CYTOLYTIC ACTION OF STREPTOLYSIN S.

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INTRODUCTION.

A HISTORICAL DESCRIPTION OF STREPTOCOCCUS PYOGENES.

Gram positive cocci were first described by Goodsir (1842) when he was examining stomach contents. Coccal organisms were found in pus by Von Recklinghausen (1871) and by Waldeyer (1871) from renal abscesses and the peritoneal pus in puerperal fever. Klebs in 1872 was the first to suggest that the coccal organism, which he called Microsporon septicum, was the cause of the morbid changes observed. He described these as necrosis of the cells and migration of leucocytes from the vessels. This view, however, was opposed by Billroth (1874) who thought that certain existing inflammatory processes, caused by a ferment-like "phlogistic zymoid", were essential for the occurence of cocci and putrefaction. He classified the coccal organisms into Monococcus, Diplococcus, Streptococcus, and Gliacoccus based on their microscopic appearance.

Classical experiments by Koch (1876) showed that infective wound processes were due to the action of bacteria and that there were bacteria of a special form for each disease. Ogston (1881) investigated eighty-two abscesses and was able to demonstrate the presence of cocci in all of them. His illustrations clearly show that he differentiated between streptococci which he called "chain-micrococci" and staphylcocci which he called "group-micrococci". The term streptococcus was first clearly defined by Rosenbach (1884) when he was describing chain-forming cocci which he had isolated from suppurative lesions in man. To this organism he gave the name Streptococcus pyogenes.

The first report of an experimental study of a streptococcal infection was made by Nocard and Mallereau (1887) who demonstrated the presence of long chains of streptocci in milk from cows suffering from mastitis. By inoculating infected milk into meat extract broth containing glucose or lactose, and bicarbonate

they were able to grow the organism and maintained it for six months by regular subculturing. They were also able to grow the streptococcus on solid medium containing gelatin, peptone and serum. On injecting the broth culture into the udder of cows and goats they produced mastitis in uninfected animals and thus proved conclusively that the streptococcus was the causative organism of the disease. This was the first time that Koch's postulates were fulfilled using a streptococcus species. Chain-forming cocci were isolated subsequently from a variety of pathological conditions in man and animals, from the mouth or faeces of healthy subjects, from milk and milk products and from numerous other sources.

A primary sub-division of the streptococci by the type of haemolysis they cause on horse blood agar was first described by Schottmüller (1903). Three groups emerged from this classification:- alpha-haemolytic, beta-haemolytic and gamma or non-haemolytic streptococci. Species in these three groups are characterised by a wide variety of serological and physiological tests, whose relevance is reviewed by Sherman (1937). The type species of the genus, Streptococcus pyogenes, is the main human pathogen and is the subject of study in this Thesis. It causes beta-haemolysis on blood and can be differentiated from other streptococcal species possessing this property by the specific soluble carbohydrate antigen which can be extracted from its cell wall i.e. by Lancefield grouping. Seventeen distinct Lancefield groups have now been differentiated; streptococci isolated from one animal species usually belong to the same Lancefield group (Table 1). The current definition of the streptococcus is that given by Topley and Wilson (1964).

TABLE 1.

The Main Lancefield Groups of Streptococcus pyogenes.

GROUP	HAEMOLYSIS	USUAL HABITAT	PATHOGENICITY
A	+	Man	Many human diseases.
B	+	Cattle	Mastitis.
C	±	Many animals	Many animal diseases.
D	±	Dairy products intestinal of tract of man & animals.	Urinary tract & wound infections, endocarditis.
E	+	Milk, swine.	Pharyngeal abscesses of swine.
F	+	Man	? respiratory tract.
G	+	Man Dogs	Respiratory infection, Genital tract infections.
H	±	Man	? respiratory tract.
K	±	Man	? respiratory tract.
L	+	Dogs	Genital tract infections.
M	+	Dogs	Genital tract infections.
N	-	Dairy products	None.
O	±	Man	Endocarditis.

After Davies, Dulbecco, Eisen, Ginsburg & Wood. "Microbiology".

The respective roles played by the bacterial cell and its extracellular products in the pathogenicity of the organism are complex. The streptococcus lies intermediate between those organisms such as Clostridium botulinum, Clostridium tetani and Corynebacterium diphtheriae which each produce extremely potent exo-toxins but have little or no invasive capacity and those organisms which do not cause death until there has been enormous systemic multiplication e.g. Bacillus anthracis. Streptococci are both invasive and toxin producing. Streptococcal toxins appear to play varying roles in the course of infection but the harmful effects of the organism cannot be wholly attributed to any single toxin or extracellular product.

The classification, structure and physiology of the streptococcus and the detailed properties of a few of its individual extracellular products have been reviewed extensively by several authors. However, to my knowledge, there has been no comprehensive account of all the streptococcal products implicated individually in the pathogenicity of the organism. Although the experimental section of this Thesis is concerned primarily with the action of Streptolysin S, I feel it is necessary, in this introduction, to describe the individual components of the toxic armouryof this important pathogen in order to appreciate the complexity of the overall picture.

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FACTORS INVOLVED IN PATHOGENICITY.

GENERAL CONSIDERATIONS.

Infections in man due to Group A streptococci exhibit a wide variety of clinical symptoms such as tonsillitis and pharyngitis, scarlet fever, osteomyelitis in infancy, cellulitis, erysipelas, puerperal sepsis, pneumonia, meningitis and endocarditis in addition to localised superficial lesions. With the exception of scarlet fever and possibly erysipelas, diagnostic terminology is dependent principally on the site of localisation of the organisms and not on any fundamental differences in the pathological physiology of the infective process. The portal of entry of the infecting streptococcus, therefore, appears to pre-determine to a considerable extent the clinical syndrome which will develop. Moreover there can be little doubt that the variation in clinical pattern of streptococcal infections can be accounted for in part, at least, in terms of the hosts' immunological response. It has been proposed that the suppurative lesions are attributable primarily to the invasive and pyogenic properties of the infecting strain and only secondarily to the immune status of the host, whereas the non-suppurative complications, such as glomerular nephritis and rheumatic fever in which the presence of the organism is not always demonstrable, are principally the expression of an unusual host response. The overall pathogenic result thus appears to depend both on the invasiveness of the streptococcus and the immune status of the host, (Keefer, 1941).

In evaluating the invasive and pathogenic properties of infecting streptococcal strains, a multiplicity of intracellular and extracellular products have been isolated and implicated in contributing to the pathogenic process. The components and products of Group A streptococci which have been related to the clinical pattern can be conveniently divided into three groups

(1.) cellular constituents (2.) extracellular enzymes and (3.) extracellular toxins. I shall review the nature and properties of these factors in three sections under the above headings and discuss their relative involvement in the pathogenicity of the streptococci. It is necessary, however, to bear in mind that despite the fact that many of these factors have been obtained in highly purified or even crystalline form, their interaction with each other and their contribution to the pathogenic process remains highly speculative.

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CELLULAR CONSTITUENTS.

Group Specific Polysaccharide.

Serologically specific and distinct polysaccharides have served as an accurate means of classifying beta-haemolytic streptococci from various sources into numerous groups (Lancefield 1933). These soluble carbohydrate antigens are located in the cell wall and are extracted from the organism by boiling at pH 2, the method used for routing typing of streptococci (Lancefield 1942). Hot formamide can also be used for extraction (Fuller 1938) of the carbohydrate antigens which can also be obtained in solution by treating the cells with a cell wall-dissolving enzyme isolated from culture filtrates of Streptomyces albus (Maxted 1948). The polysaccharide obtained by using purified preparations of this enzyme (McCarty 1952) was composed primarily of Group-specific carbohydrate but contained some mucepeptide cell wall material (Krause & McCarty 1961).

Group A carbohydrate contains 60% rhamnose and 30% glucosamine, the terminal P-N-acetylglucosamine residues being the antigenic determinants of the molecule (Krause 1963). Immunity and protection to streptococcal infection are not related to the carbohydrate antigen; however it was shown that a mucopeptide-C-polysaccharide complex of the cell wall caused chronic relapsing lesions in rabbits (Schwab & Cromartie 1960). When separated, neither the mucopeptide nor the C-polysaccharide alone produced the chronic relapsing lesion (Krause & McCarty 1961). Cell wall antigen was localised in the areas of chronic inflammation and lesions correlated with the persistence of the antigen (Ohanian & Schwab 1967). From these results it has been proposed that chronic inflammatory streptococcal diseases are the direct result of the persistence in tissues of the mucopeptide-C-polysaccharide

complex of Group A streptococcal cell walls. The mucopeptide is considered to be the "toxic" moiety while the C-polysaccharide (Fashs) the mucopeptide, thereby enabling the persistence of the irritating agent. (Schwab & Ghanian 1966).

Type Specific M - Antigen.

By somewhat similar methods to those used in Lancefield grouping, Group A streptococci have been subdivided into over thirty serological types based on type-specific proteins, the M - antigens. The type specific M - antigen is an alcohol-soluble protein, resistant to boiling at pH 2 for 20 to 30 minutes (Lancefield 1928; Lancefield & Perlman 1952). Recently highly purified preparations of M-proteins were obtained by Fox & Wittner (1965). Detailed physical chemical investigations showed it to be an elongated molecule with an average axial ratio of 15:1 having a molecular weight of 40,000. This antigen is closely associated with the cell wall and can be extracted from whole cells or isolated cell walls by acid hydrolysis or by the action of a phage-associated lysin (Salton 1952; Krause 1957; Kanter & Cole 1960). The M-protein can be destroyed by treating the cells with proteolytic enzymes. The organisms remain viable after such treatment and are capable of resynthesising the M - antigen when the enzymes are removed (Lancefield 1943). Making use of this system, Fox & Krampitz (1956) studied the synthesis of M-protein by non-proliferating streptococci and found that eight amino acids were essential for synthesis.

The loss of the M-antigen from a particular strain results in the loss of virulence (Lancefield 1928) but the purified protein is not toxic. It was shown that the presence of the M-antigen in the streptococcus hindered phagocytosis (Hirsh & Church 1960); Strains of streptococci containing little or no M-antigen were readily phagocytosed (Lancefield 1958, 1959) but the addition of homologous M-protein extract enhanced the resistance of the organisms to phagocytosis (Krasher, Young & Heitmann 1964). The host specificity of virulence was illustrated by the fact that despite the presence of M-antigen, streptococci isolated from acute infections in man were usually avirulent in mice, (Dochez, Avery & Lancefield 1919).

Type specific antibodies can be demonstrated in the sera of patients following Group A streptococcal infections (Lancefield & Perlmann 1952). Injections of M-antigen solutions induced a rapid rise in type specific antibodies in individuals who had a preceding infection with the same type of streptococcus (Stollerman & Ensted 1957) but in the absence of such a previous encounter, the antibody response was poor (Schmit 1957, 1960; Potter, Stollerman & Siegel 1962). Observations by Wannamaker, Denny, Perry, Siegel & Rammelkamp (1953) indicated that immunity in man was type specific and that there was little heterologous immunity.

T and R Antigens.

During the course of experiments designed to correlate the results obtained by means of type specific agglutination with the results of active or passive protection in mice, another protein antigen associated with agglutination was encountered (Lancefield 1940). This antigen was named the T-antigen and was distributed among the Group A streptococci independently of

the M - antigen. It is not type specific but is common to several types. Although the T - antigen stimulates antibody production during streptococcal infections, it has no demonstrable relationship to the virulence of the organism, nor does its antibodies participate in protection. A few strains of streptococci have been shown to possess a third cell wall protein antigen, the R - antigen. Like the T - antigen this does not appear to be related to the virulence of the organism.

Hyaluronic Acid.

Hyaluronic acid is located in a viscous outer capsule round the organism and is the only known surface constituent of Group A streptococci which is non-antigenic. It is composed of equimolar quantities of N-acetyl glucosamine and glucuronic acid and is indistinguishable from mammalian hyaluronate. Only some strains produce capsules which, if present, can only be demonstrated in vitro in young cultures (2 to 4 hours). By means of phagocytic, bacteriostatic and mouse protection tests Rothbard (1948) concluded that hyaluronic acid had a slight but definite influence on the virulence of Group A streptococci. On the other hand, the M - antigen had an effect 1,000 to 10,000 times as great under similar experimental conditions. Many virulent strains of streptococci produced extracellular hyaluronidase (see page 15) which destroyed any capsule produced. Since such organisms were still capable of establishing an infection, capsule formation appears to be of questionable significance in relation to the pathogenicity of the streptococcus.

