nm. The absorption maximum of delta lysin is, by contrast, at 282 nm with shoulders occurring at 275 nm and 292 nm.

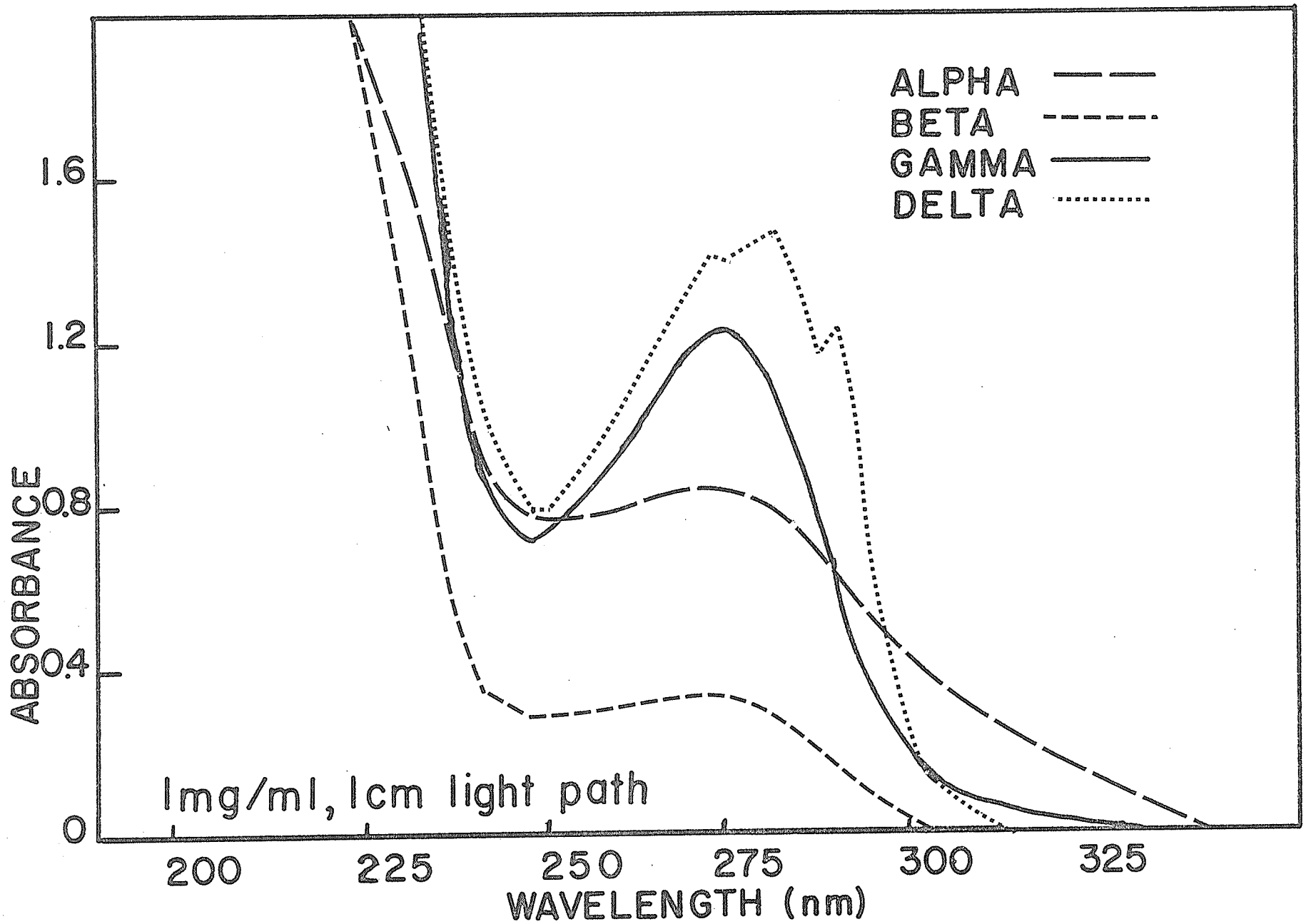
The 280:260 nm absorption ratio for alpha and beta hemolysins is 1.0 while those for gamma and delta hemolysins are 1.35 and 1.45, respectively. According to Warburg and Christian equation (123), these ratios would suggest that the amount of nucleic acids found in the four hemolysins varies from 0.6-3.0%. Such concentrations are unlikely in view of the fact that analyses of 5 mg samples of the hemolysins for pentoses, deoxypentoses and phosphate were negative in each case. Furthermore, the Dubois carbohydrate test was also negative.

Extinction Coefficients

The 1% extinction coefficients at 280 nm for the staphylococcal hemolysins were calculated from the slopes

FIGURE 20

Ultraviolet absoption spectra of the purified hemolysins


ALPHA
BETA
GAMMA
DELTA
1.6
1.2
0.8
0.4
0
ABSORBANCE
1mg/ml, 1cm light path
200
225
250
275
300
325
WAVELENGTH (nm)

of the plots of the absorbance at 280 nm vs the concentration (gm%) of each hemolysin. The lines were determined by regression analysis as shown in Fig. 21 and the extinction coefficients are given in the figure.

Amino Acid Analyses

The amino acid analysis of gamma lysin is compared with that of the other staphylococcal hemolysins in Table 18. Cysteine was present only in beta hemolysin and methionine was present only in gamma and alpha lysins but the gamma lysin had three times less methionine than the alpha hemolysin. The large number of ammonia residues suggests that much of the glutamic and aspartic acid, which represents 21-35% of the composition of the hemolysins, was present as asparagine and glutamine.

N-Terminal Amino Acid Analysis

A chromatograph of the N-terminal amino acid analysis is shown in Fig. 22. The N-terminus for gamma hemolysin was methionine while those of alpha and delta were histidine and proline, respectively. The detection of a single N-terminal amino acid for those hemolysins reinforces the conclusion that the lysins are homogeneous.

Isoelectric Focusing

All four of the purified staphylococcal hemolysins were examined by isoelectric focusing and each hemolysin

TABLE 18

STAPHYLOCOCCAL HEMOLYSINS

AMINO ACID COMPOSITION*

AMINO ACID	ALPHA	BETA	GAMMA	DELTA
Ammonia	High	441.6	141.4	218.5
Aspartic Acid	121.2	135.1	142.0	164.1
Threonine	56.9	53.8	68.0	119.9
Serine	65.1	98.7	77.4	96.3
Glutamic Acid	93.0	116.9	102.4	66.4
Proline	81.2	-	50.2	14.0
Glycine	170.1	121.2	125.0	112.4
Alanine	75.8	78.2	63.2	75.2
Valine	50.1	57.8	51.0	76.0
Cysteine	-	12.2	-	-
Methionine	12.1	-	3.5	-
Isoleucine	40.4	52.5	44.4	102.7
Leucine	46.0	58.8	57.8	61.2
Tyrosine	33.5	6.3	30.6	9.3
Phenylalanine	28.3	41.4	35.8	53.5
Lysine	70.9	98.0	78.4	24.3
Histidine	20.0	24.8	27.2	3.5
Arginine	34.5	39.6	38.6	21.4

*Residues/1,000 A.A. residues.

FIGURE 21

Extinction coefficients for the staphylococcal hemolysins

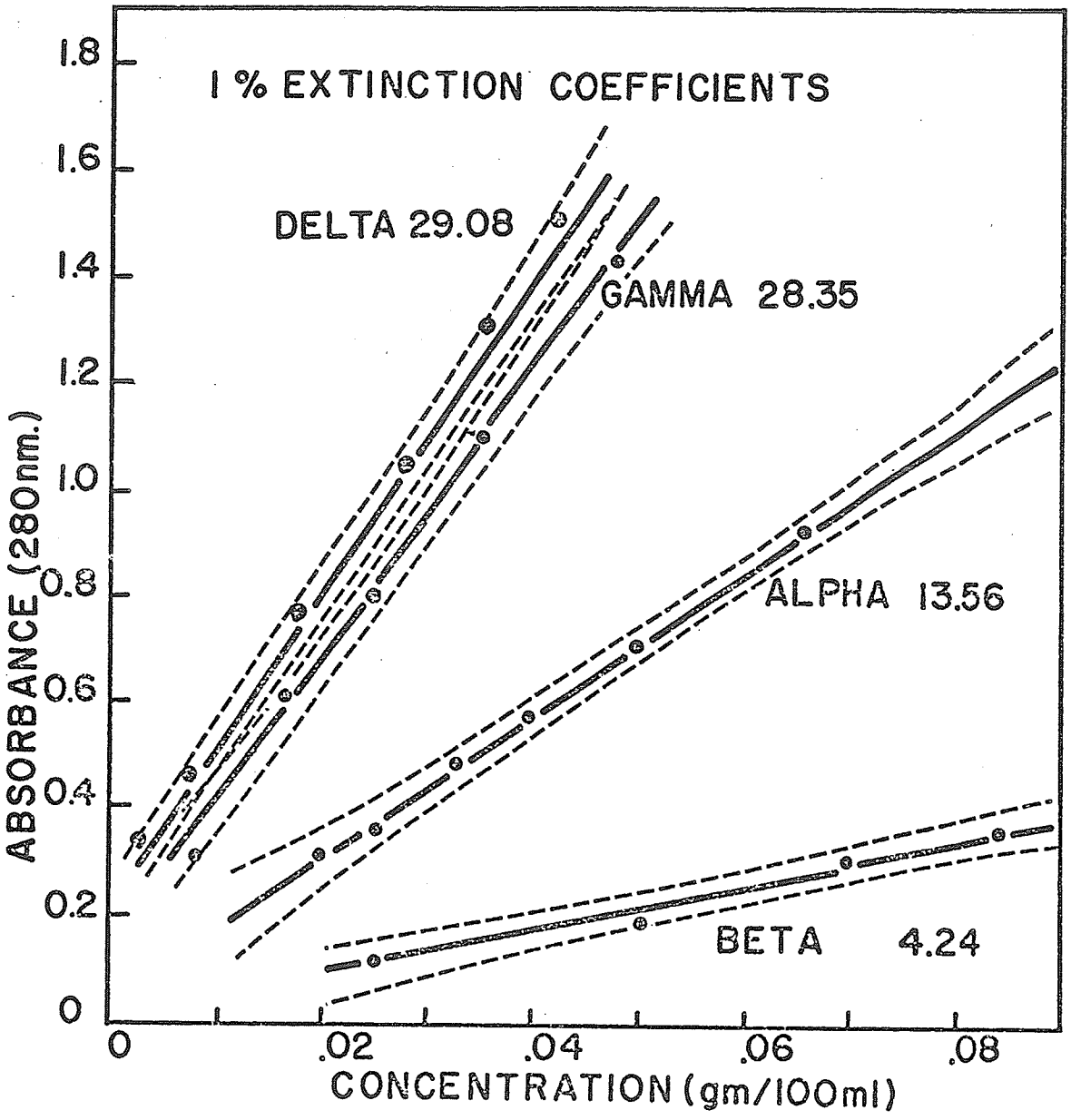
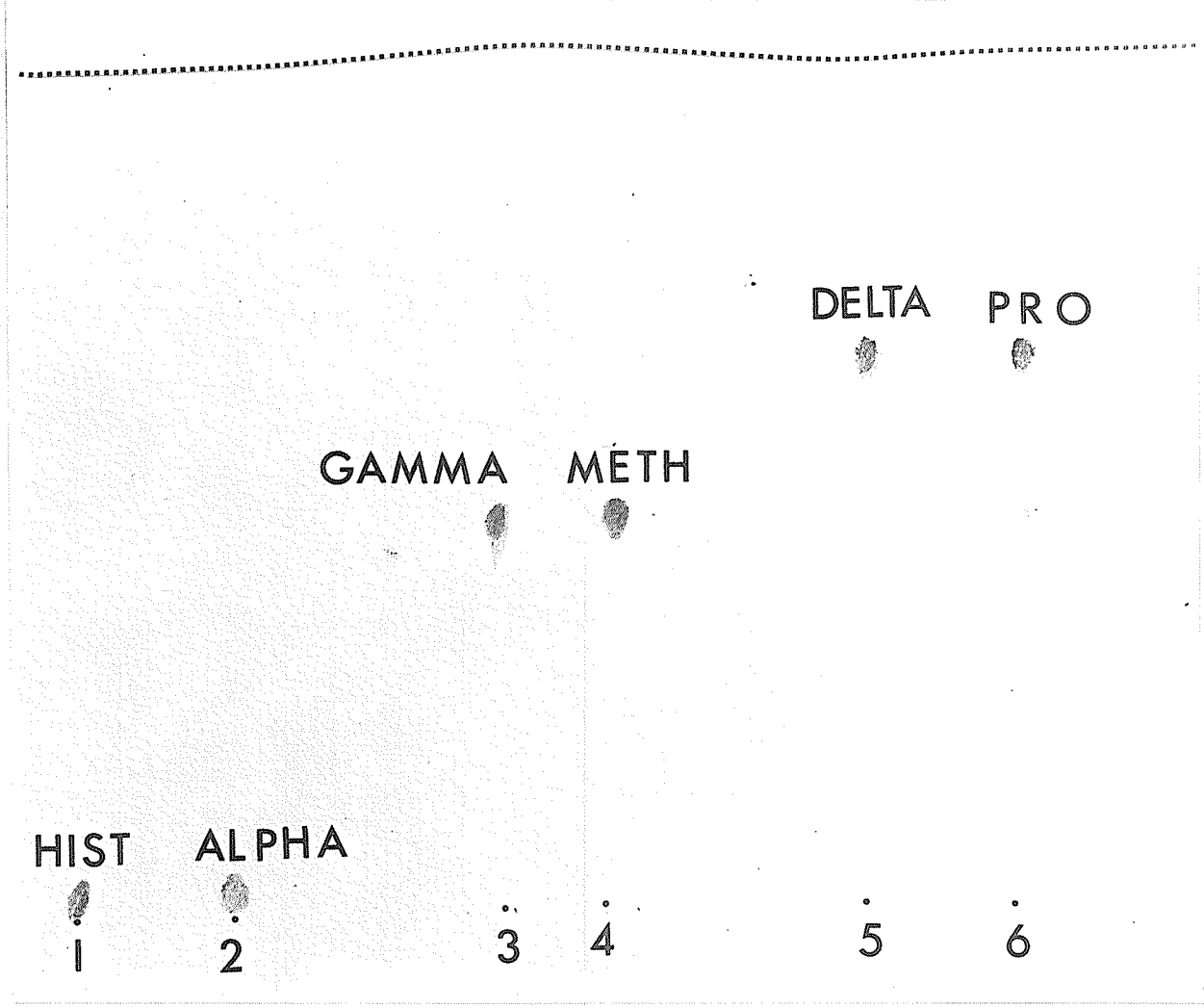

1 % EXTINCTION COEFFICIENTS
DELTA 29.08
GAMMA 28.35
ALPHA 13.56
BETA 4.24
ABSORBANCE (280nm.)
CONCENTRATION (gm/100ml)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
.02
.04
.06
.08

FIGURE 22

Thin layer chromatogram of the N-terminal amino acids of alpha, gamma and delta hemolysins. The solvent system was benzene/ pyridine/ glacial acetic acid (80:20:2) and the yellow DNP-amino acid spots were visulized by the use of a blue filter. The dotted line represents the solvent front.


DELTA
PRO
GAMMA
METH
HIST
ALPHA
1
2
3
4
5
6

gave a single protein peak by this method, confirming the homogeneity of the preparations. The protein peak coincided with the hemolytic activity in each case (Fig. 23). The isoelectric points of the hemolysins are given in the figure.

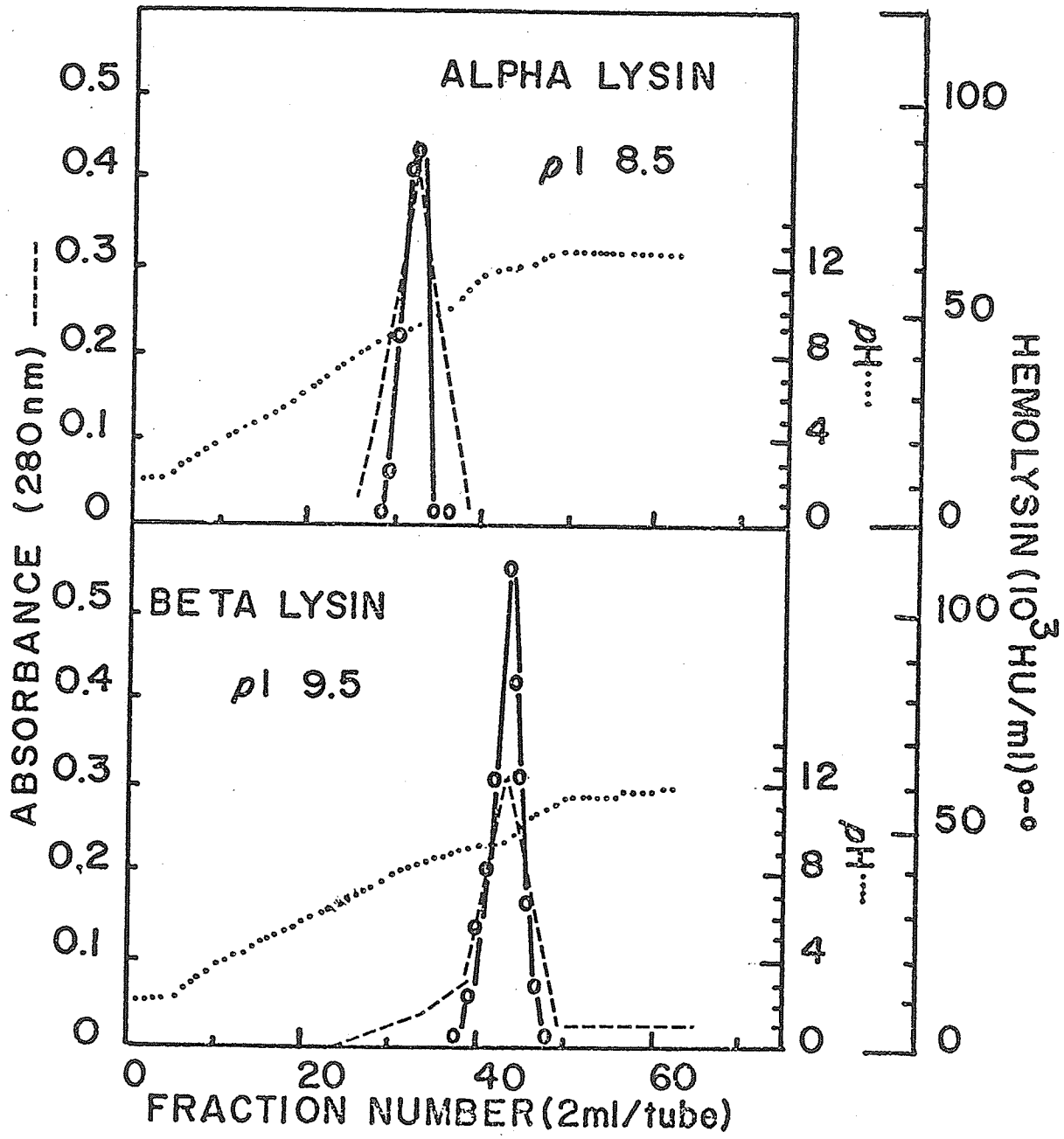
Molecular Weight

The molecular weights of the hemolysins were determined by gel filtration on a Sephadex G-75 column eluted with the buffer described by Hallander (87). A plot of the logarithm of the molecular weight of known proteins against their partition coefficients (K_{av}) established the selectivity curve for the Sephadex G-75 column, and determination of the K_{av} for the different hemolysins permitted calculation of their molecular weights from this selectivity curve as shown in Fig. 24. The K_{av} for gamma hemolysin was identical to that of alpha lysin and gave a molecular weight of 45,000 daltons while that of beta lysin was 26,000 daltons. The molecular weight of delta lysin could not be determined accurately by gel filtration because it was excluded in the void volume of both Sephadex G-75 and G-200.

