Summary

The present thesis describes the general properties, mode of action *in vivo* and *in vitro*, and a method of purification, of staphylococcal alpha toxin.

The optimum pH and temperature of the haemolytic reaction were found to be 5.5 and 31°C. respectively. In proteolytic digestion and heat sensitivity, the toxin behaved as a typical protein. Contrary to current views alpha toxin was found intrinsically heat sensitive. A revised explanation of the paradoxical "Arrhenius phenomenon" is suggested and discussed in light of this.

Alpha toxin was not used up in haemolysis; it acts as a catalyst. Kinetic experiments showed that at low concentrations of toxin the relationship between the rate of haemolysis and concentration was compatible with an enzymic reaction. At high concentrations there was a marked falling off; possible reasons for this are suggested and discussed, and the limitations of haemolysis as an indicator system are pointed out. Also the velocity of haemolysi was dependent on the concentration of rabbit red blood cells. Considering the bulk of the evidence it is concluded that alpha toxin belongs to that group of bacterial haemolysins which are thought to be enzymes.

Divalent ions are not required for haemolysis. Inhibitors of bacterial proteases had no effect. Heavy metal ions such as Hg^{2+}

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were found to inhibit at concentrations of 10^{-3}M; below this they became haemolytic by themselves. Some organic sulphydryl inhibitors also inhibited alpha toxin; it seems therefore that free SH groups may play a role in haemolysis. The trypanocidal drug Suramin and related substances were powerful inhibitors of both the haemolytic and the lethal activity of alpha toxin.

Phospholipids were tested as possible competitive inhibitors of alpha toxin in an attempt to gain information concerning the point of attack. Apart from a crude preparation of sheep brain cephalin, none of them inhibited. The possible significance of this is discussed.

Death from alpha toxin was found to be dose dependent: it was either very rapid, or occurred after considerable delay. Dose dependence and the pattern of death was largely the same for the four species examined, viz., rabbits, mice, fowl and frogs. Rapid death in seconds or minutes, without histological lesions is most likely explained by an action on heart or central nervous system. Slow death in several hours or days may result from lesions in a multiplicity of organs. Large doses (which killed rapidly intravenously) when administered subcutaneously or intraperitoneally killed much slower, probably because of the time required for diffusion of toxin to vital organs.

Alpha toxin caused local flaccid paralysis of voluntary muscle when injected into the dorsal sac of 6 weeks old mice; at high doses this occurred before any detectable histological lesion.

Muscles of the paralysed limbs did not respond to electrical stimulation *in situ*. In presence of alpha toxin the reactivity of excised voluntary muscles of mice and frogs to acetyl choline and electrical stimulation was abolished *in vitro*. Muscles of curarised mice behaved in the same way, and it is concluded that alpha toxin has a direct myotoxic action.

Heart muscle appeared less sensitive. Hearts of mice killed with alpha toxin continued to beat for a few minutes after death and hearts of frogs for up to several hours. In tissue cultures mouse heart explants were less affected than the whole animal. Whereas 128 MHD is an L.D. 50 for 20 g. mice, explants of 20 - 1,000 cells were only moderatly affected by 2,000 MHD/ml: some explants continued to beat, some stopped after 30 min., but recovered overnight. The possible significance of this is discussed.

Alpha toxin, purified by a combination of gel filtration on G.75 and DEAE A.50 Sephadex and fractional methanol precipitation, behaved as a serologically and physically homogeneous protein. The sedimentation constant was 3.1 S at 0.13% protein, which is close to that of 3.0 S suggested recently by Bernheimer and Schwartz (1963). The potency was 119,000 MHD/mg. protein. The product was unstable and evidence was obtained which suggested that on standing or dialysis alpha toxin tends to polymerise with the appearance of a heavy (16 S) component.

STAPHYLOCOCCUS ALPHA TOXIN

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"Thy natural magic and dire property
On wholesome life usurps immediately."

Hamlet, Act. III, Sc.2.

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CONTENTS

PREFACE

The use of poisons probably represents man's earliest knowledge of chemistry: the striking effects of minerals and snake venoms were known to the Egyptians, and curare to the primitive tribes of South America. Similarly the toxic effects of bacteria, e.g. the neuromuscular symptoms of Tetanus and Diphtheria, the vomiting of Cholera, the violent pains of Plague and the skin manifestations of Septicaemias, were well-known before the discovery of micro-organisms. "The little worms of the most atrocious pests are of themselves of a poisonous nature" and "as often as they enter the humoral mass, defile it with their excrement, adulterate it, pollute it" (Vallisnieri, 1713).

In the post Pasteurian era a great body of knowledge of bacterial toxins began to accumulate. In the laboratory identification of pathogens, the in vivo action of toxins proved an excellent diagnostic criterion. With the discovery that many toxins remained antigenic when deprived of their harmful effects (toxoiding), toxins became useful tools for immunisation. The recognition that bacteria produce a multiplicity of different toxins led, in turn, to numerous classifications into Gram-positive and Gram-negative, heat stable and heat labile, exo and endo-toxins.

The astronomical activity of bacterial exotoxins compared with poisons of plant or animal origin, prompted an increasing interest in their biochemistry, mode of action and point of attack. The last two decades saw the crystallisation of two bacterial toxins and the purification of a few. The tropism of neurotoxins became an established concept and their pharmacological point of attack pinpointed. The biochemical mechanisms, however, are still largely unknown. Rather surprisingly the mechanism of action was ascertained in the case of a toxin which, though highly purified, has not been crystallised: alpha toxin of Cl. welchii has been identified as a Lecithinase C.

The aim of the present work was to study the most potent toxin of the Staphylococcus, i.e., alpha toxin: to purify it, ascertain its properties, its mode of action and, if possible, its point of attack. The logical approach would seem to be to purify the toxin first. However, neither with Botulinus nor Tetanus toxin had purification led to the greater understanding of their mechanisms of action, whereas the study of the kinetics of crude preparations of Cl. welchii alpha toxin did. Also the results of each of the investigations were likely to influence the approach to the others; knowledge of the nature and properties of the toxin could give a

guide to the purification method to be used. Finally, in view of the multiplicity of toxins known to be produced by the Staphylococcus considerable difficulties were anticipated in separating and purifying one of them. It was therefore decided to proceed simultaneously with the purification of the toxin and the study of its nature and properties.

As it frequently happens in the course of work, observations were made which were neither expected at the start of the investigation nor could be fully explained in the light of present knowledge. They are incorporated in the Thesis as part of the reality of alpha toxin; it is freely admitted that they pose questions rather than answer them.

INTRODUCTION

INTRODUCTION

The acceptance of the germ theory of disease implies the existence of a mechanism whereby pathogens can establish themselves within, and then disrupt, the tissues of the host; bacterial poisons, at first an intellectual postulate (Klebs, 1872; Lister, 1880) soon became reality and part of what is called "mechanism of pathogenicity". The initial search for bacterial toxins was confused, however, by the finding, in diseased or damaged tissues, of the so-called ptomaines (Breiger, 1885), substances related to the alkaloids. It was some time before it was realised that these, though toxic, were not products of the pathogens, but resulted from break-down of host tissues.

The first evidence of a true bacterial toxin probably came from Loeffler (1884), who, from the occurrence of bacteria-free lesions in organs distant from the site of inoculation, deduced that Diphtheria bacilli killed by forming a toxin and liberating it into the blood. This was shortly afterwards isolated by Roux and Yersin (1888) and shown to be lethal for guinea pigs. In the space of a few years, Tetanus and Botulinus toxins were discovered by Behring and Kitasato (1890) and van Ermengem (1896). All three toxins reproduced (in experimental animals) the full symptoms of the natural disease; active and passive

immunisation protected not only against the toxin, but also against the organisms themselves. This success, though acting as a stimulus to future immunisation work, delayed the recognition of more complex mechanisms of pathogenicity. It was not, and indeed could not, be realised at that time that Diphtheria, Tetanus and Botulism were exceptional diseases, caused by a single toxin, and the findings led to the belief that all bacterial diseases were caused by a single toxin, which, sooner or later, would be found. This attractive generalisation was later proved to be incorrect, but the concept that toxic products could mediate in the causation of bacterial diseases has remained valid. The knowledge of the nature and mode of action of toxins is still a key to the understanding of even so complex a mechanism as that of staphylococcal infections.

Staphylococcus pyogenes

Part of the early difficulties and confusion *qua* nature and properties of staphylococcal toxins stemmed from the absence of an adequate definition of pathogenic staphylococci. Before discussing staphylococcal toxins, therefore, I would like to review very briefly some of the past and present taxonomic criteria used in defining the species.

"Micrococci" or round-shaped organisms were observed (van Recklinghausen, 1871; Waldeyer, 1871; Birch-Hirschfeldt, 1872; Klebs, 1872) in suppurative lesions and blood of patients dying of pyaemia. Pasteur (1880) was the first to grow micrococci and to reproduce suppuration in experimental animals. The "grouped micrococci" were subsequently differentiated from Streptococci (Ogston, 1881) and called Staphylococci. It was also Ogston (1880), and Verneuil (1880) who noticed that yellow coloured pus was common in severe lesions; pigment production became the basis of classification (Rosenbach, 1884) and still survives in the common name Staphylococcus aureus.

Classification on grounds of pigment production was supplemented by biochemical tests (Gordon, 1904), especially the fermentation of lactose, glucose and mannite and gelatin liquefaction. It is now, however, realised that pigment production is an unstable character (Bigger, Boland and O'Meara, 1927), that many pigmented staphylococci are non-pathogenic (Wood, 1952) and that conversely a proportion of staphylococci, pathogenic by other criteria, are deficient in pigment production (Rochaix and Rivollier, 1939; Barber, 1942); similarly, the correlation between fermentative pattern and pathogenicity

was found unsatisfactory (Cummins and Cummins, 1913) Winslow, Rothberg and Parsons, 1920; Wood, 1950).

The breakthrough came unrecognised with the discovery by Loeb (1903) and Much (1908) that staphylococci produce an enzyme which will clot plasma; it was not, however, until the work of v. Daranyi (1925, .) and Chapman (1940) that this property was correlated with pathogenicity. Today it is widely accepted as the best single criterion of potentially pathogenic staphylococci, in fact, coagulase production is now so much relied upon that the old term "Staphylococcus pyogenes" (Elek, 1959) is gradually replacing the relatively newer one of Staphylococcus aureus.

For the identification of a micro-organism, almost invariably more than one character is necessary; in this respect coagulase production is unique. False positives can arise due to clotting of citrated plasma by Gram negative organisms which utilise citrate (Harper and Conway, 1948) and by some streptococci (Evans, Buettner and Niven, 1952); in addition false negatives due to the enzymic destruction of coagulase by smooth variants (Lominski, Morrison and Smith, 1955) may be observed. Nevertheless, a properly carried out coagulase test indicative of Staphylococcus pyogenes has still

to be challenged. The production of another enzyme, phosphatase, has been found to closely parallel coagulase production (Barber, Brooksbank and Kuper, 1951) and the phosphatase test, carried out alone or in conjunction with the coagulase test, is widely used.

Modern serological methods (Cowan, 1938, 1939; Christie and Keogh, 1940, Oeding, 1953) and phage typing (Fisk, 1942a, b; Wilson and Atkinson, 1945; Williams and Rippon, 1952) are not so much concerned with identification of pathogenic staphylococci as with the defining of groups within it. At present, the concept of Staphylococcus pyogenes is one of a Gram-positive coccus of characteristic arrangement, able to clot plasma under standard conditions and producing one or more of four well-defined toxins (alpha, beta, gamma, delta).

Staphylococcal toxins

The immense bibliography of Staphylococcal toxins has been the subject of excellent reviews (Blair, 1939; Rigdon, 1940; Elek, 1959); it would be superfluous to repeat their efforts here.

In the development of knowledge of staphylococcal toxins three phases seem to emerge. An initial period of activity between 1870 and 1920 comprised the discovery

of the toxin, the antitoxin, and the first descriptions of toxic activities; factually little can be added to the observations of this period.

The second phase, between 1928 and 1940, saw the emergence of alpha toxin as a distinct entity. It was initiated by the disaster at Bundaberg, in which 12 of 21 children, died in the course of immunisation against Diphtheria. The vaccine was contaminated with a Staphylococcus and the Royal Commission (1928) investigating the tragedy concluded that "....death resulted from an overwhelming toxaemia at the early stage of the invasion of the organism." In consequence, many workers turned their attention to the toxins of the Staphylococcus in general, their multiplicity, production, properties and their possible role in pathogenicity and protection.

The emergence of a potent exotoxin, alpha toxin, which was lethal for laboratory animals and was produced by the great majority of pathogenic staphylococci, led almost logically to the belief that staphylococcal disease was analogous to Diphtheria, and that neutralisation of the toxin by its antitoxin would alleviate the symptoms of infection. Both passive immunisation with a hyperimmune sera and active immunisation with toxoid were employed.

Many conflicting and contradictory reports have been published on the effectiveness of such therapy; at first results were encouraging, but later reappraisal and use of more rigorous controls indicated that at best experimental animals could be protected for only a few days. The results in humans were again conflicting; some patients responded well, while others showed no improvement. The general opinion, around 1940, was of failure, and with the tremendous initial success of antibiotics activity in this field was virtually terminated. The background to this failure of antitoxic therapy is admirably discussed in the review of Elek (1959, pp. 348-357).

With the recent revival of interest in Staphylococci, the present phase in the history of staphylococcal toxin began. Like research into other bacterial toxins the field is now dominated by contemporary biochemistry in both approach and techniques. Though the possibility of medical application is not forgotten, it is the nature, properties and mode of action of toxins that are the primary objectives. Only when these aims have been realised will the role of the staphylococcal toxins be further understood.

Early History of Staphylococcal toxin

The first experimental demonstration of a staphylococcal toxin was probably brought by von Leber (1888); the active principle (Phlogozin) when precipitated by alcohol and injected into the anterior chamber of the eye of rabbits caused inflammation. In the same year de Christmas (1888) reported that culture fluids (sterilised by heating to 100°C. or by filtration) and their alcohol precipitates caused inflammation of the skin in the conjunctiva of rabbits; the activity disappeared on autoclaving at 120°C. Two years later Breiger and Fraenkel (1890) showed that "toxalbumins" obtained by salting out or alcohol precipitation of culture fluids of staphylococci killed guinea pigs and rabbits; the formation of sterile pus and intense inflammatory reaction with necrosis at the site of injection was also observed. Confirmations of the lethal property of "staphylococcal toxin" were soon forthcoming from Rodet and Courmont (1892), von Lingelsheim (1900) and Kraus and Pribram (1906); very large doses of toxin killed rabbits quickly on intravenous injection.

A fundamental contribution was made by van de Velde (1894); in a set of beautifully designed experiments he

showed that leucocytes in pleural effusions of rabbits infected with a virulent strain of staphylococci were badly damaged. The active principle, termed "leucocidin" could be obtained in vitro, was heat labile (destroyed on heating to 60°C.), but non-toxic for rabbits and dogs; the term leucocidin has persisted. Van de Velde also noted that his strains, one virulent and one avirulent both haemolysed rabbit blood. The haemolytic property of staphylococci was confirmed by Kraus and Clairmont (1900) and by Neisser and Wechsberg (1901) who obtained cell free haemolysin, confirmed the leucocidic action on rabbit leucocytes, and standardised the technique of assessing haemolysis around colonies of staphylococci on blood agar plates (Neisser and Wechsberg, 1903). Anti-serum to many of these preparations of staphylococcal toxins neutralised the various activities (van de Velde and Denys, 1895; Kraus and Clairmont, 1900; Neisser and Wechsberg, 1901; Kraus and Pribram, 1906).

It is difficult in retrospect to account for discrepancies in activity of different preparations obtained by using different strains and methods of preparation. The conflicting findings of van de Velde

and de Christmas regarding the heat sensitivity of staphylococcal toxin may have been due to the paradoxical effect of heat now known as the Arrhenius phenomenon (Arrhenius, 1907), which will be discussed later (p.35). However, the failure of van de Velde's toxin to kill rabbits is surprising and not easily explained. If indeed his toxin was, what later became known as, the alpha toxin and which is also known to be a leucocidin for rabbit leucocytes, then it may have been a matter of concentration, the amount of toxin present in his preparations being insufficient to kill. Certainly the production of this haemolytic toxin by both a virulent and an avirulent strain of staphylococci does not militate against it being the alpha toxin, since some strains of poor rabbit pathogenicity are now known to be good alpha toxin producers; the classical example of such a case is strain Wood 46 which is used in laboratories all over the world for the production of alpha toxin and is lethal for rabbits only at very high doses. Nevertheless, it is possible that he was dealing with an entirely different toxin, although such a haemolytic and leucocidic non-lethal factor did not subsequently emerge.

Bearing in mind all the qualifications and restrictions of interpreting pioneer work carried out almost 60 years ago, it is interesting to note that by the beginning of the 20th century the four main properties now associated with alpha toxin, namely, lethality, dermonecrosis, haemolysis and damage of rabbit leucocytes, had been described although admittedly not with the same strain, yet this factor did not emerge as a separate entity until some 30 years later.

As early as 1900 the first attempt was made to determine the mode of action of staphylococcal filtrates in vivo (Neisser and Levaditi, 1900). Multiple sublethal doses were injected into rabbits and death occurred in 5 - 15 days. Ischaemic necrosis of the cortex of the kidney was found and histology showed that the small vessels were blocked by disrupted leucocytes. Studying the mechanism of rapid death in rabbits, Russ (1916) observed a fall in blood pressure and heart failure which he attributed to obstruction of the pulmonary circulation. Yet another mechanism was suggested by Le Fevre d'Aric (1919a); the convulsive symptoms of rapid death led him to believe that the toxin acted on the central nervous system. The problem of how staphylococcal toxin causes death

attracted considerable attention in later years and research followed along lines, strikingly similar to the fundamental original work just mentioned; vascular damage with lowering of blood pressure, direction action on the heart, action on the central nervous system. These investigations will be disoussed in a later section (p.52).

The Plurality of Staphylococcal toxins

By the early 1900's then it was clearly established that cell-free products of staphylococci were toxic both *in vivo* and *in vitro*; in keeping with the time, the product was referred to as "the exotoxin". Some inconsistencies and discrepancies in the results, e.g., heat sensitivity and lethal activity (see p.13) slowly attracted the attention of some workers, but it was only some 20 years later that these inconsistencies became so consistent as to lead finally to the suspicion that more than one active principle was present in the staphylococcal toxin. Partly, as already mentioned, the discrepancies were due to the lack of a clearly defined idea of the species *Staphylococcus pyogenes*. To a large extent, however, confusion was due to the loose description of haemolytic systems and absence of serological criteria. The *in vitro* indicator system for assaying staphylococcal toxin was haemolysis, but the species of RBCs varied from worker to worker and was often unspecified. It had been

pointed out that one and the same filtrate lysed RBCs of different species to a different titre or occasionally did not lyse some of them at all (Neisser and Wechsberg, 1901). Also Le Fevre d'Arie (1919) noticed that with a given filtrate guinea pig RBCs were c. 500 times more resistant than those of the rabbit. Gradually there emerged a differentiation of haemolysins according to species of RBCs on which they acted.

In 1921 Walbum described a haemolysin which on goat RBCs had hardly any effect at 37°C. but caused rapid lysis on cooling to 0°C. This so-called "hot-cold" haemolysin was reinvestigated by Biggar, Boland and O'Meara (1927) and found active on sheep RBCs. The haemolysin was subsequently found by Glenny and Stevens (1935) to be serologically distinct from the haemolysin which acted on rabbit RBCs at 37°C; they termed the rabbit lysin "alpha" and the "hot-cold" lysin "beta" - a terminology which has survived. The unity of even the rabbit lysin was soon challenged (Dolman and Kitching, 1935; Flaum and Forsmann, 1936; Burky, 1933). Morgan and Graydon (1936) showed that two serologically distinct lysins which they called $alpha_1$ and $alpha_2$, both acted on rabbit RBCs. The

existence of two antigenically different rabbit lysins was confirmed by Smith and Price (1938) who suggested for their lysin the term gamma toxin. Although the identity of alpha$_2$ and gamma has never been entirely proved, it is probable. Gamma lysin acts on rabbit RBCs and gives a sharper end point than alpha. It lyses (in addition to rabbit RBCs) RBCs of different species, though generally to a lower titre, and is less lethal than alpha-lysin. Gamma toxin was not readily accepted as a separate entity although both Smith and Price (1938) and Smith (1956) have brought adequate proof of its existence; the latter has shown that rough variants of staphylococci produced virtually only gamma toxin.

A fourth haemolysin was recently reported by Williams and Harper (1947) and later fully investigated by Marks and Vaughan (1950) and Marks (1951). The lysin, termed delta, is non-antigenic and soluble in ethanol and ether but not in acetone, and thought to be probably a fatty acid. It is neutralised by the albumin fraction of normal serum (Marks and Vaughan, 1950).

These four haemolysins probably do not represent the full complement of staphylococcal lysins. The history of their discovery, however, illustrates clearly the

causes of divergent results and confusion in early work; the importance of using double criteria, i.e. serology and species specificity of RBC were only recently recognised. The animal experiment by itself without the use of specific antitoxins does not greatly help to differentiate toxins because all of them are capable of causing inflammatory reaction and, when injected in sufficient amounts, death; the rapid lethal action of alpha toxin is nevertheless quite outstanding.

Gradually, as the concept of one "Staphylococcus exotoxin" disintegrated, more and more activities, originally attributed to the "toxin", were shown to be separate entities frequently associated with it. So, in 1922, Julianelle showed that leucocidin activity did not always parallel haemolysis. The leucocidic, haemolytic, and dermonecrotic properties of a number of strains were later investigated by Panton and Valentine (1932) who in turn discovered that the factor responsible for the leucocidic action on human leucocytes was distinct from the rabbit haemolysin. Now, while alpha toxin is a leucocidin for rabbit leucocytes, the Panton Valentine leucocidin, usually termed P.V. leucocidin, is a separate

non-haemolytic entity and is not lethal for rabbits, although it does act on the leucocytes of the rabbit. Recently Gladstone and van Heyningen (1957) suggested that in addition to P.V. leucocidin and alpha toxin staphylococci produce yet another leucocidic substance, namely, leucolysin. This factor is reported to be thermostable and active against the leucocytes of all species tested, apart from those of the sheep.

Again, staphylococcal filtrates occasionally contained a toxin thought to be responsible for staphylococcal food poisoning (Dack, Woolpert, Corrie and Wiggers, 1930), distinct from alpha toxin, called enterotoxin (Woolpert and Dack, 1933; Dolman, 1934).

For a long time, despite its entirely different activity, but because it was commonly associated with toxin products in human strains, coagulase was thought to be identical with exotoxin. Soon, however, the not infrequent discorrelation of these characters in bovine strains and the increasing evidence in favour of differing physical and chemical properties, led to the recognition of coagulase as a separate entity. By the application of similar methods and reasoning, an ever-increasing number of staphylococcal enzymes and activators (lipases, proteases,

hyaluronidase, phosphotase, gelatinases, phospholipase, staphylokinase) often present in staphylococcal filtrates or cell-free fluids, were shown to be independent of and unrelated to any of the major toxins. It seems only reasonable to assume that more than in the case of other micro-organisms the finding of an ever-increasing number of enzymes in staphylococcal filtrates will be successful. Since the staphylococcus is a highly exacting organism, it seems only a matter of search and energy before a plurality of enzymes will be found. The converse is also possible, that is, the finding of uncharacterised proteins of as yet unknown role. Indeed, recently Bernheimer and Schwartz (1961) found some as yet uncharacterised proteins of unknown role, which seemed to correlate with the degree of pathogenicity.

Although this Thesis is concerned with alpha toxin, it was thought that the brief historical reference to staphylococcal products in general may help the reader to appreciate the difficulties attending the purification of alpha toxin and the clear demonstration of its biological properties. It is only by careful comparison and exclusion of a large number of factors that one can establish with any degree of certainty that a particular activity attributed to alpha toxin is indeed due to it.

Staphylococcal Alpha Toxin

As mentioned, it is probable that because of its outstanding haemolytic and lethal activity alpha toxin was the factor responsible for many of the effects described in the period when only a single "staphylococcal exotoxin" was recognised. Of the various activities shown by "toxic filtrates", four are still generally considered to be manifestations of the alpha toxin moiety, and the present day definition of alpha toxin is that it is haemolytic for rabbit RBCs, damaging to rabbit leucocytes, dermonecrotic, and lethal for a number of species. Attempts to dissociate these activities have either failed or are unconvincing because doubt arises whether the toxin investigated contained only alpha toxin. Thus, for instance, Parker (1924) found that some strains produced dermonecrotoxin and a potent haemolysin, but often did not kill on intravenous injection. Since it is now known that gamma toxin also necrotises but kills only at high doses, it is possible that Parker's strains produced gamma toxin and little or no alpha toxin. Burky (1933) described filtrates which were lethal but nothaemolytic; one cannot be certain which toxin was present and the cause of death can only be surmised. There is increasing

evidence however of the presence of endotoxins in staphylococci (Jensen, Neter, Gorzynski and Anzai, 1961) and autolysed preparations may contain such factors. In the case of Flaum and Forsmann (1936) who reported some filtrates which haemolysed but failed to cause dermonecrosis it is again impossible to decide which haemolysin could have been present. It is also possible, as pointed out previously, that the four toxins so far described do not represent the full complement of staphylococcal toxins.

By contrast, the identity of the four activities of alpha toxin, or even more strictly of the three characteristic ones, i.e., haemolysis of rabbit RBCs, leucocidic action on the rabbit leucocytes, and rapid death, has a great many supporters (Burnet, 1930; Gengou, 1934-5; Nolis, et al., 1934; Seiffert, 1935; Ramon and Richou, 1936 ; Parish and Clark, 1932; Rigdon, 1935-7; Hartley and Llewellyn Smith, 1935; Levine, 1939). The basis of the argument in favour of unity is the parallelism of the activities in culture fluids, their similar physical properties and the fact that they are quantitatively neutralised by alpha-anti-toxin.

By 1940 then, it was generally accepted that alpha toxin was a single moiety, however, the problem of unity or plurality was raised again by Butler, (1959), on the

grounds that in the course of purification a degree of separation of activities was observed. More recently, attempts to purify alpha toxin have not upheld this finding. Indeed, Madoff and Weinstein (1962), Bernheimer and Schwartz (1963) and Lominski, Arbuthnott and Spence (1963, in press) all conclude that highly purified alpha toxin is haemolytic, dermonecrotic and lethal. Short of decisive proof which would entail obtaining in concentrated form the lethal factor without haemolytic activity or vice versa the present position is one of unity.

The production of staphylococcal alpha toxin. The earliest studies of factors influencing the production of staphylococcal toxins aimed at obtaining enough toxin to demonstrate its various actions. The experiments of this period are difficult to interpret: the criteria of a pathogenic staphylococcus and of its toxins have radically changed. Also, the methods used are inadequately documented.

The first effort to standardise production was by Neisser and Wechsberg (1901); the basis of their medium was meat infusion broth. Although widely used for the next 30 years, this medium did not yield high-titre toxin. Fairly early on Walbum (1909) and Russ (1916) introduced the technique of extracting cultures of staphylococci

grown on solid medium; this procedure resulted in fluids rich in haemolysin and was reintroduced by Bigger, Boland and O'Meara (1927b). However, it is now obvious that Bigger, Boland and O'Meara were studying beta toxin; their preparation was a "hot-cold" lysin acting on sheep RBC.

It was not until the work of Parker, Hopkins and Gunther (1926) that a major advance was made. Using a fluid proteose peptone medium they achieved a marked increase in toxin yield in an atmosphere of 10% CO_2 which, they suggested, prevented a harmful rise in pH. Soon after this, Burnet (1930) obtained even better yields by growing staphylococci on a semi-solid agar medium in an atmosphere of 20% CO_2, and in spite of many efforts to devise an alternative to this procedure, it is still probably the most reliable.

The beneficial effect of CO_2 has until now never been fully explained. The suggestion that it acts by buffering the medium was and still is popular (Bigger, 1933; Nelis, 1933a); Nelis et al., 1934; Elek, 1959). However, replacing the CO_2 by buffers (Rigdon, 1935) has not proved entirely satisfactory; also mutants have been found which produce high yields of alpha toxin in the absence of

CO_2 (Burnet, 1930). Both these findings suggest that CO_2 acts in a different way; this is supported by the recent work of Ganczarski (1962) who demonstrated the incorporation of $C_{14}O_2$ into staphylococcal proteins including the alpha toxin moiety. It would thus appear that CO_2 is involved in the metabolic pathway of synthesis of alpha toxin, probably in a fixation reaction; mutants with high yields of alpha toxin in the absence of CO_2 probably have an alternative pathway.

Agar is thought to enhance the yield of toxin by absorbing some unidentified component of the medium or product of the staphylococcus, inhibitory to the toxin (McLean, 1937). Its action can be simulated by other absorbants; Seiffert (1935) and McLean (1937) showed that cellophane, kielselghur, kaolin and even filter paper acted in the same way. The action of agar was, however, found not to be as straightforward as first thought; the polysaccharide portion of the agar molecule was found to remove the inhibitor and the calcium portion to be inhibitory by itself (McIlwain, 1938).

The use of soft agar is technically inconvenient and introduces polysaccharide impurity. Alternative methods were therefore sought and mechanical agitation of purely liquid medium proved to be useful (Casman, 1938, 1940; Favorite and Hammon, 1941; Duthie and Wylie, 1945).

At their best, the results are of the same order as those obtained with the soft agar technique, and these methods introduced difficulties. The rate of bubbling of the CO_2/air mixture and the rate and direction of shaking proved critical (e.g., Favorite and Hammon, (1941) found that while horizontal shaking resulted in good toxin production, end-over-end shaking gave almost no toxin). Over vigorous shaking or bubbling of gas mixtures presumably destroys the toxin by denaturation (Gladstone, 1938; Rud, 1955).

The detailed recipes of the nutrient medium vary considerably from worker to worker, and individual preference plays a considerable part. Many combinations of peptones, enzymic digests and meat extracts have been recommended with varying degrees of success. Also acid hydrolysates of protein and meat have been successfully used (Mercier and Pillet; Mercier and Lehoult, 1946). Since the experimental work of the present Thesis was completed, Bernheimer and Schwartz (1963) have described a purification procedure for alpha toxin produced in a medium devised by Pinsky (unpublished) based on an acid hydrolysate of casein.

Following the work of Knight and his colleagues (Knight, 1935; Knight, 1937a; Knight, 1937b; Knight, 1937c; Knight and McIlwain, 1938) who showed that nicotinic acid or nicotinamide together with thiamine were required for the growth of staphylococci in a basal medium of amino acids, these components and crude preparations of yeast extract have been incorporated with some success (Favorite and Hammon, 1941; Bramann and Norlin, 1951; Bernheimer and Schwartz, 1963).

The addition of fermentable carbohydrate has been advocated by some workers (Bigger, 1933; Nelis, 1933; Nelis, Boukuert and Picard, 1934; Bernheimer and Schwartz, 1963) and scrupulously avoided by others (Parker, Gunther and Hopkins, 1926; Forsmann, 1933); its beneficial effect has been attributed to the lowering of the pH of the reaction. The conflicting results of different authors may be due to the different concentrations of sugar used, although it is also possible that the toxin studied was not always alpha toxin (see p. 22).

Difficulties in purification of a bacterial toxin from complex media can readily be appreciated; the medium already contains considerable amounts of protein and other components which make purification difficult. In this

respect it is interesting to note that the bacterial toxins which have been most successfully purified, viz., Tetanus and Botulinus toxins produced very potent toxin in media containing small amounts of non-bacterial protein (Mueller and Miller, 1945; Niggetal, 1947). It would be of enormous advantage, therefore, if staphylococcal alpha toxin could be produced in synthetic medium. That staphylococci will grow in synthetic medium is well known (Knight and McIlwain, 1938; Gladstone, 1937; Lominski et al., 1950). In a complete amino acid medium, containing 16 amino acids, aneurin, nicotinamide, glucose and inorganic salts, which supported the growth of staphylococci, only traces of alpha toxin were formed (Gladstone, 1937). Working on the basis of Gengou's (1935) finding that the addition of amino acids, especially arginine, to a basic medium which did not itself yield toxin, stimulated the formation of alpha toxin, Gladstone (1938) succeeded in producing relatively small amounts of alpha toxin on a fully synthetic medium. Not only the individual amino acids, but also their concentrations were important.

At a given concentration an amino acid may stimulate haemolysin production, at a higher concentration the same amino acid would inhibit the production of haemolysin. Arginine was the most important amino acid and to a lesser extent proline and glycine also affected haemolysin production. The optimum quantities were M/110 arginine, M/280 proline and M/198 glycine. Tyrosine, histidine, glutamic acid, lysine, oxy-proline and aspartic acid could be omitted and leucine was found to be inhibitory. The basic requirement of amino acids was arginine, glycine, alanine, proline, valine, phenylalamine, cystine, tryptophan, and methionine. Oxygen was essential for the production of toxin and CO_2 enhanced the yield. Unfortunately the yield of toxin from this medium is as yet not sufficient to justify its use as a preparative procedure. It is interesting that since the work of Gladstone there has been no other devoted to the modification of a completely synthetic medium for the production of alpha toxin. The results of Lominski et al., (1950) who used a synthetic medium for the production of staphylococcal coagulase, also show that at best only very low yields of this enzyme were obtained. It seems likely that the interrelationships of the basic amino acids is so complex that a great deal of effort would be

required to obtain yields of toxin comparable with those obtained in complex media; if indeed this could ever be done at all.

At the outset of the present work many different media were tried for toxin production; the most reliable and the one giving the highest titres was undoubtedly the one suggested by Burnet (1930) and for this reason it was selected.

The purification of staphylococcal alpha toxin.

Alcohol precipitation was used by early workers in the preparation of staphylococcal toxins, but it was not until the 1930's with the improved techniques of production that concentration and purification was achieved. Again the pioneer work was done by Burnet (Burnet and Freeman, 1932), who succeeded in obtaining potent preparations by precipitation of crude filtrates with glacial acetic acid at pH 5.0. For more than a decade this preparation, containing 30,000 minimum haemolytic doses (MHD)/mg. of Nitrogen was easily the most potent. Good recovery of toxin and toxoid was also achieved by Boivin and Izard (1937) using trichloracetic acid at pH 3.5 in the cold, and by Holt (1936), with a salting out procedure, employing full saturation with ammonium sulphate. Kodoma and Nishiyama (1938) achieved precipitation of alpha toxin with methanol. These methods, however, did not attempt

fractionation and were only concentration procedures. With the exception of the interesting, but also unsuccessful, attempt of Gratia and Nelis (1938) to purify the toxin by ultracentrifugation, early interest was not so much in the purification as in the preparation of potent toxoid for therapeutic use (Holt, 1936; Boivin and Izard, 1937; Kodoma and Nishiyama, 1938) or of toxin for the mechanism of the toxoiding reaction (Burnet and Freeman, 1932).

After a lapse of some ten years the problem was re-examined this time with the sole aim of purification. After their success in purifying and crystallising and Tetanus toxin (Pillemer, Wittler, Burrell and Grossberg, 1948) Wittler and Pillemer turned their attention to the alpha toxin of the staphylococcus (Wittler and Pillemer, 1948). They evolved a method involving the strict control of pH, ionic strength, temperature, protein concentration and methanol concentration; toxin was first precipitated by acidification to pH 4.3 with glacial acetic acid and addition of methanol to a concentration of 15%. This was followed by reprecipitation with acid alone and extraction of the precipitate with 0.15 M acetate buffer pH 5.5. Although potent, containing c. 40,000 MHD/mg. of protein (extrapolated from the results of their in vivo experiments) it is now known to be impure. A complex procedure, combining many of the previous

methods was described by Turpin, Relyfeldt, Pillet and Raynaud (1954); initial precipitation with metaphosphoric acid was followed by successive reprecipitations with high molarity phosphate, acid and methanol, and cellosolve. The resulting material was fairly potent and free of nucleic acid but it contained at least three antigenic components. Subsequent use of cellosolve, ethanol and ammonium sulphate (Butler, 1959) yielded neither potent nor serologically pure toxin.

In addition to precipitation procedures, other techniques, including column chromatography (Lalli and Orlani, 1953; Simonetti, 1954) have been used without success. Recently, however, electrophoresis (Robinson, Thatcher and Gangnon, 1958) and a combination of electrophoresis and column chromatography on carboxy methyl cellulose (Robinson, Thatcher, and Montford, 1960) resulted in considerable purification but, as often in the past it is not clear exactly which toxin was being studied; also their preparations were contaminated with a protease. An added limitation of the method is that only small volumes of a 100 - 200 ml. of crude toxin can be purified in a single run (Thatcher, personal communication, 1962).

Compared with the relative inactivity of the last fifteen years, the past year has brought a renewed

interest in the field. Indeed since the work for the present thesis was begun, three important communications have appeared on the subject. A complex procedure combining precipitation with zinc acetate, column chromatography on G.25 Sephadex and DEAE cellulose, and paper curtain electrophoresis has been described by Madoff and Weinstein (1962). The value of this method is difficult to assess because although the resulting protein preparation had a calculated potency of 40,000 MHD/mg. protein, the actual strength of the preparation was only 400 MHD/ml. (containing 10 μg. of protein). Similarly a method employing column chromatography on both DEAE cellulose and hydroxy apatite (Goshi, Cluff and Norman, 1963) resulted in a toxin of high calculated potency (80,000 MHD/mg. protein) but again the actual potency of 1,000 MHD/ml. is very low; also there is a discrepancy between the lethal dose of this preparation for mice and that expected for alpha toxin, which suggests that these workers were dealing with a toxin other than alpha, possibly gamma toxin. An earlier simple two stage method involving continuous paper electrophoresis at two different pH's was described by Kumar, Locken et al., (1962). Unfortunately, the potency of their preparation is not stated and can only be inferred from a previous paper

(Kumar and Lindorfer , 1962) as being in the region of 150 MHD/mg. protein.

All of these preparations are reported to give a single line of precipitation against antiserum to crude toxin (Madoff and Weinstein, 1962; Kumar, Locken et al., 1962) or to partially pure toxin (Goshi et al., 1963); Kumar, Locken et al (1962) also reported a single line in immunoelectrophoresis. The strikingly low potency of the preparations, however, does not exclude the possibility that other antigens were merely absent through dilution. Both the actual and calculated potency of the preparation of Kumar, Locken et al., is so low that the sedimentation constant of 1.4 S determined for their toxin may well be that of a protein other than alpha toxin, contaminated with a small amount of alpha toxin. On the other hand it is quite possible that though weak the toxins of Madoff and Weinstein and Goshi et al., are indeed pure.

In short two types of preparation have emerged; those which are concentrated but impure, and those which are pure but weak. Part of the present thesis has been devoted to the finding of a method which would at the same time yield toxin of high potency which was physically and immunologically pure.

(Since the experimental work for the present thesis was completed, and the relevant paper submitted to the

publishers, the comprehensive study of Bernheimer and Schwartz (1963) appeared. Using a different method which combined the use of fractional precipitation with ammonium sulphate and curtain electrophoresis, they obtained pure alpha toxin which showed essentially similar properties to the preparation obtained in the present study. The sedimentation constant of 3.0 is not significantly different from that of 3.1 obtained in the present study.)

The physical and chemical properties of alpha toxin

Little success has been achieved as yet in the characterisation of the physical properties of alpha toxin, presumably because of the difficulty in obtaining suitably pure preparations.

The effect of heat on alpha toxin. Most proteins are denatured on heating, although some, notably ribonuclease, are quite heat resistant. There have been numerous conflicting reports concerning the heat sensitivity of alpha toxin. Very early in the study of staphylococcal toxins, as already mentioned, it emerged that different preparations behaved differently when heated (see p. 13). Some workers found that the toxin was destroyed by heating to 60°C. (van de Velde, 1894; Neisser and Wechsberg, 1901)

while others found that it resisted heating even to 100°C. (Leber, 1888, de Christmas, 1888). This discrepancy attracted the attention of perhaps the greatest physical chemist of the period, Arrhenius, who observed the paradoxical behaviour towards heat now known as the "Arrhenius phenomenon" (1907); crude toxin when heated to 70°C. lost almost all its haemolytic activity, but on reheating the now inactive mixture to 100° a large proportion of this activity returned. This did not seem the behaviour expected of a typical protein. In addition he noted that an inocuous mixture of toxin and antitoxin when heated for 5 min. to 100° again became actively haemolytic.

The mechanism of this phenomenon was investigated by Lansteiner and Rauchenbichler (1909) who confirmed Arrhenius' original finding that haemolytic activity disappeared at 65°C. and reappeared on reheating at 100°C. However, they added that when toxin was diluted in broth or saline it no longer showed the paradoxical behaviour towards heat; it resisted heating both to 65°C. and to 100°C. From this they deduced that the inactivation at 65°C. in undiluted preparations was not a property of the toxin itself, but may be due to the formation of an inactive complex with a component of the culture filtrate,

which on reheating at higher temperature dissociated with the release of active toxin; in support of this they prepared autolysates of staphylococci which inactivated the toxin when heated with it at 65°C. Such explanation of the Arrhenius phenomenon assumes that intrinsically the toxin is heat stable.

The inactivation followed by reactivation on further heating was, however, not easily repeatable (Neisser, 1912; Atkin , 1910) and the work of Landsteiner and von Rauchenbichler (1909) was widely ignored. Nevertheless, throughout the years many conflicting reports concerning the heat sensitivity of the toxin have appeared. (Burnet, 1931; Gross, 1931 ; Dolman and Kitching, 1935; Flaum, 1938; Singer and Hagan, 1941). In an attempt to explain these, it was suggested that there are two lysins of differing heat sensitivity (Rigdon, 1938) and also that the sensitivity to heat depends on the age of the culture from which the toxin is prepared (Smith, 1941); haemolysin from young cultures was found more heat sensitive than that obtained from old cultures. In addition, toxin prepared in fluid medium was found to be more resistant to heat at 60°C. than that produced in agar (Beumer, 1939a) and toxin was also found more stable to heating at 60°C. in the presence of sucrose (Beumer, 1939b).

Most of this work, however, does not take into account the original paradox described by Arrhenius (1907).

The conditions necessary for the inactivation at 60°C. and the apparent reactivation on boiling have been investigated by a few workers. The importance of the pH of the reaction mixture was pointed out by Gengou (1935) and Beumer (1939a); the former found that the toxin was heat stable at a slightly acid pH and thermolabile at an alkaline pH, whereas the latter came to the opposite conclusion. A comprehensive study of the inactivation at 60°C was made by Tager (1941), who demonstrated that several autolytic products of different bacteria and other substances including pepsin (not acting in virtue of proteolytic activity) and lecithin combined with the toxin at 60°C. but not at 80°C. Recently Robinson, Thatcher and Montford (1960) found that even partially purified toxin was heat stable, and Elek (1959) concludes that "all this evidence suggests that alpha lysin itself is highly thermostable and that the apparent inactivation is due to another mechanism."