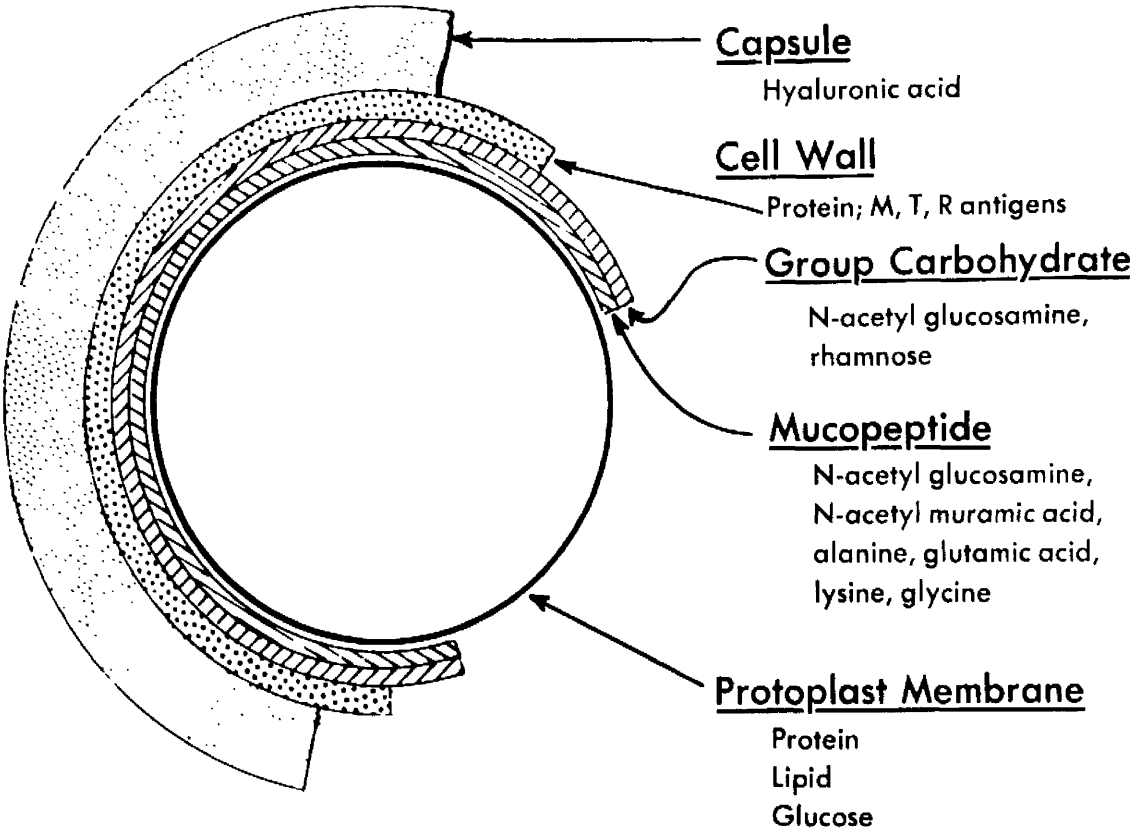
Cell-Bound Haemolysin.

Viable haemolytic streptococci lyse red blood cells, tumour cells, and tissue culture cells *in vitro*. The presence of a cell-bound haemolysin was described by Ginsburg, Bentwich & Harris (1965) and in the same year, Taketo and Taketo showed that Group A streptococci in fact contained two intracellular haemolysins. These were found to be closely related to the extracellular haemolysins, Streptolysins S and O, which were produced by the organism. Their significance will be discussed in detail in the section of the introduction dealing with streptococcal exo-toxins (page 26).

L - Forms.

Streptococcal L - forms have been produced by the treatment of cells with penicillin or muralytic enzymes and thereafter, were maintained by growth in a hypertonic medium (Crawford, Frank & Sullivan 1958; Gooder & Maxted 1961). In cultures of these forms, devoid of cell walls and lacking in Group antigen, the M - antigen was still produced and diffused into the medium. This indicated that although the end product of the intact cell was deposited in serologically active form in the cell wall and bound there, this was not the site of M-protein production. Hyaluronic acid was found to be synthesised by these forms (Markovitz & Dorfman 1962; Mortimer & Vastine 1967) which also contained haemolysins (Maruyama, Sugai & Egami 1959) and produced DNA-ase (Freimer, Krause & McCarty 1959).

L - forms of streptococci have been cultured from blood and peritoneal exudate of mice infected with streptococci (Mortimer 1964, 1965) and have also been isolated from scarlet fever patients who had received penicillin therapy (Klodnistsaja 1962). These results show conclusively that group A



Schematic diagram of capsule, cell wall, and cytoplasmic membrane of group A hemolytic streptococcal cell. (From R. M. Krause. *Bact. Rev.* 27:369, 1963.)

FIG. 1.

streptococcal L - forms can exist in vivo. They have also been reported to have a toxic effect on cardiac muscle (Ginsburg 1966), to be dermotoxic, and cytotoxic for chicken fibroblasts in tissue culture (Timakov & Kagan 1966).

Conclusions.

Most of the cellular antigens which give rise to antibodies during streptococcal infections appear to be associated with the cell wall and its adherent layers (FIG. 1.). However, the pathogenic effects of L - forms indicate that other deeper seated intracellular factors may also contribute to the pathogenicity of Group A streptococci. It would, therefore, be of interest to study toxic components specific to the bacterial cytoplasm and plasma membrane.

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EXTRACELLULAR ENZYMES.

Streptokinase (Fibrinolysin).

The low viscosity of the fluid, so characteristic of the exudate obtained in early stages of streptococcal infections, has been attributed to the lytic action of the organisms on the fibrinous exudate of the host. Tillet and Gardner (1933) found that certain strains of streptococci, mainly Group A, produced an extracellular lysin for the clot formed by the action of thrombin on human fibrin. The plasma of most animals was not susceptible, but this resistance was not a property of the fibrin itself since animal fibrin became susceptible if clotted with human thrombin; conversely human fibrin became susceptible if clotted with animal thrombin. Milstone (1941) observed that the clot formed by the interaction of purified human thrombin and fibrinogen was no longer lysed by streptococcal fibrinolysin. An essential factor was removed during the purification of the thrombin and the fibrinogen. This so called "lytic-factor" was found in the water - insoluble globulin fraction of human serum.

In a series of papers Christensen and co-workers showed that the streptococcal fibrinolysin was not proteolytic but was a kinase which catalysed the production of an active fibrinolysin (plasmin) from an inactive precursor in the plasminogen (Christensen 1945 & 1946; Christensen & Macleod 1945). Since the streptococcal factor is a kinase and not a fibrinolysin, Christensen suggested that it should be called streptokinase. The conversion of the inactive zymogen, plasminogen, to the active proteolytic enzyme plasmin, by streptokinase can be compared with many similar reactions e.g. the conversion of chymotrypsinogen to chymotrypsin. Kinases by definition are agents, generally, if

not always, enzymes which modify enzyme precursors. Some are known to be proteolytic in that their action on their zymogenic substrates results not only in the production of active enzymes but also in the appearance of non-protein nitrogen or in a reduction in the size of the zymogen. They most likely act by unmasking the active centre of the enzyme. The streptokinase system is a complex one and the question of the reactions involved cannot be answered adequately until plasminogen and Streptokinase have been obtained in a pure state. The reactions known to occur in the process are summarised in Fig. 2.

Streptokinase is a heat-stable protein which is destroyed by trypsin (Tillet 1938). It is antigenic and its activity is inhibited by specific antibody (Kaplan 1945). This enzyme has recently been purified and shown to be a protein of molecular weight 47,600 and an empirical formula of:- Asp_{68}-Thr_{30}-Ser_{24}-Glu_{46}-Pro_{20}-Gly_{21}-Ala_{23}-Met_{3}-Try_{1}-Leu_{40}-Tyr_{20}-Phe_{15}-Lys_{33}-Arg_{21} (De Renzo, Suteri, Hutchings & Bell 1967). On the other hand there are considerable difficulties in purifying plasminogen which, to date, have not been resolved.

One of the interesting aspects of this system is the finding that, of all the species tested, man appeared to possess the highest level of plasminogen and Group A streptococci the highest level of streptokinase (Christensen 1949). Thus it seems possible to attribute a specific role for this system in streptococcal diseases in man. Tillet (1938) and Neter (1949) made the important observation that the thin serous exudate of streptococcal empyema and other conditions is accompanied by the ability of these exudates to lyse fibrin clots. This lends strong support to the idea that the rapid spread of streptococci in tissues may be due, in part, to impairment of the normal walling off process of the host animal by the action of streptokinase.

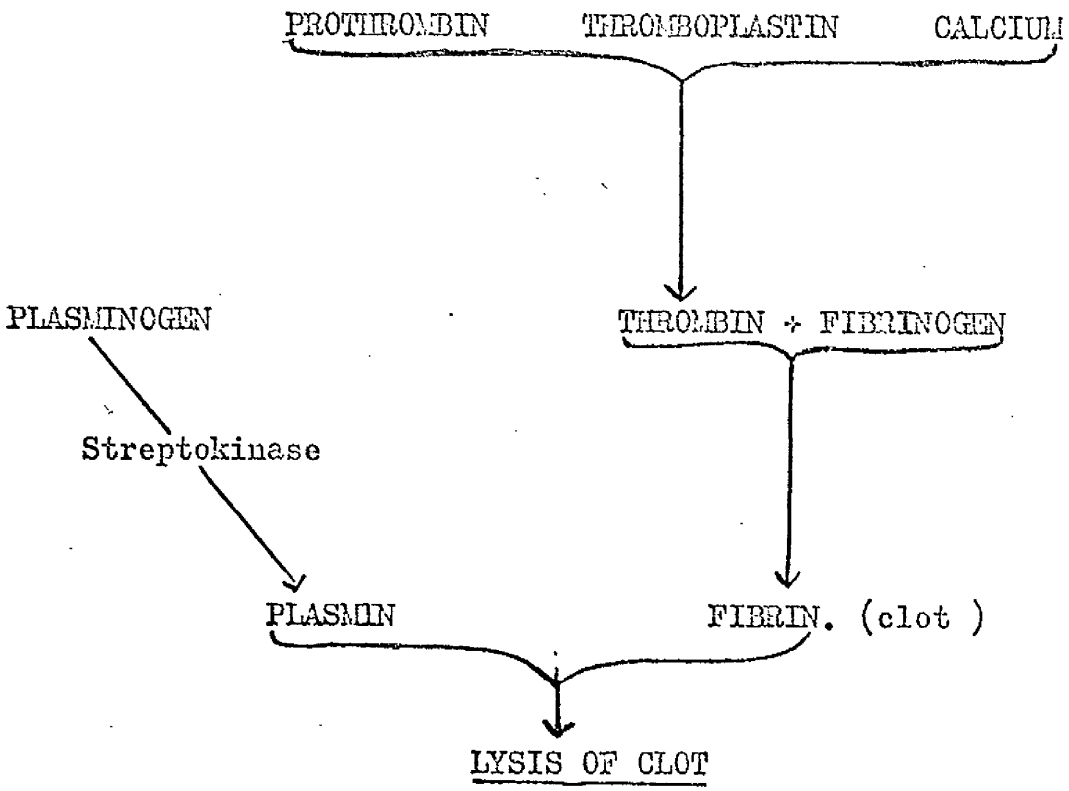

FIG. 2. The Mode of Action of Streptokinase (Fibrinolysin).

Spreading Factor (Hyaluronidase).

The presence in lysates and filtrates of invasive streptococci of a spreading factor which increased the permiability of rabbit skin to suspensions of India ink or bacterial cells was described by Duran-Reynolds (1933). A similar activity was found in testicular extracts (Chain & Duthie 1939) which were shown to hydrolyse the monopolysaccharide hyaluronic acid. At least three successive stages were observed during enzymic hydrolysis of hyaluronic acid (McClean 1943). The first stage was the destruction of the ability of hyaluronic acid to clot with acetic acid. This was followed by depolymerisation which was seen as a fall in viscosity, and finally there was liberation of reducing sugars and N-acetyl glucosamine.

Chain and Duthie (1940) showed that all samples of spreading factor tested by them, whether of animal or bacterial origin possessed hyaluronidase activity and suggested that the spreading factor of streptococci was a hyaluronidase. Similar results were found by Hobby, Dawson, Meyer & Chaffee (1941) and Meyer, Chaffee, Hobby & Dawson (1941) who showed that certain other polysaccharides e.g. chondroitin sulphate were also hydrolysed by spreading factor. All preparations containing hyaluronidase had spreading factor activity but the converse did not hold in all cases. Whether or not streptococci produce spreading factors that act on substrates other than hyaluronic acid, it is now accepted that many strains produce hyaluronidase.