In all cases, the purified hemolysins gave a single symmetrical peak when eluted from the G-75 column, and since the hemolytic activity coincided with the protein measurements, these data further confirmed that the hemolysin

FIGURE 23

Isoelectric focusing of the purified staphylococcal hemolysins


ALPHA LYSIN
pI 8.5
BETA LYSIN
pI 9.5
ABSORBANCE (280nm)
pH
HEMOLYSIN (10^3HU/ml)
FRACTION NUMBER (2ml/tube)
0
20
40
60
0.5
0.4
0.3
0.2
0.1
0
12
8
4
100
50

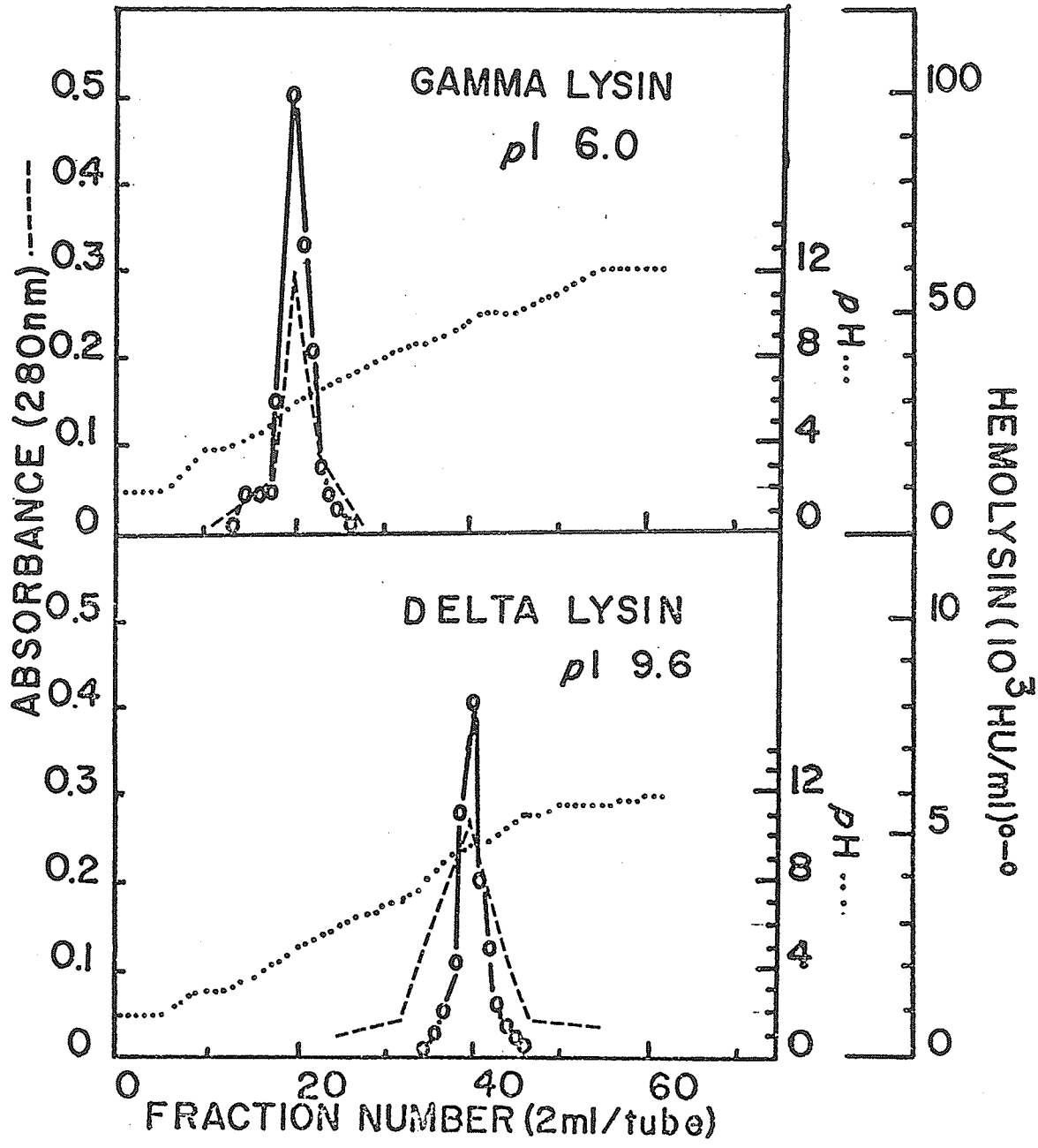
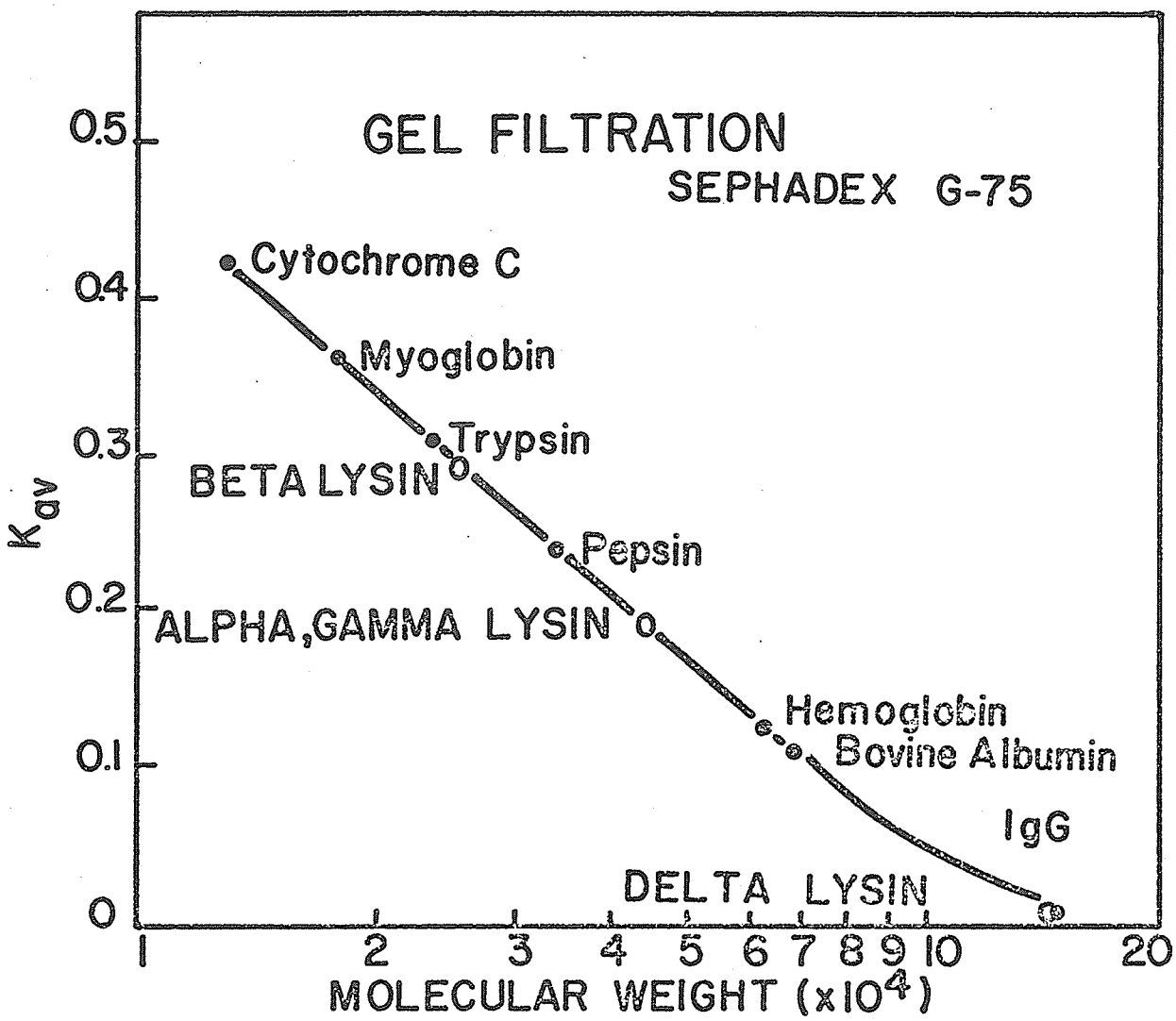

GAMMA LYSIN
pI 6.0
DELTA LYSIN
pI 9.6
ABSORBANCE (280nm)
pH
HEMOLYSIN (10³HU/ml)
FRACTION NUMBER (2ml/tube)
0
0.1
0.2
0.3
0.4
0.5
4
8
12
50
100
5
10
20
40
60

FIGURE 24

Determination of the molecular weight of the hemolysins by gel filtration


GEL FILTRATION
SEPHADEX G-75
Cytochrome C
Myoglobin
Trypsin
BETA LYSIN
Pepsin
ALPHA, GAMMA LYSIN
Hemoglobin
Bovine Albumin
IgG
DELTA LYSIN
K_{av}
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
10
20
MOLECULAR WEIGHT ($\times 10^4$)

preparations were homogeneous.

Several research workers claim that the partition coefficient, K_{av}, is more a measure of the Stokes radius than the molecular weight. The Stokes radii of the proteins used for the selectivity curve in Fig. 24 were obtained from the data of Laurent and Killander (122), and a linear plot of $(-\log K_{av})^{\frac{1}{2}}$ vs Stokes radius was constructed as shown in Fig. 25. From this plot it was determined that gamma and alpha hemolysins had similar Stokes radii of 27 Å while that of beta lysin was 21 Å.

The values of the Stokes radii suggested that the protein molecules would be visible by electron microscopy and therefore purified samples were negatively stained with potassium phosphotungstate as described by Arbuthnott *et al.* (3) for alpha hemolysin. Electron microscopy revealed no structure for gamma, alpha or delta hemolysins even when 1% solutions of the hemolysins were used. However, beta lysin formed a regular uniform structure that had the appearance of laminar arrays (Fig. 26). While the possibility exists that the structure in Fig. 26 may be an artifact of the technique, the uniform appearance reinforces the conclusion that the beta hemolysin preparation was homogeneous.

FIGURE 25

Determination of the Stokes' radii of the staphylococcal hemolysins

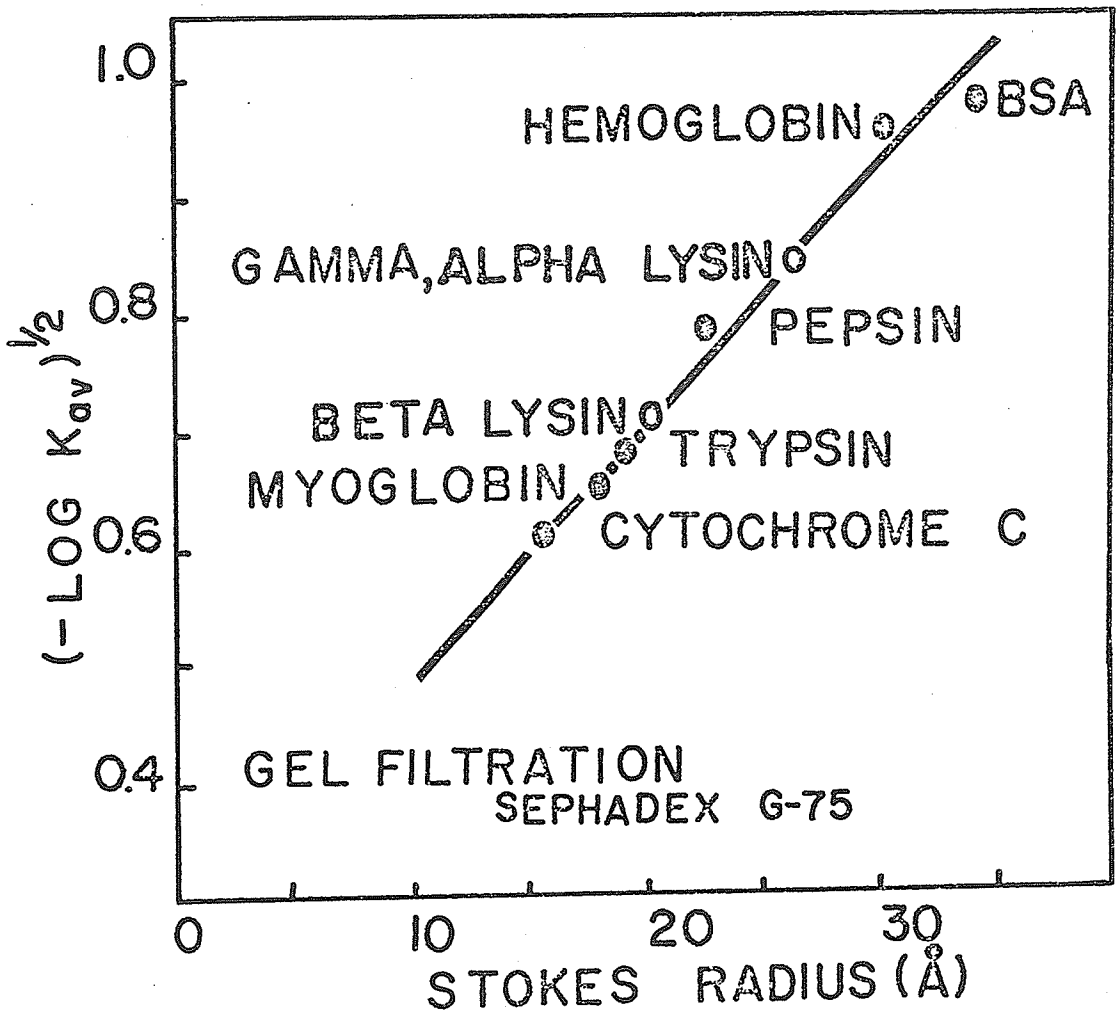

1.0
0.8
0.6
0.4
(-LOG Kav)1/2
HEMOGLOBIN
BSA
GAMMA, ALPHA LYSIN
PEPSIN
BETA LYSIN
TRYPSIN
MYOGLOBIN
CYTOCHROME C
GEL FILTRATION
SEPHADEX G-75
0
10
20
30
STOKES RADIUS (Å)

FIGURE 26

Electron micrograph of purified beta lysin.
negatively stained with potassium phosphotungstate.
total magnification: x 228,000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CHAPTER 6

IMMUNOLOGICAL STUDIES OF GAMMA HEMOLYSIN

Quantitative Precipitin Test

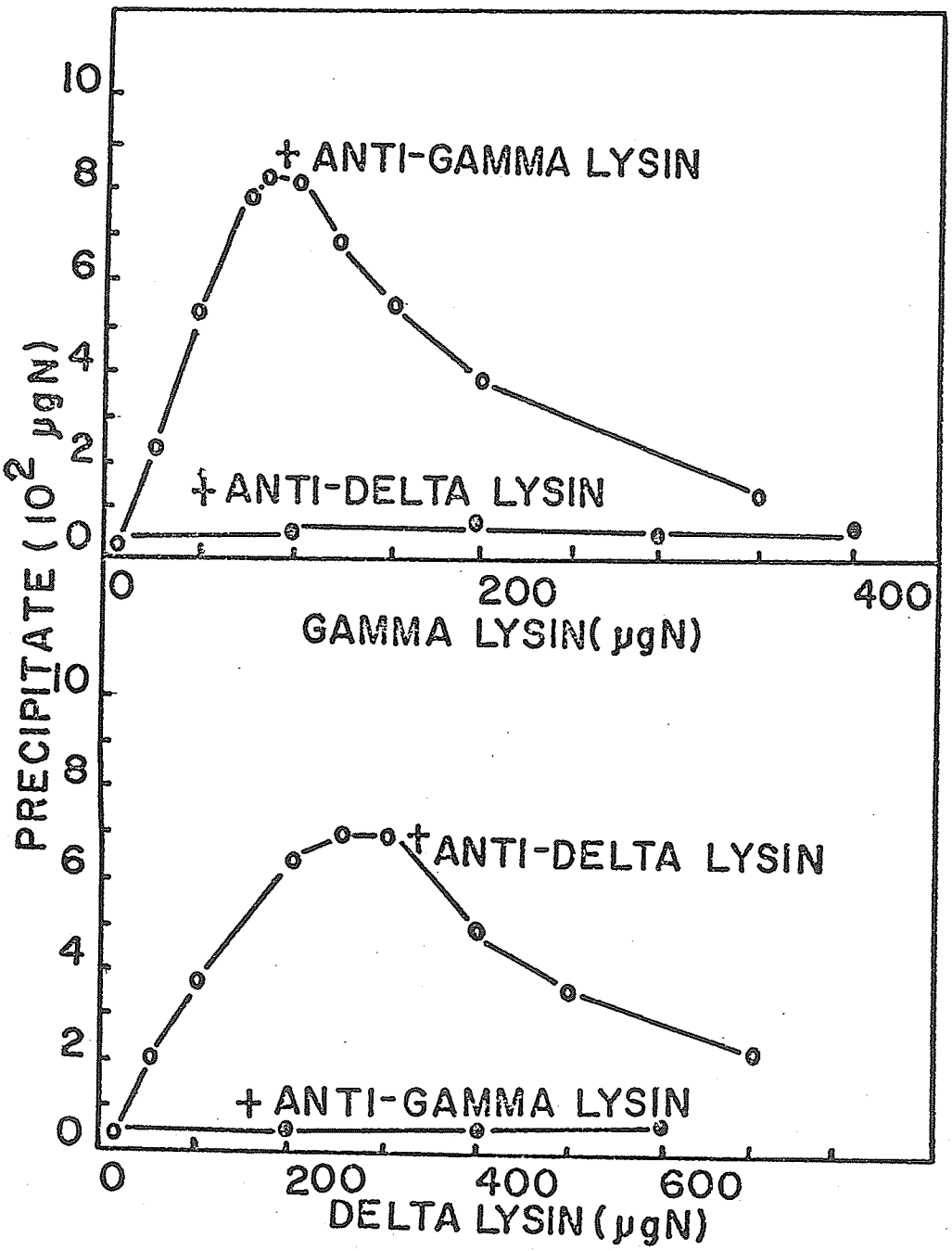
Elek (55) believed that gamma and delta hemolysins were the same entity and that antibodies could be formed against only alpha, beta and delta hemolysins. Thus, quantitative precipitin tests were performed to demonstrate that gamma hemolysin was immunologically distinct from alpha, beta and delta hemolysins. Dilutions of the purified hemolysins were incubated with specific antisera and as shown in Fig. 27, the gamma hemolysin was precipitated by its respective antiserum with the formation of a characteristic precipitin curve. None of the other hemolysins was precipitated by anti-gamma lysin. By comparison, the other hemolysins were precipitated only by their respective antisera. The supernatant solutions were qualitatively assayed for antigen or antibody, and the observation that none of the supernatants showed any evidence of both antigen and antibodies is evidence that the hemolysins are homogeneous.

Immunodiffusion, Immunoelectrophoresis

The photographs in Fig. 28 show that purified gamma hemolysin formed a single precipitin line with its homologous antiserum as did alpha, beta and delta lysins.