Recently however (Lominski and Arbuthnott, 1962) it was shown that partially purified, and purified preparations of alpha toxin (Lominski, Arbuthnott and Spence, 1963, in press) were irreversibly destroyed on heating to 60°C. for 30 min; the Arrhenius phenomenon

was found to occur with crude toxin. In view of the intrinsic heat sensitivity of alpha toxin the mechanism of the Arrhenius phenomenon needs to be reconsidered. In the present thesis experiments dealing with the heat sensitivity of crude and partially purified preparations are included, and possible explanations of the paradoxical Arrhenius phenomenon are discussed.

The effect of pH. Most workers recommend that alpha toxin should be assayed at a pH close to neutrality, probably because this is customary laboratory procedure. Possibly the first attempt to investigate the pH dependence of alpha toxin was made by Rud (1955) who found that after 1 hr. at 37°C. haemolysis was virtually unaffected over a wide pH range (5.5 - 8.5); when the degree of haemolysis was assessed after 15 min. incubation at 37°C. however, he found that the maximum haemolytic activity occurred at pH 5.5. Similar findings were reported by Mangalo and Raynaud (1959) who found that the time to 50% lysis of rabbit erythrocytes was shortest at pH 5.2 (over a range ofpH 4.4 to 7.4). By contrast the pH optimum determined by Jackson and Little (1957) was 6.8 to 7.0; these workers did not however determine the rate of haemolysis. It is also important to remember that in the assay of haemolysis the stability of the erythrocytes themselves may be considerably affected at different pH values (Rud, 1955) and that the buffer

used may influence this stability.

The effect of temperature on haemolysis. In contrast to the numerous studies on the effect of temperature on the toxin itself, only two workers have investigated the effect of temperature on the rate of haemolysis by alpha toxin. Rud (1955) found that the rate of haemolysis was maximal at a temperature of 30°C. when haemolysis was estimated after 15 min. in the presence of a small dose of toxin; again the effect of temperature was found, similar to the effect of pH, to be time dependent. After 1 hr. incubation there was a broad optimum range between 20° and 30°. On the other hand Mangalo and Raynaud in 1959 found that there was a plateau of optimum activity between 32°C. and 37°C. No activation energy, similar to that calculated by Bernheimer (1947) for other bacterial lysins has been recorded for alpha toxin.

The iso-electric point of alpha toxin. Here again there have only been two attempts to determine an important physical property of the toxin. In 1948 Wittler and Pillemer in a series of solubility studies found that maximum insolubility occurred at pH 4.3 and suggested that this was the isoelectric point. An entirely different value of 6.5 was determined by Butler (1959) who fractionated the toxin by Tisellius electrophoresis. At present until further experiments have been carried out it is im ossible to decide which is correct.

The Ultracentrifugation of alpha toxin. As previously mentioned, an early attempt to purify the toxin by ultracentrifugation (Gratia and Nelis, 1939) was unsuccessful, and the recent work of Kumar and Lindorfer (1962) suggests a value of 1.4 as the sedimentation constant of alpha toxin. This is open to considerable criticism, however (see p.34). Since the experimental work for the present thesis was completed, a value of 3.0 S has been suggested (Bernheimer and Schwartz, 1963). Experiments described in the present thesis and in press (Lominski, Arbuthnott and Spence, 1963) indicate a value of 3.1 S which is not significantly different from that of Bernheimer and Schwartz (1963).

The instability of alpha toxin. It is well-known that many crude preparations of alpha toxin are unstable (Le Fevre d'Aric, 1919a; Rud, 1955) and that highly purified preparations are even more unstable (Madoff and Weinstein, 1962; Goshi et al., 1962; Bernheimer and Schwartz, 1963; Lominski, Arbuthnott and Spence, 1963 - in press). As yet no explanation other than that the toxin is presumably toxoiding has been suggested to account for this property. It is, however, generally accepted that Tetanus toxin and Diphtheria toxin are also unstable when purified, and that this is due to polymerisation. Part of the present thesis describes experiments which suggest that the alpha toxin also tends to polymerise.

The Chemical nature of alpha toxin. Until 1959 there was very little positive evidence that alpha toxin was a protein, although it was generally accepted to be one. It did not pass through dialysis membranes, it was precipitated by protein precipitants, it was antigenic, and it was destroyed by frothing. These criteria in themselves, however, are not sufficient to establish protein nature, they could be satisfied by a polysaccharide for instance. In addition, the peculiar behaviour towards heat was not typical of a protein.

The first direct evidence came from the work of Robinson et al., (1960) who showed that the haemolytic activity of the toxin was destroyed by pepsin and trypsin. Experiments which have already been published (Lominski and Arbuthnott, 1962) and which will be described in the present thesis, showed that partially purified preparations of alpha toxin were destroyed by trypsin, and pepsin. Also the recent amino acid analysis of Bernheimer and Schwartz (1963) revealed that the purified toxin had the composition of a typical protein. By contrast the purified toxin of Goshi et al. (1963) contained a small amount of polysaccharide and was destroyed more readily by alpha and beta amylase than by trypsin. From this they conclude that the active site of the toxin is polysaccharide in nature. This finding, however, could not be confirmed by the writer, and as mentioned, there is a possibility

that this preparation may contain a toxin other than alpha, possibly gamma. In conclusion, it would seem that the bulk of the evidence is in favour of alpha toxin being a typical protein.

The action of various chemicals on alpha toxin. There has, as yet, been little systemmatic investigation of substances which either inhibit or activate alpha toxin; there exists mainly a number of scattered and unrelated findings. As early as 1919, le Fevre d'Aric (1919b) examined the effect of a number of metal colloids on a staphylococcal toxin which from his description appears to have been alpha toxin. The lethal effect was unaffected by silver, gold or platinum, but was inhibited by iron and manganese. Only manganese inhibited haemolysis. This inhibitory property of manganese was thought to be due to its oxidative properties. (A similar explanation has more recently been suggested by Petherwick and Singer (1944)). These studies were extended by Purdy and Walbum (1921) to include a great many metal salts; they found that some metals inhibited, whereas others activated haemolysis. In contrast with le Fevre d'Aric (1919b) manganese was found to stimulate activity. Also among those metals which activated the toxin were gold, silver, mercury, cobalt, magnesium, cadmium, and nickel. Inhibitors included barium calcium, zinc, lead, copper, iron and chromium. The

value of these findings is doubtful because the haemolysin was assessed on goat RBC and from experimental details it seems likely that it was beta and not alpha toxin which was being assayed.

Two vitamins have been shown to have antitoxic activity; vitamin C inhibited haemolysis at high concentrations, but not the lethal activity (Mercier, 1938a). This was explained by Mercier by the fact that the inactivation could be reversed by dialysis (Mercier, 1938b) and that probably a similar dissociation occurred in vivo. On the other hand, vitamin K has also been found to inhibit (Mule and Sedati, 1949). Both of these substances are thought to play a role in cellular oxidative metabolism and it may be that they inhibit toxin by oxidation. It is interesting to note that detoxication can be achieved also by treatment of the toxin with light and methylene blue (Li, 1936) and as has already been mentioned, by manganese. In addition, the halogens, iodine and bromine, which are powerful oxidising agents, also inhibit alpha toxin (Farkas, 1947). This property of ready oxidation may also account for the tendency of toxin to spontaneously detoxify itself. It is puzzling therefore that the amino acid analysis of Bernheimer and Schwartz, (1963) showed the complete absence of cysteine which is the most likely component of proteins to be involved in oxidation reactions.

A second group of chemicals known to affect the toxin are acids. Strong acids such as sulphuric and hydrochloric acids were found to destroy the toxin at a concentration of 5% (Nelis, 1933b). Weak acids such as lactic, acetic or tartaric, did not affect the toxin even at fairly high concentrations (Nelis, 1933b). Others, however, such as benzoic, salicylic and beta-oxy-napthoic acid destroyed the toxin only slowly (Nelis, 1933b). These results are remarkable because of the apparent resistance of the toxin to acids; for instance 5% sulphuric completely destroyed the toxin only after overnight incubation at 37° and 1% did only partially affect the toxin in the same time. Nelis himself (1933b) suggested that his medium contained protective substances, although a similar resistance was reported by Stockinger, Ackerman and Carpenter (1941) who found that the toxin retained its activity after overnight standing at pHs between 4.2 and 10.8. When inactive toxin was obtained by the treatment described above, the resulting preparation did not react with antitoxin.

The toxoiding of alpha toxin with formalin was first described by Burnet (1929), who later went on to study the chemistry of the reaction (Burnet and Freeman, 1932). These workers found the rate of toxoiding to be dependent on the concentration of formalin and proportional to the

square root of the hydroxyl ion concentration. The reaction had a high temperature coefficient. Since the 1930s the toxoiding of alpha toxin with formaldehyde has been confirmed hundreds of times. It is standard procedure to incubate the toxin at 37°C in the presence of 0.4% formalin overnight. However, some workers obtained complete toxoiding only after 5 to 10 days (Nelis, 1933). It is generally accepted that the toxoid is almost as good an immunising agent as the toxin itself and high titres of antitoxin can be attained quite readily. It is also interesting to note that toxin detoxified by treatment by methylene blue and light is also a good antigen.

Another entirely different type of inhibition is known. Rigdon (1936a) found that when 5% sodium chloride was injected into rabbits and alpha toxin then injected into the bleb that the degree of necrosis was reduced and instead of appearing on the surface was evident in the deeper layers of the skin. Similarly Smith (1937) found by accident that the necrotic action of alpha toxin was inhibited by glycerol and that similar inhibition was obtained with ethylene glycol, sucrose, and glucose; she suggested that the inhibition was due to the destruction of the toxin. The problem was re-examined by Rigdon (1937b) who found that although 5% sodium chloride reduces the rate of haemolysis, mixing toxin with 12% sodium chloride

prior to titration had no effect on the haemolytic activity. Also Avery, Rigdon and Johlin (1937) found other salts of sodium and potassium had a similar effect and that magnesium sulphate inhibited haemolysis and necrosis; peculiarly however, Lithium chloride inhibited necrosis but not haemolysis. It has been suggested that hypertonic solutions of these salts had in some way protected the cells from damage.

Phospholipids have an inhibitory action on alpha toxin. That lecithin inhibited the haemolytic action of staphylococcal toxin was noted by Weinstein (1937) who also observed the odd reversal of the inhibition by cholesterol. Recently inhibition of the lethal activity of alpha toxin by prior injection of snake venoms has been observed (North and Doery , 1958). They attributed this inhibition to the release of breakdown products of phospholipids. Recently (1961) they described a similar inhibition of the lethal action of alpha toxin by cerebrosides. The exact nature of inhibition by phospholipids is not clear, although it may be a vital clue to the finding of the biochemical point of attack of alpha toxin.

The mechanism of action of alpha toxin.

This important aspect of alpha toxin has been largely ignored; what has been done is to some extent contradictory. Most studies have been made using erythrocytes as

substrate, since this enables some degree of quantitative evaluation.

The controversy centres around whether the toxin combines stoichimetrically with the red blood cell or whether it acts enzymically. The latter concept was first suggested by Forssman (1933, 1934a,b,c) on the grounds that the reaction between staphylolysin and red blood cells of rabbit and sheep was characterised by a weak, irregular and easily reversible adsorption. On the other hand evidence in favour of a strong adsorption was put forward by Levine (1938, 1939) who found that the haemolytic, dermonecrotic and lethal effects of the toxin were reduced by incubation with "highly concentrated suspensions of red blood cells". A logarithmic plot of the amount of activity adsorbed against the amount remaining resulted in a sloping straight line; the quantitative relation between them was given by the equation:-

$$\text{Log } Y = \text{Log } 2.2 + 0.7 \text{ Log } X$$

where Y equals the number of haemolytic units adsorbed and X equals the number left. From this Levine concludes that the reaction between toxin and the red cell obeys the law of the Freundlich adsorption isotherm. This implies that the reaction between toxin and the red cell is stoichiometric.

In reply to Levine's findings, Forssman (1939) published a reappraisal of his own findings and repeated some of his earlier experiments in more detail. In conclusion he added that "To embody these phenomena in a mathematical formula is not an easy task, at any rate they cannot be classed under the formula employed by Levine". Possibly as a result of the intervention of the Second World War the controversy was not continued and the matter was allowed to rest.

Interest in this aspect was slow to return and even the important work of Bernheimer (1944, 1947) went apparently unnoticed. The kinetics of a number of bacterial haemolysins, other than staphylococcal toxins, were investigated by Bernheimer (1944, 1947) along with those of some organic lytic agents such as saponin; many of the bacterial lysins showed a characteristic direct proportionality between the rate of haemolysis and the concentration of lysin. Bernheimer concluded that "In view of the fact that all the lytic agents showing the direct proportionality appear to be proteins, it seems not improbable that some or all of the lysins of this class are enzymes".

It was not until 1957 with the work of Jackson and Little that the kinetics of staphylococcal toxins were examined. These workers using a colorimetric method

of assaying the rate of lysis observed that the alpha toxin was similar to both "other bacterial lysins and organic lytic agents". In an attempt to determine an accurate method for assessing alpha toxin activity by measuring the time to reach 50% haemolysis, Mangalo and Raynaud (1959) found that the relationship between T50 (time to 50% haemolysis) and C (concentration of alpha toxin) was given by the equation:-

$$\text{Log } T50 = -a \text{ Log } (C) + b.$$

in which a equals 0.54 ± 0.03 and b was found to have a value varying between 0.22 and 0.67. Kinetic studies of the rate of haemolysis by which alpha toxin yielded results similar to those of Bernheimer (1944;1947) were carried out by Lominski and Arbuthnott (1962) and are to be described in the present thesis. With certain limitations they support the view that the alpha haemolysin acts enzymically. Also from the results of their "split titration results" it would appear that as suggested by Foxsman (1959) the adsorption reaction, if it does take place, must be reversible.

Only a little work was devoted to the possible substrate of alpha toxin. As early as 1935 Seiffert suggested that the point of attack was the lipid portion of the red cell. Recently Robinson et al. (1960) found

that in highly purified preparations alpha toxin was inseparable from protease activity; in later work, however, separation has been achieved by further purification (Thatcher, personal communication, 1962).

The problem has been attacked in an unusual and interesting way by North and Doery and their colleagues in Australia over the past few years. As previously mentioned (p. 47) in 1958 North and Doery described the protective action of certain venoms against bacterial exotoxins, especially staphylococcus alpha toxin. Fractions of the venom of the Australian Tiger snake, rich in haemolysin and poor in neurotoxin, protected mice when injected together with, or prior to a lethal dose of alpha toxin. Similar protection could be achieved with venoms of other snakes or even of the honey bee. All of these preparations contained phospholipase A activity and they suggested that the natural protective response to the toxin involved a release of phospholipase from the tissues which then hydrolysed phospholipid to lysophosphatides and long chain fatty acids. Such fatty acids were already known to inhibit alpha toxin and also diphtheria toxin (Nelis, 1933a,). This work has been continued and extended and it was recently shown that

cerebrosides also inhibited alpha toxin *in vivo* (North and Doery, 1961). At the end of last year the same group of workers reported that crude and partially pure preparations of the toxin contained a phospholipase which catalysed the breakdown of an unspecified phosphatidyl inositol and lysophosphatidyl inositol to diglyceride and inositol phosphate (Magnusson, Doery and Gulasekarem, 1962). A similar action on phosphatidyl inositol in striated muscle of the rat and red blood cells was demonstrated. However, "evidence was obtained that the phospholipase of this toxin was not directly responsible for the lethal effect in mice and haemolysis". Nevertheless, it seems possible from the work of this group and experiments to be described here that Seiffert was correct in suggesting that the site of attack lies in the lipid portion of the cell.

The mode of action of alpha toxin *in vivo*.

In spite of the many efforts by workers of different experimental sciences, very little concerning the action of the toxin *in vivo* has been added to the findings of the early workers. The action of staphylococcal toxin on blood pressure, on the heart, on the small vessels and on the nervous system were all recorded before 1920 (van de Velde and Denys, 1895; Neisser and Levaditi, 1900;

Kraus and Pribram, 1906; Russ, 1916; le Fevre d'Aric, 1919a). Later work in addition to confirming these findings has served the important purpose of showing that all these actions can be attributed to alpha toxin; its wide spectrum of activity has been established. In addition the simple yet extremely important conclusion can be made that the action of alpha toxin depends on the dose and route of administration.

Kraus and Pribram (1906) were the first to notice that staphylococcal toxin caused a fall in blood pressure and heart failure, and that these were unaffected by section of the vagus nerve. Ten years later Russ (1916) made a comprehensive study of the effect of staphylococcal toxin in vivo and confirmed the fall in blood pressure; in heart lung preparations he found that toxin caused failure of the right heart and concluded that this resulted from obstruction of the pulmonary circulation.

These findings were extended by the work of Kellaway, Burnet and Williams (1930) who investigated the effect of alpha toxin on the blood pressure of rabbits and cats. Intravenous injection of toxin had a twofold effect: there was an initial fall, followed by an "excessive recovery" and then a rapid terminal drop in blood pressure. The initial fall was shown to be caused

by "pharmacologically active constituents of the media" and the "recovery" due to the release of adrenalin. The terminal fall was considered to result from the obstruction of the pulmonary circulation by alpha toxin. In addition the toxin was found to have an effect on the heart itself. Collapse and death of the experimental animal was due to the combined action of these two effects. On the other hand, Rigdon (1935) found no obstruction of the pulmonary circulation; in a later study he found it impossible to distinguish between the action of toxin and the constituents of the medium in causing the fall in blood pressure (Rigdon, 1936b). The blood pressure of immune dogs behaved in exactly the same way on the injection of toxin. It must be pointed out however that Rigdon's toxin appears to have been very weak. In contrast Dicker (1935) found that 15 daily injections of staphylococcal exotoxin had no effect on blood pressure.

In addition to finding a progressive fall in blood pressure Nelis and Boukaert (1933) described the appearance of cardiac irregularities on electrocardiogram tracings, such as, extrasystoles and fibrillation. The work of Dingle and his colleagues (Dingle, Hoff, Nahum and Carey, 1937) did not confirm this finding; they concluded that

"These results fail to demonstrate any causal relationship between the cardiac irregularities as such and rapid death from intravenous injection of the exotoxin of S. aureus but they do indicate that death is due to a toxic action on the myocardium". It has been generally accepted as a result of all this work that alpha toxin acts by lowering the blood pressure and damaging the myocardium. There is, however, a distinct lack of recent work on this aspect and it would seem profitable to carry out similar studies with pure toxin and modern improved apparatus.

The necrotic action of staphylococcal toxin on skin and kidney has been long known (see p. 14, 21). For many years it was believed that these toxic manifestations were due to distinct toxins (Parker, 1924; Weld and Gunther, 1931) although it is now generally accepted that they are merely examples of the many actions of alpha toxin. Whereas the effects on blood pressure and the heart were mainly studied using large and rapidly lethal doses of toxin, necrotic lesions are apparent only when the animal survives some time after challenge. In the very first description of the effect on kidney, Neisser and Levaditi (1900) attributed the cause to formation of generalised infarcts as a result of the

obstruction of the small vessels by disrupted leucocytes. Although it is now widely accepted that the action is indeed on the small vessels there is some controversy as to the exact mechanism. On injection of sub-lethal doses of alpha toxin, De Navasquez (1938) observed the typical symmetrical cortical necrosis of the kidney and suggested from histological evidence that the intravenous injection of alpha toxin causes paralysis of the interlobar arteries and efferent arterioles resulting in stasis and subsequent necrosis. Distension of the glomerular capilleries with leakage of toxin and subsequent damage of the cells of the tubular epithelium was the suggested mechanism of van Ghlan and Weld (1935). This was supported by the work of Simmonds, Linn and Lange (1946). The possibility that the ischemic effect was due entirely to vasospasm of the small vessels was put forward by Thal (1951). Recently alpha toxin has been shown to kill suspensions of kidney cells in tissue culture (Gablicks and Solotorovsky, 1961).

That the dermonecrotic reaction also involved the small vessels has been known since the work of Parker (1924). The mechanism of this reaction was investigated by Thal and Egner (1954), who showed that rather than being typical inflammatory reaction it resulted from spasm of the

small vessels. By injecting trypan blue into the blood stream they showed that the area of skin affected by toxin became ischemic. In addition by direct observation of the mesenteric blood vessels they observed that toxin caused prolonged and severe segmental spasm of the arteries and veins, and altered capillary permeability in such a way that stasis occurred. Similarly Thal and Molestina (1955) found that introduction of the toxin directly into the pancreatic duct caused a highly fatal haemorrhagic pancreatic necrosis due to the suppression of the pancreatic blood flow; injection of dye into the blood stream of animals previously injected with toxin did not result in the blackening of the pancreas. Recently the same group of workers showed that alpha toxin caused spasm of smooth muscle tissue and suggested that a similar spasm of the smooth muscle of the blood vessels would result in a fatal drop in blood pressure (Thal and Egner, 1961). It seems likely that both spasm of the blood vessels and a direct action of alpha toxin on the cellular elements of a tissue may be involved in necrosis.

In one of the earliest studies of staphylococcal toxin the cytolytic action on nerve cells was demonstrated (van de Velde and Denys, 1895). The action on nerve tissue was however ignored until le Fevre d'Aric, who was

investigating the rapid death of experimental animals from staphylococcal toxin, suggested that the dyspnoea and general convulsions which characterise rapid death may be due to its action on the nerve cells of the respiratory centre in the brain (le Fevre d'Aric, 1919a). In a series of papers on the effects of staphylococcal toxin on the central nervous system Travassos (1933a,b.) found that death from intracerebral injection of toxin is characterised by tetanic spasms, rapid respiration and finally Cheyne Stokes breathing. Also injection of toxin into the lumbar region of the spinal cord resulted in paralysis of the hind legs followed by spreading of the symptoms to the cerebral region. Similarly the direct action of alpha toxin on the respiratory centre was shown by Nelis (1935) and Nelis and Bonnet (1935); these workers observed that death often resulted before the heart had stopped beating. By injecting partially formalised crude toxin into the femoral artery, Nelis (1934) caused paralysis of the hind limb on the corresponding side in rabbits. It is possible that this was a result of the direct action of the toxin on the skeletal muscle. Although it can be seen from the above account that the action of toxin on the nervous system has not been studied

in as much detail as other actions of the toxin the impression has been gained from experiments included in the present thesis that action on the nervous system may well be one of the most important properties of alpha toxin.

In addition to the effects of alpha toxin already described it seems to act on almost every other tissue. Post mortem changes in the stomach, intestine, lungs, kidneys, liver and spleen have been described (Nelis, Boukaert and Picard, 1934). Direct action on the alimentary tract (Borthwick, 1933) and on the knee joint have also been described (Rigdon, 1937). Recently a direct action of alpha toxin on the striated muscle of the mouse has been recorded (Lominski, Arbuthnott, Scott and McCallum, 1962). Indeed the toxin has been described by Elek (1959) as a "catholic cytotoxic agent".

As yet little work has been carried out on tissue cultures. Cultures of chick heart fibroblasts are affected (Lasfargues and Delauney, 1946). However, evidence that the toxin does not act on all cells comes from the findings that laryngeal cells (Gablicks and Solotorovsky, 1961) and epithelial cells (Thal and Egner, 1961) are unaffected by the toxin. It is also interesting to note that trypanasomes are resistant to the toxin (Nelis, Boukaert and Picard, 1934).

The biochemical point of attack is as yet a mystery; indeed it is not yet known whether or not the toxin acts on the surface of cells or by disrupting an essential metabolic reaction in the interior of the cell. From the fact that it acts extremely quickly and that it has a mass of approximately 45,000 (Bernheimer and Schwartz, 1963; Lominski, Arbuthnott and Spence, 1963, in press) it seems likely that the substrate is widely distributed and is probably to be found on the surface of the cell.

The Experimental Section is presented in four parts, each dealing with a different aspect of Staphylococcus alpha toxin. The topics are so diverse that to discuss them in a single Discussion Section would be confusing. For this reason, each aspect is discussed separately following the relevant Experimental Section. An overall picture of the main conclusions will be given in the Summary.

I

THE HAEMOLYTIC ACTION OF ALPHA TOXIN

Page

RESULTS (Cont'd)

I

THE HAEMOLYTIC ACTION OF ALPHA TOXIN

This section describes experiments designed to determine the nature, properties and mode of action of crude and partially purified preparations of staphylococcal alpha toxin *in vitro* using haemolysis as indicator system.

MATERIALS AND METHODS

Production of crude alpha toxin. The organism mainly used for the production of alpha toxin throughout this study was strain Wood 46 (NCTC 7121); in some experiments another alpha toxin producing strain, 5 AS, was also used. As mentioned previously several methods were screened for the production of toxin. These included standing buffered broth cultures, shake cultures in meat extract broth in the presence and in the absence of CO_2, agitation by the method of Duthie and Wyllie (1945) using broth and semi-solid agar media, and semi-solid medium composed of casein hydrolysate and yeast extract. The best yield was undoubtedly obtained by using a modification of the method of Burnet (1930); this was used throughout the present work, except when the effect of heat was being studied, and when broth cultures were also employed.

Cultures were grown for 48-72 hr. at 37°C. in an atmosphere of 20% CO_2 and 80% air on a semi-solid medium

consisting of 0.35% Oxoid agar No.3, 1% peptone, 0.5% sodium chloride in ox heart infusion broth, which since it was prepared for diagnostic purposes contained 1 in 20,000 p-amino benzoic acid.

Toxin was then extracted by freezing at -20°C. and thawing the agar once; the cells were removed by centrifuging at 8,000 r.p.m. for 30 min. In most experiments thiomersal was added to the resulting supernatant to a concentration of 1 in 10,000; toxin was stored either at 4°C. or at -20°C.

Partial purification of alpha toxin. A modification of the method of Wittler and Pillemer (1948) was used: glacial acetic acid was added to the crude toxin at 0°C. to a pH of 4.0, followed by the addition of methanol to a concentration of 20% (v/v) at -5°C. After the mixture had been allowed to stand at this temperature overnight the precipitate was spun at 5,000 r.p.m. in a refrigerated M.S.E. centrifuge at -5°C for 30 minutes and redissolved in one tenth of the original volume of crude toxin in 0.14 M phosphate buffer pH 7.25 (Hendry, 1948). The concentrate was then acidified at 0°C. with glacial acetic acid to pH 4.0 and allowed to stand overnight at this temperature. The resulting precipitate was spun as before and redissolved in the same volume of isotonic

phosphate buffer at pH 7.25. This partially purified preparation was stored either at 4°C. in the presence of 1 in 10,000 thiomersal or at -20°C. in absence of thiomersal.

The potency of preparations at different stages in purification was assayed by estimating the haemolytic potency per mg. of Nitrogen. Nitrogen was determined by the Micro Kjeldahl method.

Titration of haemolysin. The haemolytic titre of alpha toxin was determined by making serial doubling dilutions of toxin in 0.5 ml. amounts of a diluent containing 25% ox heart infusion broth, 75% normal saline and 1 in 10,000 thiomersal; 0.5 ml. amounts of a 2% suspension of 4 times washed rabbit erythrocytes was added to each tube bringing their final concentration to 1%. The end point was usually assessed visually, the dilution of toxin causing 50% haemolysis after 1 hr. at 37°C. being accepted as comtaining 1 MHD; repeat titrations and reading by different individuals gave the same end point. In some experiments the end point was determined spectrophotometrically, estimating the amount of haemoglobin released, at an optical density of 540 mu.

Measurement of the rate of haemolysis. The rate of haemolysis was determined in four types of experiment; the effect of toxin concentration, red cell concentration,

pH and temperature on haemolysis.

Most kinetic experiments in which the relation between the rate of haemolysis and the concentration of alpha toxin was investigated, were carried out in the E.E.L. nephelometer, because at that time the department had no spectrophotometer. Mixtures of 1 ml. of a 2% suspension of rabbit erythrocytes and 4 ml. of isotonic phosphate buffer pH 6.8 - 7.0, diluted 1 in 5 in saline, containing the desired amount of toxin were prepared in 6 x $\frac{1}{2}$ in. (15 x 1.25cm.) matched glass tubes. The turbidity of mixtures was then determined at regular intervals in an E.E.L. nephelometer, previously calibrated by measuring the turbidity of graded suspensions of erythrocytes, and the turbidity units were related to the percentage erythrocytes present. Calibration and kinetics were carried out using the same suspension of red cells on the same day. All of these experiments were carried out at room temperature.

In kinetic experiments using highly purified alpha toxin and in studying the effect of pH on haemolysis the same principle was employed but the turbidity measurements were made using a Hilger and Watts spectrophotometer at a wave length of 650 mu at which the turbidity was unaffected by haemoglobin released in haemolysis (Mangalo

and Raynaud, 1959). Reaction mixtures were as follows:- 3.5 ml. of Hendry's buffer of the appropriate pH, 0.5 ml. of 2% rabbit erythrocytes and 0.5 ml. of toxin; c. 3 ml. of this was then pippeted into a 1c.m^2 cuvette. In nephelometric experiments the final red blood cell concentration was 0.4% while in spectrophotometric experiments the final concentration was 0.22%. The rate of haemolysis was calculated in each case by determining the slope of the linear portion of the time haemolysis curve (p. 89). In pH experiments the spectrophotometer was not first calibrated in terms of percentage red blood cells and the rate of haemolysis in Fig.2 is given in arbitrary turbidity units. In determing the effect of the red blood cell concentration on the velocity of haemolysis the decrease in turbidity at 650 mu was related to the actual number of red cells lysed.

The effect of temperature on haemolysis was investigated using yet another method. Temperatures were varied over the range 25°C. to 40°C. and at each value had to be strictly controlled. Measurement of the rate of haemolysis turbidimetrically either in the E.E.L. nephelometer or in the spectrophotometer would have involved removing mixtures from the water baths, with temporary change in temperature and introducing a high degree of inaccuracy. It was decided therefore

to allow the toxin and red cells to interact at the desired temperature for a given time (4 min.) then stop the reaction by adding a large excess of antitoxin and immediately remove the unlysed cells by spinning at a set speed for 3 min. The amount of haemoglobin in the supernatant was then determined spectrophotometrically at 540 mu and expressed as the percentage of the amount liberated by complete haemolysis of the suspension by alpha toxin. The relation between optical density at 540 mu and haemoglobin concentration was found to be linear.

Erythrocytes. Citrate was used as an anticoagulant in all cases. Rabbit blood was collected by bleeding from the marginal ear vein of chinchilla rabbits. Fowls were bled from the main wing vein, and guinea pigs were exsanguinated by severing the jugular vein. Frogs were anaesthetised under chloroform and the hearts held over a universal container, the tip of the ventricles was cut and blood collected. Mice were anaesthetised under chloroform, the heart exposed and the ventricles cut; blood was collected from the pericardium in pasteur pippetes. Fresh citrated human blood was kindly supplied by Dr. H. E. Hutchison of the Department of Haematology and defibrinated sheep blood ("Wellcome Brand") was used as source of sheep red cells. All blood samples were washed four times with normal saline and the red cells

kept in the packed state for up to 5 days.

Reagents. All reagents were of B.D.H. AnalaR or "laboratory standard". Thanks are due to I.C.I. Ltd., and Bayer Chemicals Ltd., for analogues of "Suramin".

RESULTS

Properties and Nature of Partially Purified Alpha Toxin

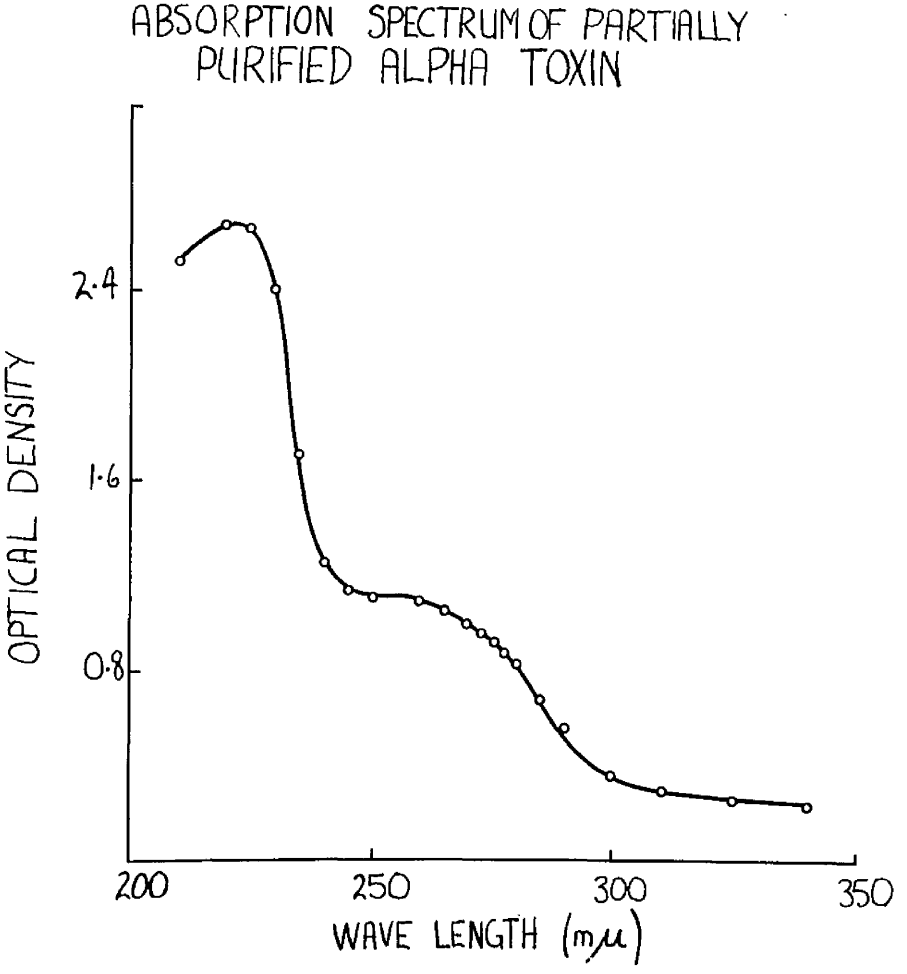
Partial purification. After the first precipitation with 15% methanol at pH 4.0, there was an 80-100% recovery of the toxin with a 15-fold increase in the number of MHD/mg. of nitrogen; at the second stage, after re-precipitation there was an overall yield of 40% with a 35-fold increase in haemolytic potency over the crude toxin. Table 1 summarises the results of a typical experiment. Such preparations contained at least 4 antigenic components in the double diffusion test (p.199).

Table 1

The number of MHD's per ml. and per mg. of nitrogen of partially purified preparations of staphylococcus alpha toxin

Stage of preparation	MHD/ml.	MHD/mg. Nitrogen
Crude filtrate	2,000	870
First precipitation (acid and methanol)	16,000	14,000
Second precipitation (methanol alone)	8,000	20,000

Fig. 1


ABSORPTION SPECTRUM OF PARTIALLY PURIFIED ALPHA TOXIN
OPTICAL DENSITY
2·4
1·6
0·8
200
250
300
350
WAVE LENGTH (mμ)

The absorption spectrum of such partially purified alpha toxin (Fig.1) has a plateau between 250 and 260 mu, indicating the presence of nucleoprotein or nucleic acid. Treatment of partially purified alpha toxin with ribonuclease for periods of up to 24 hr. had no effect on the haemolytic action of the toxin and subsequent work to be described (p. 186) showed that the nucleic acid component of such preparations was an impurity.

The effect of proteolytic enzymes on alpha toxin. As previously mentioned little is known of the nature of alpha toxin and its sensitivity to proteolytic enzymes. For this reason the effect of pepsin and trypsin was investigated. Table 2 and Table 3 summarise typical experiments.

Table 2

The effect by trypsin and pepsin on alpha toxin

System	Titre after 5hrs. at 37° C.
Alpha toxin control pH 8.0	1/4,000
Alpha toxin + trypsin(1mg/ml)pH 8.0	1/16
Alpha toxin control pH 4.0	1/128
Alpha toxin+ pepsin (3mg/ml)pH 4.0	1/4
Neither pepsin nor trypsin at these concentrations were haemolytic by themselves.	

Table 3

The effect of 0.3 mg/ml trypsin at pH 7.0 at 37°C.

Time	Haemolytic titre	
	Control	Trypsinised toxin
2 hr.	1/8,000	1/8,000
9 hr.	1/8,000	Complete haemolysis to 1/16 and partial to 1/1,000
20 hr.	1/8,000	1/64

Although in pepsin experiments the low pH at which the experiments had to be carried out caused a considerable drop in the haemolytic activity in the control (indicating either a denaturation by low pH or possibly destruction by a protease present in the preparation) the remaining haemolytic activity was destroyed by pepsin.

Trypsin also destroys the haemolytic action of alpha toxin (Table 2). However, at smaller concentrations of trypsin (Table 3) even 9 hr. incubation failed to destroy all of the toxin and a substantial "tailing" of incomplete haemolysis was noted. The significance of this finding is not yet clear. These experiments however indicate that alpha toxin is digested by proteolytic enzymes and is therefore probably a typical protein.

The effect of heat on alpha toxin. As mentioned in the Introduction (p. 35), the heat sensitivity of

alpha toxin has been a subject of considerable controversy ever since the discovery of the toxin. The toxin was found to show the paradoxical effect towards heat known as the Arrhenius phenomenon, being inactivated after 30 min. at 60°C. and reappearing on reheating at 100°C. for a few minutes. In an attempt to clarify the situation it was decided to compare the effect of heat on the crude and partially purified preparations.

In a preliminary experiment an attempt was made to examine the effect of pH on the Arrhenius phenomenon as it was felt that this may be an important factor. As can be seen from Table 4 it was found that alpha toxin was more sensitive to heating at 60°C. for 30 min. at pH 5.5 but was also more easily restored on reheating at 100°C. at this pH.

Table 4

The effect of heat on crude alpha toxin

Heat treatment	pH		
	5.5	6.4	8.6
Unheated	1/500	1/500	1/500
60°C. for 30 mins.	1/4	1/64	1/32
100°C. for 15 mins.	1/64	1/64	1/8
60°C. for 30 mins. reheated to 100°C. for 15 mins.	1/128	1/128	1/32

Table 5

The effect of heat on partially purified alpha toxin

Heat treatment	Partially pure toxin pH 7.0	Partially pure toxin pH 5.5
Unheated toxin	1/8,000	1/4,000
30 min. at 60°C.	1/4	1/4
15 min. at 100°C.	1/4	1/4
30 min. at 60°C. reheated 15 min. at 100°C.	1/4	1/4

The paradoxical effect was also found at pH 6.4 though less marked, while at pH 8.4 it was not observed. Under optimum conditions (pH 5.5) there was a 25% recovery of the toxin on reheating and this was shown by specific neutralisation with anti alpha toxin to be due to alpha toxin.

In view of the influence of the menstrum (Landsteiner and Rauchenbichler, 1909; Tager, 1941) on the effect of heat it was decided to compare the heat sensitivity of crude and partially purified preparations. A comparison of Table 4 and 5 shows that even at optimum conditions (pH 5.5) partially purified alpha toxin was completely heat sensitive; the Arrhenius phenomenon was absent and the haemolytic activity was not restored on reheating at 100°C. Since the preparations used in the experiment with partially purified alpha toxin were more potent than

those used in crude toxin experiments one might have expected a more marked demonstration of the phenomenon if it had been present. It appears therefore that alpha toxin purified by acid methanol fractionation is heat sensitive like a typical protein.

Having established that partially pure alpha toxin was heat sensitive it was decided to test the effect of broth on this sensitivity. Table 6 summarises the result.

Table 6

The effect of meat extract broth on the sensitivity of alpha toxin to heat.

Heat treatment	Partially purifed toxin in saline	Partially purified toxin in broth
Unheated	1/1,000	1/1,000
30 min. at 60°C.	1/8	1/8
Reheated for 15 min. at 100°C.	1/16	1/128

Here the toxin preparation used was different from that in previous experiments and was possibly less pure. So that even the control toxin was restored by one tube but by comparison the toxin in broth was considerably more haemolytic on reheating. Only about 12% of the total activity reappeared and this was less than the recovery observed in crude toxin (Table 4). Thus it would seem that broth is not the only factor determining the reappearance of activity. Nevertheless it does suggest that some degree of protection can be conferred on partially purified preparations by meat extract broth.

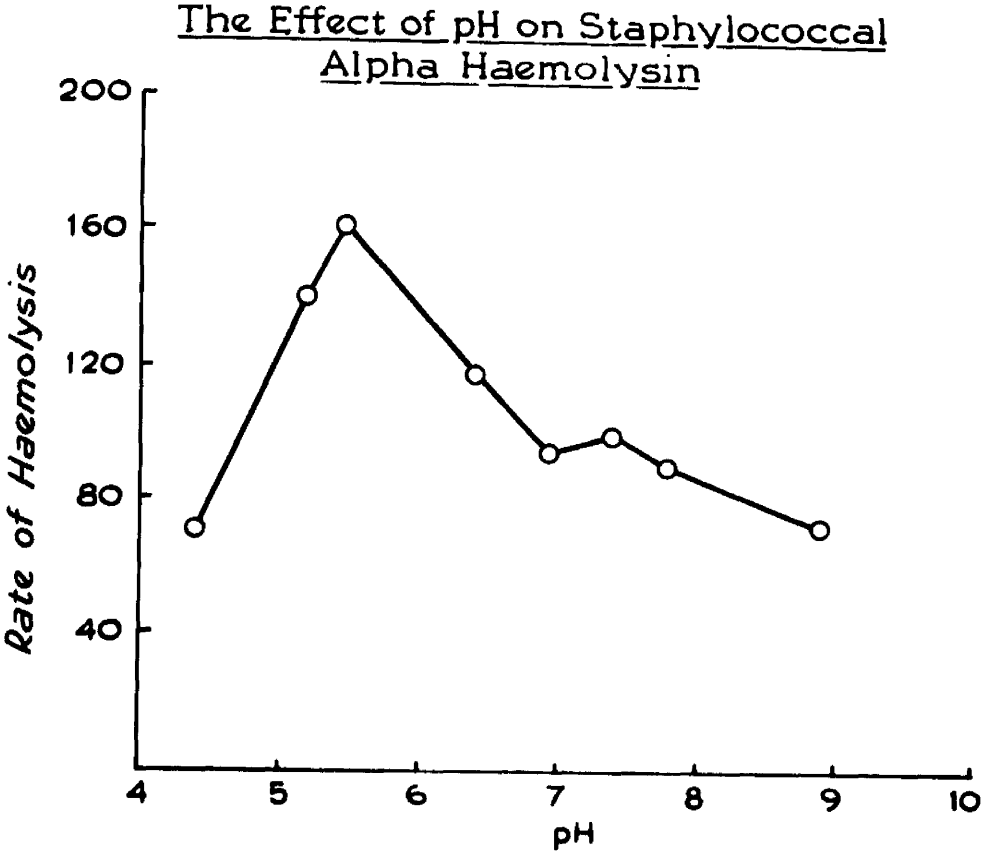
The effect of pH on haemolysis. The effect of pH was studied in two ways, by assessing the end point of alpha toxin titrations in isotonic phosphate buffer of different pHs after 1 hr. at 37°C. and by measuring the rate of haemolysis turbidimetrically over the same range of pH values.