Rogers (1946) showed that streptococcal hyaluronidase contained several enzymes each responsible for different stages in the degradation of hyaluronic acid. These were shown to be adaptive enzymes whose production was stimulated by the presence of hyaluronic acid or certain breakdown products in the culture

medium (McClean & Hale 1941; Rogers 1948). Hyaluronidase activity was neutralised in vitro by specific antibody but the spreading activity in rabbit skin was not affected (Hobby et al 1949).

Certain streptococcal bacteriophages gave rise to the formation of hyaluronidase. The serological type of this hyaluronidase was determined by the genetic constitution of the phage. Thus different phages caused the formation of antigenically different hyaluronidases even when reproducing in cultures of the same bacterial strain. On the other hand the hyaluronidases produced by non-lysogenic Group A streptococci were found to be serologically identical thus differing from the phage - associated hyaluronidases (Kjems 1958 a & b).

The part played by hyaluronidases in streptococcal infection, invasion and virulence has been reviewed in some detail by Duran-Reynolds (1942). The destruction of the hyaluronic acid capsule by spreading factor is recognised although the relative roles played by each in the pathogenicity of the organism is not clear. When rabbits are injected intradermally with a given amount of streptococcal culture, the resulting dermal lesion corresponds in dimensions to the area of spread of India ink when it is injected with culture supernatant. This suggests that the invasiveness of the organism is largely determined by the amount of spreading factor produced. Nevertheless the production of spreading factor cannot be considered an absolute criterion of virulence.

Proteinase.

Streptococcal proteinase is an extracellular enzyme and resembles papain in certain respects; it is reversibly activated in the presence of sulphydryl compounds or cyanide but is not activated by ascorbic acid. It is irreversibly inactivated by

iodoacetate, is active over a pH range extending from 4.5 to 8.5, clots milk and digests casein, haemoglobin, and fibrin of both rabbit and human origin. The enzyme first attracted attention as a result of its capacity to destroy the serological reactivity of the type specific M - antigen of Group A streptococci (Elliot 1945). With certain strains it was noticed that whereas the M - antigen could be identified in cultures grown at 22°C, this was not possible in cultures grown at 37°C. This apparent and temporary loss of the type specific M - antigen was due to the proteolytic activity in cultures grown at 37°C. It was found subsequently that proteolysis also accounted for the destruction of streptokinase in culture fluids and Crowley (1951) showed that streptococcal hyaluronidase may also be destroyed under similar conditions.

The streptococcal proteinase is derived from an inactive precursor, which like the active enzyme, is found extracellularly in the culture supernatants of most strains of Group A streptococci irrespective of serological type (Elliot & Dole 1947). Under intensive reducing conditions the precursor protein is converted to active enzyme by an autocatalytic reaction. Conversion of the precursor to active enzyme may also be achieved by the action of trypsin. Both the precursor and proteinase have been crystallised (Elliot 1950) and the two crystalline proteins are distinguishable on the basis of immunological specificity. The precursor (molecular weight 44,000) and the enzyme (molecular weight 32,000) contain only a single half-cysteine residue per molecule (Liu, Neumann, Elliot, Moore & Stein 1963).

There is a loss of about 100 amino acids when the precursor is converted to active enzyme. In the course of this reaction an intermediate product was formed which contained only 17 amino acids less than the precursor (Liu & Elliot 1965 a). This intermediate was immunologically related to both the precursor and the enzyme. Moreover, the modified precursor was almost as fully active as the proteinase after reduction with thiols. These observations refer to trypsin conversion but a similar intermediate is formed by the action of the streptococcal proteinase itself.

In the later stages of bacterial growth active proteinase was found to accumulate in the culture fluid (Elliot 1945), and from results described by Liu and Elliot (1965 b) it appeared likely that the transformation to active enzyme was initiated by the reducing activity of the streptococcal cell walls. By investigating the effect of crystalline proteinase on a number of synthetic substrates, Mycek, Elliot and Fruton (1952) were able to show that the enzyme hydrolysed peptide bonds involving the carbonyl group of arginine, lysine, glutamic acid, aspartic acid and histidine. The presence in the substrate of an adjacent glycyl residue appeared to inhibit the action of the enzyme on a sensitive peptide bond. Recently Burshtein and Beletsaya (1968) have shown that the proteinase also liberated acid mucopolysaccharides from protein complexes.

There is, however, a lack of experimental evidence for the role of this product in pathogenicity. The broad substrate specificty of this enzyme suggests that it may attack many host proteins and protein complexes during streptococcal infections thus contributing to the resultant clinical picture.

Desoxyribonuclease (DNA-ase, Streptodornase).

The presence of DNA-ase in crude culture filtrates of Group A streptococci was first reported by McCarty (1948) who also noted the production of smaller amounts of RNA-ase. In a survey of nuclease production by streptococci Brown (1950) found that 100% of the Group A streptococci which he tested produce both nucleases.

The nuclease activity of concentrated culture supernatants from Group A streptococci was markedly increased in the presence of magnesium ions (Tillet, Sol & Christensen 1948) and certain strains were shown to elaborate DNA-ase extracellularly when the washed cocci were suspended in a medium containing an energy source (maltose), phosphate and magnesium ions (Bernheimer & Ruffier 1951). Wannamaker (1958) found that three different DNA-ases were produced by Group A streptococci; these could be separated by starch zone electrophoresis. They were immunologically distinct and showed certain differences in their optimal pH and susceptibility to citrate inhibition. The relative amount of each form produced was a function of strain variation. The DNA-ases were inhibited by a variety of enzyme poisons and were destroyed by heating at 56°C for 60 minutes. One of the DNA-ases isolated by Wannamaker, DNA-ase B, was related to the streptococcal erythrogenic toxin having skin activity of 500,000 units/ml (Halbert 1958); this will be discussed later on page 23.

The pathogenic role of streptococcal DNA-ase has not been defined but it seems feasible that, like fibrolysin, it may participate in the formation of the thin serous exudate found in streptococcal lesions since the release of DNA from damaged tissues contributes to the viscosity of pus in other bacterial infections. It may also act synergistically with other

streptococcal toxins and thus contribute to the cytotoxicity of streptococci.

Streptococcal NAD-ase.

This enzyme was first isolated by Carlson, Kellner, Bernheimer and Freeman (1957) and shown to inhibit the oxidation of certain Krebs' cycle intermediates by the mitochondrion. It was often present in streptococcal preparations containing streptolysin O and has been purified and shown to hydrolyse the linkage between nicotinamide and ribose in nicotinamide adenine dinucleotide.

According to Bernheimer, Lazarides and Wilson (1957) the leucotoxic activity of streptococci is closely associated with the formation of this enzyme. Failure to produce observable damage to leucocytes by the addition of purified NAD-ase to whole blood (Carlson, et al 1957) may have been due either to the inability of this enzyme to enter the cells or to the presence of an inhibitory substance in the serum. However, if released within the leucocyte from streptococci which have been phagocytosed, NAD-ase might well impair cellular metabolism. Alternatively its access to the interior of the cell in vivo might be facilitated by the disruptive action of the cytolytic toxins, streptolysins O and S, on cell membranes.

Some of the characteristics of this enzyme have been studied (Petersen, Kroger & Rotthauwe 1961) and the presence of an intracellular inhibitor within the streptococcus has been described (Holm & Kaijser 1965, 1967). A direct relationship between NAD-ase production and pathogenicity has not been established. But Bernheimer (1960) has claimed that this enzyme might influence the development of streptococcal nephritis since raised antibody levels to this enzyme have been observed in man

following the occurrence of glomerular nephritis (Wellner, Freeman & Carlson 1958; Petersen 1962).

Conclusions.

None of these extracellular enzyres produced by Group A streptococci are lethal and it is doubtful if any one of them plays a dominant role in the pathogenicity of the organism. The combined effects of fibrinolysin, hyaluronidase, proteinase and DNA-ase, however, may well contribute to the extreme invasiveness of the streptococcus. Also, although the importance of NAD-ase is difficult to assess as a separate entity, in the section of this introduction dealing with streptococcal toxins, it is clearly seen that it may act synergistically with the cytolytic toxins.

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TABLE 2.

Streptococcal Extracellular Enzyme.

PRODUCT	PROPERTIES	MODE OF ACTION
Streptokinase	Protein, (M.W. 47,000) Enzyme - Kinase	Converts plasminogen to plasmin which lyses fibrin clots.
Proteinase	Protein - produced as zymogen (M.W. 44,000). Converted to enzyme (M.W. 32,000) by autocatalysis activated by sulphydryl compounds.	Splits peptide bonds involving carbonyl groups of arginine, lysine, glutonic acid, aspartic acid and histidine.
Hyaluronidase	Protein - complex of enzymes.	Hydrolyses hyaluronic acid with the release of 24 reducing sugars and N-acetyl glucosamine.
Desoxyribon-uclease DNA-ase (Streptodornase)	Protein - 3 antigenically distinct enzymes	Hydrolyses desoxyribonucleic acid.
Niotinamide adenine Dinucleotidase (NAD-ase)	Protein - (M.W. 25,000) Enzyme - Trypsin sensitive.	Cleaves nicotinamide - ribose linkage.

STREPTOCOCCAL TOXINS.

Erythrogenic Toxin.

Properties of erythrogenic toxin.

Some strains of Group A streptococci produce a toxin known variously as scarlet fever toxin, erythrogenic toxin or Dick toxin. This toxin causes the familiar red rash of scarlet fever, and is assayed by injecting culture filtrates intradermally to determine the smallest amount of toxin which will cause an erythematous reaction:- the skin test dose (STD). This skin reaction can be prevented by mixing the toxin with antitoxin prior to injection. Persons who have suffered from scarlet fever have a negative skin reaction following the injection of erythrogenic toxin, due to the presence of antibodies in their serum (Dick & Dick 1924 a & b).

Two distinct erythrogenic toxins, A and B, produced by strains of streptococci were described by Hooker and Follensby (1934). Both were protein in nature but were immunologically distinct. Toxin A could be identified by a neutralisation test and by a flocculation test with antibody whereas toxin B did not flocculate with antibody and could only be identified by neutralisation (Stock & Lynn 1959). The two toxins were found to also differ in their sensitivity to enzymic digestion and could be precipitated by different methods. This latter technique was used to separate and purify them.

Toxin A was purified by Stock and Verney (1952) and toxin B by Stock and Lynn (1959). The latter possessed high DNA-ase activity and was immulologically related to the DNA-ase B prepared by Wannamaker (Halbert 1958). Konikov and Krusho (1957) obtained a crystalline preparation of erythrogenic toxin with a maximum activity of 1.3×10^8 S.T.D./mg. N and a molecular weight

of 29,000. A crystalline product containing 1.04×10^9 STD/mg N was prepared by Mitrica, Pleceas and Mesrobeanu (1965); the crystals were rhombic and melted above 4°C. Recently Nauciel, Raynaud and Bizzini (1968) purified the erythrogenic toxin from culture supernatants by column chromatography. Their product was homogenous in the ultracentrifuge, in polyacrylamide gel electrophoresis and immunoelectrophoresis and had a molecular weight of 30, 500. It was a protein and its amino acid content was determined. The purified toxin withstood heating at 80°C. for 30 minutes and was not destroyed by treatment with trypsin or pepsin. This product appeared to be identical to the toxin A described by Stock and Verney (1952) which causes both a localised skin reaction and generalised scarlatina whereas toxin B causes only a localised skin reaction and these authors have suggested that the A-toxin is the only true erythrogenic toxin.

Occurrence of scarlatinal strains of streptococci.

In 1927 Frobisher and Brown demonstrated that a filtrable agent isolated from scarlatinal strains of haemolytic streptococci could induce the formation of erythrogenic toxin by non-scarlatinal strains. Krause (1957) and Kjems (1960) have since shown that lysogeny is quite common in Group A streptococci, and this finding suggested the possibility that transmissibility of toxicity observed by Frobisher and Brown might depend on the relationship between streptococci and bacteriophages similar to that described in the Corynebacterium diphtheriae complex (Freeman 1951, 1952). This hypothesis was confirmed by Zabriski (1964) who showed that non-lysogenic, non-toxigenic Group A streptococci acquired the capacity to produce erythrogenic toxin when infected by temperate bacteriophages.

isolated from known scarlatinal toxin producing strains. The production of this toxin appeared to be related to the synthesis of mature phage particles, since U.V. enhancement resulted in a concomitant increase in toxin production.