FIGURE 27

Quantitative precipitin tests with the purified staphylococcal hemolysins


10
8
6
4
2
0
PRECIPITATE (10^2 µgN)
+ ANTI-GAMMA LYSIN
+ ANTI-DELTA LYSIN
0
200
400
GAMMA LYSIN(µgN)
10
8
6
4
2
0
+ ANTI-DELTA LYSIN
+ ANTI-GAMMA LYSIN
0
200
400
600
DELTA LYSIN (µgN)

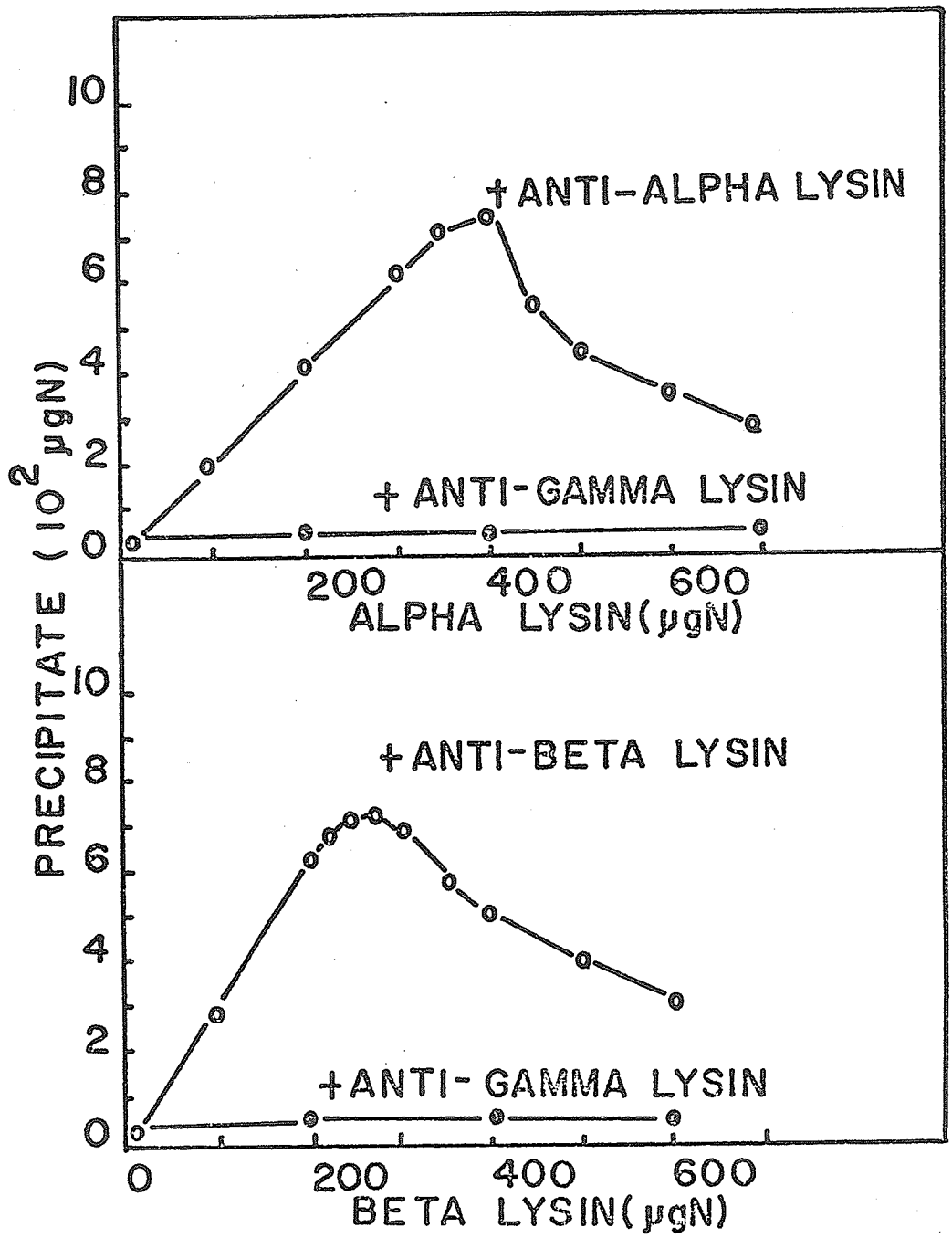
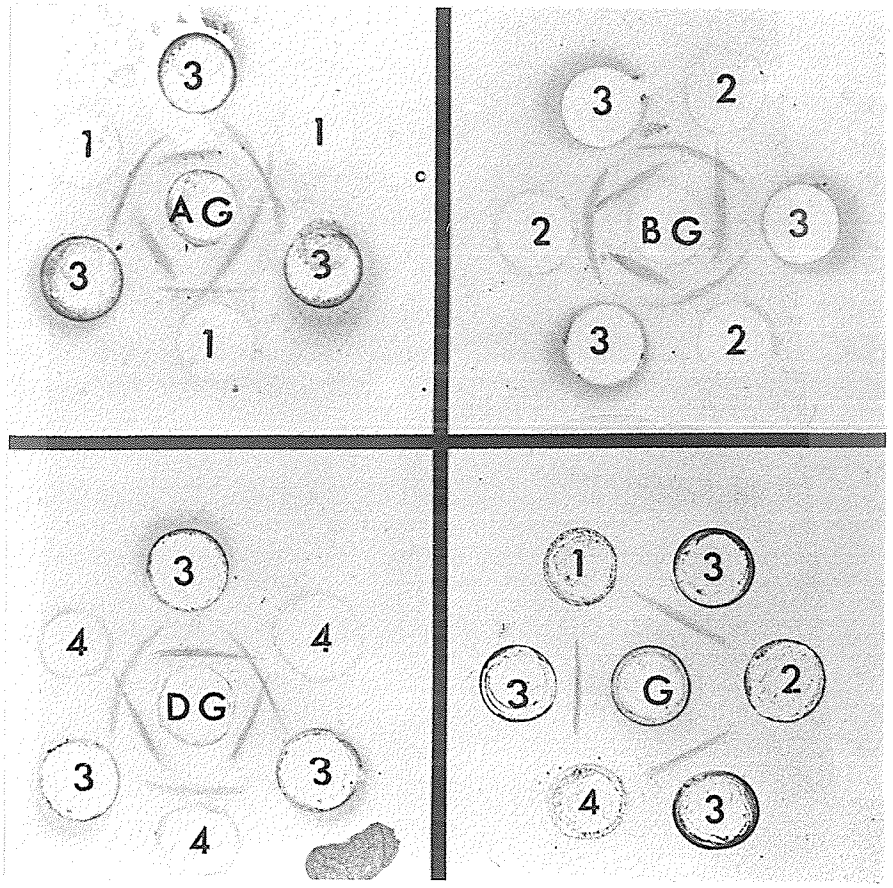

PRECIPITATE (10^2 µgN)
10
8
6
4
2
0
+ANTI-ALPHA LYSIN
+ ANTI-GAMMA LYSIN
200
400
600
ALPHA LYSIN(µgN)
10
8
6
4
2
0
+ANTI-BETA LYSIN
+ANTI- GAMMA LYSIN
0
200
400
600
BETA LYSIN(µgN)

FIGURE 28

Immunodiffusion of the purified staphylococcal hemolysins. 1=alpha lysin, 2= beta lysin, 3= gamma lysin, 4= delta lysin, AG= anti alpha, gamma lysin, BG= anti beta, gamma lysin, DG= anti delta, gamma lysin, G= anti gamma lysin


3
1
1
AG
3
3
1
3
2
2
BG
3
3
2
3
4
4
DG
3
3
4
1
3
3
G
2
4
3

Furthermore, gamma hemolysin did not form precipitin bands when diffused against the other antisera and only lines of non-identity formed between the different hemolysins when diffused against multivalent antisera.

Purified hemolysins were used as the antigen in immunoelectrophoresis and antisera to crude hemolysins were placed in the trough or conversely, crude hemolysin was used as the antigen and antibodies to purified hemolysin were placed in the troughs. In every case (Fig. 29) only a single immunoprecipitin line was obtained. These results indicate that all the hemolysins, including delta lysin, will elicit antibodies to a single immunogen when injected into a rabbit and thus are perhaps the best evidence that the hemolysins are homogeneous. Whereas delta hemolysin had moved slightly to the anode, gamma, beta and alpha hemolysins migrated towards the cathode. However, gamma lysin could be distinguished clearly from the alpha and beta lysins.

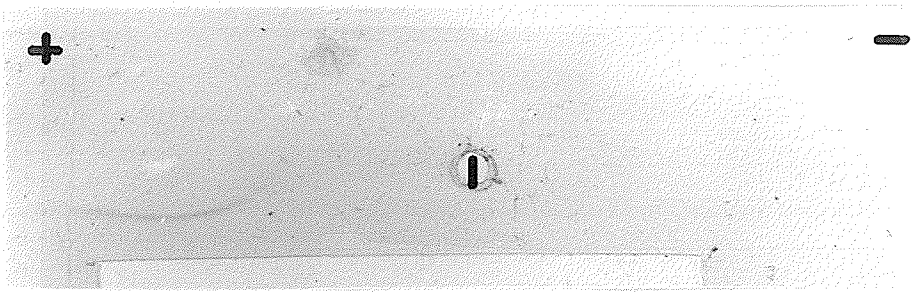
Neutralization Experiments

Although the foregoing experiments demonstrated the presence of a single immunogen in each hemolysin preparation, they did not determine whether gamma lysin or the other hemolysins were neutralized. Each hemolysin was titrated before and after incubation with the different antisera and the results are presented in Table 19. Gamma

FIGURE 29

Immunoelectrophoresis of the staphylococcal hemolysins. (1)= alpha lysin, (2) = beta lysin, (3) = gamma lysin, (4) = delta lysin. Purified hemolysin was placed in the well and homologous antisera to crude hemolysin was placed in the trough.

123b



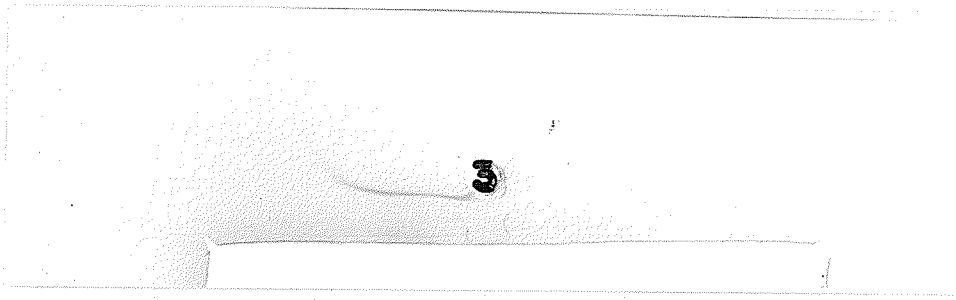


TABLE 19

NEUTRALIZATION OF *S. aureus* HEMOLYSINS

HEMOLYSIN	ANTIBODY: Alpha	Beta	Delta	Gamma	Pre-Immune
Alpha	0	2000*	2000	2000	2000
Beta	2000	0	2000	2000	2000
Delta	1100	1100	0	1100	1100
Gamma	2000	2000	2000	0	2000

* HU/ml

lysin was neutralized only by its respective antiserum as were the other hemolysins. Although delta lysin was completely neutralized by anti-delta lysin, it was also inhibited about 50% by the other antisera. However, four normal sera that did not form precipitin lines against delta hemolysin and 0.01% albumin could also inhibit to a similar extent.

The velocity of lysis by the different hemolysins was measured in the presence and absence of the various antisera. Fig. 30 illustrates that lysis of rabbit and human erythrocytes by gamma lysin was inhibited by the presence of anti-gamma hemolysin. In a similar manner, hemolysis of erythrocytes by alpha, beta and delta hemolysins was inhibited by their respective antisera. Regardless of the amount of antiserum added, the hemolytic reaction could not be stopped completely. However, the data in Fig. 30 show that antibodies to a purified hemolysin neutralize only that hemolysin.

Finally, the immunodiffusion experiments were repeated where 1% suspensions of washed erythrocytes were incorporated into agarose. Active hemolysins were used as the antigens and the plates were allowed to diffuse for 2 h at 37^{o}C, at the end of which time they were cooled to 0^{o}C and rapidly dried with blotting paper. The photographs in Fig. 31 confirm previous observations that the antisera to the purified hemolysins possess neutralizing activity

FIGURE 30

Inhibition of the rate of hemolysis by homologous antisera

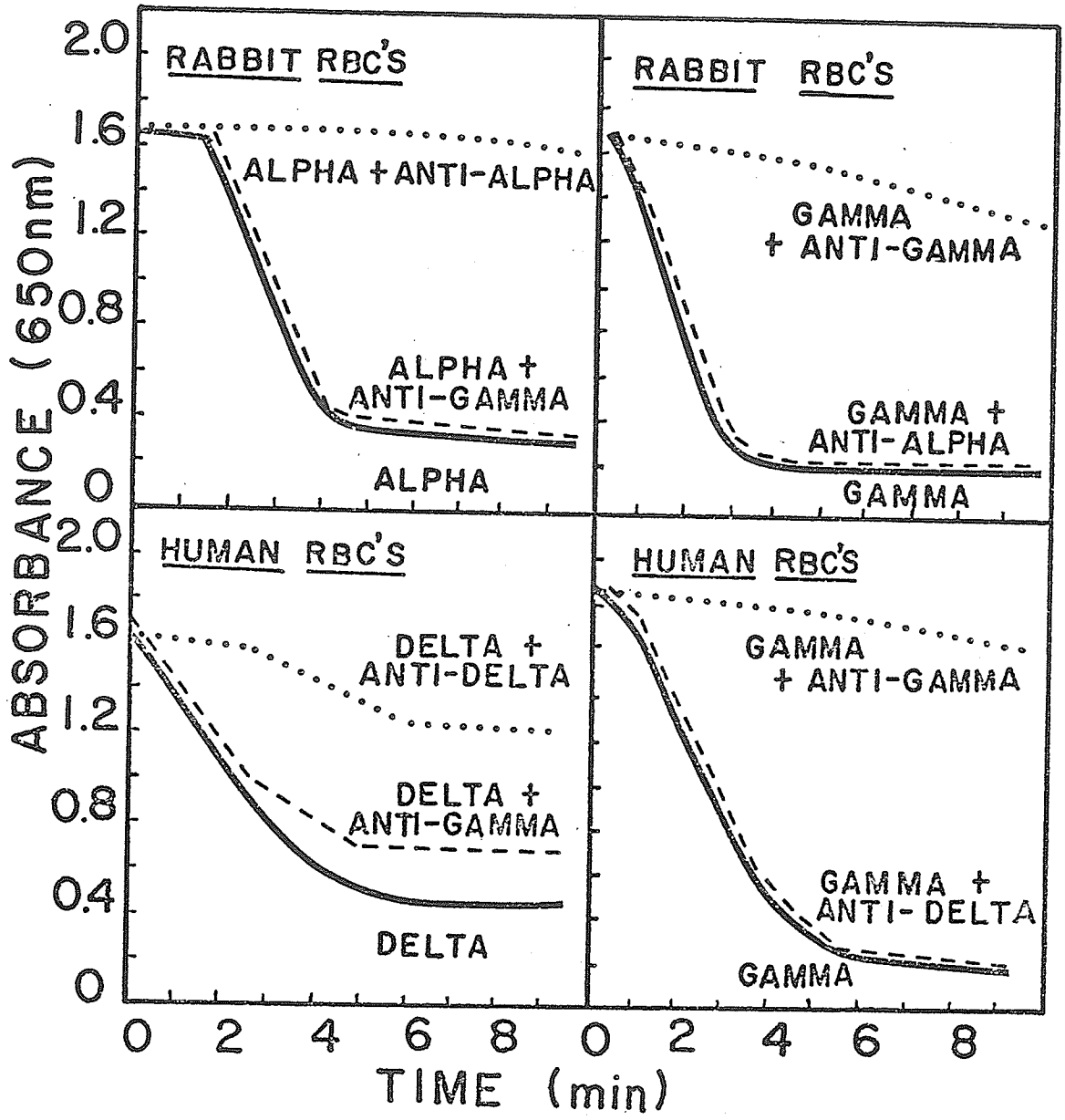

RABBIT RBC'S
ALPHA + ANTI-ALPHA
ALPHA + ANTI-GAMMA
ALPHA
RABBIT RBC'S
GAMMA + ANTI-GAMMA
GAMMA + ANTI-ALPHA
GAMMA
HUMAN RBC'S
DELTA + ANTI-DELTA
DELTA + ANTI-GAMMA
DELTA
HUMAN RBC'S
GAMMA + ANTI-GAMMA
GAMMA + ANTI-DELTA
GAMMA
ABSORBANCE (650nm)
2.0
1.6
1.2
0.8
0.4
0
0 2 4 6 8
TIME (min)

FIGURE 31

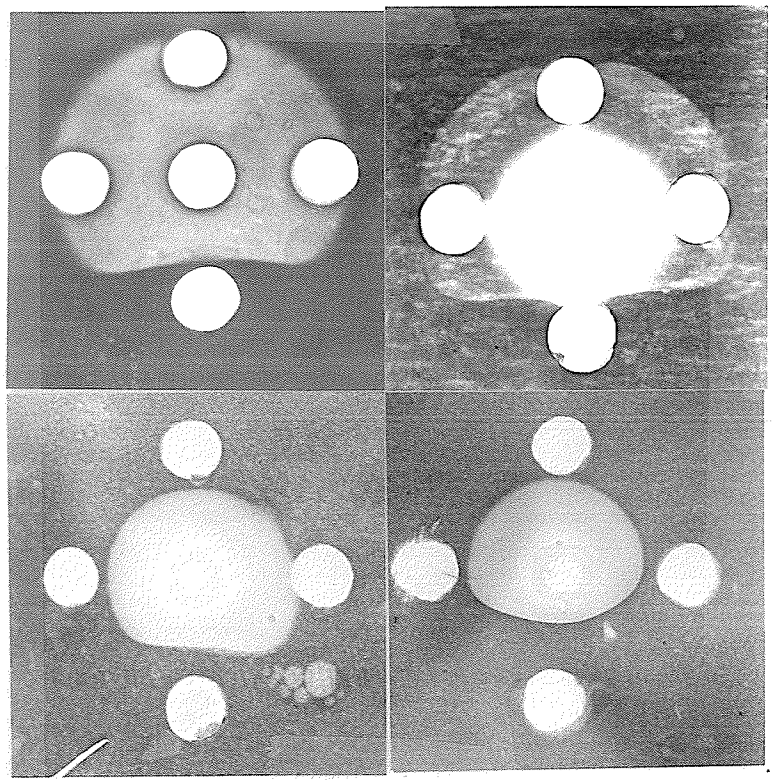
Immunodiffusion of the staphylococcal hemolysins in blood agar. In all cases the hemolysin was placed in the centre well and homologous antisera in the bottom well. The other three wells remained empty.

Top left: alpha hemolysin, rabbit blood agar

Top right: beta hemolysin, sheep blood agar

Bottom left: gamma hemolysin, human blood agar

Bottom right: delta hemolysin, human blood agar.



towards only these lysins.

Immunological Comparison with Other Hemolysin Preparations

Two vials of antisera #CPP9763 and EX1480 were gifts of the Wellcome Research Laboratories; the first was antisera to crude alpha hemolysins, the second to crude beta hemolysin. These antisera gave a single precipitin line when diffused against the appropriate purified hemolysin and a line of identity was formed with the respective monospecific antibody. Both the purified hemolysins were neutralized by the corresponding antisera from Wellcome Research Laboratories. In addition, EX1480 also neutralized gamma lysin and gave a precipitin line on immunodiffusion which formed a line of identity with anti-gamma lysin (data not shown).

A sample of purified delta hemolysin was obtained from Dr. A. Kreger and this material was used to immunize rabbits. Immunodiffusion experiments revealed the presence of at least three antigens in this preparation (Fig. 32). These antigens formed lines of identity with the purified delta, gamma and alpha hemolysins. The antisera to Dr. Kreger's delta hemolysin neutralized not only delta hemolysin but also gamma and to a lesser extent alpha hemolysin, but pre-immune sera formed no precipitin lines.

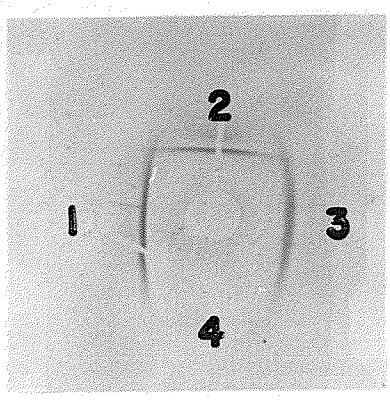
FIGURE 32

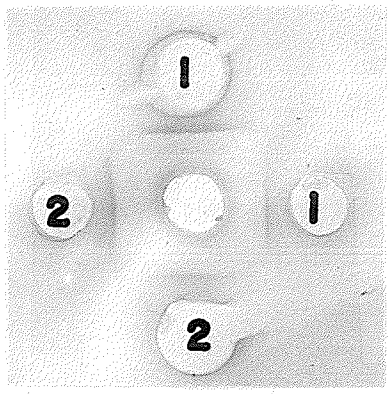
Immunodiffusion: delta hemolysin of Kreger

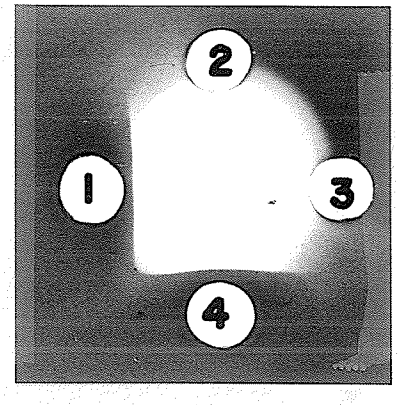
Top: Immunodiffusion, The centre well contains the delta hemolysin of Kreger and the outer wells contain antisera to (1) alpha lysin, (2) Kreger's delta lysin, (3) gamma lysin and (4) beta lysin.

Middle: Immunodiffusion: The centre well contains the delta hemolysin used by the author. The outer wells contain antisera to (1) the delta hemolysin , (2) the delta hemolysin of Kreger.

Bottom: Immunodiffusion in human blood agar. The centre well contains delta lysin. The outer wells contain (1) antisera to delta lysin, (2) pre-immune sera, (3) pre-immune sera to Kreger's delta lysin and (4) antisera to Kreger's delta lysin.


2
1
3
4


1
2
1
2


2
1
3
4

CHAPTER 7

BIOLOGICAL PROPERTIES OF GAMMA HEMOLYSIN

Comparison of the Hemolytic Spectra

Blood obtained from eleven species of animals was washed and resuspended in phosphate buffered saline supplemented with 0.001 *M* Mg++ and each blood sample was titrated in quadruplicate against the four staphylococcal hemolysins and the titrations averaged. After incubation for 1 h at 37°C, the cells were allowed to settle overnight at 4°C and then the absorbance of the supernatant at 541 nm was measured. The hemolytic spectra of the four hemolysins are quite different as shown in Table 20, which is the average of the four titrations expressed as HU/mg protein so that the sensitivity of the blood sample to the different hemolysins may be compared directly. Rabbit erythrocytes are most sensitive to the alpha and gamma hemolysins in contrast with beta and delta hemolysins which preferentially lyse sheep and human cells. Monkey cells are most resistant to beta lysin and it is worth noting that pigeon cells are refractory to alpha, gamma and delta lysins.

