THE HAEMOLYSINS OF *CHIRONEX FLECKERI* AND *CHIROPSALMUS QUADRIGATUS*

THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Submitted by

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S U M M A R Y

The modern concepts concerning the structure and metabolism of the red cell membrane have been reviewed, and the function of the membrane in relation to the transport of water, anions and cations have been discussed.

In this survey only those agents which produce haemolysis *in vitro* have been included. Such agents act directly on the red cell membrane and cause an increase in permeability and an osmotic imbalance between the cell and the surrounding medium, leading to rupture and release of haemoglobin.

Substances producing haemolysis *in vitro* are of diverse origins and compositions. They have been described under the categories of physical, chemical, biological and immunological agents. Occasionally these agents have physical and chemical properties which explain their mode of action; such as enzymes or chemical agents reacting with membrane constituents; or they have surface properties which allow them to penetrate and disorganize membrane structure. A number of model systems have been described which might be used to investigate properties of haemolysins and their mode of action.

The haemolysin of the box jellyfish *Chironex fleckeri* was initially examined using aqueous extracts of the tentacle. The haemolysin is a protein which was unstable in dilute solutions at room temperature. However, the tentacles and their extracts could be stored at temperatures of -20deg. or below for long periods without loss of activity.

Exclusion chromatography was used to separate tentacle extracts into two fractions, one which was

haemolytic (molecular weight, 70,000) and the other with lethal activity (molecular weight, 150,000). When administered parenterally the haemolytic fraction was also lethal producing cardio-respiratory failure. The skin necrotizing activity of the whole tentacle extract appeared to be confined to the haemolytic fraction.

Although the tentacle extracts contained a potent haemolysin, no clinical evidence of intravascular haemolysis has been described in human envenomation. This was considered to be due to the presence of inhibitory substances in the plasma and the instability of the lysin at normal body temperature.

The properties of the haemolysins of C. fleckeri and Chiropsalmus quadrigatus were compared. Although they were both proteins with a molecular weight of 70,000 approximately, they could be distinguished from each other by the relatively greater stability of C. fleckeri lysin at temperatures above 5 deg. There was evidence also that the mode of action of the two lysins was different. Antisera prepared against both extracts showed that the antihaemolysins were species specific and no common antigenic properties were detected.

The mode of action of the haemolysin of C. fleckeri was examined. It was not an enzyme and it did not appear to have any unusual surface properties to explain its cytolytic action. The haemolysin showed no interactions with monolayers of individual components of the red cell membrane using surface pressure and surface potential measurements. However, there appeared to be specific complexing with a component in a mixed monolayer derived from red cell lipids. This might explain the mode of action of the haemolysin, but this observation would need further verification in monolayers prepared from individual membrane lipids.

DECLARATION

The studies described in the experimental section of this thesis have been reported in several papers (see below). All the research has been carried out under my direction, and I have been responsible for the preparation of the manuscripts and the work reported in those articles which bear my name alone or in which I appear as the principal author. In those papers where I appear as the minor author with H. D. Crone, he has been responsible for the biochemical aspects of the work and I have been responsible for the toxicological investigations. All the instrumentation and techniques developed for the investigations on monolayers have been my responsibility.

I declare that the thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and that to the best of my knowledge and belief the thesis contains no material previously published or written by any other person, except when due reference is made in the text of the thesis.

THOMAS E. B. KEEN

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A. LITERATURE SURVEY

1. THE RED CELL MEMBRANE

1.1 General Structure and Function

Modern concepts of the structure of the red cell membrane are extremely tentative and are based on the morphology observed on electron microscopy and the known physical and chemical properties of the membrane. On electron microscopy, the membrane appears to have a granular surface with plaques 200 Å in diameter and 30 Å deep between which are crypts suggesting membrane pores (Hillier and Hoffman, 1953). The plaques are considered to consist of a lipoprotein-carbohydrate complex and contain the blood group substances. They can be removed from the red cell membrane with ether which dissolves the lipid and releases the lipoprotein-carbohydrate complex from an inner fibrous layer of protein called "S" protein (Moskowitz and Calvin, 1952; Firken and Wiley, 1966).

The red cell membrane possesses the property of being permeable to water, and to a less extent to anions; selectively permeable to cations, and impermeable to haemoglobin. Davson and Danielli (1943) postulated that the red cell possessed positively charged pores which repelled cations, but allowed the passage of anions and water.

In order to correlate these properties into a single concept, it has been suggested that the red cell surface consists of a mucoprotein containing sialic acid which gives the cell its negative surface charge. Under this layer are located the plaques of the carbohydrate-

lipoprotein complex which are linked through calcium ions with bimolecular leaflets of phospholipid. The membrane pores are then depicted as structures possibly protein lined and with a positive charge (Whittam, 1964).

Although numerous models have been suggested (Lucy, 1968), the structure of the membrane is still in doubt and there is a view that the constituents are in a dynamic rather than a static state. The membranes are conceived as planar aggregates of micelles, which are neither precisely constant nor specifically fixed in a pattern. The rapid turnover of membrane constituents is possibly consistent with this idea (Dawben, 1969).

1.2. Red Cell Lipids

Lipids account for 25 to 50 per cent of the dry weight of the membrane preparation, and the membrane accounts for 95 to 100 per cent of the lipid of the cell. In human red cells, the lipids consist of 65 per cent phospholipid, 23 per cent cholesterol and 2 per cent cholesterol esters, glycerides and free fatty acids. The remaining 10 per cent are probably glycolipids. The phospholipids consist of lecithin, phosphatidyl ethanolamine, phosphatidylserine, sphingomyelin and lysolecithin.

The composition of the phospholipids in the membrane vary according to the species (Rouser, Nelson, Fleischer and Simon, 1968). The phospholipid content of rabbit cells on which most of our experiments are reported subsequently is similar to that of human red cells. In human and rabbit red cell ghosts, the total lipid represents 25 to 28 per cent of the extracted material. In human cells the percentage content of lecithin; sphingomyelin and lysophosphatides: cephalins was 39, 37 and 24; and in rabbits it was 44, 29, 27 (de Gier and Van Deenen, 1961).

Alterations in diet will also cause changes in the fatty acid residues although it does not cause changes in the major classes of phospholipid.

1.3 Red Cell Carbohydrates

Carbohydrates represent about 10 per cent of the composition of the red cell membrane although the gross composition depends on the method of isolation. It is contained in the mucoprotein which lines the outside of the red cell membrane and in glycoproteins (Zahler, 1969). The terminal sialic acid bonds in the surface mucoprotein are responsible for the negative charge on the cell. (Eylar, Madoff, Brody and Oncley, 1962). This charge may be reduced or abolished by treatment with neuraminidase which splits terminal sialic acid bonds (Cook, Heard and Seaman, 1961; Seaman and Heard, 1961). Trypsin may also produce a similar change (Cook, 1962).

1.4 Red Cell Protein

Although the major proportion of the red cell membrane is protein (45 - 55 per cent), difficulties have been encountered in clarifying the protein chemistry due to contamination with haemoglobin.

Moskowitz and Calvin (1952) have provided the most comprehensive information by washing freeze-dried red cell ghosts with buffer at pH9 until the supernatant was free of haemoglobin. The residue was called "stromin" which was extracted with ether removing the cholesterol and half the cephalin. Stromin could be divided into two fractions, "elinin" which was soluble at pH9 and "stromatin" which was insoluble. By lowering the pH to 6.4 the "S" protein was precipitated from the "elinin". The relation of these structures has been described previously.

Solubilized lipoproteins have been isolated from erythrocyte ghosts treated with n-butanol (Maddy, 1966). However, the various fractions which have been isolated are heterogeneous in nature and can be shown to have different sedimentation characteristics on ultra-centrifugation, different amino-acid compositions, and different properties on electrophoresis (Rega, Weed, Reed, Berg and Rothstein, 1967).

Treatment of the erythrocyte ghosts with hypotonic buffers extracts aldolase, glyceraldehyde phosphate dehydrogenase, carbonic anhydrase and adenosine deaminase without damaging the structure of the membrane. In hypertonic buffers with salt concentrations in the region of 0.2 to 0.4 molar, portion of the ghost cell protein, stromal lipids and acetylcholinesterase may be extracted. This solubilization of stromal proteins by concentrated salt solutions suggests that electrostatic protein-protein interactions are important in maintenance of the membrane structure (Dowben, 1969).

1.5 Red Cell Metabolism

Energy is required to preserve the morphology of the red cell and this is derived from the metabolism of glucose.

The red cell contains no stores of glycogen and glucose is obtained from the plasma by transport across the red cell membrane. The glucose metabolism serves two main purposes, firstly the maintenance of oxidation reduction homeostasis and secondly the generation of adenosine triphosphate (ATP).

There are two principle pathways for the glucose metabolism, either through the "anaerobic" Embden-Meyerhof pathway, which accounts for approximately 90% of the glucose, or through the pentose phosphate shunt (Grimes, 1965).

In order to maintain normal oxidation-reduction in the cell requires the action of both pathways. The Embden-Meyerhof pathway is necessary for the reduction of methaemoglobin (Fe^{+++}) and the maintenance of normal haemoglobin (Fe^{++}) in the functional state, while the pentose phosphate shunt is responsible for the maintenance of reduced glutathione and membrane sulphydryl groups. Defects on the pentose phosphate pathway which include glucose -6- phosphate deyhdrogenase deficiency, give rise to haemolytic states when exposed to oxidant stress (Carson and Tarlov, 1962).

The Embden-Meyerhof pathway provides ATP which is the source of energy for maintaining the sodium-potassium ATPase mechanism which is believed to be essential for regulation of the sodium pump and red cell volume. (Whittam and Wheeler, 1970).

2. TRANSPORT FUNCTIONS OF THE MEMBRANE

2.1. Water

Water crosses the red cell membrane with extreme ease (Passow, 1964). Polar compounds are considered to enter the cell through water filled channels or pores. Evidence for the existence of these pores is supplied by measurement of the permeability of the cell to water under simple diffusion, or under an osmotic pressure gradient using tritiated water. The water moves twice as readily under an osmotic pressure gradient and, therefore, there appears to be a bulk flow in addition to diffusion in water movement. (Sidel and Solomon, 1957). From permeability coefficients,the pore radius in human red cells has been estimated to be 3.5 - 4.2 Å (Goldstein and Solomon, 1960).

There is a relatively high concentration of impermeable molecules in the red cell. They constitute a total of 10 milliosmols consisting of haemoglobin at a concentration of 5 mM, together with other substances such as glycolytic intermediates and glutathione. The cell copes with this inequality by pumping cations, particularly sodium, from the cell (Dunham and Glynn, 1961).

If an impermeable substance is added to the medium outside the cell, the osmotic asymmetry created by the impermeable molecules may be counterbalanced. In this situation the sodium pump mechanism may fail but swelling and lysis will not take place (Jandl, 1965).

The response to hypotonicity results in a spherical change in the red cell, but rupture does not occur until the red cell volume is approximately 160% of the original.

The behaviour of the cell in hypotonic solutions has been extensively studied and the volume changes observed are less than those expected according to the van't Hoff *equation. In order to account for these findings, it was suggested that a proportion of the water around the haemoglobin molecule was in the bound state (Perutz, 1946). However, there were some inconsistencies in this theory as the bound water served as a solvent for glucose and other non-ionic substances, and it was difficult to know why ionic materials only were excluded. It has been suggested that the net charge on the haemoglobin molecule varies with its concentration, and the resultant movement of cations resultant from the changing charge is responsible for this behaviour (Gary-Bobo and Solomon, 1968).

*The van't Hoff osmotic equation - $P \propto \frac{n}{v} T$ where P is the osmotic pressure in atmospheres, n is the number of moles of solute present in v litres, and T is the temperature in degrees K.

2.2. Anions

The principal diffusible anions are chloride, bicarbonate and phosphate ions. Both the chloride and bicarbonate anions move across the membrane readily although not as fast as water (Passow, 1964). Tannic acid may slow chloride ion penetration greatly at concentrations which would only cover a portion of the membrane. This suggests that only a small proportion of the pores are available for chloride ion transport. (Tosteson, 1959).

The rate of diffusion of phosphate ions is relatively slower, and the movements are associated with the metabolism of the cell (Whittam, 1964).

2.3. Cations, Na^+ and K^+

Any population of red cells consist of a heterogeneous group composed of cells of varying ages. As the age of the cell is an important factor in determining its metabolism and composition, results on cation movements must be interpreted in the light of this knowledge. (Allison and Burn, 1955; Sass, Levy and Walter, 1963).

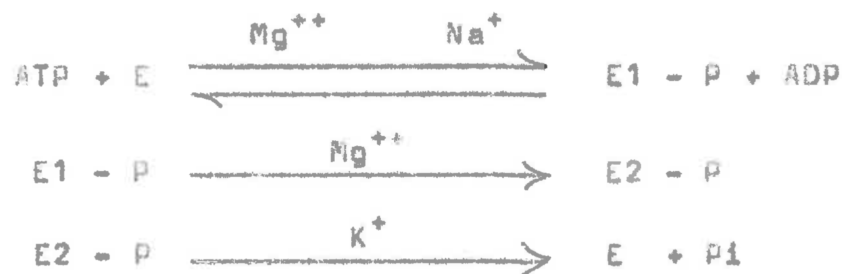
The monovalent cations K^+ and Na^+ movements are mediated by two main mechanisms, one associated with the sodium pump and the other independent of this system.

The sodium pump extrudes Na^+ from the cell and this is coupled with the uptake of K^+ from the medium in a manner such that approximately 3 Na^+ are exchanged for 2 K^+, with one Cl^- leaving the cell to maintain electric neutrality. Thus constant cation concentrations are maintained in the cell, and any leakage of cations down their concentration gradients is offset (Dunham and Glynn, 1961). The overall effect is to reduce the number of osmotically active particles in the cell, and prevents swelling due to entry of excessive Na^+.

The energy necessary to transport sodium ions against the diffusion gradient is provided by ATP. (Whittam and Wheeler, 1970). Adenosine triphosphatase (ATPase) catalyses the breakdown of the ATP and is found in the membrane. The presence of both Na^+ and K^+ are required for full activity, Mg^{++} are also necessary and the reaction may be inhibited by Ca^{++} (Dunham and Glynn, 1961).

It is significant that species, such as the cat and dog which have erythrocytes with a low K^+ and high Na^+ level, also exhibit a low level of sodium-potassium activated ATPase in the cell. (Bonting, Simon and Hawkins, 1961).

The sequence of reactions for the ATPase reaction has been represented as follows: (Whittam and Wheeler, 1970).



E1 - P and E2 - P represent two different forms of intermediates. Evidence for the presence of a phosphorylated intermediate has been demonstrated for red cells (Bader, Post and Bond, 1968).

Low concentration of cardiac glycosides, such as ouabain, specifically effect the sodium-potassium activated ATPase and inhibit Na^+ transport (Whittam, 1958). ~~However, another type of carrier system can be demonstrated~~ which is not inhibited by ouabain (Lubowitz and Whittam, 1969). ATPase activity may also be inhibited by a number

of sulphydryl reagents, such as p-chloromercurbenzoate, N-ethylmaleimide and 2 - 4 nitrofluorobenzine. Both the ouabain-sensitive ATPase and the ouabain insensitive carrier system are inhibited by ethacrynic acid.

Pathways exist for the passive diffusion of cations which are independent of the sodium pump. These are examined after inhibiting the known transport mechanisms in the membrane. Information about the penetration pathways are indefinite at the present time (Passow and Schnell, 1969).

2.4 Cations Ca^{++}

The divalent cation Ca^{++} is a normal constituent of the red cell membrane, but it penetrates the membrane with great difficulty. It may enter the cell more readily when it is depleted of metabolites, and some evidence has been presented to suggest the existence of a calcium pump (Schatzmann and Vincenzi, 1969). The process is an outward extrusion of calcium so that free ionic intracellular calcium is maintained at a lower level than the extracellular concentration. The pump is independent of the sodium pump and is unaffected by Na^+, K^+ or ouabain.

2.5 Non-Electrolytes

Non-electrolytes fall into two groups depending on whether the diffusion is simple or facilitated. In the case of simple diffusion, lipid solubility is a large factor in enhancing penetration (Jacobs, 1932). Non-polar compounds appear to pass through the lipid regions of the membrane while polar compounds pass through the water-filled channels or pores.

The transport of glucose is facilitated by a carrier system. The rate of penetration of glucose does not increase with increasing concentration in a linear manner, but it becomes progressively less as saturation of the carrier system is reached. (Le Fevre and McGinnis, 1960).

There is a marked species difference in the extent of penetration of glycerol which has been used for the storage of blood at low temperatures. The cells of man, rat, mouse, rabbit and guinea pig have a high permeability while the pig, cat, dog, ox and sheep have a low permeability (Whittam, 1964). There is some evidence for the penetration of glycerol occurring through pores as the rate is markedly affected by small concentrations of tannic acid. On the other hand, the rate of penetration is also influenced by the presence of sulphydryl inhibitors, such as copper, which may suggest a carrier system to facilitate transport.

3. HAEMOLYSIS

3.1. Haemolysis - Definitions

The term haemolysis is applied in two ways, firstly, to describe the dissolution of the membrane of the normal red cell with the liberation of haemoglobin, and secondly, to describe the shortened life span of the red cell in the vascular system of the body. The first refers to haemolysis *in vitro* while the second refers to haemolysis *in vivo*. The latter may be due to a large variety of causes, inherent or acquired, which affect the metabolism of red cells and their membranes leading to premature disruption in the body (Carson and Tarlov, 1962). The

two types of haemolysis do not commonly occur together except when a haemolytic agent is in sufficient concentration in the circulation to produce intravascular haemolysis.

The subsequent discussion refers to haemolysis *in vitro* only.

3.2. Haemolysis *in vitro*

Haemolysis *in vitro* results from a breakdown in osmotic regulation which may be due to three fundamental changes.

(1) An ionic imbalance between the cell and the bathing medium - osmotic haemolysis.

(11) Increased membrane permeability due to physical and chemical agents such as haemolysins - colloid osmotic haemolysis.

(111) Alteration in the normal metabolism in the cell membrane which leads to diminished active transport of cations.

3.3. Osmotic Haemolysis

Suspensions of red cells in an isotonic solution which is diluted with water swell until the membrane bursts and haemoglobin is liberated. Studies on the osmotic properties of the red cell have been described in detail (Jacobs, 1930, 1932). As the red cells are a heterogeneous group of different ages, their fragility to hypotonic solutions varies, and when the percentage haemolysis is plotted against the ionic concentration a sigmoid curve is obtained (Ponder, 1948). Further investigations demonstrating the different susceptibilities of red cells to hypotonic solutions have been published recently (Bowdler and Chan, 1969). Other investigations have shown that the osmotic fragility of the red cells

are markedly influenced by temperature and the time of incubation (Mortenson, 1963a) and by the pH of the medium. These investigations showed that maximum haemolysis occurred in hypotonic solutions at pH 6.7 (Mortenson,1963b).

3.4. Colloid Osmotic Haemolysis

Colloid osmotic haemolysis is seen when damage to the red cell membrane results in an increased permeability to cations with leakage of K^+ and an increased rate of Na^+ entry. As the maintenance of the ionic gradient breaks down, water enters the cell to maintain osmotic equilibrium with a gradual swelling of the cell into a sphere. A point is reached when the cell reaches approximately 160% of its original volume when rupture occurs and haemoglobin is released.

The changes which occur in the membrane may be of two types (Jandl, 1965). The first envisages the development of small holes in the membrane and may be produced by sulphydryl inhibitors. These substances alter the membrane without interfering with the glycolysis or active transport. The rate of entry of cations into the cell is so great that they cannot be compensated for by the pump mechanism and swelling occurs. As the holes are small, the addition of small molecular weight substances such as sucrose to the bathing medium may prevent further swelling and haemolysis occurring. Otherwise the process continues until cell rupture results in about one hour (Jacobs and Jandl, 1962).

A primary moderate increase in permeability may be differentiated from metabolic failure by the fact that glucose consumption is unaltered.

The second type of change is observed with haemolysis which occurs within seconds or minutes, and which is due to agents such as saponins or complement

fixing antibodies. It is postulated that they form large holes, possibly 80 - 100 Å in diameter, with the loss of K^+, phosphates and proteins (Seeman, 1967). The addition of molecules with a molecular weight in excess of 13,000 may temporarily delay haemolysis but sucrose has no effect (Jandl, 1965).

3.5. Haemolysis due to Impaired Metabolism

Cells deprived of glucose show impaired active transport of K^+ and Na^+. They may become depleted of glucose in a period of 6 hours as they have no stores of either glucose or glycogen. *In vitro* the cells undergo crenation and then swelling which will later slowly progress to haemolysis over a period of several days.

Under normal circumstances this is not an important factor when examining haemolysis *in vitro* provided fresh cells are used and an adequate supply of glucose is available in the suspending solution (Maizels, 1951).

4. HAEMOLYSINS AND HAEMOLYTIC AGENTS

4.1. Classification

A satisfactory classification of haemolysins is not possible. It would be desirable to divide them into categories based either on their chemical structure or according to their mode of action. However, in most cases neither the structure nor the mode of action is known.

For the purpose of the following discussion haemolysins are divided into the following main groups.

1. Physical or mechanical agents
2. Chemical agents
3. Biological toxins
4. Immunological agents.

4.2. Physical and Mechanical Agents

The haemolytic effects of ultraviolet and visible radiation on red cells have been examined (Green, 1956), as well as the effects of massive doses of X-rays (Sheppard and Beyl, 1951) and alternating currents (Poppendick and Hody, 1963). More recently interest has been centred on cardiac surgery and the possible changes in the red cell (Bell, Petrioglu and Frazer, 1967), and the effects of intracardiac prosthetic devices (Sears and Crosby, 1965). An *in vitro* examination of mechanical factors causing haemolysis was undertaken using agitation with quartz beads. The degree of haemolysis was influenced by the amount of trauma, pH of the medium and the presence of carbon dioxide (Po-Tum Fok and Schubothe, 1960). The presence of 1% albumin was shown to provide some protection to red cells from the trauma of extra-corporeal circulations (Garfin, Indeglia, Shea and Bernstein, 1968).

The lytic effects of freezing and thawing have been attributed to an increase in the electrolyte concentration in the unfrozen cellular fluid, although the mechanical effect of ice crystals may contribute to membrane damage. Heating on the other hand produces morphological changes in the red cell and at 48 deg. sphering and irreversible damage to the membrane occurs.

4.3. Chemical Haemolysins

These are divided into the following groups:

(a) Inorganic compounds
(b) Lipid soluble organic compounds
(c) Surfactants
(d) Steroids
(e) Vitamin A and polyene antibiotics
(f) Synthetic phospholipids
(g) Proteins and polypeptides
(h) Fluorescent dyes.

4.3(a) Inorganic Compounds

The haemolytic activity of colloidal silica was first described by Hartley and Margolis (1961) who showed that particles of a size between 3.5 and 7.0 nm were the most active. These observations were later extended to include silica dusts of varying composition (Stalder and Stöber, 1965), and asbestos powder (McNab and Harington, 1967). The lytic activity of silica was reviewed by Nash, Allison and Harington (1967), and it was considered that in aqueous solutions polymeric silicic acid acted as a hydrogen donor in forming hydrogen bonded complexes between active groups, such as quaternary and phosphate ester groups in phospholipids, and to a lesser degree between secondary amide groups of protein. Haemolytic activity may be inhibited by substances such as polyvinyl pyridine-N-oxide which forms preferential hydrogen bonding with the silica (Schnitzer and Bunescu. 1970).

The haemolytic action of mercurial and organo-mercurial compounds was described by Arbuthnott (1962). The mode of action appears to be associated with a change in the structure of the membrane followed by a reaction with sulphydryl groups in the membrane (Angelone, 1965). Glutathione has been shown to be an inhibitor. The haemolytic activity of copper (Mitral, Wahal and Bonsal, 1966), iron (Fielding, 1963), lead and silver has probably the same mode of action.

4.3(b) Lipid Soluble Organic Compounds

The number of organic compounds which produce some degree of haemolysis is extremely large and cannot be adequately covered by name in this survey. Hansch and Glave (1971) have grouped a large number of organic compounds together and examined a series of parameters which include the octanol/water partition coefficient.

They have attempted to compare the haemolytic, narcotic and antibacterial activities of the substances. They were able to demonstrate specific correlations between molecular structure and haemolytic activity which could be related to partition coefficients.

Other investigations have been described correlating the potency of phenothiazine derivatives as tranquillizers with their haemolytic activity (Freeman and Spirtes, 1962).

4.3(c) Surfactants

Both anionic (Rideal and Taylor, 1956; Rideal and Taylor, 1958) and cationic surfactants (Love, 1954, 1956) produce haemolysis which is characterized by two phases - an initial rapid phase followed by a slow haemolysis of the remaining cells. The initial rapid phase requires the presence of free phospholipid, and may be eliminated by repeated washings of the cells. The rapid phase can be induced by the addition of the cell washings or lecithin. The slow phase involves the adsorption of the surfactant into the phospholipid components of the membrane with disruption of the normal structure and lysis (Hooghwinkel, De Rooij and Dankmeijer, 1965). The reaction appears to be unaffected by the use of cells of different species or composition. The kinetics of the slow phase have been shown to be similar to saponin haemolysis (Rideal and Taylor, 1958). The non-ionic detergent Triton X-100 shows a saponin-like haemolysis without the initial fast phase. Sheep red cells show an unusual susceptibility to this surfactant (Cooney and Drake, 1969).

4.3(d) Steroids

Steroids produce haemolysis of red cells, the activity being most pronounced in the 5β - H series; the 5α - H steroids and those oxygenated at the carbon 11

position or with an α- hydroxyl at the carbon 17 position are non-lytic (Cuthbert, 1967). Non-lytic steroids augment the activity of the lytic steroids (Weissmann and Keiser, 1965). The precise mode of action of the steroids is unknown but it is presumed from the structure of active and non-active molecules that they interfere with the normal membrane configuration by diffusing into the membrane and aligning their molecules so that the polar groups are exposed on the surface. It is suggested that this forms areas of high electrophilic intensity which tend to disrupt normal membrane structure.

4.3(e) Vitamin A and Polyene Antibiotics

The polyenic compound vitamin A has been shown to be a potent haemolytic agent (Dingle and Lucy, 1962). The activity is confined to vitamin A alcohol and aldehyde but the acid has little activity. Initially it was thought to act by complexing with cholesterol but more recently it has been demonstrated that lecithin, rather than cholesterol, is the membrane constituent involved (Lucy and Dingle, 1964, Bangham, Dingle and Lucy, 1964).

The mode of action of polyene antibiotics, such as filipin, appears to be different to vitamin A and involves the presence of cholesterol (Kinsky, 1968).

4.3(f) Synthetic Phospholipids

The lytic properties of synthetic lysolecithins, lecithins and related compounds have been examined (Reman, Demel, De Gier, Van Deenan, Eibl and Westphal, 1969). The lytic properties of the lysolecithins are associated with the fatty acid carbon chain length, being optimal at 16 to 18 and losing activity above and below this figure. Double bonds in the chain appear to render the material less active. The distance between the

quaternary nitrogen and the phosphate bond is of little significence. The changes which are considered to take place are as follows: adsorption to the membrane, penetration of the lipid complex of the membrane, change in molecular organization followed by changes in permeability and lysis.

4.3(g) Protein and Polypeptides.

Protamine is a basic protein with a mol.weight of 4000 to 5000 which causes haemolysis in red cells at an optimal temperature of 37 deg. (Becker, 1961). Other basic proteins and polypeptides, such as polylysine, are also capable of causing haemolysis.

There are in addition a number of natural products which are proteins or polypeptides in structure and which are haemolytic, but these will be discussed later under their source of origin.

4.3(h) Fluorescent Dyes

The haemolytic activity of photodynamic substances has been briefly reviewed by Whittam (1964). Both light and oxygen are necessary for activity, and the speed of the reaction may vary from a few seconds to minutes. The suggested mode of action is such that light, acting on the sensitizing agent, provides the energy to cause oxidation of membrane constituents. The effect of temperature on the reaction has been examined (Blum and Kauzmann, 1954) and events leading to haemolysis have been recently described (Borgese and Green, 1962).

4.4. Biological Toxins

Although it would be desirable as taxonomists to classify the sources of the haemolysin according to phyla this is relatively unsatisfactory as the sources of these

agents are confined to a few classes of organisms. Based on our present knowledge a rather emperic subdivision has been made which is as follows:

(a) Plant haemolysins
(b) Microbiological haemolysins - viruses and bacteria
(c) Haemolysins of marine origin - protozoa, coelenterates, echinoderms and venomous fish.
(d) Haemolysins from <u>Insecta</u>
(e) Haemolysins from <u>Arachnida</u>
(f) Haemolysins from <u>Amphibia</u>
(g) Haemolysins from <u>Reptilia</u>

4.4.(a) <u>Plant Haemolysins</u> The most significant haemolysins of plant origin are the glycosides. These comprise a large series of compounds and include saponins. The aglycones which are formed by enzymatic hydrolysis are described as the genin, or in the case of saponins, as the saponogenin (Coffey, 1967). These are 17 - ketosteroids of cyclopentaperhydrophenanthrene. In aqueous solutions they have the property of concentrating at the surface and lowering surface tension. They cause membrane disruption and haemolysis by their particular affinity for cholesterol (Sollmann,1957). Although the lytic activity was originally considered to be associated with the sugar components of the molecule, work in the haemolytic activity of sapogenins indicates that the genin is the most active component of the molecule (Segal, Mansour and Zaitschek, 1966). Surface activity as measured by the ability to lower surface tension is not related to haemolytic activity.

Although the most common examples of saponins are of plant origin, an important group of saponins of marine origin have been discovered recently and will be discussed later.

An unidentified haemolysin has also been isolated from the plant *Rhus striata* (Hurtado, Burnell, Medina and Layrisse, 1965).

4.4.(b) Microbiological Haemolysins. A variety of cytolytic toxins have been isolated from bacteria and viruses. The main ones are the α, β, and δ toxins of *Staphylococcus aureus*, streptolysin S, streptolysin O, α and θ toxins of *Clostridrium welchii*, toxins of *Clostridrium septicum*, tetanolysin, pneumolysin, and the lytic toxins of *Streptococcus zymogenes* (Basinger and Jackson, 1968). These substances are proteins giving rise to neutralizing antibodies and are all lytic to erythrocytes at various concentrations. The precise mode of action of these substances is still unknown.

However, the various substrates or receptors for which the individual lytic agents have an affinity have been summarized by Weissmann, Sessa and Bernheimer (1966). They are as follows:

Toxin	Substrate or Receptor Substance
Cl. welchii α - toxin	Phosphatidyl choline
S. aureus β - toxin	Sphingomyelin (Maheswaron and Lindorfer, 1967, Wiseman, 1968)
Streptolysin O	Cholesterol
Streptolysin S	Phosphatides (Davie and Brock, 1966)
S. aureus α - toxin	Phosphatides
S. aureus β - toxin	Phosphatides

Of these substances the α- toxin of S. aureus has been investigated most thoroughly. Some initial evidence was produced to suggest that the substance had an enzymatic action (Robinson, Thatcher and Montford, 1960, Cooper, Madoff and Weinstein, 1964). This toxin shows lethal, lytic and dermatonecrotic activities which appear to be the result of a single substance only (Laminski and Arbuthnott, 1962). However, the toxin which shows only a single line on gel diffusion and immuno-electrophoresis demonstrates 3 to 4 components on electrofocusing. (Wadström, 1968). The α- toxin is characterized by loss of activity on heating to 60 deg. but is reactivated on further heating to 100 deg. This loss of activity is considered to be due to combination with an inhibitor which is destroyed at 80 deg. The lethal and dermatonecrotic activities show a similar loss of activity which suggests further that the three are the manifestations of a single substance. (Manohar, Kumar and Lindorfer, 1966). The precise mode of action of the lysin is not known, but there is evidence that it is not a phospholipase and it has no proteolytic activity (Bernheimer, 1968). More recent work suggests that the lytic action may be the result of its surface properties (Buckelew and Colacicco, 1971).

The lethal toxin of Bacillus cereus contains both haemolytic and lecithin hydrolysing activities. The latter appears to be due to a phospholipase C and is distinct from the other lytic fraction (Johnson and Bonventre, 1967).

The bacteriocidal and haemolytic agent elaborated by Streptococcus zymogenes is also a complex lysin. Kinetic studies suggest that it is an enzyme which is formed by the union of two inactive precursors (Basinger and Jackson, 1968).

Haemolysins have been described in toxins elaborated by *Escherichia coli* (Walton and Smith, 1969), *Corynbacterium ovis* (Frazer, 1961), *Leptospira pomona* (Bauer, Eames, Sleight and Ferguson, 1961), *Rickettsia* (Bovarnick and Schneider, 1960), *Cholera vibrio El Tor* (Watanabe and Seaman, 1962) and *Mycoplasma pneumoniae* (Cole, War and Martin, 1968).

The viruses of Newcastle Virus Disease and measles have also been shown to produce lytic substances, (Klemperer, 1960, Meio, 1961).

4.4.(c) Haemolysins of Marine Origin.

Protozoa. Studies have been made on the phytoflagellate *Prymnesium parvum* Carter (Reich, Bergmann and Kedron, 1966). The haemolysin can be obtained from both aqueous and methanol extracts. The substances responsible for the haemolytic and ichthyotoxic properties are similar as both are soluble in water and methanol and insoluble in acetone, they are non-dialyzable and are not retained by anionic or cationic exchange resins. Both the ichthytoxin and the haemolysin are destroyed above a temperature of 70 deg. (Shilo and Rosenberger, 1960). The toxin, which is described as a lipid-carbohydrate-protein complex, consists of several components with different activities (Reich, Bergmann and Kidron, 1966). A study of the kinetics and binding to the red cell membrane have been examined and the lysin produces a sigmoid curve of haemolysis similar to that seen with saponin (Martin and Padella, 1971).

Gymnodinium breve secreted a toxin which has also been shown to lyse the erythrocytes of the rabbit (Paster and Abbott, 1969).

Cnidaria. This phylum which is referred to as cnidaria or coelenterate is characterised by organisms containing nematocytes. Some difficulties have been

associated with the isolation of the toxins and haemolysins due to the labile nature of the active components.

Lane and Dodge (1958) described the toxicity of material derived from the nematocysts of Physalia physalia. In the original work no haemolysin was described. However, later investigations in which the toxin was injected into dogs a rise in potassium levels and slight haemolysis was noticed. The disproportionate rise in potassium levels in the serum was attributed to the toxin acting like ouabain on the ATPase system (Hastings, Larsen and Lane, 1967). The toxin has been shown to have phospholipase A and B activity, but it is doubtful whether they are of any significance from the point of view of the toxic action of the venom as the phospholipase is very heat stable, while the true toxin is heat labile. Heated toxin has little or no effect on animals. In general the physalia toxin is considered to contain other proteolytic enzymes and biologically active peptides (Stillway and Lane, 1971).

The tentacle extracts of the Box jellyfish Chironex fleckeri produces a haemolysin which was first described by Wiener (in Southcott and Kingston, 1959). The properties of the haemolysin have been examined (Keen and Crone, 1969a, Endean, Duchemin, McColm and Fraser, 1969, Baxter and Marr, 1969) and it is a protein with a molecular weight of 70,000 approximately (Crone and Keen, 1969, Crone and Keen, 1971). The degradation of the haemolysin by proteolytic enzymes has been described (Baxter and Marr, 1971). The haemolysin appears to be an important component of the nematocyst toxin, and the detailed pharmacology of its effects on the cardiovascular and respiratory systems have been described (Freeman and Turner, 1971, Turner and Freeman, 1969). Separation of the tentacle extracts by Sephadex G-200 chromatography produces two main fractions,

one lethal (molecular weight 150,000) and the other haemolytic. The relationship of the haemolytic fraction to the dermatonecrotic and lethal activities have been examined (Keen and Crone, 1969b). Some minor differences have been noted between the pharmacology of whole tentacle extracts and extracts from concentrates of nematocysts (Endean and Noble, 1971), but potent haemolysins are present in both.