Role of erythrogenic toxin in pathogenicity.

Schwab, Watson and Cromartie (1953 & 1955) reported that a reaction resembling the generalised Schwartzmann phenomenon could be produced by the injection of soluble products of Group A streptococci. American Dutch rabbits injected intravenously with an extract of a streptococcal skin lesion followed by a second intravenous injection of, either a filtrate of a culture of Salmonella typhi or a reduced culture filtrate of streptococci which had a high titre of streptolysin O, produced these symptoms. The prominent features of the reaction were death and necrosis of cardiac muscle. Watson (1960) identified three toxins in these "streptococcal lesion extracts" which were immunologically related to the erythrogenic toxins although the organisms isolated from such lesions did not produce erythrogenic toxin when grown under suitable conditions in vitro. Two of these toxins were immunologically identical to the erythrogenic toxins A and B of Stock et al (1952, 1959) and a new toxin, designated C, was also identified. The amounts of these three toxins present in the lesion extracts varied according to the strain of Group A streptococcus which had caused the lesion. Thus Watson concluded that a streptococcal strain could not be considered non-toxigenic on the grounds that it failed to produce detectable amounts of erythrogenic toxins in vitro.

In the American Dutch rabbit, 15 Lf (9000,000 SED) of purified erythrogenic toxin A gave no reaction in the skin.

It was however, lethal for these animals and when injected in sub-lethal doses caused necrosis of the heart and liver. Injection of "streptococcal lesion extract" 3 hours prior to the administration of a sub-lethal dose of purified erythrogenic toxin A increased its lethality and the amount of resultant tissue damage. Erythrogenic toxin given into the skin of sensitive rabbits at 24-hour intervals markedly enhanced the erythrogenic and nephrotoxic properties of itself (Boroff 1951). From these observations Watson suggested that the primary activity of the erythrogenic toxin was to modify the host's response to itself, to other toxins and to toxic reactions such as hypersensitivity. Moreover, the absence of an erythemateous rash in an infected individual does not preclude the possibility that the erythrogenic toxin is present and is causing damage to tissues other than skin.

Streptococcal Haemolysins.

The haemolytic activity of streptococcal culture supernatants was first observed by Marmorek (1895), and this was followed by many papers describing the production and properties of the soluble extra-cellular haemolysin. Careful quantitative studies carried out by Kruif & Ireland (1920) on the rate of haemolysin production enabled them to demonstrate a maximum haemolysin content in cultures after 8 hours at 37°C.; this rapidly declined and after 12 to 24 hours the haemolysin was no longer demonstrable. The reason for this disappearance was partly explained by Niell and Mallory (1926) who found that streptolysin, when exposed to air, undergoes an oxidation which can be reversed by suitable chemical reagents e.g. sodium hydrosulphite. Despite these observations it was not until 1938 that Todd demonstrated that Group A streptococci produce two

TABLE 3.

Some Differences Between Streptolysin O and S.

	Streptolysin O.	Streptolysin S.
1.)	Activated by SH-compounds	Not activated by SH-compounds
2.)	Antigenic and neutralised by specific antibody	Apparently non-antigenic and not neutralised by any known antibody
3.)	Formation is not markedly influenced by serum or polyribonucleotide	Formation is greatly enhanced by serum and polyribonucleotide
4.)	Destroyed by trypsin	Not destroyed by trypsin
5.)	Inhibited by minute amounts of cholesterol	Not inhibited by minute amounts of cholesterol
6.)	Not inhibited by very low concentrations. of lecithin	Inhibited by very low concentrations of lecithin
7.)	Rate of lysis is a function of lysin concentration but is not directly proportional	Rate of lysis is directly proportional to lysin concentration
8.)	Haemolysis is preceded by a relatively short induction period	Haemolysis is preceded by a relatively long induction period
9.)	Critical thermal increment (20° to 30°C) = 21,400; is higher above 30°C.	Critical thermal increment (15° to 30°C) = 14,600.

After Bernheimer A.W. in "Streptococcal Infections" 1954 Ed. McCarty.

serologically distinct extra-cellular haemolysins:- one is oxygen labile and can be reactivated by reduction and is known as Streptolysin O; the other, Streptolysin S, is more oxygen stable and can be extracted from the streptococcal cells by serum. Modern knowledge of the subject can be said to have commenced with this discovery.

Streptolysin O is a toxic protein found extra-cellularly in streptococcal cultures, and is capable of existing in either of two forms; haemolytic or non-haemolytic. Exposure of the haemolytic form to oxygen results in its slow conversion to the non-haemolytic form. The process can be readily reversed by a variety of sulphhydryl compounds. Streptolysin O is most readily identified by the use of specific antibody.

Streptolysin S is a haemolytic substance found extra-cellularly in streptococcal cultures containing serum. Unlike streptolysin O, it does not undergo reversible, oxidative inactivation and cannot be identified by any single characteristic but by a series of properties the most important of which are listed in TABLE 3 .

The familiar beta-haemolysis seen surrounding colonies of Streptococcus pyogenes growing on the surface of aerobically incubated blood agar plates is due exclusively to streptolysin S. Apparently both streptolysins contribute to the haemolysis around sub-surface colonies of anaerobic plates provided the blood used is that of an animal species that does not contain antistreptolysin O (Herbert & Todd 1944).

Streptolysin O (SLO).

Production of SLO.

Most strains of Group A haemolytic streptococci produce SLO but a few have been encountered which lack this property. SLO has been shown to be formed also by streptococci of Group G and by Group C strains of human origin but not by strains tested

which belong to Groups B, C (animal), D, E, H or K (Todd 1939). SLO is formed *in vivo* in naturally occurring as well as experimentally induced infections and *in vitro* in culture supernatants when organisms are grown in peptone-infusion broth or simple chemically defined media (Todd & Hewitt 1932; Bernheimer & Cantoni 1945; Alouf & Raynaud 1965). Reducing agents must be added to such culture supernatants before the SLO haemolytic activity can be detected. In a study of the kinetics of bacterial growth and toxin production *in vitro* Alouf and Raynaud (1965) observed a diphasic differential rate of synthesis of SLO *i.e.* initially the ratio of toxin produced to bacterial growth was low but this was followed by a phase in which this ratio markedly increased. The simple chemically defined media used by these workers gave titres of SLO constantly greater than 700 H.U./ml. compared to titres of 100 to 256 H.U./ml. in the media described by previous workers.

Structure of SLO.

SLO is a protein, being thermolabile, non-dialysable, antigenic and readily destroyed by proteolytic enzymes (Smythe & Harris 1940). It contains disulphide bonds in the oxidised state which may be reduced to -SH, by reducing agents, in the active, haemolytic form (Herbert & Todd 1941). Alouf and his co-workers (1962, 1965, 1966 & 1967) have devised a procedure to obtain highly purified SLO (10,000 fold). Their preparations contained 4×10^{6} HD_{50} (one HD_{50} representing approximately 5×10^{-3} ug protein). It formed a homogeneous peak in the ultracentrifuge, could not be further resolved by electrophoresis and gave a single line of precipitation against homologous antiserum in immunodiffusion tests. The molecular weight as determined by Sephadex gel filtration and ultracentrifugation was found to be 80,000.

Antigenicity of SLO.

SLO elicits the formation of neutralising antibody in rabbits, mice, horses and man. Both the oxidised and reduced forms are antigenic but the possibility that the oxidised form is reduced *in vivo* cannot be excluded. It has also been shown that oxidised as well as reduced SLO combines with specific antibody (Smythe & Harris 1940). Kinetic studies have shown that the neutralisation reaction is a first order reaction (Vargues 1966). SLO is antigenically related to other oxygen labile haemolysins *e.g.* the theta toxin of *Clostridium welchii*, the tetanolysin of *Clostridium tetani* and the pneumolysin of *Streptococcus pneumoniae*. Any one of these haemolysins is neutralised by a specific antitoxin to any other.

The antistreptolysin O (ASLO) titre of human sera commonly rises following an infection with haemolytic streptococci. Thus crude preparations of SLO are used in the clinical screening of sera in which the ability of the test serum to neutralise the haemolytic activity of SLO is measured (Todd 1932). Recently Klein, Addison, Boone and Foody (1968) have shown that the species of indicator red cell used affected the neutralising titre obtained. An alternative method based on the agglutination of barium sulphate particles coated with SLO has been used for the detection and quantitation of ASLO in serum (Mosley & Pickett 1965) and this may provide better standardisation. The ASLO test is widely used as an index for the detection of streptococcal antibodies; it is particularly useful as a diagnostic aid in rheumatic fever and glomerular nephritis where no infecting organisms can be detected.

Mechanism of haemolysis by SLO.

SLO is haemolytic for a wide range of mammalian, avian and amphibian erythrocytes (Howard & Wallace 1953). The time-percentage haemolysis curve for SLO, like other bacterial haemolysins, is sigmoid and the rate of haemolysis is proportional to the toxin concentration (Bernheimer 1947). The elucidation of the detailed mechanism of action of SLO has been reported in a series of important papers by Alouf and Raynaud (1965, 1966 & 1968 a & b). In 1966 these workers showed that two topologically distinct sites on the SLO molecule are involved in its lytic activity. The first site is responsible for the fixation of the molecule to a specific lipid receptor located in the surface of the cell membrane and contains cysteine residues which are required for binding. These cysteine residues must be in the reduced form since oxidised SLO does not bind to the cell. It is thought that the lipid receptor on the cell membrane is cholesterol and that this could account for the inhibition of SLO by minute amounts of cholesterol. Moreover the resistance to lysis by SLO of organisms, such as bacterial protoplasts, the membranes of which lack sterols, (Bernheimer 1966), gives strong support to the concept that cholesterol is the binding receptor for SLO. The average amount of SLO required to induce the lysis of one rabbit erythrocyte is about 140 molecules (Alouf & Raynaud 1968 a). The second site on the SLO molecule is involved in the lytic reaction proper and is thiol-independent and not inhibited by cholesterol.

Two types of neutralising antibodies seem to be elicited by the SLO molecule each directed specifically against either the fixation or the lytic site. Rabbit erythrocytes were used as a model in these studies but the observation that human, ox, sheep

and rat erythrocyte ghosts inhibited the lytic action of SLO on rabbit erythrocytes (Brusca & Mastroni 1967) suggests that the binding site for SLO at least is common to most animal erythrocyte membranes. If cholesterol is the receptor site on membranes, then its molecular arrangement in cell membranes must differ from that in cholesterol containing serum lipoproteins since the latter do not bind to or inhibit SLO. An erythrocyte membrane fraction has been isolated by isoelectric precipitation with basic protein which will inhibit SLO (Brusca & Mastroni 1967) but has not yet been identified.

The binding of SLO to the erythrocyte membrane is rapid but subsequent lysis proceeds more slowly and has been suggested by Alouf and Raynaud to involve an accurate stereochemical orientation of the attached SLO so that it can exert a disrupting action on the lipoprotein framework of the cell membrane. The molecular mechanism for this stage has not yet been determined but kinetic evidence suggests that it may be enzymic in nature since the rate of lysis is strongly dependent on erythrocyte concentration (Alouf & Raynaud 1968). The mode of action of SLO is thus extremely complex, involving non-enzymic as well as possibly enzymic stages. The non-enzymic binding stage appears dominant in that a given amount of SLO will cause a certain amount of haemolysis irrespective of the period of incubation with the red blood cells. There is increasing evidence that other cytolytic bacterial toxins, e.g. staphylococcal alpha-toxin, also exhibit extremely complex interactions with cell membranes.

Other biological activities of SLO.

Besides its haemolytic properties, reduced SLO exhibits numerous other biological effects. It is lethal for many laboratory animals and provokes severe cardiac disorders (Halbert, Dahle, Keatinge, & Bircher 1965). Intravascular haemolysis occurs,

but not to a degree sufficient to cause death, and it is the cardiotoxicity of SLO which proves fatal. This effect has also been observed in vitro on intact, isolated amphibian and mammalian hearts (Bernheimer & Cantoni 1945; Kellner, Bernheimer, Carlson & Freeman 1956; Raskova 1958) and on cardiac tissue fragments (Goullet, Coraboeuf & Breton 1963). A single dose of SLO had no effect on an isolated frog heart but, if the heart was then washed after the first dose of toxin, and a second dose of SLO applied, the heart went immediately into systolic contraction. It is thought that the first dose of SLO caused the liberation of an inhibiting substance from the heart, which was removed by washing, so that the heart was then able to react to the second dose of toxin (van Heyningen 1950). In contrast, a minute amount of SLO brought about the prompt and irreversible loss of myocardial contractility of isolated guinea pig or rabbit heart. No other bacterial toxin is known to possess such cardiotoxic properties (Raskova & Vanecek 1964; Reitz, Prager & Feigen 1968). Submicroscopical myocardial changes in rats and guinea pigs following the administration of SLO have been observed by Waldman (1966); there was swelling of the mitochondria and oedema of the capillary endothelial and parenchymal cells.