Effect of Gamma and Alpha Lysin on Human Leucocytes

Human leucocytes were prepared from fresh whole blood by centrifugation at 1,000 x g for 15 minutes and removal of the leucocyte layer. These leucocytes were

TABLE 20

HEMOLYTIC ACTIVITY* OF STAPHYLOCOCCAL LYSINS

SPECIES	ALPHA	BETA	GAMMA	DELTA
Rabbit	76,422	81,300	113,210	1,644
Dog	2,906	< 750	29,764	882
Rat	2,497	4,960	44,041	1,700
Cat	2,214	4,760	6,445	1,500
Sheep	1,026	758,830	89,906	2,929
Mouse	1,023	10,000	25,754	1,037
Chicken	83	2,950	306	803
Guinea pig	56	63,740	6,620	803
Human	53	10,360	44,041	7,527
Monkey	26	< 750	3,208	749
Pigeon	5	9,930	160	165

*HU/mg protein

washed three times in phosphate buffered saline and resuspended to a final absorbance of 1.1 at 650 nm. The washed leucocytes were treated with 10 µg of gamma lysin and the absorbance was measured at various times. The absorbance of untreated leucocytes remained constant over 35 minutes (Fig. 33) but there was a rapid decrease in the absorbance of leucocytes treated with gamma lysin. Alpha hemolysin caused a similar decrease in the absorbance of leucocytes but at a slower rate than gamma lysin.

Platelets

The effect of gamma and alpha hemolysin on human and rabbit platelets was measured spectrophotometrically (Fig. 34). Fresh, citrated blood was centrifuged at 300 x g for 15 minutes, the supernatant was removed and the platelets pelleted by centrifugation at 2,000 x g for 15 minutes. Platelets were washed three times in phosphate buffered saline and were resuspended to an absorbance of 1.0 at 650 nm. When no hemolysin was present, the absorbance of human platelets remained constant but the addition of gamma lysin (100 HU/ml, 1 µg/ml) caused it to decrease linearly with respect to time by 50% after 20 minutes. Addition of a similar amount of alpha lysin caused an absorbance decrease but to a lesser extent. Rabbit platelets were affected by alpha and gamma lysin also (Fig. 34).

Platelets treated with gamma or alpha hemolysin

FIGURE 33

Absorbance of human leucocytes incubated with alpha or gamma hemolysin

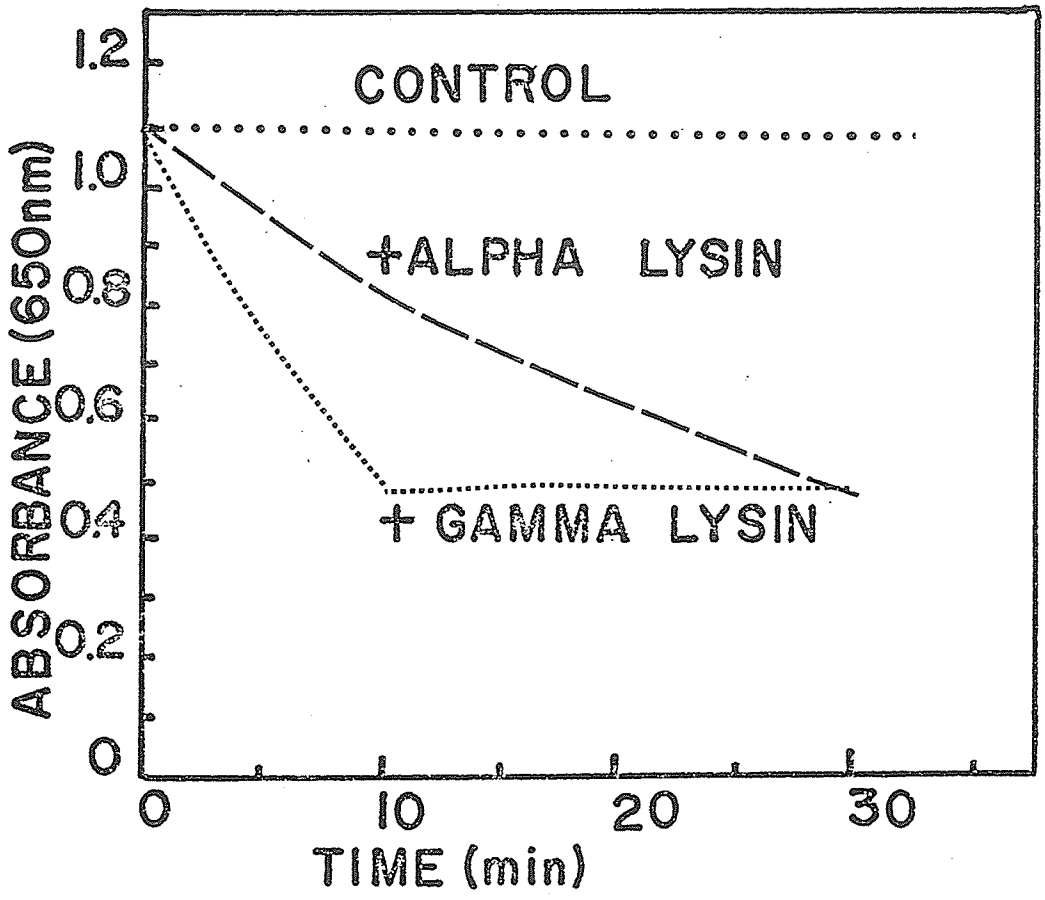
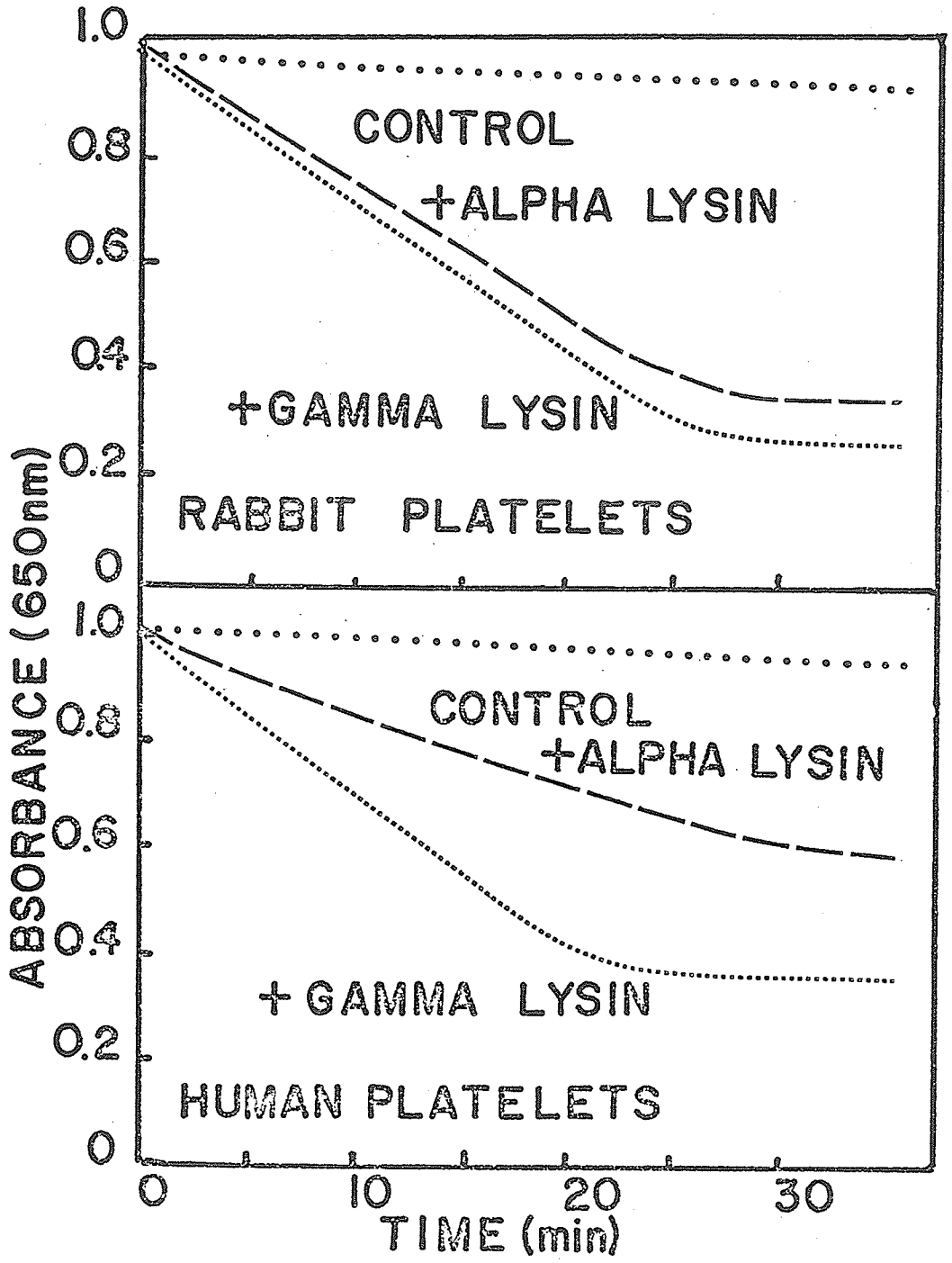

CONTROL
+ALPHA LYSIN
+ GAMMA LYSIN
ABSORBANCE (650nm)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
TIME (min)

FIGURE 34

Absorbance of human and rabbit platelets incubated with alpha or gamma hemolysin


1.0
0.8
0.6
0.4
0.2
0
CONTROL
+ALPHA LYSIN
+GAMMA LYSIN
RABBIT PLATELETS
1.0
0.8
0.6
0.4
0.2
0
CONTROL
+ALPHA LYSIN
+ GAMMA LYSIN
HUMAN PLATELETS
ABSORBANCE (650nm)
0
10
20
30
TIME (min)

were stained with osmium tetroxide and thin sections were examined under the electron microscope. Normal human and rabbit platelets have a characteristic ultrastructure similar to that shown in Figs. 35 and 36. The platelet cytoplasm was surrounded by a fine limiting membrane and contained mitochondria, microtubules and vacuoles of variable size and shape with limiting membranes. Dense osmophilic granules were eccentrically located in larger less dense membrane-bound structures (72,138,172,173). However, platelets that were exposed to gamma or alpha hemolysin for 20 minutes showed several degenerative changes. The limiting membrane remained essentially intact in all cases (Figs. 37-40) but there were few vesicles, mitochondria or microtubules present. The cell cytoplasm was noticably less dense and in some cases there was complete cell destruction and release of cellular debris. Platelets exposed to either gamma or alpha hemolysin showed granules that were more densely stained than normal platelets. The organelles tended to concentrate in the centre of rabbit platelets treated with alpha hemolysin.

Subcutaneous Injection

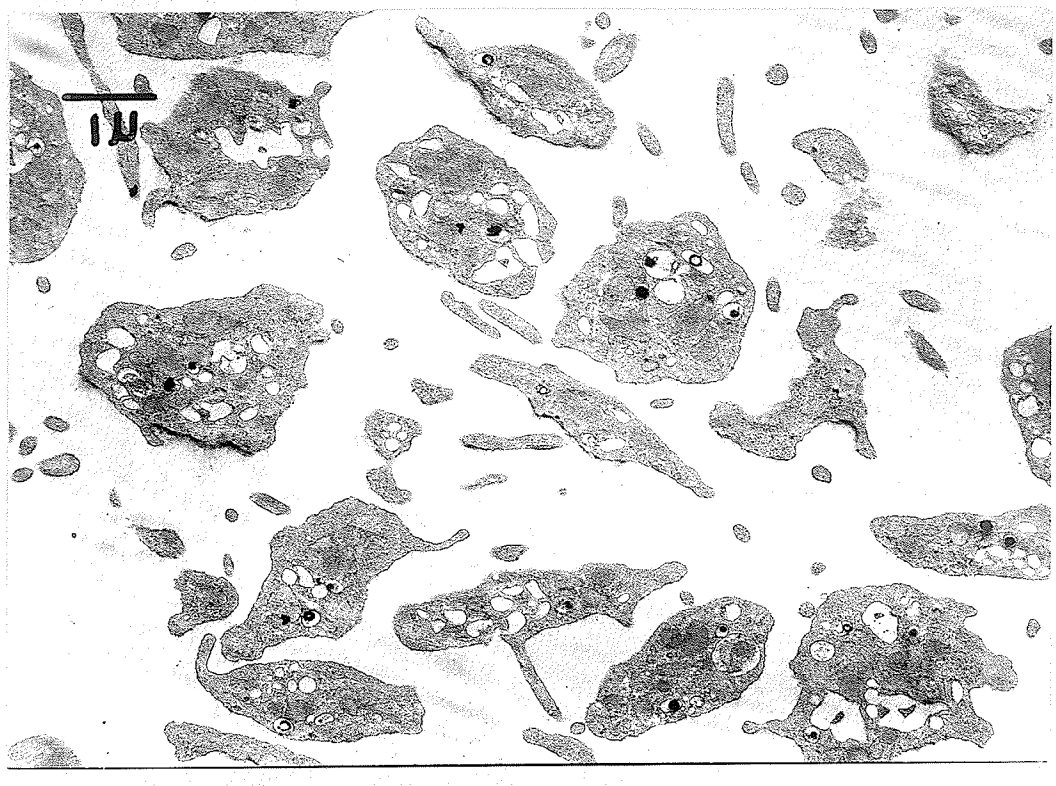
The back of a guinea pig was shaved and injected subcutaneously with 100 ug of gamma lysin and 100 µg of beta and delta lysins. Alpha lysin (10 µg) was used as a control. Gamma lysin had no overt effects but alpha

FIGURE 35

Electron micrograph of a thin section of normal rabbit platelets.

Top: total magnification x 12,600

Bottom: total magnification x 50,000



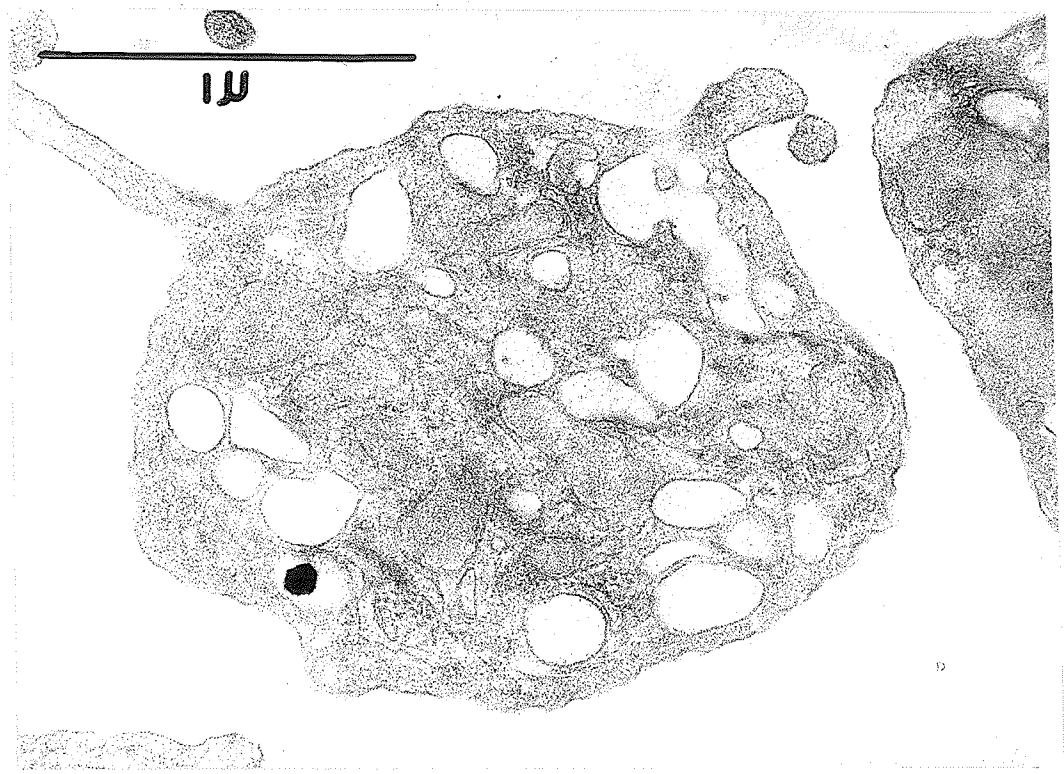
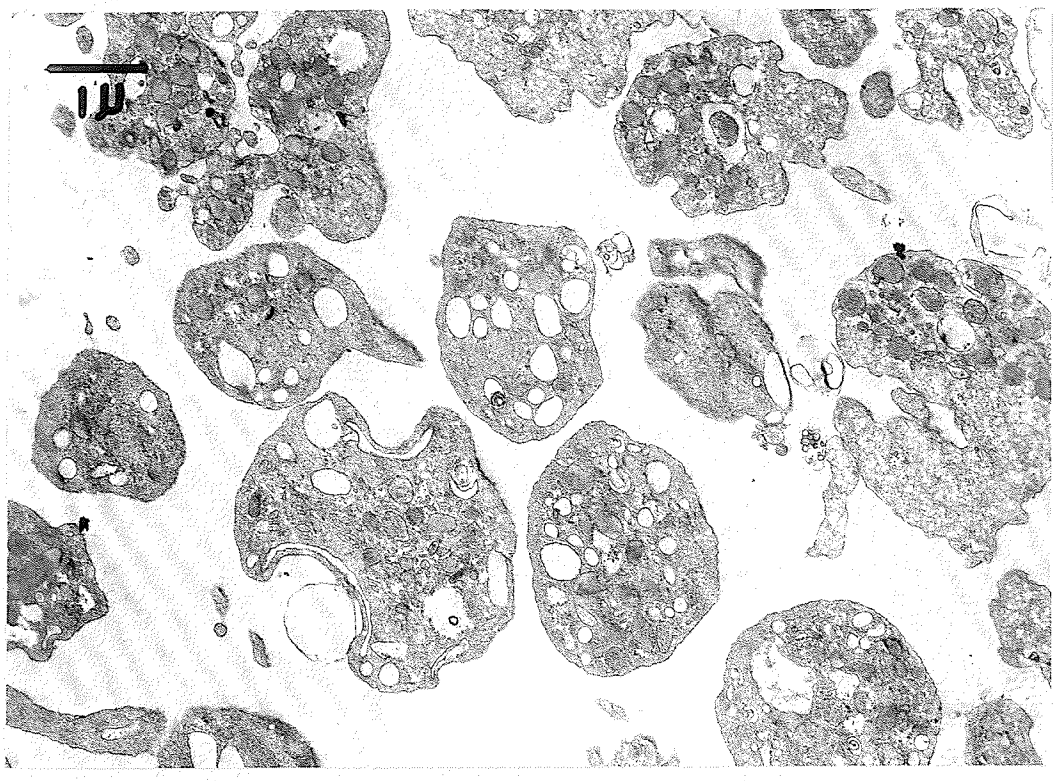