It was found that, after 1 hr. incubation, the titre was the same over a pH range of 5.2 to 7.7. It dropped to one eighth at pH 4.3 and to one half at pH 8.8. However, the titre seemed to be influenced to a greater extent after about 6 min. when the titre at pH 5.2 was considerably greater than at the other pH values; with the progress of time the titres at pH 6.3 to 7.7 increased more rapidly so that after 1 hr. the titres over the range 5.2 to 7.7 were identical.

The influence of pH on haemolysis was investigated more exactly by determining the rate of haemolysis over a similar range of pH values turbidimetrically, as described (p. 69). A dilution of toxin was chosen which would give 50% haemolysis in 12 to 15 min; the results are shown in Fig. 2. The pH optimum was found to be 5.5.

The Influence of Temperature on haemolysis. The influence of temperature on the reaction was investigated by titrating toxin at different temperatures (Table 7).

Fig. 2



As mentioned on p. 68 the rate of haemolysis in this experiment was measured in turbidity units.

Table 7

The effect of temperature on alpha toxin	
Temperature	Titre (after 1hr. at 37°C)
4°C.	1/32
20°C.	1/500
37°C.	1/1,000

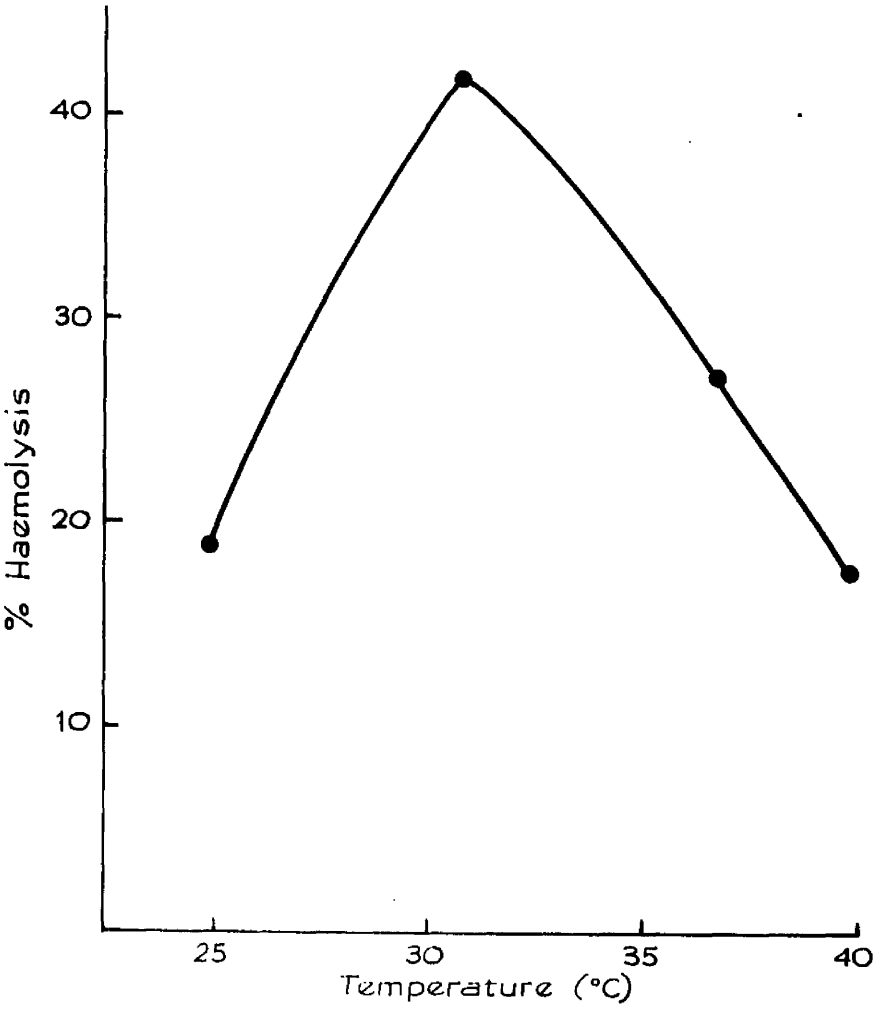
In order to determine the optimum value more accurately the rate of haemolysis was measured over a narrower range of temperature by the method described on p. 69

The average result of three consecutive experiments is given in Fig.3; the optimum temperature appears to be 31°C. with a fairly marked falling off in activity below and above this value.

Discussion

The results of proteolytic digestion with trypsin and pepsin suggest that alpha toxin is a protein and agree with the findings of Robinson et al. (1960). The nucleic acid which showed up in the absorption spectrum of partially purified preparations was absent from more highly purified toxin (see p. 203) and is therefore an impurity.

Fig. 3



The belief that alpha toxin is heat resistant was a great obstacle in accepting its protein nature; even now some aspects of the toxin's behaviour towards heat can only be explained in a speculative manner. Some conflicting reports of the past concerning heat sensitivity of the toxin may be ascribed to confusion of alpha toxin with other staphylococcal toxins.

However, where alpha toxin for certain was investigated, the Arrhenius phenomenon is still a stumbling block. Inadequately defined experimental conditions may account for some workers having missed the effect; for instance in the present studies the effect was very striking at pH 5.5 but was absent at pH 8.6 (in agreement with Gengou (1935) but conflicting with Beaumer (1939a)). Such difference in pH can account for failure to demonstrate the Arrhenius effect, but in no way helps in understanding the effect when it does occur.

Earlier explanations of the Arrhenius effect assumed that alpha toxin was intrinsically heat resistant and aimed at explaining why a toxin which can resist boiling was sensitive to heating at 65°C. With the knowledge that partially purified (Lominski and Arbuthnott, 1962) and to a greater extent highly purified alpha toxin (Madoff and Weinstein, 1962, Bernheimer and Schwartz, 1963; Lominski, Arbuthnott and Spence, 1963) is thermolabile, the position has become reversed. It is not only the

heat sensitivity at 65° but also the heat resistance (of crude toxin) to heating at 100°C. that now require explanation.

Landsteiner and von Rauchenhichler (1909) and more recently, Tager (1941) explained Arrhenius' finding by postulating that at 65°C. alpha toxin combined with a constituent of the culture fluid to form an inactive complex which on reheating at 100°C. dissociated and released active toxin; some adequate experimental evidence in support of this is available. The explanation was very satisfying as long as alpha toxin was thought to be heat stable; in fact, its first part, that is, the combination with a constituent of the culture medium, is still acceptable. The second part of the older explanation of the Arrhenius phenomenon is no longer valid, since pure alpha toxin is heat sensitive. It has now to be postulated that in crude preparations alpha toxin, when reheated to 100° either exists in a different form or else that it is protected from heat. Protection may be given by the same constituent with which it combined to give an inactive complex at 65°, or by another component of the culture fluid. Heat protection of a protein by another protein is not unknown. Coagulase is highly thermolabile when purified but can resist boiling when impure (Lominski, unpublished). Again, admittedly in a different type of system, the 'phage of B. Megatherium is inactivated at approximately 60°C in

filtrates but will withstand boiling when present in the spore of a lysogenic strain (Dooren de Jong, 1931). Such an analogy may be faulty but short of a better explanation it may help to reconcile apparently conflicting facts. What does emerge is the fact that partially purified or pure alpha toxin is, like most typical proteins thermolabile.

Also included in the present section are experiments on the optimum conditions of pH and temperature for the haemolysis reaction. The optimum pH, found in turbidimetric experiments, of 5.5 is slightly higher than that of 5.2 recorded by Mangalo and Raynaud (1959). The influence of pH on haemolysis was not demonstrated in titration experiments when the titre was read at 1hr., although it was detected when the titrations were read a few minutes after incubation. This was similar to the finding of Rud (1955) who found that lysis was rapid at about pH 5.5 over the first few minutes but that after 1 hr. there was no difference in titre over the pH range 5.5 to 7.5. Rud attributed this to increased sensitivity of rabbit red blood cells at low pH and recorded some spontaneous lysis at pH 5.5. In the present work, however, no such spontaneous lysis was noted even at pH 4.4. It seems possible that pH 5.5 is indeed the optimum pH of haemolysis although this is slightly

surprising since blood and tissues in which the toxin acts *in vivo* have a pH of about 7.4.

The optimum temperature of haemolysis in the present work was found to be 31°C. with a fairly marked falling off above and below this temperature. This contrasts with the broad plateau of activity between 32°C. and 37°C. observed by Mangalo and Raynaud (1959) but is similar to that of 30°C. determined by Rud (1955) in a comparable study. Again, it is interesting to note that 31°C. is considerably lower than mammalian body temperature, especially in a fever at the height of infection, when one would expect the toxin to be liberated, the body temperature is even higher. A fever temperature would tend to inhibit the toxin and therefore to stem its action.

Mechanism of Haemolysis

As mentioned in the Introduction (pp. 47-49) there has been considerable controversy concerning the mechanism of haemolysis by alpha toxin; some believe that the reaction between erythrocytes and toxin is stoichiometric while others believe that it is enzymic. As yet no really convincing evidence to support either hypothesis has been brought. It was decided to investigate the problem using two different techniques. The first was an extension of the early work of Forssman (1933-1939) and Levine (1938-39) the second, a more recent approach based on a study of the rate of lysis, was similar to that carried out by Bernheimer (1947) with other bacterial lysins.

Split titration experiments. The titre of alpha toxin was determined in the usual way; an intermediate dilution of the titration was then retitrated against a fresh aliquot of rabbit erythrocytes. This process was repeated until six consecutive titrations had been carried out. In each case the absolute titre remained the same (Table 8). The same type of experiment was now carried out using the organic lytic agents sodium lauryl sulphate and saponin (Table 8). With these the absolute titre dropped considerably in the process of retitration.

Alpha toxin was not therefore used up in the reaction whereas both saponin and sodium lauryl sulphate were. An enzyme as a biological catalyst should not be used up in the reaction which it catalyses; it would seem that alpha toxin fulfils this basic requirement.

Table 8

Split titrations of partially pure alpha toxin, saponin and sodium lauryl sulphate

Titration No.	Nature of Titration	Dilution in the first tube	Absolute titre
1.	Primary titration of alpha toxin	1 in 4	1 in 8,000
3.	Retitration of 2	1 in 250	1 in 8,000
6.	Retitration of 5	1 in 4,000	1 in 8,000
1.	Primary titration of saponin	1 in 80	1 in 40,000
3.	Retitration of 2	1 in 1,280	1 in 20,000
6.	Retitration of 5	1 in 10,000	1 in 10,000
1.	Primary titration of sodium lauryl sulphate	1 in 40	1 in 10,000
3.	Retitration of 2	1 in 320	1 in 5,000
5.	Retitration of 4	1 in 1,280	1 in 1,280

Although the above experiment does suggest that alpha toxin is not used up in the course of haemolysis,

it does not answer the question of whether or not it is adsorbed and then released. It was therefore decided to attempt to adsorb alpha toxin with rabbit red blood cells. Alpha toxin was mixed with suspensions of 20% and 50% rabbit red blood cells in isotonic phosphate buffer pH 7.0; controls of alpha toxin in the absence of red cells were also set up. The mixtures were incubated either at room temperature or at 4°C. and samples were taken at 0, 15, 30, and 60 min. These samples were now titrated for alpha toxin activity before and after spinning, to remove the red cells. In these experiments no adsorption could be demonstrated; the titre of the controls, and the test before and after spinning, were the same.

Kinetic Experiments

Enzyme reactions are generally characterised by investigating the kinetics of the reaction. If alpha toxin is an enzyme, then it should have the kinetics of an enzyme. It is usual in enzyme kinetics to measure either the decrease in substrate or the increase in end product; however, neither the substrate, nor the end product of alpha toxin is known. The rabbit red cell was the only substitute available for the true substrate. For this reason it was decided to study the kinetics of haemolysis, although it was fully realised that haemolysis

is only a secondary reaction and does not measure the direct interaction of substrate with toxin. Reasoning along similar lines, Bernheimer (1944,47) with bacterial haemolysins other than alpha toxin, measured the haemolysis by spinning down mixtures of red cells and toxin at intervals and estimating the amount of haemoglobin released in the supernatant. This method, however, seemed to entail two potential sources of inaccuracy; first during spinning, some red cells already rendered fragile by the action of toxin, could be lysed, and secondly, the time lag between the taking of the sample and the reading of the result would be at least 4 - 5 minutes. In addition to these possible inaccuracies, this technique also severely limits the number of measurements which can be made, especially at high concentrations of haemolysin. For these reasons a method was sought which would enable direct estimation of the concentration of red cells remaining at any given time. Measurement of the turbidity of red cell suspensions in the E.E.L. nephelometer or in the Hilger spectrophotometer seemed to provide such a method. Since the laboratory had no spectrophotometer when this work was begun, initial work was done using the nephelometer. Details of the method are outlined on p. 67

The influence of toxin concentration on haemolysis.

Under given conditions, two molecules of an enzyme should be capable of breaking down twice as much substrate in a given time as one molecule; the velocity of the reaction should in theory therefore obey the simple expression $v = k(E)$. v = velocity, k = a constant and (E) = the enzyme concentration. That is, the rate of haemolysis should be directly proportional to the amount of toxin present.

The time picture of haemolysis in a typical experiment for a number of concentrations of haemolysin is seen in Fig. 4. Each curve has an S shape. There is a time lag during which little or no haemolysis takes place, followed by an increase of the rate of haemolysis until a straight line is formed; finally, there is a slowing down of the rate of haemolysis. At high concentrations of alpha toxin the S shape is less well defined; there are merely slight inflections of the curves at the beginning and the end of the reaction. The slope of the linear portion of the curves can be taken as the rate of haemolysis as it was in the experiments of Bernheimer (1944,47). A plot of the rate so determined against the concentration of toxin is shown in Fig. 5. At lower concentrations of toxin the relation between the rate and the concentration is directly proportional and is thus compatible with an enzymic reaction. At higher

Fig. 4

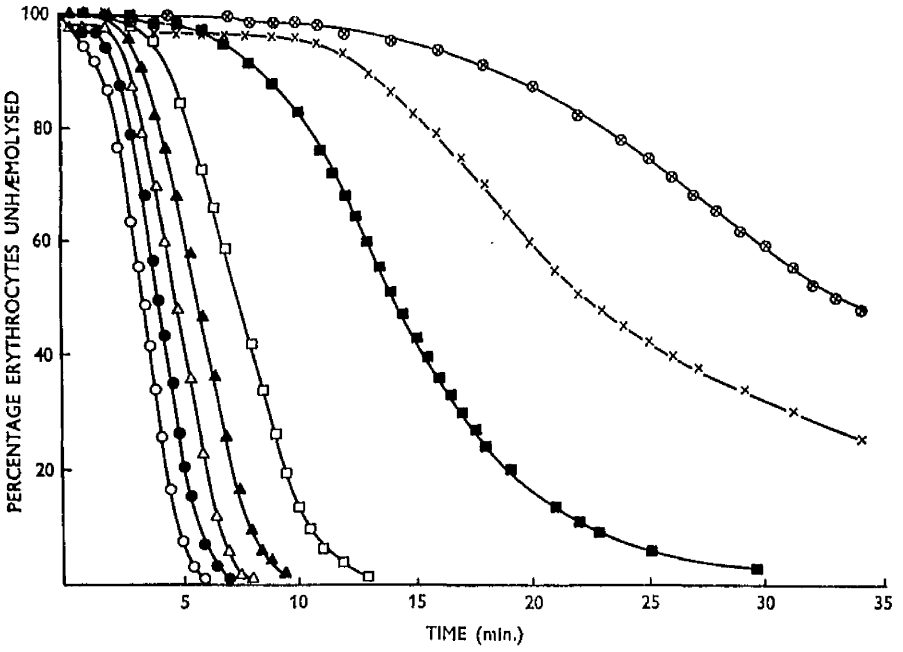


FIG. 4.—Relation between hæmolysis and time at different concentrations of partially purified staphylococcus alpha hæmolysin. The concentrations tested were ○—○, 160 M.H.D. per ml.; ● — ●, 80 M.H.D. per ml.; △—△, 40 M.H.D. per ml.; ▲—▲, 30 M.H.D. per ml.; □—□, 20 M.H.D. per ml.; ■ — ■, 10 M.H.D. per ml.; ×—×, 5 M.H.D. per ml.; ⊗—⊗, 2·5 M.H.D. per ml.

Fig. 5

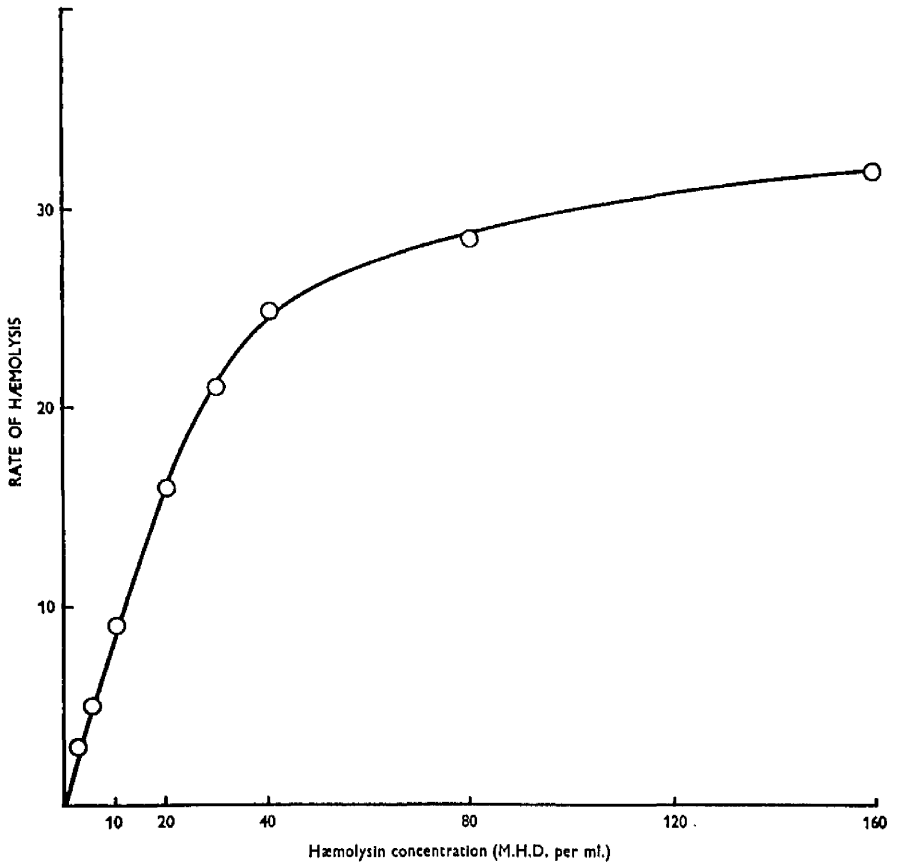
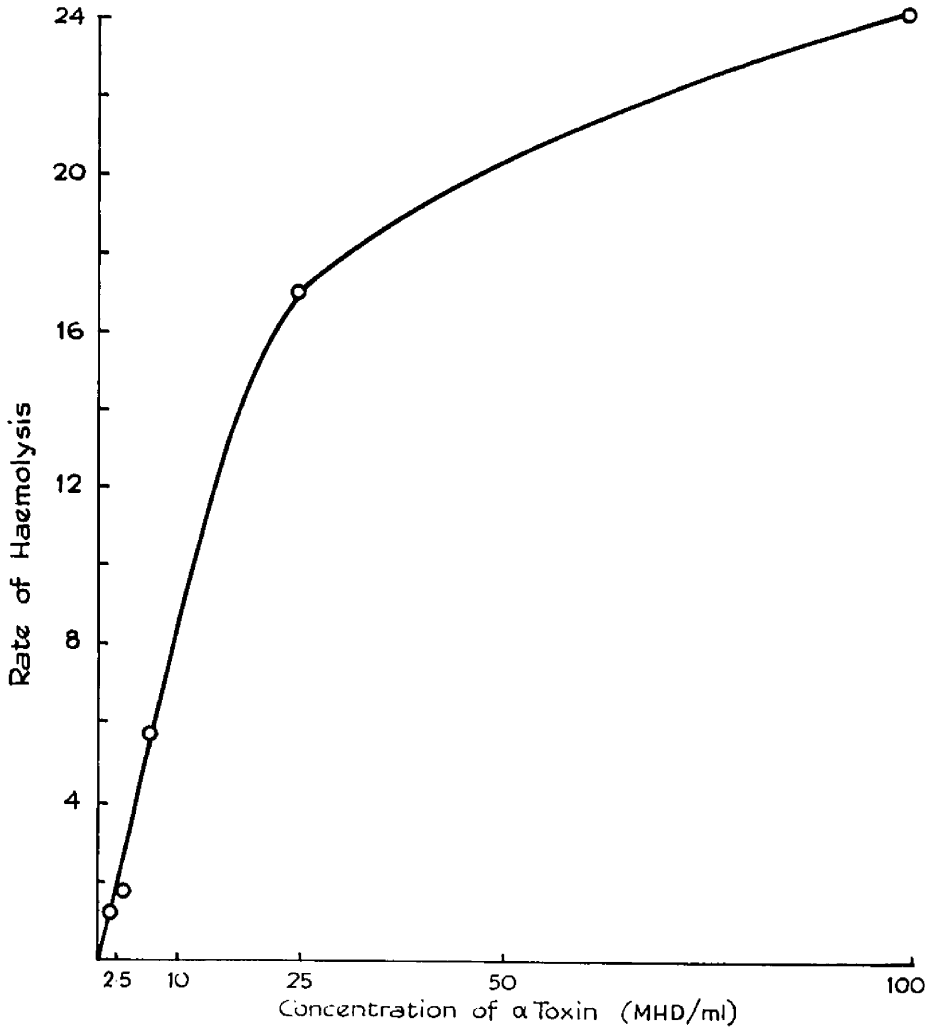


FIG. 5.—Relation between the rate of hæmolysis and hæmolysin concentration.

Fig. 6


24
20
16
12
8
4
Rate of Haemolysis
2·5
10
25
50
100
Concentration of α Toxin (MHD/ml)

concentrations, however, there is a very marked falling off. This is not uncommon and might be due to several possible reasons.

(a) Limitations in the capacity of the method due to the apparatus.

(b) Limitations in the method due to the measurement of a secondary reaction.

(c) Failure to measure the initial velocity of the reaction.

(d) Insufficient substrate.

(e) Presence of an inhibitor.

(f) Presence of a destructive protease.

Possibilities (e) and (f) were discounted when it was found that very highly purified preparations of alpha toxin (p.186) showed exactly the same characteristics (Fig. 6). It seemed that (a), (b) and (c) were therefore the most likely possibilities, and these are discussed in detail (p. 98).

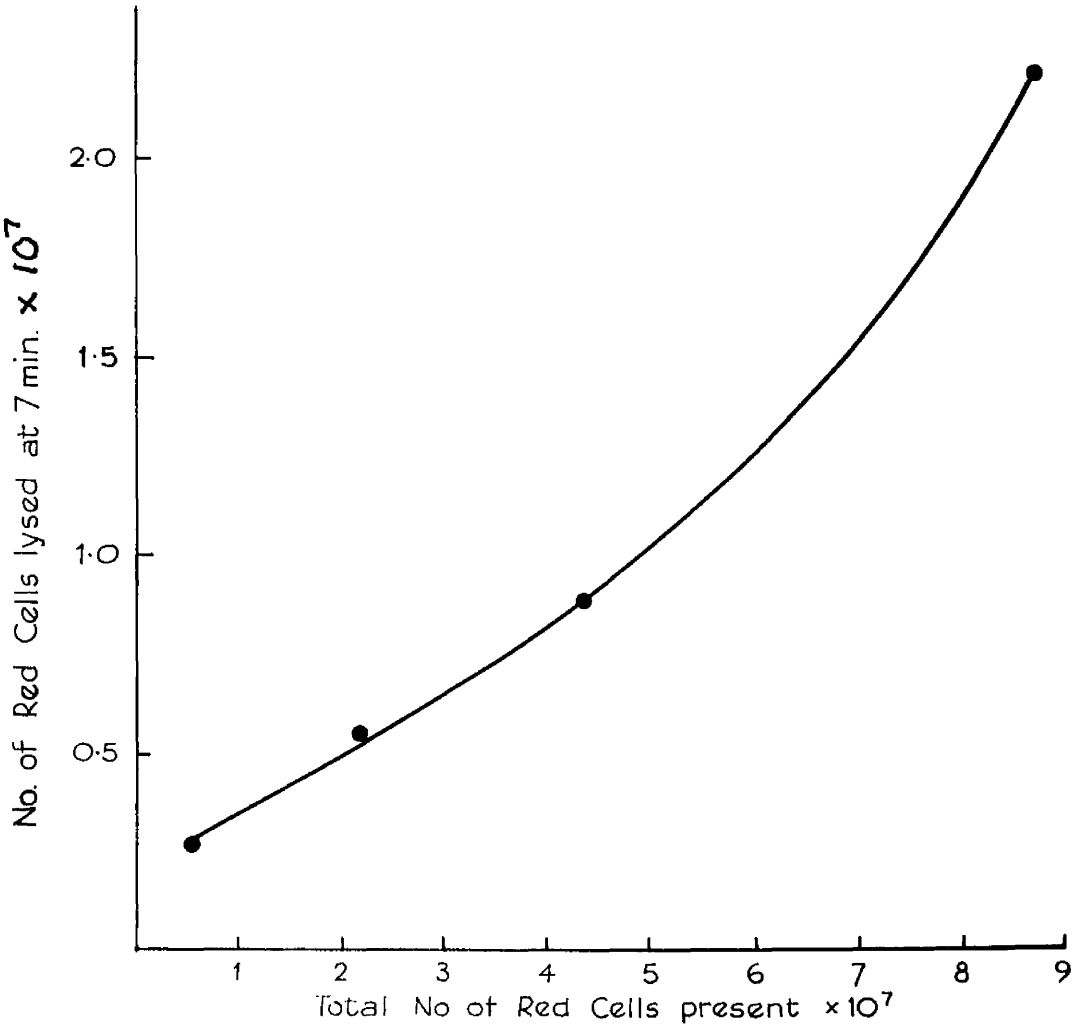
The effect of red cell concentration on the velocity of the reaction. The Michaelis Menten (1913) concept of an enzymic reaction, which is generally considered to be the simplest way of expressing the relationship between an enzyme and its substrate, states that

$$E + S \rightleftharpoons ES \longrightarrow E + \text{products}.$$

From this expression it is obvious that the rate of the reaction depends on the concentration of substrate. In order to examine the effect of the concentration of red cells on haemolysis the rate of haemolysis at different concentrations of red blood cells in the presence of a constant amount of alpha toxin was measured. Because the nephelometer lost so greatly in sensitivity above concentrations of 0.5% red cells, it was decided to carry out these experiments in the spectrophotometer, by measuring the rate of decrease in turbidity at 650 mu.

The final concentration of alpha toxin was c. 200 MHD/ml. and red cell suspensions studied were 0.055%, 0.22%, 0.44% and 0.88%. Although the percentage rate of haemolysis for all four concentrations was not widely different, when one interprets these findings in terms of the absolute amount of work done, it appears that the same time is required for the breakdown of 50% of a suspension of 0.88% red cells as is required for the breakdown of 50% of an 0.22% suspension. Thus at a constant concentration of toxin more than four times as much work has been done in the presence of four times as much substrate, and the velocity of the reaction does depend on the concentration of red blood cells (Fig. 7). The relationship between substrate concentration and the velocity of an enzyme reaction is usually a straight line which reaches a maximum and flattens out. This

Fig. 7


No. of Red Cells lysed at 7 min. × 10⁷
2·0
1·5
1·0
0·5
1
2
3
4
5
6
7
8
9
Total No of Red Cells present × 10⁷

was not observed in the present experiments. Higher concentrations of red cells could not be used to determine whether or not the curve in Fig. 7 flattened out because above 0.88% red cells the sensitivity of the instrument fell off so greatly as to make it useless. Withdrawal of samples from more concentrated suspensions followed by dilution to a level which could be read, also proved useless; it introduced a time lag, limited the number of readings to 1 per minute and for about at least 1 minute brought down simultaneously the concentration of toxin and red cells to the level one wanted to avoid.

Discussion

Partially pure alpha toxin has been used for a study of the mechanism of action *in vitro*, using haemolysis as indicator system. In split titration experiments alpha toxin was not used up during haemolysis whereas two typical organic lytic agents, saponin and sodium lauryl sulphate lost a considerable amount of their haemolytic activity on retitration (Table 8). This is evidence in favour of the hypothesis that alpha toxin acts catalytically rather than stoichiometrically. As mentioned previously, Forssman (1933-39) suggested that if there was any adsorption of alpha toxin on rabbit red cells, this was weak and easily reversed. On the contrary, Levine (1938-39) thought that alpha toxin was adsorbed by red cells according to the Freudlich adsorption isotherm.

In the present work no evidence in favour of adsorption has been obtained: no measurable decrease in activity was observed in supernatants, after interaction of toxin and large concentrations of red cells, followed by removal of red cells. In addition, although Levine's findings (1938-39) can be interpreted in terms of adsorption, he seems to have overlooked the fact that if one plots his results of haemolytic potency for a given weight of toxin, then the result is an almost perfect straight line of the type associated with enzymic reactions. In view of all this, it seems probable that if any adsorption does take place, then it must be weak and easily reversed as originally suggested by Forssman (1938-39).

The mechanism of action was further investigated by measuring the kinetics of haemolysis. The time course of haemolysis followed an S shaped curve (Fig.4). The S shape may be an expression of the statistical distribution of susceptibility of rabbit red blood cells to alpha toxin (van Heyningen, 1962, personal communication). Only a system based on the action of alpha toxin on a homogeneous substrate would be free of this drawback. It is impossible therefore to make definite conclusions regarding the mechanism of action of the toxin from the mere shape of the time haemolysis curve. Nevertheless curves for lower concentrations of alpha toxin are not symmetrical sigmoid curves; the lower shoulder of the curve is more

drawn out than the upper one. This would suggest that at lower concentrations of toxin the reaction was being inhibited after about 50% haemolysis had been reached. Such an inhibition may be due to end product inhibition. It is interesting to note that Weigerzhausen (1962a) found a marked inhibition of alpha toxin haemolysis by haemoglobin. The increase of haemoglobin with progressive lysis may therefore inhibit further lysis.

The rate of haemolysis plotted against the alpha toxin concentration is more suggestive of an enzyme reaction. At lower concentrations of toxin (Fig. 5) the relationship between the rate of haemolysis and the concentration of toxin was linear and thus compatible with the reaction being enzymic. However, at higher concentrations this relationship fell off markedly. The possibility that this falling off was due to the presence of an inhibitory protein or a destructive protease was considerably reduced when it was found that immunologically pure alpha toxin behaved in exactly the same way (Fig.6). Other reasons for the falling off are however possible. Limitations in the capacity of the method may be responsible. This may be due to either one or both of two factors. First, as can be seen from Fig.4, at high concentrations of toxin haemolysis takes place very rapidly; it is possible that errors in reading turbidities at 15 - 30 sec. intervals

at these high concentrations may cause deviation from the true values. A very small error in reading would cause a considerable deviation in slope at these high concentrations. The sluggish response of the nephelometer even when working at its best, may also cause some error in reading. Second, it must be remembered that haemolysis is a secondary reaction and measurement of it does not measure the direct interaction of toxin and its substrate or receptor. Thus haemolysis cannot give a true picture of this reaction. At high concentrations of alpha toxin the haemolytic system may be an insensitive assay. For instance, lysis may only require the breakdown of a limited amount of substrate. When this has been achieved, the red cells will tend to lyse independent of the concentration of alpha toxin; further increase in the amount of toxin would not cause the expected rise in rate of haemolysis. Ghosts resulting from haemolysis would, however, still contain a considerable amount of unchanged substrate which will provide work for the free toxin. Only at low toxin concentrations would the relative amounts of toxin and red cells be such that a direct proportional could be shown.

The fact that haemolysis is a secondary reaction precludes the measurement of the "initial velocity" of the reaction between toxin and its substrate, an essential

measurement for the proper determination of enzyme kinetics (Dixon and Webb, 1958). For a variety of reasons most enzyme reactions maintain their full efficiency for only a very short period in the reaction and it is essential to determine the so-called "initial velocity" over the first few minutes. In the present work the rate of reaction could only be determined over the linear portion of the time haemolysis curves and this probably does not measure the initial velocity. Possibly the most important reason however for the falling off in Fig.5 and 6 is the presence of insufficient substrate at high concentrations of alpha toxin. From a study of the effect of red blood cell concentration on haemolysis by 200MHD/ml. of alpha toxin (Fig.7) it was found that at a concentration of 0.88% red blood cells the amount of work done was four times that done at a concentration of 0.22% red cells; even at 0.88% there was no indication that the process had reached a maximum velocity. Thus at a concentration of 0.4% red blood cells, as used in nephelometric experiments, it seems likely that the higher concentrations of alpha toxin were far from saturated by substrate. Since an enzyme can operate at maximum activity only in the presence of an excess of substrate, this is a drawback. Unfortunately however, as pointed out, concentrations of red cells above 0.88% could not be studied because of technical difficulties.

In his study of other bacterial haemolysins Bernheimer (1944, 1947) did not observe this falling off. This may lie in the fact that the highest concentrations of haemolysin tested by Bernheimer corresponded to concentrations which would have fallen on the linear portion of the curve shown in Fig.5. Possibly Bernheimer encountered the same difficulties as mentioned in measuring the rate of haemolysis at high concentrations of haemolysin.

In conclusion it can be said that alpha toxin exhibits many of the characteristics of an enzyme. It is not used up in the reaction, the rate of the reaction over a certain range of concentrations is directly proportional to the concentration of toxin and the rate of haemolysis is dependent on the concentration of substrate (red blood cells). However, there are features which cannot yet be fully accounted for in terms of a classical enzymic reaction and will be fully understood only when the substrate is known. Nevertheless it is felt that sufficient evidence has been obtained to classify alpha toxin as one of these bacterial lysins which behave as enzymes (Bernheimer, 1947). Without postulating the existence of an entirely novel type of substance, it is intellectually more convenient at the present time to consider staphylococcal alpha toxin as an enzyme.

The Action of Inhibitors on Haemolysis

The effect of various inhibitors on haemolysis was studied for two reasons: to obtain more information concerning the mode of action of the toxin and to find a non-toxic inhibitor which could possibly be developed for use as an *in vivo* inhibitor of the lethal action of alpha toxin.

The action of sulphydryl inhibitors on haemolysis.

It was thought possible that alpha toxin acted in virtue of free sulphydryl groups and it was therefore decided to examine the effect of such inhibitors on haemolysis. The first group of sulphydryl inhibitors to be investigated were the heavy metal salts and in particular mercuric chloride.

The influence of heavy metal ions on haemolysis.

The inhibitory action of heavy metal ions was investigated by adding a constant amount of inhibitor to each tube in titrations of alpha toxin. Table 9 shows the inhibition of haemolysis by mercuric, lead and copper salts.

Table 9

Inhibition of haemolysis by heavy metal ions at a concentration of 1×10^{-3}M.

Metal salt	Haemolytic titre
Mercuric chloride	1/8
Copper sulphate	1/16
Lead Acetate	1/64
Control toxin	1/1,000

Since mercuric chloride was the most inhibitory it was selected for further study. Because of the striking inhibition achieved with 10^{-3}M mercuric chloride lower concentrations were employed in attempts to find the lower limit of inhibition. Surprisingly, concentrations lower than 1 x 10^{-3}M caused spontaneous lysis of rabbit red blood cells; this collateral finding was studied separately (Arbuthnott, 1962) and will be described later in the thesis (p.122).

The inhibition observed at 10^{-3}M could be due either to action of mercuric ions on the active group of the toxin, or to reaction with the red cell, causing blockage of the receptor. Mixtures of red cells and 10^{-3}M mercuric chloride were made and after 10 to 20 min. the red cells were spun and washed with saline. In some experiments the cells lysed spontaneously because of the carry over of small amounts of mercuric chloride in washing; however, in two experiments, where enough treated RBC red cells were harvested, they were found to be very resistant to alpha toxin. The converse was also attempted: toxin was made 2 x 10^{-3}M with mercuric chloride and subsequently precipitated by acetic acid at pH 4.2; the resulting precipitate was then washed free from traces of mercuric chloride with buffer of the same pH, before being re-dissolved in 0.15M sodium phosphate pH 7.2. A control preparation was precipitated in as near as possible

identical conditions, without first treating with mercuric chloride. The titre of the control was four times that of the "Hg-toxin". From this it would appear that both the red cell and the toxin were sensitive to mercuric ions. It was also found that the inhibition could be reversed by the addition of ethylene-diene-tetracetate to mixtures of mercuric chloride and toxin prior to the addition of red cells.

The inhibitory action of organic sulphydryl inhibitors. The inhibitory action of these compounds was assessed by examining their effect on the rate of haemolysis, turbidimetrically. The first to be studied were the halogenated thiol alkylating reagents which act in the following way:-

$$R\text{-}SH + \overset{(+)}{X} - \underset{(-)}{CH_2}(=O) - C - R \rightarrow HX + R\text{-}S\text{-}CH_2 - \underset{O}{C} - R$$

where X is the halogen atom. The compounds tested were iodoacetate, iodoacetamide, chloroacetophenone and chloroacetamide. Although iodoacetamide did not inhibit, all the other compounds of this group did to some extent (Table 10); it is well known that iodoacetate does not inhibit all SH dependent enzymes (Dixon and Webb, 1958).

Table 10

The effect of sulphydryl inhibitors on haemolysis

Inhibitor	Concentration	% Inhibition
Iodoacetate	4×10^{-3}M	0
Chloroacetophenone	2×10^{-3}M	15
Chloroacetamide	2×10^{-3}M	25
Iodoacetamide	2×10^{-3}M	42
Oxidised glutathione	2×10^{-3}M	0
Iodosobenzoate	1×10^{-3}M	18
p-iodobenzoate	8×10^{-4}M	41
p-iodobenzoate	4×10^{-3}M	82

The percentage inhibitions listed in Table 10 were determined by comparing the slope of the linear portion of the time haemolysis curves, in presence of the inhibitor, with that of a normal toxin control. Where inhibition did occur, there was also an extension of the lag phage.

Of the compounds which inhibit haemolysis by oxidation of the SH groups with the resulting formation of disulphide linkages oxidised glutathione did not inhibit, whereas iodosobenzoate inhibited only by 18% (Table 10). It is interesting to note, however, that iodobenzoate which is not one of the recognised

sulphydryl inhibitors was the most potent inhibitor tested (Table 10). The mechanism of this inhibition is not yet clear, but it seems possible that it acts in a similar way to the thiolalkylating agents such as bromo-benzyl-cyanide.

Another well known inhibitor of SH groups, p-chloromercuribenzoate, could not be studied because, like mercuric chloride, this substance was found to be a potent lytic agent by itself (see p. 125).

In short, of the substances which are known to act by inactivating SH groups, some were found to inhibit lysis of rabbit red blood cells by alpha toxin. The most potent were the heavy metal ions; others inhibited to varying degrees.

The effect of divalent ions. Clostridium welchii alpha toxin which is known to be a lecithinase C is dependent on calcium or magnesium ions for its activity (Oakley and Warrack, 1941). Recently Robinson et al. (1960) found a staphylococcal haemolysin to be dependent on divalent ions; the haemolytic activity of this toxin was considerably reduced by E.D.T.A. and the activity was restored on addition of magnesium or manganese ions.

In the present study even fairly massive amount of E.D.T.A. (2×10^{-2}M) did not inhibit haemolysis. Also addition of calcium ions did not affect the rate of

haemolysis. Dialysis of partially pure preparations did cause some decrease in haemolytic activity; it is unlikely however that this resulted from loss of divalent ions and was probably due to inactivation of the toxin (see p.211). These findings suggest that divalent ions are not required by staphylococcal alpha toxin for its maximum activity.

The influence of inhibitors of bacterial proteases. If alpha toxin is a protease as has been suggested (Robinson et al., 1960) then one would expect it to be inhibited by inhibitors of bacterial proteases. Duthie and Lorenz, (1949) investigated a number of substances which inhibit bacterial proteases and some of these were tested in the present work. Concentrated solution of human gamma globulins, egg white, a saline extract of soya bean meal were examined for their effect on haemolysis; none of them was found to inhibit.

Inhibition of haemolysis by hypertonicity. In his investigation of the mechanism of haemolysis by Cl. septicum haemolysin, Bernheimer (1944) found that haemolysis was inhibited by hypertonic solutions of sucrose. It was decided to carry out similar experiments with staphylococcal alpha toxin in a further attempt to classify the reaction. The following typical titration experiment shows the effect of different concentrations of sucrose on haemolysis (Table 11).