Many other tissues or cells have been reported to be killed or damaged by exposure to SLO in vitro; these include chick embryo (Gabrielli, Saletti & Piazzi 1960), leucocytes (Hirsh, Bernheimer & Weissmann 1963; Zucker-Franklin 1965), macrophages (Fauve, Alouf, Delaunay & Raynaud 1966), Ehrlich ascites tumour cells (Ginsburg & Grossowicz 1960); mammalian skin cells (Lawrence 1959) and blood platelets (Bernheimer & Schwartz 1965). Microscopic observation of all these cells after SLO treatment showed striking cytological alteration of the cell membrane and intracellular organelles, particularly mitochondria and lysosomes. Suspensions of isolated organelles were also directly damaged by

exposure to SLO. and liberated some of their hydrolytic enzymes into the suspending medium (Weissmann, Keiser & Bernheimer 1963; Weissmann, Beecher & Thomas 1964; Bernheimer & Schwartz 1964).

Role of SLO. in pathogenicity.

Most of the studies of the biological action of SLO. have been carried out with crude or partially purified preparations and this has led to a certain amount of confusion. For instance, initially it was claimed that SLO inhibited mitochondrial respiration (Carlson, Kellner & Bernheimer 1956). Subsequent work showed this to be a property of contaminating amounts of NAD-ase present in the preparation of SLO (Carlson et al 1957). Nevertheless several lines of evidence indicate that most of the biological effects which have been reported are due to the activity of a single entity. It appears that, in distinction to many other bacterial toxins, the action of which is restricted to one kind of cell and very often to a single or very few animal species, SLO is a pantrophic cytopathogenic toxic protein It can be inferred from this property that this toxin must damage a basic cellular structure common to all sensitive animal cells. Recent evidence (Mastroeni, Pisfari & Nacci 1969) showed that an erythrocyte membrane fraction inhibited both the cytotoxic and haemolytic activity of SLO. Given its disruptive properties and its rapidity of action an obvious possibility is that the lipoprotein membranes bounding both the cell itself and intracellular organelles must be the primary target of attack of SLO.

Streptolysin S (SLS).

SLS was first identified by Todd (1938) and was so named because he thought that it was produced only in the presence of serum. This oxygen stable haemolysin, specific for Group A streptococci, is a heterogenous product and its production can be induced by albumin, alpha-lipoprotein, ribonucleic acid and RNA-ase digested oligoribonucleotide, Tween or Triton detergents and serum. Two extensive reviews have been written on the subject of SLS (Okamoto 1962; Ginsburg & Harris 1964), and I therefore, intend to review briefly those aspects relevant to this Thesis, concentrating on more recent observations.

Production of SLS.

Supernatants from cultures grown in the presence of serum were the first source of SLS until Weld (1934,1935) found that it could be extracted by shaking "resting" streptococci with serum. At first this property of serum appeared to be associated with the lipoprotein fraction (Herbert & Todd 1944). However, "resting" streptococci incubated with albumin in the presence of an energy source e.g. maltose, and a reducing agent have also been shown to elaborate haemolysin.

Subsequently, Okamoto (1939) discovered that yeast nucleic acid induced the formation of a potent haemolysin in growing cultures and further work (Ito, Okami & Yoshimura 1948; Bernheimer 1949; Hoysoya, Hayasni, Mori, Homma, Egami, Shimamura, Yagi & Suzuki 1949) showed that yeast nucleic acid promoted haemolysin production in "resting" suspensions of Group A streptococci in the presence of an energy source e.g. maltose or glucosamine, and magnesium and phosphate ions. Digestion of yeast RNA with pancreatic ribonuclease markedly increased its haemolysin-inducing capacity (Bernheimer & Rothbard 1948; Bernheimer 1949; Hoysoya et al 1949; Tanaka 1958). The active fraction (AF) associated with the ribonuclease-resistant core of RNA had approximately 100 times

the activity of undigested RNA. Maximal haemolysin production under these conditions was obtained if the cells were harvested from the beginning of the stationary phase of growth." (Barheimer 1954)

Considerable confusion has arisen as a result of the different extraction procedures employed for the production of oxygen-stable haemolysin from Group A streptococci. In 1958 Ginsburg and Grossowicz showed that various strains of Group A streptococci possessed a cell bound haemolytic factor which could be demonstrated by the addition of red blood cells to washed streptococci incubated with glucose, magnesium ions and sulphydryl compounds. Under these conditions, haemolysis of the red blood cells occurred but no extracellular haemolysin was found. Moreover, the haemolytic factor was not released from the cells by sonic oscillation or by grinding with glass beads but could be extracted by serum albumin, Tween 40, 60, or 80, or Triton X-205. More recently it has been shown that the haemolytic moiety of serum or albumin SLS can be transferred to the RNA active factor resulting in the formation of RNA-SLS (Taketo & Taketo 1964 b). Conversely transfer of SLS from RNA to serum or albumin has also been shown. The many hitherto confused reports of the nature of the oxygen stable streptococcal haemolysin can be explained on the basis of these findings. In brief, the similarities of these haemolysins are a reflection of a single biologically active prosthetic group of polypeptide nature, while the various discrepancies are due to the different carrier substances.

Two distinct intracellular haemolysins have been obtained by disrupting streptococcal cells (Taketo & Taketo 1965). One, termed intracellular streptolysin O (ICH-O) was neutralised by ASLO serum and had no relation to the formation of RNA-SLS. The other, intracellular streptolysin S (ICH-S) was oxygen stable and was not affected by ASLO. These two intracellular haemolysins were separated by gel filtration on Sephadex G 200. The production

of RNA-SLS by streptococcal protoplasts has been demonstrated (Maruyama, Sugai & Egami 1959). The toxin can also be produced by protoplast ghosts (Sugai & Egami 1960; Koyama, Sokawa & Egami 1963) and by a disrupted cell-free system in the presence of AF. (Taketo & Taketo 1964 a). Toxin production in the cell-free system was insensitive to various agents known to inhibit protein synthesis and this suggests that the haemolysin was not formed by de novo synthesis. It has been postulated by Taketo and Taketo that RNA-SLS is formed in the cell-free system by the transfer of the haemolytic moiety or the prosthetic group of ICH-S from a cellular carrier protein to AF. RNA itself is not active in the cell free system and in the whole cell system, presumably functions after its conversion to AF. by streptococcal nuclease.

Thus during the growth of streptococci in media devoid of any of the carriers normally used, the haemolysin presumably remains associated with the bacterial cell. Upon addition of a substance which has greater affinity for the haemolytic moiety than does the streptococcal cellular carrier, the haemolytic moiety is transferred to the extracellular carrier. The cell membrane appears to be the most likely site of binding for the haemolysin within the cell since protoplasts and "ghosts" have been shown to yield haemolysin under suitable conditions.

Structure of SLS.

Most studies of the structure of SLS have been carried out using the RNA-SLS form. As commonly prepared by the method of Bernheimer (1949) SLS exists as a polypeptide attached to an oligoribonucleotide (Koyama & Egami 1963, 1964; Koyama 1963). As mentioned earlier the haemolytic moiety appears to be the polypeptide and the oligoribonucleotide functions only as a carrier. This carrier, however, confers stability on the

polypeptide moiety which is incapable of existing alone in an active state. The molecular weight of purified preparations of the RNA-SLS complex estimated by gel filtration was 12,000 and a value of 20,000 was obtained by ultracentrifugation in a sucrose gradient (Bernheimer 1967). This discrepancy may be due to polymerisation of the molecules under the conditions of ultracentrifugation.

According to Koyama (1963) the molar ratio of polypeptide to oligoribonucleotide is 0.3. Accepting the molecular weight of the carrier-protein complex obtained from gel filtration as being the most accurate, it can be calculated that the molecular weight of the polypeptide portion is 2,800. This would suggest a polypeptide consisting of 28 amino acid residues. Glutamic acid or glutamine and serine have been shown to be the most abundant but aspartic acid, threonine, proline, alanine, valine, iso-leucine, leucine, lysine and histidine were also detected (Koyama 1963). The oligoribonucleotide portion of the molecule was composed of aggregated heterogeneous oligoribonucleotides rich in guanine which dissociated in 8M urea and could be separated on DEAE cellulose (Koyama 1964).

Inhibition of SLS.

Agents known to inhibit the haemolytic activity of SLS in vitro include lecithin and to a lesser extent other phospholipids (Hewitt & Todd 1939; Cinader & Pillemer 1950; Humphrey 1949; Stollerman, Bernheimer & MacLeod 1950), analine dyes, trypan blue and congo red (Ito 1940; Rosendal & Bernheimer 1952), extracts of certain plant and animal tissues (Stollerman et al 1950) and the sera of man and animals (Todd 1939; Humphrey 1949; Stollerman et al 1950). Papain and chymotrypsin destroy the toxin but it is unaffected by trypsin, pepsin or cholesterol (Bernheimer 1949).

Non-antigenicity of SLS.

SLS. in any of its extracellular forms has never been shown to be antigenic and this is one of the major differences between it and ICH--S. The low molecular weight of RNA-SLS alone could account for its lack of antigenicity. Toxin neutralising activity has been shown in the sera of some patients recovering from streptococcal infections using extracellular SLS for the assay. This neutralising activity was not predominantly associated with the beta or gamma-globulin fractions in which antibodies are found (Humphrey 1949). Also treatment of inhibitory sera with ether or ether/alcohol, in such a manner as to cause only minor changes in the titres of known antibodies, caused major reductions in the ability to neutralise SLS. The degree of inhibition of SLS was related to the total phospholipid content of the sera (Stollerman, Brodie & Steel 1952), and it appeared that the phospholipids, in the form of lipoprotein complexes, played a major role in SLS. inhibition by such sera.

A tabular comparison of some of the similarities and differences reported for the different forms of SLS are given in TABLE 4 .

TABLE 4.

Materials and Methods Which Have Been Used To Obtain

Oxygen-Stable Haemolysins From Streptococci.

Haemolysin	Compound required for production	Optimal conditions and time	Inhibition by	Anti-genicity	Chemical nature reported
Streptolysin S Serum - SLS	Whole serum Albumin alpha-lipoprotein Lecithovitelline	Growing and resting cells 5 to 15 by resting cells	Lecithin serum lipo-proteins	?	Lipoprotein
Streptolysin S RNA-SLS	RNA-native or RNA-ase digested, Oligonucleotides Biosynthetic Polynucleotides Sugars, Mg^{2+} PO_4^{3-}	Growing and resting cell-free extracts 60 to 90 by resting cells	Lecithin, Trypan blue, Congo red Serum lipo-proteins Papain Chymotrypsin Ficin	?	Polynucleotide. Polysaccharide. Protein complex Oligonucleotide. Polypeptide Complex
Intracellular Haemolysin ICH		Sonication 1 to 6 hours	K^+ Antiserum	+	
Cell bound Haemolysin CBH	Sugars, Mg^{2+} SH-compounds	Growing and resting cells 5 to 10 by resting cells	Trypan blue tetracylines chloramphenicol Iodoacetic acid, NaF	?	
Streptolysin D	Sugars or Amino acids, Mg^{2+}, SH-compounds Serum, Albumin or Detergents	Growing and resting cells 5 to 10 by resting cells	Lecithin Trypan blue, Congo red. Whole serum Papain Lipoproteins	?	

Modified from Ginsburg I and Harris T.N. (1964) Ergeb, Mikrobiol, Immunol, Exptl. Therap. 38, 198

Mechanism of haemolysis by SLS.

The time haemolysis curves for SLS are sigmoid in shape consisting of three phases:- (a) the lag time (phase 1), (b) the period of maximum rate of haemolysis (phase 2) and (c) the period of decreasing rate of haemolysis (phase 3). (Bernheimer 1949; Cinader & Pillemer 1950; Koyama 1965). During phase 1 there is always a large proportion of SLS in the supernatant. For instance, erythrocytes treated with SLS for 10 minutes at 10°C. and then removed prior to the onset of haemolysis, washed, resuspended in buffer and incubated at 37°C. for 30 minutes were found to haemolyse although 90% of the SLS was recovered from the supernatant (Koyama 1965). These results suggest that, during phase 1, there is a progressive, irreversible modification of the erythrocyte membrane, which, after reaching a critical point, results in haemolysis.