FIGURE 36

Electron micrograph of a thin section of normal human platelets

Top: total magnification x 12,600

Bottom: total magnification x 40,000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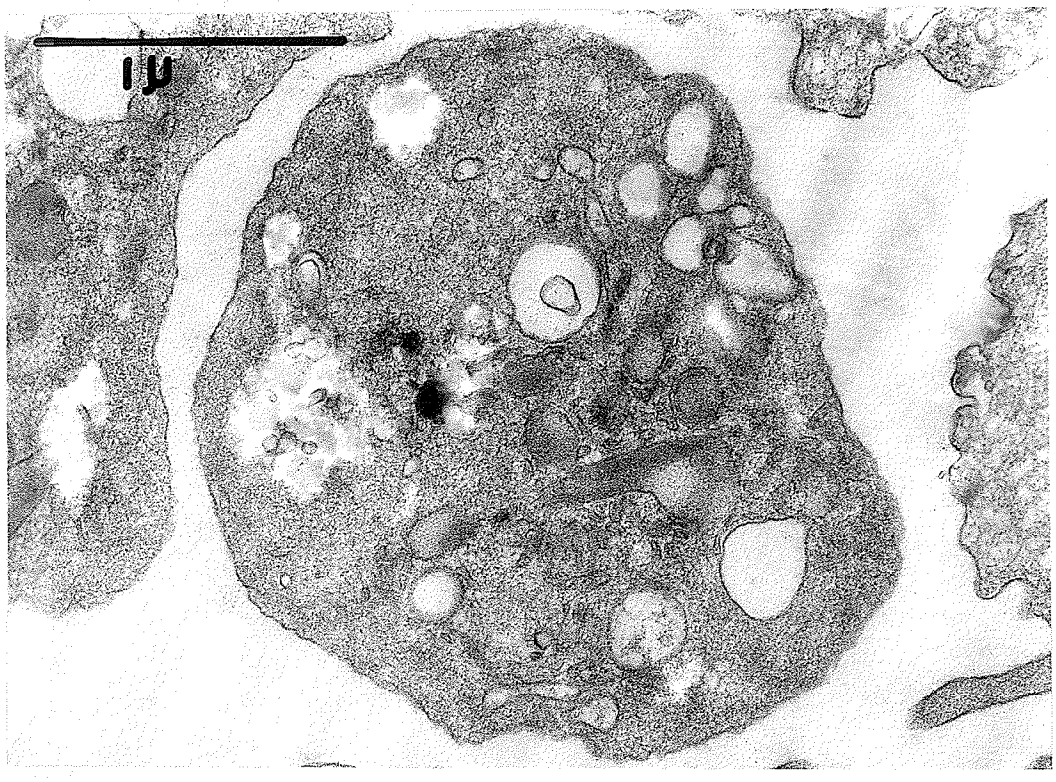
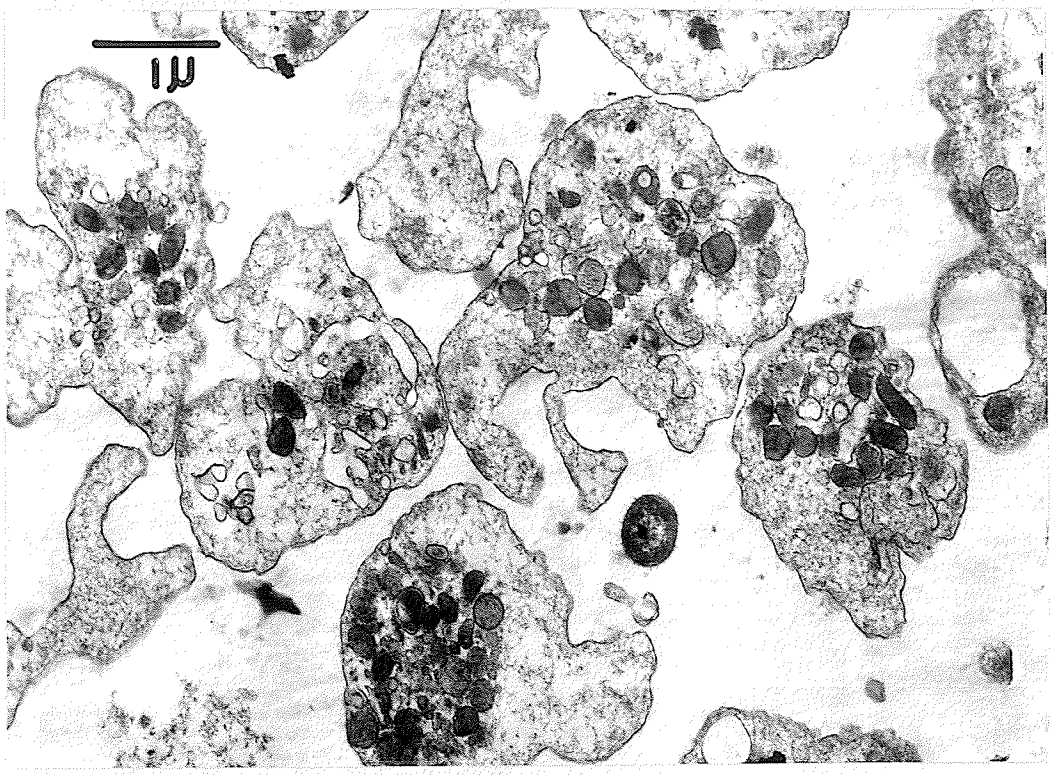

١٢

FIGURE 37

Electron micrograph of thin sections of human platelets after exposure to gamma hemolysin. Note loss of structure of internal organelles and less dense cytoplasm

Top: total magnification x 17,010

Bottom: total magnification x 40,000


1μ

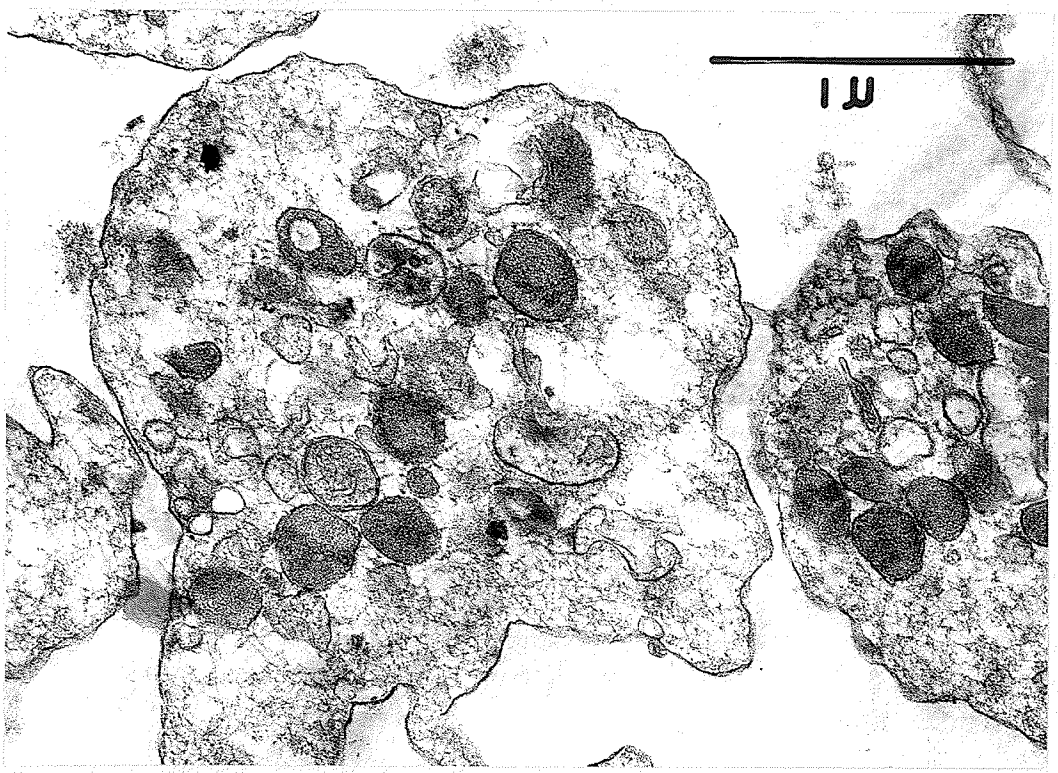

1μ

FIGURE 38

Electron micrograph of thin section of human platelets after exposure to alpha hemolysin. There is a complete loss of cell structure and organization.

Top: total magnification x 12,600

Bottom: total magnification x 40,000

1μ

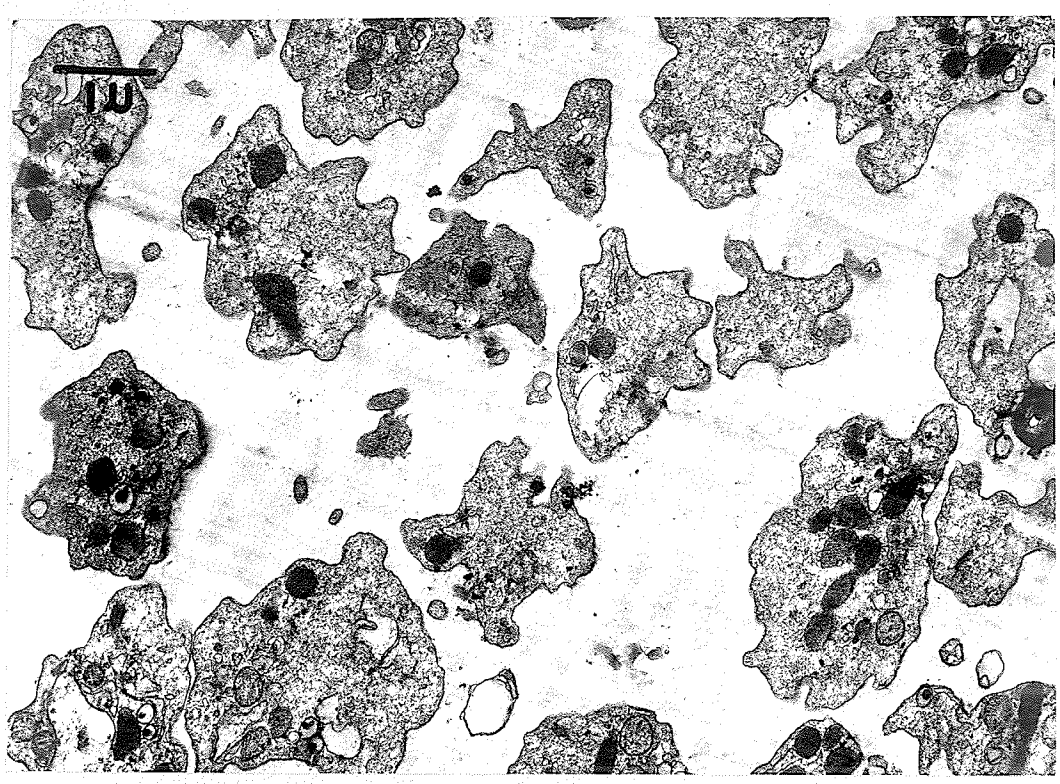
1μ

FIGURE 39

Electron micrograph of thin section of rabbit platelets after exposure to gamma lysin. The cytoplasm is less dense and the internal organelles are stained more densely.

Top: total magnification x 14,175

Bottom: total magnification x 48,000


١٤

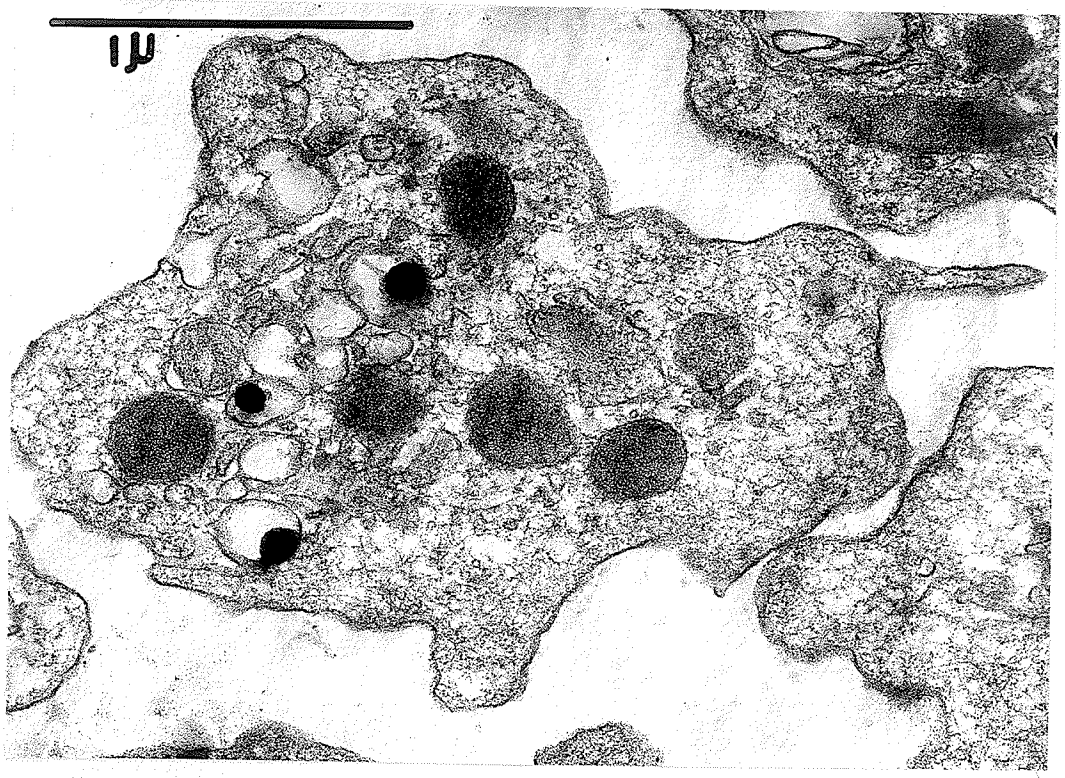
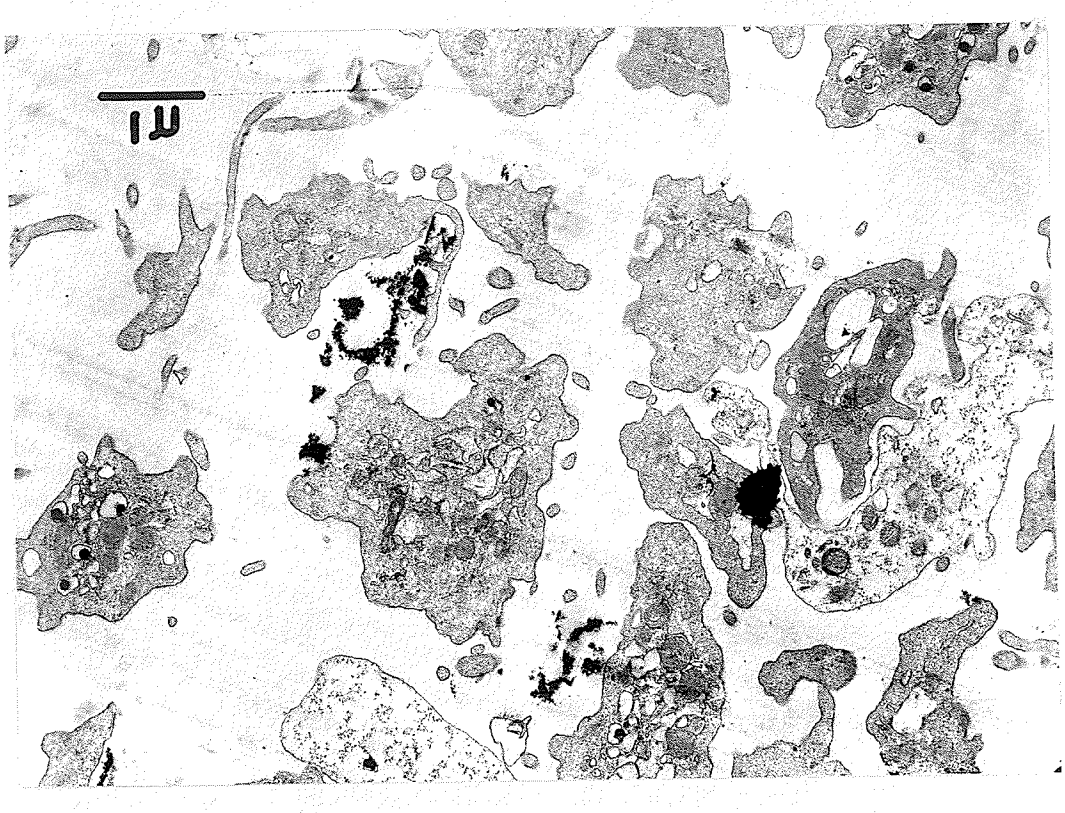

١٢

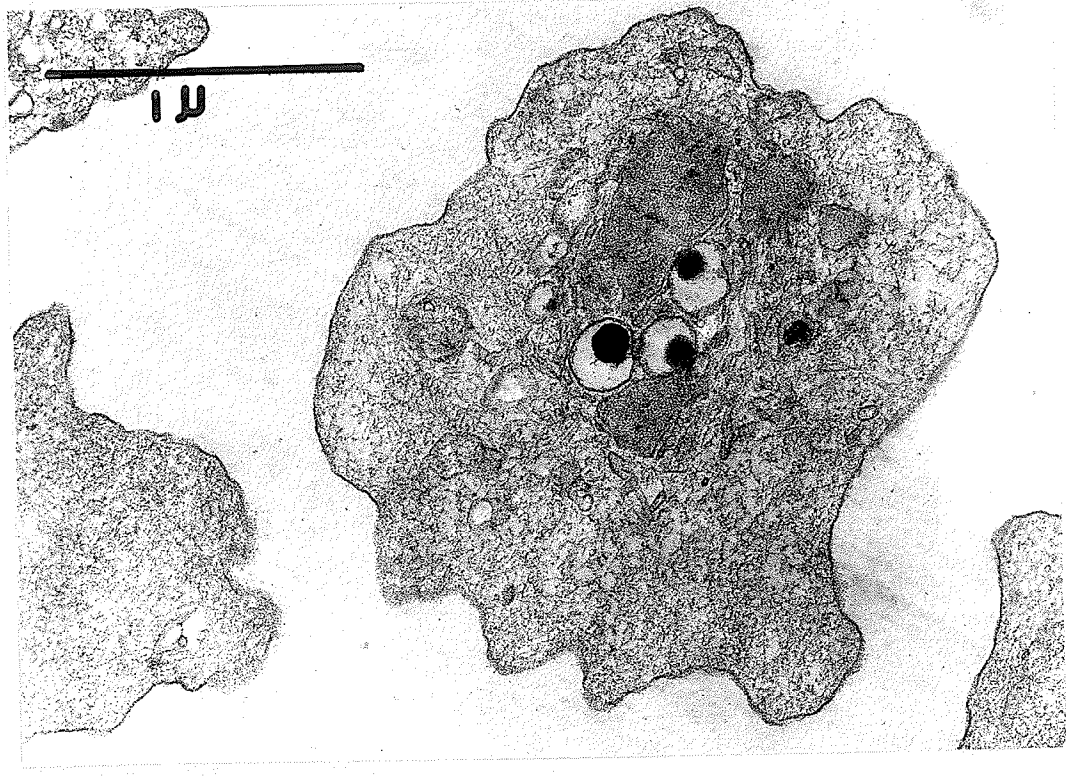
FIGURE 40

Electron micrograph of thin section of rabbit platelets after exposure to alpha lysin. There is disorganization of the cell structure and cellular debris.

Top: total magnification x 14,175

Bottom: total magnification x 42,000


1μ


1μ

hemolysin caused visible dermonecrosis 72 h after injection (Fig. 41). Beta hemolysin caused only a slight swelling and delta lysin was without effect. Similar effects were observed when the hemolysins were injected subcutaneously into rabbits (Fig. 42) except that the same concentration of alpha hemolysin consistently gave larger areas of dermonecrosis than in the guinea pig.

Lethality

The toxicity of gamma lysin was investigated by intravenous and intraperitoneal injection of mice. The gamma lysin as well as beta and delta lysins had no effect at 100 ug dosages. However, as expected, alpha lysin was lethal for mice, the LD_{50} being 0.68 ± 0.22 µg or 27-34 µg/kg of mouse (Fig. 43).

In contrast to the negative results obtained with mice, gamma lysin killed guinea pigs instantly when they were injected intracardially with 50 µg amounts. The same result was obtained with alpha lysin but beta and delta lysins had no effect by this route.

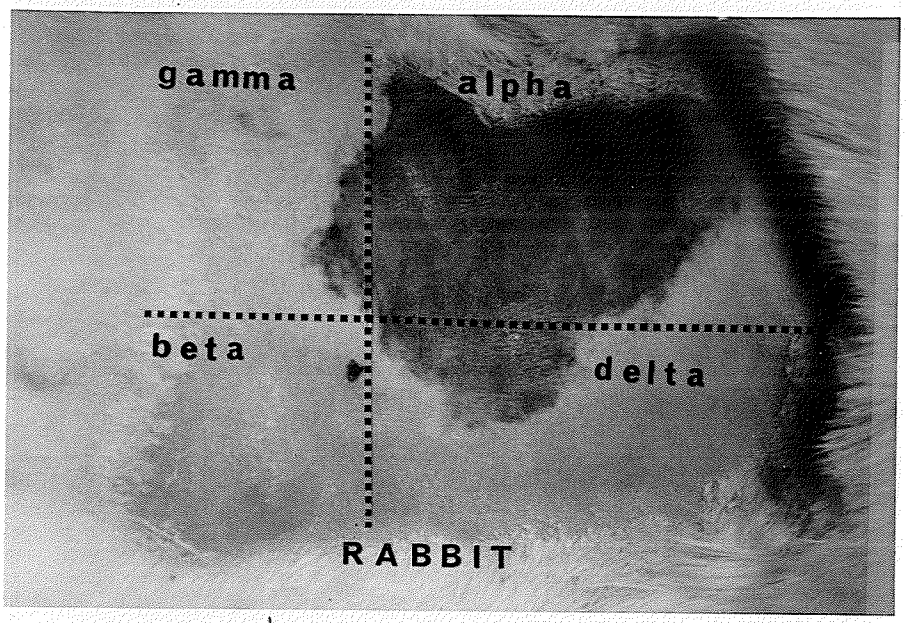
Autopsy findings obtained by Dr. H. Sayed of this Department were that the guinea pigs which received gamma lysin intracardially showed massive hemorrhage of the kidney and serosal surfaces of the intestines and frank lysis of red cells in the major veins and arteries. Histologically, there was evidence of congestion in the tissues.