Fig.8

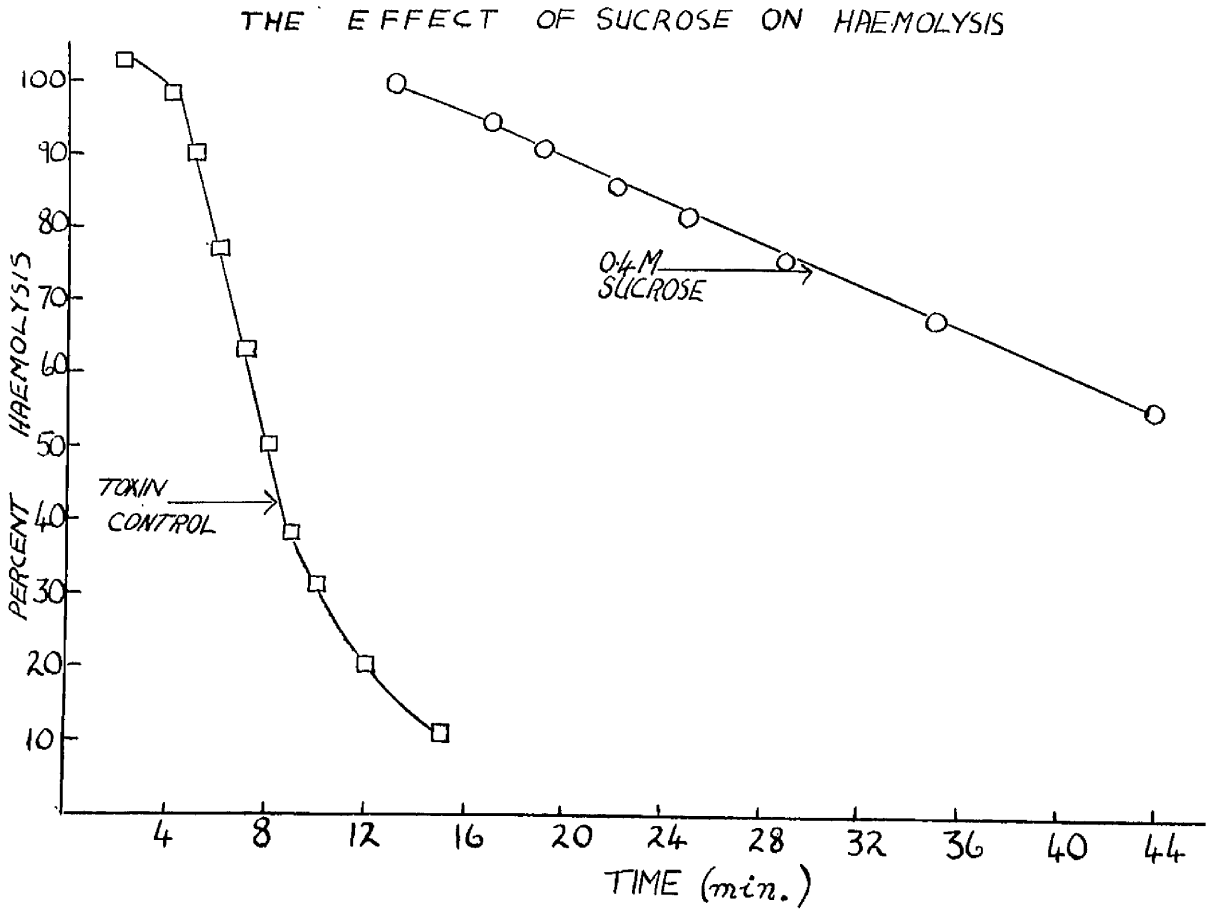

THE EFFECT OF SUCROSE ON HAEMOLYSIS
PERCENT HAEMOLYSIS
100
90
80
70
60
50
40
30
20
10
TOXIN CONTROL
0·4M SUCROSE
4
8
12
16
20
24
28
32
36
40
44
TIME (min.)

Table 11

The effect of sucrose on haemolysis

Sucrose concentration	Titre at various time intervals					
	15 min.		25 min.		60 min.	
	50%	Some	50%	Some	50%	Some
0.5 M	128	250	128	500	128	500
0.25 M	128	250	128	500	250	500
0.125 M	250	1,000	250	1,000	500	1,000
0.0625 M	500	1,000	500	2,000	500	2,000
Control	1,000	2,000	1,000	16,000	4,000	16,000

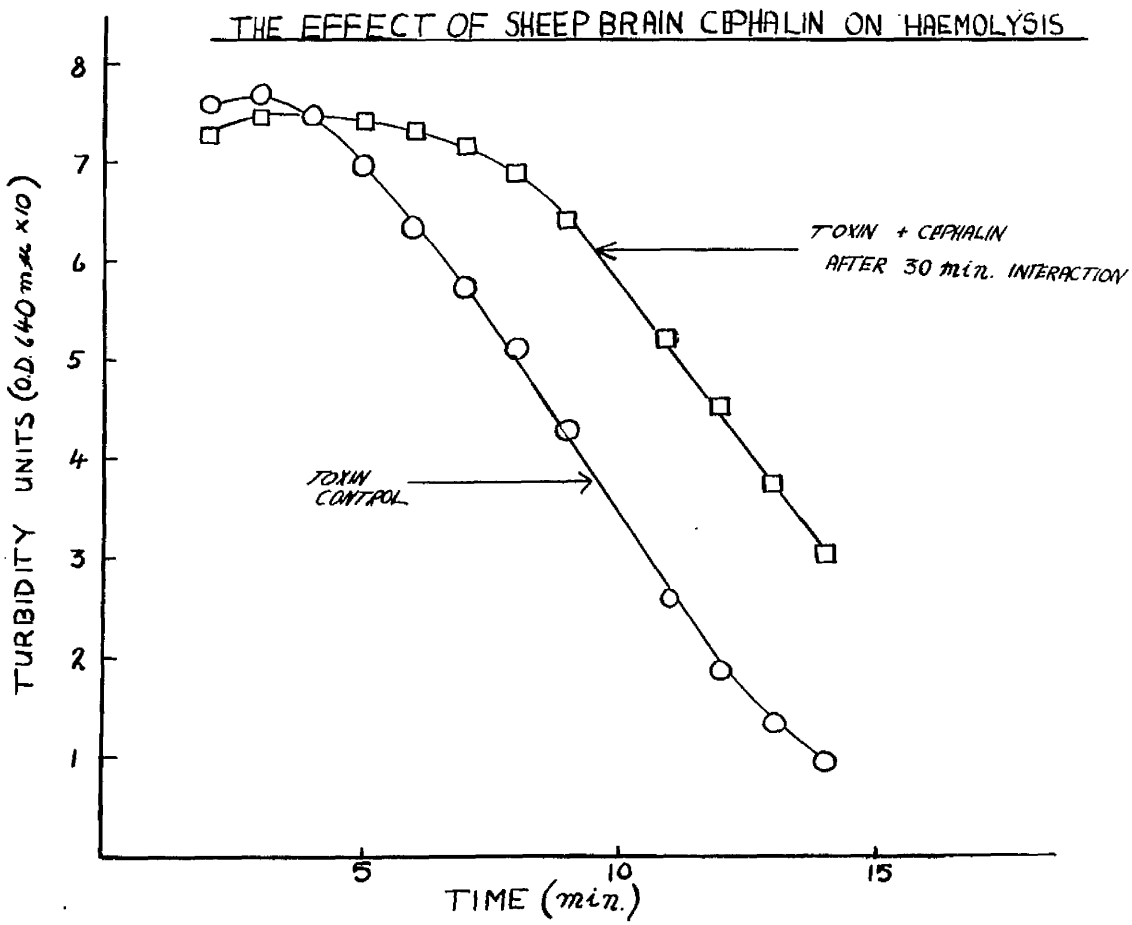
The influence of sucrose on the rate of haemolysis can also be seen from Fig.8. Thus hypertonic solutions of sucrose inhibit haemolysis by alpha toxin.

The effect of phospholipids on haemolysis. In some respects the alpha toxin of Cl. welchii is similar to that of the staphylococcus; they are both for instance rapidly lethal, haemolytic toxins. It was considered possible therefore, that they may act on the same or similar substrates. As yet, biochemical alteration of a synthetic phospholipid substrate by staphylococcal alpha toxin has not been demonstrated. The approach of examining the inhibitory effect of possible substrates or end products by competitive inhibition seemed worth some investigation.

Premixing crude alpha toxin with 5 mg. of lecithin had no effect on the titre of the haemolysin and there was no apparent alteration in the appearance of the lecithin suspension. Also, serial doubling dilutions of alpha haemolysin were set up in constant concentrations of lecithin . Even at a concentration of 1 mg./ml. lecithin did not inhibit haemolysis. In addition, neither Cohn's fraction III of serum lipoprotein nor sphingomyelin had any inhibitory effect on haemolysis; also, their appearance was not changed by alpha toxin.

Incubation of alpha toxin with suspensions of sheep-brain-cephalin however, did cause a slight clearing of the suspension; the effect was not dramatic. Nevertheless, it was decided to examine the effect of sheep brain cephalin on haemolysis. On premixing alpha toxin with 2 mg./ml. of sheep brain cephalin and withdrawing samples at different time intervals, a considerable degree of inhibition was observed (Fig. 9) at 30 min. Inhibition was not time dependent, since after 2 min. almost the same degree of inhibition was found. Neither heating of the cephalin at 100°C. for 15 min. nor extraction of the suspension with ether removed the inhibitor. However, alpha toxin did not have any effect on suspensions of synthetic DL-palmitoyl-cephalin; there was no solubilisation or alteration of the granular nature of the suspension, and it did not

Fig. 9


THE EFFECT OF SHEEP BRAIN CEPHALIN ON HAEMOLYSIS
TURBIDITY UNITS (O.D. 640 mμ x10)
8
7
6
5
4
3
2
1
5
10
15
TIME (min.)
TOXIN + CEPHALIN
AFTER 30 min. INTERACTION
TOXIN
CONTROL

inhibit haemolysis even at a concentration of 2.5 mg./ml. It seems possible, therefore, that the effect of sheep brain cephalin on haemolysis is due to the presence of an impurity, possibly to cerebroside, which, as previously mentioned, has been shown to inhibit the lethal effect by North and Doery (1961).

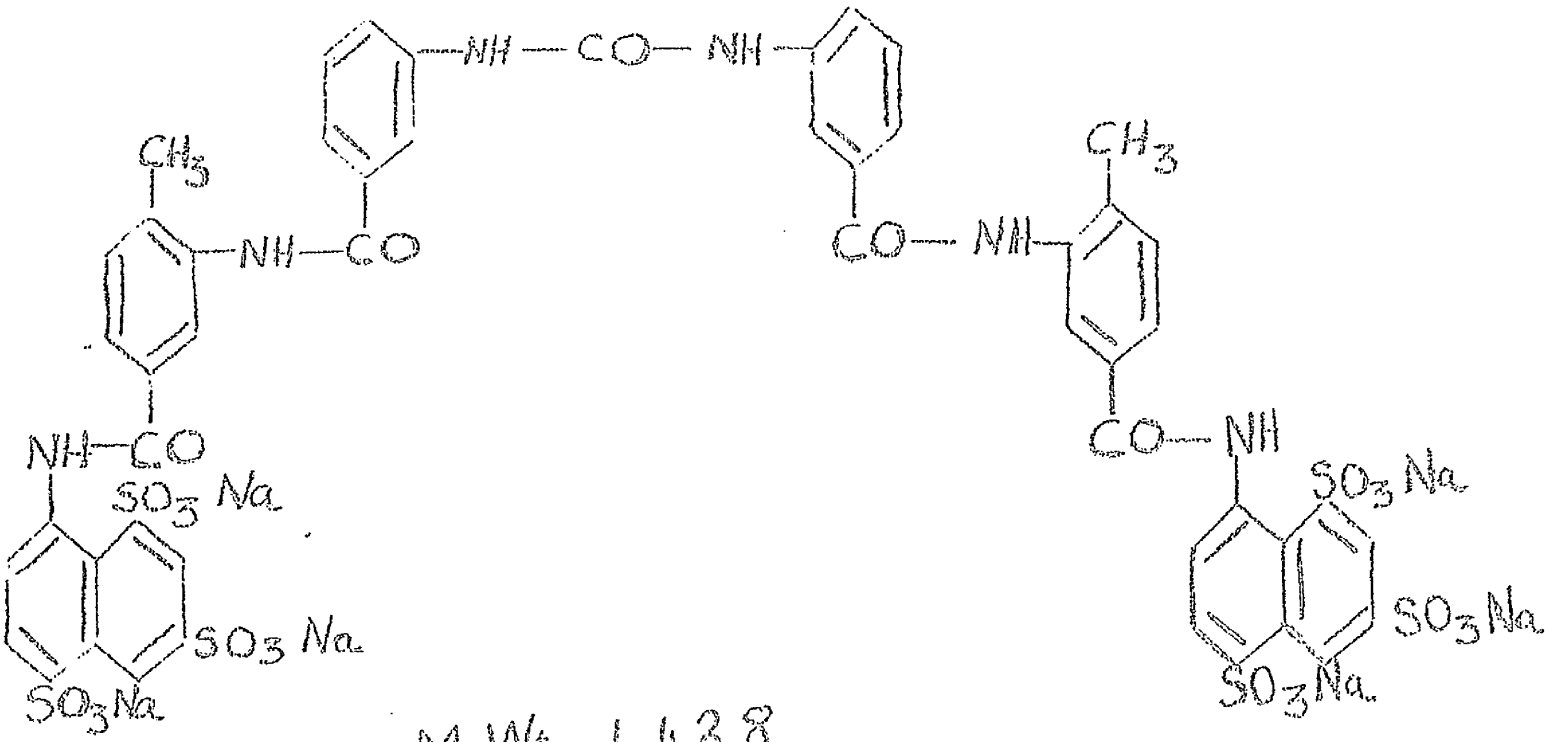
Inhibition of haemolysis by Suramin and related compounds. As well as investigating inhibitors which would give a clue to the mechanism of action of alpha toxin, it was decided to search for a non-toxic group of inhibitors which could then be used to inhibit alpha toxin in vivo. The ultimate aim of this approach is to find a chemotherapeutic agent for alpha toxin.

Suramin (Antrypol) is a well known inhibitor of some enzyme systems, e.g. the lytic enzyme lysozyme and the enzyme responsible for bacterial cell separation (Lominski and Gray, 1961). This compound is also used in the treatment of trypanosomiasis and is non-toxic even in fairly large quantities. It was decided to investigate the action of this drug and also some of its related compounds (Fig. 10) on haemolysis.

At pH 5.5 both Suramin and Bayer 1 (Fig.10) were very potent inhibitors (Table 12).

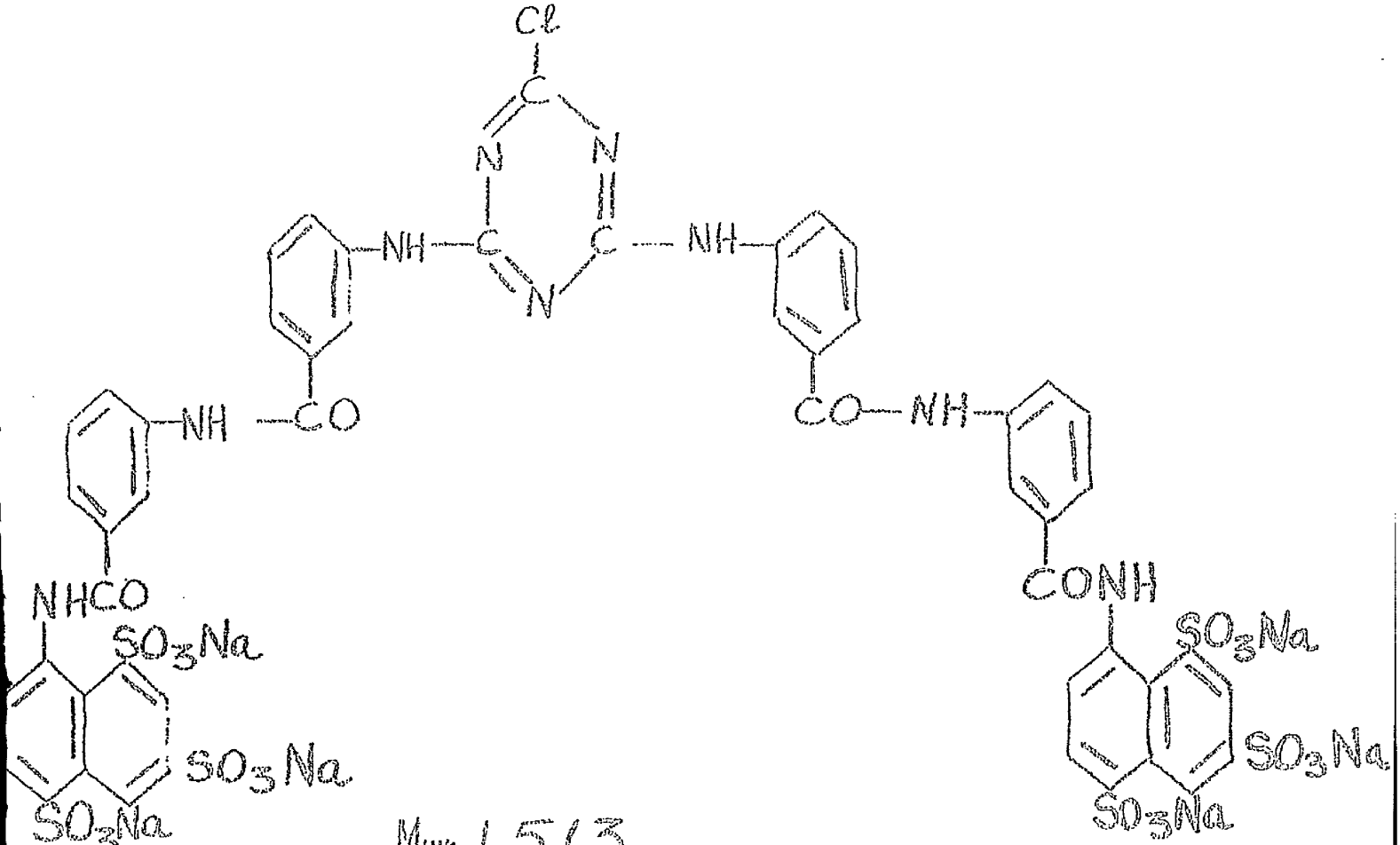
FIG. 10

SURAMIN



M Wt. 1,428

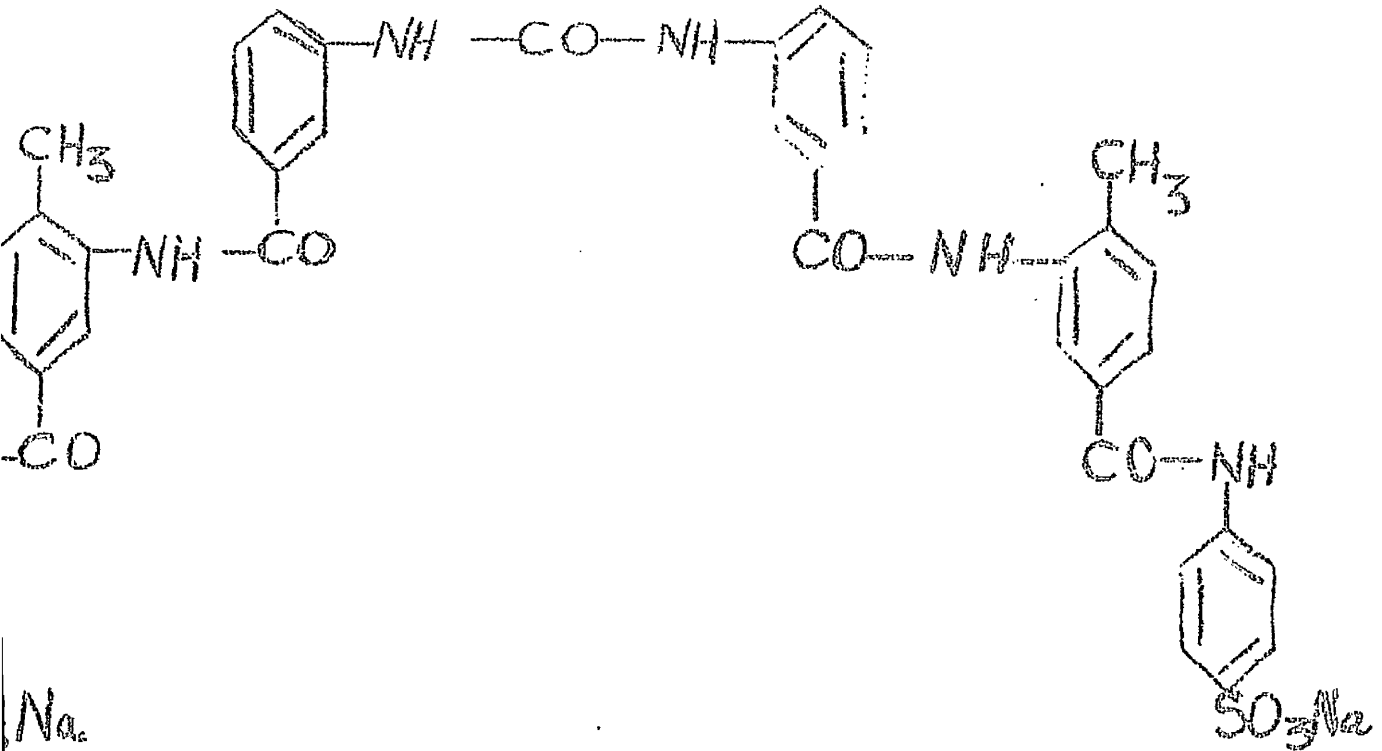
BAYER I



Mwt 1,513

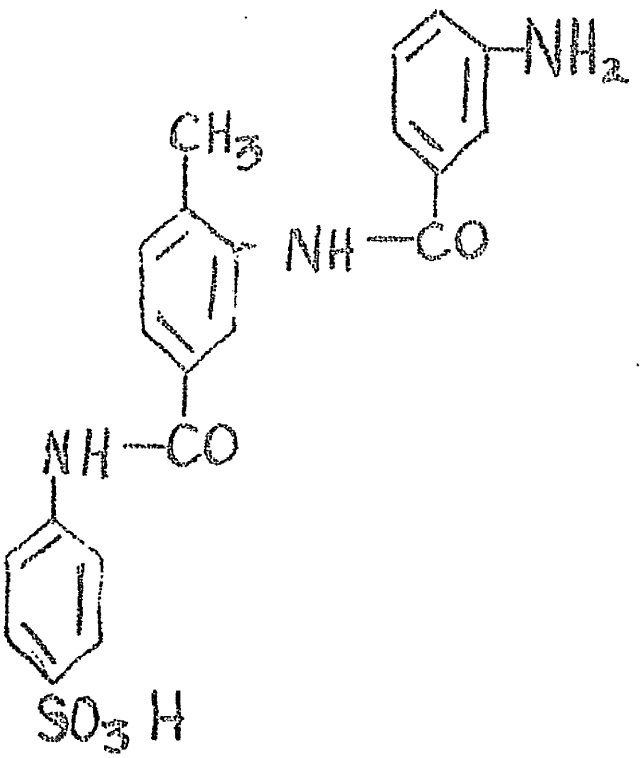
I.C.I. ONB/25

FIG 10



Mwt 1024

I.C.I. ONB/24



Mwt 452

Table 12

The Inhibitory Action of Suramin and Bayer I at pH 5.5

Compound	Concentration	Haemolytic titre
Suramin	8×10^{-4}M	1/4
"	4×10^{-4}M	1/8
"	2×10^{-4}M	1/16
Control	-	1/250
Bayer I	8×10^{-4}M	1/4
"	4×10^{-4}M	1/4
"	2×10^{-4}M	1/4
Control	-	1/250

In an attempt to further characterise the mechanism of action and to find an inhibitor which might act at a more physiological pH, Suramin and its related compounds were tested for antihaemolytic activity in titrations at pH 7.0 - 7.2. The drugs were dissolved in isotonic buffers. Two, I.C.I. ONB/24 and 25, which formed insoluble precipitates with chloride and phosphate ions, were dissolved in 0.15 M Tris buffer pH 7.2. Toxin was titrated in doubling dilutions in the appropriate isotonic buffer and 0.2 ml., containing the desired amount of drug, was added. After 5 - 15

minutes interaction, 0.3 ml. of a 3% suspension of rabbit red cells was added, and the titrations were incubated at 37°C. (For results see Table 13).

Table 13

The action of Suramin and its analogues on haemolysis at pH 7.0

Compound	Concentration	Haemolytic titre
Suramin	8×10^{-3}M	1/4
"	4×10^{-3}M	1/8
Control	-	1/500
Suramin	8×10^{-4}M	1/128
"	4×10^{-4}M	1/128
"	2×10^{-4}M	1/250
Control	-	1/1,000
Bayer 1	8×10^{-3}M	1/4
"	4×10^{-3}M	1/4
Control	-	1/500
Bayer 1	8×10^{-4}M	1/32
"	4×10^{-4}M	1/64
"	2×10^{-4}M	1/64
Control	-	1/1,000
I.C.I. ONB/25	2×10^{-3}M	1/8
"	6×10^{-4}M	1/16
"	3×10^{-4}M	1/32
Control	-	1/128
I.C.I. ONB/24	2×10^{-3}M	1/128
Control	-	1/250

The end point of the control titration, by definition contains 1 MHD of alpha toxin. Thus in these experiments where each tube in the series of dilutions contains a constant amount of inhibitor, an inhibition of, for example, 1/1,000 to 1/32 means that 16 MHD of alpha

toxin have been completely inhibited. Thus at pH 7.0 and at a concentration of 8 x 10^{-3}M both Suramin and Bayer compound I inhibited at least 64 MHD of alpha toxin. On dilution it appeared that Bayer I was more active than Suramin itself. Compound I.C.I. ONB/25 was at least as active as Suramin, while ONB/24 the "half" molecule of ONB/25, was almost without activity. Because of insolubility in the isotonic solutions used in the present work ONB/24 and 25 could not be tested at higher concentrations. (I.C.I. who synthetised these drugs supplied them with the proviso that they may be insoluble). In view of the differing degrees of inhibition caused by the compounds tested, it seems unlikely that sulphonic acid groupings are the sole determinants of inhibitory capacity. Some attempts to use these drugs as in vivo inhibitors of alpha toxin are described in a later section (p. 139).

Discussion

The inhibition of haemolysis by a number of substances, generally considered to act by blocking free SH groups, suggests that such groups play a role in the haemolysis of red blood cells by alpha toxin. Whether such substances act on the red cell, on the toxin or on both is not yet clear. The very recent work of Bernheimer and Schwartz (1963) suggests that pure alpha toxin contains no cysteine and thus no free SH groups;

although they found large amounts of methionine, it seems unlikely that any of the inhibitors tested would be capable of splitting the $-CH_2S-CH_3$ bond and reacting with the sulphydryl group. Nevertheless, there is increasing evidence that the so-called SH inhibitors react with other grouping of protein molecules (Dixon and Webb, 1958). It is also possible that the substrate of alpha toxin on the cell membrane contains a sulphydryl grouping and that this plays a role in the formation of the enzyme substrate complex.

Unlike the lethal, necrotic and haemolytic alpha toxin of Clostridium welchii (Oakley and Warrack, 1941) staphylococcal alpha toxin is not dependent on divalent metal ions for its activity. Fairly massive amounts of EDTA does not inhibit haemolysis and addition of calcium ions also does not affect the haemolytic activity. Further it has since been reported that although staphylococcal beta toxin does require divalent metal ions, alpha toxin does not (Robinson, personal communication, 1962).

Inhibitors of bacterial proteases do not inhibit haemolysis and thus it seems unlikely that alpha toxin is a protease. The recent dissociation of alpha toxin from protease activity (Robinson, personal communication) supports this conclusion.

Crude preparations of sheep brain cephalin were found to have an inhibitory action on haemolysis. In addition, alpha toxin caused a slight clearing of suspensions of this. From experiments with synthetic cephalin it seems unlikely that this effect is due to cephalin. An impurity, possibly a cerebroside, is perhaps the active constituent. Cerebroside has already been shown to have an inhibitory action on the lethal action of alpha toxin (North and Doery, 1961). Whether this inhibition is indeed due to competitive inhibition by a substrate, and product inhibition, or simply by non-specific inhibition awaits further work with synthetic phospholipids. The inhibitory action of lecithin (Weinstein, 1937) could not be demonstrated in titration experiments. Since Weinstein incorporated the lecithin into the agar and demonstrated the inhibition of haemolysis around colonies of staphylococci, it seems possible that a product of the metabolism of lecithin was the inhibitor; it is known that fatty acids, for instance, inhibit alpha toxin (North and Doery, 1958).

The finding that hypertonic solutions of sucrose inhibit haemolysis are similar to those of Bernheimer (1944) for Cl. septicum toxin. Bernheimer suggested that the initial stage of haemolysis, the enzymic degradation of a component of the red cell surface, is followed by swelling of the red cell and subsequent

rupture of the membrane. Hypertonic solutions of sucrose probably prevent the later stage by maintaining an osmotic balance between the interior of the cell and its environment; the stronger the sucrose concentration, the greater the inhibition (Table 11). This probably also explains the findings of Rigdon (1936a) and Smith (1937) who found that hypertonic solutions of salts and glycerol inhibited the dermonecrotic action of alpha toxin.

In attempts to find non-toxic inhibitors of alpha toxin, it was observed that Suramin and related compounds inhibit haemolysis. Although Suramin does inhibit at neutral pHs it is more active at pH 5.5. This suggested that the inhibition was dependent on the sulphonic groups of Suramin. However, it was also noted that there was a considerable variation in the inhibitory capacity among structurally, closely related compounds. At pH 7.0 Bayer compound I was more inhibitory, and I.C.I. ONB/25 was at least as active as Suramin. This would suggest that the mechanism of inhibition is not simply a function of the sulphonic groups since Bayer I is structurally identical having exactly the same number of such groups as Suramin and only differs by the presence of an additional ring.

I.C.I. ONB/25 has only 2, where Suramin itself has 6 sulphonic acid groups. However, I.C.I. ONB/24 which is "half" of the ONB/25 molecule is considerably less effective at a molar concentration of twice ONB/25. All this suggests that the spatial configuration of the sulphonic acid groupings is of considerable importance in the mechanism of inhibition. It is also possible that other groups such as the Cl of Bayer I and the -CO-NH- groupings play an important part in bonding proteins (Rose, 1963, personal communication). Whether the inhibition described in the present work is due both to inhibition of the toxin and to an action on the surface of the red cell is not yet known, but from the inhibition of the lethal effect (p. 139) it can be said that Suramin does have a direct action on the toxin. It is also realised that a number of other analogues must be tested before the mechanism of inhibition will be fully understood.

Haemolytic Action of Mercurials

While studying the mechanism of action of alpha toxin *in vitro* certain collateral findings came to light which were investigated. One such finding was the haemolytic action of small amounts of mercuric chloride and other mercurial compounds (Arbuthnott, 1962).

In attempts to determine the lower limits of inhibition of alpha toxin by mercuric chloride, it was found that at concentrations of 5×10^{-4}M and below, this salt was haemolytic. The lytic effect of mercuric chloride along with certain organo-mercurials and other heavy metal salts was examined on human, sheep, guinea pig and rabbit red blood cells. These compounds were dissolved in isotonic acetate buffer pH 6.8 - 7.2 and titrated in serial doubling dilutions in isotonic buffer of the same pH; 0.5 ml. of a 2% suspension of four times washed erythrocytes were then added to each tube. Readings were made at different time intervals after incubation at 37°C. Haemolysis was assessed visually.

Not only was mercuric chloride lytic, but lysis spread to both higher and lower concentrations (Table 14). With rabbit red cells lysis began after 30 min. at a concentration of 6.75×10^{-5}M mercuric chloride; with

the progress of time lysis spread to higher and lower concentrations of mercuric chloride, until at 4½ hr. lysis extended from 5 x 10^{-3}M to 2.1 x 10^{-6}M. The red blood cells of other species were found to be less sensitive than those of the rabbit. At 1 hr. no haemolysis was observed. However, at 2 hr. lysis had begun with human, sheep and guinea pig red cells and this progressed as described in Table 14. This selectivity of action is similar to some extent to that of the staphylococcal toxins, although the order of sensitivity, rabbit human sheep guinea pig, does not correspond with that of any of the known toxins. This differential sensitivity may be a manifestation of structural differences in the membranes of the red cells of different species. Although it is widely assumed that red cells behave in the same way to physical agents, such as osmotic pressure and surface active agents, it is obvious from the review of Ponder (1948) that there are considerable chemical differences between species and the widely differing electrophoretic mobilities (Abramson, 1929) indicate that the surface charges are also different. All this points to a considerable variation on the structural nature of the red cells of species. The differing sensitivity of red cells to staphylococcal toxins and such agents as

Table 14

The haemolytic action of mercuric chloride on the red cells of different species

Species, Time		Mercuric chloride concentrations x 10^{-4}M					Mercuric chloride concentrations x 10^{-5}M					
		20	10	5	2.5	1.25	6.75	3.4	1.7	0.85	0.42	0.2
Rabbit	1 hr.	-	-	-	A.C.	C.	A.C.	<50%	<50%	-	-	-
Sheep	"	-	-	-	-	-	-	-	-	-	-	-
Human	"	-	-	-	-	-	-	-	-	-	-	-
Guinea Pig	"	-	-	-	-	-	-	-	-	-	-	-
Rabbit	3 hr.	-	-	S	C	C	C	A.C.	A.C.	<50%	Tr.	-
Sheep	"	-	-	-	-	-	S	S	-	-	-	-
Human	"	-	-	-	A.C.	>50%	<50%	S	Tr.	-	-	-
Guinea Pig	"	-	-	-	S	C	S	-	-	-	-	-
Rabbit	4½ hr.	-	-	50%	C	C	C	C	A.C.	50%	50%	Tr.
Sheep	5 hr.	-	-	-	-	-	50%	50%	S	-	-	-
Human	"	-	-	50%	>50%	>50%	50%	S	S	Tr.	-	-
Guinea Pig	"	-	-	>50%	C	50%	S	-	-	-	-	-

C = complete; A.C.= almost complete; >50% = greater than 50%; <50% = less than 50%; S = some; Tr.=trace

mercuric chloride only serves to emphasise this point.

Lytic effect was not restricted to inorganic mercuric salts, it was shared by organomercurials such as p-chloromercuribenzoate, ethyl mercuric chloride and thiomersal. In contrast with mercuric chloride these substances had no upper limit of haemolysis at the concentration tested. After 1 hr. incubation p-chloromercuribenzoate and ethyl mercuric chloride caused lysis down to a concentration of 10^{-3}M with rabbit red cells and thiomersal to a concentration of 4×10^{-3}M; at 2 - 3 hr. p-chloromercuribenzoate lysed to a concentration of 1.25×10^{-3}M and thiomersal to a concentration of 5×10^{-4}M. In addition to rabbit red blood cells these compounds also lysed human and sheep red cells though again less well than rabbit cells. The order of susceptibility was rabbit>human>sheep. The lytic reaction is temperature dependent; only after 3 hr. at 4°C. does mercuric chloride begin to lyse rabbit red cells. The optimum pH is around 7.9 and the reaction appears to depend on the concentration of red blood cells.

Other heavy metal salts such as copper and lead are also haemolytic although not as active as mercuric chloride. However, the property is not typical of all heavy metal salts as barium ions did not lyse even after

19 hr. incubation with rabbit red blood cells.

The lytic capacity of these compounds seems to be dependent on the number of positive mercuric charges present on the ionised molecule. Mercuric chloride itself which contains two such charges is more potent than p-chloromercuribenzoate and ethyl mercuric chloride which have only one; these in turn are more active than thiomersal which has no free positive charge, but it is thought to split in the presence of tissue proteins to reveal one free positive charge.

The property is probably not simply due to the combination of the heavy metal ion with SH groups on the red cell surface since iodoacetamide and iodobenzoate both of which combine with SH groups had no lytic action. There is an optimum concentration of mercuric chloride (Table 14) lysis spreading in both directions. This along with the fact that the reaction is also dependent on the concentration of red cells suggests that a chelate or coordination complex is formed between the heavy metal ion and an active grouping on the red cell surface.

The action of alpha toxin on the red blood cells of different species.

Since part of the present work was a study of the effect of alpha toxin on rabbits, mice, frogs and fowls in vivo it was of interest to compare the sensitivity of

the red cells of these species to alpha toxin. In addition the sensitivity of human and sheep red cells was also compared. Erythrocytes were prepared as described on p. The findings are summarised in Table 15.

Table 15

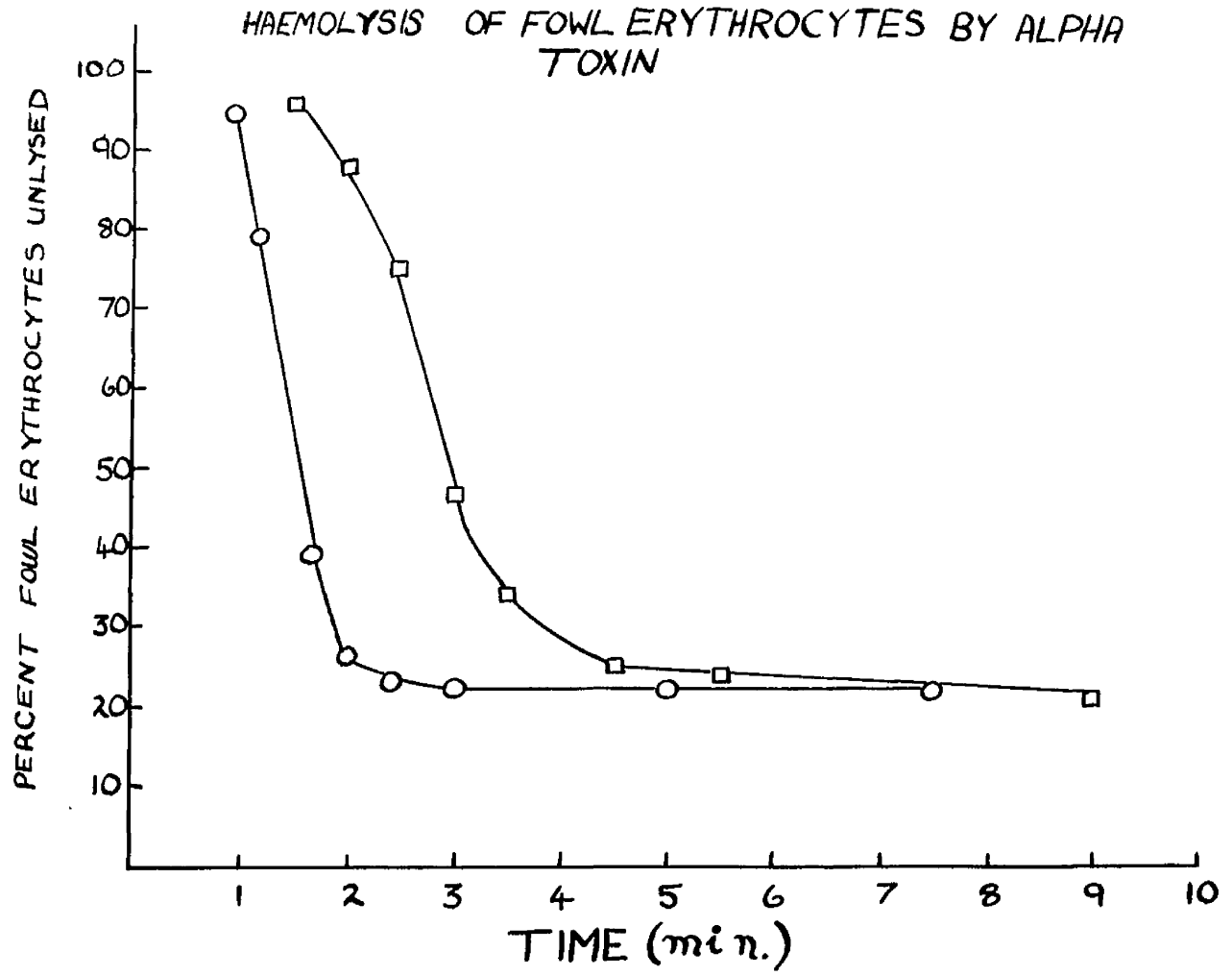
The effect of alpha toxin on the red cells of different species

Expt. No.	Species	Haemolytic Titre	
		Crude alpha toxin	Partially pure alpha toxin
1 *	Rabbit	1/8,000	1/32,000
	Mouse	1/500	1/2,000
2	Rabbit	1/8,000	1/128,000
	Sheep	1/128	1/4,000
	Human	1/16	1/500
	Fowl	1/16	1/500
	Frog	1/16	Not done

*In this case the partially purified toxin was less active than that used in Experiment 2.

Thus mouse red cells which have been reported insensitive (Elek, 1959) were about 16 times less sensitive, sheep about 40 times less sensitive and human, fowl and frog very much less sensitive. In addition it was noted that red blood cells of different animals of the

Fig.11


HAEMOLYSIS OF FOWL ERYTHROCYTES BY ALPHA TOXIN
PERCENT FOWL ERYTHROCYTES UNLYSED
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
TIME (min.)

same species varied greatly in sensitivity, and as far as possible in quantitative work the same animal was used as donor. As far as is known the sensitivity to frog RBC has not been previously recorded. There were various points of interest observed while carrying out this part of the work.

The titre against sheep red cells did not increase on cooling to 4°C; indicating the absence of beta toxin. The end point of titrations with mouse cells were sharper than those using rabbit red cells. Perhaps the most interesting finding however was that at no dilution of toxin were fowl red cells completely lysed. When investigated turbidimetrically the rate of lysis stopped sharply (Fig.11).

It was decided to investigate this. Samples of a reaction mixture containing fowl red cells were taken at regular intervals, stained with bromrocresol blue and examined microscopically. It was observed that although alpha toxin lysed the cell membrane, the nucleus remained intact. These intact nuclei were responsible for the residual opacity; apart from the frog, the fowl was the only species noted with nucleated red blood cells. On the other hand, lysis of fowl red cells by sodium lauryl sulphate caused complete lysis not only of the red cell membrane but also of the nucleus. The

resulting solution became extremely viscous after about 7 min., possibly as a result of polymerisation of the DNA from the lysed nuclei. Lysis by distilled water presented yet another picture; lysis was immediately followed by the formation of a fibrous precipitate which settled out rapidly. Microscopical examination revealed that the nuclei had become agglutinated into large groups linked to one another by long strands.

The apparent resistance of fowl red cell nuclei to alpha toxin supports the view that alpha toxin acts only on the cell membrane. A comparison of the chemical nature of the nuclear membrane with that of the cell membrane may yield a useful clue as to the component which is attacked by alpha toxin.

II

THE LETHAL ACTION OF ALPHA TOXIN

II.

THE LETHAL ACTION OF ALPHA TOXIN

In this section observations on the lethal action of alpha toxin in four species are presented. The aim was to throw light on the mechanism of action of alpha toxin *in vivo* by ascertaining the symptoms of intoxication. Two mammallian species were chosen, the mouse because large numbers of relatively inbred animals were available, and the rabbit because it is the most sensitive known species. The fowl was chosen as the representative of birds; the frog was studied because, as a cold-blooded animal, it seemed likely to allow interesting comparison with warm-blooded animals.

MATERIALS AND METHODS

Mice used in the present study were mainly 4 - 6 week old Porton white mice weighing 20-25g. However in some experiments older mice weighing 30-40g. were used. Injections of 0.5 ml. or less of crude, partially purified and highly purified alpha toxin were administered intravenously (I.V.) into the tail vein, intraperitoneally (I.P.) and subcutaneously (S.C.) into the dorsal sac. Rabbits were 3-4 Kg. chinchilla rabbits. Toxin was given I.V. into the marginal ear vein in doses of 0.5 ml - 1 ml.

Dermonecrosis was assayed by injecting 0.1 ml. intradermally (I.D.).

Fowls were of mixed breeds and weighed c. 1.5 Kg. Toxin was administered I.V. into the wing vein, S.C. under the loose skin of the wing and intramuscularly (I.M.) into the pectoral muscle.