In phase 2 the rate of haemolysis is proportional to the concentration of SLS present. However, an increase in the initial erythrocyte concentration results in a decrease in the rate of haemolysis. In such an experiment the degree of modification of each erythrocyte membrane probably decreases as the initial concentration of erythrocytes increases and, therefore, haemolysis occurs more slowly. Phase 3 is primarily due to the decrease in the numbers of unhaemolysed erythrocytes.

Erythrocyte ghosts inhibited haemolysis by the toxin suggesting that at some stage in the haemolytic reaction the SLS is bound to or inactivated by erythrocyte membrane. The receptor or substrate of SLS differs from that of SLO. Cholesterol does not inhibit SLS and this toxin lyses membranes which lack cholesterol including the protoplasts of Bacillus megaterium, Sarcina lutea and Escherichia coli and three species of Micrococcus lysodeikticus (Bernheimer & Schwartz 1965; Davie & Brock 1966). Also Bangham, Standish and Weissman (1965) have

shown that SLS. caused leakage of potassium and sodium from artificial lipid spherules and that its action resembled lysolecithin. If lecithin or phospholipid are the receptors for SLS. on the erythrocyte this would explain the inhibition of haemolysis by minute amounts of lecithin.

Other biological activities of SLS.

SLS. is cytopathic for a number of mammalian cell types in vitro. The types of cell which have been shown to be susceptible include leucocytes (Weld 1934; Bernheimer & Schwartz 1960; Ginsburg & Grossowicz 1960), Ehrlich ascites tumour cells (Ginsburg 1959; Ginsburg & Grossowicz 1960; Symington & Arbuthnott 1969), various tissue culture cells (Snyder & Hamilton 1963; Marcus, Davies & Ginsburg 1964) and blood platelets (Bernheimer & Schwartz 1965). The changes induced by the haemolysin were characterised by swelling, the appearance of blebs, cytoplasmic vacuolation and reduced viability of the cells. Subcellular membrane-bound organelles such as mitochondria and lysosomes were also affected by SLS. resulting in enzyme leakage (Weissmann et al 1963; Bernheimer & Schwartz 1964; Keiser et al 1964). The visible cytopathic changes in mammalian cells caused by SLS. were almost identical to those caused by living streptococci (Havas, Donnelli & Porreca 1963; Taketo & Taketo 1966; Khovanova 1967 a & b; Quinn and Lowery 1967).

These changes described above are in agreement with the membrane being the site of attack and Taketo and Akira (1967) have suggested that the oncolytic activity of Group A streptococci is due to the action of cell-bound SLS. (ICH-S). The cytopathic effect of SLS. can be abolished by lecithin (Marcus et al 1964) and it has been postulated that phospholipids may be the membrane receptor site.

Role of SLS in pathogenicity.

Despite its obvious toxicity the role of SLS in streptococcal infections remains unclear. Whether SLS is produced *in vivo* remains to be shown. The lack of demonstrable antigenicity of this haemolysin has hampered attempts to follow its production *in vivo*. There is a major discrepancy concerning the lethality of SLS. The injection of serum extracts containing 15 to 30H.U. killed mice within 24 hours causing pathological changes in spleen, liver and kidney (Weld 1933, 1935; Hare 1937; Barnard & Todd 1940). On the other hand 300 µg. of RNA-SLS (equivalent to 12,000H.U.) was necessary to kill mice (Rosendal & Bernheimer 1952). It is hard to conceive of such large amounts of SLS being produced *in vivo*, therefore, it seems unlikely that this toxin alone is lethal.

SLS, like SLO, is a pantropic cytopathogenic agent but its stability in the presence of oxygen would possibly extend the range of susceptible cells and the conditions under which it could act. The diversity of carriers which can be attached to the haemolytic moiety of SLS makes it seem feasible that extracellular SLS could be formed *in vivo* by the transfer of SLS from the streptococcal cells to various carriers in the body. Since a large proportion of the resultant extracellular haemolysin would be host material, it probably would not stimulate antibody production. .... Such a product would thus be able to persist in the body for long periods and might contribute significantly to the chronic streptococcal diseases.

The contribution of SLS to the pathogenicity of Group A streptococci is a complex one which is still not understood and there is a lot of scope for further investigation in this field.

AIMS OF THIS THESIS.

The pantrophic cytological effects of streptolysins O and S are now well documented and it is generally accepted that the primary action of these toxins is on cellular membranes causing disorganisation. These toxins also appear to penetrate to the interior of cells and disrupt the membranes bounding intracellular organelles such as lysosomes, nuclei and mitochondria.

The cytolytic activity of living streptococci on mammalian cells closely resembles the effect of SLS itself; This suggests a dual role for SLS in vivo. It may play a role in pathogenicity both as a cell-bound toxin and as an extracellular diffusible product; the action of the latter could result in an extensive increase in the area of host tissues being damaged during a streptococcal infection. At the cellular level, the action of this toxin on the cell's membrane systems must result in secondary changes which presumably cause cell death.

The object of this Thesis was to determine what metabolic changes occur within the cell as a result of the cytotoxic action of SLS. To study this the respiratory centre of the cell, the mitochondrion, was used as a model since it manufactures all the energy, stored in the form of adenosine triphosphate (ATP), which is necessary for the diverse synthetic processes carried out in the cell. The adequate functioning of mitochondria is, therefore, essential for cellular survival. The first section of the results will deal with the action of SLS on isolated mitochondria.

The effect of SLS on the respiration of whole cells was also investigated in order to find out whether its cytotoxicity can be explained solely by an effect on mitochondria or whether more complex changes are involved. Krebs 2 ascites tumour cells were selected as a model for the study of cellular respiration and the second part of the results section deals with this aspect.

A study of another cytolytic toxin, staphylococcal alpha-toxin, on Krebs 2 ascites tumour cells was also carried out and the findings are included in the third results section. It is now generally accepted that both SLS and staphylococcal alpha-toxin act on the limiting cell membrane causing the loss of its normal semi-permeable properties. Therefore, a comparison of these toxins in this phase of the work seemed likely to yield results which would facilitate the understanding of the mode of action of SLS. The value of using staphylococcal alpha-toxin for comparison was enhanced by the fact that it is known not to impair mitochondrial respiration. (Lominski, Arbuthnott, Gemmell, Gray & Marshall 1964; Lominski, Gemmell & Arbuthnott 1968). This to some extent allowed distinction to be made between effects resulting from an action on the limiting cell membrane from those on mitochondria.

The detailed properties of staphylococcal alpha-toxin are well documented (Bernheimer 1968; Arbuthnott 1969) and it is considered unnecessary to review staphylococcal alpha-toxin at length here. Staphylococcal alpha-toxin is an oxygen-stable, extracellular protein excreted by Staphylococcus aureus during the logarithmic phase of growth and unlike SLS, it does not have to be extracted from the cells by a carrier. This toxic protein is haemolytic in the presence of molecular oxygen although the range of erythrocyte species susceptible to lysis by staphylococcal alpha-toxin is somewhat narrower than that lysed by SLS. For instance the species of erythrocyte most sensitive to staphylococcal alpha-toxin is rabbit while human erythrocytes are 100 times more resistant to the toxin; SLS lyses both human and rabbit erythrocytes to the same extent. Like SLS, in addition to being haemolytic, staphylococcal alpha-toxin damages the membranes of several other biological structures, (TABLE 5).

TABLE 5.

In Vitro Sensitivity (+) and Insensitivity (-)

Of Various Biological Structures

To Streptolysin S and Staphylococcal Alpha-Toxin.

Test Biological Material	Streptolysin S	Staphylococcal Alpha-Toxin
Rabbit erythrocytes	+	+
Rabbit polymorphonuclear leucocytes	+	+
Rabbit platelets	+	+
Tissue Culture cells (various)	+	+
Mitochondria (rabbit or mouse)	+	-
Rabbit lysosomes	+	+
Whole bacteria	-	-
Staphylococcus protoplasts	+	-
E. coli Spheroplasts	+	-
Mycoplasma neurolyticum	+	-
Ciliated protozoa	-	-

Modified from Bernheimer A.W. (1968) Science, 159, 847.

Of particular relevance to this work is the fact that staphylococcal alpha-toxin has been reported to cause gross morphological damage to Ehrlich ascites tumour cells which resulted in the leakage of small and large molecular weight compounds from the cells. Such treatment also rendered the cells incapable of proliferating when injected intraperitoneally into mice (Madoff, Artenstein & Weinstein 1962).

Having reviewed the toxic armoury of the Group A streptococcus and having outlined the broad aims of this Thesis it is now necessary to give a brief account of the biological models employed, namely the mitochondrion and Krebs 2 ascites tumour cells.

- o -

BIOLOGICAL MODELS.

THE MITOCHONDRION.

The mitochondrion is the "powerhouse" of the cell. It contains the enzymes of the tri-carboxylic acid cycle and the complex enzyme system which transmits to oxygen the electrons removed from the various intermediates during metabolism and traps part of the energy so released in the form of high energy phosphate (ATP) which is then utilised by the cells in biosynthetic reactions. Comprehensive reviews of the isolation, structure and metabolic activities associated with the mitochondrion have been made by Lehninger (1964) and Gemmell (1968). I shall, therefore, only outline the properties of the mitochondrion which are pertinent to its use as a model for the action of SLS.

Structure of the Mitochondrion.

An electron micrograph of cross sections of mitochondria is shown in FIG. 3 . The mitochondrion is bounded by a double membrane, the inner layer of which is invaginated to form characteristic cristae which vary in conformation from mitochondria from different sources. The interior of the mitochondrion contains a "matrix" of dense granules. The generalised structure of the mammalian mitochondrion is represented diagrammatically in FIG. 4 . The number of mitochondria in any one cell varies according to the function of the cell; rat liver cells contain as many as 2,500 mitochondria whereas sperm cells contain only about 20.

When mitochondria are isolated from disrupted tissues they are very sensitive to changes in osmotic pressure and the composition of the fluid used to suspend them is critical. Sucrose solutions (Hogeboom, Schneider & Palade 1948) are particularly valuable because they prevent aggregation and allow preservation of the cytological properties of the mitochondria. Mitochondria isolated in sucrose show close morphological resemblance to those

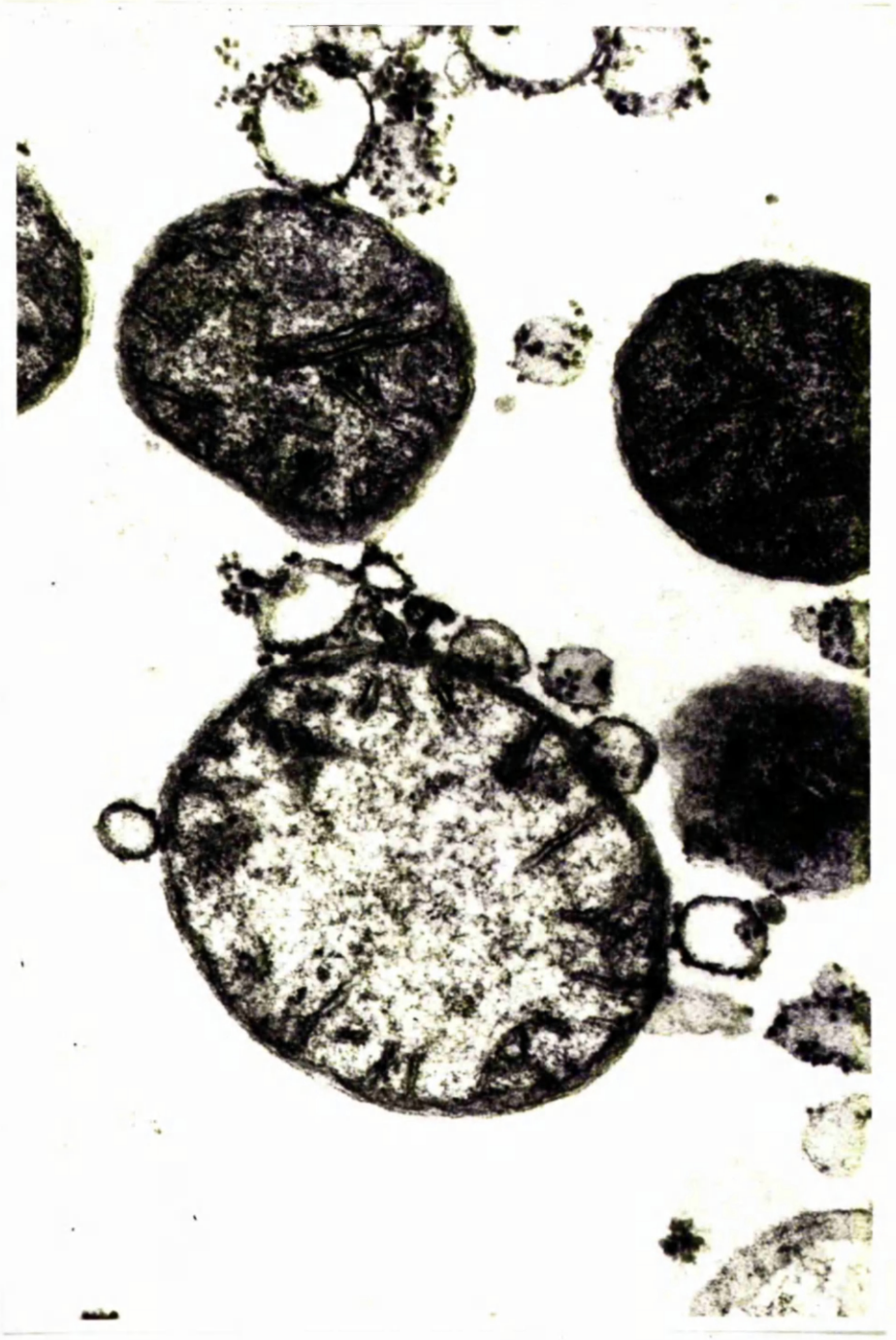


FIG. 3. Electron Micrograph of sectioned Mouse Liver Mitochondria.