FIGURE 41

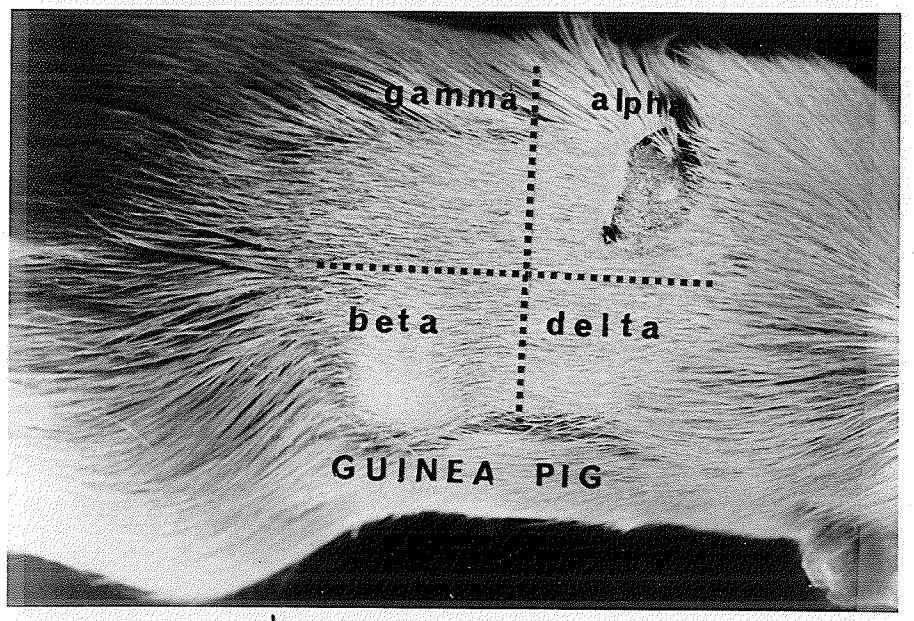
Subcutaneous injection of a rabbit with the purified staphylococcal hemolysins

FIGURE 42

Subcutaneous injection of a guinea pig with the purified staphylococcal hemolysins


gamma
alpha
beta
delta
RABBIT

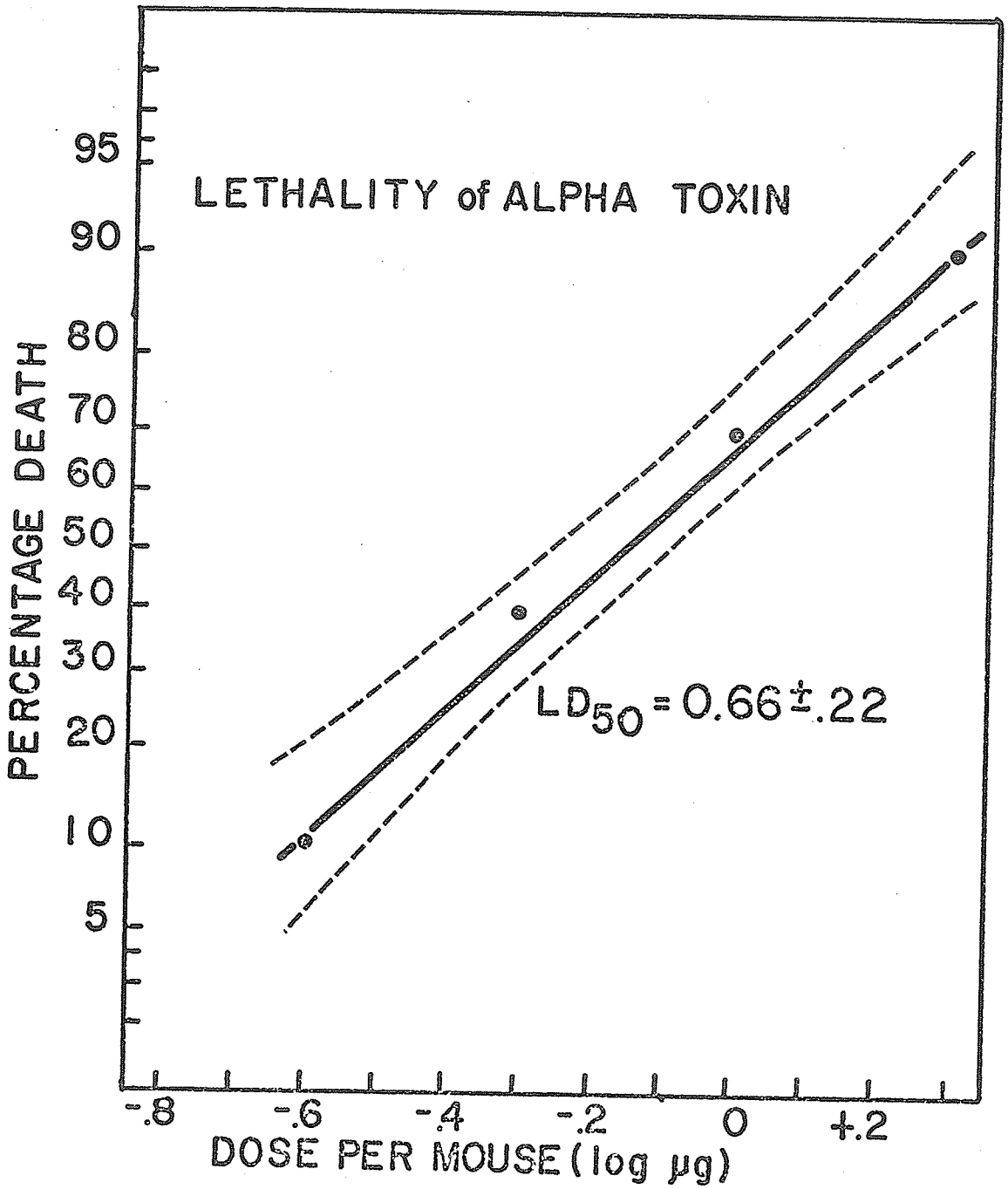
1


gamma
alpha
beta
delta
GUINEA PIG

1

FIGURE 43

Determination of the LD_{50} for alpha hemolysin injected intraperitoneally into Swiss white mice

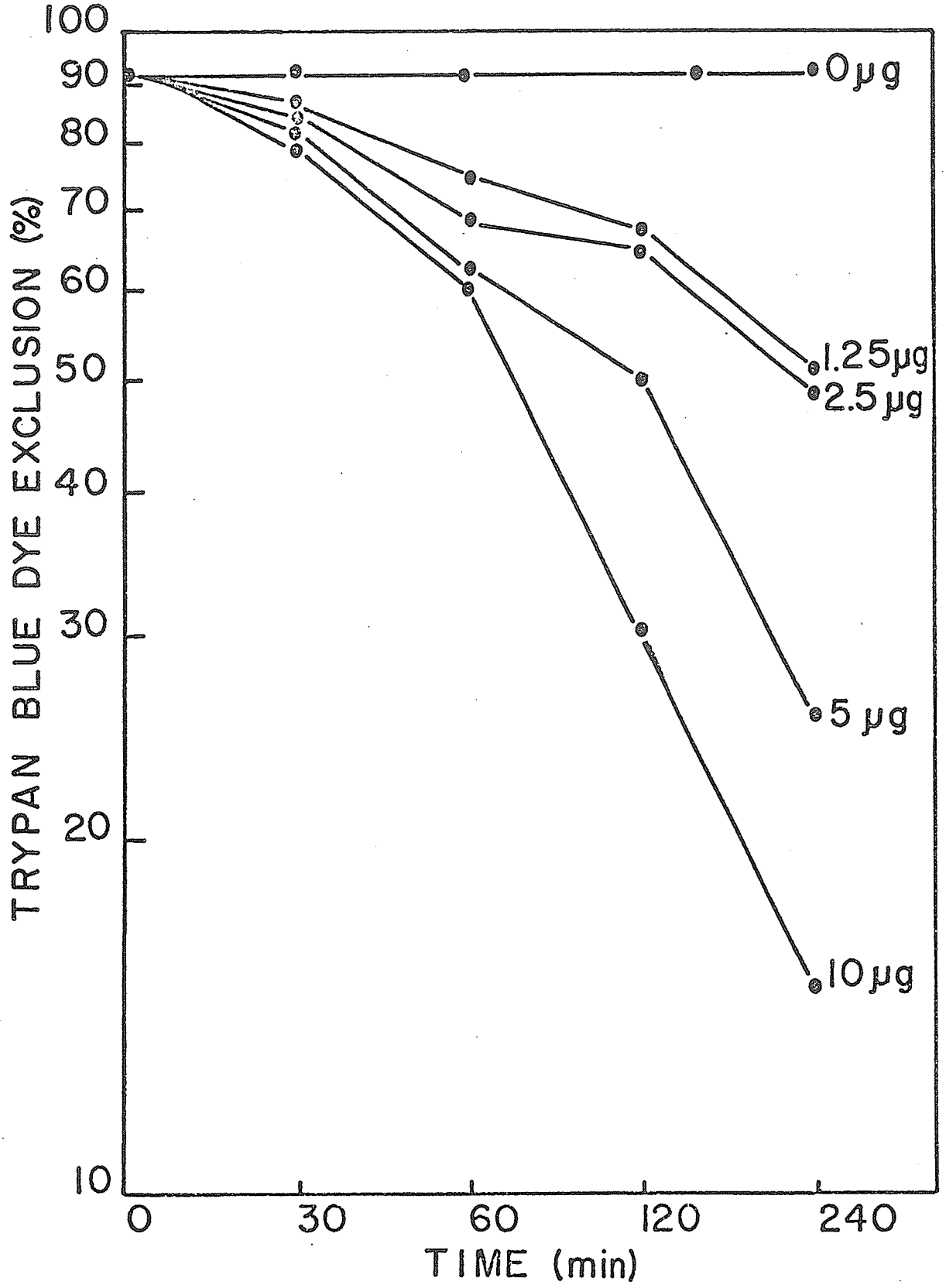

LETHALITY of ALPHA TOXIN
PERCENTAGE DEATH
95
90
80
70
60
50
40
30
20
10
5
$LD_{50} = 0.66 \pm .22$
-.8
-.6
-.4
-.2
0
+.2
DOSE PER MOUSE (log µg)

Tissue Culture

The effect of gamma lysin on tissue cultures was measured with stationary cultures of C-6 cells. Gamma hemolysin (0.5 ml) was added to 4.5 ml of cells and at various time intervals, 0.5 ml of the cells was removed and mixed with 0.2 ml of trypan blue. At least 700 cells per sample were counted and their ability to exclude trypan blue was recorded.(Fig. 44). About 93% of the untreated cells were capable of excluding trypan blue but after 4 h incubation with 10 µg/ml (700 HU/ml) of gamma lysin, only 15% of these cells still excluded the dye. As shown in the figure, higher concentrations of hemolysin increased the rate at which the C-6 cells were affected but calculation of the rate of trypan blue exclusion vs the hemolysin concentration showed that there was no simple linear correlation as that shown in Fig. 7 for human red cells.

FIGURE 44

Tissue culture: exclusion of trypan blue by C-6 cells incubated with various concentrations of purified gamma hemolysin.


100
90
80
70
60
50
40
30
20
10
TRYPAN BLUE DYE EXCLUSION (%)
0 µg
1.25 µg
2.5 µg
5 µg
10 µg
0
30
60
120
240
TIME (min)

CHAPTER 8

MECHANISM OF ACTION OF GAMMA HEMOLYSIN

Comparison of Gamma Lysin with Alpha Lysin

Wiseman and Caird (208,209) have shown that alpha lysin is secreted by *S. aureus* as a proenzyme which, after being activated by proteolytic enzymes in the red cell membrane, degrades membrane protein. Furthermore, they have shown (unpublished data) that activated alpha lysin hydrolyses toluene sulphonyl-L-arginine methyl ester (TAME). The author has not been able to "activate" gamma lysin by any of the methods of Wiseman and Caird, nor has he been able to hydrolyse TAME in the presence of this lysin (Fig. 45).

Comparison of Gamma Lysin with Beta Lysin

Doery *et al.* (48) and others (134,136,204-206) have demonstrated that beta lysin degrades sphingomyelin in red cell membranes. As shown by the author in Fig. 46, his preparation of beta lysin released acid-soluble phosphorus from sphingomyelin in contrast with gamma lysin which had no effect. The methods used in this experiment were those of Wiseman and Caird (206). Thin layer chromatography of beta lysin-treated sphingomyelin confirmed the absence of this phospholipid from the reaction mixture, but sphingomyelin was still present in the mixtures treated

FIGURE 45

Hydrolysis of TAME by purified alpha and gamma hemolysins (○)= " activated"alpha lysin, (●)= " activated" gamma lysin, (▼)= gamma lysin, (✗)= alpha lysin

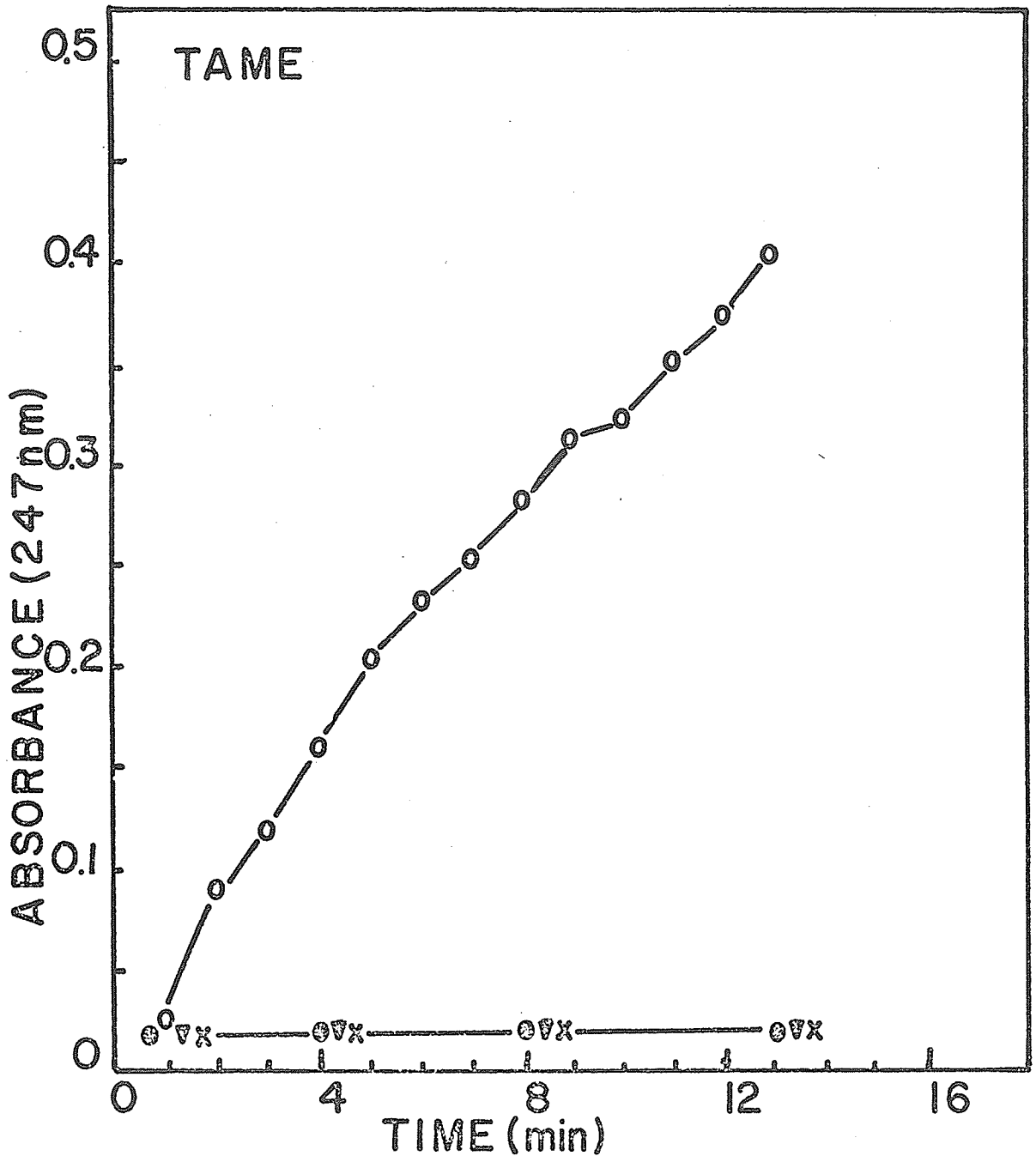
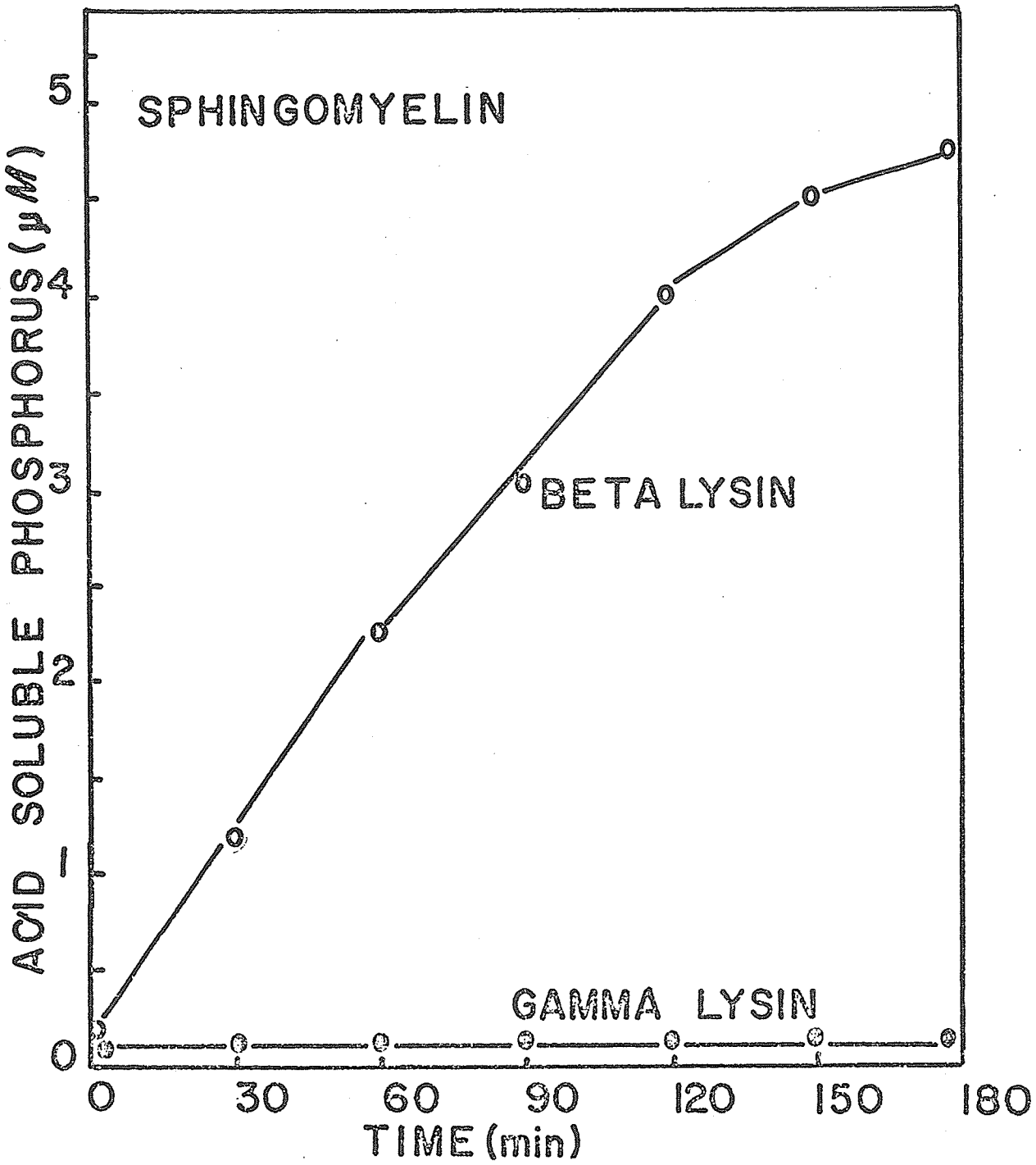

TAME
ABSORBANCE (247nm)
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
TIME (min)

FIGURE 46

Hydrolysis of sphingomyelin by purified beta and gamma hemolysins


SPHINGOMYELIN
BETA LYSIN
GAMMA LYSIN
ACID SOLUBLE PHOSPHORUS (μM)
0
1
2
3
4
5
0
30
60
90
120
150
180
TIME (min)

with gamma lysin.

Comparison of Gamma Lysin with Delta Lysin

According to Wiseman and Caird (31,207) delta lysin degrades phosphatidylinositol. The author used the methods of Wiseman and Caird and found that his purified delta lysin released acid-soluble phosphorus from phosphatidylinositol after 3 h incubation (Fig. 47). However, as shown in the figure gamma lysin had no effect.