Extracts from the tentacle of the related box jellyfish *Chiropsalmus quadrigatus* have been examined and shown to have a haemolysin which is similar to that seen in *C. fleckeri*. However, there are differences of heat stability and the two lysins appear to be immunologically distinct (Keen, 1971).

The toxin of the sea nettle *Chrysaora quinquecirrha* has a number of similar features possessing haemolytic, dermatonecrotic and lethal activities. Also the toxin affects the cardiovascular system of the rat in a manner similar to that seen with *C. fleckeri* and *C. quadrigatus* toxin (Burnett and Goldner, 1969). The haemolysin may be separated from the lethal and dermatonecrotic activities and is very labile and of low potency (Burnett and Goldner, 1971).

Echinodermata. Almost all cells contain sterols which are essential for the structure and integrity of the membrane. However, many land plants and some animals have evolved steroids which have both a poisonous and haemolytic activity.

Saponin-like steroids have only recently been obtained from certain marine organisms such as sea cucumbers. The material is concentrated in the Cuverian tubules and the active principle has been called holothurin (Nigrelli and Zahl, 1952). The material has been characterized as a glycoside with a steroidal aglycone and four molecules of monosaccharide (Nigrelli,

Chanley, Kohn and Sobotka, 1955). It falls into the same category as a cardiac glycoside (Chanley, Perlstein, Nigrelli and Sobotka, 1960), and its haemolytic activity has been compared with that of saponins and digitonin (Thron, 1964). In general they each appear to have a similar mode of action although the activity of the holothurin is greater than that of either of the other two substances.

Steroidal glycosides have also been isolated from the poisonous starfish *Asterias amurensis* (Yasumoto and Hashimoto, 1965, 1967). Two saponins called Asterosaponin A and Asterosaponin B have been isolated and their pharmacological properties examined (Freiss, Durant and Chanley, 1968).

Venomous Fishes. The haemolysin elaborated by the box fish *Ostracion centigenosus* has been examined in detail (Boylan and Scheuer, 1967). The material has a formula of $C_{23}H_{46}NO_4Cl$ and analysis suggests that it is a choline chloride ester of 3-acetoxyhexadeconic acid. Therefore there is a structural similarity to a cationic surfactant agent so that its haemolytic properties can be understood.

Haemolytic activity has been described in the venom glands of other fishes. The poison spines of the Weever fish (*Trachinus Draco*) were described by Evans (1907), and the haemolytic activity in the venom demonstrated. The haemolytic activity was enhanced by the presence of serum and was stable to heat at 75 deg. for one hour. The lytic activity has been noted *in vivo* (Skeie, 1962). However, the precise chemical composition has not been defined (Halstead, 1970).

The haemolysin in the venom obtained from the dorsal and pectoral spines of the catfish (*Plotosus lineatus*) requires no lecithin or serum to activate it; results

suggest that these substances act as inhibitors. Haemolysis occurs at an optimal temperature of 37 deg. and the material is relatively heat stable. It is probably a protein as it is antigenic (Halstead, 1970).

The haemolysin obtained from the venom of the stonefish (Synanceja horrida) is inhibited by serum but reactivated by lecithin (Duhig and Jones, 1928). Wiener (1959) showed that the venom lysed guinea pig red cells, but large quantities were required and lysis was rarely complete. The absence of haemolysis in animals dying of envenomation suggests that this activity was not an important manifestation of intoxication.

4.4.(d) Haemolysins from Insecta. The venom of the honey bee (Apis mellifera) is a complex mixture of peptides, proteins, sugars and lipids (O'Connor, Henderson, Nelson, Parker and Peck, 1967). The haemolytic activity may be divided into two types - direct and indirect; an indirect lysin requiring the presence of lecithin has been identified as a phospholipase A, and a direct lysin, which is in the polypeptide mellitin fraction of the venom (Rothschild, 1965). The precise mode of action of the active polypeptide fraction has been examined and it appears that it may penetrate the lipid components of the red cell membrane causing loss of normal structure (Sessa, Frier, Colacicco and Weissmann, 1969). A further fraction which has surface activity has also been identified and this is separate from both the phospholipase A and mellitin (Shipman and Cole, 1969). A phospholipase B has also been isolated but its relation to the other factors is not known (Doery and Pearson, 1964).

An unusual haemolysin has been obtained from the venom of the fire ant (Solenopsis saevissima rickteri Forel).

The whole venom is called solenamine and consists of two amines which are derivations of 2 methyl - 3 hexadecyl-pyrroline and 3 - pyrroline. Both solamine and a synthetic pyrroline derivative similar in structure to a component in the toxin are haemolytic, but their precise mode of action is unknown (Sonnet, 1967).

The Australian bulldog ant (Myrmecia pyriformis) has a venom containing both a direct and indirect lysin (Lewis, Day and De La Lande, 1968). The indirect haemolysin is due to a phospholipase A, but a direct lytic factor is also present, being demonstrated by the ability of the substance to lyse cells in the absence of lecithin. The venom has been separated into seven major protein components. However, the identity of mode of action of the direct lytic factor has not been fully identified (Wanstall, 1969).

Haemolytic activity in scorpion venom has generally been attributed to the presence of a phospholipase A. A number of species haye been examined and it appears that the lytic activity is not a universal finding (Rosin, 1969). An examination of the scorpion Leiurus quinquestriatus found in the Sudan showed that it had no phospholipase activity, although no attempt was made to demonstrate any other lytic activity (Ibrahim, 1967).

4.4.(e) Haemolysins from Arachnida. A number of clinical descriptions of bites from the genus Loxoscales have been reported with haemolysis in vivo (Minton and Olsen, 1964, Denny, Dillaha and Morgan, 1964). A comparative study of the venom in three species has been made. All have haemolytic activity but L. rufescens was the most potent (Smith and Micks, 1968). The venom contains a phospholipase, as lysophosphatides could be demonstrated after incubating the venom with lecithin (Denny, Dillaha and Morgan, 1964).

further work has been undertaken to analyse the fractions in the venom and identify their activity. (Morgan, 1969, Kniker, Morgan, Flannagan, Reagan and Dillaha, 1969).

4.4.(f) Haemolysins from *Amphibia*. Skin secretions of newts *Triturus cristatus*, *T. vulgaris*, *T. pyrrhogaster* and the orange speckled toad *Bombina variegata* show a haemolytic activity which is attributed to basic polypeptides. There is also a lysin in the skin secretions of *Hyla arborea* (European tree frog) and *R. dalmatina* (jumping frog), which is less potent (Kiss and Michl, 1962). The separation of the active fractions in newt secretions has been achieved (Bachmayer and Michl, 1964), and a detailed investigation of the haemolysin in *B. variegata* has been made (Kaiser and Kramer, 1967). The authors believe that the lysin may act like mellitin in bee venom, by causing disruption of membrane lipoprotein.

4.4.(g) Haemolysins from *Reptilia*. The literature on the haemolysins of snake venoms is extensive and has been reviewed by a number of workers (Rosenfeld, Kelen and Nudel, 1960-2; Condrea, De Vries and Mager, 1964). The lytic activity of the venoms can be divided into two types direct (DLF) and an indirect, which requires the presence of lecithin and is a phospholipase A. A similar composite lytic activity has been described for bee venom. The haemolytic properties of the venoms are complicated by the varying susceptibility of the red cells of different species (Condrea, Mammon, Aloof and De Vries, 1964, Condrea, Kendrzersky and De Vries, 1965), and the presence of inhibitors in serum (Luzzio, 1967).

The DLF is a basic protein and the amount of both phospholipase A and DLF in most snake venoms is small when compared with bee venom. Separation of the two fractions

has proved difficult even when using modern methods of chromatography (Slotta, Gonzalez and Roth, 1967).

The DLF is essential for the action of the phospholipase A when washed red cells are used. The DLF apparently makes the phospholipid of the red cell membrane available for hydrolysis. Digitonin and surfactants can act in a similar manner. Consequently the effect of different species of red cell on the haemolytic activity resides mainly in the ability of the DLF to prepare the cells for phospholipase activity. Thus sheep and camel cells are resistant to the haemolytic activity of cobra venom, but the phospholipids of ghosts of these cells are readily hydrolysed by cobra venom (Kelen, Rosenfeld and Nudel, 1960-2, Condrea, Mammon, Aloof and De Vries, 1964). More recent work has implicated calcium ions in the reaction (Lankisch, Lege, Oldigs and Vogt,1971).

There have been some recent reviews on the relationship of the DLF and phospholipase A in various venoms. There have been reports on Crotoxin (Hendon and Fraenkel-Conrat, 1971), *Naja Naja* venom (Colacicco and Rapport, 1966), *Echis coloratus* venom (Klibansky, Ozcan, Joshua, Djaldelhi, Bessler and De Vries, 1966), *Habu* snake venom (Kurashige, Hara, Kawakami, and Mitsuhashi, 1966), and similar investigations have been made on Australian snake venoms.

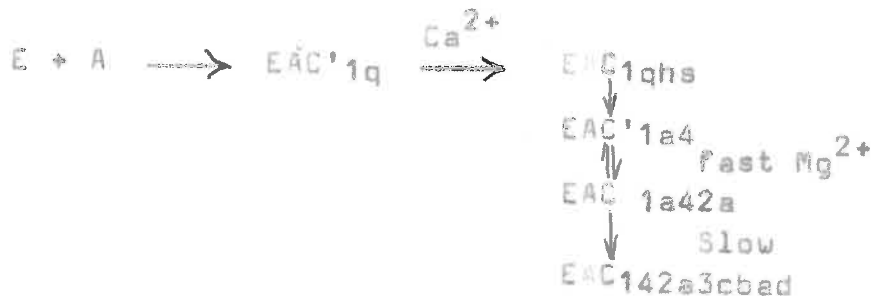
Early reports on haemolysins in Australian snake venoms were made by Holden (1934, 1935), and the relative proportion of phospholipase A and DLF in a number of Australian snake venoms were described by Doery and Pearson (1961). Later the same workers described a phospholipase B in the Australian black snake (*Pseudechis Porphyriacus*) and a variety of other indigenous snake venoms (Doery and Pearson, 1964.).

4.5. Immunological Agents

In 1896 Bordet showed that there was a factor in normal serum which was necessary for the lysis of foreign red cells. The quantity of this lytic material was not increased by immunization. Later it was shown that the lytic power of the serum was dependent on heat labile "complement", together with heat stable specific antibody.

It soon became apparent that complement was not a single factor, and over the past twenty years more and more components of the system have been isolated, the present total being eleven (Polley and Muller-Eberhard, 1966).

The changes which occur follow the initial reaction of binding of the specific antibody onto the red cell surface. A subcomponent C'1 is attached to the cell and the remaining components of the C'1 system, namely, C'1$_a$ and C'1$_s$ are fixed provided Ca^{2+} is present. This results in the generation of C'1 esterase and activated C'1$_a$ and then C'$_4$ binds onto the cell. The esterase attacks C'2 splitting it into C'2$_i$ and C'2$_a$ and the latter is added to the complex on the cell surface. The latter two steps are dependent on esterase and may be inhibited by di-isopropylphosphofluoridate. This reaction is rapid but the succeeding steps are slow, with the attachment of C'3 with its four subcomponents. If C'142a does not react with C'3 it decays. The final product EAC142a3cbad represents the stage where the cell is irreversibly damaged (Firken and Wiley, 1966).



These changes have been worked out for sheep red cells, rabbit antiserum and guinea pig complement.

The initial effect of complement on the red cell membrane is to produce increased cation permeability with cell swelling. Only later in the reaction do large holes appear in the membrane with the liberation of haemoglobin. Although it has been suggested that only a specific site on the membrane is affected, electron microscopy shows many areas of damage (Frank, Dourmashkin and Humphrey, 1970).

Human red cells sensitized by reactions other than those associated with antibody can bind complement with subsequent haemolysis (Yachnin and Ruthenberg, 1965a,b). It is thought that the binding of complement to human red cells occurs following on a change in net charge on areas of the cell surface (Polley and Muller-Eberhard, 1966).

5. EVENTS ASSOCIATED WITH HAEMOLYSIS

5.1 Utilization of Lysin

The changes which are associated with the addition of the lysin to the red cell suspension are complex and difficult to interpret in a general manner.

It appears that the lysin is first adsorbed onto the cell membrane so that only a proportion of the added lysin may be utilized. The lysin then reacts with the membrane, or a specific component of the membrane, producing instability and leakage of intracellular contents. This may be one of adsorption and penetration of the cell membrane in a manner similar to that described for monolayers (Doty and Schulman, 1949, Matalon and Schulman, 1949).

On the other hand there may be a chemical complexing in the membrane, or degradation of a component due to enzymatic action which subsequently interferes with the stability of the membrane.

5.2 Prolytic Phase.

This is the period from the time the lysin is added to the stage where haemolysis commences. The alterations which occur in the cell result from loss of intracellular potassium and replacement with sodium. This produces volume changes and spherocytosis. As the lysin causes further damage to the cells, the membrane becomes permeable to haemoglobin and haemolysis occurs.

5.3 Lytic Phase.

This stage lasts from the time of haemolysis of the least resistant cells to the time of haemolysis of the most resistant cells, with the rate of haemolysis dependent on the lysin concentration. The loss of haemoglobin converts the cell into a ghost but in many cases the loss of haemoglobin is not complete. In the case of hypotonic haemolysis, the red cell ghost assumes its discoidal form immediately after haemolysis. A mass of precipitated haemoglobin may be seen around a single membrane break which may be as large as two microns in diameter (Baker and Gillis, 1969). During the course of haemolysis the erythrocytes are permeable both to small and large molecular weight materials (Seeman, 1967).

The morphological changes accompanying haemolysis have been examined by electromicroscopy (Dourmashkin and Rosse, 1966). Osmotic haemolysis produces tubular extrusions from the membrane, or large clear rings may be seen. Saponin haemolysis also produces holes, while

digitonin produces a rigid crystalline appearance. Phospholipase C produces large defects in the membrane approx. 500 A^{0} in diameter surrounded by a dense ring.

As the changes are produced by indirect methods, their precise significance is not known. Nevertheless it is felt that one defect only is necessary to permit the efflux of haemoglobin.

5.4 Stromatolytic Phase

This phase consists of the continued reaction of the lysin with the stroma of the red cell ghost. Although Ponder (1948) considers that this phase commences only after complete haemolysis has occurred, the process actually commences immediately after the first cells have haemolysed. This part of the haemolytic reaction has received little attention. It consists of further utilization of the haemolysin, liberation of material which may be inhibitory to the lytic process and disintegration of the stroma.

6. METHODS OF ESTIMATING HAEMOLYSIS

6.1 Haemolysis Curves

Methods are usually chosen to provide both quantitative and qualitative measurements of the lytic process.

The three main variables in the reaction are the concentration (or dilution) of the haemolysin, the number of red cells, and the time the reaction is allowed to proceed. Using these three variables, three standard combinations may be investigated.

(a) Time-dilution curves. The concentration of red cells is maintained constant while the time to reach a fixed level of haemolysis is examined over a range of lysin concentrations. For technical reasons the level of haemolysis chosen is usually 100% haemolysis. This type of relation was used extensively in the examination of the haemolytic reactions of saponin (Ponder, 1948), and anionic and cationic detergents (Love, 1954, Rideal and Taylor, 1956).

(b) Percentage haemolysis curves. These are obtained by measuring the percentage of cells haemolysed at the end of a given time using a fixed concentration of lysin. However, there are technical difficulties encountered in rapidly measuring the number of red cells haemolysed.

(c) Percentage haemolysis - concentration curves. These are obtained by allowing the haemolytic reaction to proceed for a theoretically infinite time so that haemolysis is 100 per cent complete. This may provide useful information on the mode of action of surface active agents (Thron, 1964) and was employed to compare the haemolysin of *C. fleckeri* with *C. quadrigatus* (Keen, 1971).

6.2 Methods of Measuring the Percentage of Haemolysed Cells

Two standard methods may be employed, one measuring the amount of haemoglobin liberated from the lysed red cells, and the other the particle density of the remaining non-lysed cells in the red cell suspension.

Methods which employ estimations of liberated haemoglobin are frequently used, and have the advantage

of accuracy as there is a linear relation between the optical density (OD) at 540 nm and the haemoglobin in solution. However, the method has the disadvantage that the red cells must be removed before the readings are taken and in fast reactions this may provide some practical difficulties.

The other method employs the measurement of particle density of the remaining red cells. It is simple if 100 per cent haemolysis is only required, the end point being estimated by eye when the suspension becomes completely transparent. However, the method can be used with a spectrophotometer for measuring percentages less than 100, provided the OD at a wave length above 600 nm is employed (Baudler and Chan, 1969). The method is not as accurate as the first and is subject to errors of altered absorption by cells in the swollen prelytic phase. However, it may be used in self-recording spectrophotometers which provide a continuous reading of changes in OD and the rate of haemolysis under varying experimental conditions. A disadvantage is the non-linear relation between the particle density and the OD and estimation of actual percentage haemolysis must be read off a calibration curve.

6.3 Haemolysis in Gels.

This is a method employed by microbiologists to distinguish cells producing lytic endotoxins (Elek and Levy, 1950). The method may be employed for qualitative investigations and for approximate quantitative measurements. It has the advantage that the red cells can be maintained over a period of several days and allows the use of double diffusion techniques (Crowle, 1961) for the

possible identification of haemolysins when allowed to diffuse against specific antisera (Keen, 1971).

6.4 Inhibitors.

Certain substances may be inhibitors when introduced into the haemolytic system. They may act on the lysin in the bulk phase or on the membrane of the red cell, making it less susceptible to haemolysis (Ponder and Ponder, 1954, 1956). In practice differentiation can be readily achieved by examining the rate of haemolysis after incubation of the suspected inhibitor with either the lysin or the red cells. The examination of the lysin in association with known inhibitors, such as cholesterol, serum or sugars, may assist in identifying the mode of action of the lysin.

6.5 Accelerators.

These rarely act on the lysin in the bulk phase, except in rather specialized circumstances where the action of lecithin increases the activity of phospholipases. The effect of an accelerator is usually on the red cell membrane making it more susceptible to the action of the lysin. In saponin haemolysis, benzene and its halogenated derivatives may act as accelerators (Ponder, 1948). It is considered that they act by penetrating the molecules of the membrane lipoprotein complex of the red cell wall facilitating the action of the lysin (Rideal and Taylor, 1958, Klibansky, London, Frenkel and De Vries, 1968).

7. KINETICS OF HAEMOLYSIS

In general two types of processes may occur; either an enzymatic degradation of the membrane which follows first order enzyme kinetics, or adsorption of the lysin

occurs onto the surface of the cells according to Gibbs adsorption equation. In some instances both types of reactions are proceeding at the same time with the formation of inhibitors, and acting on a population of cells with a varying susceptibility to the lysin. An analysis of the kinetics, using formal chemical equations, may therefore become complicated.

In a more general approach, the kinetics are dependent on a number of factors.

1. The nature and concentration of the haemolysin.
2. The number and type of red cells.
3. The temperature of the reaction.
4. Miscellaneous factors such as pH and the constitution of the suspending medium.

7.1. The Nature and Concentration of the Haemolysin

Bernheimer (1947) examined a number of haemolytic agents, many of bacterial origin, and other surface active agents. He examined the reactions in relation to the degree of haemolysis as a function of time, and the rate of haemolysis, as a function of concentration. Bacterial lysins, such as pneumolysin, tetanolysin and streptolysin S had a rate of haemolysis directly proportional to lysin concentration, and these reactions could be analysed using enzyme kinetics. On the other hand, the haemolysis produced by saponin and sodium taurocholate had an exponential relation to the concentration, a feature which is common to the reaction of surface active agents. A detailed analysis has been made of the kinetics of haemolysis produced by anionic (Rideal and Taylor, 1956) and cationic (Love 1954, 1956) surface

active agents. In the case of toxins and venoms, the processes may be complicated by the presence of more than one lytic agent each with a different mode of action but often acting in sequence.

7.2 The Number and Type of Red Cells

The chemical compositions of red cells of different species have been reviewed by Prankerd (1961). The nature of the membrane may influence the rate of lytic activity when one specific component is involved. However, this aspect tends to modify rather than control the reaction.

The number of cells in a lytic system is important when examining agents which act on the membrane surface (Croes and Ruyssen, 1951 a, b). Haemolysis is dependent on two specific features, the amount of lysin taken up by the cell, and the quantity required to bring about haemolysis. When using dilute cell suspensions only small portions of the lysin are utilized in the reaction, so that the concentration is represented by the amount in the surrounding medium.

Using these principles, Thron (1964) examined the cellular uptake of holothurin, digitonin and saponin, and compared the relative potency and mode of action of these substances. In order to produce a given degree of haemolysis (50% was chosen, C50) in a time in which the reaction was complete (6 hours), a given concentration of free lysin (bx) was required. The C50 is related to the number of cells by the following equation: $C50 = axN + bx$; where N is the number of cells, ax the total quantity of the lysin taken up by the cells and bx the free lysin. When a series of experiments are undertaken with different values of N, the graph relating C50 to N is a straight line

with a slope of ax. The figure for ax may be used to give some indication of the number of molecules per cell required to produce haemolysis, while bx gives some indication of the potency of the haemolysin.

7.3 The Temperature of the Reaction

Experimental results indicate that the effect of temperature on haemolysis cannot be related adequately by the Arrhenius equation.* Plotting the logarithm of the rate of the reaction against the reciprocal of the absolute temperature does not produce a straight line except in those cases where the agent is acting as an enzyme, and here a straight line is only obtained over a limited temperature range (Bernheimer, 1947). In those cases where the haemolytic process consists first of adsorption onto the red cell followed by a second process of lysis, the effect of temperature on each process may be different. In these cases lysis proceeds at an optimal temperature which is usually determined experimentally.

7.4 Effect of pH

The effect of pH is also complicated as it may affect the surface of the membrane or produce a change in the properties of the lysin. In systems containing saponin, haemolysis is increased at pH below 4 and above 9.5 (Ponder, 1948).

* The Arrhenius equation $\frac{d \ln k}{dT} = \frac{E}{RT^2}$, or its integrated form $\ln k = -\frac{E}{RT}$ + constant; where k is the rate constant, R is the gas constant, T the absolute temperature and E the activation energy (Glasstone, 1947).

8. MODE OF ACTION OF HAEMOLYSINS

8.1 Biological Considerations.

Haemolysis *in vitro* is the result of a change in the normal protein-lipid-cholesterol complex which constitutes the red cell membrane brought about by substances which react with one or other of these major groups of components. Haemolysins may, therefore, fall into three major categories:

1. Enzymes such as phospholipases or proteases.
2. Substances which chemically bond with the membrane, such as glycosides or polyene antibiotics.
3. Substances which produce physicochemical changes in the membrane, such as surface active detergents.

In the case of substances with enzymatic activity, the kinetics of the haemolytic reaction and the identification of the breakdown products provide clear evidence of the mode of action of the substance. However, in group 2 and 3, it is more difficult to identify the precise nature of the reaction, and for this reason a number of model systems have been devised to assist in elucidating this information.

8.2. Model Systems

For a number of years lysis and haemolysis have been regarded as the end result of an interfacial phenomenon, and the approach to the problem has been based on either the surface properties of the lytic agent or its ability to penetrate artificial membranes.

8.3 Surface Activity of the Lytic Agent

The surface activity of a substance in an aqueous solution is based on its ability to concentrate at the air-water interface and lower surface tension. In this way free energy of the system is reduced (Glasstone,1947).

The exact relation between the adsorption of a substance at the surface and the surface tension was defined by Gibbs in 1878. For dilute solutions, the relationship between surface concentration (Γ), concentration in solution (c) and surface tension (γ) is as follows:

$$\Gamma = - \frac{C}{RT} \frac{d\gamma}{dC}$$ where R and T have the usual connotations.

Using this approach Pethica and Schulman (1953) examined the relation between the haemolytic activity of surface active agents and their ability to lower surface tension.

8.4 The Ability to Penetrate Monolayers.

Monolayers of lipids, protein or cholesterol may be prepared at the air-water interface (Gaines, 1966). The lytic agent may be injected under the monolayer at a given surface pressure Π and the rise in surface pressure ($\Delta\Pi$) estimated as a function of time. The magnitude of $\Delta\Pi$ is a measure of the interaction in the monolayer which is referred to as penetration. (Doty and Schulman, 1949, Matalon and Schulman, 1949).

The properties of proteins (Bull, 1947; Evans, Mitchell, Mussellwhite and Irons, 1970) and lipids (Shah, 1970) at the air water interface have been reviewed in detail. In the case of protein monolayers (Arnold and Pak, 1962) cholesterol monolayers (Schulman and Rideal

1937a, 1937b; Pethica and Schulman, 1953) and lipid monolayers (Doty and Schulman, 1949) the change on surface pressure has been taken as evidence of the degree of penetration.

However, more recently the penetration of lipid monolayers by protein in particular has been divided into three types which are important in the interpretion of the magnitude of $\Delta\Pi$ (Colacicco, 1969, 1970). The three types are:

(a) Free penetration which represents a diffusion into the surface and the development of a two dimensional micelle with protein molecules surrounding clusters of lipid molecules. This is similar to the original concept of penetration. We envisage a $\Delta\Pi$ value which is the mean of the surface activities of the two components, and on compression of the monolayer the less surface active material will be extruded from the film.

(b) Binding-mediated penetration which is seen with films of cholesterol, cerebrosides and other mixed lipids. This process consists of hydrogen bonding or electrostatic attraction of the lytic agent in the monolayer with an initial drop in $\Delta\Pi$ followed by penetration and a marked increase in the $\Delta\Pi$ values.

(c) Binding-inhibited penetration where a similar mechanism of bonding occurs as in (b) above, but the complex formed inhibits penetration of the monolayer. In these cases the $\Delta\Pi$ values may be small.

The measurement of surface tension and surface potential have been applied to the understanding of the action of a number of haemolytic agents. These include the enzymatic hydrolysis of lecithin monolayers by phospholipase A (Shah

and Schulman, 1967; Colacicco and Rapport, 1966) and the degradation of protein monolayers by proteases (Colacicco, 1970); the penetration of cholesterol monolayers by glycosides (Schulman and Stenhagen, 1938) and surface active agents (Pethica and Schulman, 1953); the penetration of mixed lipid monolayers by polyene antibiotics (Demel, Van Deenan and Kinsky, 1965; Kinsky, Demel and Van Deenan, 1967), local anaesthetics (Skou, 1958) and psychoactive drugs (Van Deenan and Demel, 1965); and protein monolayers with surfactants (Pearson and Alexander, 1967).

In the case of haemolysins of biological origin, the interaction of mellitin with lipid monolayers has been investigated (Sessa, Frier, Colacicco and Weissman, 1969), and a similar study was undertaken with the α-toxin of Staphylococcus aureus (Buckelew and Colacicco, 1971). In the latter investigation, the α-toxin was shown to have a high surface activity, and the degree of penetration of the monolayer, depending on the type of lipid monolayer employed, was greatest with cholesterol and least with ganglioside. The small increase in $\Delta\Pi$ with ganglioside had the characteristics of binding mediated penetration.

A similar study has been carried out with the haemolytic fraction of C. fleckeri toxin obtained by chromatography of tentacle extracts. The fraction showed marked surface activity but it was not as great as that of the α-toxin. A reaction suggesting binding-mediated penetration was observed with a mixed lipid monolayer derived from rabbit red cells, but the precise component involved was not determined.

8.5 The Ability to Penetrate Lipid Bilayers.

Lipid bilayers are theoretically more representative of the cell membrane than monolayers. Although considerable work has been undertaken on their physical

properties, investigations of lytic action have been uncommon. Polyene antibiotics have been shown to disrupt bilayers containing lecithin and cholesterol in the ratio of 1:1. The action of the antibiotics could only be observed when the bilayer contained at least 10% cholesterol (Zutphen, Van Deenan and Kinsky, 1966) which confirmed the previous findings in monolayers.

8.6 The Ability to Disrupt Spherulites

The preparation of spherulites consisting of liquid crystals of phosphatidyl choline swollen in salt solutions, was first described by Bangham, Standish and Watkins (1965). The spherulites were shown to have properties which were similar to biological membranes and their structures could be shown to be affected by sterols, organic solvents and local anaesthetics (Bangham, Standish and Miller, 1965; Cuthbert, 1970). They have been used to investigate the mode of action of the mellitin fraction of bee venom (Sessa, Frier, Colaccico and Weissmann, 1969).

8.7 Binding and Partition Systems

Techniques to evaluate binding or complexing of the lysin may be employed in systems used for the routine investigation of the properties of the haemolysin. The addition of cholesterol, lecithin, proteins, sugar or sterols to the haemolytic system may influence the reaction so that some information on the mode of action of lysin may be obtained. Prior incubation of these substances with either the red cells or the lysin alone may provide additional information. Methods such as these have been used to demonstrate the presence of phospholipse A activity which is markedly accelerated by the presence of lecithin, or the presence of glycoside haemolysis which are specifically inhibited by cholesterol.

The use of partition systems have been mentioned previously. In the case of anaesthetic agents, there is a reasonable connection between solubility, surface activity and the oil/water partition coefficient. This is a logical association as the three parameters represent a distribution between heterogeneous phases (Ferguson, 1939). In the case of those anaesthetic agents with haemolytic activity, an indication of potency may be achieved by measuring the octanol/water partition coefficient (Hansch and Glave, 1971).

9. MEMBRANE STABILIZATION: SPECIFIC AND NON-SPECIFIC HAEMOLYSINS

The subject of membrane stabilization has been reviewed by Seeman (1966). It is demonstrated on the red cell membrane by the use of sublytic doses of the haemolysin which will protect the cell from hypotonic haemolysis. The available evidence indicates that for anaesthetics (Seeman, Kwant, Sauks and Argent, 1969) and tranquillizers (Seeman, Kwant and Sauks, 1969; Seeman and Kwant, 1969), stabilization is the result of membrane expansion which increases the critical volume required to produce lysis of the red cell.

Membrane stabilization can be demonstrated by a large proportion of haemolysins, such as local anaesthetics, tranquillizers, antihistamines, steroids and detergents (Seeman and Weinstein, 1966; Seeman, 1966a, b).

Seeman has divided haemolysins into two groups dependent on their ability to produce membrane stabilization. The first group he describes is non-specific haemolysins. These have low levels of haemolytic activity and produce membrane stabilization at sublytic

levels. The second group is specific haemolysins which are very potent lytic agents and show no evidence of membrane stabilization even at very low concentrations. Typical specific haemolysins are saponins, glycosides, filipin (Seeman, 1966) and haemolysins of C. fleckeri.

If we refer to our model systems, we conclude that stabilization by non-specific haemolysins is represented by the early stages of free penetration where expansion of the film may occur without disruption of the normal function of the membrane. Specific haemolysins probably act in a different manner based on binding or complexing in the membrane. Consequently, small areas of activity lead to large areas of disruption. Some support for this concept may be obtained by estimating the intrinsic activity of the haemolysin (cell membrane surface area corresponding to each lysin molecule taken up by the cell). For specific haemolysins holothurin and digitonin, the intrinsic activities are 8700 $Å^2$ and 1050 $Å^2$ respectively, while for the non-specific alkyl sulphates the activity is 100 $Å^2$ approximately (Croes and Ruyssen, 1951; Thron, 1964).

B. EXPERIMENTAL SECTION

1. Chapter 1: The characterization of the haemolysin.

2. Chapter 2: The haemolysin of *Chironex fleckeri* and its relation to the lethal and dermatonecrotic activities; antigenic properties of tentacle extracts.

3. Chapter 3: The role of the haemolysin of *Chironex fleckeri* in envenomation in animals and humans.

4. Chapter 4: Comparative study of the haemolysin derived from tentacle extracts of *Chiropsalmus quadrigatus* and *Chironex fleckeri*.

5. Chapter 5: Examination of the possible mode of action of the haemolytic fraction derived from tentacle extracts of *Chironex fleckeri*.

6. Conclusions:

CHAPTER 1

1. CHARACTERIZATION OF THE HAEMOLYSIN OF *CHIRONEX FLECKERI*

1.1. I N T R O D U C T I O N

In reviews of jelly fish stingings occurring around the coast-line of Australia, Cleland and Southcott (1965) and Barnes (1966) have recorded 50 - 60 deaths which could be attributed to the marine organism commonly known as the box jelly fish or sea wasp. Fatalities are confined to the area north of the Tropic of Capricorn and occur during the summer months, usually between November and February.

Stingings with these organisms are a concern to public health authorities. Consequently, a number of workers in Australia have undertaken to investigate the problem along a number of lines; to define the causative organism, to isolate the lethal toxin, to characterize the toxin chemically and pharmacologically and to review human symptomatology of injuries and the possible methods of treatment.

Kingston and Southcott (1960) demonstrated initially in two fatal cases of jelly fish stingings that the nematocysts attached to the skin matched the principal nematocysts of *Chironex fleckeri* and *Chiropsalmus quadrigatus*. Both the organisms belong to the phylum coelenterata or cnidaria. The phylum is divided into three main classes; the Anthozoa, the sea anenomes and corals; the Hydrozoa, the hydroids and hydromedusae, and the Scyphozoa, the 'true' jellyfish. In the latter class *C. fleckeri* and *C. quadrigatus* are included in the family of Chirodropidae.

The morphological appearance of the two organisms *C. fleckeri* and *C. quadrigatus* is very similar but they can be identified by differences in the periradial nucleus and gonads. Investigations on the size and stinging potential show that *C. fleckeri* is of larger size and has a greater density of nematocysts in the tentacle (Barnes, 1966). It is the more important of the two species and the major portion of our work was concerned with the toxin of this organism. The investigations of *C. quadrigatus* have been reported as a comparison.

Following the positive identification of the causative organisms/isolation of the active principle from the nematocysts was hampered by the labile nature of the toxin. Some toxicological studies were initially undertaken and reported by Weiner (in Southcott and Kingston, 1959). He used extracts of frozen tentacle and demonstrated their lethality in mice and guinea pigs. He reported the presence of a haemolysin but did not elaborate on this finding. In an attempt to isolate the active principle in the nematocysts, Barnes (1967) reported a novel method of collecting the contents of the nematocysts by causing them to rupture through human amniotic membrane. Endean *et al.* (1969) and Endean and Noble, (1971), reported on the pharmacology of extracts prepared from nematocyst concentrates. Freeman and Turner (1969) were able to demonstrate that the material prepared by Barnes by discharge of nematocysts through the amniotic membrane had the same pharmacological properties as crude tentacle extracts and the properties of both of these extracts were similar to concentrates of nematocysts.

Whether the tentacle extracts can be considered pharmacologically representative of the toxin is a matter of some significence. The available evidence suggests

that the tentacle extracts of C. fleckeri are suitable for examining the properties of the toxin for the following reasons:

(i) Extracts of the tentacle, extracts prepared by discharge of nematocysts, and concentrates of nematocysts have identical pharmacological properties.

(ii) The activity of the tentacle extracts is considerably greater than that of discharged nematocysts or nematocyst concentrates due to the simplicity of preparation. Tentacle extracts may therefore be used at such high dilutions that any activity from non-specific contaminants is unlikely to be significant or observed experimentally.

(iii) The pharmacological properties of the extracts when administered to animals resemble the clinical signs of human envenomation.

Recently an attempt has been made to differentiate the detailed pharmacological properties of concentrated extracts of nematocysts and tentacle extracts (Endean and Noble, 1971). Both are mixtures consisting of at least two known toxic fractions with different stabilities. Although minor differences were reported, these may readily be due to different methods of preparation of the two extracts. It is significant that the haemolysin was observed in both preparations.

During the course of the examination of the whole tentacle extract, it was noted that there appeared to be a relation between the lethal and the haemolytic activity of the extract. The haemolysin was so potent that it enabled studies to be made of the active principle in such concentrations that detection by chemical means would have been impossible.

The initial investigation was undertaken to characterize the properties of the haemolysin and to attempt to relate quantitatively the haemolytic and lethal activities of the tentacle extract.

1.2 METHODS

Collection, Identification and Preparation of Extracts.

The specimens of *C. fleckeri* had been collected and identified by Dr. J. Barnes of Cairns, Queensland, Australia. They were kept in a cool room until the tentacles could be removed and then transferred to a liquid nitrogen container for transport to the laboratory. Using this procedure the tentacles were well preserved and small quantities could be removed as required.