Frogs were of the common British species Rana temporaria, weighing 15-30g. and were of both sexes. The stock was maintained in a galvanised tank in the open air with fresh running water. Smaller batches were kept at room temperature and at 4°C. Injections were made S.C. into the dorsal lymph sac; it has been reported that tetanus toxin injected by this route was totally absorbed in as little as 3 hr. (Rowson, 1961). In some experiments injections were also given intracerebrally (I.C.) using a gauge 31 needle in doses of 0.0075 - 0.015 ml. with a calibrated "Agla micro-syringe". It was first established that control frogs would tolerate 0.03 ml. of solaine without harmful effect.

RESULTS

The Mouse

For many years the mouse has been used as a convenient laboratory animal for assaying the lethal potency of alpha toxin preparations (L.D. or L.D.50); the time to death varying from a few hours to 3 days.

By contrast, little attention has been devoted to the study of alpha intoxication. In the present work the symptoms of intoxication and the mode of death were closely studied; it was hoped that information derived in this way in conjunction with histology experiments on isolated organs and comparison with other species may throw light on the point of attack of alpha toxin.

The relationship between the dose of toxin and the time to death. The relation between dose of toxin administered I.V. and the time to death was investigated; a summary of typical experiments can be seen in Table 16. The time to death was ascertained taking death as occurring at the end of the terminal spasms, although it is fully realised that the animal may have been dead a few seconds before then.

Table 16.

Dose (MID)	Time to death		Average	% dead
	Minimum	Maximum		
32,000	11 seconds	32 seconds	17 seconds	100
4,000	20 seconds	40 seconds	36 seconds	100
1,000	1½ min.	2 min.	2 min.	100
500	4 min.	6 min.	5 min.	100
250	5 min.	3 hr.	1½ hr.	100
128	3 hr.	4 days.	-	50

From Table 16 it can be seen that the time to death is markedly influenced by the dose of toxin. The most striking finding was the extreme rapidity of action of high doses; 32,000 MHD killed in a few seconds. With an eighth of this (4,000 MHD) the time to death was doubled. Further reduction of the dose however caused a marked increase in the time to death; this was especially noticeable when the dose was reduced to about 250 MHD. Whereas the average time to death at 500 MHD was 5 min., that at 250 MHD was 1½ hr; here a halving of the dose caused a 25 times increase in the time to death. A "threshold dose" above which death was extremely rapid and below which death was comparatively slow was observed in all the species studied, as will be seen later.

Rapid death was observed only when the toxin was administered by the I.V. route; when injected I.P. or S.C. the time to death even with large doses was considerably extended. For instance a dose of 2,500 MHD, which I.V., killed in less than a minute, killed in 40 to 50 minutes when administered by the I.P. route. Similarly the time to death with a dose of 500 MHD was extended from 5 min. (when injected I.V.) to about 1¾ hr. when given I.P. This length of time probably reflects time required for the toxin to diffuse across the peritoneal membrane.

Time to death was even longer when the toxin was administered S.C. A dose of 4,000 MHD killed in an average of 3 hr.40 min. when injected by this route, and the time to death with a dose of 500 MHD was increased to 9 hr. In this case the delay is most probably due to the time required for the toxin to diffuse into the capillaries.

Symptoms of intoxication. When large doses of toxin (32,000 MHD) were injected I.V. death was so rapid that little information could be derived concerning the symptoms. Animals collapsed within a few seconds and a series of spasms followed which terminated some 10 seconds later. With a dose of 4,000 MHD animals collapsed in about 15 sec. and spasms ended in 25 to 40 sec. Spasms were tetanic, with stretching and arching of the back; however at death the body and limbs were completely flaccid. At 1,000 MHD animals became unsteady after about half a minute and subsequently irregular breathing with gasping was observed; death occurred after intermittent spasms. The same slightly extended general picture was observed at 500 MHD.

Further reduction in dose had an interesting and striking effect. With 250 MHD a few animals died in the first 15 min. in a manner not unlike that just described. The remainder on the other hand were normal

for a short period and then became slowly and progressively weaker. The coat stared, the skin temperature dropped considerably and the rate of respiration became so slow that at times it was difficult to tell whether or not the animal was dead or alive. They remained in this comatose state for periods of up to an hour and sometimes even longer. At even lower doses (128 MHD) no animals died quickly; those which did eventually die showed the slow type of death, and some, which showed signs of intoxication to begin with, gradually recovered and about 50% of these animals survived.

Possible causes of death. With doses of alpha toxin which killed in a few hours death was too rapid to allow the development of histological lesions. However, the symptoms preceding death suggested that the toxin possibly acted upon the central nervous system. Certainly it appeared that the prime cause of death was not due to cardiac arrest since the hearts of animals killed by large doses of alpha toxin when exposed and perfused with saline containing 1% glucose continued to beat for 6 - 8 min. after death. However, electrocardiograms could not be done and therefore the possibility of cardiac irregularities could not be ruled out. The slow death, taking several hours was

characterised by a fall in the surface temperature, suggesting that a fall in blood pressure due to peripheral collapse may play a major role as put forward by Thal and Egner (1961).

By contrast, when small doses were given some animals survived for up to 24 hr. or even longer before dying; the time to death was then long enough to enable detection of histological lesions. These were found almost solely in the kidneys. The cells of the lining of Bowmans capsule were severely necrosed; the glomerular tuft was shrunken and compressed. Indeed in one case necrosis was so complete that not a single normal nephron was seen. No lesion was observed in the liver and the lungs, although occasionally patchy necrosis of the heart was noted. In most of these animals it seemed that death may be due to renal failure.

The effect of central nervous system stimulants. As mentioned it was considered possible that alpha toxin may act on the central nervous system and especially on the respiratory centres. It was thought that if indeed this were so, then the symptoms may be to some extent counteracted by prior injection of drugs which are known to act by stimulating these sites. Three such drugs, ephedrine, dexamphetamine and nikethamide were tested. The first two act on the higher cortical

centres, on the hypothalamus, the medulla including the medullary respiratory centres and the brain stem (Lewis, 1960). Nikethamide has a direct action on the medullary and spinal respiratory centres (Lewis, 1960). None of these drugs extended the time to death - if anything, dexamphetamine and nikethamide shortened it - ephedrine and dexamphetamine however had an interesting effect. Control animals were injected with about 500 MID of alpha toxin I.P. and died in the usual manner with a period of guarding in response to the injection, followed by gradual onset of general weakness until they became comatose; they remained in this condition until death in about 2 hr. By contrast, those animals which received ephedrine or dexamphetamine S.C. or I.V. prior to toxin were less affected. They did not exhibit such a marked guarding action and continued to behave normally although slightly nervous as a result of the drug. Even until a few minutes before death they attempted to carry out normal actions, such as washing themselves, eating and climbing. Then quite suddenly they became ill. They went into convulsions, alternating with periods in which they attempted to wash themselves; they jumped jerkily around the cage; a few minutes later they died. There was no extended comatose period which was observed in the controls. Ephedrine was more active in this way than dexamphetamine.

The effect of eserine Botulinus type A toxin is thought to act by inhibiting the release of acetyl choline at the end plates (Ambache; Burgen et al., 1949). Although staphylococcal alpha toxin is quite unlike Botulinus toxin in the mode of death and in the speed of action (Botulinus toxin acts only after a considerable incubation period of some hours, even in large doses) it seemed possible that it may act by inhibiting the release of acetyl choline at a different level of the central nervous system. If indeed this were the case, injection of physostigmine (eserine) which blocks the action of acetyl choinesterase may cause an increase in acetyl choline and to some extent relieve the symptoms. However, neither prior injection of the drug nor administration some time after the toxin caused any alleviation of symptoms.

Inhibition of the lethal action of alpha toxin in mice.

As mentioned earlier in section I (p.112) Suramin and analogues of this drug were found to inhibit the action of alpha toxin in vitro. It was therefore decided to investigate the effect of these compounds on the lethal action of alpha toxin.

In early experiments alpha toxin was premixed with the drug both at pH 5.5 and 7.0 for 30 min. before injection of the mixture I.V. into mice. The results

are summarised in Table 16.

Table 16

The effect of premixing alpha toxin with Suramin and Bayer 1

Compound	Concentration/ml.	pH	Deaths at 4 hr.	Deaths at 24 hr.
Suramin	2.5 mg.	5.5	1/5	1/5
"	"	7.0	5/5	5/5
Control (no drug)			3/5	4/5
Suramin	2.5 mg.	5.5	0/7	1/7
"	"	7.0	1/6	2/6
Control (no drug)			12/14	13/14
Bayer 1	1.0 mg.	7.0	1/6	5/6
Controls (no drug)			6/6	6/6

Thus it appears that premixing with Suramin and Bayer 1 does have a sparing action on the lethal effect of alpha toxin. The next step was therefore to determine whether a similar sparing action occurred when the drug was administered prior to toxin. Table 17 summarises the result of injecting Bayer compound 1 I.P. before 500 MHD of alpha toxin by the same route.

Bayer 1 does protect mice against a fairly high dose of alpha toxin when both the drug and the toxin were given by the I.P. route.

Table 17

The effect of prior injection of Bayer 1 - I.P.

	Death at 1½ hr.	5 hr.	18 hr.
5 mg. Bayer 1; then 500 MHD alpha toxin	0/4	1/4	3/4
Controls (no drug)	4/4	4/4	4/4
7.5 mg. Bayer 1; then 500 MHD alpha toxin	0/10	0/10	0/10
Controls (no drug)	10/10	10/10	10/10

However, when the drug was injected I.V. and toxin subsequently given I.P. no protection or extension of life was observed with Suramin, Bayer 1, or any of the other drugs which were found to inhibit haemolysis (p.112). In these experiments the drug was injected about 20 min. before a dose of toxin which killed controls in about 2 hr.

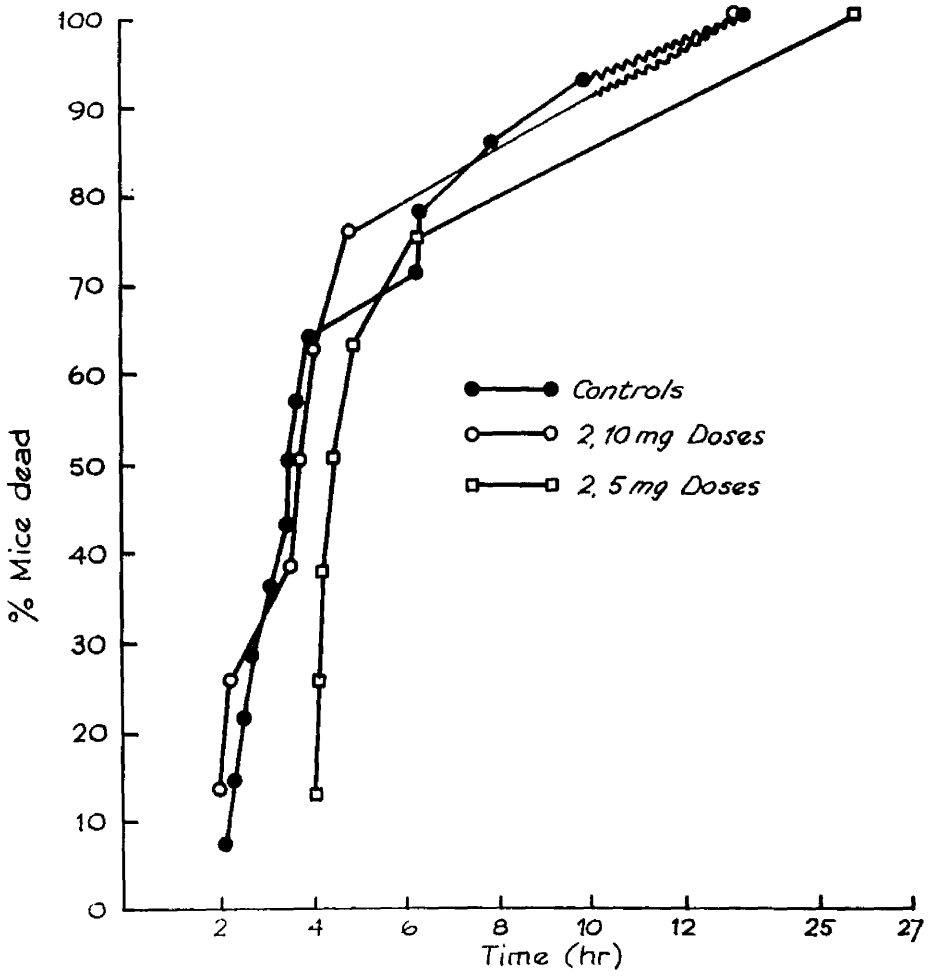
Nevertheless in one experiment some evidence of extension of life was observed. Mice received 2 doses of 5 mg. or 10 mg. of Suramin prior to an I.V. challenge with 1 LD of alpha toxin. The first dose of drug was was given I.V. 18 hr., and the second S.C. 2 hr., before challenge. Table 18 and fig. 12 show time to death for tests and controls. Although 10 mg. doses had virtually no effect on the time to death, mice which received 5 mg.

Table 18

Time sequence of death after preinjection of Suramin

Time (hrs)	Number Dead		
	Controls (Toxin alone)	2, 10 mg. doses	2, 5 mg. doses
2	-	1/8	-
2.2	1/14	-	-
2.25	-	2/8	-
2.3	2/14	-	-
2.6	3/14	-	-
2.75	4/14	-	-
3.2	5/14	-	-
3.5	-	5/8	-
3.55	6/14	-	-
3.6	7/14	-	-
3.7	-	4/8	-
3.8	8/14	-	-
4.1	9/14	5/8	1/8
4.2	-	-	3/8
4.5	-	-	4/8
4.6	-	-	5/8
5.0	-	6/8	-
5.25	-	7/8	-
6.3	10/14	-	7/8
6.4	11/14	-	-
8.0	12/14	-	-
10.0	13/14	-	-
15.0	14/14	8/8	-
26.0	-	-	8/8

Figure 12


100
90
80
70
60
50
40
30
20
10
0
% Mice dead
2
4
6
8
10
12
25
27
Time (hr)
Controls
2, 10 mg Doses
2, 5 mg Doses

doses began to die only after 50% of the controls had died. It may well be therefore, that the dose of drug and the route and time of administration of toxin used are critical in demonstrating the in vivo inhibition.

The Rabbit

The symptoms of death in the rabbit have been described by many workers in the past. However, in order to compare its sensitivity and behaviour with that of the other animals investigated, it was decided to re-examine the lethal action of alpha toxin in this species.

The effect of dose on time to death. Table 19 summarises the effect of different doses of alpha toxin in chinchilla rabbits.

As in mice the time to death was dependent on the dose of toxin administered, with a sharp increase in the time to death when the dose was reduced below 1,000 MHD. The approximate LD_{50} for rabbits (the number tested was too small to allow accurate determination of LD_{50}) was 200 MHD/Kg. of rabbit tissue. Thus the rabbit is considerably more sensitive than the mouse where the LD_{50} is 128 MHD for a 25 g. mouse or 5,000 MHD/kg. of mouse tissue.

Table 19

The effect of alpha toxin in rabbits when injected I.V.

Weight of rabbit	Dose (MHD)	Time to death	Ave.
3.7 Kg.	8,000	1 min. 35 sec.	2.35 min.
2.15 Kg.	8,000	2 min.	
1.865 Kg.	8,000	1 min. 30 sec.	
4.36 Kg.	8,000	4 min. 30 sec.	
3.0 Kg.	4,000	2 min. 15 sec.	-
3.25 Kg.	1,000	11 min. 30 sec.	18.1 min.
2.4 Kg.	1,000	12 min.	
2.9 Kg.	1,000	41 min.	
2.13 Kg.	1,000	8 min.	
2.5 Kg.	700	2 hr. 46 min.	94 min.
3.84 Kg.	700	28 min. 30 sec.	
2.75 Kg.	500	19 hr.	-
3.3 Kg.	500	Lived.	

Here again the toxin killed very rapidly when introduced in large doses I.V. The symptoms at such doses were similar in nature to those observed in the mouse; the animal was quiet for a short time and then the legs gave way and the animal sank to the ground.

Breathing became irregular and terminal spasm followed. With smaller doses the animal remained fairly normal, though quiet until the onset of symptoms of intoxication.

Intravascular haemolysis. It was decided to investigate whether intravascular haemolysis took place and if so whether it could account for death. Chinchillla rabbits were killed in three minutes by a high dose of alpha toxin. The heart, which in some cases continued to beat after death, was exposed, blood taken with a No.1 serum needle and citrate added as an anti-clotting agent. This was now diluted 50 times in saline and 1 ml. spun; at the same time blood from a control rabbit killed by pentothal was also diluted and spun. The supernatant of the control rabbit was completely free of haemoglobin, whereas that of the test animal contained a trace of haemoglobin, indicating that some intravascular haemolysis had taken place. This amounted to less than 10% haemolysis. There were then two possibilities; that 10% haemolysis was sufficient to cause fatal anaemia, or that the products of lysis were lethal. The first possibility was discounted on the grounds that up to 20% of the total blood volume was routinely taken from animals in obtaining red blood cells and normal and immune sera. To investigate the second possibility, 50 ml. of blood (c. 20%) was taken and the red cells

spun; plasma was discarded. The red cells were then lysed by addition of 20 ml. of distilled water. The volume was now restored to 50 ml., the mixture made isotonic with 0.4 g. of sodium chloride and injected I.V. into the same rabbit. The animal did not die and showed no signs of distress.

The Fowl

As far as is known, the sensitivity of the fowl to alpha toxin has not been previously recorded. It was therefore decided to investigate the fowl as a typical avarian species and to compare its behaviour with the other species.

The effect of dose on time to death. From Table 20 it can again be seen that the time to death is dependent on the dose; in this respect the fowl behaved in the same way as the mouse and rabbit. Again large doses were found to kill rapidly when injected I.V., but reduction of the dose below 16,000 MHD resulted in a considerable extension in time to death. This dependence of time to death on dose, similar to that observed in mice and rabbits, now appeared to be a characteristic of the toxin. Also when injected S.C. into the wing, the time to death was considerably extended and the wing became "paralysed", i.e., it was no longer kept in the normal position and was allowed to hang by the side

of the animal.

Table 20

The effect of varying doses of alpha toxin I.V. in the fowl.

Weight of the fowl in Kg.	Dose in MHD	Time to death
1.45	64,000	3 min.
1.37	64,000	3 min.
1.4	32,000	4 min.
1.31	32,000	4 min.
1.35	16,000	8⅓ min.
1.47	16,000	11 min.
1.3	16,000	16 min.
1.7	16,000	4⅓ min.
1.4	8,000	44 min.
1.3	8,000	Survived 24 hr.

From Table 20 it can be seen that the fowl is considerably less sensitive than the rabbit; the MLD from the few birds used was of the order of 8,000 MHD per bird of 6,000 MHD/Kg. of fowl tissue, and is therefore of the same order of sensitivity as the mouse.

Toxin inactivated by heating at 60°C. for 30 min. and toxin just neutralised by anti-alpha toxin did not kill fowls. In addition immunologically and physically

pure toxin (p.186) killed in the same way as partially pure toxin. Thus it seems that the lethal action in fowls was indeed due to alpha toxin.

Symptoms of intoxication. Injection of a large dose (64,000 MHD) killed very rapidly. Almost immediately after the injection the animal sank to the sitting position and the head began to droop. The regular jerking movements of the neck, characteristic of normal animals, ceased. On attempting to stand, the bird staggered drunkenly around the cage, as if it had completely lost its sense of balance. Just before death there was an outburst of kicking and flapping which lasted some 10-15 seconds; this was followed by a few spasmodic contractions of the body, and death. The same pattern of events repeated itself regularly in animals which received 16,000 MHD or more. When the dose was lowered to 8,000 MHD however, the picture was different. There was the same "sit down" response and head droop; then respiration became deep and laboured, until the animal was almost gasping. One animal died at 44 min. with characteristic terminal symptoms, while the other survived for 24 hours. This bird showed complete loss of the sense of balance and staggered around the cage. Its head was almost permanently in the droop position, and the eyes were kept shut. The impression was gained that this animal had lost its ability to make coordinated

movements. It was killed to avoid further suffering.

Thus the symptoms in the fowl are not unlike those observed in the rabbit and the mouse; the loss of sense of balance suggests that the toxin acts on the central nervous system. The fowl being a two-legged animal is probably more dependent on a correct sense of balance than is a four-legged animal and, in this respect, is possibly more obviously affected than the four-legged animals.

The Frog

It seemed of interest to determine whether or not cold-blooded animals were sensitive to staphylococcal alpha toxin, and if so, to compare the mode of death with that in warm-blooded animals. Since it is much easier to demonstrate survival of individual organs in the frog than in, for instance, the mouse, it was also hoped that further information would be obtained concerning the mechanism of action of alpha toxin *in vivo*.

The effect of different doses of alpha toxin in the frog. A large dose of 32,000 MHD killed frogs in 18-26 min., and the time to death was again found to be dependent on the dose of toxin administered but not quite in the same way it was for warm-blooded animals. The typical time sequence of death at different doses of alpha toxin can be seen in Table 21.

Table 21

The effect of alpha toxin in the frog.

Dose (MHD)	Time to death.
32,000	18 - 26 min.
8,000	4 hr, 5 hr, 5 hr, 50 hr.
4,000	5½hr, 5½hr, 6hr, 18hr, 36hr, 36hr.
1,000	5hr, 5hr, 5hr, 24hr, 48hr, 72hr, 1 survivor.
500	All survived.

At doses below 32,000 MHD there was a considerable scatter in time to death. Some animals died shortly after the injection of toxin and others survived for some considerable time. The L.D. for frogs was 1,000 MHD/25g. frog or 40,000 MHD/Kg. of frog tissue: thus the frog is considerably less sensitive than the rabbit, mouse and fowl.

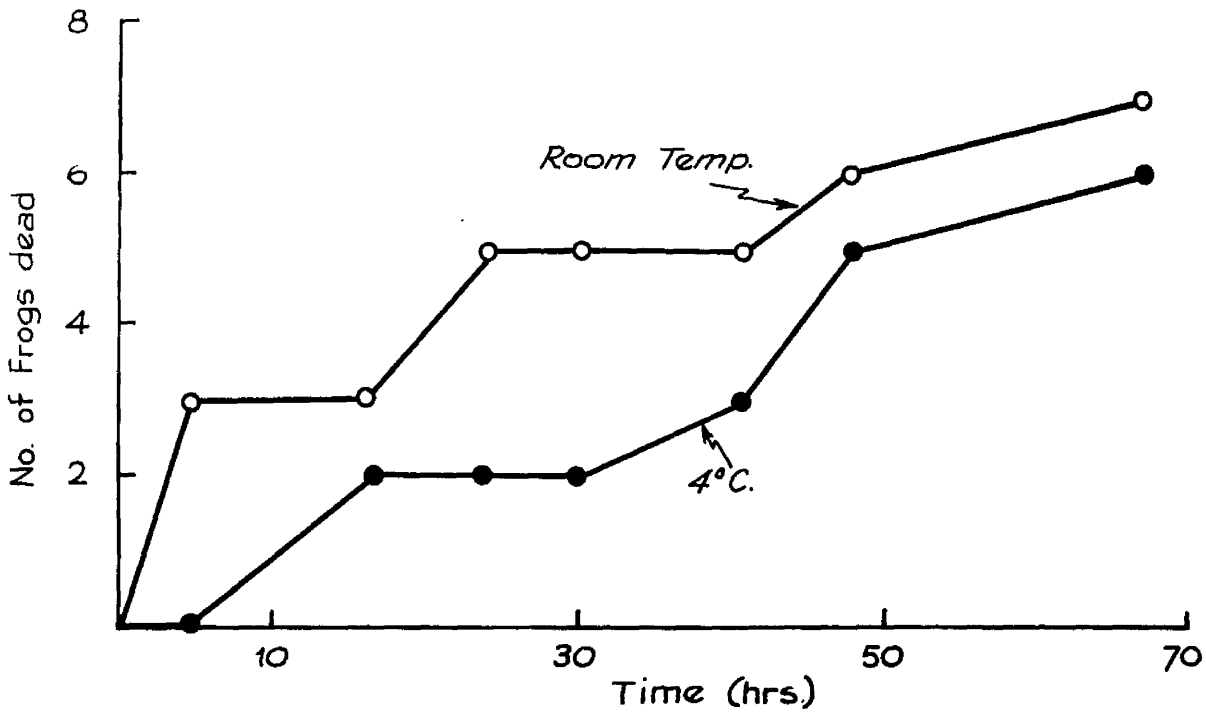
The symptoms preceding death in the frog. Very shortly after injection of large doses, the mandibular and the flank respiratory movements became irregular and eventually stopped. The frogs then became distressed, attempting to climb from the container. They became weak and when placed on their back could not right themselves, nor could they swim. It was difficult to decide just when death occurred and it was (rather arbitrarily) decided that when the respiratory movements had completely ceased, when the corneal reflex was negative, and when the frog

failed to right itself after 10 minutes on its back, then the frog was indeed dead. The symptoms preceding death at low doses of toxin were similar to those observed for high doses, only the process was slower. Since toxin heated to 60°C. for 30 min., autoclaved, and toxin just neutralised with anti-toxin were not lethal it seemed that alpha toxin was the factor responsible for death; also it was later found that pure alpha toxin was lethal.

The effect of environmental temperature on the time to death. Recently Rowson (1961) described the increased resistance of frogs to tetanus toxin when they were kept at low environmental temperature. It was decided to examine the influence of temperature on the susceptibility of frogs to staphylococcal alpha toxin. Frogs were kept at 4°C. for 24 hr. prior to injection of alpha toxin. A control batch were kept at room temperature. It can be seen from Fig.13 that those kept at 4°C. died less quickly than those at room temperature. This finding was reproducible and also held for high doses of toxin. For instance at a dose of 32,000 MHD the time to death was increased from 25 min. at room temperature to 3 hr. at 4°C. As described in an earlier section the haemolytic action of alpha toxin was dependent on the temperature, being considerably less

Figure 13

The effect of environmental temperature on the action of alpha toxin in frogs.



at 4°C. than at room temperature. Thus the frog experiment shows that the lethal action of the toxin is also dependent on the temperature.

Possible cause of death in the frog. From the symptoms of death it appeared that respiratory paralysis could be the cause of death. When frogs were killed by a large dose of alpha toxin and the heart exposed, this continued to beat for a considerable period after death; thus cardiac arrest was not then the primary cause of death. It was considered possible that alpha toxin acted on the central nervous system and especially on the respiratory centres. In a cross experiment frogs were killed by large doses of alpha toxin, dissected, and the muscles, nerves, and spinal cord exposed and stimulated electrically with a 4½ volt battery through platinum electrodes in situ. All stimulations produced strong positive responses of either the fore or hind limbs. Contraction of the fore limbs could be elicited by stimulation as high as the brain stem. However, stimulation of the higher levels of the brain produced no response, suggesting that alpha toxin may act on the brain itself.

In order to further test this hypothesis injections of very small volumes of highly potent purified toxin were made intracerebrally. Table 22 summarises the results of injecting varying doses of toxin by this route.

Control frogs received up to 0.03 ml. of normal saline without harmful effects. Injections were made through a "weak-point" one-quarter inch behind the orbits along the mid-line of the head. Two frogs were tested at each dose.

Table 22

Volume administered	Dose (MHD)	Times to Death
0.015 ml.	4,500	7 min., 8 min.
0.0075 ml.	2,250	8 min., 9 min.
0.0075 ml.	375	10 min., 18 min.
0.0075 ml.	300	15 min., 18 min.
0.0075 ml.	175	24 hr., 1 survivor.

When given by this route death occurs much more rapidly and to a considerably lower dose, 175 MHD killing, whereas 1,000 MHD was required in S.C. injections. Immediately following the injection the animals became highly excited, then rolled on their backs and showed tetanic spasms; in many cases the mouth gaped open.

DISCUSSION

Alpha toxin causes death either very rapidly or only after a considerable delay; there seems to be a threshold dose below which death is slow and above which it is extremely rapid. Rapid death does not seem to be

merely an accelerated form of slow death; clinically the two differ so greatly that they will be considered separately.

When death occurs within a few seconds or minutes, lesions at the organ level have had no time to develop. However, they must clearly have developed at the cellular level in order to account for loss of function and death.

On the other hand, many workers have recorded extensive histological lesions in the soft tissues when death is slow. Indeed in the present work, severe kidney necrosis was noted in animals which died after 24 hr. Some of these lesions however, may be secondary, and where lung congestion is found, this may result from heart damage. A striking symptom of slow death is the fall in skin temperature; animals are cold to the touch. This again may result from a direct action on the heart or vascular failure, or perhaps from action on the central nervous system. In fact, in slow alpha intoxication no organ can, with certainty, be stated not to have been affected. Kidney failure, or liver damage may account for death, although such an explanation has yet to be proved experimentally; biochemical tests, e.g. blood urea, transaminase estimation etc., have never been done.

It is, however, the strikingly different symptomology that prompts a separation of fast and slow death. In

rapid death symptoms preceding or accompanying death in rabbits, mice, fowls and frogs are fairly uniform: collapse, respiratory distress, ataxia and convulsions were observed. The process, especially in warm-blooded animals is so rapid that it is difficult to determine with certainty the cause of death. Extrapolation of pharmacological experiments on isolated perfused organs (Weigershausen, 1960, 1962) is informative but open to the criticism that the mechanism of death in different species and the behaviour of organs in vitro and in vivo may differ.

Basically all the symptoms of rapid death could be covered under the general term anoxia. Anoxia in turn could be due to several causes: intravascular haemolysis, cardiac failure, vascular failure, central respiratory failure, or poisoning of the essential respiratory enzymes could all be involved.

Intravascular haemolysis with loss of oxygen carrying capacity or release of a toxic product from lysed red cells seemed a possible explanation especially in view of the extreme sensitivity of rabbit erythrocytes. However, experimental evidence is against it. In rabbits, not more than 10% haemolysis was found in animals which died rapidly; removal of an equivalent volume of blood followed by injection of the lysed red

cells caused neither death nor even discomfort.

Cardiac arrest was often thought to be the cause of death (see Elek, 1959). E.C.G. changes indicative of an action on the sinuses, were reported by Nelis et al. (1934) while Dingle et al. (1937) suggested a direct action on the myocardium. However, these findings could equally be secondary results of general anoxia. More recently however, Thal and Egner (1961) reported that cardiac muscle was resistant to alpha toxin. Such differences may be due to differences in the purity of toxin preparations. By contrast, Wiegershausen (1960,1962b) found perfused isolated hearts of the rabbit, fowl and cat to be sensitive, while that of the common snail and to a lesser extent the guinea pig were resistant. Along with systolic arrest, alpha toxin caused constriction of coronary arteries and it is not easy to conclude from Wiegerhausen's experiments whether cardiac arrest resulted from a restricted flow of nutrient through constricted coronaries, whether the effect was direct on the muscle, or whether both contributed. An added difficulty in the interpretation of Wiegershausen's findings is the fact that he postulates (a) an indirect action of the toxin on smooth muscle of blood vessels causing release of serotonin and (b) release of an unidentified "polypeptide" which acts directly on heart muscle.

These experiments were carried out with only partially purified alpha toxin and Wiegershausen's views as to the cause of death must await experimental confirmation.

Experiments presented in this thesis are not readily integrated into the "cardiac arrest" hypothesis. In all species, most markedly in the frog, the heart continued to beat after death. It is of course unknown to what extent the heart function was impaired and whether it ensured adequate oxygenation and survival of the animal. Then again, cardiac arrest *in vitro* is preceded by a considerable lag time, up to 20 minutes, while death, say in mice, occurs in seconds. It is open to doubt whether under these conditions the *in vitro* results can be literally transposed into the *in vivo* system. It is felt that the argument centres *not* on the sensitivity of the heart, but upon its role in bringing about rapid death. It is therefore very possible, but by no means proved, that cardiac failure may in some species be the cause of or contribute to death.

Death as rapid as that observed when alpha toxin is injected into mice is rare. It is well-known with potassium cyanide which acts by poisoning the respiratory enzymes. But it seems unlikely that a substance of molecular weight around 40,000 (Bernheimer and Schwartz, 1963; Lominski, Arbuthnott and Spence, 1963, in press)

could be expected to diffuse so rapidly through the cell membrane and poison by acting on the respiratory enzymes. Also, it was found that alpha toxin did not inhibit respiration of mouse muscle tissue (p.180).

Another possibility is an action on the central nervous system. Action on the central nervous system would be compatible with the speed of action of the toxin when given intravenously and would account for all symptoms. There is some supporting experimental evidence. In frogs, intracerebral injection leads to death in a few minutes, while injection into the dorsal lymph sac acts much slower. Admittedly Elek (1959) is correct in stating that any markedly cytotoxic substance when introduced directly into the brain will cause death. However, while fully accepting this, one is forced to accept the possibility that when the toxin is introduced into the blood stream it reaches the brain and in sufficient quantity may cause death. It is also to be borne in mind however, that vascular failure, if taking place in the central nervous system, would equally well result in death with similar symptoms.

Tentatively, and in the nature of a working hypothesis, the following mechanism of death from alpha toxin is suggested. Alpha toxin is capable of acting upon a multiplicity of cells and tissues. According to species, dose and route of introduction, the toxin may come in

contact with more, or less, vital organs. A small dose may kill by gradually affecting parenchymatous organs; death will only occur as a secondary process through functional failure of those organs, e.g. kidney. With rapidly introduced large doses a vital organ which controls vital functions must be affected. This may be the heart, the vascular system, or the brain.

III

THE MYOTOXIC ACTION OF ALPHA TOXIN

III

THE MYOTOXIC ACTION OF ALPHA TOXIN

In the course of assaying alpha toxin it was noticed that mice which received intraperitoneal injections of alpha toxin frequently showed flaccid paralysis of the hind legs, while the forelegs remained normal. This occurred shortly before death and it was at first thought to be part of the general weakness due to alpha intoxication. However, an even more marked flaccid paralysis was observed when toxin was injected subcutaneously either into the scruff of the neck, when the forelegs and neck became paralysed, or into the lumbar region at the base of the tail, when paralysis of the hind legs was observed; under these conditions general weakness and death were delayed. A comparable paralytic phenomenon was seen in fowl and frogs, but not in rabbits. Since a similar effect has not been previously described, it was decided to investigate it further; the study falls into two parts (a) the paralytic action *in vivo* and (b) the effect of alpha toxin on isolated muscles *in vitro*.

MATERIALS AND METHODS

Injections of varying amounts of crude, partially purified and highly purified alpha toxin were injected S.C. into the dorsal sac either in the scruff of the neck

or at the base of the tail.

Mice were generally 20 g. in weight, although in a few experiments adult mice weighing 30-40 g. were used. In some experiments mice were killed by cervical dislocation and muscles and nerves stimulated *in situ* through platinum electrodes from a 4½ volt battery. Also the muscles, nerves and spinal cords of some animals were submitted for histological examination.

In order to attribute the paralytic effect to alpha toxin it was necessary to exclude other staphylococcal products in crude and partially purified preparations. Preparations from strains producing predominantly some of the other factors commonly produced by staphylococci were tested for paralytic action in mice; some factors were excluded because they could not be correlated with the paralytic factor; finally some factors were excluded by means of specific inhibition.

Beta toxin was obtained by growing a high beta toxin producing strain (3197) in soft agar in the presence and absence of CO_2. The crude toxin was extracted in the same way as alpha toxin. This strain produced large amounts of beta toxin and only traces of alpha toxin. The small amounts of alpha toxin were inactivated by heating at 60^{o} for 30 min.

Gamma toxin was prepared in the same way from strain 5R,

a rough variant of strain 5, which has been shown to produce a rabbit lysin which is almost solely gamma toxin (Smith, 1958). However, only low titres of 1/64 to 1/128 were obtained. Crude toxin was therefore concentrated by the same procedure as used for alpha toxin; the titre of the concentrate was 1/1,000.

Delta toxin was prepared according to the method of Marks and Vaughan (1950) from N.C.T.C. strain 9715, the classical delta toxin producing strain. The resulting preparation had a titre of 1/640 against human red cells.

Gelatinase was prepared according to the method of Lominski (unpublished). Overnight cultures of strain KSC on agar slopes in 4 oz. medical flat bottles were extracted with 3 ml. of saline and the bacteria removed by spinning. The resulting fluid was titrated in doubling dilutions in 0.2 ml. amounts of diluent; 0.8 ml. 15% gelatin was then added. The highest dilution which caused liquifaction after 18 hr. was 1/160. Such preparations contained no detectable alpha toxin.

Coagulase. Large amounts of coagulase detected by the method of Lominski et al. (1962) were present in gamma toxin preparations from strain 5R.

Staphylokinase. Small amounts of staphylokinase were present in partially purified alpha toxin. This was detected by titrating alpha toxin in 0.2 ml. amounts of diluent and adding 0.8 ml. of 1/10 normal human plasma.

A clot was formed by the addition of a few drops of thrombin, the highest dilution causing lysis of the clot after 18 hr. incubation was 1/10. This small amount of fibrinolysin (staphylokinase) activity was inhibited by epsilon amino-caproic-acid at a concentration of 8×10^{-5} M. An excess of this was added to partially purified alpha toxin before injection into mice.

Phosphatase. Strains which produced the paralytic factor and those which did not were streaked on agar containing phenolphthalein diphosphate; the presence of phosphatase was detected by the formation of a pink coloration on exposing the plate to ammonia vapour.

Protease. Strains were streaked on agar plates containing 10% plasma heated at 56°C. for 30 min. Protease activity was determined by the presence of a zone of clearing.

Egg yolk factor. Gillespie and Alder's (1952) egg yolk factor was detected by streaking strains on egg yolk agar. Opacification showed the presence of the egg yolk factor.

Assay of the action of alpha toxin on excised muscles in vitro. Most experiments were carried out on excised muscles of mice, although some were carried out on frog muscles. Mice were killed by cervical dislocation, and the thigh muscles of the hind limbs were dissected out. One set was used as control while the other was used as test. They were immersed in 5 - 10 ml. of a modified

Krebs Ringer solution which contained partially purified toxin, no toxin, autoclaved toxin, or toxin just neutralised with anti-toxin. The formula of the Krebs-Ringer solution was:- 100 parts 0.85% sodium chloride, 4 parts 1.15% potassium chloride, 1 part 3.38% magnesium sulphate ($MgSO_4.7H_2O$), 20 parts 0.1 M sodium dihydrogen phosphate pH 7.4 and 2 parts of 1.22% calcium chloride (added dropwise to prevent precipitation). 100 ml. of this mixture was now made 1% with glucose and the whole was gassed for 10 min. with 100% oxygen before the experiment. The mixture was made fresh daily.

First experiments were done on excised muscle of mice; later voluntary muscles of the frog were used. Finally, by way of comparison, the effect of alpha toxin on embryonic mouse heart explants was examined. No tracing equipment, comparable to that used for guinea pig ileum or frog rectus, was available for mouse muscle preparations and so a visual assay system was used. A 4½ volt battery with platinum electrodes was used to stimulate the muscles. Visible contraction of the muscle was recorded as a positive response; responses to such stimulations were always checked by two observers.

Frog gastrocnemius muscles were studied in the same way, but the response of frog rectus abdominis muscle, to acetyl choline stimulation, was recorded on a smoked drum.

In a few experiments the effect of alpha toxin on respiration of mouse muscles was examined in the following way. The muscles were excised in the usual manner, placed in isotonic phosphate buffer pH 6.8, made 1.5mM with $MgSO_4$ at 0^oC. and immediately chopped into small pieces; approximately equal amounts of chopped tissue were then placed in 25 ml. capacity Warburg flasks containing 1.9 ml. of the buffer. 10 u moles of sodium citrate in distilled water was then added to the outer well, 0.1 ml. of 20% KOH placed in the centre well and 0.5 ml. of toxin or control fluid into the side arm. Mannometric readings were taken at regular time intervals.

By way of comparison with the effect of alpha toxin on voluntary muscle, its action on mouse heart explants, kindly supplied by Dr. H. Morag McCallum, was also examined. An aliquot of highly purified alpha toxin was added to the culture of explants to bring the concentration of alpha toxin to 2,000 MHD/ml. ; beating of individual groups of cells was followed under the microscope.

RESULTS

The Paralytic Action of Alpha Toxin in vivo

Paralytic action in mice. Injection of 250 MHD or more alpha toxin caused flaccid paralysis of the limbs nearest to the site of the injection. For instance,

injection into the lumbar region caused flaccid paralysis of the hind legs; these were dragged uselessly behind the animal which moved only by dragging itself, using the front legs (Plate 1(a), 1(b)). The hind limbs were unable to grip the sides of the cage while the fore limbs were unaffected. By contrast injection into the scruff of the neck caused flaccid paralysis of the fore limbs and the neck (Plate 2(a)). Here the animals moved only on the hind limbs by Kangaroo-like movements while the fore limbs completely lost the power of grip; the hind legs could still grip cotton wool (Plate 2(b)).

The time necessary for paralysis to appear depends on the dose of toxin administered (Table 23).

Table 23

Time to onset of paralysis in mice injected with alpha toxin S.C.

Dose (MHD)	Onset	Complete Paralysis
16,000	17 - 30 min.	45 min. to 1 hr.
4,000	40 - 60 min.	1½ - 3 hr.
500/1,000	2 - 3 hr.	5 hr.