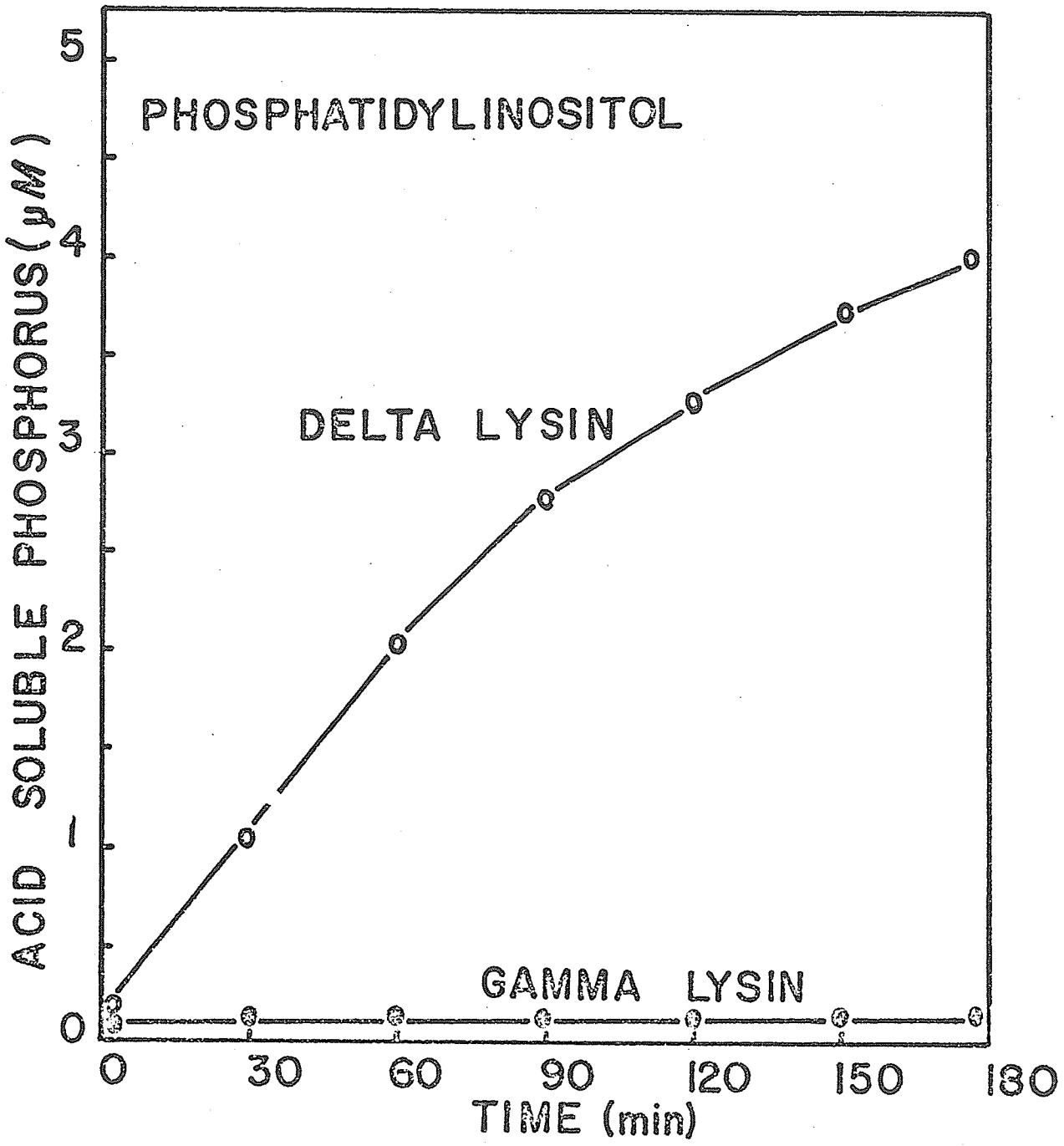
In summary, gamma lysin is without effect on any of the substrates that are associated with alpha, beta and delta hemolysins. These observations confirm that the gamma lysin is free of alpha, beta and delta lysins and that it is distinct from them.

Effect of Gamma Hemolysin on Human Erythrocyte Membranes

In this experiment, human erythrocyte membranes were isolated by the method of Dodge *et al.* (47). The membranes were added to gamma lysin such that the final concentration of membranes in terms of available nitrogen was 500 µg/ml while that of the hemolysin was 50 µg/ml or 6,000 HU/ml. The suspensions were incubated together at 37^{o}C for 3 h and the reaction was stopped by the addition of trichloroacetic acid. Control and test supernatants showed no difference in total carbohydrates, pentoses, deoxypentoses, reducing sugars, hexosamines, sialic acid or Lowry protein. Furthermore, there was no difference

FIGURE 47

Hydrolysis of phosphatidylinositol by purified delta and gamma hemolysins


PHOSPHATIDYLINOSITOL
DELTA LYSIN
GAMMA LYSIN
ACID SOLUBLE PHOSPHORUS (μM)
0
1
2
3
4
5
0
30
60
90
120
150
180
TIME (min)

in absorbance at 260 nm or 280 nm. However, nitrogen content of the gamma lysin supernatants after 3 h incubation increased 7% in relation to the control. In addition to this, the amount of acid-soluble phosphorus liberated by the hemolysin increased by 33%. These preliminary results indicated that phospholipids may be the substrate of gamma lysin but as shown previously, the substrates cannot be sphingomyelin or phosphatidylinositol.

Further experimental work investigated the rate of release of nitrogen and phosphorus from human erythrocyte membranes by gamma lysin. The experiment was performed as described above except that the samples for nitrogen and phosphorus analysis was taken over a time period of 0-180 minutes in the presence of twice the amount of substrate. The results, shown in Fig. 48, indicate that the rate of nitrogen released from the membranes by gamma hemolysin was linear over the 3 h period. However, the rate of release was not comparable to that obtained with pronase. Results obtained with phosphorus (Fig. 49) were similar to those for nitrogen in terms of linearity but its liberation was quantitively greater. Comparison of the figures shows that the rate of release of phosphorus was greater than that of nitrogen. Phospholipase C (Worthington) released a similar amount of phosphorus from erythrocyte membranes.

FIGURE 48

Release of nitrogen from human erythrocyte membranes during incubation with gamma hemolysin

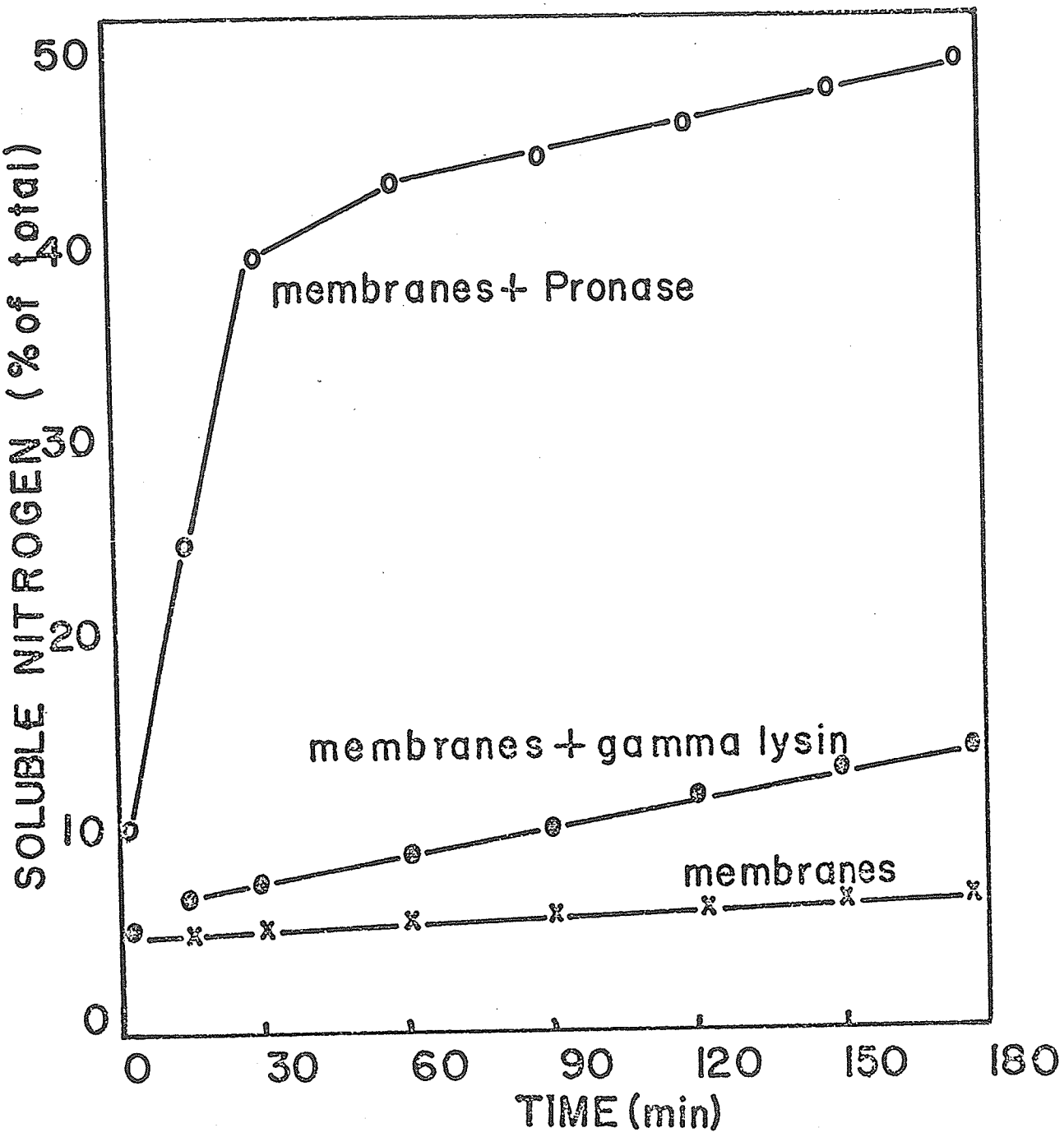
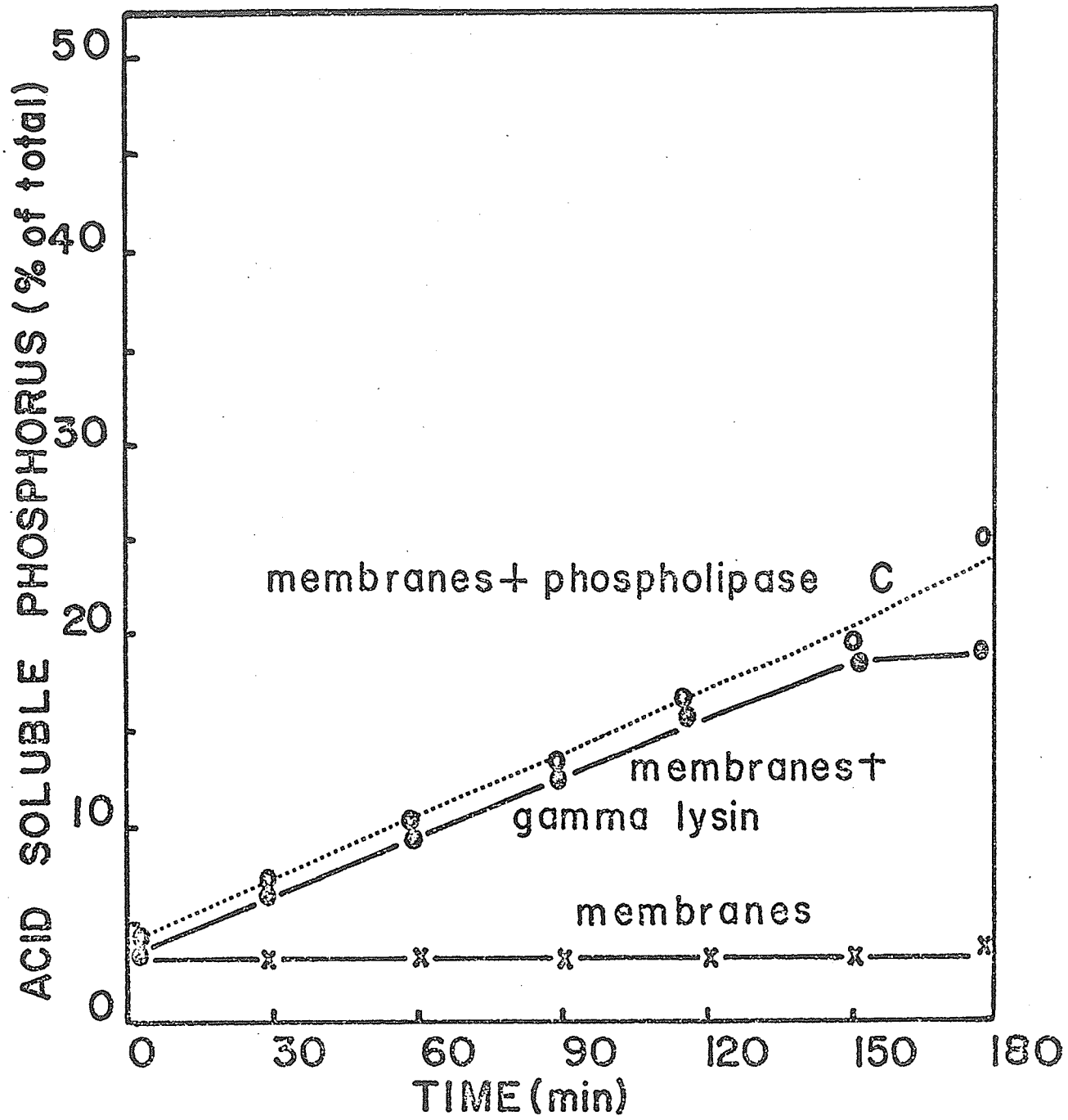

SOLUBLE NITROGEN (% of total)
50
40
30
20
10
0
membranes + Pronase
membranes + gamma lysin
membranes
0
30
60
90
120
150
180
TIME (min)

FIGURE 49

Release of phosphorus from human erythrocyte membranes during incubation with gamma hemolysin


ACID SOLUBLE PHOSPHORUS (% of total)
50
40
30
20
10
0
membranes + phospholipase C
membranes + gamma lysin
membranes
0
30
60
90
120
150
180
TIME (min)

The phospholipids of human erythrocyte membranes were extracted by the method Rose and Oklander (168) and washed with 1 *N* HCl to remove contamination by other materials. These crude phospholipids (1 mg/ml) were dialysed against Tris-saline buffer and then incubated with 10,000 HU/ml (0.12 mg/ml) of gamma lysin, but over a 3 h period, there was no detectable release of acid-soluble phosphorus. None of the phospholipids detected by thin layer chromatography disappeared after treatment with gamma lysin. Gamma lysin also failed to hydrolyse purified preparations of sphingomyelin and phosphatidyl-inositol, as noted earlier, nor did it have any effect upon phosphatidylserine, phosphatidylethanolamine or phosphatidylcholine.

Lipid-free proteins were prepared from human erythrocyte membranes by the method of Rosenburg and Guidotti (169). A suspension of protein (2% w/v) in phosphate buffered saline was incubated with 10,000 HU/ml (0.12 mg/ml) of gamma lysin, but no detectable acid-soluble nitrogen was released after 3 h incubation. No breakdown products were detected by N-terminal analysis, amino acid analysis or thin layer chromatography.

Inhibition of Hemolysis

A Lineweaver-Burk plot of 1/v vs 1/S for gamma lysin and human erythrocytes showed a straight line

relationship. Therefore any fractions of the erythrocytes that contained the substrate should competitively inhibit the rate of hemolysis. The velocity of lysis of human erythrocytes exposed to gamma lysin was measured with various concentrations of erythrocytes and then in the presence of either human erythrocyte membranes or erythrocyte "cytoplasm". The results (Fig. 50) indicated that erythrocyte cytoplasm (1 mg/ml) did not inhibit the rate at which human erythrocytes were lysed by gamma hemolysin. In contrast, hemolysis by gamma lysin was inhibited by as little as 20 μg N/ml of erythrocyte membrane. The common Y intercept for the different amounts of membrane used as an inhibitor indicates a competitive inhibition.

The experiment was repeated with different components of the erythrocyte membrane as inhibitor rather than the entire membrane. Crude phospholipids from human erythrocyte membranes exhibited a competitive inhibition whereas lipid-free membrane protein had no effect (Fig. 51).

FIGURE 50

Competitive inhibition of gamma hemolysin by human erythrocyte membranes and cytoplasms

(●) = gamma lysin + human erythrocytes, (0) = gamma lysin + human erythrocytes + membranes, (X) = gamma lysin + human erythrocytes + red cell cytoplasm

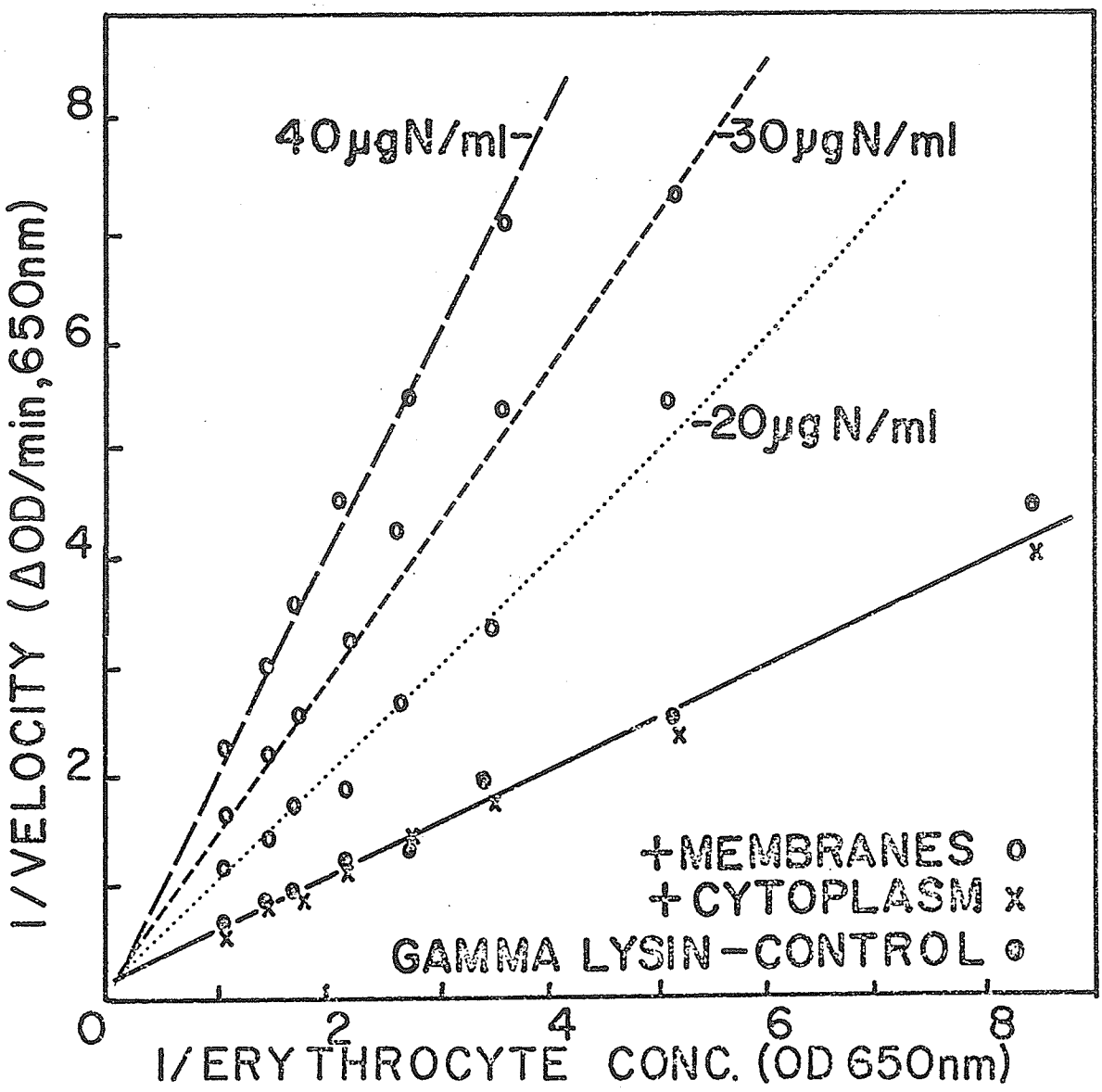

40µgN/ml
30µgN/ml
20µgN/ml
+MEMBRANES o
+CYTOPLASM x
GAMMA LYSIN-CONTROL o
1/VELOCITY (ΔOD/min,650nm)
1/ERYTHROCYTE CONC. (OD 650nm)
0
2
4
6
8

FIGURE 51

Lineweaver-Burk plot to demonstrate the competitive inhibition of gamma hemolysin by human erythocyte membrane phospholipids.