Dr. Barnes also supplied a quantity of material which was obtained by discharge of nematocysts through human amniotic membrane (Barnes, 1967). This material was employed in comparisons between haemolytic and lethal properties of the extract. This material had been stored in the frozen state for 20 months and this may account for its relatively low potency at the time of use.

The haemolysin appeared labile at room temperatures and attempts to make a stable and concentrated extract from the nematocysts as described for Physalia (Lane and Dodge, 1958) were unsuccessful. The haemolysin could not be extracted with alcohol or ether and it would not pass through a dialyzer membrane.

For these investigations a small quantity of tentacle was homogenized at 5 deg. and extracted with a phosphate buffer at pH 6·3, which was initially thought to be the optimum pH for stability of the extracts (Freeman and

and Turner, 1969). The material was centrifuged at 2500g for 20 min. and the supernatant removed, dispensed into 1·0 ml quantities and placed in a deep freeze at -20 deg. Two batches were made - the first by extracting 648 mg of tissue in 10·0 ml of buffer, and the second by extracting 2·9g in 15·0 ml of a similar buffer solution. The first batch was used for most of the experiments but there was insufficient to complete the investigation. The second batch was mainly used for studies on the rate of haemolysis. The extracts were then diluted with phosphate buffered saline (Ponder, 1948) at pH 7·20 to give the initial dilution required for the haemolytic studies.

Preparation of Red Cells

Red cell suspensions, except those from humans, were normally obtained by intracardiac puncture and the blood collected into heparinized tubes. Human red cells were obtained by puncture of the median cubital vein. The cells were washed three times in 20 times their vol. of 0·146 M sodium chloride and were used within 24 hr. of collection. Rabbit cells were used for all routine assays.

Estimation of Haemolytic Activity

The method of measuring the dilution of extract producing 50 per cent haemolysis (D_{50}) was similar to that described by Thron (1964). Ten tubes were used for each estimation and the haemolytic system consisted of a total vol. of 3·0 ml. The initial 1·0 ml in the tube consisted of serial twofold dilutions of the venom and buffered saline. To each tube was added a further 1·0 ml of buffered saline, or other test solution, and 1·0 ml of rabbit red cells, the concentration being adjusted to give a final dilution of 3·0 - 3·5 X 10^7 cells per ml.

The number per unit volume and the species of the red cells were kept constant in all experiments except in those where these two variables were independently investigated.

Haemolysis was allowed to occur at 25 deg. for 5 hr. when the numbers of non-haemolysed cells were estimated in a spectrophotometer (Unicam SP600) by their absorbance at 700 nm. A calibration curve had been prepared using suspensions of cells with varying proportions haemolysed by freezing and thawing. From this graph the optical absorbances could be converted into figures for per cent haemolysis and when these were plotted against the dilutions of venom the D_{50} could be calculated. The haemolysin titre (D_{50}) was expressed as the logarithm of the reciprocal of the highest dilution of extract which produced 50 per cent haemolysis in 5 hours. By relating this dilution to the wet weight of tentacle from which extract was prepared, an approximate figure for the concentration causing 50 per cent haemolysis (C_{50}) could also be estimated.

Estimation of Rate of Haemolysis

Investigations on the rate of haemolysis were undertaken in a spectrophotometer (Unicam SP 800) with an automatic cell changer and programme controller. The temperature was maintained at 25 deg. in all experiments unless stated otherwise. The total volume of the haemolytic system was again 3·0 ml, consisting of 1·0 ml of red cell suspension, 1·0 ml of buffered saline, or other test solution, and 1·0 ml of the appropriate dilution of haemolysin. The same principle was adopted of converting the apparent absorbance at a fixed wave length of 700nm into per cent haemolysis by means of a calibration curve. When it was necessary to add test substances during the course of the haemolysis,

solutions in 0·1 ml were used. The alteration in the dilution of the red cell suspension caused by the addition of 0·1 ml caused very slight changes in the absorbance recorded and any major variation could be attributed to the substance added.

Estimation of Toxicity LD_{50}

The toxicities of the extracts by the i.v. route were estimated by measuring the LD_{50} for male albino mice weighing 18 - 20 g. Four groups of 4 animals were used for each estimation and four dose levels were employed in a constant vol. of 0·1 ml except at the highest dose level in the least potent extract where it was necessary to use 0·15 ml. The LD_{50} was calculated from the method described by Weil (1952) and expressed as the logarithm of the dilution contained in 1·0 ml of the extract.

The buffers used in the stability tests were as follows: between pH 4·0 and 8·0 they were mixtures of citric acid and disodium phosphate, and between pH 8·0 and 9·0 they were mixtures of Tris-(hydroxymethyl) aminomethane (Tris) and hydrochloric acid. The isotonic buffers used to measure the effect of pH on rate of haemolysis were mixtures of sodium chloride, disodium phosphate, potassium dihydrogen phosphate for values between pH 6·0 and 7·5 and sodium chloride and Tris-hydrochloric acid between pH 7·5 and 9·0.

1.3 R E S U L T S

Estimation of the D_{50} and C_{50} values for the haemolysin of *C. fleckeri*

Figure 1 shows the percentage haemolysis plotted against the dilution of the extract and the wet weight of

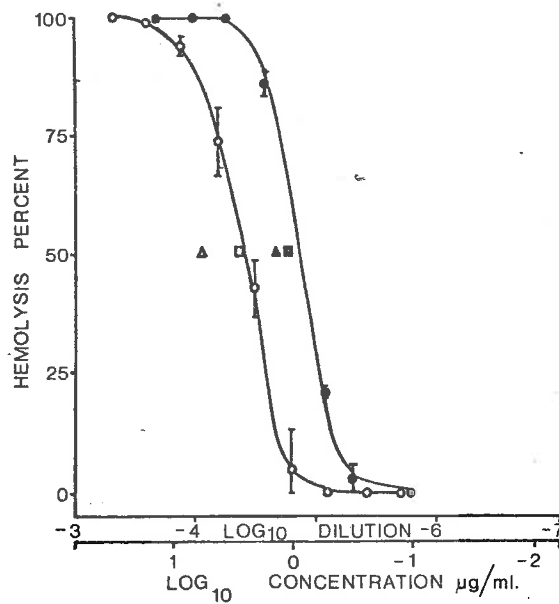


FIG.1 LOG_{10} DILUTION AND LOG_{10} CONCENTRATION PLOTTED AGAINST per cent HAEMOLYSIS.

Each point on the curve is the mean of five experiments ±SD. ○ -<u>C.fleckeri</u> haemolysin; ● -digitonin. D_{50} values for different species of red cells using <u>C.fleckeri</u> haemolysin; △-guinea pig; □-mouse; ▲ -human; ■ -rat.

the tentacle from which the material was obtained. The figures are those obtained for the first batch of extract which has a C_{50} value of 2·3 μg per ml. Using the same haemolytic system the value obtained for digitonin was 0·89 μg, which is in the same range as that reported previously (Thron, 1964).

When supplies of the first batch were depleted, a second was prepared which had a considerably higher potency and gave a C_{50} level of 0.23 μg per ml.

Factors affecting the D_{50} values of the haemolysis.

1. The species of red blood cells. The susceptibility of different types of red cells to the haemolysis varies from one species to another. Of the types tested those from the guinea pig appeared to be the most resistant and those from the rat were the most sensitive. For comparison the D_{50} values for the different cell types are included in Fig. 1.

2. The concentration of red blood cells. The D_{50} values obtained using different concentrations of cells are shown in Fig. 2. Using the spectrophotometric method it was difficult to obtain absorbance values for cell concentrations below 5×10^6 per ml, and above $1 \cdot 0 \times 10^8$ per ml. However, within this range there appears to be a linear relationship between the D_{50} values and the cell concentration. A similar type of relationship has been described for the haemolysis produced by saponin and anionic detergents (Rideal and Taylor, 1958).

3. The addition of cholesterol, lecithin, plasma and sucrose. The replacement of 1·0 ml of buffered saline with 1·0 ml of the following substances: cholesterol sol. (0·05 mg per ml), lecithin (0·1 mg per ml) or plasma 10 per cent, produced no effect on the D_{50} values obtained.

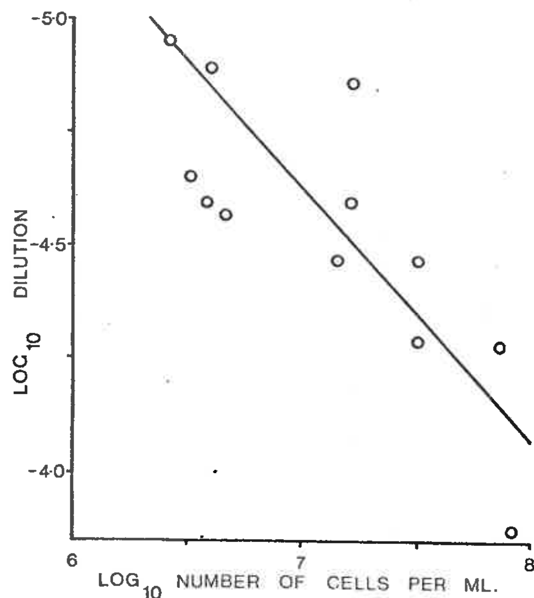


FIG.2 DILUTION OF C.FLECKERI HAEMOLYSIN REQUIRED FOR 50 per cent HAEMOLYSIS PLOTTED AGAINST THE RED CELL COUNT.

Each point represents the result of one experiment with line of best fit drawn to show correlation.

Incubation of the same concentrations of these substances with either the red cells or the extract at 25 deg. for 1 hour prior to addition to the system was also ineffective.

On the other hand, the replacement of 1.0 ml of buffered saline with a similar quantity of sucrose 5 per cent caused a two - to fourfold reduction in the D_{50} values and this could be further increased if the sucrose was incubated with the haemolysin of 25 deg. for 1 hour prior to addition to the system.

4. The potency of the haemolysin. The haemolysin proved to be relatively labile at room temperatures and this was partly dependent on the dilution; the more dilute solutions tended to deteriorate more rapidly. In practice the stock material was removed from the deep freeze, thawed and diluted just before making up the haemolytic system. For reproducible results it was always necessary to use fresh dilutions.

As it was thought that the stability of the material might be related mainly to the pH of the diluent and the temperature at which it was held, the effect of both these parameters on the potency of the haemolysin were examined. The D_{50} values obtained after 1 hour at different temperatures and at different pH values are shown in Fig. 3.

Factors affecting the rate of haemolysis.

1. The dilution of the extract. The effect of dilution of the extract on the time to produce 100 per cent haemolysis of a standard cell suspension is shown in Fig.4. A more detailed examination of these figures indicated a non-linear relationship between the dilution of extract and the rate of haemolysis. In this respect it appeared to be similar to lysis produced by saponin and ionic detergents (Rideal and Taylor, 1956, 1958) and differed from that produced by proteins or enzymes (Bernheimer, 1947).

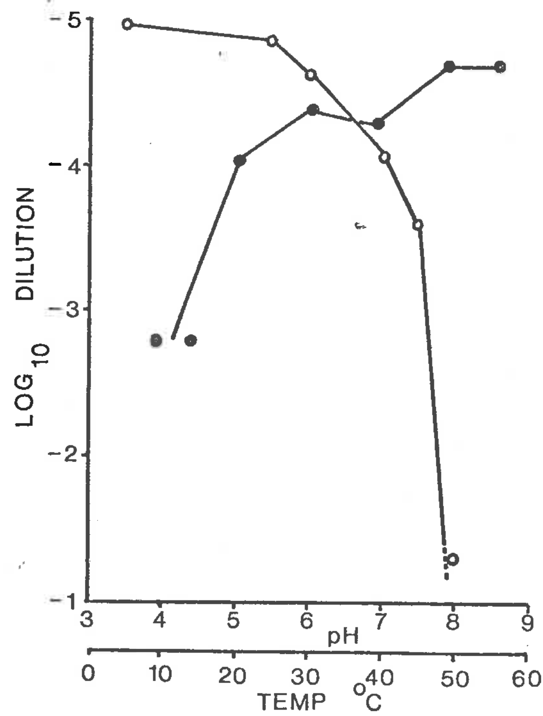


FIG.3 THE EFFECT OF pH AND TEMPERATURE ON THE DILUTION OF C.FLECKERI HAEMOLYSIS REQUIRED FOR 50 per cent HAEMOLYSIS.

○ -D_{50} values after 1 hour exposure at different temperatures; ● -D_{50} values after 1 hour exposure to diluents of different pH, at constant temperature of 25 deg.

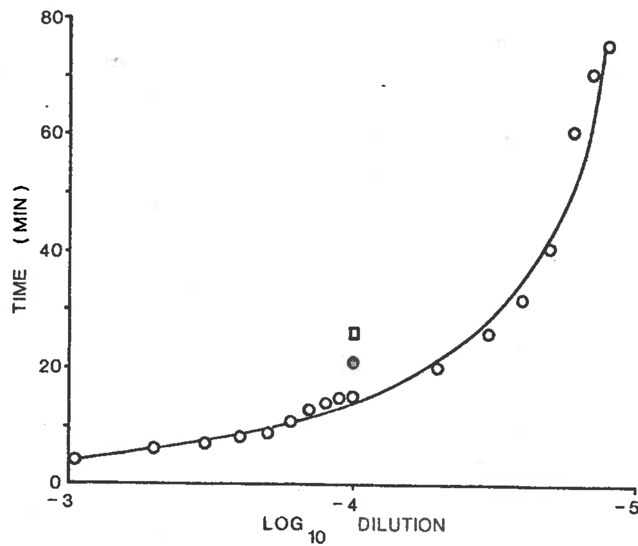


FIG.4 THE EFFECT OF DILUTION OF THE EXTRACT ON THE TIME FOR COMPLETE HAEMOLYSIS OF STANDARD CONCENTRATION OF RED CELLS ($3.0-3.5 \times 10^7/cm^3$) AT 25 DEG.

Also shown is the effect of temperature at a fixed dilution of extract. ● -10deg. ○ -25deg. □ -37deg.

An examination of the curves relating optical density (700 nm) and time to complete haemolysis indicated that with increasing dilution of the extract there is an increase in both prolytic and lytic phases of the haemolysis curve (Fig. 5).

2. Temperature. When dilute solutions were used haemolysis appeared to proceed more rapidly at temperatures below 20 deg., but this was later considered to be due to the greater stability of the haemolysin at the lower temperatures.

Using more concentrated preparations where complete haemolysis was achieved within 30 minutes the optimum temperature appeared to be between 25 and 30 deg. Above 35 deg. the rate fell markedly but this was largely due to lack of stability at the higher temperatures.

For comparison the time taken for complete haemolysis at temperatures of 10, 25 and 37 deg. for a standard dilution are shown in Fig. 4.

3. The effect of pH. A series of isotonic diluents at various pH values between 5·2 and 9 were used and the rate of haemolysis estimated by the time required for complete haemolysis. The rate was considerably reduced at pH level below 6·5 and appeared greatest between pH 8 and 9. At the lower pH levels the effect on rate might have been partly due to the reduced stability of the extract under these conditions.

4. The effect of added substances. The replacement of 1·0 ml of buffered saline with cholesterol sol. (0·05 mg per ml) or lecithin (0·1 mg per ml) caused no alteration in the rate of haemolysis.

On the other hand, if sucrose 5 per cent or plasma 10 per cent was added there was a marked reduction in the

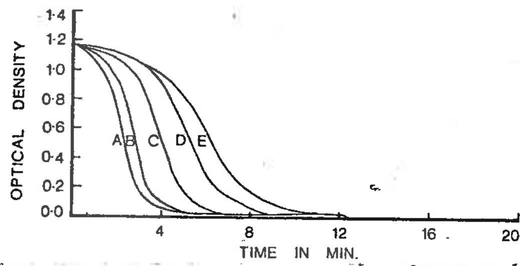


FIG.5 EFFECT OF DILUTION OF TENTACLE EXTRACT ON RATE OF HAEMOLYSIS.

A - 1:100; B - 1:200; C - 1:400; D - 1:750; E - 1:1000.
Red cell concentration 3.0 x 10^7 cells per cm^3; temperature 25deg; optical density at 700 nm.

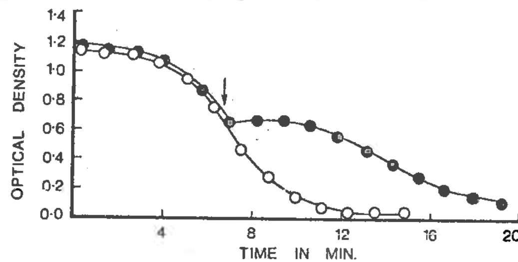


FIG.6 EFFECT OF ADDING SUCROSE ON THE RATE OF HAEMOLYSIS OF 1:1000 DILUTION OF TENTACLE EXTRACT.

0.1 cm^3 of sucrose (50 per cent) added as indicated; red cell concentration 3.0 x 10^7 cells per cm^3; temperature 25deg; optical density at 700 nm.

rate of haemolysis. The addition of equivalent quantities of plasma after haemolysis had commenced did not result in any delay, while sucrose added in the same manner was still able to cause a reduction in this rate (Fig. 6).

The replacement of 1·0 ml of buffered saline with 2×10^{-4} to 5×10^{-5} M sodium dodecyl-sulphonate or 10^{-5} to 10^{-6} M cetyltrimethylammonium bromide resulted in inhibition of haemolysis by both the anionic and cationic detergent at the highest concentration employed.

The addition of benzene or bromo-benzene appeared to increase the rate of haemolysis.

Relation between haemolysin titre and lethal properties of the extract

Four batches of extract with known varying titres of haemolysin were examined. The first was the standard extract, the second was the product of discharge of nematocysts through the amniotic membrane, the third was prepared by diluting the standard extract in a buffer at pH 5·5 and the fourth by diluting in buffer at pH 7·2 and then heating at 37 deg. for 30 minutes. In the latter two batches the D_{50} and LD_{50} were estimated on the same day. If haemolysis and lethality were due to different factors their stability to heat or changes in pH might vary and this difference would result in separation of the two activities. The result of the comparative assays of the 4 batches are shown in Table 1.

There is a definite correlation between the haemolysin and lethal properties of the extract although this relationship is non-linear. This suggests that the haemolysin is a more specific manifestation of the toxin which *in vivo* appears to have more general effects on the heart and central nervous system (Freeman and Turner, 1969).

TABLE 1

Type of extract	Haemolysin titre ± SD	LD_{50} for mice expressed in $1/(\log_{10} D \pm S.D.)$
Standard batch	5·9 ± 0·03 (5)	4·1 ± 0·08
Material obtained by discharge of nemato-cysts through amniotic membrane	4·0 ± 0·08 (5)	2·8
Standard batch diluted in buffer pH 5·5	3·3 ± 0·06 (4)	1·9 ± 0·11
Standard batch, diluted and heated 37 deg.	3·0 ± 0·11 (5)	1·1 ± 0·09

Figures in parentheses represent number of observations. As there was no gravimetric means of estimating the LD_{50}, it was expressed as a calculated dilution of the extract which would be contained in 1·0 ml.

1.4 DISCUSSION

The cell membrane is considered to be made up of a cholesterol-phospholipid-lipoprotein complex.

In the initial part of this investigation the presence of a phospholipase was excluded by examining the lipid products of haemolysis of the red cell membrane by thin-layer chromatography. No lysophosphatides could be detected. An examination was also made for proteolytic agents by using paper chromatography to identify products of protein breakdown. No proteolytic activity could be observed which agreed with the findings of Endean *et al*. (1969).

The subsequent investigation was undertaken to

determine the characteristics of haemolysis in the hope that this knowledge might assist in the broad chemical identification of the substance. The fact that it did not pass through a dialyzer membrane showed that it had a molecular weight greater than 1000 or it is coupled to a substance of high molecular weight. Although a number of other features were defined which might later assist in the isolation of the active material, no precise chemical classification was possible.

The most important of these features was the high activity of the haemolysin when care was taken to prevent deterioration in dilute solutions at room temperatures. The C_{50} for the two batches prepared was 2·3 μg per ml and 0·23 μg per ml respectively based on the wet weight of the tentacle from which they were extracted. If the haemolysin is contained in the venom of the nematocysts only, the active component must represent a very small proportion of the total weight of the tentacle. These figures should then be considered a most conservative estimate only.

A more detailed investigation of the haemolysin showed that the D_{50} was proportional to the red cell concentration, and at a given concentration it was unaffected by cholesterol or plasma. There was a non-linear relation between the rate of haemolysis and the dilutions of the extract over the range employed and the rate appeared to increase both with temperatures up to 30 deg. and with values of pH up to 9. The haemolysin was impaired by sucrose, plasma and both anionic and cationic detergents while benzene and bromo-benzene acted as accelerants.

While the above evidence may suggest that the material is an enzyme or other protein, in the case of an enzyme one would expect the rate of haemolysis to be proportional to its concentration. On the other hand, there is little

to suggest that the material would fall into the category of a steroid (Weissmann and Keiser, 1965), vitamin A (Dingle and Lucy, 1962) or an ionic detergent.

However, the high haemolytic activity might suggest that it could fall into the category of saponin or other glycoside. A precedent for saponins of marine origin with high haemolytic activity has been achieved by the chemical isolation of Holothurin A (Nigrelli et al., 1955). A haemolytic process whose D_{50} is directly proportional to the concentration of red blood cells and whose rate of reaction has a small positive temperature coefficient up to 30 deg. and is influenced by inhibitors such as sucrose and plasma and by accelerators such as benzene suggests a mechanism similar to that produced by saponin or the slow reaction of ionic detergents.

On the other hand, the lack of association with cholesterol suggests that it is not chemically similar to either saponin or digitonin. Similarly, the relatively greater activity at an alkaline pH differs from the haemolysis produced by saponins which normally proceed more rapidly in acid solutions.

As the preceding information was inconclusive, a further investigation of the haemolysin was undertaken using column chromatography (Crone and Keen, 1969). A number of procedures were used but the most concise information was obtained using exclusion chromatography on Sephadex G-200. The haemolysin was eluted a little in advance of a marker of albumin (molecular weight 67,000) and therefore it was estimated to have a molecular weight of 70,000 approximately (see Fig. 7). This was confirmed by using other marker systems. No other haemolytic component could be detected.

The examination of the Sephadex G-200 fraction for toxicity revealed the presence of toxic material

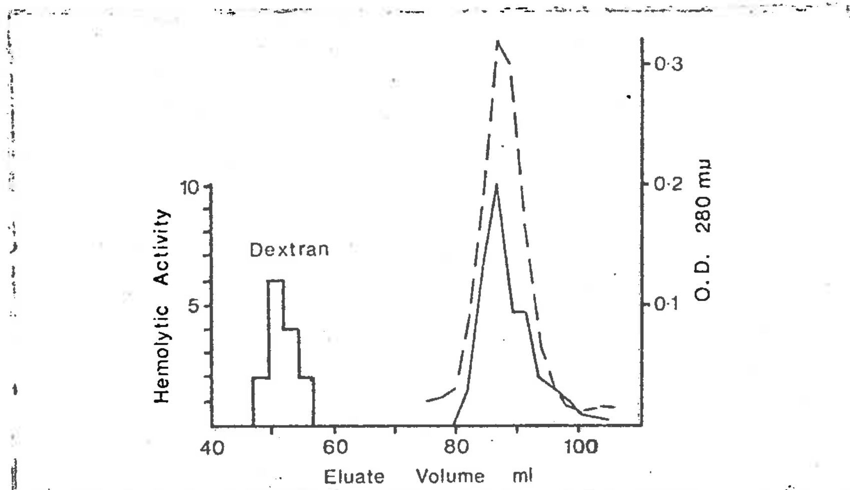


FIG.7 CO-CHROMATOGRAPHY OF THE HAEMOLYTIC EXTRACTS WITH BOVINE ALBUMIN ON SEPHADEX G-200.

The position of the haemolytic material is shown by a solid line, that of the bovine albumin by a broken line (estimated by the optical density at 280 mu). Also shown is the elution position of Blue Dextran 2000, marking the void volume of the column. Fraction volume 2.8 ml.

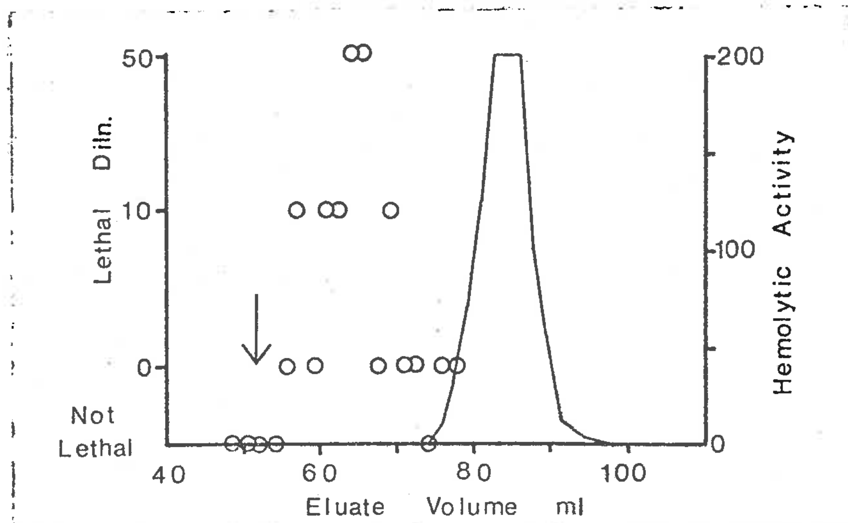


FIG.8 SEPARATION OF A SECOND LETHAL FACTOR FROM THE HAEMOLYSIN ON SEPHADEX G-200.

The solid line shows the position of haemolytic activity. The open circles show the maximum dilutions at which 0.1 ml samples of the fractions were lethal to mice. This monitoring of lethality was not carried on into the haemolytic fractions, but these were also lethal (see text).

The arrow indicates the void volume of the column.

between the void volume and the haemolytic peak. The results of an attempt to locate the material are seen in fig. 8. The position of this second lethal component was found to correspond very approximately to a molecular weight of 150,000. Although it was not demonstrated in the figure the haemolytic peak also contained lethal activity.

These results show that the haemolysin was not the only toxic material in the extracts. The haemolytic material was also lethal, but whether this lethality was an integral property of the haemolysin or due to other unresolved components could not be decided at this stage. Further work was therefore undertaken to resolve the relationship of the haemolysin to the other toxic activities.

CHAPTER 2

2. THE RELATION OF THE HAEMOLYSIN OF C.FLECKERI TO THE LETHAL AND DERMATONECROTIC ACTIVITIES - ANTIGENIC PROPERTIES OF THE EXTRACT.

2.1 I N T R O D U C T I O N

When administered to experimental animals, tentacle extracts of *C. fleckeri* may be shown to have haemolytic, lethal and dermatonecrotic activities.

Previous work has described the chromatography of tentacle extracts using Sephadex G-200 and characterization of two fractions, one with lethal activity (molecular weight 150,000) and the other with both haemolytic and lethal activity (molecular weight 70,000). The pharmacology of the fractions has been examined in detail (Freeman and Turner, 1969) and each produced cardiovascular and respiratory failure. Also both toxins reduce the rate, amplitude of contraction and the coronary flow in the isolated perfused guinea pig heart (Turner and Freeman, 1969). However, using a mouse unit as a measure of lethality these workers found that the fraction containing the haemolysin appeared to be less potent than the other higher molecular weight fraction.

The two fractions cause a rise in arterial pressure in anesthetized rats and rabbits which was shown to be due to a direct action on vascular musculature. These changes were followed by hypotension, bradycardia and cardiac irregularities. An increase in respiratory rate is followed by apnoea and fluctuations in arterial pressure. The cardiovascular effects of the two toxins

were thought to be due to direct vasoconstriction, cardiotoxicity, baroreceptor stimulation and possibly depression of the vasomotor centre (Freeman and Turner, 1971).

The lethal properties of tentacle extracts and the two fractions were therefore well defined, but it was necessary for further work to be undertaken to characterize the dermatonecrotic activity and to establish a method of assay. It would then be possible to make a quantitative estimate of the three activities in material prepared in different ways and also in fractions obtained by chromatography. This would assist in establishing whether the haemolysin was a separate component, or only a single property of a component with diverse activities.

Further information could also be obtained by preparing antisera against the tentacle extracts. Investigations could then be undertaken using immunodiffusion studies in agar gels to observe whether precipitating antibodies to the various components were formed. At the same time titres of antibodies to the three components could be estimated in the various fractions and the whole extracts.

2.2 M E T H O D S

The method of preparing the aqueous extracts from the whole tentacle and the estimation of the dilution required to produce 50 per cent haemolysis of a standard concentration of red cells (D_{50}) has been described in Chapter 1.

Chromatography on Sephadex G-200

The preparation of the active fractions of the extracts by chromatography on Sephadex G-200 was made on

a column of internal diameter 2.5 cm and the bed height was 33 cm. The buffer employed for this column was 25 mM Tris-HCl, 150 mM NaCl, pH 8.0. The flow rate at the operating pressure of 20 cm of water was 5.6 ml per hour, and 2.8 ml fractions were collected.

The void volumes of each column were estimated by the use of Blue Dextran 2000 and all operations were carried out at 5 deg.

Fraction I consisted of the eluate collected between the void volume and the commencement of the haemolytic peak. Fraction II was a small quantity of material collected at the beginning of the haemolytic peak, while fraction III consisted of the major volume of fluid containing haemolysin. In order to obtain fractions of smaller volume a second chromatographic separation of tentacle extract on Sephadex G-200 was undertaken. Fractions IA and IIIA represent the volume of eluate collected over a range which was estimated to represent the peaks of activity in fractions I and III.

Column Chromatography on Cation Exchange Sephadex

CM-Sephadex C-50 was allowed to swell in a buffer of 50 mM NaCl, 10 mM sodium phosphate pH 6.5 and equilibrated with this buffer by repeated decantation and resuspension. A column was then packed with the gel (bed size 1.6 cm diameter by 16 cm high) and the sample applied. The column was eluted with 65 ml of the above buffer at a flow rate of 16 ml/hr. After this a linear gradient of NaCl was applied which was formed by 200 ml of the above mentioned buffer in a mixing chamber, replenished by 200 ml of 0.5 M NaCl, 10 mM sodium phosphate pH 6.5. Finally the column was eluted with buffer containing 1M NaCl. All operations were carried out at 5 deg. (Crone and Keen, 1971).

Estimation of Dermatonecrotic Activity

The minimum dilution of extract causing skin necrosis was determined either in guinea pigs weighing 400 - 600 g or in Wistar rats weighing 200 - 300 g. The skin used was on the dorsal surface, and the area was prepared by shaving on the day of the experiment. Serial double dilutions of the extract or venom were prepared in isotonic phosphate buffered saline at pH 7.2. As a routine, eight dilutions were used, the initial dilution being selected by preliminary experiments. Using a microsyringe, 0.05 ml of each was injected i.c. into separate areas outlined on the skin. Controls of buffered saline and heat inactivated extract were used in each case. After inoculation the animals were observed at 30 min, 1 hour, 4 hour and 24 hour for changes in colour, swelling, vesiculation and necrosis around the sites of administration. The final readings were made after 48 hours when the minimum dilution producing at least 5.0 mm of necrosis in its maximum diameter was taken as the end point. This was expressed as the logarithm of the dilution of extract or toxin.

Estimation of Toxicity (LD_{50})

The i.v. toxicities of the extracts were estimated by measuring the LD_{50} in albino mice weighing 15 - 20 g. Four groups of four animals were used for each estimation and four dose levels were employed in a constant volume of 0.1 ml. The LD_{50} estimations were calculated from tables and expressed as the logarithm of the reciprocal of the dilution in 1.0 ml of the original extract (Weil, 1952).

Preparation of Antisera and Estimation of Antibody Titres

The preparation of antisera was undertaken in rabbits. The procedure proved to be difficult as a

number of animals died during the course of the immunization. An initial dose equivalent to three LD_{50} for mice was given i.v. to rabbits twice weekly for a month and then they were rested for a month. A further series of injections were given, increasing the dose slowly up to 10 LD_{50} by the end of a further 4 weeks. Serum was obtained by bleeding the animals by intracardiac puncture, allowing the blood to clot overnight, and decanting the serum. It was then cleared by centrifugation, heated at 56 deg. for 30 minutes and stored at -20 deg. until required.

The antiserum was tested for antibodies against the lethal, haemolytic and skin necrotizing properties of the extracts.

Tests for antibodies against the lethal factor were made in a series of 10 tubes using double dilutions of the antisera in buffered saline. A dilution of the extract was added to each tube so that five LD_{50} was present in 0.1 ml of the mixture. The mixture was allowed to stand for 30 minutes at a temperature of 25 deg. and it was then tested for lethality by injecting 0.1 ml into the lateral tail vein of mice. The minimum dilution of serum neutralizing five LD_{50} of extract could then be estimated.

A similar technique was used to measure antihaemolysins. Serial double dilutions of antisera were made up in 10 tubes and to each was added 1.0 ml of a dilution of extract containing 50 times the estimated O_{50} of haemolysin. This was incubated for 30 minutes at 25 deg. and then 1.0 ml of a standard red cell concentration was added. The tubes were allowed to stand for 5 hours in a water bath at 25 deg. The maximum dilution of serum which could completely neutralize the haemolysin was estimated. Controls of normal serum and buffered saline were used.

Titres of antibodies to the dermatonecrotic factor were estimated by making double dilutions of venom in a series of eight tubes, adding an equal volume of one in four dilution of antiserum and incubating at 25 deg. for 30 minutes. 0.05 ml from each was then injected intradermally in the manner previously described and compared with two series of controls using normal serum and buffered saline.

Immunodiffusion in Agar

Immunodiffusion studies on the haemolysin and chromatographic fractions were carried out in a medium containing either 1 per cent special grade agar (Oxoid), or in a similar medium to which 10 per cent rabbit red cells had been added. The red cells were prepared from heparinized blood, washed three times and then reconstituted to their original volume with phosphate buffered saline and added to the agar at 48 deg. Sufficient agar was used to give a thickness of 1 - 1.2 mm in the petri dishes.

Double diffusion experiments were carried out on both types of plates using extracts and antisera. The plates were left for 72 hours at 5 deg.C and then stained with Amidoblack 10B (Wieme, 1965) and examined for haemolysis.

2.3 R E S U L T S

The dermatonecrotic activity of extracts.

Necrosis and damage to the skin of experimental animals could be produced by extracts obtained from homogenized tentacle, toxin obtained by discharge of

nematocysts through the amniotic membrane, and with one of two active fractions prepared by chromatography on Sephadex G-200. The types of lesions produced by the three types of preparations were identical and with the exception of fractions I and IA obtained on chromatography the minimum necrotic dose appeared to follow the over-all potency of the extracts as measured by both lethal and haemolytic activity. The minimum necrotic dose of the various materials is shown in Table 1.

After injection of 0.05 ml of an active extract into either guinea pigs or rats no immediate reaction was observed in the skin. There was a discrete swelling in the skin which was mainly due to the volume of the inoculum with some reddening around the periphery. Approximately 15 minutes after the administration of very active extracts, a bluish colouration was often seen under the site of inoculation in those areas which would later progress to necrosis. This appearance was most frequently observed in guinea pigs and it was a less consistent finding in rats. Vesiculation over the area of injection was a most uncommon finding.

After 4 hours some areas already showed patches of brownish discolouration and the skin appeared devitalized. At the end of 24 hours these areas had become discrete brown patches having a firm texture of dead skin, although in rats it was occasionally necessary to wait for 48 hours for this appearance to be complete.

After death of the skin there is a slow process of separation from the viable tissues which occurs over the next 10 to 14 days. Provided there is no secondary infection the inflammatory reaction is not marked.

An examination of the effects of the toxin in rats in which 1 per cent trypan blue had been injected 30

minutes before the i.c. inoculation confirms the previous observation that there is little or no general increased vascular permeability at the site of the lesion. At the end of 4 hours the central area appears as a pale plaque surrounded by a blue rim, indicating increased vascular permeability at the periphery only.

The relation between necrotic, lethal and haemolytic activities.