The first signs of the mice being affected were the appearance of an unsteady walk and inability to grip the sides of the cage properly. The animals then showed a reluctance to use their limbs, tending to drag

Plate 1 (a)

Plate 1 (b)

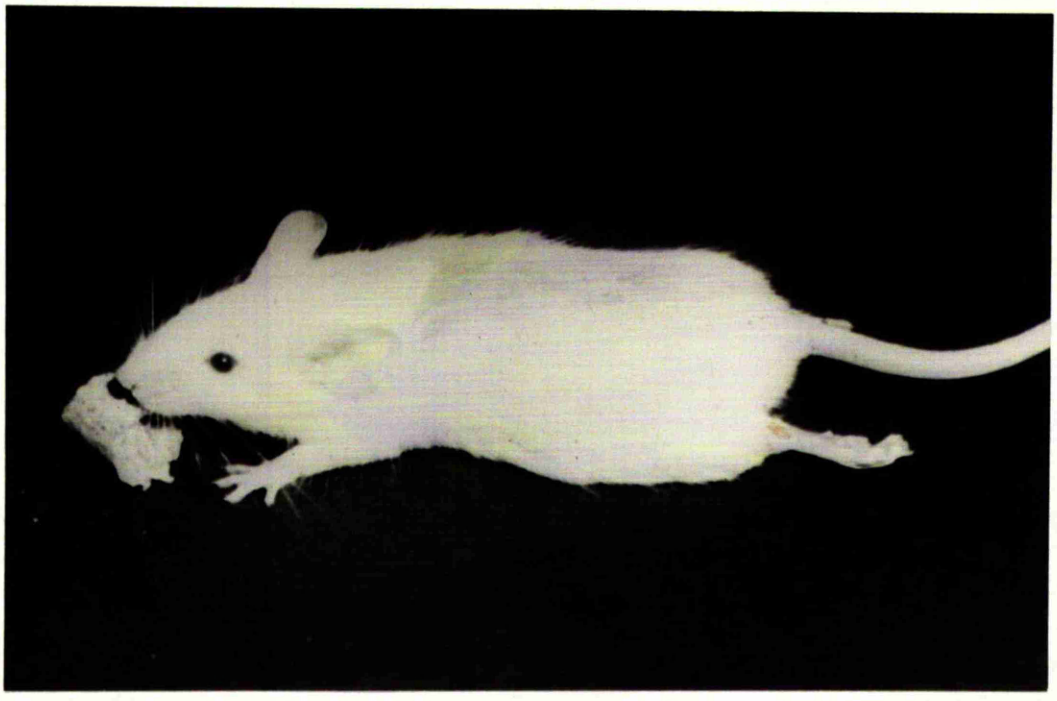


Plate 2 (a)

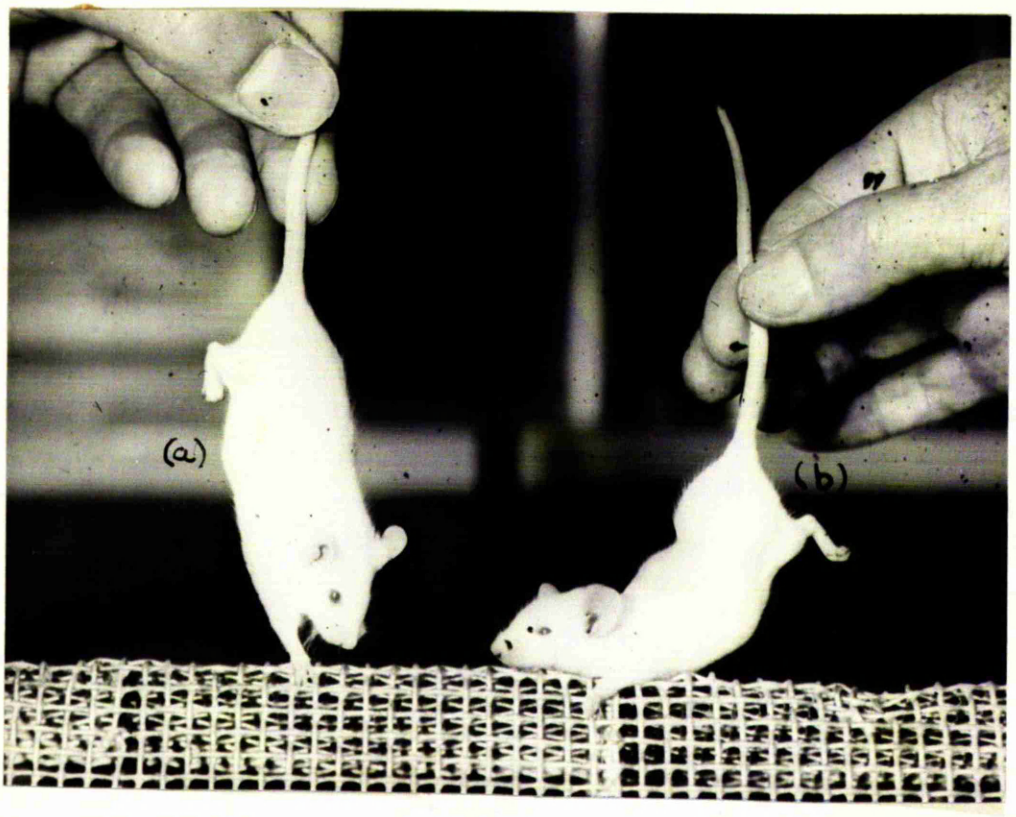


Plate 2 (b)

(a) DENOTES CONTROL (b) DENOTES ANIMAL WHICH RECEIVED TOXIN

them, although they occasionally showed periods of normal walking. Thereafter the condition worsened, until they could no longer use their limbs. The injection was seldom directly along the mid-line and the toxin went either to the right or to the left; the limb nearest to the direction of the injection was first to be affected. Paralysis was flaccid with no signs of rigidity.

When (with large doses) paralysis occurred shortly after injection, there were no detectable histological lesions, except slight engorgement of the capillaries. Where 4 - 5 hr. elapsed before complete paralysis, there was evidence of slight inflammatory reaction. In some animals which survived for 18 hr. there was extensive muscle necrosis. Occasionally after a small dose a mouse survived for 72 hr; strikingly even in these animals there was no detectable evidence of nerve or cord damage.

Older mice, weighing some 30 - 40 g. were very much more difficult to paralyse; even after 7 hr. 32,000 MHD failed to paralyse. They limped and had a poor grip but there was no paralysis as there was in young mice.

Paralysis in species other than mice. As noted in experiments on the lethal action of alpha toxin, fowls which received a large dose subcutaneously into the loose skin under the wing developed "paralysis", and after a

few hours the wing was allowed to hang by the side. Also, injection of alpha toxin into the leg muscles of frogs caused flaccid paralysis. On the other hand, chinchilla rabbits failed to develop paralysis even when large doses (8-16,000 MHD) were injected into the scruff of the neck or into the lumbar region.

Control experiments with factors other than alpha toxin. The paralytic action was inhibited completely by just neutralising the haemolytic activity of the preparation with anti-alpha toxin. Also, heating to 60°C. before injection abolished the paralytic action. These findings strongly suggest that the paralytic action was indeed due to alpha toxin, but in order to establish this conclusively, fluids containing staphylococcal products other than alpha toxin were injected and the properties of strains which did and did not produce the paralytic factor were compared and contrasted.

No beta toxin was found in the partially pure preparations which caused paralysis. Also 1000 MHD of beta toxin from strain 3197 from which traces of alpha toxin were removed by heating at 60°C. when injected did not cause paralysis. Controls injected with the same amount of alpha toxin were paralysed within 3 hr.

Since strain Wood 46 produces at most, traces of gamma toxin, not exceeding 1/16th of the amount of alpha

toxin (Smith, 1956) it is unlikely that gamma toxin causes paralysis; also, animals injected with 500 MHD of gamma toxin from strain 5R showed no flaccid paralysis while the same dose of alpha toxin caused complete paralysis in 5 hr.

Delta toxin was excluded on two counts; (a) mice injected with 320 MHD of delta toxin (from strain 9715) only developed a slight limp after 5 hr. while controls injected with the same dose of alpha toxin were completely paralysed in the same time. Even after 18 hr. mice which received delta toxin showed no signs of flaccid paralysis although there was an inflammatory response. (b) Normal horse serum neutralises delta toxin (Marks and Vaughan, 1950) and the traces of delta toxin present in partially pure preparations was inhibited by a 1/16 dilution of such serum. The resulting mixture injected into mice caused flaccid paralysis at the same time as animals which received alpha toxin without added serum.

Some staphylococcal extracellular enzymes were also eliminated. Coagulase was excluded on the grounds that preparations of strain 5R which is a high coagulase producing strain did not cause paralysis. Gelatinase preparations from strain KSC were not paralytic and anyway partially purified alpha toxin contained no gelatinase activity. The small amount of fibrinolytic activity present in such preparations was inhibited by epsilon-

amino-caproic acid but paralysis was not. Phosphatase, egg yolk factor and protease were excluded because they were either not produced by strain Wood 46 or else were also produced by strains which did produce the paralytic factor.

Possible cause of paralysis. At first it was considered possible that paralysis resulted from an action of alpha toxin on the nerves supplying the voluntary muscles of the limbs. To test this it was decided to stimulate the muscles of paralysed mice in situ. Mice were completely paralysed by injecting large doses of alpha toxin into the lumbar region and the animals were killed by breaking the neck; muscles were exposed and stimulated with platinum electrodes in situ. The muscles of the paralysed hind limbs did not react to stimulation whereas non-paralysed muscles of the fore legs responded well. This finding, although not excluding a possible action of toxin on the nerve suggested that the muscle itself was paralysed. However, paralysis may also have been due to an action on the blood supply to the affected muscle and in order to further investigate the action of alpha toxin on voluntary muscle it was decided to examine its effect on excised muscles.

Action of Alpha Toxin on Isolated Muscle Preparations.

The action of alpha toxin on excised voluntary mouse muscle. The thigh muscles of normal mice were excised and immersed in Krebs Ringer solution containing various amounts of alpha toxin, as described previously. Table 24 summarised the effect of different doses of alpha toxin on the time during which the muscle preparations reacted positively to electrical stimulation.

Table 24

The effect of alpha toxin on excised muscles of mice

	Reaction mixtures	Time to negative response
1)	250 MHD/ml.	54 min.
	No toxin	72 min.
2)	1,000 MHD/ml.	35 min.
	Neutralised toxin	50 min.
3)	5,000 MHD/ml.	19 min.
	Autoclaved toxin	72 min.
4)	5,000 MHD/ml.	47 min.
	No toxin	105 min.

The survival time of control muscle preparations varied, from experiment to experiment, but in all cases the test preparation containing alpha toxin reacted

to electrical stimulation for a considerably shorter period than in controls. The muscle preparations were made as free as possible from attached nerves, but it was still possible that small nerve endings in the muscle were being stimulated by the electrodes and not the muscle itself. For this reason it was decided to do the same experiment on muscles of a mouse which had first been completely paralysed by injection of curare, which is known to block nerve endings; 0.1 mg. of curare was injected I.V. and the mouse was almost instantly completely paralysed. After killing the animal by cervical dislocation, the muscles were excised in the usual way and stimulated at regular intervals. The muscle when immersed in 5,000 MHD/ml. of alpha toxin ceased to respond after 38 min. while the control in the absence of alpha toxin continued to react for a total of 68 min. Thus the results were similar to those obtained in non-curarised mice and strongly suggest that alpha toxin has a direct action on muscle.

In some experiments the muscles were weighed and the amount of tissue present was found to be considerable. For instance in experiment No.4 (Table 24) the wet weight of the muscle exposed to toxin was 696 mg. and that in the control fluid 675 mg. It was also found that if after 5 min. contact with toxin the muscles were then washed

free of toxin in Krebs Ringer solution and then placed in Krebs Ringer solution in the absence of toxin, the time to negative response in the presence of toxin was still considerably less than in control. Thus suggested that the toxin was either rapidly adsorbed or that damage during the first 5 min. contact was sufficient to ensure that the muscle would later lose its reactivity to electrical stimulation.

The effect of alpha toxin on voluntary muscle of frog. The gastrocnemius muscle of the frog was excised and tested in the same way as the mouse muscles; they were considerably heavier than the mouse muscle preparations. After 82 min. the test was negative to electrical stimulation, while the control continued to contract for 180 minutes, after which the experiment was discontinued. It was next decided to test the effect of alpha toxin on frog rectus muscle in the "rectus-bath", using acetyl choline as the stimulator. A frog rectus muscle was excised and separated into two halves. Each was attached in a rectus bath and connected to a lever which traced the response to stimulation on a smoked drum. The preparations were stimulated regularly at intervals with acetyl choline until a regular control contraction was observed. The test muscle was now immersed for 15 min. in 10 ml. Ringer solution containing approximately

Figure 14

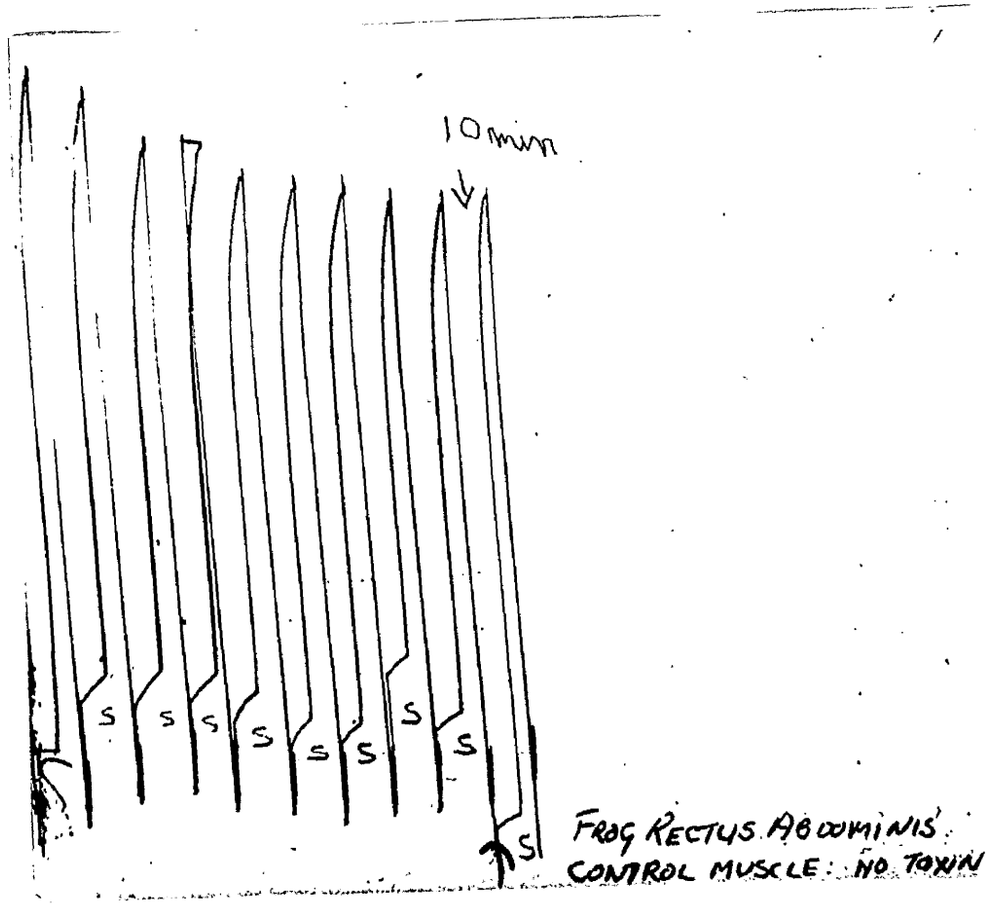
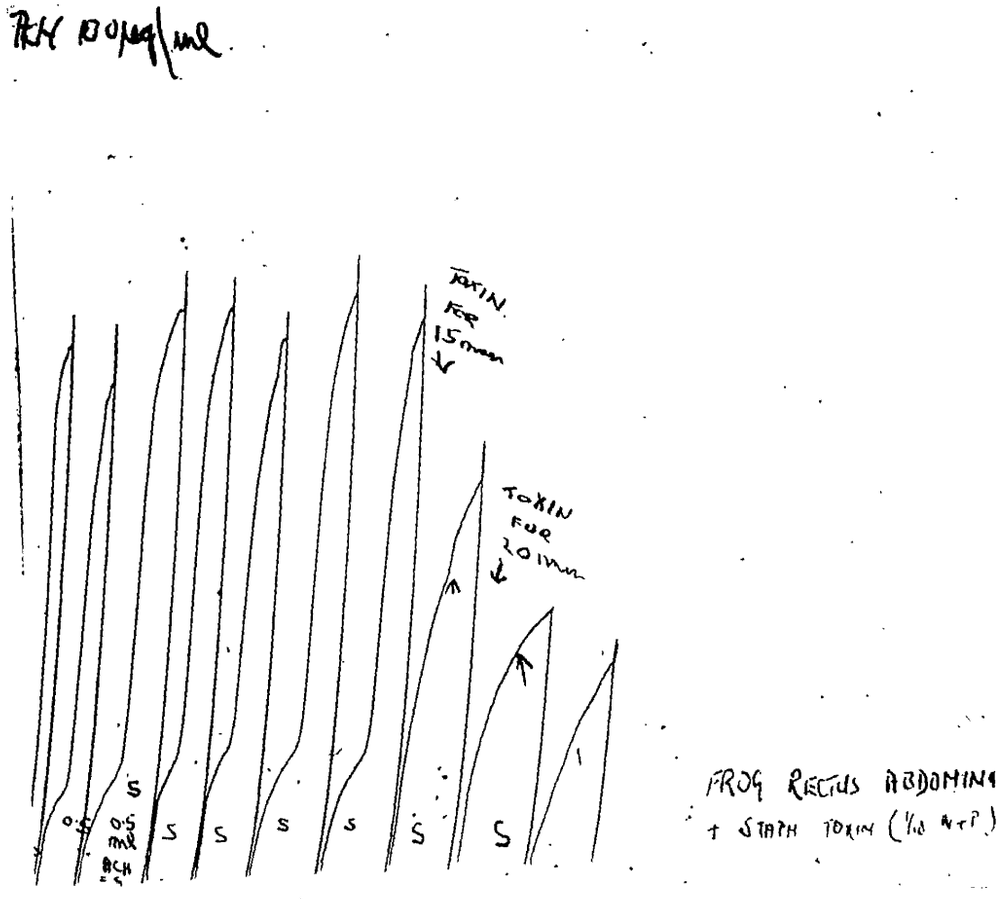


Figure 15



3,000 MHD/ml. while the control muscle was allowed to stand in Ringer solution for about the same length of time. On subsequent stimulation with acetyl choline the control responded normally (Fig.14) while the test showed a very much reduced response (Fig.15). After a further 20 min. in a fresh solution of toxin the response was further reduced. After washing the muscle free of toxin the response was still negligible. It was noted, however, that after contact with toxin the muscle had contracted some distance spontaneously; the distance between the base line and the arrow in fig. 15 represents the contraction by the muscle itself, before stimulation with acetyl choline. To cause a slight shortening of the muscle is a property normally associated with myotoxic substance (Lewis, 1962, personal communication). Thus it would appear that alpha toxin has a myotoxic action on the striated muscle of the mouse and the frog.

The effect of alpha toxin on respiration of mouse muscle tissue. In the previous section the argument against alpha toxin acting inside the cell membrane is put forward, on the basis of the rapidity of death and the size of the toxin molecule. However, the action on muscle tissue is not a rapid process and it was thought possible that alpha toxin may, after some time, penetrate the cell membrane and act on some essential metabolic reaction within the cell. For this reason it was decided to

investigate the effect of alpha toxin on the respiration of mouse muscle tissue. This was done in the Warburg apparatus, as outlined. Citrate was used as energy source in the present experiments. Crude alpha toxin was added to a concentration of 2,500 MHD/ml. of the reaction mixture: apart from a slight interruption of the O_2 uptake immediately following the addition of toxin, which may be a technical artefact, alpha toxin had no effect on the respiration of mouse muscle tissue under the conditions used.

The effect of alpha toxin on embryonic mouse heart explants.

The cultures of embryonic mouse heart explants contained clumps of cells which contracted spontaneously. The frequency of the contraction could be conveniently counted under the microscope. It was decided to study the effect of alpha toxin in such preparations. This was carried out as described.

Prior to the addition of alpha toxin the average rate of beating for a sample group of cells was 78/½ min. For ten minutes after the addition of toxin the rate of beating remained 78/½ min. After 20 min. the rate of beating per ½ min. began to increase, as follows:- 92, 102, 100, 115, 120, then the beating became shallow and some explants stopped beating altogether, some showed severely impaired heating and some continued to beat fairly normally. However, after overnight

incubation all but one of the explants had restarted and were beating quite strongly. A sample of the fluid was taken and titrated for alpha toxin activity. The titre was found to have dropped by 90% to 1/160 from 1/2,000. It would appear from this that heart muscle is not irreversibly damaged by alpha toxin, and not rapidly affected.

DISCUSSION

From the evidence obtained in the present work, it seemed that the factor responsible for causing flaccid paralysis in mice on subcutaneous injection was indeed alpha toxin. It was found only in alpha toxicogenic strains and in heat lability and neutralisation experiments behaved like alpha toxin. Also, other staphylococcal products were either absent from paralysing preparations or were produced by strains which did not produce the paralytic factor. Rather than postulate the existence of a previously unidentified staphylococcal myotoxin, it was accepted - for the time being at least - as yet another manifestation of the general cytoxicity of alpha toxin.

Although not investigated as fully as the mouse, frogs and fowls were found to develop a comparable paralysis, but even when large doses (16,000 MHD) were administered, the rabbit was not paralysed. Recently

Thal and Egner (1961) also reported that the voluntary muscle of the rabbit was resistant to the action of alpha toxin. On the other hand, Nelis (1934) found that injection of an unspecified staphylococcal toxin into the femoral artery caused paralysis of the corresponding limb in rabbits; again, differences in purity may be responsible.

Since high doses caused paralysis before any inflammatory lesions developed, and since delta toxin caused an inflammatory response totally unlike flaccid paralysis, inflammation can be discounted as a cause of paralysis. Paralysis *in vivo* was accompanied by a loss of the ability of the muscles to respond to electrical stimulation; this finding suggested that alpha toxin had a direct action on the muscle itself. The effect of alpha toxin on muscle was emphasised by the finding that mice which survived 2 or 3 days showed extensive muscle necrosis but no nerve or cord damage, when examined histologically. Neither of these findings however, excluded the possibility that damage to the nerves supplying the limb was responsible in part for paralysis *in vivo*. Whatever the cause, flaccid paralysis is probably the first functional lesion of alpha toxin which does not result in death.

Further evidence of direct involvement of the voluntary muscle came from experiments on isolated muscles of mice and frogs; alpha toxin abolished the

response to electrical or acetyl choline stimulation. Even this was not however conclusive; it was possible to argue that response to electrical stimulation resulted from stimulation of the neuromuscular junction or small attached nerves, and not to direct stimulation of the muscle itself. This criticism was largely removed when it was shown that muscles from mice previously paralysed with curare (known to block nerve endings and neuromuscular junction) behaved in the same way as those of non-curarised mice. Thus it is concluded that alpha toxin is indeed myotoxic for voluntary muscle.

By contrast, explants of embryonic mouse hearts, comprising not more than a few thousand and sometimes as little as 20 or 30 cells were not very sensitive to a large dose of alpha toxin. Some stopped beating after 20 - 30 min. and some only after a much longer time; but after overnight incubation most were beating again. Thus, heart muscle is not irreversibly damaged by alpha toxin. Unfortunately the technique used in the present work did not enable the maintenance of isolated voluntary muscle long enough to determine whether or not damage to it was also reversible. However, pretreatment with toxin for 5 min. followed by washing and placing in fresh fluid did not prevent loss of the response to electrical stimulation. Also,

taking into account the extremely small weight of the heart explants in comparison with adult mouse voluntary muscle, the former emerges as being less sensitive. This would not be surprising since in the present work the mouse heart was found to beat for some time after death, and as mentioned earlier Thal and Egner (1961) found cardiac muscle resistant to alpha toxin.

IV.

PURIFICATION OF STAPHYLOCOCCAL ALPHA TOXIN

V.

PURIFICATION OF STAPHYLOCOCCAL ALPHA TOXIN

None of the methods described, resulted in preparations which were at the same time highly potent and pure (see p. 34). A method is described here for the preparation of serologically and physically pure toxin which was potent *in vivo* and *in vitro*. The purification work proceeded more or less concurrently with the other studies in the present thesis but success came late and for this reason it occupies the last section.

MATERIALS AND METHODS

Immunological analysis. A single antiserum (anti-Wood 46, batch 3102, potency 135 units/ml.) kindly supplied by The Wellcome Research Laboratories, was used throughout. Double diffusion tests were carried out by a modification of the technique outlined by Elek (1948) and Ouchterlony (1948, 1949). Cups of *c.* 0.03 ml. capacity containing undiluted serum and toxin preparations were placed at about 7 mm. distance on the surface of 1.5% (v/v) agar plates which were kept at 37°C. for 24 hr. and thereafter for a further 7 days at room temperature.

Immunoelectrophoresis was carried out in 2 mm. thick layers of 1.7% agar in borate buffer pH 8.6 (675 ml. 0.04 M boric acid and 300 ml. 0.1 M sodium borate made

up to 1 litre with distilled water). Undiluted toxin was introduced into a round well and electrophoresed for 2 hr. at 110 v. and 20 m.amp; undiluted antiserum c. 0.03 ml. was then introduced into an oblong trough c. 2.5 mm. x 65 mm. cut parallel to the direction of electrophoresis. Plates were kept for several days at 20°C.

Ultracentrifuge measurements were made in a Spinco Model E ultracentrifuge. In most experiments the analytical cell of 0.75 ml. capacity was used; in some, an interference or a fixed partition cell were used.

Protein estimations. The nitrogen content was measured by the micro Nessler method (Paul, 1958) and the protein concentration calculated from this by assuming the value of 6.25 as a conversion factor (Jones, 1941) i.e. assuming that the protein contained 16% nitrogen. A rough guide to the protein concentration was obtained by measuring the optical density of preparations at 280 mu in a Hilger and Watts H 700 spectrophotometer.

Reagents were of B.D.H. AnalaR or "Laboratory standard".

Sephadexes were manufactured by Pharmacia, Uppsala, Sweden.

Biological assay of toxin. The haemolytic titre was measured as described previously (p.66); a single rabbit was used throughout as donor of erythrocytes. The lethal activity was determined in mice, rabbits, frogs and fowls, and in some experiments the dermonecrotic

activity was assayed by intradermal injection of 0.1 ml. into rabbits.

The Purification Procedure

Pilot experiments. The final method was evolved gradually from pilot experiments, which are outlined below.

Concentration and partial purification by a modification of the method of Wittler and Pillemer (1948) gives serologically impure material with at least 4 antigenic components and contained large amounts of nucleic acid.

To purify this material further it was decided to apply the technique of gel filtration, using Sephadex, recently introduced by Porath and Flodin (1959). Sephadex is an insoluble three-dimensional network of cross-linked dextran chains. It is believed that small molecular weight substances diffuse into the network and become bound to the numerous hydroxyl groups, while large molecular weight components, which because of their large mass, cannot enter the network, are washed out in the eluate and not retained. By varying the degree of crosslinkage different limits of exclusion are attained.

G 75 Sephadex is said to have a limit of exclusion of around 40,000 to 50,000; that is all substances of this molecular weight and above are allowed to pass. When partially purified preparations were applied to

columns of G 75 Sephadex alpha toxin appeared as soon as the fluid volume of the column had been displaced, indicating that toxin was not retained in the column. However, the spectrum still had an absorption maximum of 260 - 270 mu and contained 3 antigenic components. Nevertheless the potency per mg. protein was increased, indicating that some purification had occurred. Attempts to remove the nucleic acid by fractional precipitation failed.

It then was thought that the nucleic acid component might be removed by combining gel filtration with ion exchange chromatography. This was done by passing the eluate from G 75 Sephadex through a column of DEAE A 50 Sephadex, which is an ion exchange form of the simple Sephadex structure into which diethyl-amino-ether groups are incorporated; the procedure was carried out at pH 6.5. The experiment proved successful. The eluate from DEAE A 50 Sephadex was spectrophotometrically free of nucleic acid, and the potency per mg. of protein considerably increased. The preparation, however, was still serologically impure.

Attempts were now made to purify further by increasing the ionic strength and lowering the pH of the material with 0.14 M dihydrogen phosphate buffer. This was not successful; alpha toxin was not precipitated and 75% of the haemolytic activity of the supernatant was lost.

Fractional precipitation with methanol was then tried. The best fractions from DEAE A 50 Sephadex were bulked and stepwise precipitated with methanol at various temperatures. This led to a considerable increase in the potency/mg. protein and at the same time enabled concentration. This concentrated material still showed the presence of several antigenic components on double diffusion test.

Further purification of the methanol fractionated material on aluminium hydroxide and magnesium trisilicate columns was attempted but proved unsuccessful; alpha toxin was irreversibly adsorbed on both of these substances and was not eluted even at pHs as high as 8.0.

It is known that purification is often accomplished by repeating a step under slightly different conditions. It was decided to attempt a further purification of methanol fractionated material by once more passing it through DEAE A 50 Sephadex, this time at pH 5.8. The resulting fractions showed an improved potency/mg. protein, but the preparation now had a low protein concentration. In order to assess the purity of the material it was necessary to concentrate the toxin. This was again achieved by fractional precipitation with methanol which also served as an additional purification step. The best methanol fractions were serologically pure and very potent.

From these preliminary experiments the final method evolved as described below. It is not claimed that this is the most satisfactory method; it is time-consuming and tedious, and it may well be that further changing of conditions of pH and ionic strength as well as the use of the newly available Sephadexes may simplify it. Nevertheless it results in an end product which is potent and pure. Indeed until the recent work of Bernheimer and Schwartz (1963) which was published after the experimental part of the present thesis had been completed, it was the only method.

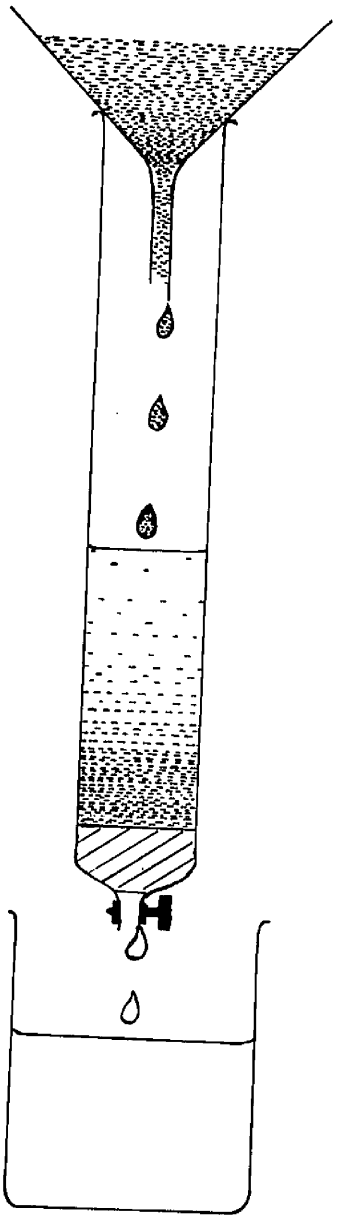
Crude alpha toxin was prepared as described in an earlier section (p. 64). In one experiment the crude toxin was supplied by The Wellcome Research Laboratories. In the course of work it was found that prolonged storage of crude toxin resulted in a considerable loss of activity, probably due to spontaneous toxoiding. Use of the flocculation technique would have enabled the estimation of inactive as well as active toxin, but as the aim of the present study was primarily the purification of active alpha toxin this was avoided. Thus freshly prepared batches were processed as rapidly as possible. For this reason and because of technical considerations (the largest number of soft agar plates which could be processed in a single experiment was 300) the volume of batches was limited to about 2 - 3 litres.

First Stage. Crude toxin was cooled to 0°C. and the pH adjusted to 4.3 with glacial acetic acid; methanol was added to a final concentration of 15% (v/v) and the temperature lowered to -5°C. After overnight standing at this temperature the resulting precipitate was spun at 8,000 r.p.m. for 15 min. at -5°C. and redissolved in one-tenth of the original volume in 0.14 M sodium phosphate buffer (Hendry, 1948) ionic strength 0.3, pH 7.2.

Second Stage. Columns of G 75 Sephadex were prepared in the following manner. 20 g. of Sephadex were suspended in c. 800 ml. of 0.03 M sodium phosphate buffer pH 7.0, ionic strength 0.06, and the suspension allowed to settle for 30 min. The supernatant was discarded and the slurry resuspended in the same volume of buffer. After settling out for a second time the supernatant was discarded and the slurry packed to give a column of 14 - 16 cm. x 5 cm. The glass column was first filled one third full with buffer and the slurry was allowed to drip slowly from a filter funnel, as shown in fig. 16, with the stop cock open until the Sephadex had settled to a constant level. 100 to 200 ml. of toxin from Stage One were now applied to the top of the column and the breakthro' volume determined, i.e., the volume of buffer which passes before alpha toxin activity begins to appear in the eluate. This varied from 70 - 90 ml.

Figure 16

The packing of a Sephadex column.



for different columns and was determined by testing drops of eluate at 10 ml. intervals for haemolytic activity in 0.5 ml. of a 2% suspension of rabbit erythrocytes. When 50% haemolysis was observed in 1 - 2 minutes fractions of 15 - 50 ml. were collected by hand.

Third Stage. Fractions with the highest haemolytic activity were now bulked and applied in columns of DEAE A 50 Sephadex prepared in the following way. 20 g. of DEAE A 50 Sephadex were suspended in c. 800 ml. of distilled water; enough 2N NaOH was now added to bring the pH of the supernatant, after settling of the slurry to 6.5. The supernatant was now discarded and the Sephadex washed several times with c. 800 ml. of sodium phosphate buffer (0.03 M, ionic strength 0.06, pH 6.5). Columns were packed and the breakthro' volume determined as in Stage Two. The eluate was again fractionated by hand.

Fourth Stage. Fractions of maximum haemolytic potency per mg. of protein which were spectrophotometrically free of nucleic acid and which had an E_{max} 280 of greater than 0.4 were bulked. (When, in one experiment using Wellcome Laboratories toxin, the E_{max} at this stage was found to be 0.25 or even less, considerable difficulty was experienced in the further purification and the preparation had to be concentrated by freeze-drying.)

Stepwise methanol fractionation was now carried out. Methanol was added to a concentration of 15% (v/v) and

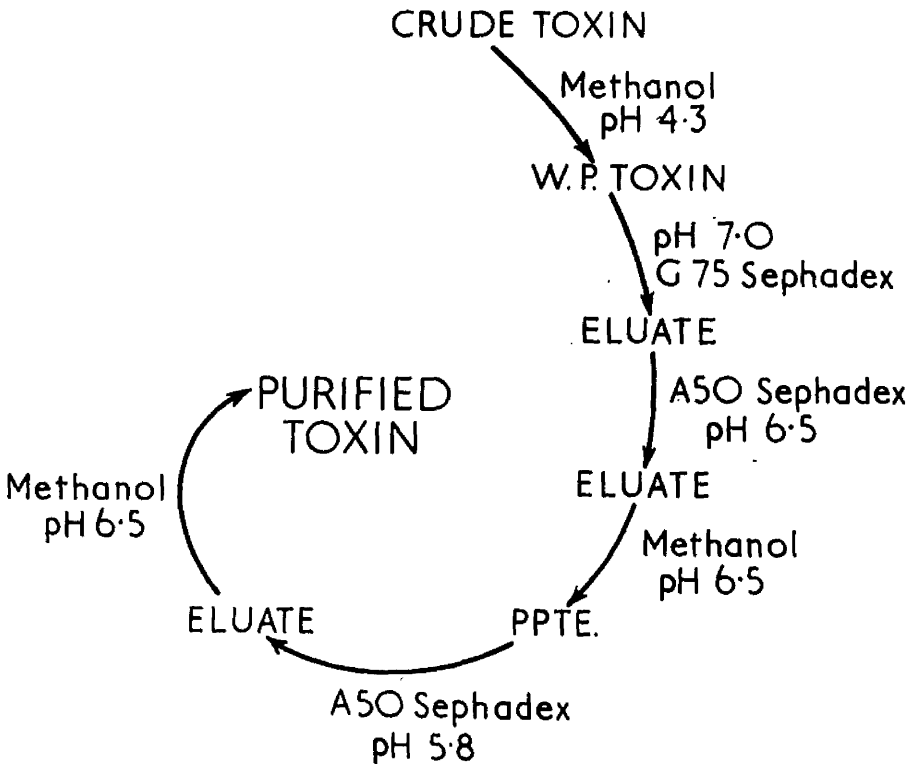
precipitation allowed to take place at -5°C. for 18 to 24 hours. This precipitate was spun and redissolved in one-twentieth to one-fortieth of the original bulked volume in sodium phosphate buffer pH 7.0 diluted 1/2 with saline. The methanol concentration of the supernatant was now increased to 30% (v/v) and the procedure repeated. In early experiments the methanol concentration of the supernatant was then increased to 40% (v/v) and the mixture placed at -20°C. Later, however, it was found that similar precipitation was achieved by merely lowering the temperature to -20°C., without increasing the methanol concentration. After spinning and redissolving in the usual manner, the concentration of methanol in the supernatant was further increased to 60% (v/v) at -20°C. The fractions at each methanol concentration were retained for further investigation.

Fifth Stage. The best fractions were now further processed by applying to columns of DEAE A 50 Sephadex prepared in the following way. According to the volume of the material to be passed, 2 - 10 g. of Sephadex were suspended in 75 - 300 ml. of distilled water, the pH of the supernatant was adjusted to 5.8 with 2N NaOH, after washing with sodium phosphate buffer pH 5.8 (0.03 M, ionic strength 0.05); the slurry was packed in the usual way using columns of 1.5 cm. or 3.0 cm.

Figure 17

SUMMARY OF

PURIFICATION PROCEDURE



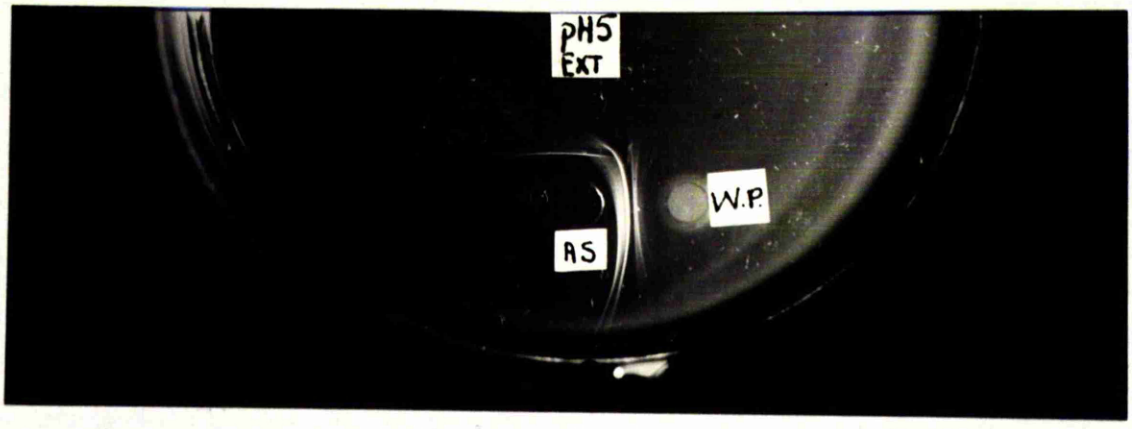
diameter. The breakthro' volume was determined as in previous stages and the eluate collected in fractions of 3 - 4 ml.

Sixth Stage. The fractions showing maximum haemolytic activity per mg. of protein were bulked and further processed by a stepwise methanol fractionation as in Stage Four. In 2 experiments Stage Six toxin was pure by all the criteria. However in 4 experiments there were two components at this stage and these were separated either by closer methanol fractionation or by spinning in a partition cell in the ultracentrifuge. Fig. 17 shows a diagrammatic summary of the purification procedure.

RESULTS

First Stage. This was essentially a concentration procedure; not much purification was achieved. As can be seen from fig. 18 the spectrum hms a plateau between 250 and 260 mu, indicating the presence of nucleic acid impurity. Also Plate 3 shows that Stage One toxin is serologically very impure. The haemolytic potency of these preparations was of the order of 10,000 MHD/mg. protein.

Plate 3



W.P. = Stage One Toxin. A.S. = Antiserum.

Second Stage. The haemolytic activity and the O.D. at 260 mu for Stage Two fractions in a typical experiment can be seen in Table 25.

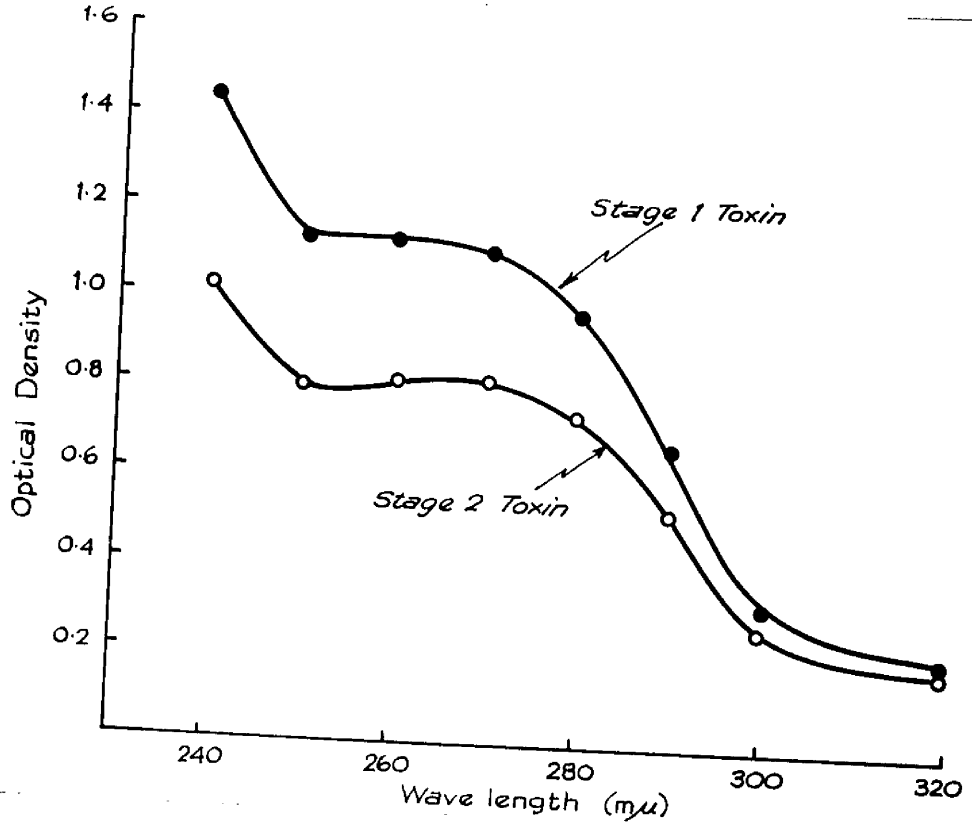
Table 25

Haemolytic activity and O.D. at 260 mu and 280 mu of G 75 fractions

Fraction No.	Volume (c.c)	O.D. 260	Titre
1	15	0.205	500
2	15	0.460	4,000
3	15	0.610	10,000
4	15	0.685	32,000
5	15	0.775	32,000
6	15	0.817	32,000
7	15	0.777	64,000
8	15	0.887	32,000
9	30	0.835	32,000
10	30	0.920	64,000
11	30	1.018	64,000
12	30	0.740	32,000
13	22	-	8,000
14	14	-	4,000

Absorption spectra and O.D. of Stage 1 and 2 toxin were determined on 1 in 5 diluted material.

Figure 18


1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Optical Density
Stage 1 Toxin
Stage 2 Toxin
240
260
280
300
320
Wave length (mμ)

In this experiment fractions 4 to 14 were bulked, and the titre of the bulked preparation was 1/32,000. As can be seen from Fig.18 the adsorption spectrum has a plateau in the nucleic acid region. Also in double diffusion tests 3 antigenic components were detected. Thus the preparation is still far from pure. In these experiments the flow rate through G 75 Sephadex was extremely slow and the collection of fractions was not complete until 24 hr.