(●)= gamma lysin + human erythrocytes, (◎)= gamma lysin + human erythrocytes + phospholipids (x) = gamma lysin + human erythrocytes + membrane protein

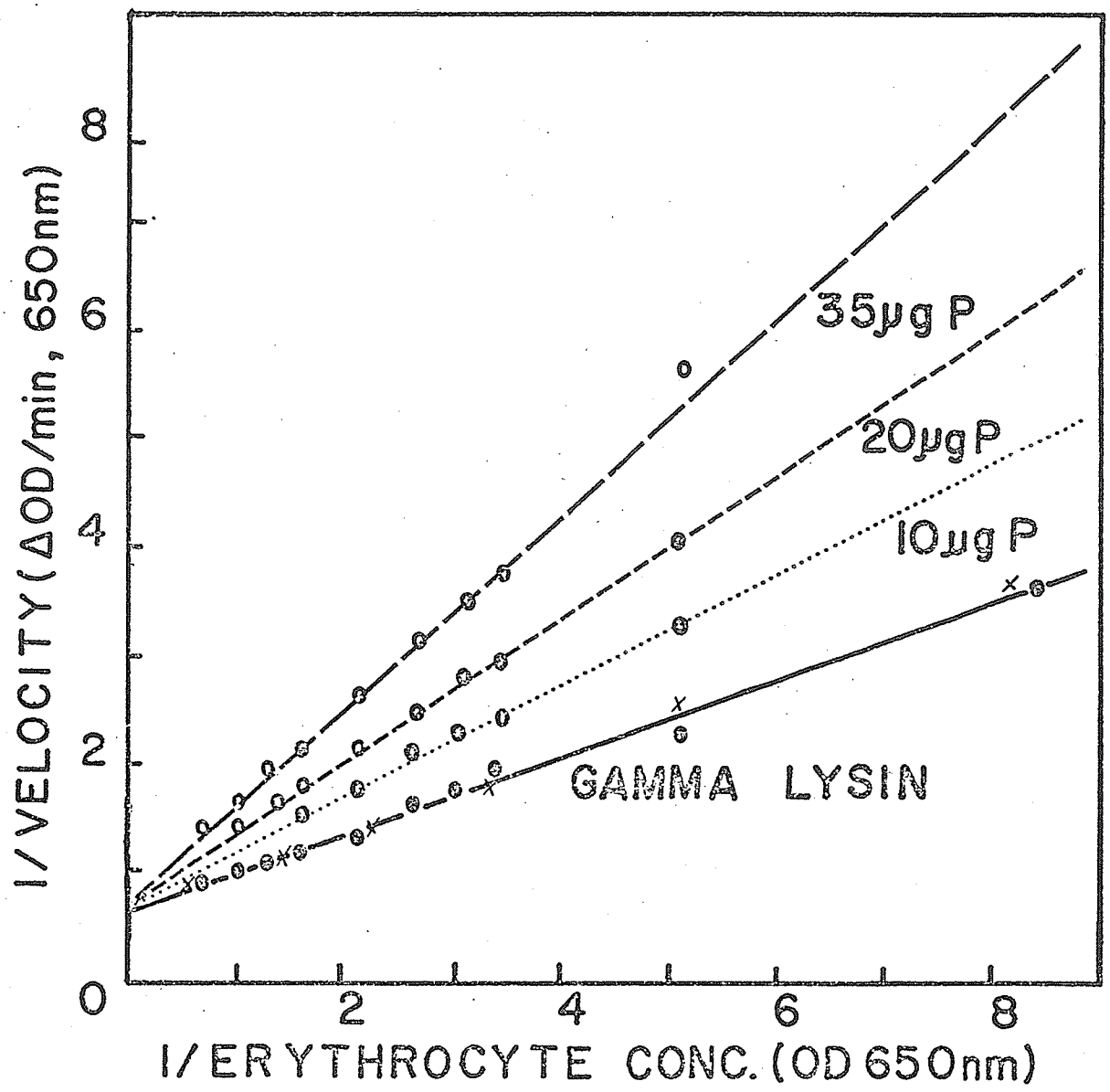

1/VELOCITY (ΔOD/min, 650nm)
1/ERYTHROCYTE CONC. (OD 650nm)
0
2
4
6
8
35μg P
20μg P
10μg P
GAMMA LYSIN

TABLE 21

SUMMARY OF PHYSICAL-CHEMICAL DATA FOR THE STAPHYLOCOCCAL HEMOLYSINS

PARAMETER	HEMOLYSIN			
	ALPHA	BETA	GAMMA	DELTA
Sedimentation constant, S_{20w}	1.4,3.0	1.8	2.6	2.8,9.8
Diffusion coefficient, D_{20w}	7.3×10^{-7}	*	13.9×10^{-7}	*
Extinction coefficient, 1% 280nm	13.56	4.24	28.35	29.08
Molecular weight gel filtration	45,000	26,000	45,000	200,000
Stokes radius, nm	2.7	2.1	2.7	3.0
Partial specific volume, ml/gm (ref 216)	.715	.711	.719	.729
Isoelectric point	8.5	9.5	6.0	9.6
N-terminal amino acid	histidine	*	methionine	proline

*not determined

DISCUSSION

This investigation has shown that the factors which influence the growth of strain Smith 5R of *S. aureus* are not necessarily those which mediate production of gamma hemolysin. The Smith 5R strain grew well over a pH range of 4.5 to 8.5 but hemolysin production was maximal at pH 7.0. Furthermore, a wide variety of carbohydrates was used successfully by the organism as an energy source for growth but gamma hemolysin was formed only on β-glycerophosphate and lactate. Finally, growth of the cells was greater in air than in carbon dioxide-air mixtures, but hemolysin production was enhanced if 10% carbon dioxide was present during incubation. A carbon dioxide requirement has been observed by Wiseman (202) for alpha and beta hemolysins and for beta hemolysin by Riaz-ul Haque (90). Arbuthnott (2) has also noted that alpha hemolysin titres are enhanced in the presence of carbon dioxide. It has been suggested by various investigators that the beneficial effect of carbon dioxide may be explained on the basis of its effect on pH, but in the case of gamma lysin production by strain Smith 5R, incubation of the cultures at pH levels ranging from 5.0 to 9.5 in the absence of carbon dioxide did not facilitate production of the hemolysin as would be expected if this were the correct explanation.

The present study has shown that gamma lysin is formed by the strain Smith 5R in the late log phase of growth and released into the medium. Although some lysin can be found intracellularly, the bulk of it is extracellular at a time when cell autolysis is not important. This raises the question of the hemolysin's function in the cells which produce it. Although this author has no data, Fritsche (73), in the only study of the problem, was unable to find the substrate of beta hemolysin in staphylococci even though it is known to be a sphingomyelinase. Fritsche concluded that the beta lysin was not involved in phospholipid metabolism of the cells but served rather as a means by which the cells released metabolites from host cell membranes which contained the substrate.

With regard to reaction kinetics, the author has shown that the activation energies of gamma lysin reacting with red cell membranes were 1,600 and 4,800 cal., which are within the range of 1,000-25,000 cal. observed for most enzyme-substrate interactions, by Sizer (174). In comparison, Wiseman (201) found that the activation energy for beta lysin reacting with sheep red cells was 14,100 cal., while that for delta lysin reacting with phosphatidylinositol was 18,750 cal. Dixon and Webb (46) believe that the activation energy is more characteristic of an enzyme than its substrate. In connection with the observed discontinuity of the Arrhenius plot

for the gamma lysin, this may be the result of reversible inactivation of the hemolysin (see 113). Kinetic studies have also shown that Na+ and K+ ions are required by the gamma lysin for optimal hemolysis. This suggests that these ions assist in the preservation of a critical structure in the red cell membrane by which the interaction with the hemolysin is facilitated. It is worth pointing out that while delta and alpha lysins require no known cations, Mg++ at a concentration of 10^{-3} *M* is required by beta lysin for optimal hemolysis of sensitive red cells (204).

In the course of this research, gamma lysin from strain Smith 5R was purified 2,700-fold with a 75% recovery. The purified hemolysin was homogeneous by the criteria used and its specific activity was 10^5 HU/mg protein, which compares very favourably with the gamma lysin preparation of Guyonnet and Plommet (85,86), the specific activity of which was 1,000-2,500 HU/mg protein. These authors (85,86) produced low titre gamma lysin in broth and purified it on hydroxylapatite to yield two synergistic hemolytic fractions both of which contained at least two other proteins. Purification of gamma lysin with the same strain of *S. aureus*, in our laboratory, did not confirm their observation and it is possible that their fractions were contaminated with delta and beta lysins which are known to act synergistically (55). More recently, Mollby and Wadstrom (149) have partially purified gamma

lysin from the Smith 5R strain by the techniques of ion exchange chromatography and isoelectric focusing. These workers (189) noted that separation according to charge and molecular size is not sufficient to obtain homogeneous proteins from *S. aureus* since many are basic proteins of the same molecular weight. Their 1972 comments contrast with their 1971 work in which they used such techniques in the isolation of gamma lysin. Regrettably, Wadstrom and Mollby (189), after emphasizing the importance of using highly purified hemolysin which fulfills several criteria of purity, reported on the biological activity of a gamma lysin preparation for which there was no evidence of homogeneity.

Physical-chemical data obtained by the author for the gamma hemolysin confirm that it is distinct from the alpha, beta and delta lysins and that it is a typical protein. The N-terminal amino acid of gamma lysin is methionine in contrast with histidine for alpha lysin (43,208) and proline for delta lysin (31). Others (92,104, 119) could not detect proline as a component of delta lysin but we found small amounts and it is conceivable that these workers failed to detect the amino acid because of the use of low concentrations of hemolysin in their analysis. We feel that the small amounts of proline present in our amino acid analysis of the delta lysin may indicate that proline is present only as the N-terminus.

Gamma lysin may be distinguished from the other hemolysins by its ultraviolet absorption spectrum, although it has no unexpected features. The sedimentation constant of gamma lysin is 2.6S and its molecular weight is about 45,000 daltons. These values are similar to data obtained for alpha toxin by Bernheimer and Schwartz (10) and by Arbuthnott (21) and various other workers (43,215), and it is doubtful if alpha and gamma lysins could be distinguished on the basis of their molecular weights and sedimentation constants. Contrasting with these values are a 1.8S molecule and a molecular weight of 26,000 daltons for beta lysin. The sedimentation constant of beta lysin is the same as that reported by Gow and Robinson (83) and the molecular weight is in the same range as that given by Chesbro and Kucic (35).

It should be mentioned that data presented in this thesis suggest that the 3S alpha hemolysin may be composed of smaller 1.4S components and it may be possible on this basis to differentiate alpha from gamma lysin. This observation provides support for the finding of Kumar *et al*. (121) that alpha lysin is a 1.4S molecule. Arbuthnott (2) and Forlani *et al*. (215) also stated that the 3S alpha hemolysin may breakdown to a 2.0S component. In this connection, we were unable to confirm that the 3S alpha hemolysin aggregates to 12S or 16S molecules as shown by others (2,3,10,215) although this failure is probably

the result of technical difficulties.

Turning to the isoelectric point (pI) of gamma lysin, we obtained a value of 6.0 which contrasts sharply with the results of Mollby and Wadstrom (149) who claimed that its pI was 9.5. It is difficult to get agreement about the isoelectric points of the hemolysins, an example of the problem being the range of values found for the delta hemolysin by various authors (105,119,189). Surprisingly our observed pI of 8.6 for alpha hemolysin agrees well with the value obtained by McNiven *et al.* (146) for $alpha_1$ and with that obtained for $Alpha_a$ by Wadstrom (188). In spite of this our opinion is that isoelectric points contribute little to the characterization of the hemolysins at present.

Amino acid analysis of the gamma lysin indicated that it contained a large number of residues of aspartic and glutamic acids, large amounts of ammonia, and that cysteine was absent or not detected. The alpha, beta and delta lysins also contain large numbers of aspartic and glutamic acid residues as well as ammonia although they are basic proteins, it seems, in contrast with the gamma lysin which is acidic. At first inspection the amino acid analysis contradicts this statement but whether a staphylococcal hemolysin is basic or acidic probably depends upon the number of asparagine and glutamine residues present. The beta and gamma lysin analyses are

the first to appear in print and unfortunately there are no other analyses with which to compare them. The analyses for delta lysin compare favourably with those given by others (92,105,119,214) in that arginine, proline, tyrosine and cysteine are either absent or present in low concentration. Also the same amino acids, lysine, isoleucine and aspartic acids are present in high concentrations. The amino acid analysis of alpha lysin also compares favourably with those of Coulter (43) and Bernheimer and Schwartz (10) although the analysis presented in this thesis shows a higher concentration of glycine.

In summary, reference to Table 21 indicates that the physical-chemical parameters of characterization of the gamma lysin are distinct from those of the alpha, beta and delta lysins. By and large, values obtained for the latter three hemolysins agree fairly well with other published data.

We have shown in this study that the gamma lysin is immunogenically distinct from the alpha, beta and delta lysins. It is now clear from our own work and the work of Mollby and Wadstrom (149) and Guyonnet and Plommet (85,86) that Elek (55) was incorrect in assuming that the gamma and delta lysins were identical. His claim was based on a study of specific neutralization by antisera of hemolytic patterns on blood agar. We now know that agar inhibits the activity of gamma lysin as shown in the

present investigation and also by Jackson (96) and Wadstrom and Mollby (189). The sample of highly purified delta lysin sent to us by Dr. Kreger elicited antibodies to alpha, delta and gamma lysins. Thus, the reported differences between the delta lysin preparations of Kreger and Wiseman are probably the result of contamination and interaction of the three hemolysins present in Kreger's material. Our finding also calls into question the validity of many of Kreger's analyses of his "purified" delta lysin. Indeed, Kreger's preparation is probably no better than that of Yoshida (214) which has been shown to be contaminated with beta lysin and ribonuclease (78).

There has been some controversy regarding the immunogenicity of delta lysin itself. We have shown in this thesis that antibodies to delta lysin produce a characteristic quantitative precipitin curve with purified hemolysin. Since the antibody preparation was purified gamma globulin, this curve cannot be attributed to non-specific precipitation and the neutralization experiments confirm that the antiserum to delta hemolysin was specific. A difficulty was the presence of non-specific serum inhibitors of delta lysin activity as shown by (31,76,105,119). It is believed that the inhibitors are β-lipoproteins (52,53,108). However, we removed these inhibitors from the antiserum to delta lysin by purifying the globulins and thus had no difficulty in demonstrating the lysin's immunogenicity.

Some of the biological properties of gamma lysin deserve comment. It has been possible to observe directly the hemolytic spectra of all four hemolysins of *S. aureus*. Nevertheless, these results must be interpreted with caution, since we have shown that a five-fold difference or more in sensitivity of different samples of human erythrocytes to gamma lysin can occur, and that this variability is greater than that between red cells of different species. Cooper *et al*. (41) have reported an eight-fold variation in the sensitivity of rabbit red cells to alpha hemolysin and Bernheimer (7) believes that the difference in sensitivity of rabbit and human erythrocytes to alpha hemolysin may vary 15-150 fold. However, information concerning hemolytic spectra are of some value if an attempt is made to relate them to the substrate (206-208).

Studies of the toxicity of gamma lysin for laboratory animals showed that where 1 µg of alpha hemolysin caused dermonecrosis in the skin of rabbits, 100 times this amount of beta, gamma or delta lysin had no effect. Kreger *et al*. (119) claimed that delta lysin was dermonecrotic for rabbits, but as we have already pointed out, their preparation was contaminated with alpha lysin. Since various investigators have used a wide range of concentrations of lysins in attempting to demonstrate either necrosis or lethal effects, it is difficult to make cross comparisons of data. We found, as with dermonecrosis, that 0.5 µg

of alpha hemolysin kills mice but that 100 ug of beta, gamma and delta lysins has no effect. Calculations based on the data of Kreger *et al.* (119) give an LD_{50} of 2.0 mg delta lysin for mice and 7.17 mg for guinea pigs. These high doses are quire compatible with contamination by a microgram or less of alpha lysin. Wadstrom and Mollby (189) claim an LD_{50} dose of 125 mg for delta lysin in mice and that doses of less than a milligram of gamma lysin are lethal for these animals. Here again, the dosages are meaningless without the presentation of additional evidence. Contamination of these preparations with alpha hemolysin at concentrations of less than 1 part in 2,000 would give the same results and this amount of contamination might be difficult to detect. If mice can be protected with a specific antibody and not by anti-alpha hemolysin, then the assertion that large amounts of the lysin are lethal (or dermonecrotic) is valid. A further point that must be made in connection with toxicity has to do with the variations in susceptibility of different animal species with regard to a given hemolysin. According to Van Heyningen (186), the guinea pig is 1,000-fold more susceptible to dysentery neurotoxin than the mouse. Arbuthnott (2) states that all small animals are susceptible to alpha hemolysin but there is a difference of about 200-fold in the LD_{50} dose for rabbits and chickens. By comparison, then, it is not surprising that guinea pigs

in our experience succumbed to a dose of gamma lysin which had no effect on mice. In spite of the pitfalls in interpretation of the LD_{50} doses, it is interesting to note that the LD_{50} of alpha hemolysin determined by Bernheimer and Schwartz (10) and Lominski *et al.* (127) is very close to the LD_{50} dose we obtained in the present work.

Unfortunately, it has not been possible to elucidate the precise mode of action of the gamma lysin. However, kinetics of hemolysis are compatible with a catalytic reaction and since the gamma lysin is a highly purified protein, it is likely in our view to be an enzyme. Liberation of nitrogen and phosphorus from gamma lysin-treated red cell membranes suggested that the hemolysin is a phospholipase and these data are corroborated by the fact that membrane phospholipids competitively inhibit hemolysis but membrane proteins do not. Against this observation is the fact that none of the extracted or purified phospholipids was attacked by the gamma lysin. Nevertheless, if a phospholipid were the substrate of the hemolysin, the extraction procedure may have denatured the substrate so that it cannot be hydrolysed. For example, without special precautions, lysophospholipids from membranes are degraded to phospholipids non-enzymatically during the extractions (1). Another possibility is that conformation of the phospholipid molecule changes during extraction rendering it insusceptible to the action of gamma lysin. It may be

that indirect demonstrations of the gamma lysin's mode of action will be required. Analysis of erythrocyte membrane components before and after treatment with gamma lysin by polyacrylamide gel electrophoresis may be helpful. It may be possible to demonstrate that pre-treatment of red cell membranes with gamma lysin destroys the substrate required for another enzyme, but such an approach even at its best is tenuous.

SUMMARY

The conclusions derived from this research are the following:

1. Gamma hemolysin from the Smith 5R strain of *S. aureus* was best produced by the Birch-Herschfeld (19) technique with Dolman-Wilson agar at pH 7.0. Maximal yields were recovered after 24 h incubation at 37°C in 10% carbon dioxide and air.
2. Hemolysis occurred optimally at pH 7.0 in phosphate buffered saline at 37°C with a 1% suspension of erythrocytes.
3. Gamma lysin required Na+ or K+ and hemolysis was inhibited by EDTA.
4. Gamma hemolysin was purified by ultrafiltration, gel filtration, two ammonium sulphate fractionations and NaCl extraction.
5. A new technique, developed for purification of alpha hemolysin, involved methanol precipitation, gel filtration, two ammonium sulphate fractionations and ion exchange chromatography on carboxymethyl cellulose.
6. The gamma hemolysin was shown to be a protein and was homogeneous when subjected to isoelectric focusing, disc gel electrophoresis and immunodiffusion. One peak of 2.6S was observed in the analytical ultracentrifuge and methionine was identified as the only N-terminal amino acid.
7. The absorption spectra of gamma lysin were characteristic

of a protein and the 1% extinction coefficient was 28.35. The lysin had an amino acid composition distinctive from the alpha, beta and delta lysins and a molecular weight of about 45,000 daltons.

8. Gamma lysin was differentiated from the other hemolysins by quantitative precipitin tests, immunodiffusion, immunoelectrophoresis and neutralization tests.

9. Evidence was presented to show that delta lysin was immunogenic and that the delta lysin of Kreger formed a line of identity with that of Wiseman in immunodiffusion but Kreger's preparation also contained alpha and gamma lysins.

10. The hemolytic spectra of the four hemolysins were compared directly. Rabbit red cells were most sensitive to alpha and gamma hemolysins but beta and delta hemolysins preferentially lysed sheep and human erythrocytes.

11. Investigation of the biological properties of gamma lysin showed that it attacked human and rabbit platelets, human leucocytes and C-6 cells in tissue culture. Intravenous injection of guinea pigs and mice with 50 µg of gamma lysin killed the guinea pigs but had no effect on the mice, nor was it dermonecrotic for rabbits or guinea pigs.

12. Gamma lysin released acid-soluble nitrogen and phosphorus from red cell membranes. Phospholipids derived from membranes competitively inhibited hemolysis by gamma

lysin but the phospholipids were not hydrolysed. In this connection, sphingomyelin and phosphatidylinositol were not attacked by the gamma lysin confirming that it was free of beta and delta lysins.

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