The estimation of the relative activities for several batches of extract are shown in Table 1. These results show that there is a correlation between the three types of activity in both crude extracts prepared from tentacle and also in purified preparations obtained by discharge of nematocyst toxin through human amniotic membrane.

TABLE 1. THE RELATION BETWEEN DERMATONECROTIC, HAEMOLYTIC AND LETHAL ACTIVITIES OF VARIOUS PREPARATIONS.

Batch No.	Minimum skin necrotising dose Log_{10}(dilution)*	LD_{50} in mice** Log_{10}(dilution) ± S.D.	Haemolysin titre Log_{10}(dilution) ± S.D.
Batch 2 of tentacle extract	2.1 (2)	4.1 ± 0.08	5.9 ± 0.3(5)
Batch 4 of tentacle extract 23/10/68	1.6 (1)	3.7 ± 0.08	5.4 ± 0.11(5)
24/ 3/69	1.5 (6)	N.E.***	4.4 ± 0.03(3)
Batch 5 of tentacle extract	2.0 (6)	3.2	5.3 ± 0.13(5)
Material obtained by discharge of nematocyst through amniotic membrane	0.9 (2)	2.8	4.0 ± 0.08(5)
Sephadex G-200 Fractions			
Fraction I	N.A.(2) @	2.5 ± 0.01	1.6 (2)
Fraction IA	N.A.(2) @	N.E. ***	<1.5 (2)
Fraction III	0.9 (2)	2.7 ± 0.01	4.8 (2)
Fraction IIIA	0.6 (2)	N.E. ***	4.5 (2)

* The minimum skin necrotizing dose is expressed as the logarithm of the dilution of extract or toxin contained in 0.05 ml which will cause 5.0 mm of skin necrosis in rats or guinea pigs.

** The LD_{50} was expressed as a calculated dilution of the extract or toxin which would be contained in 1.0 ml.

♀ N.A. No activity in undiluted samples.

*** N.E. Not examined.

Figures in parentheses represent the number of experiments.

These findings alone could suggest that the three types of activity might be the manifestation of a single substance. However, previous work employing chromatography with Sephadex G-200 has shown that two lethal fractions can be obtained, one with a mol. wt. of 70,000 approximately and the other with a mol. wt. 150,000 approximately (Crone and Keen, 1969). The former contained in fractions III and IIIA has been shown to have both haemolytic and dermatonecrotic activity, while fractions I and IA have little or no haemolytic activity and no dermatonecrotic activity.

The antigenic properties of tentacle extracts.

It was difficult to produce high titre antiserum in rabbits; a course of twice weekly injections being required for 8 weeks before satisfactory material was obtained.

When 2.0 ml of this antiserum was given 24 hours before an intradermal inoculation of extract it failed to prevent or modify the necrosis. However, this may be partly due to the relatively low titres of the antiserum for the dermatonecrotic factor.

When the same antiserum was mixed with an extract of a known potency and held at 25 deg. for 30 minutes prior

to inoculation a definite increase in the minimum necrotizing dose was observed. This is shown in Table 2.

The antiserum had been shown to have antibodies to the lethal and haemolytic activities, the titre against these components being shown in Table 2.

TABLE 2

Component		Neutralising titre
Lethal factor	Whole extract	40
	Sephadex fraction I	320
	Sephadex fraction III	80
Haemolysin		3000
Dermatonecrotic Factor		16

The neutralizing titre is expressed as follows: The number of LD_{50} neutralized by 1.0 ml of undiluted serum; the number of D_{50} of haemolysin neutralized by 1.0 ml of undiluted serum, and the number of minimum skin necrotizing doses neutralized by 1.0 ml of undiluted serum.

Diffusion of Extracts in Agar

No precipitation bands could be detected after staining with Amidoblack 10B and perhaps this was due to the low protein content of the extract and the Sephadex fractions (Fig. 1).

On the blood agar plates circular areas of haemolysis could be observed around the wells containing the extracts and the fractions IA and IIIA, which constituted the peak of activity of the cardiotoxic lethal activity (C) and the peak of the haemolytic activity (H). Inhibition of lytic

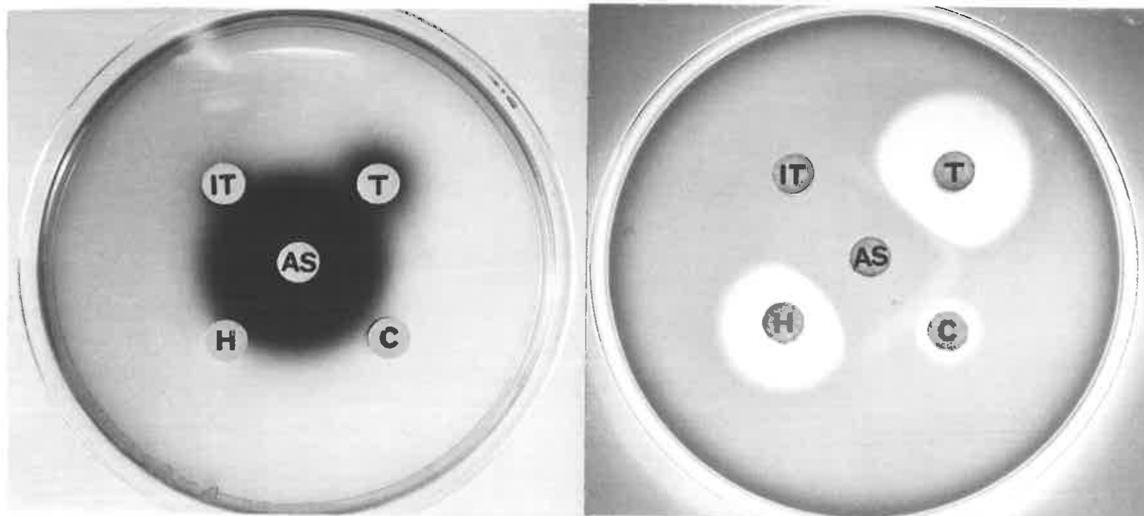


FIG 1 FIG 2

FIG.1 DOUBLE DIFFUSION IN 1 per cent AGAR AT 5DEG. FOR 72 HOURS.

T - tentacle extract (undiluted); IT - heat inactivated tentacle extract; C - lethal fraction; H - haemolytic fraction; AS - C.fleckeri antiserum; 25 μ litre of each antiserum and extract.

FIG.2 DOUBLE DIFFUSION IN AGAR CONTAINING 10 per cent BLOOD AGAR.

T - tentacle extract (undiluted); IT - heat inactivated tentacle extract; C - lethal fraction; H - haemolytic fraction; AS - C.fleckeri antiserum; 25 μ litre of each antiserum and extract.

activity occurs in the areas adjacent to the well containing the antiserum. The significance of the band in front of the cardiotoxic fraction has not been determined (Fig.2).

Similar types of lytic activity are seen with four representative samples prepared by CM-Sephadex-C50 chromatography. Fraction 4 was the beginning of the eluate, fraction 30 the cardiotoxic peak, fraction 41 the haemolytic peak and fraction 60 the end of eluation volume approximately (Fig. 3). Inhibition occurs in areas adjacent to the antiserum. Again some lytic activity is seen around the cardiotoxic fraction and there is a pale band between it and the well containing the antiserum.

2.4 D I S C U S S I O N

From the results of these experiments it is still uncertain whether the three activities of the extracts and the toxin, namely dermatonecrotic, haemolytic and lethal are due to one component or separate components. So far, chemical analysis of the extracts has produced two fractions with approximate molecular weights of 70,000 and 150,000. Dermatonecrotic activity could only be demonstrated in the material with the lower molecular weight and in this respect it is similar to the material with haemolytic activity.

The antiserum proved difficult to prepare and satisfactory titres could only be obtained after several weeks of carefully graded doses of extracts. In order to achieve a satisfactory immunological response, it was necessary to inject doses at a level just below the calculated lethal dose and as a result some animals died from accidental overdosage. Attempts to reduce the dose or to employ material which had been "toxoided" resulted in a

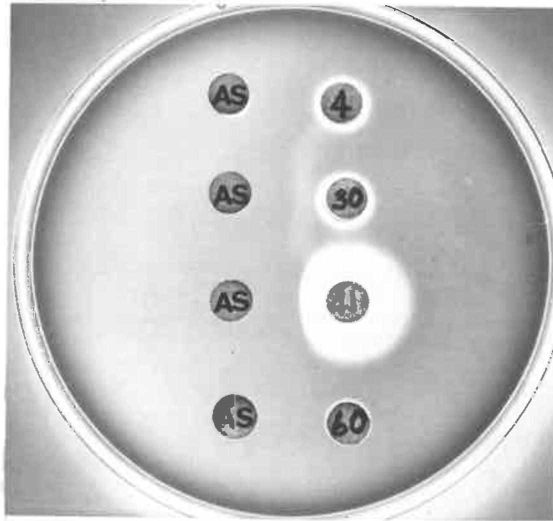


FIG.3 DOUBLE DIFFUSION IN AGAR CONTAINING 10 per cent BLOOD AGAR.

4 - early eluate; 30 - lethal fraction;

41 - haemolytic fraction; 60 - late eluate;

AS - C.fleckeri antiserum; 25 μ litre of each antiserum and extract.

poor response to immunization. It would have been advantageous to produce antisera against the two chromatographic fractions. However this was impractical as both fractions were very labile and satisfactory supplies of potent antigen could not be maintained.

The antiserum could be shown to have neutralizing properties against the lethal, haemolytic and dermatonecrotic activities. High titre serum was required to inhibit the dermatonecrotic activity while the haemolytic activity was the most sensitive to neutralization and for practical purposes provided the easiest method of checking the activity of extracts and titres of antisera.

The inability to obtain a diffusion pattern with precipitin bands excluded the method of double diffusion in agar as a means of identifying the different protein fractions in the tentacle extracts. Parameters such as the concentration of the antiserum and the toxin, diffusion distance and time, pH of medium, and the temperature were varied without successfully producing resolution into bands. It was concluded that the concentration of protein in the active fractions was so low that detection of bands was not possible.

The use of 10% red cells in the agar provided a means of identifying fractions with haemolytic activity and haemolysis could be shown to be specifically inhibited by antiserum. Bands of haemolysis in front of the main haemolytic zone were observed and they could be accentuated by allowing the diffusion to proceed at temperatures of 25 deg. or higher. Liesegang bands (Glasstone, 1947) could also be seen in some cases. The precise nature of these changes would require further investigation.

Other workers (Baxter and Marr, 1969) reported findings on the "milked" toxin which are difficult to reconcile

with these results, e.g. the assignment of molecular weights in the range 10,000 to 30,000 for the biologically active molecules. As a result a comparative examination of the tentacle extracts and the "milked" toxin were undertaken using different chromatographic techniques (Crone and Keen, 1970).

The results indicated that tentacle extracts and the "milked" toxin contained the same active components in approximately the same proportions.

The chromatography on CM-Sephadex was the best separatory method found for isolating the two toxins, yielding sharp peaks in a very reproducible manner. Since the two toxins are not separated by chromatography on CM-cellulose the separation on CM-Sephadex must be mainly a size effect, the larger non-haemolytic toxin being denied access to some of the exchange groups on the matrix and thus being eluted earlier. The further chromatography of this toxin on G-200 Sephadex confirmed its higher molecular weight.

CHAPTER 3

3. THE ROLE OF THE HAEMOLYSIN OF *C. FLECKERI* IN ENVENOMATION IN HUMANS AND EXPERIMENTAL INTOXICATION IN ANIMALS.

3.1 I N T R O D U C T I O N

Cleland and Southcott (1965) have reviewed the histories and clinical symptoms associated with approximately 50 deaths which have been attributed to *C.fleckeri* and *C. quadrigatus*. Barnes (1966) has described the signs and symptoms of stingings in humans which consist of severe pain around the site of injury, and local lesions consisting of multiple transverse weals with discolouration and oedema of the skin, which later may become necrotic. In more extensive stings, death may occur within minutes of injury. Collapse is associated with respiratory arrest, but the clinical appearance of the victim suggests cardiovascular rather than respiratory failure. Recovery from a severe state of collapse may occur in occasional cases, and those patients surviving for more than 20 to 30 minutes after the injury usually recover uneventfully. However there have been no reports suggesting intravascular haemolysis either as an immediate effect of the stinging or as a delayed effect in survivors.

In experimental animals Freeman and Turner (1969) have reported elevated potassium levels in the blood following doses of tentacle extract. They have attributed the changes to intravascular haemolysis.

In view of the established potency of the haemolysin the absence of haemolysis in clinical cases appeared unusual. However, certain properties of the haemolysin

have been described in Chapter 1 which might provide possible reasons for the lack of haemolysis *in vivo*. The most important would appear to be the inhibition of the rate haemolysis produced by serum proteins and glucose. The lack of stability at body temperature, particularly in dilute solutions, would also contribute to the absence of findings.

The purpose of this investigation was, therefore, to examine the combined effect of temperature and serum proteins on the activity of the haemolysin and the release of potassium from the red cells. Further experiments were carried out also to confirm the observations of Freeman and Turner.

3.2 M E T H O D S

Methods of collection and identification of specimens, the preparation of extracts, and the technique of estimating the rate of haemolysis and the factors affecting it have already been described in Chapter 1.

Assay method for per cent haemolysis.

A haemolytic system contained 2.0 ml of red cells (concentration 3.0 - 3.5 x 10^7 cells per millilitre), 2.0 ml of phosphate-buffered saline at pH 7.2, and 2.0 ml of a dilution of a batch of tentacle extract (1:50,000), which was known to produce 100% haemolysis at 25 deg.C in 60 minutes approximately. Twenty tubes with a control were used for each experiment. A tube was removed from the bath at four-minute intervals, and a fraction of the contents taken for estimation of the O.D. in a spectro-

photometer (Unicam SP600) at 700 nm. Using a calibration curve the per cent haemolysis could be obtained.

Estimation of potassium release from red cells.

The remainder of the cell suspension in the tube used to measure haemolysis was centrifuged at 3,000g, and the supernatant removed immediately for estimation of the potassium levels by flame photometry (EEL). The total time of centrifugation was confined to 4 minutes. Experiments were carried out at 25 deg. and 37 deg.C.

Effect of temperature and plasma in per cent haemolysis and release of potassium.

In the experiments carried out at 37 deg., a 1:10 dilution of plasma in 2.0 ml of phosphate-buffered saline was included in order to observe the effects of both plasma and body temperature on the haemolysis and release of potassium.

Intravascular release of potassium.

This was undertaken on Wistar rats weighing between 200 and 400g. They were anaesthetized by administration of pentobarbital (40 mg/kg) given intraperitoneally. Samples of blood were obtained by an indwelling needle in the caudal artery. A lethal dose of tentacle extract was administered intravenously and blood samples were taken at approximately 2 minute intervals. The blood was spun at 3000g for a total time of 4 minutes and the plasma separated. Potassium levels were estimated as described previously.

At the same time the electrocardiogram was recorded from leads connected by skin electrodes to the right and left forelegs, and the abdominal respirations were monitor-

ed by a colloidal-carbon-in-rubber-tube pneumograph placed across the abdomen below the xiphisterum. The output from the electrocardiogram and pneumograph couplers was recorded on a Beckman type R dynograph recorder.

3.3 R E S U L T S

The effect of temperature and plasma on the percent haemolysis and the release of potassium.

In vitro, the haemolysis is accompanied by the release of intracellular potassium. Figure 1 shows that the level of potassium released by the red cells at 25 deg.C is related to the degree of haemolysis. There is no evidence that the release of potassium precedes haemolysis. When the temperature is raised to 37 deg.C and the haemolysis is allowed to occur in the presence of plasma, there is a considerable reduction both in the percentage of haemolysis and in the amount of potassium released.

The release of potassium from red cells *in vivo*.

In vivo a lethal dose of tentacle extract produced a rise in the serum potassium level. In two experiments it rose from a level of 3.1 mM to 6.6 and 5.0 mM respectively. Whether this was due to the haemolysin only could not be ascertained as the degree of haemolysis in the samples could not be estimated.

The changes in the E.C.G. could not be directly related to the levels of potassium in the plasma. The E.C.G. showed a decrease in rate with an increase in the

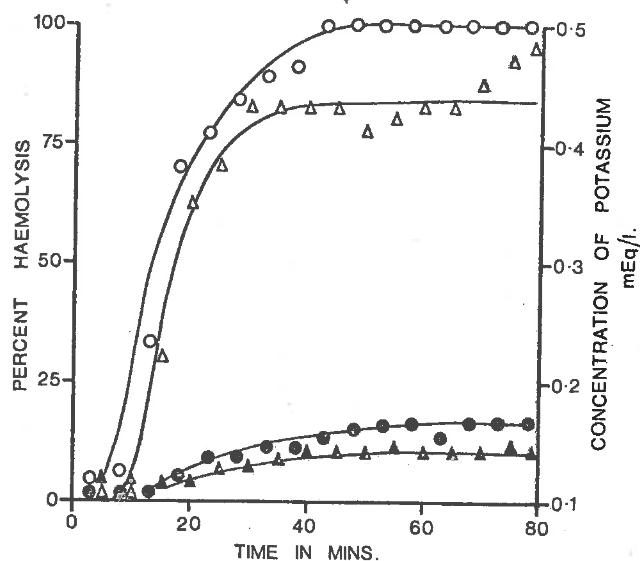


FIG. 1 The effect of temperature and the presence of plasma on the release of haemoglobin and potassium from rabbit erythrocytes by Chironex fleckeri tentacle extract. At 25 deg. ○-haemolysis percentage; △-potassium released. At 37deg. in the presence of plasma (1:10 dilution); ●-haemolysis percentage; ▲-potassium released.

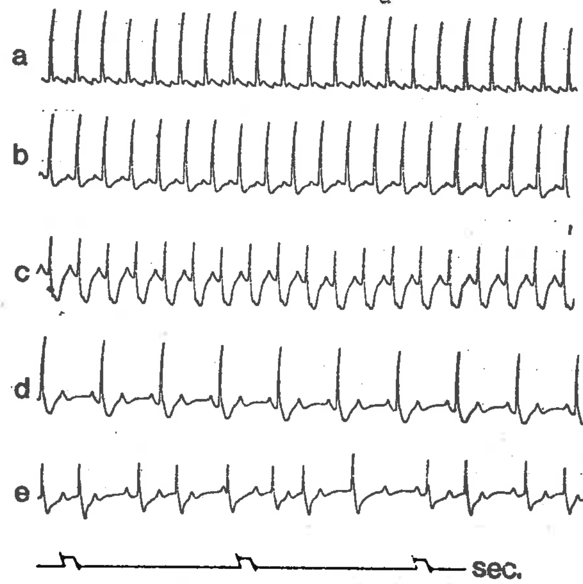


FIG.2 Changes in the electrocardiogram of the rat following the administration of a lethal dose of Chironex fleckeri tentacle extract.

(a) normal; (b) and (c), degrees of S-T depression; (d) and (e), bradycardia and partial heart block.

P-R interval, and these changes progressed to partial and then complete heart block. There was usually little alteration in the QRS complex, but there was usually an increase in the Q-T interval with elevation or depression of the S-T segment, suggesting changes in repolarization of the cardiac muscle. These are shown in Figure 2.

3.4 D I S C U S S I O N

These results confirmed the absence of haemolysis in vivo following the administration of tentacle extracts to experimental animals. This was apperently due to the inhibitory effect of plasma constituents on the lysin and the effect of temperature on its stability.

However, there was a rise in the level of potassium in the plasma following a lethal dose of extract which confirmed the observations of Freeman and Turner (1969). Although it was presumed that the elevated levels were the result of potassium released from red cells, there was no complete assurance that this was the case for detectable haemolysis did not accompany the rise. The elevated potassium levels might arise from cells other than red cells as the administration of tentacle extracts has also been shown to have effects on other cells in the body. Likewise the changes could be produced by the lethal cardiotoxic fraction rather than the haemolytic fraction as the former has been shown to produce changes in the cell membrane particularly cardiac muscle.

Neither the haemolytic nor cardiotoxic components of the extract produced a change in the plasma potassium levels which would be adequate to explain the gross

alterations in the E.C.G. These changes were attributed to the direct result of both components on the heart. Separate effects of the fractions on the heart could not be identified as their modes of action have been shown to be almost identical (Turner and Freeman, 1969; Freeman and Turner, 1971).

Therefore, haemolysis appears to be mainly a property of the toxin which has been observed in vitro and although some evidance of red cell damage might be seen in vivo haemolysis is not a marked feature of envenomation. The absence of intravascular haemolysis in clinical cases tends to be consistent with the above findings.

CHAPTER 4

4. COMPARATIVE STUDY OF THE HAEMOLYSIN DERIVED FROM TENTACLE EXTRACTS OF *CHIROPSALMUS QUADRIGATUS* AND *CHIRONEX FLECKERI*

4.1 INTRODUCTION

The two species of box jellyfish *Chironex fleckeri* and *Chiropsalmus quadrigatus* are toxic hazards to man. Both are similar in appearance although *C. fleckeri* appears to reach a larger size (Barnes, 1966). They can be distinguished on a morphological basis, but the differences are slight and may become apparent only after fixing in a suitable preservative.

The pharmacological properties of extracts of the tentacles of *C. fleckeri* and *C. quadrigatus* are also very similar. Chromatographic separation of an extract of *C. quadrigatus* on a column of Sephadex G-200 produced two peaks of activity; one haemolytic and the other lethal when injected into mice and rabbits. The activity of the haemolytic fraction was insufficient to establish whether it was lethal also (Freeman and Turner, 1972). The elution behaviour of the two peaks indicated that the molecular weight of the haemolytic fraction was approximately 70,000 and that of the lethal fraction approximately 150,000 which was identical to the molecular weight of the two fractions obtained on chromatography of extracts of *C. fleckeri*.

The purpose of this investigation was to examine extracts of the tentacle of *C. quadrigatus* for haemolytic activity and to compare this property with that previously described for *C. fleckeri*.

4.2 METHODS

The specimens of C. quadrigatus were collected and identified by Dr. J. Barnes, Cairns, Queensland, Australia. The collection and storage was similar to that described for specimens of C. fleckeri in Chapter 1.

Preparation of Extracts

Whole tentacle extracts were used for all experiments. The extracts of C. quadrigatus were prepared by mincing the frozen tentacle and suspending it in a buffer containing 150 mM sodium chloride and 5 mM tris-(hydroxymethyl)aminomethane(tris)(pH 8.0). This buffer was chosen since a portion of the extract was to be used for chromatographic studies. The suspension was centrifuged at 2500g for 20 minutes at 5 deg. and the supernatant stored at -20 deg. until required. The initial batch which was used for most of the quantitative assays was prepared from 4.35g of tentacle and 10 ml of buffer. Two other batches were prepared similarly, containing 400 mg of tentacle per ml of buffer. The extract of C. fleckeri had been prepared by homogenizing 8.716g of tentacle in 12 ml of phosphate buffered saline (Ponder, 1948) pH 7.20 at 5 deg. The material had been centrifuged at 2500g for 20 minutes and the supernatant stored at -20 deg.

Estimation of Haemolysin Titre (D_{50}), Lethal Activity (LD_{50}) and Skin Necrotizing Dose (SND)

The methods have been described previously in Chapters 1 and 2.

Preparation of Antisera

In order to prepare antisera an initial dose of extract equivalentto 3 LD_{50} (mice) was given i.v. to rabbits twice a day, 3 days a week for the first week. Injections were then given three 3 times a week, the dose being gradually increased until it was ten times the initial dose. At the end of 4 weeks immunization, animals were bled by intracardiac puncture. The blood was allowed to clot and the serum decanted. It was then cleared by centrifugation, heated to 56 deg. for 30 minutes and stored at -20 deg. The preparation of C. fleckeri antiserum has been described previously in Chapter 2. The procedures for estimating antibodies against the lethal and dermatonecrotic factors, and haemolysins have also been described.

Immunodiffusion in Blood Agar

The preparation of blood agar plates has been described previously in Chapter 2.

Reagents and Buffer Solutions

The isotonic solutions used to measure the effect of pH on stability of the extracts and the rate of haemolysis were buffered with disodium phosphate and potassium dihydrogen phosphate between pH 6 and 8 and with tris-hydrochloric acid between pH 7.5 and 9. Lecithin was prepared from egg yolk by the method of Dawson (1963).

4.3 R E S U L T S

Haemolytic Activity and Estimation of D_{50} values for C. quadrigatus Extracts

Estimations of the D_{50} were carried out at temperatures of 5, 15, 25 and 35 deg. (Fig. 1). The values

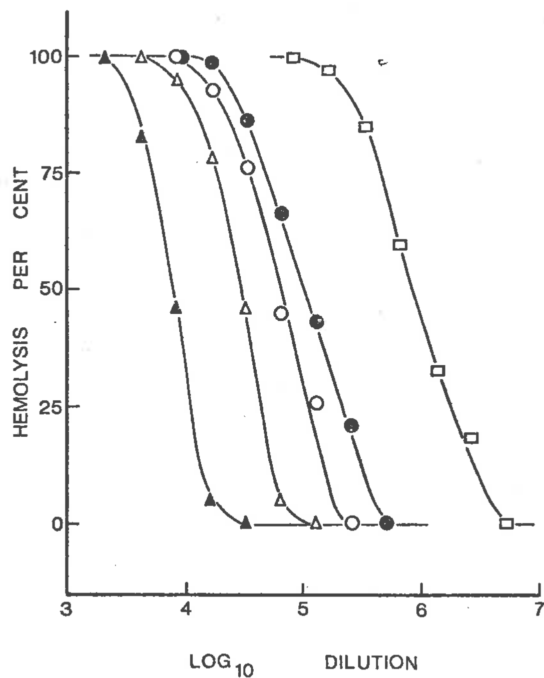


FIG.1 HAEMOLYSIS PRODUCED BY <u>C.QUADRIGATUS</u> AND <u>C.FLECKERI</u> AT DIFFERENT TEMPERATURES .

Log_{10} dilution plotted against per cent haemolysis ($3.0 - 3.5 \times 10^7$ cells per cm^3).

<u>C.quadrigatus</u> ○ - 5deg; ● -10deg; △ -25deg. ▲ -35deg.

<u>C.fleckeri</u> □ -25deg.

were 4.7, 5.0, 4.5 and 3.9 respectively indicating that the optimal temperature for haemolysis was 15 deg. A similar experiment was carried out with the _C. fleckeri_ extract which gave D_{50} values of 5.2, 6.0, 6.0 and 5.4 respectively.

The sigmoid curves obtained when graphing per cent haemolysis against dilution indicate the variable susceptibility of red cells to the haemolysin of _C. quadrigatus_. The shape of the curves is therefore not markedly affected by change in temperature. However, the displacement of the curves along the abscissa indicates that the activity of the extract is temperature-dependent.

The haemolysis curve produced by the extract of _C. fleckeri_ at 25 deg. (Fig. 1) is comparable with those of _C. quadrigatus_. This suggests that the susceptibility of the red cells to the haemolysin of both extracts is similar.

Factors affecting the D_{50} values of the haemolysin of _C. quadrigatus_

The stability of _C. quadrigatus_ and _C. fleckeri_ haemolysins was examined at 35 deg. and 40 deg. The fall in D_{50} values for the two extracts was plotted against the time of exposure at either 35 deg. or 40 deg. (Fig. 2). Statistical analysis of the results indicates that there is a significant difference in the stability of the haemolysin of _C. quadrigatus_ and _C. fleckeri_ at 35 deg. ($p<0.001$) and 40 deg. ($p<0.01$). There also appeared to be variations in stability of different batches of extracts of _C. fleckeri_ and _C. quadrigatus_ but the exact cause of this is not known.

The stability of _C. quadrigatus_ extracts was examined over a range of pH values from 4 to 9. Samples

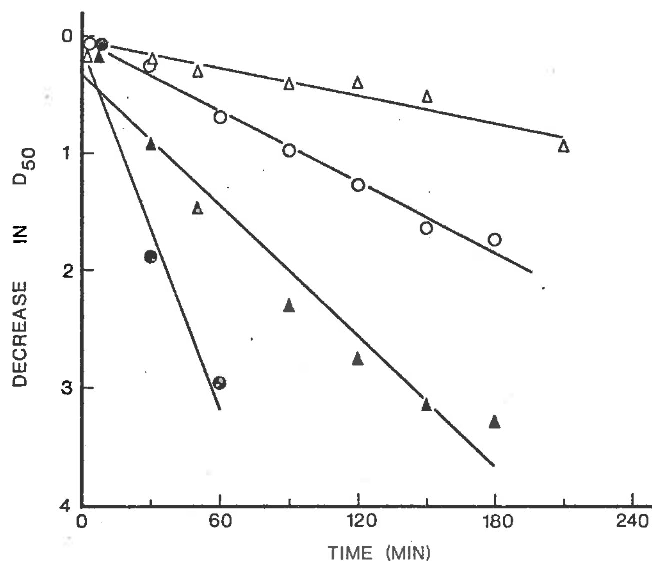


FIG.2 EFFECT OF TEMPERATURE ON THE STABILITY OF THE HAEMOLYSIN OF C.QUADRIGATUS AND C.FLECKERI.

Each point represents the result of one experiment. Lines fitted by the method of least squares regression. *C.quadrigatus*: ○ =35deg; ● =40deg. *C.fleckeri*: △=35deg; ▲ =40deg. Extract of *C.quadrigatus* in tris buffered saline, pH 8.0, and extracts of *C.fleckeri* in phosphate buffered saline, pH 7.2.

of the extract were diluted in the appropriate buffer to give a dilution of 1:30. They were kept at 5 deg. for 16 hours and estimations of D_{50} made. The haemolysin was unstable below pH 5, but between pH 5 to 9 there was little effect on stability.

Various additions replaced 1 ml of buffered saline in the haemolytic system (cholesterol 0.05 mg per ml, lecithin 0.1 mg per ml, serum 10 per cent and sucrose 5 per cent). Each caused a reduction in the D_{50} values (approximately 4-fold reduction for sucrose and lecithin and an 8-fold reduction with serum and cholesterol).

The haemolysin of *C. fleckeri* is not affected by lecithin, cholesterol, or serum although sucrose interferes with its activity; the significance of these findings has been reported.

The Effect of Cell Concentration on D_{50} values of *C. quadrigatus* and *C. fleckeri*

A linear relationship between haemolytic concentration and cell count has been demonstrated for saponins and ionic detergents (Ponder, 1948; Rideal and Taylor, 1958; Thron, 1964).

Although the structure of the haemolysin of *C. fleckeri* has not been elucidated, it appears to be a protein and unrelated to saponins. However, the kinetics of haemolysis appear to be similar with the rate of haemolysis being an exponential function of the concentration or dilution of the substance. For *C. fleckeri* extracts it has been shown that there is a linear relation between the number of cells in the system and the concentration of the haemolysin required to obtain the haemolysis of a fixed percentage of the cells. This relationship is based on the concept that the uptake of haemolysin per cell is a function of the free

haemolysin concentration provided sufficient time is given for the reaction to be completed (Thron, 1964). That is $Ch = KN + bh$, where Ch is the concentration required to produce haemolysis, N is the number of cells, bh the concentration of free haemolysin in solution and K a constant. In these experiments Ch could not be estimated. However, the D_{50} value is inversely proportional to log Ch and can be related to the logarithm of N by the equation: $D_{50} = K1 \log N + b$ where $K1$ is a constant of negative value and b is second constant.

This type of relationship has already been shown to exist for *C. fleckeri* haemolysin when haemolysis was allowed to occur at 25 deg. for 5 hours. A similar experiment was carried out at the same temperature with the haemolysin of *C. quadrigatus*. The results are shown in Figure 3.

Statistical analysis of the data indicates that the slopes are significantly different ($p<.01$). This suggests that the affinity of the two haemolysins for rabbit red cells, and possibly the haemolytic process for the two extracts are different.

Estimation of the Lethal, Dermatonecrotic and Haemolytic Potency of *C. quadrigatus* Extracts.

Measurements of the D_{50}, LD_{50} and the SND were made. The D_{50} was 5.0 ± 0.04 ($\pm$ S.D. of seven experiments), the LD_{50} 2.7, and the SND 1.5 (mean of five experiments). The properties and relative proportions of each activity compare with the values for whole extracts of *C. fleckeri*.

Dermatonecrotic Activity

The lesions produced by extracts of *C. quadrigatus* are identical to those reported with *C. fleckeri*. However, the dermatonecrotic factor of *C. quadrigatus* appeared to

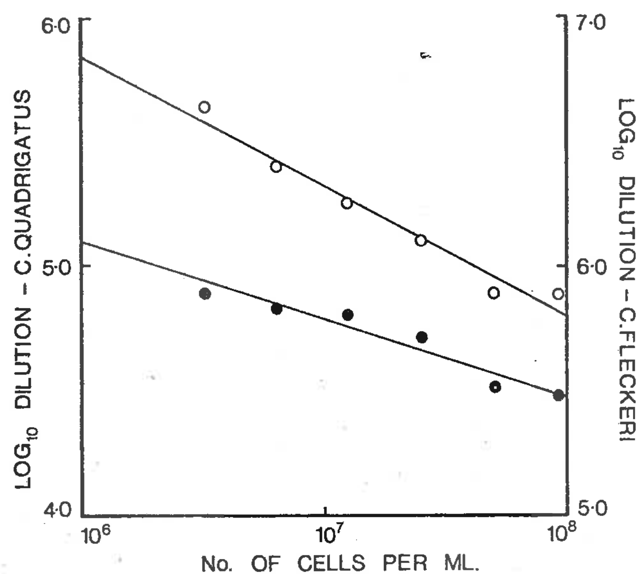


FIG.3 DILUTION OF EXTRACTS REQUIRED FOR 50 per cent HAEMOLYSIS PLOTTED AGAINST THE RED CELL COUNT (TEMPERATURE 25DEG)

O- C.fleckeri; ●- C.quadrigatus. Each point is the result of a single experiment. Lines for both C.fleckeri and C.quadrigatus were plotted by the method of least squares regression.

be more stable than the haemolysin and considerable activity could be detected after heating at 35 deg. for several hours. By this method an extract can be obtained with dermatonecrotic but no haemolytic activity. Therefore in C. quadrigatus extracts, the two activities do not appear to be related as in C. fleckeri.

Antigenic Properties of Tentacle Extracts

Rabbit antiserum prepared against C. quadrigatus contained antibodies to haemolytic, lethal and dermatonecrotic activities. The antiserum was capable of neutralizing 2500 times the D_{50} value of haemolytic activity, and 320 times the LD_{50} dose. The dermatonecrotic activity was completely neutralized by an equal volume of a 1 in 4 dilution of antiserum.

On the other hand, using C. fleckeri antiserum we were not able to demonstrate any neutralizing antibodies to C. quadrigatus extracts. The minimum quantities of extract employed to detect antibodies were as follows: 50 times the D_{50} value for haemolytic activity, 2.5 LD_{50} for lethality, while undiluted antiserum caused no decrease in the SND.

A similar series of experiments was carried out with the antiserum of C. quadrigatus against extracts of C. fleckeri. No neutralizing antibodies against the haemolytic, lethal or dermatonecrotic factors of this species could be found.

Diffusion of Extracts in Agar

In order to identify the haemolysin 10 per cent washed red cells were incorporated into the agar. The specific antihaemolysin for the appropriate extract could be demonstrated by inhibition of haemolysis in double diffusion experiments. This is shown in Fig. 4. The

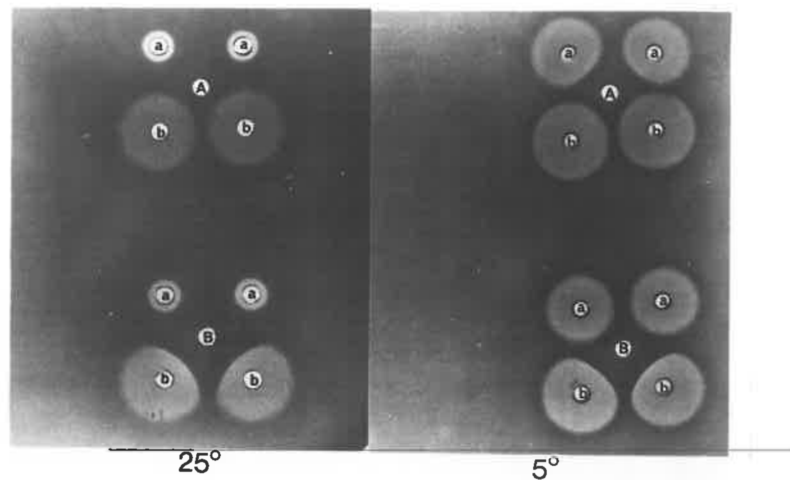


FIG.4 DOUBLE DIFFUSION IN 10 per cent BLOOD AGAR DEMONSTRATING INHIBITION OF HAEMOLYSIS BY SPECIES SPECIFIC ANTISERUM, AND CHANGE IN HAEMOLYSIS WITH TEMPERATURE.