Third Stage. Passage through DEAE A 50 Sephadex considerably purified the toxin. From the spectra shown in Fig. 19 it can be seen that in this experiment fractions 1 - 6 had the spectra of a typical protein with an absorption maximum at 280 mu. However, the spectra of the last three fractions (Fig.20) showed a shift towards the nucleic acid region; also as can be seen from Table 26 the potency of these fractions per mg. protein and their haemolytic titres were low in comparison with fractions 1 - 6. Fractions 1 - 6 inclusive were therefore bulked for further processing. The potency of the bulked material in this experiment was about 21,000 MHD/mg. protein. However, the material was still serologically impure.

Fourth Stage. The bulked fractions from Stage Three were now diluted to c. $O.D_{280}$ of 0.4 - 0.5 and then fractionally precipitated with methanol (Table 27).

Figure 19

The absorption spectra of Stage Three fractions 1 - 6

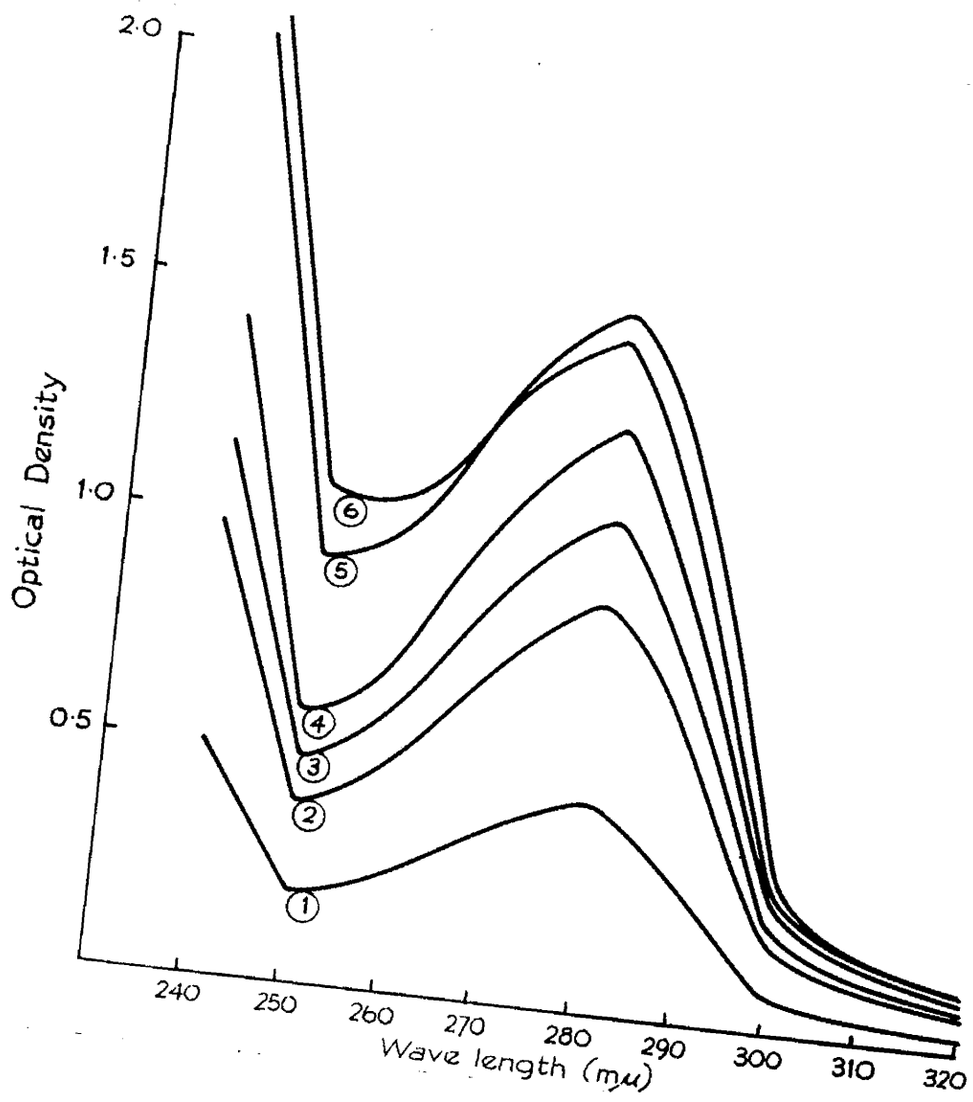


Figure 20

The absorption spectra of Stage Three fractions 7 - 9

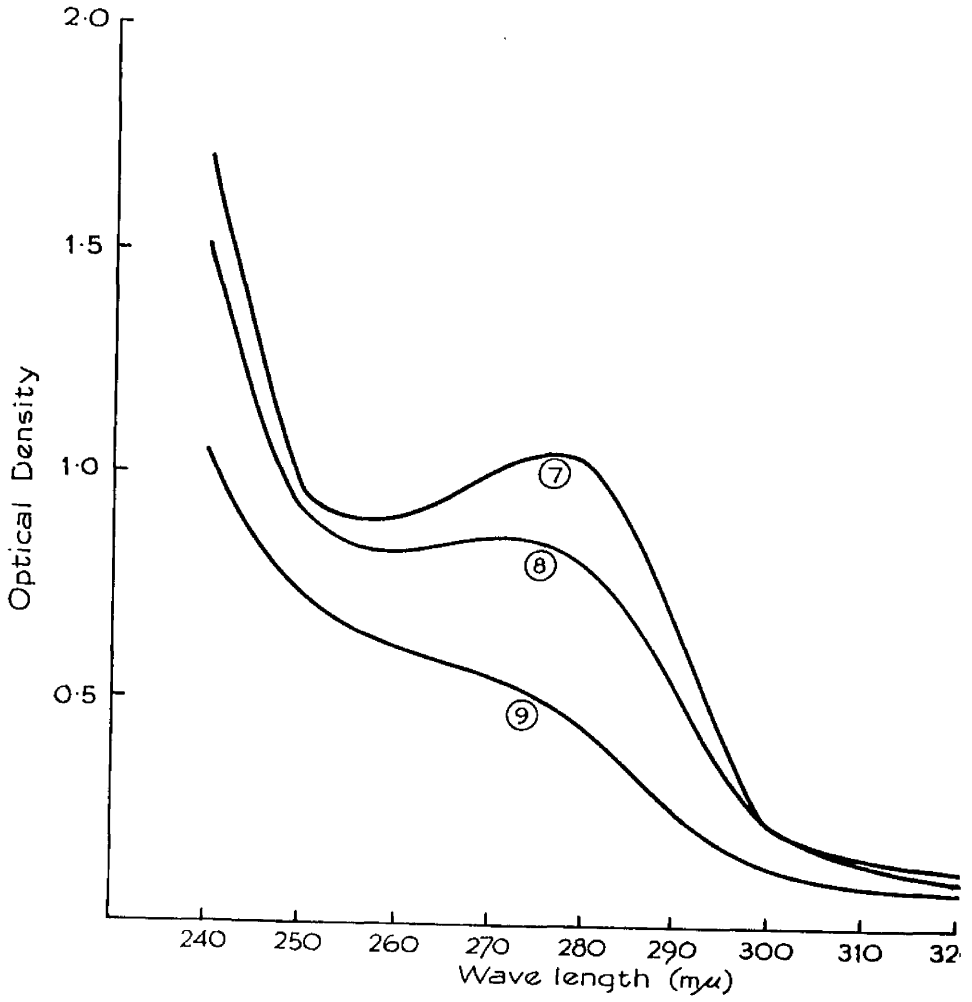


Figure 21

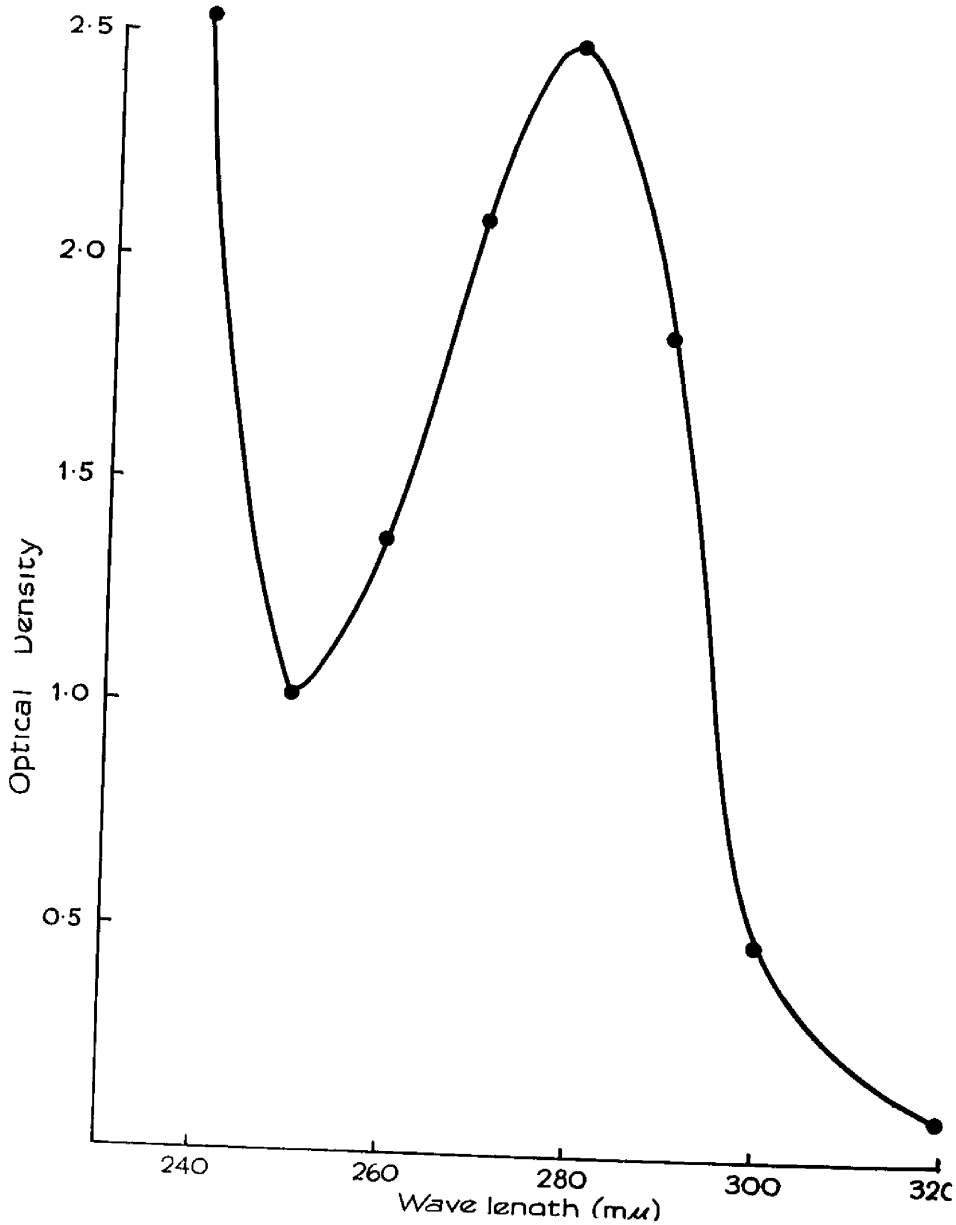

2·5
2·0
1·5
1·0
0·5
Optical Density
240
260
280
300
320
Wave lenath (mμ)

Table 26

Properties of Stage Three Fractions

Fraction Number	Volume ml.	Titre/ml.	ug.N/ml.	Potency: MHD/mg.N
1	15	8,000	60	130,000
2	38	16,000	100	160,000
3	50	16,000	144	110,000
4	50	32,000	220	145,000
5	53	32,000	250	125,000
6	24	32,000	230	140,000
7	26	8,000	175	45,000
8	26	8,000	150	50,000
9	29	1,000	75	13,000

Table 27

The stepwise fractionation of alpha toxin in Stage Four by Methanol

Fraction No.	Methanol concentration v/v	Haemolytic titre/ml.	OD 280	Potency/mg. protein
1)	15%	2,500	1.720	1,300
2)	30%	32,000	1.470	19,000
3)	40%	500,000	2.480	45,000
4)	60%	32,000	1.839	-

Thus maximum precipitation of alpha toxin took place at 40% (v/v) methanol. This fraction had a titre of 1/500,000 and was the most concentrated preparation of

toxin obtained in the present work. The absorption spectrum, as can be seen from Fig. 21, was that of a typical protein with an absorption maximum at 280 mu. Also the potency of 45,000/mg. protein was twice that of the previous stage. However, it was still far from pure in serological and ultracentrifugal analysis (Plates 4(c) and 5).

Fifth Stage. Fractions from Stage Four were further processed by passing through DEAE A 50 Sephadex at pH 5.8; the resulting fractions were immediately brought to pH 6.5 by adding N NaOH. In early experiments this resulted in a 2 - 3 fold increase in potency in comparison with Stage Four toxin; for instance, in one such experiment the $O.D._{280}$ of Stage Four toxin was 2.96 and the haemolytic titre was 1/128,000, and Stage Five toxin had a titre of 1/100,000 and an $O.D._{280}$ of only 1.1 (Fig.22). In later experiments such an increase in potency was not obtained. This may be due to an alteration at Stage Four. In early experiments material from Stage Three was used without dilution; later Stage Three toxin was adjusted to 0.4 - 0.5 before fractionation with methanol.

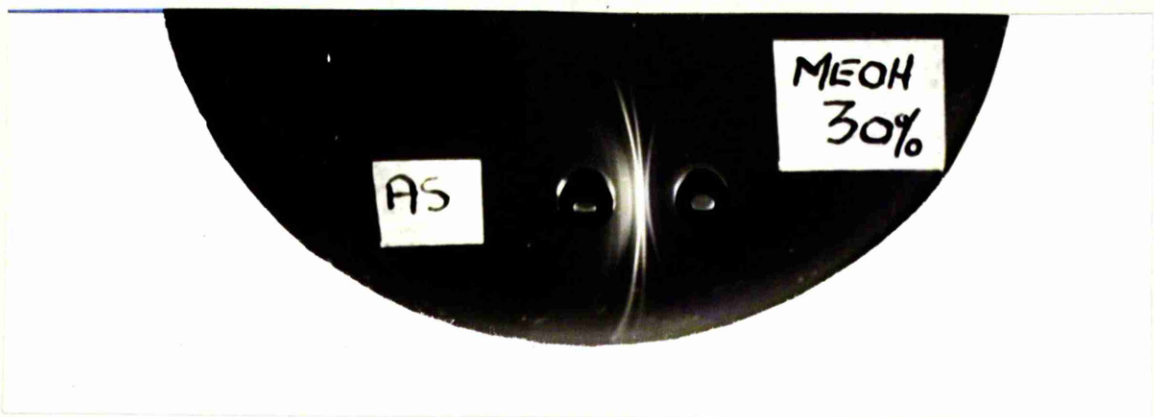
In spite of this finding it was decided to retain Stage Five since it was considered possible that it played a role other than that of removing a protein impurity. For instance it is known that Sephadex

Plate 4

Immunological Analyses of Stage Four fractions

(a)

(b)



(c)

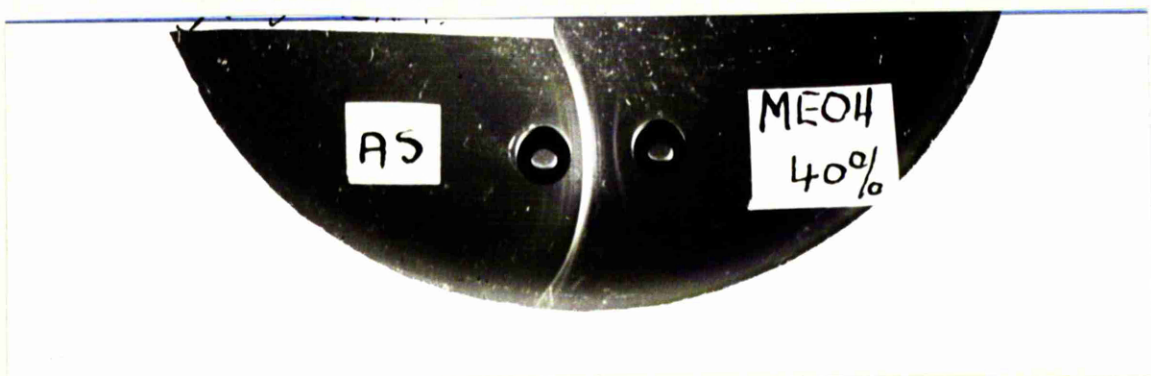
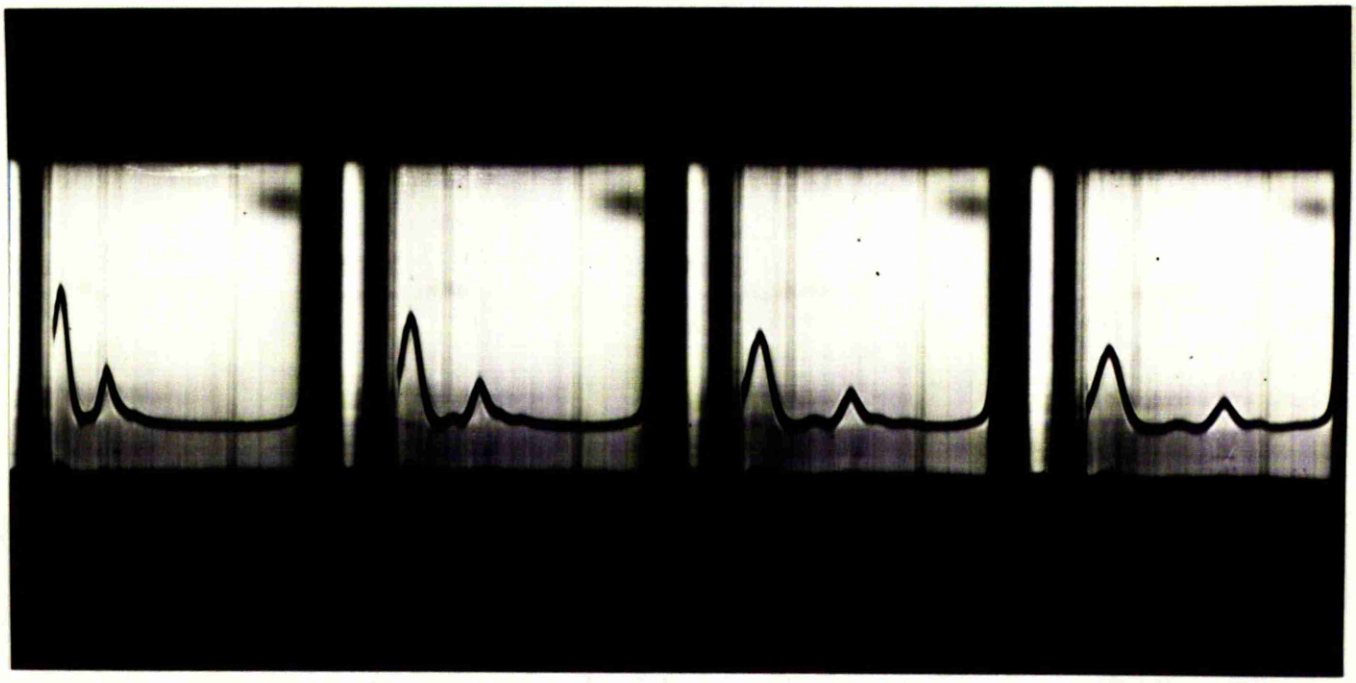


Plate 5

Ultracentrifugation of Stage Four toxin (Fraction 3)



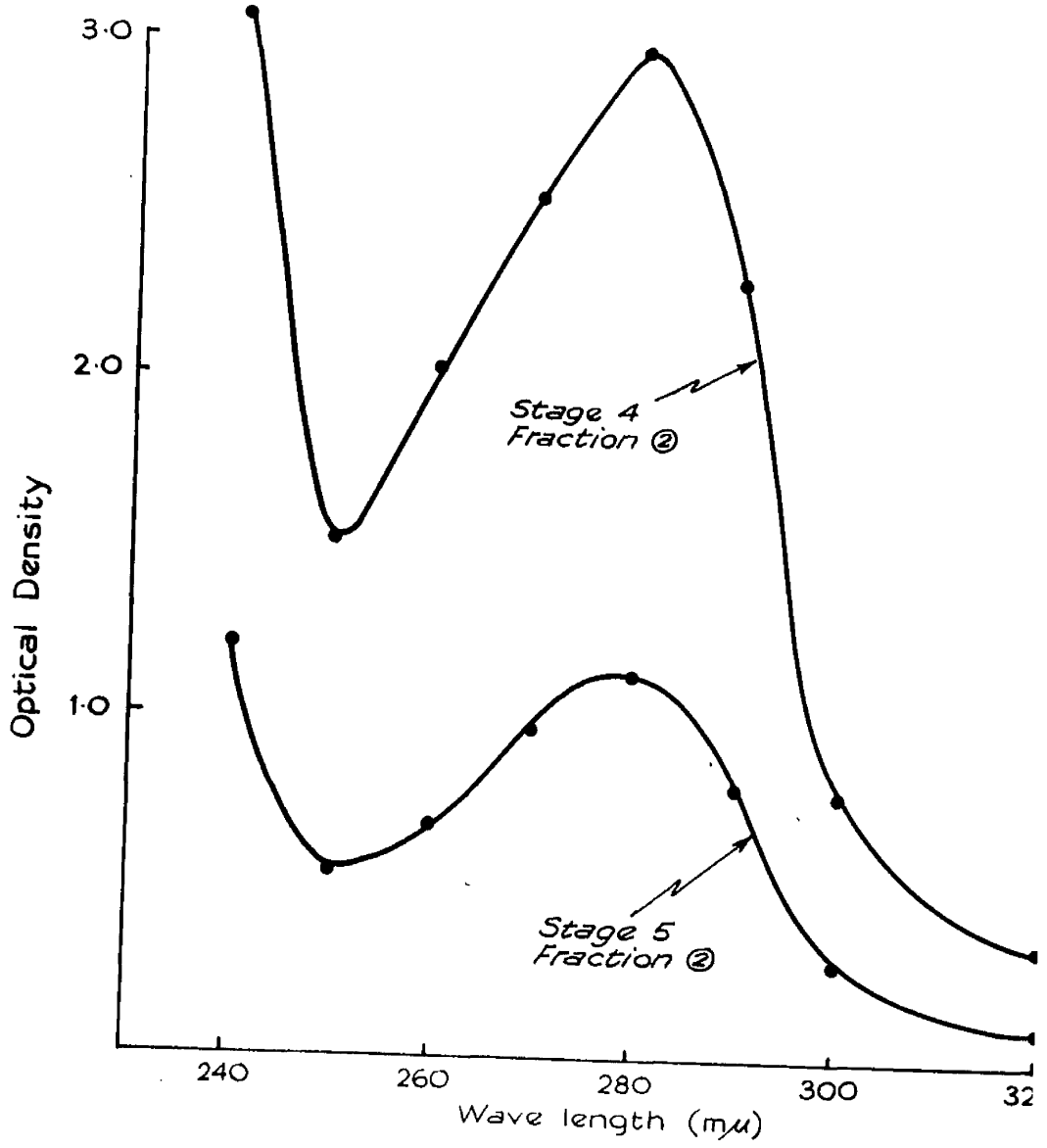
(a) (b) (c) (d)

Time after start a=22 min; b=30 min; c= 38 min;d= 46 min.

Speed 59,780 r.p.m. at 240,000 x g. Bar angle 65°

Temp. = 10°C.

Figure 22


3·0
2·0
1·0
Optical Density
Stage 4
Fraction ②
Stage 5
Fraction ②
240
260
280
300
32
Wave length (mμ)

adsorbs inorganic salts such as phosphates; indeed one of its main uses is as a desalting agent. It was therefore thought that Stage Five may serve to "dialyse" the Stage Four toxin, and for this reason it was retained.

Sixth Stage. The bulked fractions of Stage Five were now stepwise precipitated with methanol in a similar procedure to that carried out in Stage Four. The results of a typical experiment are shown in Table 28.

Table 28

The MHD/ml. and the potency/mg. protein of Stage Six fractions

Fraction	% Methanol	Temperature	O.D. 280	MHD/ml.	Potency/ mg. protein
1	15	-6°C.	1.13	2,500	20,000
2	30	-6°C.	0.332	10,000	-
3	30	-20°C.	1.173	160,000	119,000
4	40	-20°C.	0.474	40,000	100,000

In two experiments Stage Six toxin gave a single line of precipitation in double diffusion (Plates 6(a) and 6(b)) and in ultracentrifugal analysis (Plate 7). The protein concentration of these preparations was about 0.1%, and the sedimentation constant of the homogeneous protein boundary corrected for viscosity and temperature (S_{20}) was 3.1S, which suggests a molecular weight of the order of 40,000 - 50,000 (without determining the diffusion constant this can only be an approximation). This

also resulted in a single line of precipitation in immunoelectrophoresis.

However in four experiments Stage Six toxin contained 2 components (Plates 8 and 9) which were separated by further close methanol fractionation at 10% (v/v), 20% (v/v), 30% (v/v) and 40% (v/v) methanol, and by ultracentrifugation in a fixed partition cell (Plate 10(a)). As can be seen from Plate 10(b) the top half of the partition cell contained the light component which gave a single peak.

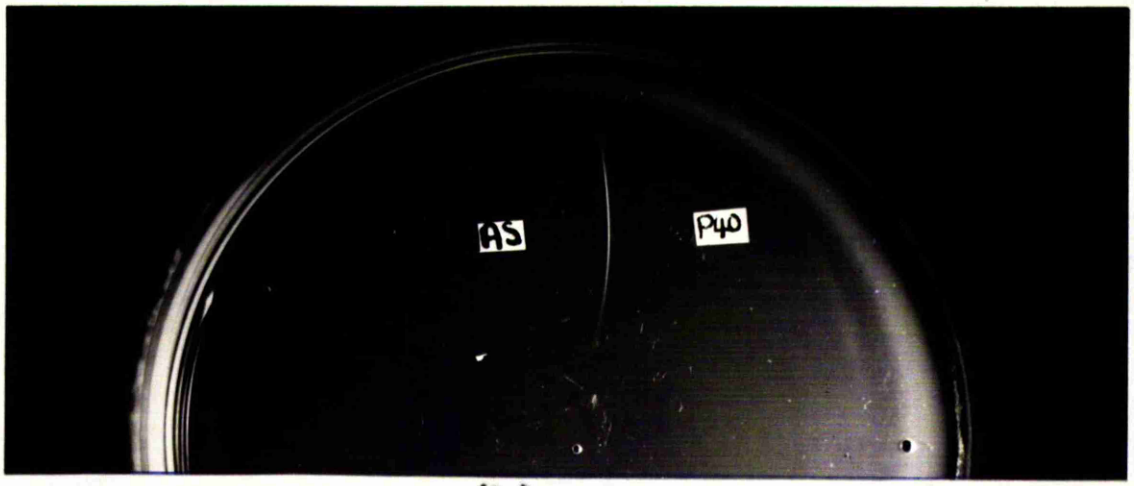
In fractional precipitation the heavy component was maximally precipitated at 10% methanol and gave a single line in double diffusion and immunoelectrophoresis (Plate 11 (a),(b)); this material had a potency of 4,000 MHD/mg. of protein. The light component was precipitated at 30% and 40% methanol, and also gave a single line of precipitation in double diffusion and immunoelectrophoresis (Plate 12(a),(b)).

Dialysis of purified toxin. Highly purified toxin with a single line of precipitation in immunoelectrophoresis, double diffusion and ultracentrifugation was now dialysed against running water at 12°C. for 18 hr. with the aim of crystallising the toxin from a salt free solution. After dialysis the pH was brought to neutrality with a drop of N NaOH and the volume reduced to $\frac{1}{2}$ by freeze-drying. A precipitate formed which on

Plate 6

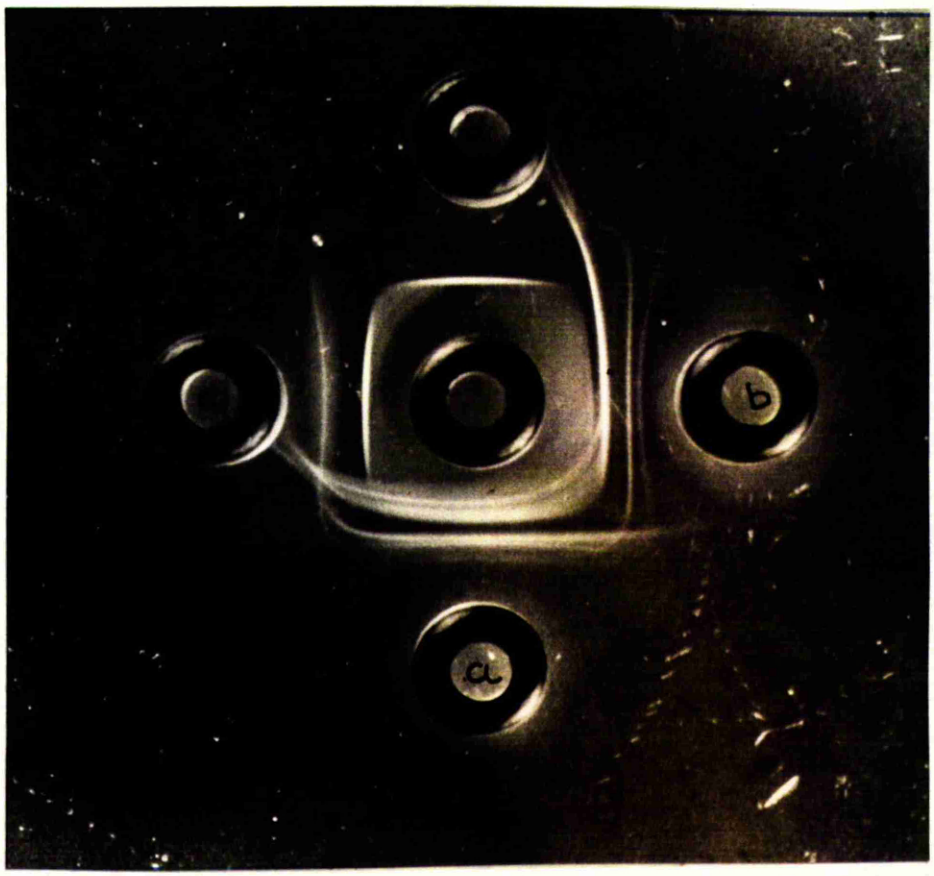
Double diffusion analysis of Stage Six.toxin.

(a)



(b)

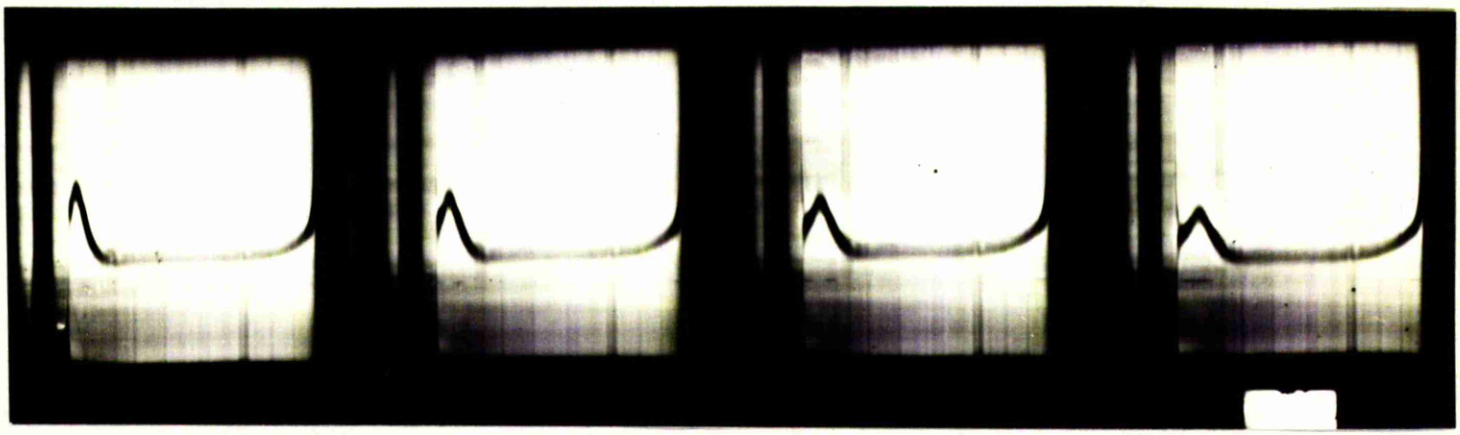
Double diffusion analyses of different stages.



a = 1st stage; b = 3rd stage; c = 5th stage; d = 6th stage.

Plate 7

Ultracentrifugation of Stage Six toxin



(a) (b) (c) (d)

Time after start: a = 27 min; b = 35 min;

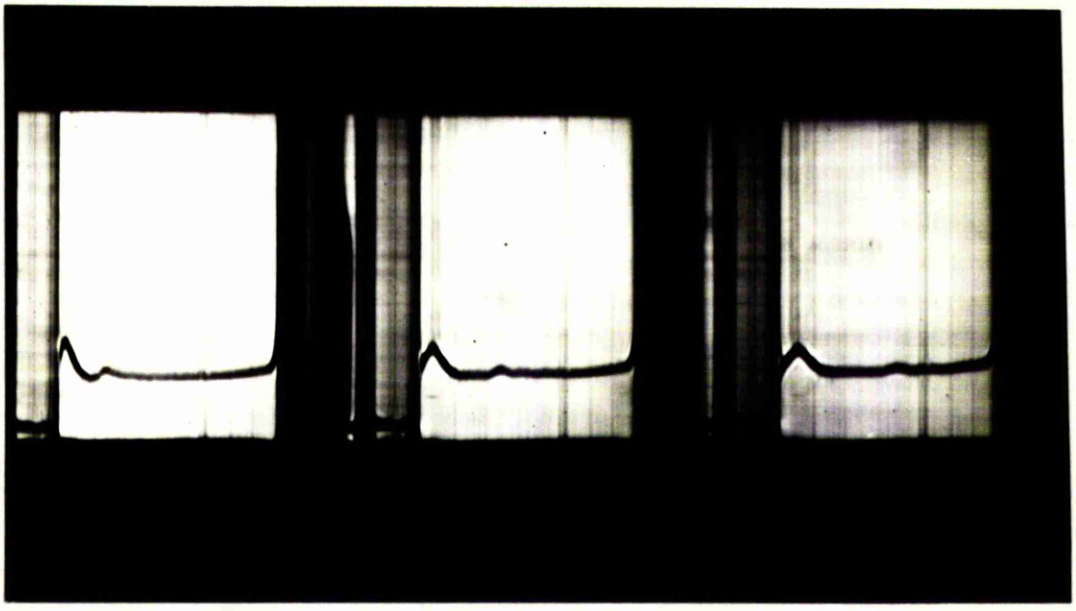
c = 43 min; d = 51 min.

Speed 59,780 at 240,000 x g. Bar angle 45°.

Temp = 10°C

Plate 8

Ultracentrifugation of Stage Six toxin, showing 2 components



(a) (b) (c)

Time after start: a = 28 min ; b = 33 min ; c = 41 min ;

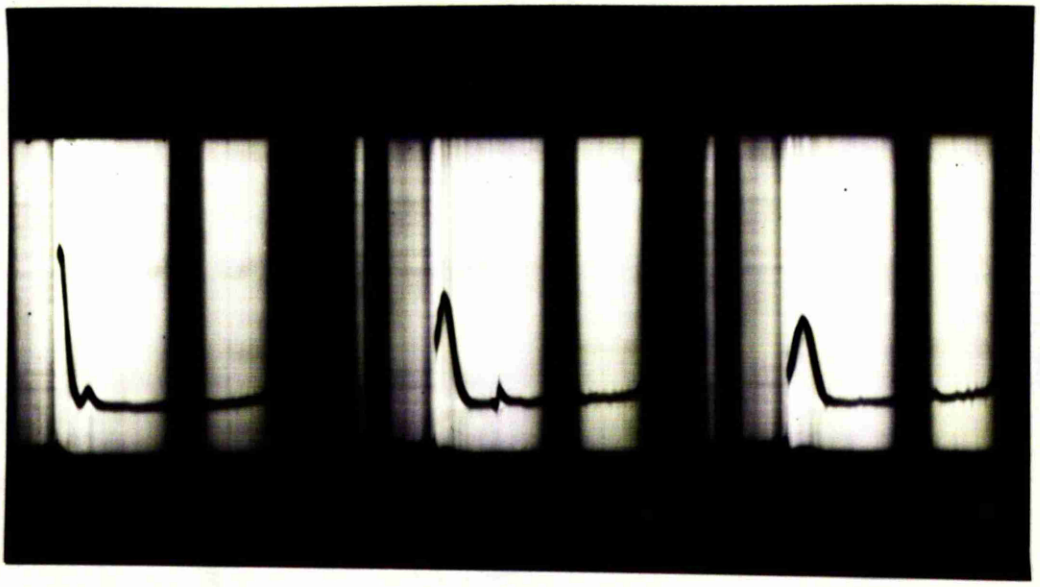
Speed 59,780 Bar angle 60° Temp = 17.5°C .

Plate 9

Fraction 4, Stage Six showing 2 components in double diffusion.

Plate 10 (a)

Separation of heavy and light components in a fixed partition cell.



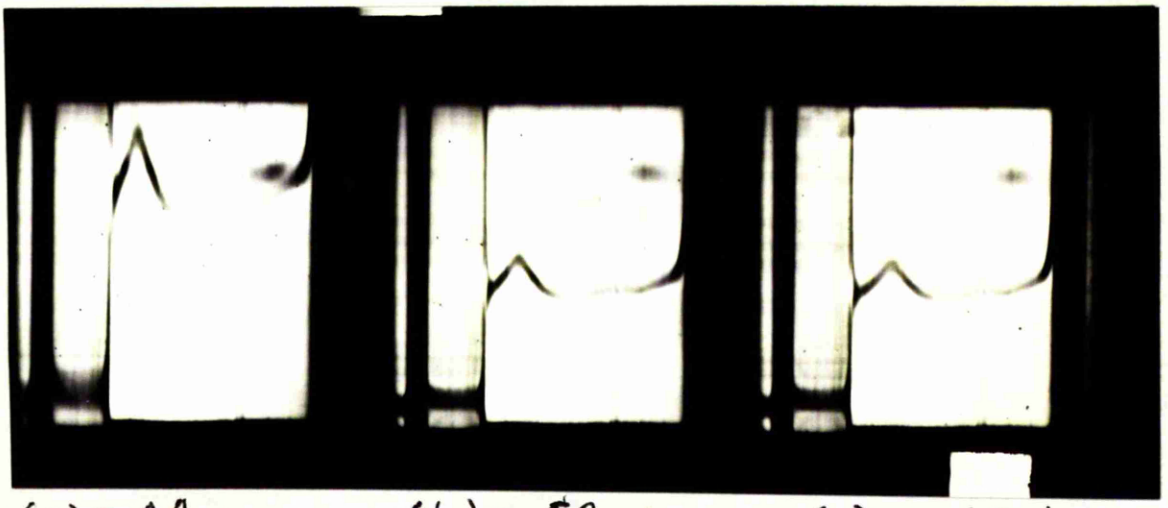
(a) (b) (c)

Time after start: a = 19 min ; b = 27 min ; c = 35 min;

Speed 59,780 Bar angle 65°

Plate 10 (b)

Top half of partition cell after separation.



(a) = 44 min (b) = 52 min (c) = 60 min

Bar Angle = 25° 40° 40°

Plate 11 (a)

Double diffusion analysis of 10% (v/v) methanol fraction.

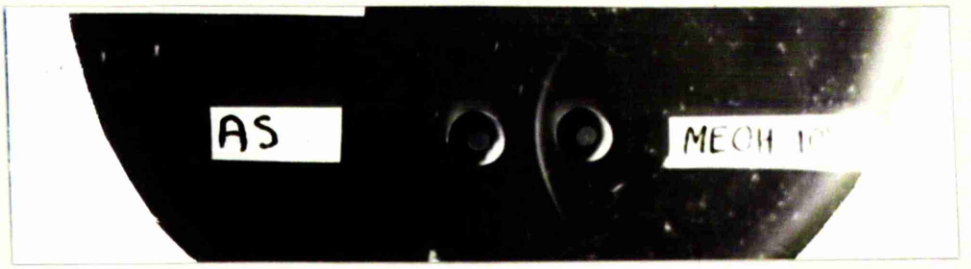


Plate 11 (b)

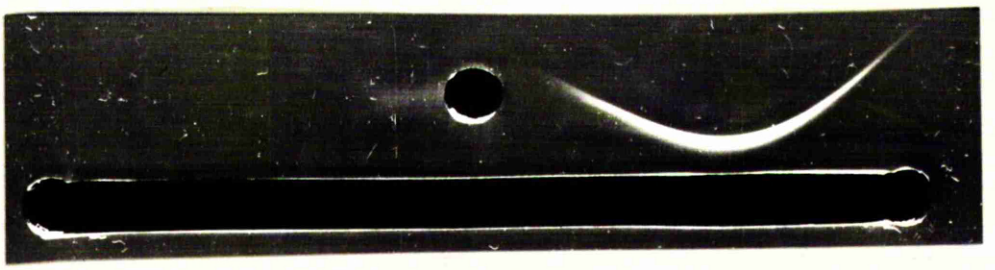
Immunoelectrophoretic analysis of 10% (v/v) methanol fraction.

Plate 12 (a)

Double diffusion analysis of 30% (v/v) methanol fraction.

Plate 12 (b)

Immunoelectrophoretic analysis of 30% (v/v) methanol fraction.



examination under the microscope appeared to consist both of amorphous and crystalline material. Typical examples of this crystalline material can be seen in Plate 13. Since dialysis would be expected to remove all traces of salts and small molecular weight substances, such as amino acids, it is difficult to escape the conclusion that these crystals were indeed of alpha toxin. If so, then this would be the first time that staphylococcal alpha toxin had been crystallised. Unfortunately due to scarcity of material it has been so far impossible to further characterise the crystals.

The precipitate was now redissolved by adding distilled water until the original volume was reached and the solution was submitted for ultracentrifugal analysis. Surprisingly the only component of the preparation now apparent had a sedimentation constant of 16 S (Plates 14 and 15); in double diffusion and immunoelectrophoretic analysis two components were found (Fig.23). The line of precipitation in immunoelectrophoresis was in the shape of a "Cupid's bow"; Dr. J. R. Anderson of the Department of Pathology, The University, suggested that this resembled patterns obtained from preparations which contained a mixture of two forms of the same antigen with different electrophoretic mobilities. These changes were

Plate 13

Crystals observed in partially freeze-dried preparations.