(x 50,000)

This section was prepared for me by Dr.P.G.Toner.

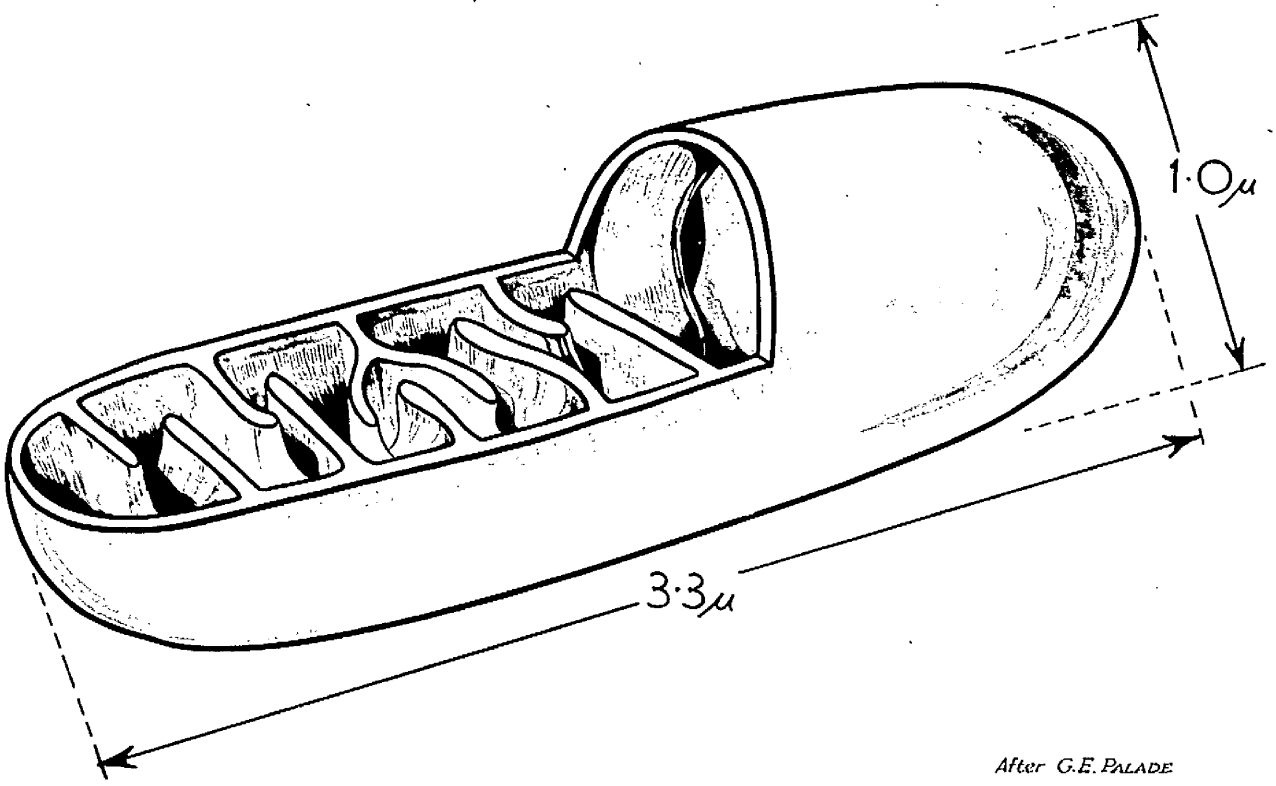


FIG 4. Diagramatic Representation of the Mammalian Mitochondrion.

found in intact tissues when examined in ultra-thin sections under the electron microscope (Witter, Watson & Cotton 1955; Huff, Hogeboom & Dalton 1956; Watson & Sickevitz 1956; Zeigler, Linnanne, Green, Dass & Riss 1958). Modifications to this suspending medium have been made by adding a number of different substances particularly buffers.

Metabolic Activities Associated with the Mitochondrion.

Although the entire complex of enzymes and co-factors necessary for the Tri-carboxylic acid cycle is present in the mitochondrion (Schneider & Hogeboom 1958) as well as the fatty acid oxidation enzymes (Green, 1952 ; Kennedy & Lehninger 1948-1949), the respiratory activity of the mitochondrion is generally regarded as its central property. All cellular oxidations do not occur in the mitochondrion but the terminal enzymes of the respiratory chain have been established, without a doubt, to be localised in the mitochondrion (Schneider 1946; Schneider & Hogeboom 1950).

Following the discovery of dehydrogenases by Thunburg (1909), the rediscovery of cytochromes by Keilin (1925), and the flavo-proteins by Warburg and Theorell (1939), Keilin formed his first reconstruction of the electron transport system using heart particles. This was as follows:-

dehydrogenases ---> flavoproteins ---> cytochromes ---> oxygen.

The basic concept that the electron carriers are arranged in a chain of increasing oxygen-reduction potentials, with electrons flowing from the relatively electronegative substrates via the pyridine nucleotides and the flavoproteins to the increasingly electropositive cytochromes and finally to oxygen, still stands today. Since then Chance and his colleagues using spectroscopy to examine the respiratory carriers in the intact mitochondrion,

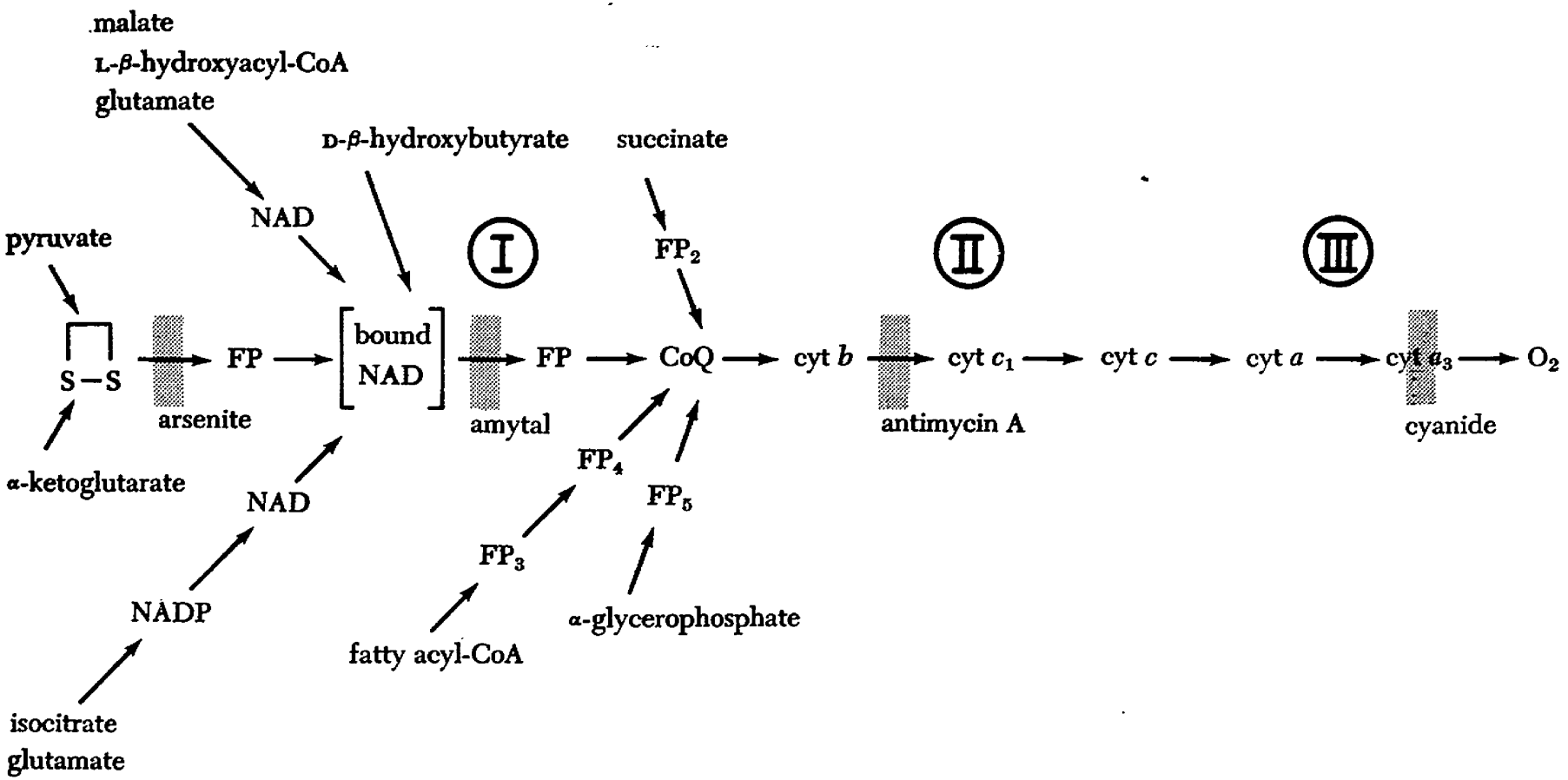
have provided a precise picture of the composition, sequence and dynamics of the respiratory chain. FIG. 5 shows how various substrates are oxidised within the mitochondrion.

The constituents of the respiratory chain are closely integrated with the mitochondrial membrane. This contains 30 to 40% lipids, largely phosphatides, and 60 to 65% protein of two types, structural and enzymic. Undoubtedly, the great bulk of the membrane lipids serve a purely structural function. However, certain lipids in the membranes have been identified as having quite specific functions in electron transport, translocation mechanisms, and swelling and contraction. Most of the mitochondrial phospholipid can be extracted with 90% acetone in H_2O (Lester & Fleisher 1961). Such a procedure leads to loss of the ability to carry out electron transfer but this capacity can be restored by adding mitochondrial phospholipid and ubiquinone. Most of the proteins in the mitochondrion, with the exception of the primary dehydrogenase complexes, are quite hydrophobic, forming water-insoluble polymeric arrays in the absence of lipids (Green & Fleischner 1963). It has been suggested that the hydrophobic proteins of the electron transfer chain require phospholipid as a bridge to the aqueous phase.

On the basis of kinetic studies Chance, Estabrook and Lee (1963) concluded that the catalysts of the respiratory chain are distributed within or over the surface of the mitochondrial membrane system. It is thought that the primary dehydrogenase complexes are associated with the outer membrane while the electron transport chain for succinic oxidation is located in the cristae. Purified preparations of rat liver mitochondria have been prepared by swelling followed by differential centrifugation (Parsons, Williams & Chance 1966) and appeared as thin bag-like structures under the electron microscope. The specific activity of cytochrome oxidase in these outer membrane preparations was less than 10% of that found in the inner membranes. Chance and his

From "The Mitochondrion" Lehninger (1964)

FIG 5



Schematic diagram of the respiratory chain. The scheme shows points of inhibition as well as sites of phosphorylation (Roman numerals).

co-workers postulated that each component of the respiratory chain possessed some individual mobility but together they formed a macromolecular assembly of enzymes capable of carrying out all the functions of electron transport and oxidative phosphorylation.