(a) C.quadrigatus extract; (b) C.fleckeri extract (1:4 dilution)

(A) C.quadrigatus antiserum;(B) C.fleckeri antiserum. Temperature of diffusion indicated. Five μ litre of each extract and antiserum used.

plates were kept at a constant temperature of either 5 deg. or 25 deg. Other discrete bands of haemolysis could be produced by exposing the plates at different temperatures. In these cases the changes are similar to the multiple precipitation bands observed in routine immunodiffusion experiments, which may be attributed to sudden changes in the rate of diffusion or varying solubilities of the antigen-antibody complex (Crowle,1961).

A comparison of the plates at 5 deg. and 25 deg. provides a simple means of differentiating the two haemolysins. The haemolysin of *C. fleckeri* is not affected by the higher temperature, whereas there is considerable inhibition of the zone of haemolysis around *C. quadrigatus*. As there is only a slight difference in the D_{50} value for *C. quadrigatus* at 5deg. and 25 deg. the findings are attributed to the lower stability of the extracts at the higher temperature.

The D_{50} value of *C. fleckeri* at 25 deg. was not affected by serum, cholesterol or lecithin. However, each of these compounds caused inhibition of the haemolysis produced by *C. quadrigatus* extracts. This inhibition of haemolysis could be demonstrated on blood agar plates when extracts of *C. quadrigatus* were allowed to diffuse against normal rabbit serum. The inhibition of haemolysis was considerably less than that produced by the specific antiserum and, at 5 deg, it occurred only after several days.

4.4 D I S C U S S I O N

Examination of the haemolysin of *C. quadrigatus in vitro* showed that it was more labile than the haemolysin of *C. fleckeri*. The optimal temperature for haemolysis

was 15 deg. rather than 25 deg. and the haemolysin in this extract deteriorated more rapidly at higher temperatures.

Serum, cholesterol and lecithin interfered with the activity of the haemolysin of C. quadrigatus and these factors were without effect on extracts of C. fleckeri. It would appear that the haemolysin of C. quadrigatus and its mode of action are different from that of C. fleckeri. The two extracts despite certain chemical similarities which have been described produce neutralizing antibodies for a particular species and no cross immunity could be detected for the lethal, dermatonecrotic or haemolytic activities of the two extracts.

From a practical point of view a ready distinction between the two can be made on the basis of their haemolytic activity at different temperatures. There are several methods of demonstrating this difference but the use of blood agar plates appears to be a simple method which is both reliable and reproducible. Diffusion of the extract occurs on plates held at 5 deg. and 25 deg. At the end of 24 hours extracts of C. quadrigatus showed a marked difference in the haemolysis on the two plates, the area being considerably larger on the plates held at 5 deg. This difference is attributed to the reduced stability of C. quadrigatus extracts at the higher temperature. On the other hand, with extracts of C. fleckeri the difference is not marked, and the area of haemolysis at 25 deg. may be slightly larger. Double diffusion experiments in which the specific antisera are used may further confirm the identity of the extracts. These experiments should be carried out at 5 deg. in order to prevent loss of activity of the haemolysin during the period of the diffusion.

The above tests might be applied to the tentacle fragments which are available on the skin of the injured victims. If the fragments were collected and kept at low temperature, sufficient activity might be present to allow identification of the organism.

CHAPTER 5

5. AN EXAMINATION OF THE MODE OF ACTION OF THE HAEMOLYTIC FRACTION DERIVED FROM TENTACLE EXTRACTS OF *C. FLECKERI*.

5.1 I N T R O D U C T I O N

Previous investigations on the haemolysin derived from tentacle extracts of *Chironex fleckeri* have shown that it is a protein with a molecular weight of approximately 70,000 (Crone and Keen, 1969). *In vitro* the haemolysin showed no phospholipase or apparent proteolytic activity, and it has no interaction with cholesterol, lecithin or protein which might explain the effects on the red cell membrane. An examination of the kinetics of the haemolytic reaction indicated that the rate of haemolysis was non-linear and exponentially related to the lysin concentration (Keen and Crone, 1969). In this respect it was similar to saponins and other surface active agents (Ponder, 1948). Methods to elucidate further the mode of action of the haemolysin were restricted by an inability to concentrate and isolate the protein due to the poor stability of the active material.

A number of other lytic agents of protein origin have been described (Bachmayer and Michl, 1965; Becker, 1961; Bernheimer, 1968) but few have been examined in sufficient detail to explain their mode of action. One exception has been the α-haemolysin of *Staphylococcus aureas*. A detailed investigation of the properties of this substance had previously led to the conclusion that it was a lytic enzyme (Laminski and Arbuthnott, 1962).

More recent work suggested that this was not so (Bernheimer, 1968) and that the biological activity of the α-haemolysin was related to its unusual surface properties (Buckelew and Colacicco, 1971).

In view of these findings it was of interest to determine whether more precise information could be obtained on the activity of the haemolysin of C. fleckeri using techniques similar to those employed by Buckelew and Colacicco. Previous work had shown that a haemolytic fraction of tentacle extract could be obtained by chromatography on either Sephadex G-200 or CM-Sephadex C-50 (Crone and Keen, 1970). Although the latter method provided better separation, the material obtained was too unstable for examination. For this reason Sephadex G-200 was employed but only those fractions containing the maximum activity were included in the pooled material used for the investigation. In the case of C. quadrigatus it was impossible to obtain on chromatography material which was sufficiently stable to allow investigation. For this reason the following experiments were confined to the haemolysin of C. fleckeri only.

The surface properties of the haemolytic fraction of C. fleckeri were examined; firstly for the ability of the protein to spread at the air-water interface and secondly for the ability to interact with and penetrate lipid and protein monolayers. The results of these experiments have been reviewed to see whether any general explanation could be made to account for the cytolytic action of the haemolysin.

5.2 M E T H O D S.

Methods for the collection, identification and storage of specimens, the preparation of fractions by

chromatography on Sephadex G-200, and the estimation of the haemolysin titre (D_{50}) has been described in Chapters 1 and 2.

<u>Estimation of Protein Levels in Extracts and Fractions</u>

These were undertaken using the method of Lowry, Rosebrough, Farr and Randall (1951). The concentration of protein per cm^3 at the haemolysin titre (D_{50}) was calculated to give the C_{50}.

<u>Measurement of Surface Tension and Surface Pressure</u>

Surface tension was measured by the Wilhelmy method using a sand blasted platinum plate suspended from a modified displacement transducer (Phillips PR 9310). The displacement was amplified (Phillips Bridge PR 9300) and the output recorded continuously on an X-Y recorder (Hewlett Packard Type 7004). All measurements were made in an enclosed cabinet at 25 deg. ± 0.5 deg. Under these conditions the surface tension which is expressed in SI units could be measured to ± 0.3 mN per m (dynes per cm).

The surface activity was expressed as the total surface pressure (π), i.e. the surface tension of the subphase minus the surface tension of the film; or the total increase in surface pressure ($\Delta\pi$) of the monolayer resulting from penetration of the film at an initial surface pressure (πi).

<u>Measurement of (π-A) and (π-C) Isotherms</u>

The surface pressure-area (π-A) isotherms were measured in a Teflon trough (area 116 cm^2) using a Teflon coated barrier which allowed compression of the surface to 20 per cent of the original area at a linear rate. The compression cycle was displayed on the X axis, and π on the Y axis of the X-Y recorder. The total time for

compression in all cases was 100 min. However in those cases where the stability of the monolayer was examined after penetration with haemolysin the surface was compressed rapidly over a period of 2 min. The compressibility of the protein monolayers was obtained from the Π-A isotherms using the formula $\frac{1}{A} \frac{\Delta A}{\Delta \Pi}$. (Evans at al, 1970). The compressibility was expressed in SI units by Km per N (cm per dyne).

The surface pressure-concentration (Π-C) isotherms were obtained in a circular pyrex glass dish with a Teflon coated rim with a small area for the introduction of materials into the subphase. The area for spreading the monolayer was 22.7 cm^2. The Π was measured continuously with the X-axis of the recorder being used as a time base so that both Π and $\Delta\Pi$ could be measured as a function of time (Π-t).

Materials were thoroughly mixed with magnetic stirrers for one minute after introduction into the subphase contained in either the Teflon trough or the pyrex dishes.

Measurement of Surface Potential

The surface potential (ΔV) was measured using an air ionizing electrode containing ^{242} curium (R.C.C. Amersham). This was connected through an electrometer (Keithley 610C) to a calomel electrode introduced into the trough behind the service barrier in the dish, or moveable barrier of the trough (Gaines, 1966). ΔV was measured continuously on a pen recorder (Varian, G1000). The measurement ΔV represents the difference between the surface potential of the monolayer and the aqueous subphase and could be measured to an accuracy of $\pm$ 10 mv.

Preparation of Red Cell Stromal Proteins and Lipids

Red cell stromal proteins and lipids were derived from rabbit red cells by the method of Maddy (1964), modified by Rega et al. (1967). The final aqueous solution of proteins was thoroughly dialysed against distilled water to remove the butanol when a stable solution with a protein concentration of 1 mg per cm^3 was obtained. This material showed a maximum absorbance at 275 nm.

The butanol phase containing the major portion of the membrane lipids was dried at 40 deg, and the residual butanol removed by washing with ethyl alcohol and redrying. The material was held in the dried state at -20 deg. until required when it was redissolved in a solution of hexane-ethyl alcohol (9:1) before spreading as a mixed lipid monolayer. An estimation of total phosphorus was undertaken by the method of Bartlett (1959) after digestion of the lipid in perchloric acid. Total phosphorus was 110 μg per cm^3 which gave a calculated level of 2.75 mg of phospholipid per cm^3.

Preparation of Monolayers

The haemolysin and the stromal protein of the rabbit red cells were spread by the method of Trurnit (1960). The monolayers were allowed to stabilize for 30 minutes before π was measured or before material was introduced into the subphase under the monolayer. The lipids L-α-dipalmitoyl lecithin and cholesterol were spread from a solution of chloroform methanol (3:1) for π-A estimations, or from hexane ethanol (9:1) for π-C estimations. The solutions were prepared on the day of use. As a routine all monolayers were spread on a subphase of 16.7 mM phosphate buffer-154 mM sodium chloride (pH 7.2). The distilled water used to make up the solutions had a conductance of 1.3×10^{-6} ohm^{-1} cm^{-1}.

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5.3 RESULTS

Haemolytic Fraction prepared by Chromatography on Sephadex G-200.

Four pools of haemolytic fraction were prepared with protein concentrations of 79, 70, 100 and 42.5 μg per cm^3. The C_{50}'s were calculated and these were .0028, .0019, .0022 and .0023 μg per cm^3 respectively. The stability of the haemolysin was estimated at temperatures of 25 deg. and 40 deg. At 25 deg. there was little loss of activity, with the D_{50} falling by 0.1 unit only over a period of 4 hours. However at 40 deg. almost all activity was lost in 1 hour. As the investigations were carried out at 25 deg. and completed in less than 1 hour it is presumed that the loss of activity due to heat lability would be of little practical significance during the course of the experiments.

Surface Properties of the Haemolytic Fraction

The π-A isotherms were obtained by layering the protein solution onto the trough at an initial area of approximately 2 m^2 per mg. After 30 minutes the surface was compressed (Fig. 1). Minimum compressibility occurred at an area of 0.80 m^2 per mg which is characteristic of a dilute protein film (Bull 1947). The π-C isotherms were obtained by spreading varying quantities of the haemolytic fraction onto a fixed area (22.7 cm^2). The monolayers showed a typical collapse pressure of 15-17 mN per m which occurred at concentrations greater than 1.5 μg per cm^2 (Fig. 2). The π-A and the π-C isotherms were not identical; the formation of monolayers at a fixed area producing a more concentrated film (Fig. 1).

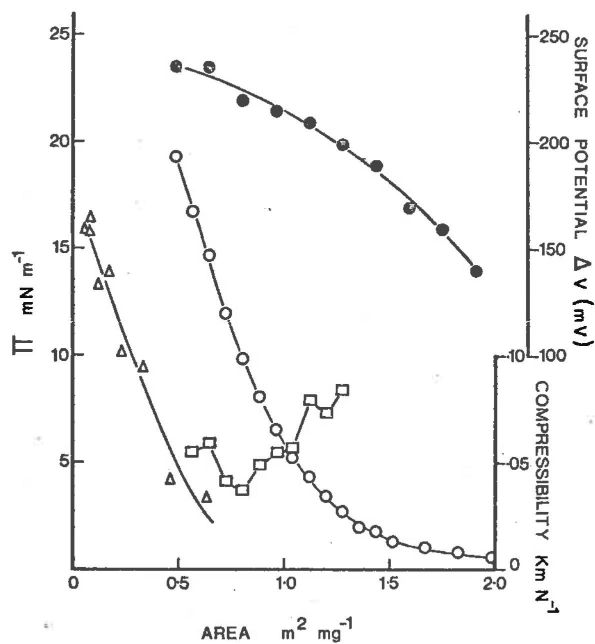

FIG.1 Π- A; Π- C; COMPRESSIBILITY; AND SURFACE POTENTIAL OF HAEMOLYSIN.

○-○ , Π- A; △-△ , Π- C; □-□, Compressibility; ●-●, - Surface potential on subphase of 154 mM sodium chloride, 16.7 mM phosphate buffer; pH 7.2, temperature 25deg.

The ability of the haemolytic fraction to adsorb at the air-water interface was examined at varying concentrations of the protein in subphase up to a level of 2 μg per cm^3. At 2 μg per cm^3 no adsorption could be demonstrated and it was not practical to prepare more concentrated solutions. However after leaving for 1 hour a significant increase in surface pressure of 16 mN per m was observed when the surface was compressed to 20 per cent of the original area indicating the formation of a dilute film at the air-water interface.

In order to examine the possible relation between surface properties and biological activity, the π-C characteristics of haemolysin which had been heated at 40 deg. for 4 hours were compared with those of the original material. Several points for the π-C isotherm were obtained and plotted on Fig. 2. The C_{50} of the heated material was 2.2 μg per cm^3 compared with .0028 μg per cm^3 for the original unheated fraction. Although there had been approximately a thousand-fold reduction in biological activity only a slight reduction in surface activity could be observed.

Interaction with Lipid and Protein Monolayers

The lipid monolayers examined were cholesterol, lecithin and the mixed lipids extracted from the rabbit red cell membrane. They were each tested at a πi of approximately 2, 5, 10, 20 and 30 mN per m. The increase in surface pressure was measured 30 minutes after the introduction of the haemolysin into the subphase (0.5 μg per cm^3). At low πi the $\Delta\pi$ was greatest and became progressively less as the πi increased (Fig. 3).

The π-t isotherms showed a slow rise in $\Delta\pi$ suggesting a simple diffusion of the material into the surface. Only in the monolayer formed by the red cell

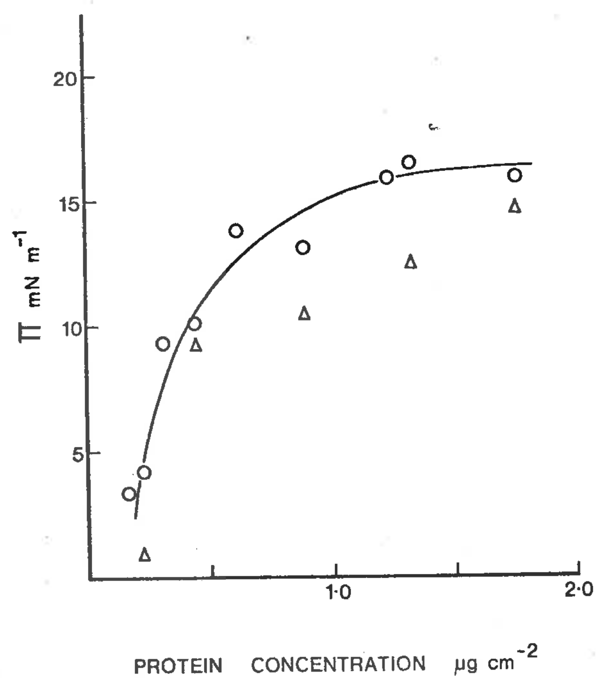

FIG.2 FORMATION OF PROTEIN MONOLAYERS AFTER SPREADING DIFFERENT QUANTITIES OF HAEMOLYSIN.

O— untreated material; Δ— material heated at 40deg. for four hours.

Subphase 154 mM sodium chloride, 16.7 mM phosphate buffer; pH 7.2, temperature 25deg., film area 22.7 cm^2.

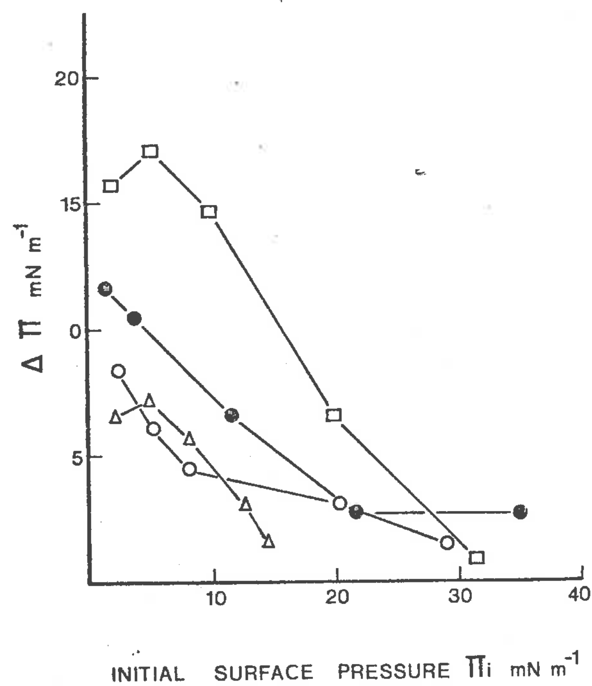

FIG.3 EFFECT OF INITIAL FILM PRESSURE (π_i) IN THE PENETRATION ($\Delta\pi$) ON DIFFERENT MONOLAYERS BY HAEMOLYSIN (0.5 u g per cm^3).

□-□ , cholesterol; ●-● , mixed lipids from rabbit red blood cells; O-O , lecithin; Δ-Δ , red cell stromal protein. Subphase 154 mM sodium chloride, 16.7 mM phosphate buffer, pH 7.2, temperature 25deg, film area 22.7 cm^2.

lipids were there any changes suggesting a specific interaction. In this case there was an initial drop in surface pressure of 0.5 to 3.0 mN per m which occurred rapidly usually within the first 2 minutes of the introduction of the haemolysin into the subphase. This was followed by an increase in surface pressure due to the haemolysin penetrating the monolayer (Fig. 4).

The effect of altering the concentration of the material in the subphase was also studied using cholesterol monolayers at πi of 3 mN per m. At 2.1 μg per cm^3 $\Delta\pi$ was 19.5 mN per m which just exceeded the equilibrium spreading of 15-17 mN per m for the haemolysin monolayer (Fig. 5).

The protein monolayer used was obtained from rabbit cell stroma. This was spread at different πi as for the lipid monolayers (Fig. 3). The maximum πi was limited to less than 15 mN per m which was the approximate equilibrium spreading pressure of the stromal protein. As seen in the lipid monolayers, $\Delta\pi$ was greatest at low πi. There was no evidence of specific interaction between the stromal protein and the haemolytic fraction.

In the case of both the lipid and protein monolayers the ΔV measurements followed a simple algebraic summation of the potential of the monolayer and the penetrant. There were no changes suggesting molecular breakdown as described for the action of phospholipases on lecithin membranes (Colacicco and Rapport, 1966).

5.4 D I S C U S S I O N

Chromatography of the tentacle extracts on Sephadex G-200 provided a separation based on molecular size. The haemolytic fraction might therefore contain a mixture of proteins, but estimations of activity indicated that the material must be largely haemolytic protein. This method

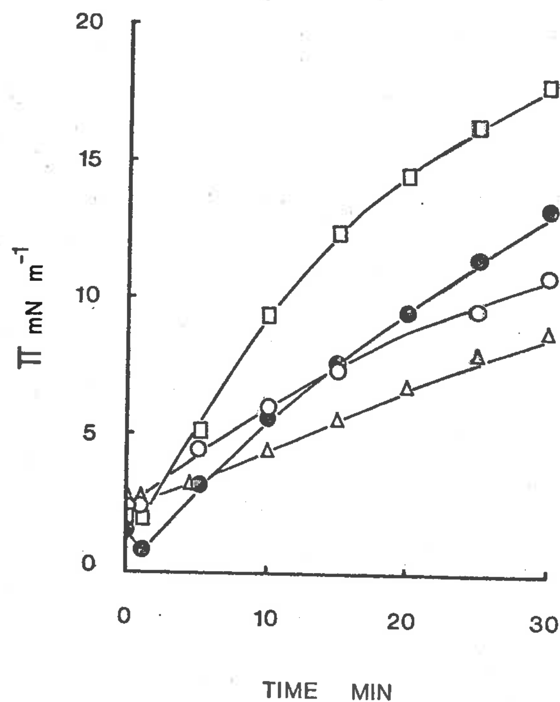

FIG.4 (π - t)CURVES FOR THE PENETRATION OF DIFFERENT MONOLAYERS BY HAEMOLYSIN (0.7 μg per cm^3)

□-□ -cholesterol; ●-● -mixed lipids from rabbit red blood cells; ○-○ -lecithin; △-△ -red cell stromal protein.
Subphase 154 mM sodium chloride; 16.7 mM phosphate buffer; pH 7.2; temperature 25deg; film area 22.7 cm^2.

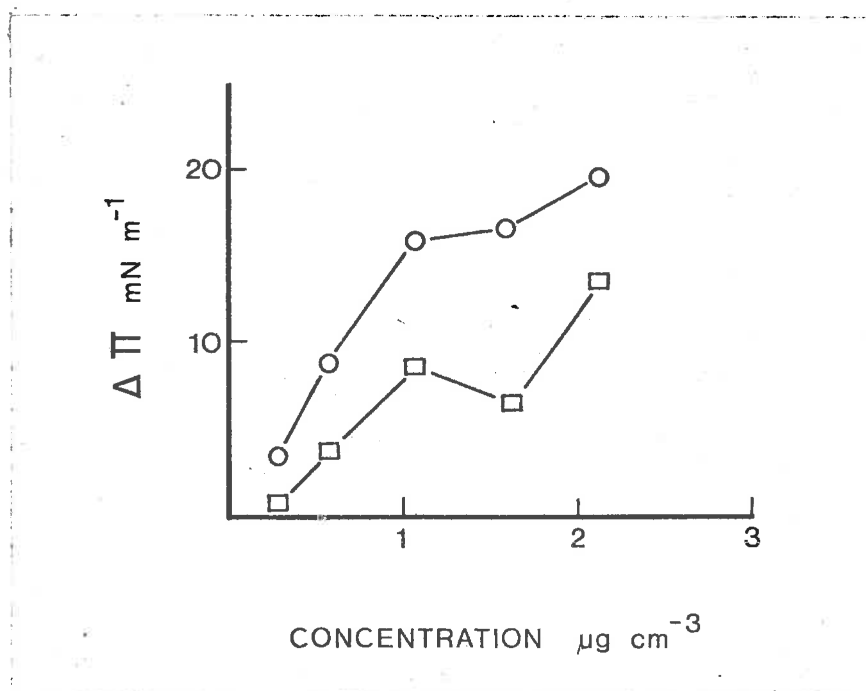

FIG.5 EFFECT OF CONCENTRATION OF HAEMOLYSIN ON THE PENETRATION ($\Delta\Pi$) OF CHOLESTEROL MONOLAYERS (Πi 3mN PER m).

□-□, 10 minutes; ○-○, 30 minutes
Subphase 154 mM sodium chloride; 16.7 mM phosphate buffer; pH 7.2; temperature 25deg; film area 22.7 cm^2.

was chosen as it provided a relatively stable product. However, loss of activity was still a problem and appeared to be due to two factors. Firstly, loss of activity occurs with heating to 40 deg. and is due to denaturation of protein, and secondly, there is a loss of protein from solution. This is seen when successive protein estimations of the pools were made several days apart. There is a fall in protein levels presumably due either to adsorption onto the surface of the container or to adsorption at the air-water interface. The difference between the Π-A and Π-C isotherms suggests that some conformational changes occur in the haemolysin when spread at low surface pressures and this may be associated with denaturation, loss of solubility and precipitation.

The protein of the haemolysin behaves in a similar fashion to other structural proteins showing an ability to spread at the air-water interface on relatively dilute salt solutions and a limited capacity to adsorb at the surface from its own solution. It forms dilute films although more concentrated monolayers may be obtained when spread at a fixed area. However, very concentrated films as described for the α-haemolysin of S. aureus do not occur.

The changes in surface pressure in monolayers following injection of a surface active substance into the subphase as described in these experiments has been called penetration. For protein interactions in lipid monolayers three types of penetration have been defined (Colacicco, 1969, 1970). The first is "free penetration" in which the type of protein structure is the important factor in determining the surface properties; the second is "binding mediated penetration" in which binding to the lipid monolayer produces an initial fall in surface pressure followed by rapid penetration into the lipid film; and the third "binding inhibited penetration" where the initial binding

prevents further penetration of the monolayer. The latter is characterised by small $\Delta\pi$ values.

Therefore care was taken to observe the following features which might be representative of specific interactions between the haemolytic fraction and the lipid monolayers; the rate of change of surface pressure following the introduction of the material in the subphase, the magnitude of the $\Delta\pi$ particularly at levels of πi at or near the equilibrium spreading pressure of the protein, and the total rise in surface pressure when πi was low. Similar methods were also employed to examine possible protein-protein interactions in the monolayer of red cell stromal protein.

In the case of lecithin monolayers there was no evidence of any interaction other than a simple diffusion into the surface. The development of the maximum $\Delta\pi$ was slow and related to πi. The absence of specific interactions was further suggested by compression of the monolayer when an inflection occurred in the isotherm in the region of 35-45 mN per m. indicating extrusion of the haemolysin into the subphase (Fig. 6). There were no substantial changes in ΔV which supports previous observations that the haemolysin was not a phospholipase.

With cholesterol the $\Delta\pi$ values were greater than with lecithin but this might be due to the fact that monolayers of cholesterol are less compressible. At haemolysin concentrations in the subphase of 2 μg per cm^3 values were obtained which just exceeded the equilibrium spreading pressure of the haemolysin and their significance was doubtful. Evidence for lack of specific interaction could again be obtained with compression of the monolayer, when an infliction in the isotherm occurred suggesting that the haemolysin was being expelled from the surface (Fig. 6).

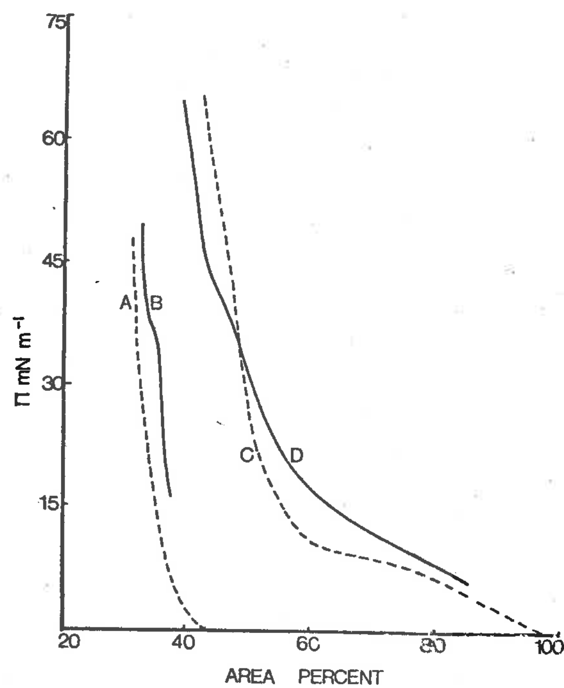

FIG.6 COMPRESSION (AFTER 30 MINUTES) OF CHOLESTEROL AND LECITHIN MONOLAYERS PENETRATED BY HAEMOLYSIN (0.34 ug PER cm^3) SHOWING INFLECTIONS IN Π - A ISOTHERMS.

A - cholesterol; B - cholesterol-haemolysin monolayer; C - lecithin; D - lecithin-haemolysin monolayer.
Trough area 116 cm^2; compression time 2 minutes.
Subphase 154 mM sodium chloride; 16.7 mM phosphate buffer; pH 7.2, temperature 25deg.

The monolayer derived from the red cell mixed lipids showed changes which might be attributed to "binding mediated penetration". There was an initial drop in surface pressure which occurred rapidly and this was attributed to an initial monolayer contraction due to binding of the haemolysin. This was followed by a rise in $\Delta\pi$ which was due to the subsequent penetration of this film (Colacicco, 1969). At low πi values the development of maximum $\Delta\pi$ values was slow and the final levels reached were not high. It is not possible to determine the component involved as a full analysis of the red cell lipids was not undertaken. However, as phospholipids and cholesterol represent 55 per cent of the total rabbit red cell lipids (Prankerd, 1961) any specific interaction would be with a component in low concentration in the monolayer. This might explain the relatively small values of $\Delta\pi$ observed.

Monolayers derived from the red cell stromal protein showed no specific protein-protein interactions. The values were low and the maximum levels were reached slowly. Collapse of the red cell stromal protein monolayer prevented any examination at high π i.

These experiments suggest that the activity of the haemolysin of <u>C. fleckeri</u> might be associated with specific complexing with a lipid component in the red cell membrane. However, this concept needs to be confirmed by further examination in monolayers prepared from individual membrane lipids.

6. CONCLUSIONS

6.1 The haemolysin of *C. fleckeri* has been shown to be a protein with a molecular weight of 70,000. The active material was stable when stored as a concentrated extract of the tentacle at temperatures below 0 deg. However, it was much less stable in dilute solutions and activity was rapidly lost at temperatures above 5 deg. Heating for one hour at 50 deg. caused a loss of all three types of activity - haemolytic, dermatonecrotic and lethal. Fractions containing the haemolysin could be prepared by exclusion chromatography on Sephadex G-200 and also on ion-exchange Sephadex. The haemolysin in the purer preparations was extremely labile and lost activity even when stored below 0 deg.

6.2 The lack of stability appeared to be associated with two main characteristics of the haemolysin. The first was the ability of the protein to be denature at relatively low temperatures, and the second was due to the surface activity of the material, so that it was adsorbed at the air-water interface where it denatured and precipitated. Attempts to separate fractions of the haemolysin by other methods, such as electrophoresis, were unsuccessful as the haemolysin was also adsorbed onto supporting media such as filter paper and cellulose polyacetate.

6.3 As it was not possible to obtain a pure preparation of the haemolysin only an approximate quantitative estimate of haemolytic activity could be achieved. Nevertheless, estimates made on the various batches showed that in comparison with other lysins the haemolysin of *C. fleckeri* was a very potent lytic agent. The precise level of

activity varied with different species of red cell used in the assay, although all species tested were readily lysed. The optimal temperature for lytic activity was between 25 deg. and 30 deg. in a medium of pH between 8 and 9.

6.4 An examination of the kinetics of haemolysis showed that the rate of the reaction was non-linear and exponentially related to the lysin concentration. This finding suggested a non-enzymatic process which was further confirmed by an inability to obtain any evidence of breakdown of membrane proteins or lipids. The addition of plasma or sucrose to the haemolytic system during the course of the process temporarily slowed the rate of haemolysis. However, this could be a non-specific process and an osmotic effect of the added materials only.

6.5 Chromatography on Sephadex separated the tentacle extracts into two fractions which were arbitrarily called the lethal and haemolytic fractions. The lethal fraction had an approximate molecular weight of 150,000, while the haemolytic fraction had an approximate molecular weight of 70,000. When administered parenterally to animals both fractions produce cardiorespiratory failure and death, although the higher molecular weight fraction appeared to be more potent. The haemolytic fraction also contained dermatonecrotic activity, but it was impossible to determine whether lethal, haemolytic and dermatonecrotic activities were the result of a single substance or three separate materials.

6.6 An antiserum was prepared against the whole tentacle extract and this could be shown to contain antihaemolysins. However, some non-specific inhibition occurred if the

amount of protein was increased by concentrating the anti-serum.

6.7 The haemolysin could be demonstrated in whole extracts of the tentacle, in the "milked toxin" of Barnes and in concentrates of nematocysts which had been prepared and described by other workers. Therefore, it appeared to be a component of the toxin but it was unusual that haemolysis had not be described either in clinical cases or as a feature of envenomation in experimental animals. These investigations suggested the absence of haemolysis to be due to three factors; the dilution of the venom and haemolysin following inoculation, the lack of stability at normal body temperature and the inhibitory effects of plasma proteins. It was also possible that death may supervene before haemolysis occurs.

6.8 The haemolysin of C. fleckeri was compared with the haemolysin of the related box jellyfish Chiropsalmus quadrigatus. The haemolytic fraction of C. quadrigatus also contained dermatonecrotic activity, but the latter could be distinguished from the haemolytic activity by its greater heat stability. The haemolytic fraction of C. quadrigatus was not prepared in sufficient concentration to demonstrate whether it was lethal also.

6.9 The haemolysin of C. quadrigatus differed from that of C. fleckeri being more labile and having an optimal temperature of haemolysis at 15 deg. rather than 25 deg. A comparison of the kinetics suggested that the mode of action of the two haemolysins was similar but not identical. The lack of similarity was further confirmed by demonstrating that antihaemolysins to the two extracts were species specific and no cross antigenicity could be detected.

6.10 An attempt to elucidate the mode of action of *C. fleckeri* haemolysin was undertaken using surface pressure and surface potential measurements. In the case of *C. quadrigatus* haemolysin it was impossible to obtain material which was stable enough to allow investigation. The surface properties of the haemolysin of *C. fleckeri* were examined firstly for the ability of the protein to spread at the air-water interface and secondly for the ability to interact with, and to penetrate lipid and protein monolayers. The haemolysin was surface active but the ability to concentrate at the air-water interface was not unusual and was similar to a number of other structural proteins. When injected under protein monolayers of red cell stromal proteins, or lipids such as lecithin or cholesterol, no specific interactions could be demonstrated. However, there appeared to be complexing with a component in a mixed monolayer of lipids derived from rabbit red cells. This observation will require confirmation by further examination in monolayers prepared from individual membrane lipids.

APPENDIX 1

LIST OF DRUGS AND REAGENTS

Agar Special Grade	Oxoid
Blue Dextran 2000	Pharmacia
Cetyl trimethylammonium bromide	I.C.I.
C.M. Sephadex C-50	Pharmacia
Cholesterol	B.D.H.
Digitonin	Kock Light Labs.
L-α-lecithin	K. and K. Laboratories
Sephadex G-200 Superfine Grade	Pharmacia
Sodium dodecylsulphonate	Gardinol Chem. Co.
Trypan Blue	K. and K. Laboratories

Suspending Medium for Red Blood Cells and Aqueous Subphase for Spreading Monolayers.

Sodium chloride	154 mM
Disodium hydrogen phosphate	12.5 mM
Potassium dihydrogen orthophosphate	4.2 mM

Adjusted to pH 7.2

When potassium efflux from red cells was being examined sodium dihydrogen orthophosphate 4.2 mM replaced the potassium salt in the above medium.

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APPENDIX 3

Reprints of published papers attached.

T.E.B. Keen and H.D. Crone (1969). The hemolytic properties of extracts of tentacles from the Cnidarian *Chironex fleckeri*. Toxicon 7. 55.

H.D. Crone and T.E.B. Keen (1969). Chromatographic properties of the hemolysin from the Cnidarian *Chironex fleckeri*. Toxicon 7, 79.