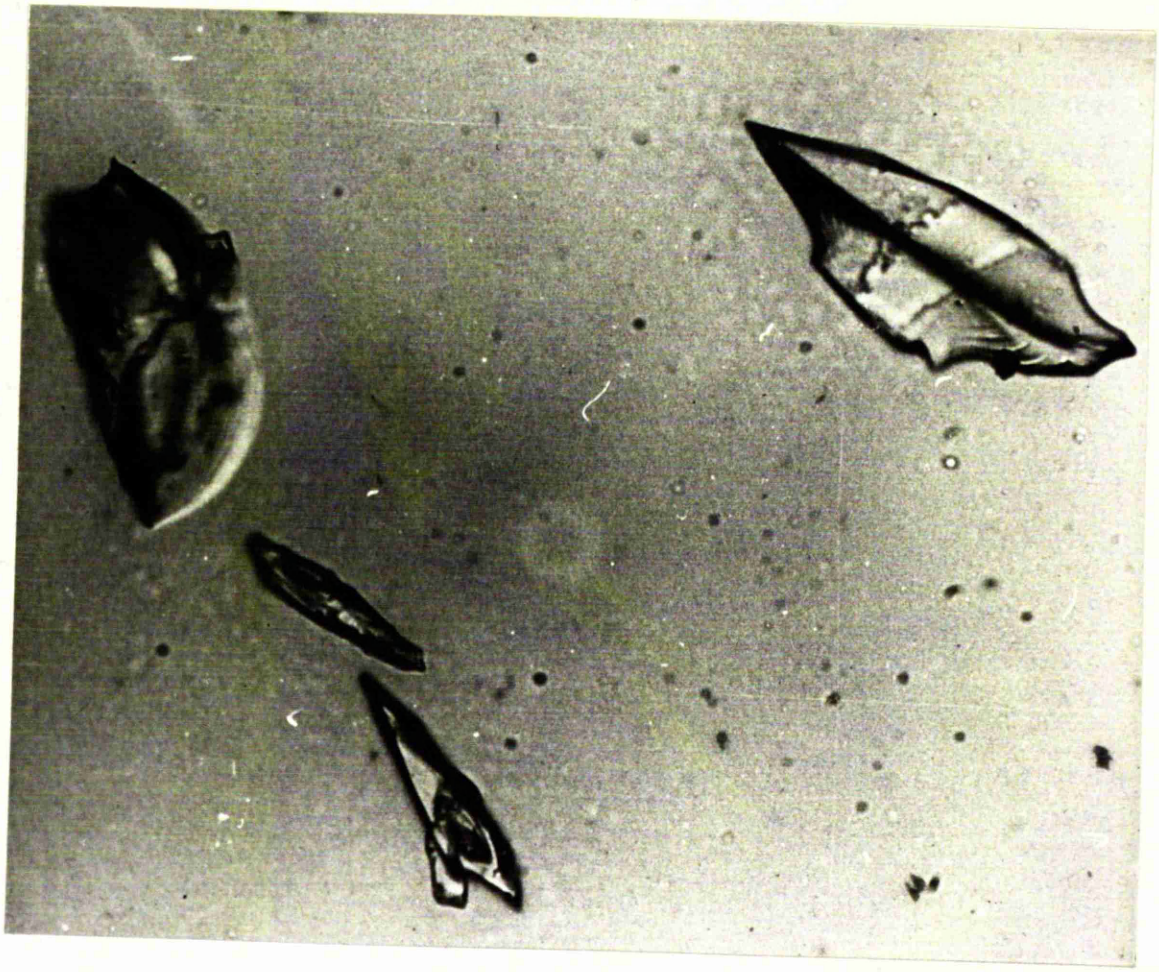
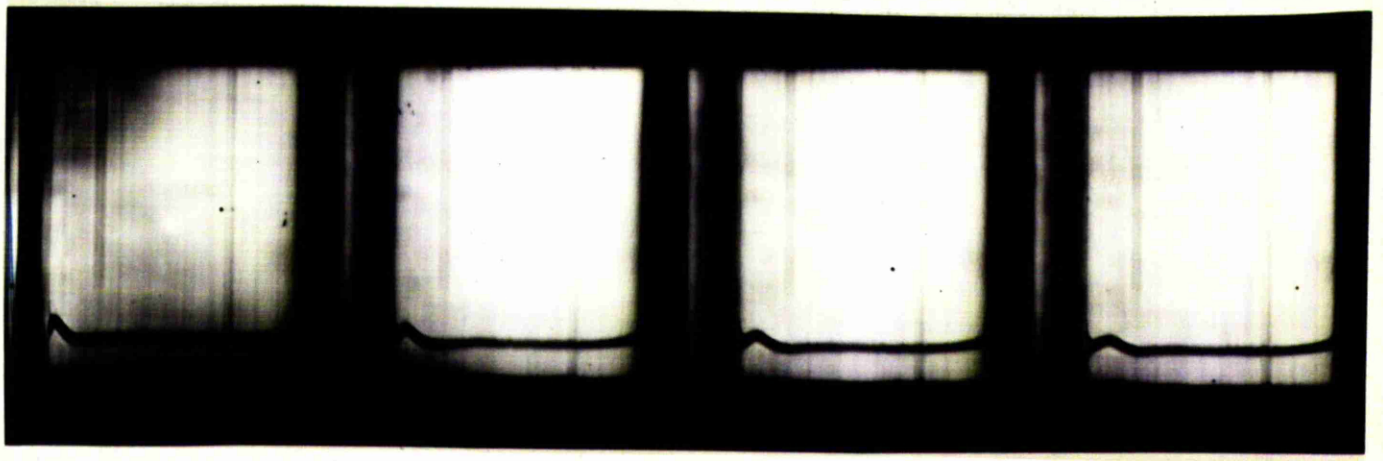


Plate 14

Ultracentrifugation of purified alpha toxin before dialysis



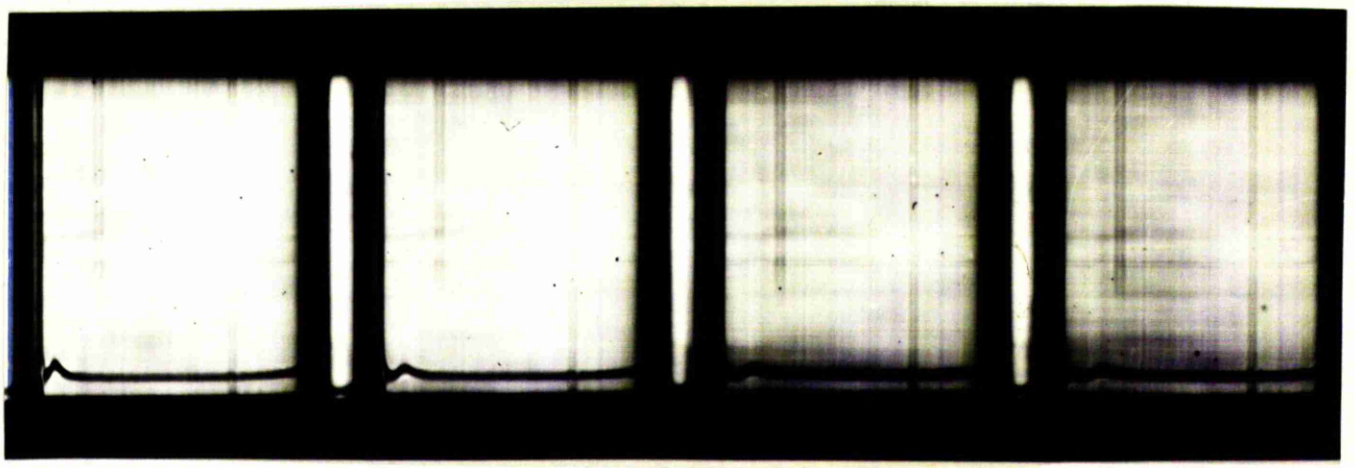
(a) (b) (c) (d)

Time after start: a = 22 min; b = 30 min; c = 38 min; d = 46 min.

Speed 59,780 r.p.m. Bar Angle = 65°. Temp = 5°C

Plate 15

Ultracentrifugation of purified alpha toxin after dialysis



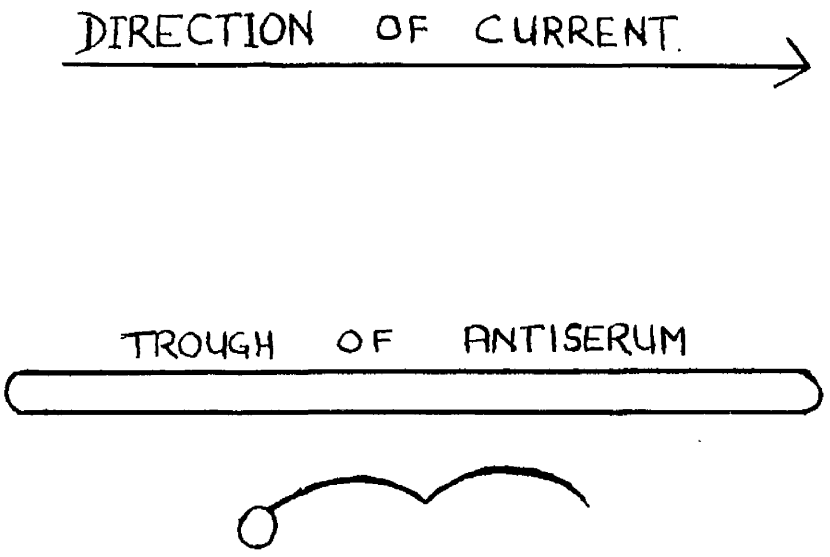
(a) (b) (c) (d)

Time after start: a = 15 min; b = 23 min; c = 31 min; d = 39 min.

Speed 31,410 r.p.m. Bar Angle = 65°. Temp = 5°C

Figure 23

Diagram of immunoelectrophoretic pattern of dialysed toxin.



accompanied by an eight times reduction in haemolytic potency.

The above findings suggested that during dialysis alpha toxin underwent polymerisation with the resulting formation of a large molecular weight compound which has a sedimentation constant of 16S. Indeed it is difficult to conceive an explanation other than polymerisation. As will be discussed later, Tetanus toxin is known to polymerise readily in the highly purified state. Here it is interesting to reconsider the heavy component, mentioned previously, which was present in the final stage of some experiments. This also had a sedimentation constant of 16S. It seemed a possibility therefore that this heavy component was also formed in the course of purification due to polymerisationand that it may be identical with that formed on dialysis. There are two pieces of evidence in favour of this possibility: highly purified preparations rapidly lose their haemolytic activity on standing, even at low temperatures, and a 16S component appeared in the course of purification. Possible mechanisms of polymerisation will be discussed later, but it is fully realised that more work is required to clarify this aspect.

Yields. The potency at different stages in purification and the yields for a typical experiment are given in Table 29. From column 4 it can be seen that there is

a considerable loss of activity in the course of purification. Indeed the final yield is only about 3.5%. This loss may be partially accounted for in terms of polymerisation in the course of purification. But the greatest loss which occurs between Stage Five and Stage Six may be due to the almost equal distribution at Stage Six between fractions 3 and 4 (Table 28).

Table 29

The haemolytic activity and potency of different stages.

Stage	MHD/ml.	MHD/mg. protein	Total No. of MHD/ preparation
Crude	8,000	270	18,000,000
1st stage	64,000	9,000	14,000,000
2nd stage	32,000	12,800	7,000,000
3rd stage	16,000	25,000	3,300,000
4th stage	500,000	45,000	3,000,000
6th stage	160,000	119,000	640,000

Difficulties experienced in purification. It must be stressed that, to obtain pure toxin by the method outlined above, it is necessary to adhere strictly to the details of the procedure. This was appreciated when in one experiment, using a large volume of toxin kindly supplied by The Wellcome Research Laboratories as starting material, considerable difficulty was encountered.

The toxin used was prepared by an entirely different method and the purification was scaled up to deal with large volumes of starting material. In spite of these difficulties pure toxin was obtained by slight alteration of the procedure involving freeze-drying.

Biological properties of purified alpha toxin. Alpha toxin purified to the degree of a single homogeneous protein preparation possessed virtually the same biological properties as crude alpha toxin. It was haemolytic, lethal and dermonecrotic. The lethal dose in terms of MHD was the same for both purified and crude toxin. Table 30 shows a summary of the potency of pure toxin in terms of MLD/kg. for different species.

Table 30

The potency of alpha toxin/kg. of tissue/u mole

Animal	MLD/ug.	MLD/u mole
Frog	0.003	120
Fowl	0.02	800
Mouse	0.025	1,000
Rabbit	1.0	40,000

These values were calculated accepting 40,000 as the molecular weight of alpha toxin.

The MHD for rabbit red blood cells was calculated as being 0.008 ug. and 0.5 - 1 ug. of toxin were found to be dermonecrotic when injected in 0.1 ml. intradermally in the rabbit.

By contrast with crude toxin however, these preparations were highly thermolabile, and did not show the Arrhenius phenomenon.

DISCUSSION

The purification procedure itself is a long and somewhat tedious method. Bearing in mind, however, the extremely complex nature of the medium used for the production of crude toxin and the considerable number of extracellular products of the staphylococcus, it seems likely that any effective method will of necessity consist of numerous stages. Here it is interesting to note that the other toxins which have been purified, such as tetanus, botulinus and diphtheria were all prepared in a relatively simple medium containing little non-bacterial protein, and the resulting crude toxin contained only one major bacterial protein, the toxin itself. This may explain why these toxins were purified some 15 - 20 years ago.

The yield of the purified alpha toxin was very low. Again however, the separation of a complex mixture of many components will almost certainly entail a considerable loss of any one of them in purification due to the spreading of its activity in fractions containing substances with similar physical properties. In spite

of these drawbacks, the present method did result in the production of a serologically pure and physically homogeneous preparation which was potent *in vivo* and *in vitro*. In this it offers considerable advantage over the previous methods outlined in the Introduction (p.30), which resulted either in preparations which are extremely weak, or which are potent but impure. At the same time the final product contained only 0.15% protein and it is possible that further improvement of the method to yield a more concentrated protein preparation at the final stage will reveal the presence of impurities, which are not absent, but merely undetectable in the present preparations. It is not claimed that the method described here is the best method for the purification of alpha toxin, but that it will provide a basis for further fruitful investigation of the properties of pure alpha toxin. For instance, the electrophoretic mobility, the diffusion constant, and the molecular weight from osmotic and solubility measurements have still to be determined.

The sedimentation constant of 3.1 S observed in the present work is almost identical with that of 3.0 S described by Bernheimer and Schwartz (1963) whose comprehensive study of the purification of alpha toxin by a method different from that described in the present thesis reached this laboratory after the experimental

work had been completed; indeed it appeared on the morning that the relevant paper was submitted for publication. Both of these values differ considerably from the value of 1.4 S which was recorded by Kumar and Locken et al. (1962); however, as mentioned previously, these workers may have purified a concomitant protein which was contaminated with small amounts of alpha toxin. The value of c. 3.0 S is in good agreement with a molecular weight of 44,000 suggested by Bernheimer and Schwartz (1963) from a study of the amino acid composition of alpha toxin; determination of the diffusion constant would however strengthen this argument. A comparison of alpha toxin with other bacterial toxins which have been purified can be seen in Table 31.

Table 31

A comparison of the physical properties of alpha toxin with other bacterial toxins.

Toxin	Crystallised	Sedimentation const.	Diffusion const.	Molecular Weight
Botulinus Type A	Yes	17.3S	2.14×10^{-7}	900,000
Botulinus Type B	No	-	7.22×10^{-7}	60,000
Tetanus	Yes	4.5S	-	c. 70,000
Diphtheria	No	4.6S	6.0×10^{-7}	72,000
Streptococcus Group A	No	2.7S	-	27,000
Staphylococcus Alpha	?	3.0S	-	44,000

Properties of toxins other than staphylococcus alpha toxin reprinted from Greenberg (1951).

Serological examination of the purified material which gave a single peak in ultracentrifugation showed the presence of a single line of precipitation in double diffusion tests and also in immunoelectrophoresis. Double diffusion tests by themselves are not sufficient to show that the preparation is serologically pure because two different antigens may diffuse at the same rate and the mixture will show the presence of only one line of precipitation. However, immunoelectrophoresis separates the antigens present in a mixture by virtue of their charge and it is highly unlikely that two antigens will both diffuse and electrophorese at the same rate. This in fact is one of the most sensitive tests available for assessing the purity of preparations. Indeed preparations which are apparently pure in physicochemical tests are often impure in immunoelectrophoresis.

Purified alpha toxin was unstable even when stored at low temperatures and lost 90% of its activity when dialysed; instability in dialysis has also been observed by Bernheimer and Schwartz (1963). This instability was associated with the appearance of a second component in serological and ultracentrifugal analysis, with a sedimentation constant of 16 S. It seems reasonable to suggest that this represents a polymerised form of alpha toxin. The heavy component when separated by

methanol fractionation was found to have a haemolytic potency of 4,000 MHD/mg. protein. Whether the 16 S component is an inactive polymer contaminated with traces of alpha toxin, or a polymer which has retained a small amount of its original activity is, as yet, impossible to say. Tetanus toxin is also unstable on storage at 0°C. and a second component with a sedimentation constant of 7.0 S appeared on standing (Pillemer et al., 1948). These workers suggested that the 7.0 S component was a dimer of the active toxin. However, in the case of alpha toxin it would seem that polymerisation by a factor of some 5 or 6 fold has occurred. It is unlikely, though not impossible, that the polymerisation is a single step reaction; a more likely mechanism would be the formation of first a dimer, followed by a four-fold and then a six-fold polymerisation. If this were so, then one would expect to find intermediates with the appropriate sedimentation constant. Although these have not yet been seen it may be that the intermediates are present only in undetectable amounts. A 12 S component has been observed in highly purified preparations by Bernheimer and Schwartz (1963) and this may be such an intermediate polymer.

No uniform crystalline preparations of alpha toxin were observed during the fractionation procedure, but after dialysis and reduction of the volume some crystals

were seen along with the amorphous material. It seems likely that these are crystals of alpha toxin since inorganic salts should have been removed in dialysis.

In biological characteristics purified alpha toxin is similar to the crude toxin. It is haemolytic, dermonecrotic and lethal. The lethal dose of pure alpha toxin in terms of MHD is the same as that for crude toxin. These findings are strongly in favour of the unitarian hypothesis (Introduction, p.22) that all three properties are manifestations of a single factor, and are in agreement with the findings of Madoff and Weinstein (1962) Kumar and Locken et al. (1962) and Bernheimer and Schwartz (1963). However, the lethal properties differ from those described by Goshi, Cluff and Norman (1963); as mentioned previously (p. 33) these workers may have purified a toxin other than alpha toxin, possibly gamma toxin.

By comparison with Botulinus type A and B toxin, Tetanus toxin and Diphtheria toxin, staphylococcal alpha toxin is not highly potent. With a potency of about 44,000 MLD/kg. rabbit tissue/u mole it is very close to the value for Cl. welchii alpha toxin in van Heyningen's (1950) classification of toxic substances.

SUMMARY

The present thesis describes the general properties, the mode of action *in vivo* and *in vitro*, and a method of purification, of staphylococcal alpha toxin.

The optimum pH and temperature of the haemolytic reaction were found to be 5.5 and 31°C. respectively. In proteolytic digestion and heat sensitivity, the toxin behaved as a typical protein. Contrary to current views, alpha toxin was found to be intrinsically heat sensitive. A revised explanation of the paradoxical Arrhenius phenomenon is suggested and discussed in the light of this finding.

An investigation of the mechanism of action *in vitro* showed that alpha toxin is not used up in the course of haemolysis; it acts as a catalyst. Kinetic experiments revealed that the relationship between the rate of haemolysis and the concentration of alpha toxin was compatible with an enzymic reaction, at low concentrations of toxin. At high concentrations there was a marked falling off; possible reasons for this are suggested and discussed, and the limitations of haemolysis as an indicator system are pointed out. In addition the velocity of the reaction was shown to be dependent on the concentration of the rabbit red blood cells present. Taking into account the bulk of the evidence it is

concluded that alpha toxin belongs to the group of bacterial haemolysins which are thought to be enzymes.

Divalent ions are not required for haemolysis. Inhibitors of bacterial proteases had no effect. Heavy metal salts such as mercuric chloride were found to inhibit haemolysis at concentrations of 10^{-3}M; (below this, rather surprisingly, they became haemolytic by themselves). Some organic sulphydryl inhibitors also inhibited alpha toxin; it seems therefore that free SH groups may play a part in haemolysis. A group of substances related to the trypanocidal drug, Suramin, also acted as potent inhibitors of both the haemolytic and the lethal activity of alpha toxin.

Phospholipids were tested as possible competitive inhibitors of alpha toxin in an attempt to gain information concerning the point of attack. Apart from a crude preparation of sheep brain cephalin, none of them inhibited. The possible significance of this finding is discussed.

Death from alpha toxin was found to be dose dependent: it was either very rapid, or occurred after considerable delay. Dose dependence and the pattern of death was largely the same in the four species examined, viz.,

rabbits, mice, fowl and frogs. Rapid death in seconds or minutes, without histological lesions, is most likely explained by action on heart or central nervous system. Slow death occurring after several hours or days may result from lesions in a multiplicity of organs, liver, kidney, or vascular system. Large doses (which killed rapidly intravenously) when administered subcutaneously or intraperitoneally killed much slower, probably because of the time required for diffusion of toxin to vital organs.

When injected into the dorsal sac of 6 weeks' old mice, alpha toxin caused local flaccid paralysis of voluntary muscle; at high doses this occurred before any detectable histological lesions were formed. Muscles of the paralysed limbs did not respond to electrical stimulation in situ. In the presence of alpha toxin the reactivity of excised voluntary muscles of mice and frogs to acetyl-choline and electrical stimulation were abolished in vitro. Since muscles of curarised mice behaved in the same way, it is concluded that alpha toxin has a direct myotoxic action on voluntary muscle.

By contrast, heart muscle appeared less sensitive. Hearts of mice killed with alpha toxin continued to beat after death for a few minutes, and hearts of frogs for periods up to several hours. In tissue cultures mouse-

heart explants were less affected than the whole animal. Whereas 128 MHDs is an LD 50 for 20 g. mice, explants of 20 - 1,000 cells were only moderately affected by 2,000 MHD/ml. of tissue fluid: some cells continued to beat, some stopped after 30 minutes, but recovered overnight. The possible significance of these findings is discussed.

Alpha toxin, purified by the combined use of gel filtration on G 75 and DEAE A 50 Sephadex columns and fractional methanol precipitation, behaved as a serologically and physically homogeneous protein. The sedimentation constant of preparations containing 0.13% protein was 3.1 S which is in good agreement with the value of 3.0 S recently suggested by Bernheimer and Schwartz (1963). The potency of such preparations was 119,000 MHD/mg. of protein. The resulting product was unstable and evidence was obtained which suggested that on standing and dialysis alpha toxin tends to polymerise with the appearance of a heavy (16 S) component. The method is time-consuming and tedious and results in a low yield. However, this is possibly an inherent drawback in attempting to isolate a toxin from a complex mixture containing constituents of the medium and a large number of other staphylococcal products.

BIBLIOGRAPHY

Abramson, H.A. (1929) J.gen.Physiol., 12, 711.
Ambache, N. (1949) J. Physiol., 108, 127.
Arbuthnott, J.P. (1962) Nature, 196,277.
Arrhenius, S. (1907) Immunochemistry, New York.
Atkin, E. E. (1910) Z. Immunforsch., 7, 656.
Avery, R.C., Rigdon, R.H. & Johlin, J.M. (1937) Proc.Soc. exp. Biol., 37, 131.
Barber, M. (1942) Brit. med J., 1, 407.
Barber, M., Brooksbank, B.W.L. & Kuper, S.W.A. (1951) J. Path. Bact., 63, 57.
Behring, E. von. & Kitasato, S. (1890) Dtsch. med. Wschr., 16, 1113.
Bernheimer, A.W. (1944) J. exp. Med., 80, 333.
Bernheimer, A.W. (1947) J. gen. Phys., 30, 337.
Bernheimer, A.W. & Schwartz, L.L. (1961) Proc.Soc. exp. Biol., 106, 776.
Bernheimer, A.W. & Schwartz, L.L. (1963) J. gen. Microbiol., 30, 455.
Beumer, J. (1939a) C.R. Soc. Biol., 130, 283.
Beumer, J. (1939b) C.R. Soc. Biol., 130 286.
Bigger, J.W. (1933) J.Path. Bact., 36, 87.
Bigger, J.W., Boland, C.R. & O'Meara, R.A.Q. (1927,a) J.Path. Bact., 30, 261.
Bigger, J.W., Boland, C.R. & O'Meara, R.A.Q. (1927,b) J.Path. Bact., 30, 271.
Birch-Hirschfeldt, F.V. (1872) Schmidt's Jb., 155, 97.
Blair, J.E. (1939) Bact. Rev., 3, 97.
Boivin, A. & Izard, Y. (1937) C.R. Soc. Biol., 124, 25.
Borthwick, G.R. (1933) Brit. J. exp. Path., 14, 236.
Bramann, J. & Norlin, G. (1951) Acta. path. microbiol. scand., 29, 127.
Broiger, L. (1885) Untersuchungen uber Ptomeines, Berlin.
Broiger, L. & Fraenkel, C. (1890) Berl. klin. Wschr., 27, 241.
Burgen, A.S.V., Dickens, F. & Zatman, J.L. (1949) J. Physiol., 109, 10.
Burkey, E.L. (1933) J.Immunol., 24, 93.
Burnet, F.M. (1929) J. Path. Bact., 32, 717.
Burnet, F.M. (1930) J. Path. Bact., 33, 1.
Burnet, F.M. (1931) J. Path. Bact., 34, 471.
Burnet, F.M. & Freeman, M. (1932) J. Path. Bact., 35, 477.
Butler, L.O. (1959) Biochem. J., 71, 67.
Gasman, E.P. (1938) J. Bact., 35, 13.
Gasman, E.P. (1940) J. Bact., 39, 84.
Chapman, (1940) J. Bact., 39, 5.
Christie, R. & Keogh, E.V. (1940) J. Path. bact., 51, 189
Cowan, S.T. (1938) J. Path. Bact., 46, 31.
Cowan, S.T. (1939) J. Path. Bact., 48, 169.
Cummins, S.L. & Cummins, C.C. (1913) J.Roy. Army Med. Crps., 20, 499
Dack, G.M. Woolpert, O. Corrie, W.E. & Wiggers, H. (1930) J.prev. Med., 4, 167
De Christmas, M.J. (1888) Ann. Inst. Past., 2, 469
De Navasquez, S. (1938) J. Path. Bact., 46, 47.
Dicker, E. (1935) C.R. Soc. Biol., 120, 793.
Dinglo, J.H., Hoff, H.E., Nahum, L.H. & Carey, B.W. (1937) J.Pharmacol., 61,121
Dixon, M. & Webb, E.C. (1958) Enzymes, London.
Dolman, C.E. (1934) J. infect. Dis., 55, 172
Dolman, C.E. & Kitching, J.S. (1935) J.Path. Bact., 41, 137
Dooren de Jong, L.E. den (1931) Zent. Bakt. 1. Abt. Orig., 120, 1.

Duthie, E.S. & Lorenz, L. (1949) Biochem. J., 44, 173.
Duthie, E.S. & Wylie, J.A.H. (1945), Brit. J. exp. Path., 26, 130.
Elek, S.D. (1948) Brit. med. J., 1, 493.
Elek, S.D. (1959) Staphylococcus Pyogenes, Edinburgh and London.
Evans, J.B. Buttner, L.G. & Niven, C.F. Jr. (1952) J. Bact., 64, 433 .
Farkas, H. (1947) J. Bact., 53, 401.
Favorito, G.D. & Hammon, W. McH. (1941) J. Bact., 41, 305.
Fisk, R.T. (1942 a) J. infect. Diz., 71, 153.
Fisk, R.T. (1942 b) J. infect. Dis., 71, 161
Flaum, A. (1938) Acta. path. microbiol. scand. Suppl., 35, 1.
Flaum, A. & Forsmann, J. (1936) Acta. path. microbiol. scand., 13, 263
Forsmann, J. (1933) Biochem. Z., 265, 291.
Forsmann, J. (1934 a) Acta. path. microbiol. scand., 11, 214.
Forsmann, J. (1934 b) Acta. path. microbiol. scand., 11, 452.
Forsmann, J. (1934 c) C.R. Soc. Biol., 117, 639
Forsmann, J. (1939) Acta. path. microbiol. scand., 16, 335
Gablicks, J. & Solotorovsky, M. (1961) J. Immunol., 88, 505.
Ganczarski, A. (1962) Lodz. Towarzystwo. Naukowe. Soc. Sci., 44,
Gengou, O. (1934-35). Arch. int. med. exp., 2, 413
Gengou, O. (1935) Ann. Inst. Past., 55, 129
Gillespie, W.A. & Alder, V.G. (1952). J. Path. Bact., 64, 187.
Gladstone, G.P. (1937). Brit. J. exp. Path., 18, 322.
Gladstone, G.P. (1938). Brit. J. exp. Path., 19, 208.
Gladstone, G.P. & van. Heyningen, W.E. (1957) Brit. J. exp. Path., 38, 123.
Glenny, A.T. & Stevens, M.F. (1935) J. Path. Bact., 40, 201.
Gordon, M. (1904) 33rd Ann. Rept. Local. Govt. Board. Suppl. Rept. of Med. Officer, 388.
Goshi, R., Cluff, L.E. & Norman, P.S. (1963). Johns Hopk. Hosp. Bull.,112, 15.
Gratia, A. & Nelis, P. (1938) C.R. Soc. Biol., 127, 350.
Greenberg, D.M. (1951) Amino Acids and Proteins, Illinois.
Gross, H. (1931) Zent. Bakt. 1 Abt. Orig., 123., 212.
Harper, E.M. & Conway, N.S. (1948) J. Path. Bact., 60, 247.
Hartley, P. & Smith, M.L. (1935) League of Nations, Quart. Bull. Hlth., 68.
Hendry, E.B. (1948) Edinb. med. J., 55., 142.
Holt, L.B. (1936) Brit. J. exp. Path., 17, 318.
Jackson, A.W. & Little, R.M. (1957) Canad. J. Microbiol., 3, 47
Jensen, K. Neter, E. Gorzynski, E.A. & Anzai, H. (1961) Acta, path. microbiol. scand., 53, 191.
Jones, D.B. (1941) Circular U.S. Dept. Agr. (Revised No. 183).
Julliannelle, L.A. (1922) J. infect. Dis., 31, 256
Kelloway, C.H., Burnet, F.M. & Williams, F.E. (1930) J. path. Bact., 33, 889
Klebs, E. (1872) Beitrage zur pathalogischen Anatomie der Schusswunde, Leipzig.
Knight, B.C.J.G. (1935) Brit. J. exp. Path., 16,315.
Knight, B.C.J.G. (1937 a) Nature, 139, 628.
Knight, B.C.J.G. (1937 b) Biochem. J., 31, 731.
Knight, B.C.J.G. (1937 c) Biochem.J., 31, 996
Knight, B.C.J.G. & McIlwain, H. (1938) Biochem J., 32, 1241
Kodoma, T. & Nishiyama, S. (1938) Kitasato Arch. exp. Med., 15, 247.

Kraus, R. & Clairmont, P. (1900) Wein. klin. Wschr., 13, 49.
Kraus, R. & Pribram, E. (1906) Wein. klin. Wschr., 19, 493.
Kumar, S. & Lindorfer, R.K. (1962) J. exp. Med., 115, 1095.
Kumar, S., Loken, K.I., Konyon, A.J. & Lindorfer, R.K. (1962) J. exp. Med., 115, 1107.
Lalli, G. & Orlandi, A. (1953) Nuovi. Ann. Ig. Microbiol., 4, 182.
Landsteiner, K. & Rauchenbichler, R. von. (1909) Z. Immunforsch., 1, 439.
Lasfargues, E. & Delauney, A. (1946) Ann. Inst. Past., 72, 38.
Le Fevre D'Arric, (1919) (a) C.R. Soc. Biol., 82, 1313.
Le Fevre D'Arric,(1919) (b) C.R. Soc. Biol., 82, 1331.
Levine, B.S. (1938) J. Immunol., 35, 131.
Levine, B.S. (1939) J. Path. Bact., 48, 291.
Lewis, J.J. (1960) An Introduction to Pharmacology, 1st Ed. Edinburgh and Glasgow.
Li, K-H. (1936) Proc. Soc. exp. Biol., 34, 659.
Lister, J. (1880) Quoted by Elek (1959).
Loeb L. (1903) J. med. Res., 10, 407.
Loeffler, F. (1884). Mitth. a.d.k. Gsndhtsamte., 2, 421.
Lominski, I. Arbuthnott, J.P. (1962) J. Path. Bact., 83, 515.
Lominski, I. Arbuthnott, J.P. Scott, A.C. & McCallum, H.M. (1962) Lancet, 2, 590.
Lominski, I. Arbuthnott, J.P. & Spence, J.B. (1963). in press J. Path. Bact. (has now been published: J. Path. Bact., 86, 258.)
Lominski, I. & Gray, S. (1961) Nature, 192, 683.
Lominski, I., Morrison, R.B. & Smith, D.D. (1955) J. gen. Microbiol., 13, 446
Lominski, I., O'Hea, A.J., Goudie, J.G. & Porter, I.A. (1950) Nature, 166,309
Lominski, I., Smith, D.D., Scott, A.C., Arbuthnott, J.P., Gray, S. Muir, D., Turner, G.H. & Hedges, C.K. (1962) Lancet, 1, 1315.
Madoff, M.A. & Weinstein, L. (1962) J. Bact., 83, 914.
Magnusson, B.J. Doory, H.M. & Gulasekaram, J. (1962) Nature, 196, 270.
Mangalo, R. & Raynaud, M. (1959) Ann. Inst.Past., 97, 188.
Marks, J. (1951). J. Hyg., 49, 52.
Marks, J. & Vaughan, A.C.T. (1950) J. Path. Bact., 62, 597.
McIlwain, H. (1938) Brit. J. exp. Path., 19, 411.
McLean, D. (1937). J. Path. Bact., 44, 47.
Mercier, P. (1938 a). C.R. Soc. Biol., 127, 188.
Mercier, P. (1938 b) C.R. Soc. Biol., 127, 297.
Mercier, P. & Lehoult, Y. (1946) Ann. Inst. Past., 72, 468.
Mercier, P. & Pillet, J. (1946) Ann. Inst. Past., 72, 659.
Michaelis, L. & Menten, M.L. (1913) Biochem. Z., 49, 333.
Morgan, F.G. & Graydon, J.J. (1936) J. Path. Bact., 43, 385.
Much, H. (1908) Biochem. Z., 14, 143.
Mueller, J.H. & Miller, P.A. (1945) J. Immunol., 50, 377.
Mule, F. & Sedati, P. (1949) Riv. Ist. siertor Ital. (sez.1)., 24, 235.
Neisser, M. (1912) Hanbush der Pathogenen microorganismen, Kolle, W. & Wassermann, A. Vol.4, p. 121, Jena.
Neisser, M. & Levaditi, C. (1900). 13th Cong. Internat. Med. Pathol., 475.
Neisser, M. & Wechsberg, F. (1901) Z. Hyg. Infect., 34, 299.
Neisser, M. & Wechsberg, F. (1903) Hanbush der Pathogenen Microorganismen, Kolle W. & Wassermann, A. Vol. 3 p. 121, Jena.

Nelis, P. (1933 a) C.R. Soc. Bio., 113, 7.
Nelis, P. (1933 b) C.R. Soc. Biol. 114, 598.
Nelis, P. (1934) C.R. Soc. Biol., 117, 756.
Nelis, P. (1935) Bull. Acad. Med. Belg., 15, 539.
Nelis, P. & Bonnet, H. (1935) C.R. Soc. Biol., 118, 136.
Nelis, P. & Boukaert, J.J. (1935) C.R. Soc. Biol., 113, 1157.
Nelis, P. & Boukaert, J.J. & Picard. (1934) Ann. Inst. Past., 52, 59.
North, E.A. & Doery, H.M. (1958) Nature, 182, 1374.
North, E.A. & Doery, H.M. (1961) Brit. J. exp. Path., 42, 23.
Oakley, C.L. & Warrack, G.H. (1941). J. Path. Bact., 53, 335.
Oeding, P. (1953) Acta. path. microbiol. scand., 33, 312.
Ogston, A. (1880) Arch. klin. Chir., 25, 588.
Ogston, A. (1881) Brit. med. J., 1, 369.
Ouchterlony, O. (1948) Acta. path. microbiol. scand., 25, 186.
Ouchterlony, O. (1949) Lancet, 1, 346.
Panton, P.N. & Valentine, F.C.O. (1932) Lancet, 1, 506.
Parish, H.T., Clark, W.H.M. (1932) J. Path. Bact., 35, 251.
Parker, J.T. (1924) J. exp. Med. 40, 761.
Parker, J.T., Hopkins, J.G. & Gunther, A. (1926) Proc. Soc. exp. Biol., 23, 34
Pasteur, L. (1880) C.R. Acad. Sci., 90, 1033.
Paul, J. (1958). The Analyst, 83, 37.
Petherwick, M.H. & Singer, E. (1944). Aust. J. exp. biol. med. Sci., 22, 21.
Pillemer, L., Wittler, R.G., Burrell, J.I. & Grossberg, D.B. (1948). J. exp. Med., 88, 205.
Porath, J. & Flodin, P. (1959) Nature, 183, 1657.
Purdy, H.A. & Walbum, L.E. (1921) C.R. Soc. Biol., 85, 374.
Ramon, G. & Richou, R. (1936) C.R. Soc. Biol., 121, 1064.
Rigdon, R.H. (1935) Arch. Path., 20, 201.
Rigdon, R.H. (1936 a) Arch. Path., 22, 763.
Rigdon, R.H. (1936 b) Arch. Path., 22, 806.
Rigdon, R.H. (1937 a) Arch. Path., 24, 215.
Rigdon, R.H. (1937 b) J. infect. Dis., 60, 25.
Rigdon, R.H. (1938) J. Lab. clin. Med., 24, 142.
Rigdon, R.H. (1940) Amer. J. Med. Sci., 2, 412.
Robinson, J. Thatcher, F.S. & Gangnon, J. (1958) Canad.J. Microbiol., 4, 345.
Robinson, J. Thatcher, F.S. & Montford, J. (1960) Canad. J. Microbiol., 6,183.
Rochaix, & Rivollier. (1939) Progres. med., 67, 1045.
Rodet, A. & Courmont, J. (1892) Sem. med., 12, 32.
Rosenbach, F.J. (1884) Mikroorganismen bei den Wundinfectionskrankheiten des Menschen. Weisbaden.
Roux, E. & Yersin, A. (1888) Ann. Inst. Past., 2, 629.
Rowson, K.E.K. (1961) J. gen. Microbiol., 4, 315.
Royal Commision (1928) Med. J. Aust., 2, 2.
Rud, E. (1955). Acta. path. microbiol. scand., 36, 441.
Russ, V.K. (1916) Z. exp. Path. Therap., 18, 220.
Seiffert, W. (1935) Zent. Bakt. 1 Abt. Orig., 135, 100.
Simmonds, J.P. Linn, H.J. Lange, J. (1946) Arch. Path., 41, 185.
Simonetti, A. D'A. (1954) Nuovi. Ann. Ig. Microbio., 5, 17.
Singer, A. Q. & Hagen W.A.C. (1941) J. Bact., 41, 74.
Smith, D.D. (1956) Nature, 178, 1060.
Smith, D.D. (1958) M.D. Thesis, The University, Glasgow.

Smith, G.H. (1941) Yale. J. Biol. Med., 13, 409.
Smith, M.L. (1937) Brit. J. exp. Path., 18, 265.
Smith, M.L. & Price, S.A. (1938) J. Path. Bact., 47, 379.
Stokinger, H. E. Ackerman, H. & Carpenter, C.M. (1941). J.Bact., 42, 135.
Tager, M. (1941) Yale. J. Biol. Med., 14, 68.
Thal, A.P. (1951) Fed. Proc., 10, 372.
Thal. A.P. (1955) Amer. J. Path., 31, 233.
Thal. A.P. & Egner, W. (1954) Arch. Path., 57, 392.
Thal. A.P. & Egner, W. (1961) J. Exp. Med., 113, 67.
Thal. A.P. & Molestina, J.E. (1955) Arch. Path., 60, 212.
Travassos, J. (1933 a). C.R. Soc. Biol., 114, 369.
Travassos, J. (1933 b). C.R. Soc. Biol., 114, 371.
Turpin, A., Relyfeldt, E.H., Pillet, J. & Raynaud, M. (1954) Ann. Inst. Past., 87, 185.
Vallisnieri (1713) Quoted from W.E. van Heyningen 5th Symposium of the Soc. of gen. Microb., 1955.
Van de Velde, H. (1894) Cellule, 10, 401.
Van de Velde H. & Denys, J. (1895) Cellule, 11, 358.
Van Ermengem, E. (1896) Rev. Hyg., 18, 761.
Van Heyningen, W.E. (1950) Bacterial Toxins, Oxford.
Van Recklinghausen, N.D. (1871) Quoted by von Lingelsheim (1900)
v. Daranyi, J. (1925) Arch. Hyg., 96, 182.
Verneuil (1889) Arch. gen. Med., 146,641.
Von Ghlan, W.C. & Weld J.T. (1935) J. exp. Med., 61, 1.
Von Leber, T. (1888) Forsch der. Med., 6, 460.
Von Lingesheim, W. (1900) Aetiologie und Therapie der Staphylokokken Infectionen, Berlin and Vienna.
Walbum, L.E. (1909) Z. Immunforsch., 3, 70.
Walbum, L.E. (1921) C.R. Soc. Biol., 85, 1205.
Waldeyer, (1871) Arch. Gynak., 2, Quoted by Flaum 1938.
Weinstein, L. (1937) J. infect Dis., 60, 209.
Weld, J.T.P. & Gunther, A. (1931). J. exp. Med., 54,315.
Wiegershausen, B. (1960) Arch. exp. Pathol. Pharmacol., 238,144.
Wiegershausen, B. (1962 a) Arch. int. Pharmacodynam., 136, 174.
Wiegershausen, B. (1962 b) Acta. Biol. med. germ., 9, 517.
Williams, R.E.O. & Harper, J.G. (1947) J. Path. Bact., 59, 69.
Williams, R.E.O. & Rippon, J.E. (1952) J. Hyg., 50, 320.
Wilson, G.S. & Atkinson, J.D. (1945) Lancet, 1,647.
Winslow, C.E.A. Rothberg, W. & Parsons, E.I. (1920) J. Bact., 5, 145.
Wittler, R.G. & Pillemer, L. (1948) J. biol. Chem., 174, 23.
Wood, E.J.F. (1950) Proc. Linn. Soc. N.S.W., 75, 158.
Wood, E.J.F. (1952) J. gen. Microbiol., 6, 205.
Woolpert, O.C. & Dack, G.M. (1933) J. infect. Dis., 52, 6.