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THE HEMOLYTIC PROPERTIES OF EXTRACTS OF TENTACLES FROM THE CNIDARIAN *CHIRONEX FLECKERI*

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Abstract—The properties of an extract of the tentacles from *Chironex fleckeri* were examined to determine the nature of the hemolysin. The hemolysin was labile at room temperatures but when care was taken to prevent deterioration it had a high degree of activity which was unaffected by cholesterol. There was a non-linear relation between the rate of hemolysis and the dilution of the extract and this rate was dependent on temperature and pH. The activity of the hemolysin was inhibited by sucrose and plasma and accelerated by benzene. There was a correlation between the hemolysin titre and the LD_{50} of a series of crude extracts.

INTRODUCTION

FATAL stings from Cubomedusae which occur along the coast line of Australia, north of the Tropic of Capricorn, appear to be due to either *Chiropsalmus quadrigatus* or *Chironex fleckeri* (CLELAND and SOUTHCOTT, 1965). However an examination of the morphology of both organisms suggests that the stinging potential of the latter is greater and it is probably the more dangerous of the two species (BARNES, 1966).

Extracts from the frozen tentacles of *C. fleckeri* have been shown by Wiener (in SOUTHCOTT and KINGSTON, 1959) to be rapidly fatal to mice and guinea pigs and to contain a hemolysin which was destroyed by boiling.

Work on the pharmacology of the toxin obtained from the frozen tentacles has demonstrated two important features of these preparations (FREEMAN and TURNER, 1969). Firstly, the most toxic material is obtained by homogenizing the whole tentacle and extracting with a sucrose or buffer solution, while concentrates of intact nematocysts obtained by centrifugation and disrupted by grinding or sonic disintegration showed little activity. Secondly, the pharmacological properties of the extracts from the whole tentacle or an active fraction prepared by Sephadex filtration were identical to those obtained from material collected by discharge of nematocysts through the amniotic membrane (BARNES, 1967). As the latter material should contain the venom of the nematocysts only, it was concluded that either during the process of freezing and thawing, or during the period of storage the venom leaked out of the nematocysts even though microscopic examination established that only a small proportion had discharged.

During the course of the above pharmacological investigations on these extracts it was noted that some degree of hemolysis was a frequent finding in animals dying of envenomation. Therefore an investigation of this property of the extract was undertaken to characterise the hemolysin in more detail.

METHODS

The specimens of *C. fleckeri* had been collected and identified by Dr. J. Barnes of Cairns, Queensland, Australia. They were kept in a cool room until the tentacles could be removed

and then transferred to a liquid nitrogen container for transport to the laboratory. Using this procedure the tentacles were well preserved and small quantities could be removed as required.

Dr. Barnes also supplied a quantity of material which was obtained by discharge of nematocysts through human amniotic membrane (BARNES, 1967). This material was employed in comparisons between hemolytic and lethal properties of the extract. This material had been stored in the frozen state for 20 months and this may account for its relatively low potency at the time of use.

The hemolysin appeared labile at room temperatures and attempts to make a stable and concentrated extract from the nematocysts as described for Physalia (LANE and DODGE, 1958) were unsuccessful. The hemolysin could not be extracted with alcohol or ether and it would not pass through a dialyzer membrane.

For these investigations a small quantity of tentacle was homogenized at 5° and extracted with a phosphate buffer at pH 6·3, which was initially thought to be the optimum pH for stability of the extracts (FREEMAN and TURNER, 1969). The material was centrifuged at 2500 *g* for 20 min and the supernatant removed, dispensed into 1·0 ml quantities and placed in a deep freeze at −20°. Two batches were made—the first by extracting 648 mg of tissue in 10·0 ml of buffer, and the second by extracting 2·9 g in 15·0 ml of a similar buffer solution. The first batch was used for most of the experiments but there was insufficient to complete the investigation. The second batch was mainly used for studies on the rate of hemolysis. The extracts were then diluted with phosphate buffered saline (PONDER, 1948) at pH 7·20 to give the initial dilution required for the hemolytic studies.

Red cell suspensions, except those from humans, were normally obtained by intracardiac puncture and the blood collected into heparinized tubes. Human red cells were obtained by puncture of the median cubital vein. The cells were washed three times in 20 times their vol. of 0·146 M sodium chloride and were used within 24 hr of collection. Rabbit cells were used for all routine assays.

The method of measuring the dilution of extract producing 50 per cent hemolysis (D_{50}) was similar to that described by THRON (1964). Ten tubes were used for each estimation and the hemolytic system consisted of a total vol. of 3·0 ml. The initial 1·0 ml in the tube consisted of serial twofold dilutions of the venom and buffered saline. To each tube was added a further 1·0 ml of buffered saline, or other test solution, and 1·0 ml of rabbit red cells, the concentration being adjusted to give a final dilution of $3{\cdot}0$–$3{\cdot}5 \times 10^7$ cells per ml.

The number per unit volume and the species of the red cells were kept constant in all experiments except in those where these two variables were independently investigated.

Hemolysis was allowed to occur at 25° for 5 hr when the numbers of non-hemolysed cells were estimated in a spectrophotometer (Unicam SP 600) by their apparent absorbance at 700 mμ. A calibration curve had been prepared using suspensions of cells with varying proportions hemolysed by freezing and thawing. From this graph the optical absorbances could be converted into figures for per cent hemolysis and when these were plotted against the dilutions of venom the D_{50} could be calculated. By relating this dilution to the wet weight of tentacle from which extract was prepared, an approximate figure for the concentration causing 50 per cent hemolysis (C_{50}) could also be estimated.

Investigations on the rate of hemolysis were undertaken in a spectrophotometer (Unicam SP 800) with an automatic cell changer and program controller. The temperature was maintained at 25° in all experiments unless stated otherwise. The total volume of the hemolytic system was again 3·0 ml, consisting of 1·0 ml of red cell suspension, 1·0 ml of buffered

saline, or other test solution, and 1·0 ml of the appropriate dilution of hemolysin. The same principle was adopted of converting the apparent absorbance at a fixed wave length of 700 mμ into per cent hemolysis by means of a calibration curve. When it was necessary to add test substances during the course of the hemolysis, solutions in 0·1 ml were used. The alteration in the dilution of the red cell suspension caused by the addition of 0·1 ml caused very slight changes in the absorbance recorded and any major variation could be attributed to the substance added.

The toxicities of the extracts by the i.v. route were estimated by measuring the LD_{50} for male albino mice weighing 18–20 g. Four groups of 4 animals were used for each estimation and four dose levels were employed in a constant vol. of 0·1 ml except at the highest dose level in the least potent extract where it was necessary to use 0·15 ml. The LD_{50} was calculated from the method described by WEIL (1952) and expressed as the logarithm of the dilution contained in 1·0 ml of the extract.

The buffers used in the stability tests were as follows: between pH 4·0 and 8·0 they were mixtures of citric acid and disodium phosphate, and between pH 8·0 and 9·0 they were mixtures of Tris-(hydroxymethyl) aminomethane (Tris) and hydrochloric acid. The isotonic buffers used to measure the effect of pH on rate of hemolysis were mixtures of sodium chloride, disodium phosphate, potassium dihydrogen phosphate for values between pH 6·0 and 7·5 and sodium chloride and Tris–hydrochloric acid between pH 7·5 and 9·0.

Analytical grade chemicals were used for all experiments. The digitonin (Koch Light Labs.) was prepared at a concentration of 0·1 mg per ml approximately 30 min before use. The lecithin used was prepared from egg yolk by the method described by DAWSON (1963). The solutions of sodium dodecyl sulphonate (Gardinol Chem. Co.) and cetyl trimethyl ammonium bromide (I.C.I.) were used within 24 hr of preparation.

RESULTS

Estimation of the D_{50} and C_{50} values for the hemolysin of C. fleckeri

Figure 1 shows the percentage hemolysis plotted against the dilution of the extract and the wet weight of the tentacle from which the material was obtained. The figures are those obtained for the first batch of extract which has a C_{50} value of 2·3 μg per ml. Using the same hemolytic system the value obtained for digitonin was 0·89 μg, which is in the same range as that reported previously (THRON, 1964).

When supplies of the first batch were depleted, a second was prepared which had a considerably higher potency and gave a C_{50} level of 0·23 μg per ml.

Factors affecting the D_{50} values of the hemolysis

1. *The species of red blood cells.* The susceptibility of different types of red cells to the hemolysis varies from one species to another. Of the types tested those from the guinea pig appeared to be the most resistant and those from the rat were the most sensitive. For comparison the D_{50} values for the different cell types are included in Fig. 1.

2. *The concentration of red blood cells.* The D_{50} values obtained using different concentrations of cells are shown in Fig. 2. Using the spectrophotometric method it was difficult to obtain absorbance values for cell concentrations below 5×10^6 per ml, and above $1{\cdot}0 \times 10^8$ per ml. However, within this range there appears to be a linear relationship between the D_{50} values and the cell concentration. A similar type of relationship has been described for the hemolysis produced by saponin and anionic detergents (RIDEAL and TAYLOR, 1958).

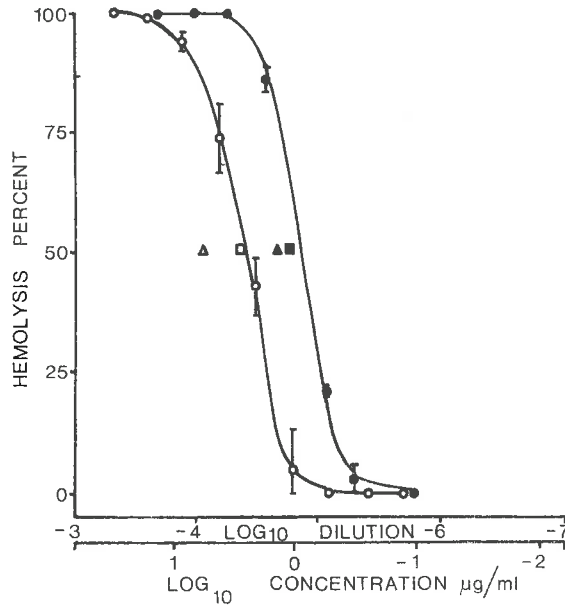

FIG. 1. LOG$_{10}$ DILUTION AND LOG$_{10}$ CONCENTRATION PLOTTED AGAINST per cent HEMOLYSIS. Each point on the curve is the mean of five experiments ± SD. ○—*C. fleckeri* hemolysin; ●—digitonin. D$_{50}$ values for different species of red cells using *C. fleckeri* hemolysin; △—guinea pig; □—mouse; ▲—human: ■—rat.

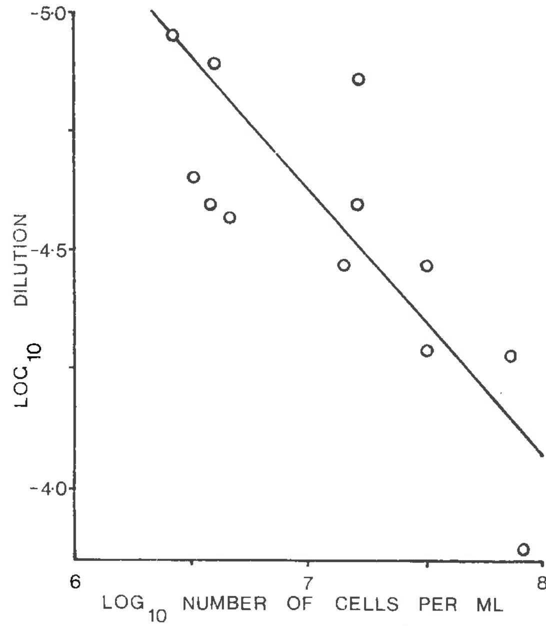

FIG. 2. DILUTION OF *C. fleckeri* HEMOLYSIN REQUIRED FOR 50 per cent HEMOLYSIS PLOTTED AGAINST THE RED CELL COUNT.

Each point represents the result of one experiment with line of best fit drawn to show correlation.

3. *The addition of cholesterol, lecithin, plasma and sucrose.* The replacement of 1·0 ml of buffered saline with 1·0 ml of the following substances: cholesterol sol. (0·05 mg per ml), lecithin (0·1 mg per ml) or plasma 10 per cent, produced no effect on the D_{50} values obtained. Incubation of the same concentrations of these substances with either the red cells or the extract at 25° for 1 hr prior to addition to the system was also ineffective.

On the other hand, the replacement of 1·0 ml of buffered saline with a similar quantity of sucrose 5 per cent caused a two- to fourfold reduction in the D_{50} values and this could be further increased if the sucrose was incubated with the hemolysin at 25° for 1 hr prior to addition to the system.

4. *The potency of the hemolysin.* The hemolysin proved to be relatively labile at room temperatures and this was partly dependent on the dilution; the more dilute solutions tended to deteriorate more rapidly. In practice the stock material was removed from the deep freeze, thawed and diluted just before making up the hemolytic system. For reproducible results it was always necessary to use fresh dilutions.

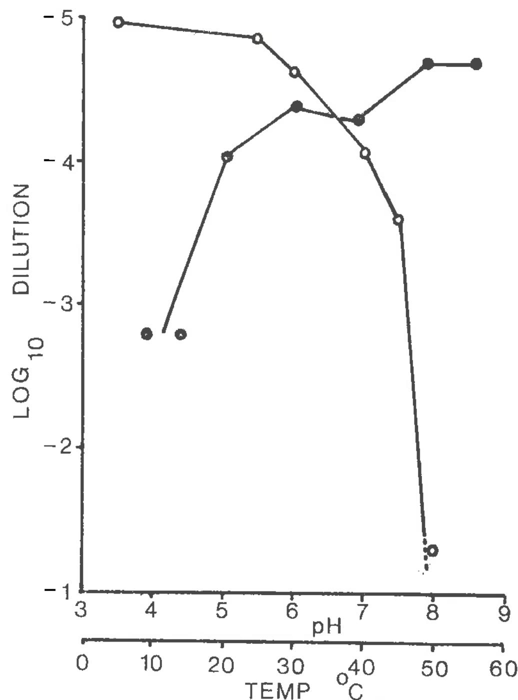

FIG. 3. THE EFFECT OF pH AND TEMPERATURE ON THE DILUTION OF *C. fleckeri* HEMOLYSIS REQUIRED FOR 50 per cent HEMOLYSIS.
○—D_{50} values after 1 hr exposure at different temperatures; ●—D_{50} values after 1 hr exposure to diluents of different pH, at constant temperature of 25°.

As it was thought that the stability of the material might be related mainly to the pH of the diluent and the temperature at which it was held, the effect of both these parameters on the potency of the hemolysin were examined. The D_{50} values obtained after 1 hr at different temperatures and at different pH values are shown in Fig. 3.

Factors affecting the rate of hemolysis

1. *The dilution of the extract.* The effect of dilution of the extract on the time to produce 100 per cent hemolysis of a standard cell suspension is shown in Fig. 4. A more detailed

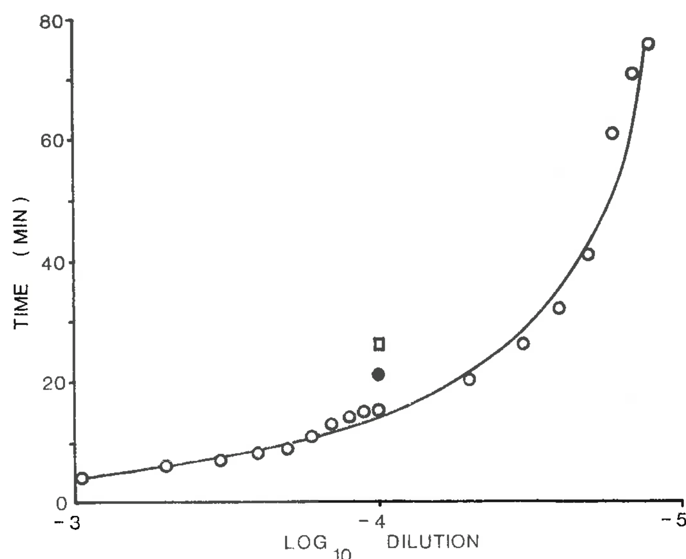

Fig. 4. The effect of dilution of the extract on the time for complete hemolysis of standard concentration of red cells ($3{\cdot}0$–$3{\cdot}5 \times 10^7$/ml) at 25°.
Also shown is the effect of temperature at a fixed dilution of extract. ●—10°. ○—25°. □—37°.

examination of these figures indicates a non-linear relationship between the dilution of extract and the rate of hemolysis. In this respect it appears to be similar to lysis produced by saponin and ionic detergents (Rideal and Taylor, 1956, 1958) and differs from that produced by proteins or enzymes (Bernheimer, 1947).

2. *Temperature.* When dilute solutions were used hemolysis appeared to proceed more rapidly at temperatures below 20°, but this was later considered to be due to the greater stability of the hemolysin at the lower temperatures.

Using more concentrated preparations where complete hemolysis is achieved within 30 min the optimum temperature appears to be between 25 and 30°. Above 35° the rate fell markedly but this is largely due to lack of stability at the higher temperatures.

For comparison the time taken for complete hemolysis at temperatures of 10, 25 and 37° for a standard dilution are shown in Fig. 4.

3. *The effect of pH.* A series of isotonic diluents at various pH values between 5·2 and 9 were used and the rate of hemolysis estimated by the time required for complete hemolysis. The rate is considerably reduced at pH level below 6·5 and appears greatest between pH 8 and 9. At the lower pH levels the effect on rate may have been partly due to the reduced stability of the extract under these conditions.

4. *The effect of added substances.* The replacement of 1·0 ml of buffered saline with cholesterol sol. (0·05 mg per ml) or lecithin (0·1 mg per ml) caused no alteration in the rate of hemolysis.

On the other hand, if sucrose 5 per cent or plasma 10 per cent is added there is a marked reduction in the rate of hemolysis. The addition of equivalent quantities of plasma after

hemolysis had commenced did not result in any delay, while sucrose added in the same manner was still able to cause a reduction in this rate.

The replacement of 1·0 ml of buffered saline with 2×10^{-4} to 5×10^{-5} M sodium dodecyl sulphonate or 10^{-5} to 10^{-6} M cetyl trimethyl ammonium bromide resulted in inhibition of hemolysis by both the anionic and cationic detergent at the highest concentration employed.

The addition of benzene or bromo-benzene appeared to increase the rate of hemolysis.

Relation between hemolysin titre and lethal properties of the extract

Four batches of extract with known varying titres of hemolysin were examined. The first was the standard extract, the second was the product of discharge of nematocysts through the amniotic membrane, the third was prepared by diluting the standard extract in a buffer at pH 5·5 and the fourth by diluting in buffer at pH 7·2 and then heating at 37° for 30 min. In the latter two batches the D_{50} and LD_{50} were estimated on the same day. If hemolysis and lethality were due to different factors their stability to heat or changes in pH might vary and this difference would result in separation of the two activities. The result of the comparative assays of the 4 batches are shown in Table 1.

TABLE 1

Type of extract	Hemolysin titre $\frac{1}{}$ (Log$_{10}$ (D_{50}) ± S.D.)	LD_{50} for mice expressed as 1·0 ml of $\frac{1}{}$ (Log$_{10}$ dilution ± S.D.)
Standard batch	5·9±0·03 (5)	4·1±0·08
Material obtained by discharge of nematocysts through amniotic membrane	4·0±0·08 (5)	2·8
Standard batch diluted in buffer pH 5·5	3·3±0·06 (4)	1·9±0·11
Standard batch, diluted and heated 37°	3·0±0·11 (5)	1·1±0·09

Figures in parentheses represent number of observations.
As there was no gravimetric means of estimating the LD_{50}, it was expressed as a calculated dilution of the extract which would be contained in 1·0 ml.

There is a definite correlation between the hemolysin and lethal properties of the extract although this relationship is non-linear. This suggests that the hemolysin is a more specific manifestation of the toxin which *in vivo* appears to have more general effects on the heart and central nervous system (FREEMAN and TURNER, 1969).

DISCUSSION

The cell membrane is considered to be made up of a cholesterol–phospholipid–lipoprotein complex. Lysis of the membrane may be produced by enzymes such as lipases or by lysins such as steroids, vitamin A, detergents or saponins (CUTHBERT, 1967).

In the initial part of this investigation the presence of phospholipase A was excluded by examining the lipid products of hemolysis of the red cell membrane by thin-layer chromatography. No lysophosphatides could be detected.

The subsequent investigation was undertaken to determine the characteristics of hemolysis in the hope that this knowledge might assist in the broad chemical identification of the

substance. The fact that it does not pass through a dialyzer membrane shows that it has a molecular weight greater than 1000 or it is coupled to a substance of high molecular weight. Although a number of other features were defined which might later assist in the isolation of the active material, no precise chemical classification was possible.

The most important of these features was the high activity of the hemolysin when care was taken to prevent deterioration in dilute solutions at room temperatures. The C_{50} for the two batches prepared was 2·3 μg per ml and 0·23 μg per ml respectively based on the wet weight of the tentacle from which they were extracted. If the hemolysin is contained in the venom of the nematocysts, only the active component must represent a very small proportion of the total weight of the tentacle. These figures should then be considered a most conservative estimate only.

A more detailed investigation of the hemolysin showed that the D_{50} was proportional to the red cell concentration and at a given concentration it was unaffected by cholesterol or plasma. There was a non-linear relation between the rate of hemolysis and the dilutions of the extract over the range employed and the rate appeared to increase both with temperatures up to 30° and with values of pH up to 9. The hemolysin was impaired by sucrose, plasma and both anionic and cationic detergents while benzene and bromo-benzene acted as accelerants.

While the above evidence may suggest that the material is an enzyme or other protein, in the case of an enzyme one would expect the rate of hemolysis to be proportional to its concentration. On the other hand, there is little to suggest that the material would fall into the category of a steroid (WEISSMANN and KEISER, 1965), vitamin A (DINGLE and LUCY, 1962) or an ionic detergent.

However, the high hemolytic activity might suggest that it could fall into the category of saponin or other glycoside. A precedent for saponins of marine origin with high hemolytic activity has been achieved by the chemical isolation of Holothurin A (NIGRELLI *et al.*, 1955). A hemolytic process whose D_{50} is directly proportional to the concentration of red blood cells and whose rate of reaction has a small positive temperature coefficient up to 30° and is influenced by inhibitors such as sucrose and plasma and by accelerators such as benzene suggests a mechanism similar to that produced by saponin or the slow reaction of ionic detergents.

On the other hand, the lack of association with cholesterol suggests that it is not chemically similar to either saponin or digitonin. Similarly, the relatively greater activity at an alkaline pH differs from the hemolysis produced by saponins which normally proceed more rapidly in acid solutions. For the present time, therefore, the broad chemical classification of the hemolysin is in doubt.

The material examined in this study was from extracts of the whole tentacle and it was of obvious interest to know whether this resembled the venom discharged by the nematocysts. The pharmacological studies referred to previously (FREEMAN and TURNER, 1969) have shown that the material from crude extract and nematocysts was similar, while this study showed that hemolytic activity could be demonstrated in material from both sources.

The range of pharmacological activities shown by the venom (FREEMAN and TURNER, 1969) raises the question of whether they are the result of a single factor or several unassociated components. The correlation which appears to exist between the hemolysin and the lethal properties of a series of extracts suggests that there may be only a single factor involved. If we assume that this is the case, the hemolysin provides an *in vitro* method of detecting the venom during attempts to concentrate and purify the extract.

SUMMARY

1. The properties of an extract of *C. fleckeri* were examined to determine the nature of the hemolysin.

2. The hemolytic activity was measured in terms of D_{50} or C_{50} values which represented the dilution or concentration of extract producing 50 per cent hemolysis.

3. The D_{50} values were dependent on the species and concentration of red cells used. They were unaffected by cholesterol, lecithin or plasma, but were reduced by sucrose.

4. The hemolysin was labile at room temperatures and relatively unstable above 35° and at pH below 5·0.

5. There was a non-linear relationship between dilution of extract and rate of hemolysis.

6. The rate of hemolysis was dependent on temperature and pH and inhibited by sucrose and plasma and accelerated by benzene and bromo-benzene.

7. There appeared to be a relationship between the titre of hemolysin and the LD_{50} of a group of extracts.

Acknowledgements—We wish to thank Dr. J. Barnes who generously supplied the specimens and the extracts of *Chironex fleckeri* which would have been impossible to obtain otherwise; and to acknowledge the advice of Mr. G. White on statistical procedures and Mr. M. Poretski for his technical assistance.

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CHROMATOGRAPHIC PROPERTIES OF THE HEMOLYSIN FROM THE CNIDARIAN *CHIRONEX FLECKERI*

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(*Accepted for publication* 6 *April* 1969)

Abstract—Extracts of tentacles from the jellyfish *Chironex fleckeri* which possessed strong hemolytic properties were examined by ion exchange chromatography on carboxymethyl and diethylaminoethyl cellulose and by exclusion chromatography on Sephadex G-75 and G-200. In some experiments the hemolytic activity was related quantitatively to the lethality of the samples to mice, before and after the separatory procedure used.

It was concluded that the hemolytic activity of the extracts was due to a single component, a protein of molecular weight of approximately 70,000. At least one other toxic component was present, which had a molecular weight of approximately 150,000. Methods for purifying the hemolysin are suggested.

INTRODUCTION

THERE are many reports of fatalities from jellyfish stings off the North Australian coast (CLELAND and SOUTHCOTT, 1965), and the cnidarian *Chironex fleckeri* (the box jellyfish or sea wasp) has been assigned by BARNES (1966) as the probable cause of the majority of these. The pharmacological actions of extracts from this animal have been described by FREEMAN and TURNER (1969), and the hemolytic properties of similar extracts by KEEN and CRONE (1969). In the latter study it was noted that the potency of the hemolysin would enable studies to be made of the active principle in concentrations so low that detection by chemical means would be impossible. The present study makes use of this fact to derive information about the hemolysin from a study of its behaviour during column chromatography.

It was found during the course of this work that the hemolysin could be separated from a second toxic component which had no hemolytic properties. However, this paper is confined to a description of the hemolysin only.

MATERIAL AND METHODS

Extraction of tentacles

The tentacles used had been obtained from *Chironex fleckeri* as described previously (FREEMAN and TURNER 1969), and were stored in liquid nitrogen. A portion of tentacle weighing approximately 4 g was ground in a mortar with 5 ml of a 10 mM phosphate, 10 mM NaCl buffer (pH 6·5) at 5°. The mixture was then centrifuged at 9000 ***g*** for 10 min and the supernatant was retained. The residue was again extracted with 5 ml of the buffer, centrifuged, and this supernatant was combined with the first. The combined supernatants formed an opalescent, frothy solution which was stored at −20° until required for use. Two extracts, prepared in an identical manner, were used for the present studies; they did not vary greatly in hemolytic potency and were relatively stable at −20° in this form.

Assay methods for the hemolysin

A rapid method which estimated rate of hemolysis was used semi-quantitatively to locate the hemolysin after chromatography, and a second method based on finding the dilution giving 50 per cent hemolysis after 5 hr was employed to compare hemolytic activity with lethality.

The semi-quantitative method was based on observing the time to complete hemolysis at 25°, the end point being judged by eye (PONDER, 1948). Samples of 0·1 ml were added to 1 ml of rabbit red cell suspension (3×10^7 cells per ml) at pH 7·4. The observed time to hemolysis was converted to a relative dilution by means of a calibration curve, and the reciprocal of this value multiplied by 1000 was used as an arbitrary estimate of the hemolytic activity. The original extracts when diluted 1:10 caused complete hemolysis in 4 min; samples not showing complete hemolysis after 30 min were regarded as inactive by this rapid method. This procedure gave a good estimate of the relative distribution of activity in a single series of tubes, but it is emphasized that any comparison of the various values given for different experiments will be unreliable and misleading.

The quantitative assay for hemolytic activity, based on noting the dilution giving 50 per cent hemolysis of a given cell suspension in 5 hr, was performed as described previously (KEEN and CRONE, 1969).

Toxicity tests

Samples were examined for lethality by injecting 0·1 ml into the tail veins of male albino mice weighing 18 to 20 g. For each of the separatory procedures described below, two samples were tested for lethality from fractions containing the hemolysin, and two samples from each side of the hemolytic peak. Thus for the separation on CM cellulose (Fig. 1), two samples were used from the region 20–60 ml of eluate, two from the hemolytic peak (60–110 ml), and two from the region 110–200 ml. Results were recorded simply as positive (death in 30 min) or negative.

For the separations on CM cellulose and Sephadex G-200 in which a quantitative hemolytic assay was employed, the LD_{50} values of bulked fractions were determined by using four groups of four animals each, and calculating the LD_{50} values from the tables of WEIL (1952).

The hemolytic and lethal activities were both expressed as dilutions of the original extracts or fractions being investigated. In the case of the hemolysin this was the dilution causing 50 per cent hemolysis of a standard cell suspension in 5 hr. The lethality was expressed in the form of a LD_{50} value as the dilution at which 1·0 ml produced a calculated 50 per cent mortality in mice. To relate the two dilutions in a simple manner, reciprocals of the two values were used, and then the hemolytic value was taken as unity and the LD_{50} expressed as a multiple of this.

Column chromatography on carboxymethyl cellulose

Carboxymethyl (CM) cellulose powder (Serva, Heidelberg, 0·72 m-equiv per g) was suspended in 10 mM phosphate, 10 mM NaCl buffer at pH 6·5 and allowed to settle for about 5 min. The supernatant with the fines from the cellulose was decanted and the residue was resuspended in buffer. This procedure was repeated ten times over a period of 24 hr. The CM cellulose was formed into a slurry with the buffer and poured into a column 7 mm i.d., bed height 8 cm. The column was run at a pressure difference of 80 cm of water, which gave a flow rate of 0·7 ml per min. Samples of 1 ml of the extracts were applied to the column

at 5°, washed through with 20 ml of the 10 mM NaCl phosphate buffer, and then eluted by a linear gradient of increasing NaCl concentration. The gradient was formed by putting 100 ml of the 10 mM NaCl buffer in a mixing chamber and having 100 ml of 500 mM NaCl buffer (pH 5·95) in a connecting reservoir so that the buffer lost to the column was replaced with the more concentrated solution. Fractions of 2·7 ml were collected.

Column chromatography on diethylaminoethyl cellulose

Diethylaminoethyl (DEAE) cellulose (Schleicher and Schuell, for thin-layer chromatography, 0·86 m-equiv per g) was suspended in 0·1 N NaOH and stirred for 1 hr. The coarser particles were separated out and washed free from fines by many decantations from distilled water. The residue was then equilibrated with 5 mM Tris–phosphate buffer, pH 7·96, and poured into a column of dia. 11 mm, bed height 15 cm. The buffer of 2 ml of the original *Chironex* extract was exchanged for the Tris–phosphate buffer on a small Sephadex G-75 column and 2·5 ml of eluate containing the hemolytic activity was applied to the DEAE cellulose column at 5°. The column was eluted with 50 ml of the 5 mM Tris–phosphate, then with a gradient of increasing NaCl concentration and decreasing pH (175 ml of Tris–phosphate pH 7·96 in the mixing chamber, 175 ml of 50 mM phosphate, 1 M NaCl, pH 6·51 in the reservoir). Flow rate was 0·9 ml per min, and fractions of 5·5 ml were collected. The pH of each fraction was measured at 5°.

Exclusion chromatography on Sephadex columns

A column of G-75 Sephadex, regular grade, was used initially. The diameter was 2·5 cm and the bed height was 41 cm. The eluting buffer was 10 mM phosphate, 150 mM NaCl at pH 6·3. The flow rate was 0·95 ml per min and fractions of 3·8 ml were collected.

Later a column of G-200 Sephadex, superfine grade, was used. The column i.d. was 2·5 cm and the bed height was 33 cm. The buffer employed for this column was 25 mM Tris–HCl, 150 mM NaCl, pH 8·0. The flow rate at the operating pressure of 20 cm of water was 5·6 ml per hr, and 2·8 ml fractions were collected.

The void volumes of each column were estimated by the use of Blue Dextran 2000 (Pharmacia), and all operations were carried out at 5°.

Other methods

The u.v. spectra of samples were obtained using a Unicam S.P. 800 spectrophotometer. Investigation of fluorescence was done in an Aminco–Bowman spectrofluorimeter. Protein in samples from *Chironex* extracts was estimated by the method of Lowry *et al.* (1951). The quantity of protein in the extracts, as detected by this method, was low and the procedure served only to indicate the position of the main protein components after chromatography. Marker proteins were estimated by the absorption at 280 mμ. Measurements of pH were made using a Radiometer pH meter model 25SE; the values given are those at 20° unless otherwise noted.

RESULTS AND DISCUSSION

Preliminary examination of extract

The crude extracts were examined in the u.v. region. Despite the slight turbidity, an unusual absorption peak at 311 mμ could be discerned. After Sephadex G-200 chromatography this material was eluted at a position corresponding to the total bed volume,

indicating a molecular weight below 10,000. The spectrum of the column eluate at pH 8 showed maxima at 213 and 311 mμ, with a minimum around 250 mμ. The O.D. of this compound in the crude extracts was high enough to mask any of the normal protein or nucleic acid absorption peaks.

Since the instability of the hemolysin (KEEN and CRONE, 1969) would present problems in the more lengthy chromatographic procedures, this property was examined in a general way to gain an idea of the feasibility of the procedures to be used. When the original extract was diluted 1:100 with the extraction buffer at pH 6·5, the hemolytic activity was lost between 24 and 48 hr at 5°. The presence of 1 per cent albumin or 0·1 M Tris at pH 7·2 increased the stability slightly. The previous work (KEEN and CRONE, 1969) had shown that the stability of the hemolysin decreased at temperatures above 5°, with very rapid loss of activity above 20°.

The hemolysin at this dilution of 1:100 was found to adsorb onto glass and sand. The adsorption onto sand was reduced by the presence of urea or albumin, but not by Tris. Adsorption onto the cellulose esters of Millipore filters was strong, a lesser affinity being found for filter paper. These results suggest that the apparent loss of hemolytic activity from dilute solutions could be due to adsorption onto any surfaces present, in addition to change in the hemolysin molecule.

Because of these observations, the *Chironex* extracts were always handled at 5°, enough extract was employed to allow for a large degree of inactivation, and transfer to different containers or passage through tubing was limited to the minimum necessary.

Ion exchange chromatography on substituted celluloses

The results of chromatography on CM cellulose are shown in Fig. 1. The major part of the hemolytic activity was eluted when the sodium chloride concentration exceeded 0·1 M (pH about 6·3). There was some tendency for the hemolysin to tail out in the later fractions, a situation which might be improved by use of a steeper or concave gradient. Most of the protein in the extract, together with the visible turbidity, passed straight through the column

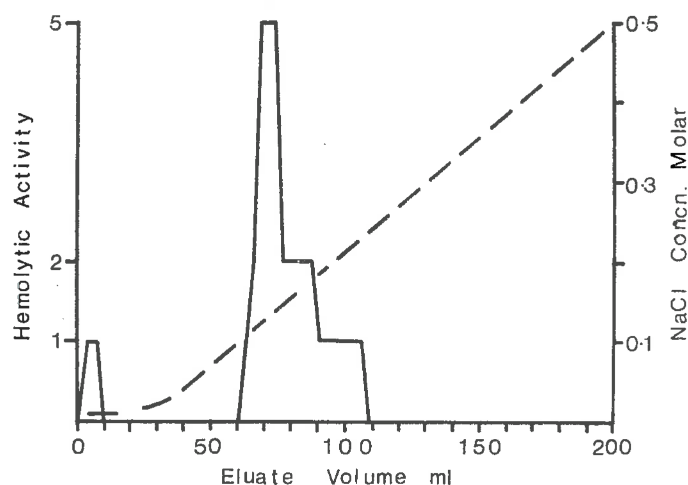

FIG. 1. CHROMATOGRAPHY OF THE HEMOLYTIC EXTRACT ON CM CELLULOSE.
The solid line represents the hemolytic activity in the fractions (volume 2·7 ml each) as assayed by the semi-quantitative method. The broken line shows the concentration gradient of sodium chloride. Other details are given in the text.

on loading, and some hemolytic activity accompanied this. However, during other runs this hemolytic activity was low or not observed (Table 1), and probably did not represent material different from the main peak. Examination of the hemolytic fractions did not show any u.v.-absorbing material, and the only fluorescence observable was a very weak emission at 350 mμ when excited at 285 mμ.

Table 1 shows results from a separate quantitative run on CM cellulose. The total recovery of hemolytic activity was less than 22 per cent and 18·4 per cent was recovered in the peak

TABLE 1. DISTRIBUTION AND RECOVERY OF HEMOLYTIC ACTIVITY FROM A CM CELLULOSE COLUMN, BASED ON A QUANTITATIVE PROCEDURE

Sample	Position on gradient as concentration of NaCl (mM)	Hemolytic activity		
		% of starting activity	% of recovered activity	% of recovered activity (semi-quantitative procedure)
1. Eluate recovered as extract was applied to column	10	0·1	0·5	0
2. Gradient before hemolytic peak	10–120	1·9	8·7	7·5
3. Leading edge of peak	120–190	12·6	58·4	71
4. Trailing edge of peak	190–260	5·8	26·9	18
5. After hemolytic peak	260–500	1·2	5·5	3·5
	Totals	21·6	100·0	100·0

Individual fractions from a CM cellulose column separation similar to that of Fig. 1 were first examined for hemolytic activity by the semi-quantitative procedure. On the basis of these results the fractions were bulked in five main fractions, in which the hemolytic activity was assayed by the quantitative procedure.

between NaCl concentrations of 120 to 260 mM (50 ml of eluate). This low recovery is consistent with the known instability of the hemolysin. The table also allows a comparison between the quantitative and semi-quantitative assays for the hemolysin; the latter method is obviously accurate enough to serve as a guide to the location of the hemolysin. The LD_{50} values of the original extract and of fraction 3 (Table 1) were determined and compared with the hemolysin titre by the quantitative method. The ratio of the two dilutions (see Methods section) was 1:81 for the whole extract and 1:48 for fraction 3. Since the errors in the determinations would be considerable, this result only allows the conclusion that no great separation of lethality from hemolytic activity occurred.

A further sample of *Chironex* extract was chromatographed on CM cellulose and then every third fraction was examined for lethality to mice. Fractions from the hemolytic peak were lethal at dilutions of 1:10, whereas no lethality was observable at full strength in fractions free of hemolysin, Therefore in this system the lethality cannot be effectively resolved from the hemolysin.

Anion exchange chromatography on DEAE cellulose (after buffer exchange on G-75 Sephadex) also resulted in the isolation of a single hemolytic peak (Fig. 2). This material was eluted along with other material from the column, as shown by the irregular pH changes and also by the determination of protein in the fractions.

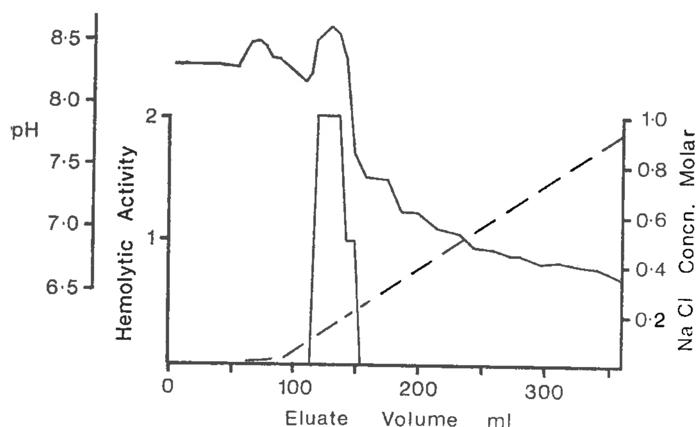

FIG. 2. CHROMATOGRAPHY OF THE *Chironex* EXTRACT ON DEAE CELLULOSE.
The lower solid line represents hemolytic activity (determined for each fraction of 5·5 ml), and the upper solid line represents the pH of each fraction. The broken line indicates the gradient of sodium chloride.

The fact that the hemolysin can be chromatographed by both cation and anion exchange methods is evidence that the substance has an isoelectric point near neutrality. Chromatography on CM cellulose was found to be a good method of isolating the hemolysin from the other material in the crude extract, whereas DEAE cellulose chromatography was less effective in this respect. It was found that the chromatography of small amounts of *Chironex* extract on columns of CM-cellulose (0·8 ml bed volume) was a rapid method of preparing the hemolysin free of detectable contamination by u.v.-absorbing material. This procedure would be suitable for daily preparation of samples for pharmacological work.

Exclusion chromatography on G-75 Sephadex

When the crude extract was chromatographed on G-75 Sephadex, the hemolysin was eluted at the void volume along with the visibly opalescent material and some of the protein. Other protein material was eluted later, but no further hemolytic activity appeared. It was thought possible that this might be a false result due to absorption of the hemolysin onto the fine suspended material in the extract. To test this, 2 ml of the extract was chromatographed on CM cellulose, then 10 ml of eluate from the hemolytic peak (now free of insoluble matter) was applied to the G-75 Sephadex column. The hemolytic activity was once again eluted with the void volume, showing that the molecular weight of the hemolysin was above the exclusion limits of G-75 Sephadex (about 65,000 for spherical proteins).

Exclusion chromatography on G-200 Sephadex

The chromatography of the *Chironex* extract together with bovine serum albumin as a marker is illustrated in Fig. 3. It can be seen that the hemolysin is eluted with, or a little in advance of, the albumin (molecular weight 67,000). This indicates a molecular weight for the hemolysin of approximately 70,000. The crude extract was also chromatographed twice in the absence of added protein, with the same result, that is a ratio of elution volume to void volume (Ve/Vo) of 1:71. The ratio for bovine albumin in two experiments averaged 1:73. When cytochrome-*c*, bovine albumin monomer and dimer, and human γ-globulin were used to give an approximate calibration of the column, the results for the hemolysin

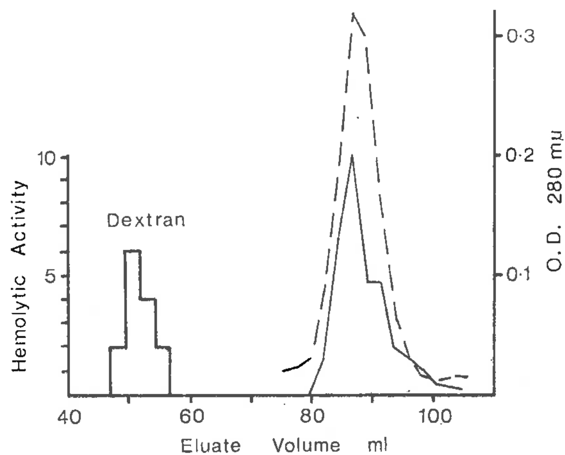

FIG. 3. CO-CHROMATOGRAPHY OF THE HEMOLYTIC EXTRACTS WITH BOVINE ALBUMIN ON SEPHADEX G-200.
The position of the hemolytic material is shown by a solid line, that of the bovine albumin by a broken line (estimated by the optical density at 280 mμ). Also shown is the elution position of Blue Dextran 2000, marking the void volume of the column. Fraction volume 2·8 ml.

were consistent with a molecular weight from 64–76,000. These results suggest strongly an approximate molecular weight of 70,000 for the hemolysin, with the usual reservation that if the molecule is elongated in shape or contains large non-protein groups, this result might be erroneous. No other hemolytic component was observed during the Sephadex chromatography.

Examination of the Sephadex G-200 fractions for toxicity revealed the presence of toxic material between the void volume and the hemolytic peak. The result of attempts to locate this material more precisely are shown in Fig. 4. By the injection of various dilutions

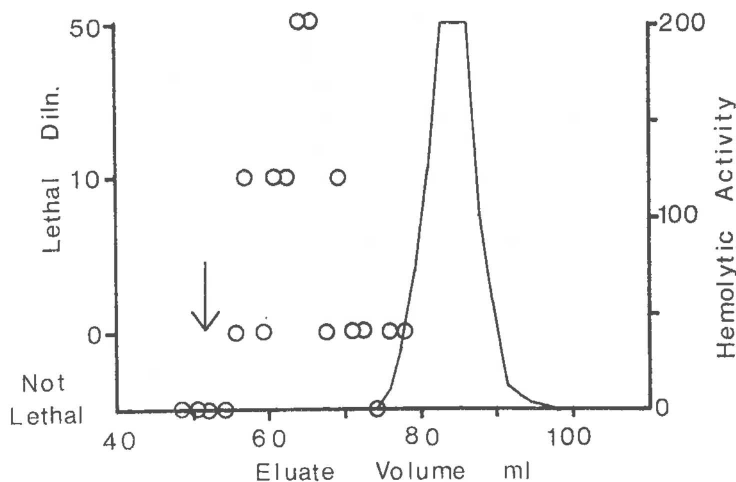

FIG. 4. SEPARATION OF A SECOND LETHAL FACTOR FROM THE HEMOLYSIN ON SEPHADEX G-200.
The solid line shows the position of hemolytic activity. The open circles show the maximum dilutions at which 0·1 ml samples of the fractions were lethal to mice. This monitoring of lethality was not carried on into the hemolytic fractions, but these were also lethal (see text). The arrow indicates the void volume of the column.

of the fractions into mice, the position of this second lethal component was found to correspond very approximately to a molecular weight of 150,000. Although not shown in the figure, the fractions of the hemolysin peak were also lethal (see below).

A quantitative estimation of hemolytic activity after Sephadex G-200 chromatography was performed as described for CM cellulose previously. The total recovery of hemolytic activity was 45·3 per cent of which 93 per cent was present in the single, sharp peak, The ratio of hemolytic activity to LD_{50} was 1:120 for the whole extract and 1:140 for the hemolytic peak, whereas the ratio was 1:0·1 for the second toxic peak. This last ratio reflects the virtual absence of hemolytic activity in this peak. The LD_{50} of the hemolytic fraction (as a dilution of 1 ml) was 480, whereas that of the fraction containing the non-hemolytic toxin was 340.

These results show that the hemolysin was not the only toxic material in the extracts. The hemolytic material was still lethal, but whether this lethality was due to an integral property of the hemolysin or to contamination with further unresolved toxins cannot be decided on the present evidence. Certainly at least one other toxic material is present in the extracts.

In this experiment the lethal activity of the second component was found to be approximately equal to that of the fraction containing the hemolysin. However, since it is possible that the two compounds have different stabilities, this equality of activity may not apply to the original extract.

Chromatography on Sephadex G-200 would complement CM cellulose chromatography as an additional means of purification of the hemolysin, based on completely different separatory principles.

Toxicity tests on separated fractions

The test procedure described under Methods afforded a rough means of monitoring the various fractions for toxicity; in some cases a more thorough study was made (CM cellulose and Sephadex G-200 separations). In all cases the fractions containing the hemolysin were found to be lethal, whereas the fractions on either side were not toxic, except on the Sephadex G-200 columns. Since neither ion exchange chromatography nor exclusion chromatography separated the hemolysin from all lethal properties, there is evidence that the hemolysin may be similar or identical to the factor responsible for this lethality. However, there is at least one other lethal component which chromatographs with the hemolysin on CM cellulose but is resolved on Sephadex G-200. The presence of two components would not conflict with the immunological observations of other workers (BAXTER *et al.*, 1968). It should be noted here that the toxic compounds of the extracts from whole tentacles, as used in the present study, are not necessarily the same as those present in the venom discharged by the nematocysts. However, there is evidence that extracts of whole tentacle and the venom from nematocysts are similar pharmacologically (FREEMAN and TURNER, 1969; KEEN and CRONE, 1969).

CONCLUSIONS

1. The *Chironex* extracts contain only one hemolytic compound; a variety of separatory procedures failed to resolve this into more than one component.

2. The hemolysin is protein in nature. No other class of compound is known which can fill all the requirements of high molecular weight, great potency coupled to high

sensitivity towards inactivation, and amphoteric behaviour at pH values near neutrality. It remains possible that the biological activity of the compound could be due to a prosthetic group attached to the main protein molecule.

3. The isolation of the hemolysin in a high degree of purity, as judged by the low levels of u.v.-absorbing or fluorescent materials, can be effected by column chromatography on CM cellulose and Sephadex G-200. Loss of activity remains the main problem to be solved in the preparation of the hemolysin in amounts suitable for direct chemical investigation.

4. The hemolysin is not the only toxic principle in the *Chironex* extracts. The *Chironex* venom appears to resemble other venoms in possessing both lytic and more directly toxic factors.

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DERMATONECROTIC PROPERTIES OF EXTRACTS FROM THE TENTACLES OF THE CNIDARIAN *CHIRONEX FLECKERI*

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Abstract—Whole extracts of the tentacles of *Chironex fleckeri*, active fractions obtained by Sephadex chromatography, and the toxin from nematocysts were examined for dermatonecrotic activity in rats and guinea pigs. The necrosis of skin appears to be the direct effect of the active material on the cells of the epidermis and dermis. Antihistamine and anti-5-HT compounds, cortisone acetate and parenteral rabbit antisera did not influence the development of skin necrosis. The dermatonecrotic, hemolytic and lethal activities appear to be related except for one fraction separated by chromatography, which had a high lethality but in which the other activities were low. The dermatonecrotic activity is antigenic and neutralizing antibodies can be produced in rabbits. The findings are discussed in relation to the symptoms occurring in human injuries.

INTRODUCTION

A COMPREHENSIVE review has been made of the clinical histories and findings associated with jellyfish stingings in Australia (CLELAND and SOUTHCOTT, 1965). The most severe stings and those resulting in death have been attributed to *Chiropsalmus quadrigatus* and *Chironex fleckeri*. On morphological examination *C. fleckeri* appears the more dangerous of the two species, being of larger size and having a greater density of nematocysts in the tentacles (BARNES, 1966).

In both fatal and non-fatal stings from *C. fleckeri*, local lesions are produced on the skin. These have been described as a series of transverse lines which may become rapidly red, swollen and vesicular. In non-fatal cases, necrosis and sloughing of the full thickness of the skin may occur and, later, scarring with pigmentation is common. Intense pain is also felt around the site of the injury, which may be so severe that an individual may become temporarily irrational.

Recent studies on the pharmacology of the toxin have demonstrated that most of the features of human stingings can be reproduced in the experimental animal (FREEMAN and TURNER, 1969; ENDEAN *et al.*, 1969). It has also been shown that extracts of the whole tentacle have similar pharmacological properties to preparations of toxin obtained by discharge of nematocysts through the amniotic membrane (FREEMAN *et al.*, 1969). Both types of material caused cardiac and respiratory failure and some intravascular hemolysis, but these findings could only be produced consistently when the material was introduced by the i.v. route. When either preparation was given i.c. or into the superficial subcutaneous tissues of experimental animals, death rarely occurred but areas of skin necrosis occurred around the site of inoculation, which appeared to be similar to those described in human injuries.

The purpose of this investigation was to define the mode of action of the extracts on the skin and to attempt to relate the skin necrotising properties of the toxin with the lethal and hemolytic activities which have been described (KEEN and CRONE, 1969).

MATERIALS AND METHODS

Supplies of the whole tentacle, and toxin prepared by discharge of nematocysts through the amniotic membrane, were obtained from Dr. J. Barnes, Cairns, Queensland (BARNES, 1967). The whole tentacles were stored in liquid nitrogen, and the prepared extracts at −20°.

The method of preparing the aqueous extracts from the whole tentacle and the estimation of the dilution required to produce 50 per cent hemolysis of a standard concentration of red cells (D50) has been described (KEEN and CRONE, 1969). The preparation of the active fractions of the extracts by chromatography on Sephadex G-200 has also been described (CRONE and KEEN, 1969). Fraction I consisted of the eluate collected between the void volume and the commencement of the hemolytic peak. Fraction II was a small quantity of material collected at the beginning of the hemolytic peak, while fraction III consisted of the major volume of fluid containing hemolysin. In order to obtain fractions of smaller volume a second chromatographic separation of tentacle extract on Sephadex G-200 was undertaken. Fractions IA and IIIA represent the volume of eluate collected over a range which was estimated to represent the peaks of activity in fractions I and III.

The minimum dilution of extract causing skin necrosis was determined either in guinea pigs weighing 400–600 g or in Wistar rats weighing 200–300 g. The skin used was on the dorsal surface, and the area was prepared by shaving on the day of the experiment. Serial double dilutions of the extract or venom were prepared in isotonic phosphate buffered saline at pH 7·2. As a routine, eight dilutions were used, the initial dilution being selected by preliminary experiments. Using a microsyringe, 0·05 ml of each was injected i.c. into separate areas outlined on the skin. Controls of buffered saline and heat inactivated extract were used in each case. After inoculation the animals were observed at 30 min, 1 hr, 4 hr and 24 hr for changes in color, swelling, vesiculation and necrosis around the sites of administration. The final readings were made after 48 hr when the minimum dilution producing at least 5·0 mm of necrosis in its maximum diameter was taken as the end point. This was expressed as the logarithm of the dilution of extract or toxin.

In another group of rats and guinea pigs, undiluted extracts were injected intracutaneously in the same manner, and samples of skin were removed at various intervals for the preparation of sections.

The effects of various substances were observed in rats; diphenhydramine hydrochloride (Parke Davis) 5 mg per kg, D-lysergic acid diethylamide tartrate (Sandoz) 0·6 mg per kg were given into the lateral tail vein 30 min before commencing the inoculations, and cortisone acetate (Roussel) 10 mg per kg was administered as a suspension i.p. in three daily doses. Antiserum prepared in rabbits against the extracts was given in a dose of 2·0 ml i.v. 24 hr prior to the experiment.

The assessment of vascular permeability at the site of injections was made in rats by injecting 1 per cent trypan blue 4·0 ml per kg i.v. 30 min before skin inoculations (JUDAH and WILLOUGHBY, 1962). Estimations of dye leakage were made by the macroscopic appearance of the skin 1 to 4 hr later.

The i.v. toxicities of the extracts were estimated by measuring the LD_{50} in albino mice weighing 15–20 g. Four groups of four animals were used for each estimation and four

dose levels were employed in a constant volume of 0·1 ml. The LD_{50} estimations were calculated from tables and expressed as the logarithm of the dilution in 1·0 ml of the original extract (WEIL, 1952).

The preparation of antisera was undertaken in rabbits. The procedure proved to be difficult as a number of animals died during the course of the immunization. An initial dose equivalent to three LD_{50} for mice was given i.v. to rabbits twice weekly for a month and then they were rested for a month. A further series of injections were given, increasing the dose slowly up to 10 LD_{50} by the end of a further 4 weeks. Serum was obtained by bleeding the animals by intracardiac puncture, allowing the blood to clot overnight, and decanting the serum. It was then cleared by centrifugation, heated at 56° for 30 min and stored at −20° until required.

The antiserum was tested for antibodies against the lethal, hemolytic and skin necrotizing properties of the extracts.

Tests for antibodies against the lethal factor were made in a series of 10 tubes using double dilutions of the antisera in buffered saline. A dilution of the extract was added to each tube so that five LD_{50} was present in 0·1 ml of the mixture. The mixture was allowed to stand for 30 min at a temperature of 25° and it was then tested for lethality by injecting 0·1 ml into the lateral tail vein of mice. The minimum dilution of serum neutralizing five LD_{50} of extract could then be estimated.

A similar technique was used to measure antihemolysins. Serial double dilutions of antisera were made up in 10 tubes and to each was added 1·0 ml of a dilution of extract containing 50 times the estimated D_{50} of hemolysin. This was incubated for 30 min at 25° and then 1·0 ml of a standard red cell concentration was added. The tubes were allowed to stand for 5 hr in a water bath at 25°. The maximum dilution of serum which could completely neutralize the hemolysin was estimated. Controls of normal serum and buffered saline were used.

Titres of antibodies to the dermatonecrotic factor were estimated by making double dilutions of venom in a series of eight tubes, adding an equal volume of one in four dilution of antiserum and incubating at 25° for 30 min. 0·05 ml from each was then injected intradermally in the manner previously described and compared with two series of controls using normal serum and buffered saline.

RESULTS

The dermatonecrotic activity of extracts

Necrosis and damage to the skin of experimental animals could be produced by extracts obtained from homogenized tentacle, toxin obtained by discharge of nematocysts through the amniotic membrane, and with one of two active fractions prepared by chromatography on Sephadex G-200. The types of lesions produced by the three types of preparations were identical and with the exception of fractions I and IA obtained on chromatography the minimum necrotic dose appeared to follow the over-all potency of the extracts as measured by both lethal and hemolytic activity. The minimum necrotic dose of the various materials is shown in Table 1.

After injection of 0·05 ml of an active extract into either guinea pigs or rats no immediate reaction was observed in the skin. There was a discrete swelling in the skin which was mainly due to the volume of the inoculum with some reddening around the periphery. Approximately 15 min after the administration of very active extracts, a bluish coloration was often seen under the site of inoculation in those areas which would later progress to

necrosis. This appearance was most frequently observed in guinea pigs and it was a less consistent finding in rats. Vesiculation over the area of injection was a most uncommon finding.

After 4 hr some areas already showed patches of brownish discoloration and the skin appeared devitalized. At the end of 24 hr these areas had become discrete brown patches having a firm texture of dead skin, although in rats it was occasionally necessary to wait for 48 hr for this appearance to be complete.

After death of the skin there is a slow process of separation from the viable tissues which occurs over the next 10 to 14 days. Provided there is no secondary infection the inflammatory reaction is not marked.

An examination of the effects of the toxin in rats in which 1 per cent trypan blue had been injected 30 min before the i.c. inoculation confirms the previous observation that there is little or no general increased vascular permeability at the site of the lesion. At the end of 4 hr the central area appears as a pale plaque surrounded by a blue rim, indicating increased vascular permeability at the periphery only.

Microscopic changes in the skin

Samples of skin were taken from the trunk of guinea pigs at varying intervals up to 24 hr after inoculation. Before being placed in fixative the skin was examined by transmitted light when the outline of thrombosed skin vessels could be seen readily against the translucent skin. The sections were stained with hematoxylin and eosin.

Sections obtained during the first 4 hr usually showed changes in the epidermis. The nuclei of the basal cells stained more deeply and there were some signs of separation of the thin horny layer from the granular cell layer. In the dermis there was some extravasation of blood into the tissue spaces and the capillaries were prominent and packed with red cells. The reaction in the surrounding tissues was not marked, with only a moderate increase in tissue histiocytes and a few polymorphs.

Although the findings in the early lesions were mild, after 24 hr the changes in both the dermis and epidermis were quite marked. The epidermal cells lost their normal outline and at the end of the areas of necrosis they appeared as cellular ghosts. Towards the centre of the damaged area their structure was completely lost, merging with the large bulk of eosinophilic necrotic tissue. The signs of necrosis extended into the dermis where there were areas of hemorrhage. There was a profuse cellular reaction with some of the cells in close proximity to the damaged tissue showing nuclear fragmentation and death. The cells which extend around the whole boundary of the necrotic tissue were mainly tissue histiocytes, small round cells and polymorphs.

Effect of drugs on the dermatonecrotic activity

Previous work has shown that there appears to be no acetylcholine, 5-hydroxytryptamine (5-HT) or histamine in extract of the tentacle (FREEMAN and TURNER, 1969).

However it was considered that either 5-HT or histamine could be liberated in the tissue following the administration of the extract and that either one or the other might contribute to the development of the lesion. Rats were given diphenhydramine hydrochloride 5 mg per kg and D-lysergic acid diethylamide tartrate 0·6 mg per kg 30 min prior to the administration of the extract in the skin but showed no unusual feature when compared with controls.

Animals given cortisone acetate 50 mg per kg by the i.p. route for 3 days and 2·0 ml of rabbit antiserum 24 hr prior to the i.d. dose of extract also showed lesions similar to controls.

The minimum necrotic dose for a given batch of extract was estimated in animals given each of the above substances. No change in the dilution of extract required to produce necrosis could be observed in any animal.

The relation between necrotic, lethal and hemolytic activities

The estimation of the relative activities for several batches of extract are shown in Table 1. These results show that there is a correlation between the three types of activity in both crude extracts prepared from tentacle and also in purified preparations obtained by discharge of nematocyst toxin through human amniotic membrane.

TABLE 1. THE RELATION BETWEEN DERMATONECROTIC, HEMOLYTIC AND LETHAL ACTIVITIES OF VARIOUS PREPARATIONS

Batch No.	Minimum skin necrotizing dose Log_{10} (dilution)*	LD_{50} in mice† Log_{10} (dilution) ±S.D.	Hemolysin titre Log_{10} (dilution) ±S.D.
Batch 2 of tentacle extract	2·1 (2)	4·1±0·08	5·9±0·3 (5)
Batch 4 of tentacle extract			
23/10/68	1·6 (1)	3·7±0·08	5·4±0·11 (5)
24/ 3/69	1·5 (6)	N.E.§	4·4±0·03 (3)
Batch 5 of tentacle extract	2·0 (6)	3·2	5·3±0·13 (5)
Material obtained by discharge of nematocyst through amniotic membrane	0·9 (2)	2·8	4·0±0·08 (5)
Sephadex G-200 Fractions			
Fraction I	N.A. (2)‡	2·5±0·01	1·6 (2)
Fraction IA	N.A. (2)‡	N.E.§	< 1·5 (2)
Fraction III	0·9 (2)	2·7±0·01	4·8 (2)
Fraction IIIA	0·6 (2)	N.E.§	4·5 (2)

*The minimum skin necrotizing dose is expressed as the logarithm of the dilution of extract or toxin contained in 0·05 ml which will cause 5·0 mm of skin necrosis in rats or guinea pigs.

†The LD_{50} was expressed as a calculated dilution of the extract or toxin which would be contained in 1·0 ml.

‡N.A. No activity in undiluted samples.

§N.E. Not examined.

Figures in parentheses represent the number of experiments.

These findings alone could suggest that the three types of activity might be the manifestation of a single substance. However, previous work employing chromatography with Sephadex G-200 has shown that two lethal fractions can be obtained, one with a mol. wt. of 75,000 approximately and the other with a mol. wt. 150,000 approximately (CRONE and KEEN, 1969). The former contained in fractions III and IIIA has been shown to have both hemolytic and dermatonecrotic activity, while fractions I and IA have little or no hemolytic activity and no dermatonecrotic activity.

The antigenic properties of tentacle extracts

It was difficult to produce high titre antiserum in rabbits; a course of twice weekly injections being required for 8 weeks before satisfactory material was obtained.

When 2·0 ml of this antiserum was given 24 hr before an intradermal inoculation of extract it failed to prevent or modify the necrosis. However, this may be partly due to the relatively low titres of the antiserum for the dermatonecrotic factor.

When the same antiserum was mixed with an extract of a known potency and held at 25° for 30 min prior to inoculation a definite increase in the minimum necrotizing dose was observed. This is shown in Table 2.

The antiserum had been shown to have antibodies to the lethal and hemolytic activities, the titre against these components being shown in Table 2.

TABLE 2.

Component		Neutralizing titre
Lethal factor	Whole extract	40
	Sephadex fraction I	320
	Sephadex fraction III	80
Hemolysin		3000
Dermatonecrotic	Factor	16

The neutralizing titre is expressed as follows:
The number of LD_{50} neutralized by 1·0 ml of undiluted serum; the number of D_{50} of hemolysin neutralized by 1·0 ml of undiluted serum, and the number of minimum skin necrotizing doses neutralized by 1·0 ml of undiluted serum.

Pain and dermatonecrotic activity

The current pharmacological investigations have not demonstrated any of the known pain-producing substances such as acetylcholine, 5-HT, histamines or kinins in extracts from or prepared toxins (FREEMAN and TURNER, 1969; ENDEAN *et al.*, 1969).

At the same time this investigation suggests that there is no release of histamine, 5-HT or vaso-active kinins into the tissues. Most of the experimental animals used in this investigation, mice, rats and guinea pigs, showed little or no signs of discomfort when relatively large doses of extract or toxin was given into the subcutaneous or intracutaneous tissues.

Although there are unidentified substances in the skin which are capable of producing pain the present work would suggest that the most likely cause of pain which accompanies accidental injuries in humans is due to tissue damage or possibly the release of potassium from damaged cells (FREEMAN and TURNER, 1969).

DISCUSSION

The experiments indicate that in rats and guinea pigs the dermatonecrotic activity of tentacle extracts and pure toxin preparation of *Chironex fleckeri* are the result of a direct effect on the cells of the epidermis and dermis. Although there is capillary damage with thrombosis within the area of damage there appears to be only a mild vascular response around the lesion. It is suggested that the toxin acts directly on the cell membrane in a manner similar to that which produces hemolysis, resulting in rapid death of the cells. The damage to capillary endothelium with thrombosis appears to be part of this over all effect rather than a factor contributing to the cell necrosis. In view of the type of response it is not

unexpected that neither antihistamines nor 5-HT antagonists had any effect on the development of the lesions.

It is still uncertain whether the diverse activities of the extracts and the toxin, namely dermatonecrotic, hemolytic and lethal are due to one component or separate components. So far, chemical analysis of the extracts has produced two fractions with approximate molecular weights of 75,000 and 150,000. Dermatonecrotic activity could only be demonstrated in the material with the lower molecular weight. In this respect it is similar to the material with hemolytic activity (KEEN and CRONE, 1969).

When the whole extract of the tentacle is injected into rabbits, antiserum which is capable of neutralizing the dermatonecrotic activity *in vitro* is obtained. The antiserum proved difficult to prepare and satisfactory titres could only be obtained after several weeks of carefully graded doses of extract. The antiserum could be shown to have neutralizing properties against both the lethal and hemolytic activities. However, as skin necrosis is the least sensitive method of detecting activity in an extract relatively high titre serum is required to neutralize dermatonecrotic factor. For practical purposes the hemolytic system is the most sensitive test system and provides the easiest method of checking the activity of extracts and the titres of antisera.

The above results on the antigenicity of extracts differ in certain respects to a previous report on immunity to the venom of *Chironex fleckeri* (BAXTER *et al.*, 1968). However, this may be due to the different antigenic properties of the extracts of the tentacle which were used in these experiments for the preparation of antiserum.

In humans, skin lesions are the most common manifestations of stingings due to *Chironex fleckeri*. Severe constitutional symptoms and death are considered to be relatively uncommon and occur only in a small proportion of accidental injuries (BARNES, 1966).

The dermatonecrotic response in experimental animals differs in degree to that described in humans where there is marked reddening, swelling and vesiculation. This may be entirely due to the physical differences; human skin is thicker and more vascular than the skin of guinea pigs and rats and has a well developed keratin layer.

In animals it has been shown that many times the i.v. dose of extract or toxin can be given into the tissues of the skin, or by other parenteral routes without causing severe symptoms or death. Despite the absence of systemic effects, when given by the i.c. route necrosis may occur around the site of the injection indicating that the extract is potent. It appears that the active fractions are fixed in the tissues and rapidly destroyed while any remaining toxic material is poorly absorbed into the circulation.

In humans, therefore, fatal cases may be explained on the basis of the fortuitous inoculation of venom directly into the circulation through a superficial vessel in the skin. The fact that the everted threads are long enough and are capable of penetrating into the dermis has been previously demonstrated (KINGSTON and SOUTHCOTT 1960; ENDEAN *et al.*, 1969).

This investigation has been of little help in defining the relationship between the dermatonecrotic activity and the severe pain reported in human injuries. In the absence of any known pain-producing substance in either the extracts or the toxin, or the liberation of similar substances in the skin, it is suggested that the pain is mainly due to tissue damage with the liberation of potassium from damaged cells (FREEMAN and TURNER, 1969).

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NOTE:

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FURTHER STUDIES ON THE BIOCHEMISTRY OF THE TOXINS FROM THE SEA WASP *CHIRONEX FLECKERI*

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Abstract—Extracts from the tentacles of *Chironex fleckeri* (Cnidaria) were resolved into two lethal fractions by chromatography on CM-Sephadex C-50. These were apparently identical to the two fractions previously resolved on Sephadex G-200. The hemolysin in the extracts was also examined by thin-layer chromatography on Sephadex G-200 and by various electrophoretic techniques. It was shown that the hemolysin associated markedly with the various support media, which made electrophoresis of little use. The tentacle extracts and a toxin obtained by a milking technique were compared by chromatography and shown to be very similar if not identical in their biological activities. Some data on the stability of the hemolysin was also obtained.

INTRODUCTION

A PREVIOUS study at this laboratory (CRONE and KEEN, 1969) has shown that extracts of the tentacles from the sea wasp or box jellyfish *Chironex fleckeri* contain at least two toxins. One of these is hemolytic with a mol. wt. of 70,000 and the second one of mol. wt. 150,000 is lethal on injection into mice but not hemolytic. The pharmacological effects of these two toxins have also been studied (Freeman and Turner, unpublished observations).

The present work is concerned with extending the separatory techniques used to study this material. Also, since other workers (BAXTER and MARR, 1969) have reported apparently different results from a *Chironex* preparation obtained by a technique analogous to the milking of snake venom, we have compared the tentacle extracts with a milked toxin preparation.

MATERIALS AND METHODS

Extracts from tentacles of *Chironex fleckeri* were prepared as described in the previous paper (CRONE and KEEN, 1969). A preparation referred to here as 'milked toxin' was kindly prepared and supplied by Dr. J. H. Barnes. This was obtained by causing *Chironex* nematocysts to discharge through amniotic membrane and collecting the venom which had penetrated to the other side (BARNES, 1967).

Biological assay methods

The rapid semi-quantitative method (CRONE and KEEN, 1969) was used to estimate hemolytic potency. This method, based on the relationship between time to complete hemolysis and dilution, yielded values related to the particular whole extract being employed, which was assigned the arbitrary value of 1000. Since the time against dilution curves of various extracts were different, direct comparison of results between different preparations (e.g. tentacle extract and milked toxin) was not possible.

Lethal activity was located and estimated by injection of 0·1 ml of samples into the tail veins of mice. Approximate quantitation was achieved by finding the maximum dilution which caused death on injection. Usually five dilutions were tested, each in one mouse.

Column chromatography on cation exchange Sephadex

CM-Sephadex C-50 (product of Pharmacia, Sweden) was allowed to swell in a buffer of 50 mM NaCl, 10 mM sodium phosphate pH 6·5 and equilibrated with this buffer by repeated decantation and resuspension. A column was then packed with the gel (bed size 1·6 cm dia. by 16 cm high) and the sample applied. The column was eluted with 65 ml of the above buffer at a flow rate of 16 ml/hr. After this a linear gradient of NaCl was applied which was formed by 200 ml of the above mentioned buffer in a mixing chamber, replenished by 200 ml of 0·5 M NaCl, 10 mM sodium phosphate pH 6·5. Finally the column was eluted with buffer containing 1M NaCl. All operations were carried out at 5°.

Thin-layer chromatography on Sephadex G-200

Sephadex G-200, superfine grade, was allowed to swell in 150 mM NaCl, 5 mM Tris HCl pH 8·0, and spread on 20 × 20 cm glass plates as a layer 0·5 mm thick. The plate was equilibrated with buffer by eluting overnight at 5°. Samples were formed by soaking 2 mm square pieces of Whatman No. 1 (small sample) or No. 3 (large sample) filter paper in the specimen, and the paper squares were then applied to the thin-layer plates. Six samples were run on each plate, one of the centre samples being Blue Dextran 2000 (Pharmacia). The plates were then eluted with buffer until the Blue Dextran had reached the far side (about 4 hr).

To detect hemolytic activity, a strip of Whatman No. 3 paper was soaked in the washed red blood cell suspension used for the hemolysis assay and then applied to the developed chromatogram. The paper was left in contact with the Sephadex layer at 5°, and inspected after 1 and 18 hr. Rapid hemolysis was indicated by a clear area surrounded by a bright red colour after 1 hr, slow hemolysis appeared after 18 hr as a white zone against a pale pink background.

Protein markers were detected by applying Whatman No. 3 paper to the chromatogram for 2 min, then drying the paper transfer and staining for protein with Amido Black (WIEME, 1965).

Electrophoretic techniques

Trial electrophoretic separations were made on Whatman No. 1 paper in pyridine–acetic acid–water buffer pH 6·5, or on cellulose polyacetate (Sepraphore III, Gelman Instrument Co.) in various buffers of pH from 6·5 to 8·8. Detection of hemolytic activity was effected by incubating 0·5 cm wide segments of the electrophoretic support in tubes of the normal hemolytic assay system.

Electrofocussing in polyacrylamide gels was performed by the method of AWDEH *et al.* (1968).

Other methods used were described previously (CRONE and KEEN, 1969).

RESULTS

Stability of the hemolysin

Since the lack of stability of the hemolysin is a decisive factor in determining what procedures the material can be subjected to, the stability was examined under conditions likely

to occur in normal chromatographic and electrophoretic procedures. The experiment was carried out at 5°, mostly in a medium of 150 mM NaCl supplemented with 10 mM buffer salts or other additions. Table 1 summarizes the results as percentage hemolytic activity

TABLE 1. THE STABILITY OF *Chironex* HEMOLYSIN IN SOLUTIONS OF VARYING COMPOSITION AT 5°

Addition*	Activity remaining after 48 hr (as % of activity at zero time)
Tris	50
Sodium phosphate	20
Sodium barbitone	10
Imidazole	10
Alanine	80
EDTA	70
1M Urea	20
2% Ampholine	80
No addition (150 mM NaCl only)	90
Distilled water	100

**Chironex* tentacle extract 0·1 ml was added to 2·0 ml of 150 mM NaCl solution at 5° containing one of the above compounds (10 mM) and adjusted to pH 7·4. A sample of 0·1 ml was removed immediately for assay of hemolytic activity. Subsequent samples were removed and assayed at 2, 4, 24, 48 and 72 hr. The best estimate of activity remaining after 48 hr was made from the curve of activity against time. EDTA=Ethylenediaminetetra–acetic acid (adjusted to pH 7·4 with NaOH). Ampholine=Carrier ampholytes for isoelectric focusing (pH range 3–10), product of LKB—Produkter, Sweden.

remaining after 48 hr. The pattern of stability was not different after the short time periods. Tris was apparently the most favourable buffer salt, barbitone and imidazole were definitely unfavourable. Unbuffered media (saline or distilled water) caused least loss of activity. There was no marked loss of activity due to EDTA, contrary to the results of other workers using a milked toxin (BAXTER and MARR, 1969). These results show that one can expect a recovery of hemolytic activity better than 10 per cent after the usual chromatographic fractionation, which takes about 2 days.

Chromatography on cation exchange Sephadex

The results of chromatography on carboxymethyl Sephadex are illustrated in Figs 1 and 2. Separation of the tentacle extract (Fig. 1) into two lethal fractions was achieved, one of them being hemolytic. Most of the protein in the extract passed straight through the column, resulting in considerable purification of the two active fractions. Compared to the whole extract, the ratio of lethal activity to protein content of the non-hemolytic toxin was increased fourfold, and the ratio of hemolytic activity to protein was increased sevenfold for the hemolysin. Recovery of hemolytic activity in the main peak was 34 per cent. The approximate value for recovery of lethal activity was 70 per cent, assuming that the two separated activities were additive in the whole extract; 40 per cent was in the first peak and 30 per cent in the second, hemolytic peak. Minor amounts of hemolytic activity were present in fractions other than the main peak; at fractions 4, 5, 60 and 61. However the sum of these activities did not amount to 1 per cent of the total activity.

The chromatography of the milked toxin (Fig. 2) resulted in a very similar pattern to that of the tentacle extract. The two active fractions were eluted at the same points of the NaCl

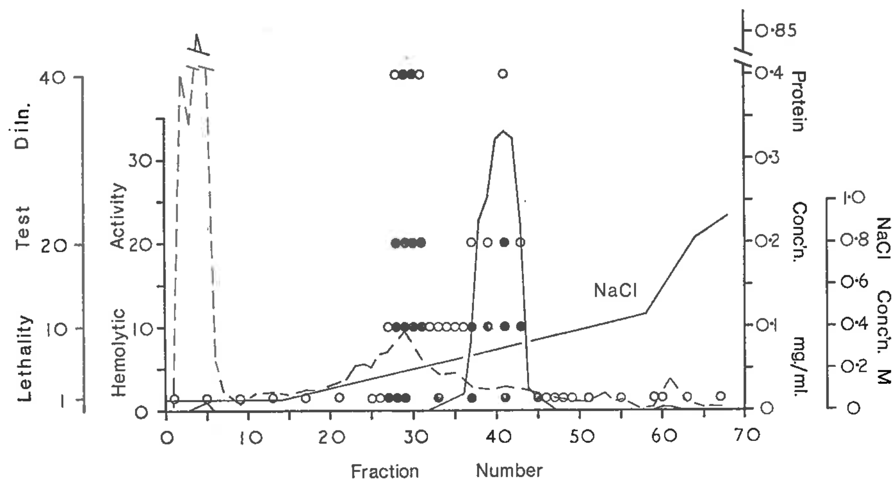

FIG. 1. THE CHROMATOGRAPHY OF A *Chironex* TENTACLE EXTRACT ON A COLUMN OF CM-SEPHADEX C-50.

Hemolytic activity is shown by solid line, and protein concentration by broken line. The salt gradient applied to the column is also shown as a solid line, labelled NaCl. Lethality tests are shown by circles, the open ones indicating survival and the solid ones death. The dilution of the column fractions used in the lethality test are shown on the scale at the extreme left-hand side. The fraction volume was 8 ml. Details of the technique are given in the Methods section.

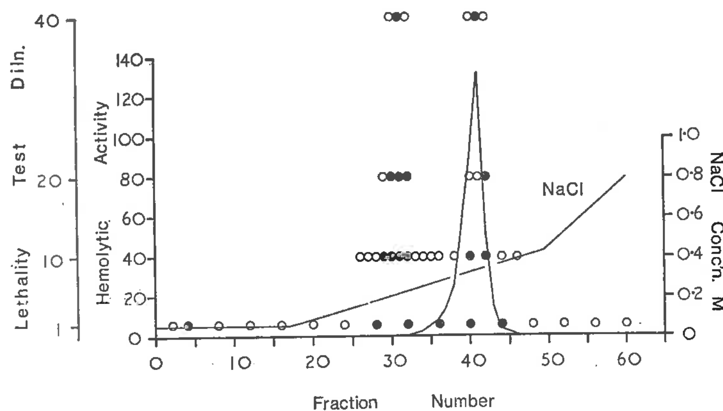

FIG. 2. THE CHROMATOGRAPHY OF *Chironex* MILKED TOXIN ON A COLUMN OF CM-SEPHADEX C-50.
The symbols and conventions used are described under Fig. 1.

gradient, and no other biological activity was detectable, except lethality in fraction 4, which probably represents some leakage through the column on loading. The recovery of hemolytic activity from this column was 160 per cent, based on the apparent activity of the original extract.

Since the estimates of biological activity, especially lethality, were only approximate, a close quantitative comparison of the distribution of activity in each preparation was not possible. However, when a survey was attempted the following picture emerged. The two whole preparations were equal in lethal potency (0·1 ml of a 1 : 600 dilution was fatal) whereas hemolytic activity in the milked toxin was estimated as a tenth of that in the tentacle

extract. Since 4 ml of tentacle extract and 2 ml of milked toxin were applied to the columns, the observed sharper outline and reduced heights of the lethal peaks from the milked toxin were commensurate with the smaller sample, and did not indicate a difference between the preparations. The hemolytic peak of the milked toxin (in terms of the dilution units) was higher than expected, resulting in the apparent recovery of 160 per cent mentioned earlier. It appears that the hemolytic activity of the whole milked toxin was depressed yielding a value much lower than expected relative to the lethality. This apparent depression may have been due to an inhibitor in the milked toxin which was removed during chromatography, but the nature of this component is not known. Apart from this depression of the initial hemolytic activity in the milked toxin, the two preparations were very similar.

Chromatography of separated fractions on Sephadex G-200

A sample of 5 ml of the non-hemolytic lethal peak from CM-Sephadex C-50 (fractions 28–32, Fig. 2) was mixed with 1 ml of 50 mM Tris pH 8·5. The mixture was applied to a column of Sephadex G-200 and eluted as described previously (CRONE and KEEN, 1969). Lethality was found in three adjacent fractions at $V_e/V_0=1{\cdot}28$ (elution volume divided by void volume). This was the same position as that of the non-hemolytic lethal fraction of mol. wt. 150,000 previously described ($V_e/V_0=1{\cdot}24$) and was distinct from the lethality associated with the hemolysin (V_e/V_0 1·63–1·76).

When the same procedure was tried with the hemolytic peak from the CM-Sephadex, no hemolytic activity was detectable in the elute from the Sephadex G-200 column, presumably due to the instability of the hemolysin.

Thin-layer chromatography of tentacle extract on Sephadex G-200

The results of this work are summarized in Fig. 3. The best conditions found for chromatography were use of a buffer at pH 8·0 containing NaCl at 150 mM or above. Even at a concentration of 0·5 M NaCl, the hemolysin showed a tendency to trail. Comparison of the main spot in these buffers with markers of hemoglobin and serum albumin confirmed the original assignment of a molecular weight of 70,000 for the hemolysin (CRONE and KEEN, 1969). A loading effect was also seen on these chromatograms; smaller samples did not move as far from the origin.

When either the salt concentration or the pH was lowered, the trailing effect became more evident. At low salt concentration the bulk of the hemolysin remained near the origin and only a slight amount of activity moved forward. Clearly, to obtain reasonable results a moderate salt concentration, alkaline pH and correct degree of loading are required. The hemolysin of the milked toxin behaved very similarly to that of the tentacle extract.

When hemolytic fractions from the G-200 Sephadex or CM-Sephadex columns were chromatographed on the thin layer, very little movement forward was noted. In some cases the zone of hemolysis covered only the paper square on which the sample had been applied. Use of G-25 Sephadex in place of G-200 did not alter this result.

Electrophoresis of the hemolysin

Electrophoresis experiments were unsatisfactory in that much trailing of the hemolysin was observed and very little hemolytic activity survived even after runs of 4 hr or less. Compared with rabbit serum, the hemolysin appeared to behave as a γ-globulin.

Gel electrofocussing produced the same unsatisfactory results as the electrophoresis experiments.

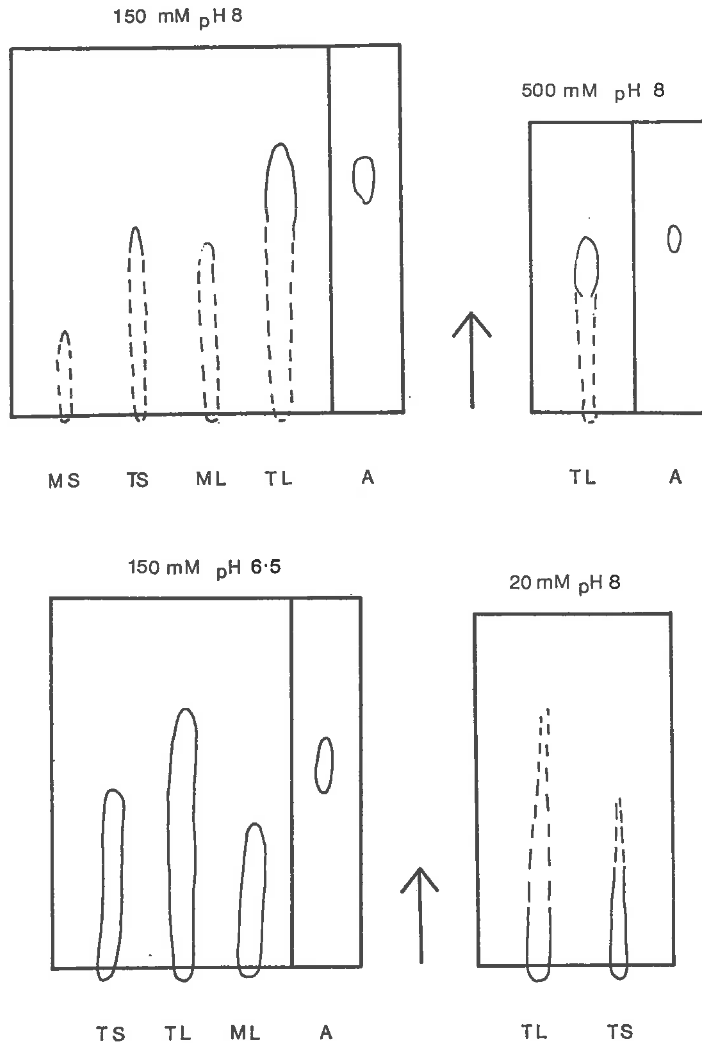

FIG. 3. THE CHROMATOGRAPHY OF *Chironex* TENTACLE EXTRACT ON THIN LAYERS OF SEPHADEX G-200 UNDER DIFFERENT CONDITIONS OF pH AND SALT CONCENTRATION

The diagram shows tracings from the paper transfers of the chromatograms. Samples were applied on the lower horizontal line, and the chromatograms developed until marker Blue Dextran 2000 reached the position shown by the upper line. Areas of rapid hemolysis (1 hr) are outlined by a continuous line, slow hemolysis (18 hr) by a broken line. Above each chromatogram is given the NaCl concentration and the pH (buffered by 10 mM phosphate or 5 mM Tris). T=Tentacle extract, M=Milked toxin, A=Serum albumin, L=Large sample, S=Small sample.

DISCUSSION

The poor separations obtained on electrophoresis or thin-layer chromatography of the hemolysin can be explained on the basis of an early observation (CRONE and KEEN, 1969), i.e. that the hemolysin shows a marked affinity for various support media including filter paper and cellulose polyacetate. In the case of chromatography on Sephadex layers, higher salt concentrations and alkaline pH reduced ionic bonding to weak acidic groups of the

Sephadex and decreased the cationic nature of the hemolysin, thus reducing the interaction with the support and the consequent trailing. Even under reasonable conditions of pH and salt concentration it seems that very small samples (such as the dilute column fractions) are adsorbed to such an extent near the site of application that none moves forward to the expected position. For meaningful results to be obtained from column chromatography on Sephadex, a suitable eluting buffer must be used, and sharp peaks of activity only considered. Our previous work on Sephadex G-200 (CRONE and KEEN, 1969) conforms to these requirements, and the method has been used several times with complete reproducibility for the preparation of the two active components. A second possibility, that the hemolysin is a small molecule adsorbed strongly to a larger molecule in the less pure extracts, is unlikely since it is not dialysable and no effect indicating even slight dissociation has been seen. There is little doubt that the previous assignments of molecular weight values to the two active toxins were correct.

The tentacle extract and the milked toxin contained the same active components in approximately the same proportion. Some reported properties of the milked toxin (BAXTER and MARR, 1969) are difficult to reconcile with these results, e.g. the instability of the hemolysin in the presence of EDTA, and the assignment of molecular weights in the range 10,000 to 30,000 for the biologically active molecules.

The chromatography on CM-Sephadex was the best separatory method yet found for isolating the two toxins, yielding sharp peaks in a very reproducible manner. Since the two are not separated by chromatography on CM-cellulose (CRONE and KEEN, 1969) the separation on CM-Sephadex must be mainly a size effect, the larger non-hemolytic toxin being denied access to some of the exchange groups on the matrix and thus being eluted earlier. The further chromatography of this toxin on G-200 Sephadex confirmed its higher molecular weight.

The nature of the *Chironex* toxins can be summarized in the following way. Three biological activities have been examined; lethality, hemolysis (KEEN and CRONE, 1969*a*) and dermatonecrosis (KEEN and CRONE, 1969*b*). Two active fractions have been prepared. One, of mol wt. 150,000 displays only lethal activity and is eluted first from Sephadex G-200 and CM-Sephadex. The second, mol. wt. 70,000 has lethal, hemolytic and dermatonecrotic activity when eluted from Sephadex G-200, and is apparently the same fraction as that which is eluted second from CM-Sephadex, although this has not been conclusively proved. It is of course possible that further work will resolve these two fractions into further biologically active components.

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COMPARISON OF TENTACLE EXTRACTS FROM *CHIROPSALMUS QUADRIGATUS* AND *CHIRONEX FLECKERI*

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Abstract—The dermatonecrotic, lethal and hemolytic activities of extracts of the tentacles of *Chiropsalmus quadrigatus* and *Chironex fleckeri* were examined. The activity of the dermatonecrotic and lethal components appeared to be similar. The hemolytic activity of the two extracts could be distinguished by the relative greater stability of *C. fleckeri* extracts at higher temperatures. The experiments suggest that the mode of action of the two hemolysins is different. Antisera prepared against the individual extracts showed neutralizing antibodies against the three types of activity but they were specific for each species and no common antigenic properties could be detected. A suggested method for the identification of tentacle fragments is described.

IN AUSTRALIA two species of box jelly fish, *Chironex fleckeri* and *Chiropsalmus quadrigatus*, are a toxic hazard to man. During the summer months they are commonly present in the waters around the coastline north of the Tropic of Capricorn and are considered to be responsible for all the major incidents of stingings and human fatalities reported (CLELAND and SOUTHCOTT, 1965). Both *C. quadrigatus* and *C. fleckeri* are similar in appearance, although the latter appears to reach a larger size (BARNES, 1966). They can be distinguished on a morphological basis, but the differences are slight and may only become apparent after fixing in a suitable preservative. The principal nematocysts of both species are cigar-shaped and similar in appearance and both types are classified as microbasic mastigophores. In general the density of nematocysts in *C. fleckeri* is greater and this is considered the more dangerous of the two species as far as human injuries are concerned.

The pharmacological properties of extracts of the tentacles of *C. fleckeri* and *C. quadrigatus* are very similar. Chromatographic separation of an extract of *C. quadrigatus* on a column of Sephadex G-200 produced two peaks of activity; one hemolytic, and the other lethal when injected into mice and rabbits. The activity of the hemolytic fraction was insufficient to establish whether it was lethal (Freeman and Turner, unpublished data). The elution behaviour of the two peaks indicated that the molecular weight of the hemolytic fraction was approximately 70,000 and that of the lethal fraction approximately 150,000 which was identical to the molecular weight of two fractions obtained on chromatography of extracts of *C. fleckeri* (CRONE and KEEN, 1969).

The purpose of this investigation was to examine extracts of the tentacle of *C. quadrigatus* for hemolytic, dermatonecrotic and lethal activities and to compare these properties with those previously reported for *C. fleckeri* (KEEN and CRONE, 1969*a*).

METHODS

The specimens of *C. fleckeri* and *C. quadrigatus* were collected and identified by Dr. J. Barnes, Cairns, Queensland, Australia. The method of collecting and storing samples has

been described (KEEN and CRONE, 1969*a*). In the case of *C. quadrigatus* the extracts were prepared from pooled samples which were taken from several organisms.

Whole tentacle extracts were used for all experiments. The extracts of *C. quadrigatus* were prepared by mincing the frozen tentacle and suspending it in a buffer containing 150 mM sodium chloride and 5 mM tris-(hydroxymethyl) aminomethane (tris) (pH 8·0). This buffer was chosen since a portion of the extract was to be used for chromatographic studies. The suspension was centrifuged at 2500 *g* for 20 min at 5° and the supernatant stored at −20° until required. The initial batch which was used for most of the quantitative assays was prepared from 4·35 g of tentacle and 10 ml of buffer. Two other batches were prepared similarly, containing 400 mg of tentacle per ml of buffer. The extract of *C. fleckeri* had been prepared by homogenizing 0·716 g of tentacle in 12 ml of phosphate buffered saline (PONDER, 1948) pH 7·20 at 5°. The material had been centrifuged at 2500 *g* for 20 min and the supernatant stored at −20°.

The hemolysin titre (D_{50}) was estimated as the logarithm of the reciprocal of the highest dilution of extract which produced 50 per cent hemolysis of rabbit red cells ($3{\cdot}0$–$3{\cdot}5 \times 10^7$ cells per ml) in 5 hr. Estimations were carried out at 15° unless otherwise stated. The lethal activity (LD_{50}) was expressed as the logarithm of the reciprocal of the dilution of extract contained in 1 ml which when injected into mice will kill 50 per cent of the animals. The skin necrotizing dose (SND) is expressed as the logarithm of the reciprocal of the highest dilution of extract or toxin which when injected in a 0·05 ml dose will cause 5 mm of skin necrosis in guinea pigs. These measurements have been described (KEEN and CRONE, 1969*a*, 1969*b*). To prepare antisera an initial dose equivalent to $3LD_{50}$ (mice) was given i.v. to rabbits twice a day, 3 days a week for the first week. Injections were then given three times a week, the dose being gradually increased until it was ten times the initial dose. At the end of 4 weeks immunization, animals were bled by intracardiac puncture. The blood was allowed to clot and the serum decanted. It was then cleared by centrifugation, heated to 56° for 30 min and stored at −20°. The preparation of *C. fleckeri* antiserum has been described previously (KEEN and CRONE, 1969*b*). The procedures for estimating antibodies against the lethal and dermatonecrotic factors, and hemolysins have been described (KEEN and CRONE, 1969*b*).

Immunodiffusion studies on the hemolysin were carried out in a medium containing 1 per cent special grade agar (Oxoid) and 10 per cent rabbit red cells. The red cells were prepared from heparinized blood, washed three times and then reconstituted to their original volume with phosphate buffered saline, and added to the agar at 48°. Sufficient agar was used to give a thickness of 1 to 1·2 mm in the Petri dishes.

The isotonic solutions used to measure the effect of pH on stability of the extracts and the rate of hemolysis were buffered with disodium phosphate and potassium dihydrogen phosphate between pH 6 and 8 and with tris–hydrochloric acid between pH 7·5 and 9. Analytical grade chemicals were used for all experiments. Lecithin was prepared from egg yolk by the method of DAWSON (1963).

RESULTS

Estimation of the lethal, dermatonecrotic and hemolytic potency of C. quadrigatus *extracts*

Measurements of the D_{50}, LD_{50} and the SND were made. The D_{50} was $5{\cdot}0 \pm 0{\cdot}04$ (mean of seven experiments), the LD_{50} 2·7, and the SND 1·5 (mean of five experiments). The properties and relative proportions of activity compare with the published values for whole extracts of *C. fleckeri* (KEEN and CRONE, 1969*b*).

Hemolytic activity and estimation of D_{50} *values for* C. quadrigatus *extracts*

Estimations of the D_{50} were carried out at temperatures of 5, 15, 25 and 35° (Fig. 1). The values were 4·7, 5·0 4·5 and 3·9 respectively indicating that the optimal temperature for hemolysis was 15°. A similar experiment was carried out with the *C. fleckeri* extract which gave D_{50} values of 5·2, 6·0, 6·0 and 5·4 respectively.

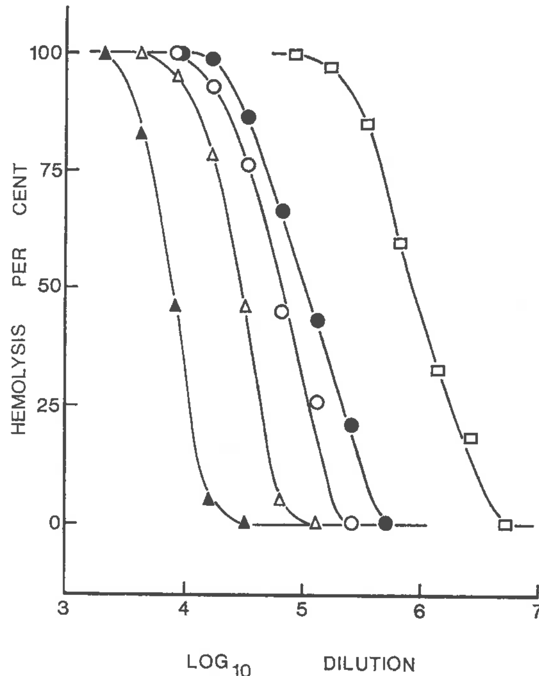

FIG. 1. HEMOLYSIS PRODUCED BY *C. quadrigatus* AND *C. fleckeri* AT DIFFERENT TEMPERATURES. Log_{10} dilution plotted against per cent hemolysis (3·0–3·5 × 10^7 cells per ml). *C. quadrigatus*: ○=5°; ●=15°; △=25°; ▲=35°. *C. fleckeri*: □=25°.

The sigmoid curves obtained when graphing per cent hemolysis against dilution indicate the variable susceptibility of red cells to the hemolysin of *C. quadrigatus*. The shape of the curves is therefore not markedly affected by change in temperature. However, the displacement of the curves along the abscissa indicates that the activity of the extract is temperature-dependent.

The hemolysis curve produced by the extract of *C. fleckeri* at 25° (Fig. 1) is comparable with those of *C. quadrigatus*. This suggests that the susceptibility of the red cells to the hemolysin of both extracts is similar.

Factors affecting the D_{50} *values of the hemolysin of* C. quadrigatus

The stability of *C. quadrigatus* and *C. fleckeri* hemolysins was examined at 35 and 40°. The fall in D_{50} values for the two extracts was plotted against the time of exposure at either 35 or 40° (Fig. 2). Statistical analysis of the results indicates that there is a significant difference in the stability of the hemolysin of *C. quadrigatus* and *C. fleckeri* at 35° ($p < 0{\cdot}001$) and 40° ($p < 0{\cdot}01$). There also appeared to be variations in stability of different batches of extracts of *C. fleckeri* and *C. quadrigatus* but the exact cause of this is not known.

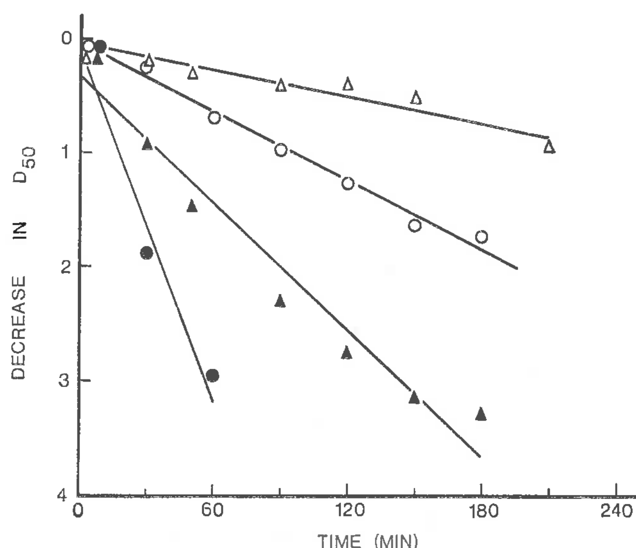

FIG. 2. EFFECT OF TEMPERATURE ON THE STABILITY OF THE HEMOLYSIN OF *C. quadrigatus* AND *C. fleckeri*.

Each point represents the result of one experiment. Lines fitted by the method of least squares regression. *C. quadrigatus*: ○=35°; ●=40°. *C. fleckeri*: △=35°; ▲=40°. Extract of *C. quadrigatus* in tris buffered saline, pH 8·0, and extracts of *C. fleckeri* in phosphate buffered saline, pH 7·2.

The stability of *C. quadrigatus* extracts was examined over a range of pH values from 4 to 9. Samples of the extract were diluted in the appropriate buffer to give a dilution of 1:30. They were kept at 5° for 16 hr and estimations of D_{50} made. The hemolysin was unstable below pH 5, but between pH 5 to 9 there was little effect on stability.

Various additions replaced 1 ml of buffered saline in the hemolytic system (cholesterol 0·05 mg per ml, lecithin 0·1 mg per ml, serum 10 per cent and sucrose 5 per cent). Each caused a reduction in the D_{50} values (approximately 4-fold reduction for sucrose and lecithin and an 8-fold reduction with serum and cholesterol).

The hemolysin of *C. fleckeri* is not affected by lecithin, cholesterol, or serum although sucrose interferes with its activity; the significance of these findings has been reported (KEEN and CRONE, 1969*a*).

Dermatonecrotic activity

The lesions produced by extracts of *C. quadrigatus* are identical to those reported with *C. fleckeri* (KEEN and CRONE, 1969*b*). However, the dermatonecrotic factor of *C. quadrigatus* appeared to be more stable than the hemolysin and considerable activity could be detected after heating at 35° for several hours. By this method an extract can be obtained with dermatonecrotic but no hemolytic activity. Therefore in *C. quadrigatus* extracts the two activities do not appear to be related as in *C. fleckeri* (KEEN and CRONE, 1969*b*).

Antigenic properties of tentacle extracts

Rabbit antiserum prepared against *C. quadrigatus* contained antibodies to hemolytic, lethal and dermatonecrotic activities. The antiserum was capable of neutralizing 2500 times the D_{50} value of hemolytic activity, and 320 times the LD_{50} dose. The dermatonecrotic activity was completely neutralized by an equal volume of a 1 in 4 dilution of antiserum.

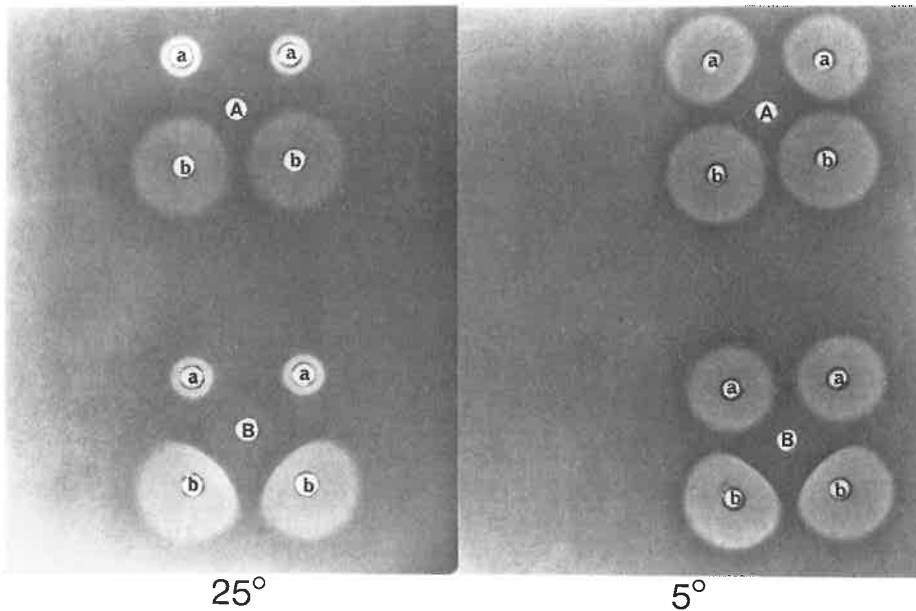

FIG. 3. DOUBLE DIFFUSION IN 10 PER CENT BLOOD AGAR DEMONSTRATING INHIBITION OF HEMOLYSIS BY SPECIES SPECIFIC ANTISERUM, AND CHANGE IN HEMOLYSIS WITH TEMPERATURE.
(a) *C. quadrigatus* extract; (b) *C. fleckeri* extract (1 in 4 dilution).
(A) *C. quadrigatus* antiserum; (B) *C. fleckeri* antiserum.
Temperature of diffusion indicated. Five μl of each antiserum and extract used.

On the other hand, using *C. fleckeri* antiserum we were not able to demonstrate any neutralizing antibodies to *C. quadrigatus* extracts. The minimum quantities of extract employed to detect antibodies were as follows—50 times the D_{50} value for hemolytic activity, 2·5 LD_{50} for lethality, while undiluted antiserum caused no decrease in the SND.

A similar series of experiments was carried out with the antiserum of *C. quadrigatus* against extracts of *C. fleckeri*. No neutralizing antibodies against the hemolytic, lethal or dermatonecrotic factors of this species could be found.

Diffusion of extracts in agar

Double diffusion experiments were carried out in agar plates using both extracts and their antisera. Plates were stained with Amidoblack 10B (Wieme, 1965). No precipitation bands could be detected and perhaps this was due to the low protein content of the extracts.

In order to identify the hemolysin 10 per cent washed red cells were incorporated into the agar. The specific antihemolysin for the appropriate extract could be demonstrated by inhibition of hemolysis in double diffusion experiments. This is shown in Fig. 3. The plates were kept at a constant temperature of either 5° or 25°. Other discrete bands of hemolysis could be produced by exposing the plates at different temperatures. In these cases the changes are similar to the multiple precipitation bands observed in routine immunodiffusion experiments, which may be attributed to sudden changes in the rate of diffusion or varying solubilities of the antigen–antibody complex (Crowle, 1961).

A comparison of the plates at 5 and 25° provides a simple means of differentiating the two hemolysins. The hemolysin of *C. fleckeri* is not affected by the higher temperature, whereas there is considerable inhibition of the zone of hemolysis around *C. quadrigatus*. As there is only a slight difference in the D_{50} value for *C. quadrigatus* at 5 and 25° the findings are attributed to the lower stability of the extracts at the higher temperature.

The D_{50} value of *C. fleckeri* at 25° was not affected by serum, cholesterol or lecithin (Keen and Crone, 1969*a*). However, each of these compounds caused inhibition of the hemolysis produced by *C. quadrigatus* extracts. This inhibition of hemolysis could be demonstrated on blood agar plates when extracts of *C. quadrigatus* were allowed to diffuse against normal rabbit serum. The inhibition of hemolysis was considerably less than that produced by the specific antiserum and, at 5°, it occurred only after several days.

DISCUSSION

Only in the past decade has it been possible to elucidate the cause of fatalities due to sea wasp stings along the northern coastline of Australia. The two important causative organisms *C. quadrigatus* and *C. fleckeri* resemble one another in morphological appearance and from reports of injuries in humans the actions of their toxins appears to be similar.

In order to prevent and treat injuries it is desirable to know whether the two toxins are identical. This investigation indicates that the properties of the extracts of the tentacles which have previously been shown to contain the active principles of the toxin (Freeman and Turner, 1969; Keen and Crone, 1969*b*) are very similar. Although extracts of *C. fleckeri* were more potent overall, both extracts have lethal, dermatonecrotic and hemolytic activities in the same relative proportions. The active materials in the extract appear to be chemically related and each extract can be separated chromatographically into two fractions, one lethal and the other hemolytic. In *C. fleckeri* extracts the hemolytic fraction is also lethal but so far this has not been established in fractions of *C. quadrigatus* toxin due to instability and low

activity of the material obtained (Freeman and Turner, unpublished). In both extracts the hemolysins have a molecular weight of 70,000 approximately and the lethal components a molecular weight of 150,000.

In this investigation the lethal and dermatonecrotic activities of *C. fleckeri* and *C. quadrigatus* extracts appeared to be pharmacologically identical although in estimating the LD_{50} in mice it was noticed that death was slower in those animals given lethal doses of *C. quadrigatus* extract. However, despite this finding the mode of death, which is associated with cardiovascular failure and respiratory arrest, is virtually identical with both extracts.

Examination of the hemolysin of *C. quadrigatus in vitro* showed that it was more labile than the hemolysin of *C. fleckeri*. The optimal temperature for hemolysis was 15° rather than 25° and the hemolysin in this extract deteriorated more rapidly at higher temperatures.

Serum, cholesterol and lecithin interfered with the activity of the hemolysin of *C. quadrigatus* and these factors were without effect on extracts of *C. fleckeri*. It would appear that the hemolysin of *C. quadrigatus* and its mode of action are different from that of *C. fleckeri*. The two extracts despite certain chemical similarities which have been described produce neutralizing antibodies for a particular species and no cross immunity could be detected for the lethal, dermatonecrotic or hemolytic activities of the two extracts.

From a practical point of view a ready distinction between the two can be made on the basis of their hemolytic activity at different temperatures. There are several methods of demonstrating this difference but the use of blood agar plates appears to be a simple method which is both reliable and reproducible. Diffusion of the extract occurs on plates held at 5 and 25°. At the end of 24 hr extracts of *C. quadrigatus* showed a marked difference in the hemolysis on the two plates, the area being considerably larger on the plates held at 5°. This difference is attributed to the reduced stability of *C. quadrigatus* extracts at the higher temperature. On the other hand, with extracts of *C. fleckeri* the difference is not marked, and the area of hemolysis at 25° may be slightly larger. Double diffusion experiments in which the specific antisera are used may further confirm the identity of the extracts. These experiments should be carried out at 5° in order to prevent loss of activity of the hemolysin during the period of the diffusion.

The above tests might be applied to the tentacle fragments which are available on the skin of the injured victims. If the fragments are collected and kept at low temperature, sufficient activity may be present to allow identification of the organism.

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