Using a different approach, Green (1962) was able to isolate four complexes from mitochondria (TABLE 6). These four complexes could be recombined in a 1:1:1:1 molar ratio to form a unit which exhibited integrated electron transport activity provided that two mobile components, ubiquinone and cytochrome c were present. Lipids were also essential for recombination. In this model all the components appeared to be rigidly aligned except for ubiquinone and cytochrome c.

A third type of structural subunit has been isolated from mitochondria more recently by Blair, Oda, Green, Silver and Fernandez-Moran (1963), the elementary particle. Negative staining with phosphotungstic acid has shown that these elementary particles, whilst still present in the mitochondrion, consist of a spherical head-piece (80 to 100 Å in diameter) and a cylindrical stalk (30 to 50 Å) which is attached at one end to the head-piece and the other end to the cristae (Blair et al 1963; Fernandez-Moran 1963). The elementary particles have been shown to contain the complete electron transport chain for the oxidation of succinateor NADH by molecular oxygen. The internal arrangement of the enzymes in mitochondria is, therefore, a matter of controversy although a fairly rigid spatial arrangement of most of the respiratory enzymes is supported by the most recent work.

Effect of Streptococcal Products on Mitochondrial Function.

SLS has been demonstrated to cause structural alterations in cellular membranes including those of mitochondria which result in permeability changes (Keiser et al 1964). It seems possible that such structural disorganisation of mitochondria would result in impairment of respiratory function. To my knowledge this has

TABLE 6.

The Four Complexes of the Electron Transfer Chain.

Complex	Designation	Electron transfer Reaction catalysed
1	NADH-ubiquinone (Q) reductase	NADH → Q
11	Succinate--ubiquinone (Q) reductase	Succinate → Q
111	QH_2 - cytochrome c reductase	QH_2 → cytochrome c
1V	Cytochrome oxidase	Reduced cytochrome c → O_2

not been demonstrated nor has a possible site of toxin attack on the electron transport chain been determined.

Streptococcal NAD-ase has been shown to split the nicotinamide-ribose linkage of NAD and therefore inhibits the utilisation by mitochondria of intermediates of the Tri-carboxylic acid cycle which require NAD as a co-enzyme (FIG. 5). It was decided to investigate the action of SLS on the functioning of the electron transport chain beyond NAD. Facilities available in this department allowed the assay of succinic oxidase and cytochrome oxidase to be carried out. Succinic oxidase activity was assayed since it necessitated the functioning of all the intermediate components of the electron transport chain beyond NAD while the assay of cytochrome c reflected the function of the terminal portion of the chain. These enzyme complexes were also chosen because they were known to contain components which functioned only when lipid bridges were present to maintain their spatial arrangement.

KREBS 2 ASCITES TUMOUR CELLS.

Much of the work on the action of cytolytic toxins has been done using erythrocytes as cellular models. However, since the erythrocytes lack most features of intracellular organisation (e.g. nucleus and mitochondria), haemolysis reflects the action of cytolytic toxins on the limiting cell membrane. It seemed desirable to select a type of cell which possessed a full complement of intracellular organelles, in order to assess the overall action of cytolytic toxins on cellular integrity. For several reasons ascites tumour cells were chosen as a model for this study. These cells possess a subcellular organisation which is absent in erythrocytes and the ease with which they can be propagated, by intraperitoneal passage in mice, presents a distinct practical advantage over tissue culture cells.

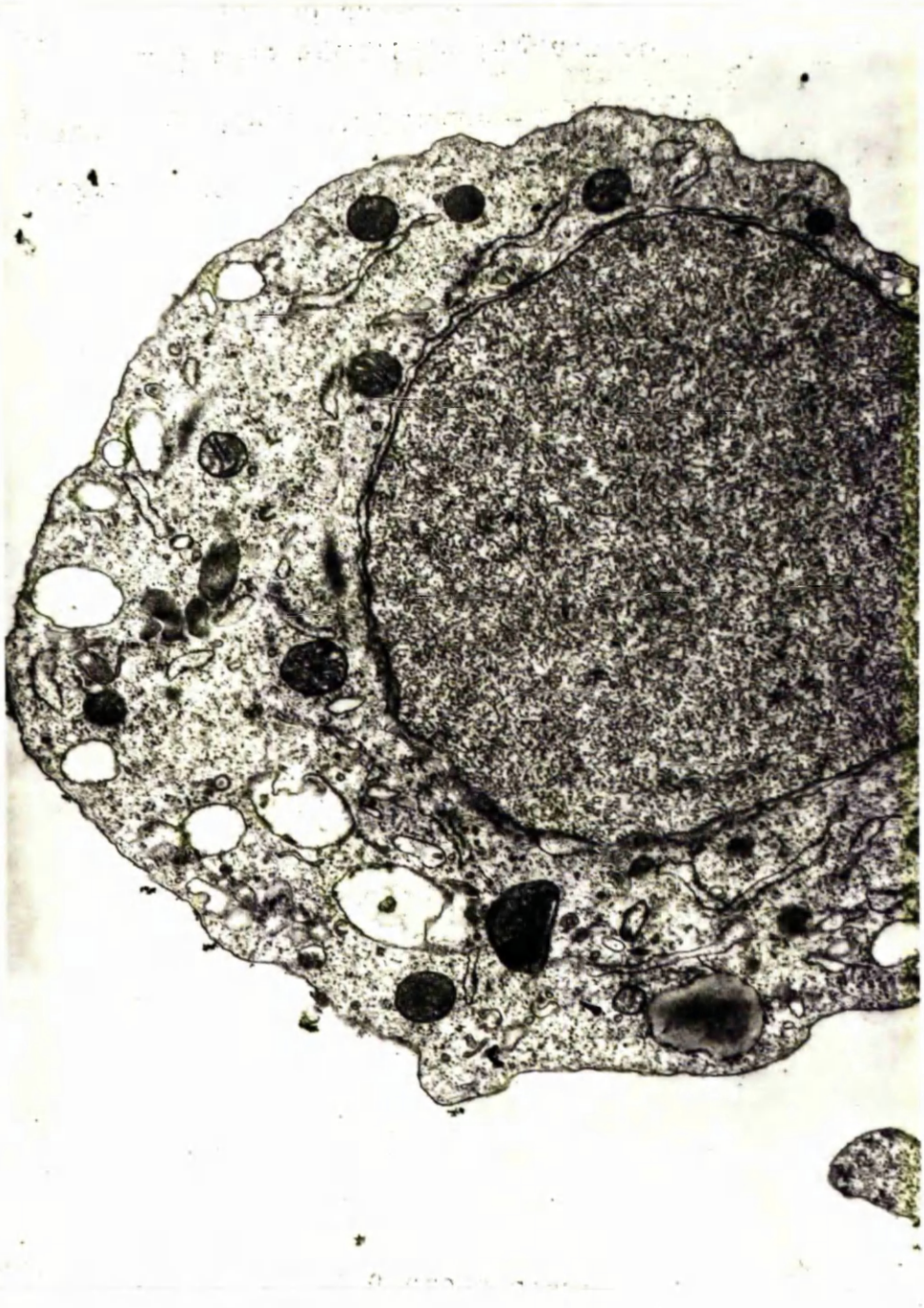


FIG. 6. Electron Micrograph of a cross section of a Krebs 2 Ascites Tumour cell (x 20,000).

This section was prepared for me by Dr. P.G.Toner. Dept. Pathology Glasgow University.

The term "ascites tumour" refers to neoplasms growing in the form of suspensions of free and homogeneously distributed living tumour cells in the ascites fluid, in cases where the proportion of tumour cells (after a certain stage in development) is very high representing almost a pure culture. The metabolism of ascites tumour cells has been extensively studied and the enzymes of Glycolysis and the Tri-carboxylic acid cycle have been isolated from them. Their mitochondria appear to respire in the same way as those from normal mammalian cells.

The Krebs 2 line of ascites tumour cells originated from a solid carcinoma which arose spontaneously in the inguinal region of a hybrid male mouse and the cells were converted to the ascitic form by Klein and Klein (1951). Mice containing Krebs 2 ascites tumour cells were kindly donated to me by Miss Janet Smillie and Dr. R.H. Burdon of the Biochemistry Department, University of Glasgow. An example of the Krebs 2 cells used in this work is illustrated in FIG. 6 .

It is necessary to bear in mind that, being tumour cells, the Krebs 2 cells are abnormal but perhaps not more so than other cells grown in vitro e.g. tissue culture cells. These cells have been extensively used as models for the study of cellular metabolism because of the ease of producing large numbers. For the purpose of this Thesis, Krebs 2 ascites tumour cells were therefore, used to study the effects of SLS and staphylococcal alpha-toxin on their respiration using exogenous substrates which were known to be intermediates of Glycolysis and the Tri-carboxylic acid cycle.

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MATERIALS AND METHODS.

PREPARATION OF STREPTOLYSIN S.

Test organism.

Most preparations of Streptolysin S were made using Streptoccus pyogenes strain C 203S which was kindly sent to me by Dr. A. W. Bernheimer. A mutant of this strain, C 203U, known not to produce Streptolysin S was also used for comparison. Four strains of Group A streptococci from the National Collection of Type Cultures, NCTC 3359, 5163, 9994 and 8198 were also used in the preparation of serum Streptolysin S. All cultures were maintained in freeze dried ampoules which were opened when required.

Extraction procedures.

Serum Streptolysin S was prepared by the method of Todd (1938). The organisms were grown overnight at 37°C in Brain Heart Infusion broth (Difco) containing 20% horse serum (Wellcome Laboratories Ltd.) and harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C. The cocci from 500 ml of culture were suspended in 8 ml inactivated horse serum (56°C for 30 minutes) and shaken with large glass beads (5 mm diameter) in a Mickle disintegrator using the slowest speed of shaking for 60 minutes. The liquid was decanted and the cocci removed by centrifugation; these cells were resuspended in 8 ml of horse serum and extracted in the same way. The serum extracts were bulked and Seitz filtered. The resulting haemolysin was stored at -20°C.

R.N.A. Streptolysin S was prepared by a modification of the method described by Bernheimer (1949). A fresh freeze-dried ampoule of the organism was inoculated into 200 ml of Brain Heart Infusion broth (Difco) containing 0.1% maltose and incubated overnight at 37°C. 20 ml of this overnight culture was inoculated into 180 ml of the same medium, which had been pre-heated to 37°C, and incubated for 5 hours until the end of the logarithmic phase of growth was reached. The cells were harvested by centrifugation and washed once in 50 ml of phosphate buffered saline, containing 0.9% NaCl, and 0.15M sodium phosphate having a pH of 7.0; these resting cells were then incubated for 3 hours in 3 ml of suspending medium prepared according to the method of Bernheimer (1949). This contained 0.02M maltose, 0.11M KH_2PO_4, 0.12M $Mg_2SO_4,7H_2O$ and 0.6 mg/ml "Active Factor" (a ribonuclease resistant R.N.A. core commercially prepared by Worthington Biochemicals Ltd.). After standing overnight at 4°C the cells were removed by centrifugation and the supernatant fluid assayed for haemolytic activity. This was then stored at -20°C in small volumes (2 ml). Aliquots of toxin were thawed when required and used immediately; repeated freezing and thawing was found to inactivate the toxin.

Albumin Streptolysin S Resting cells were prepared as for R.N.A. Streptolysin S and suspended in 3 ml of a medium containing 5mM glucose, 6mM cysteine HCl, 5mM Mg_2SO_4, $7H_2O$ and 120 mg/ml bovine serum albumin, and incubated for 20 minutes at 37°C. After centrifugation the supernatant fluid was assayed for haemolytic activity.

Tween Streptolysin S Again cells were prepared as for R.N.A. Streptolysin S and suspended in a solution containing

4 mg/ml Tween 40 or 80. An incubation period of 20 minutes at 37°C was allowed prior to centrifuging the cells and assaying the supernatant fluid for haemolytic activity.

PREPARATION OF KREBS 2 ASCITES TUMOUR CELLS.

These cells were propagated by intraperitoneal injection of 0.02 ml of freshly aspirated ascitic fluid into Porton white mice (weighing 25 to 35 g.). Cells for all experiments were harvested by needle aspiration from mice 7 to 10 days after the animals were inoculated. Only clean preparations free of blood were used. A series of pilot experiments showed that the following washing procedure gave cell suspensions suitable for respiration studies. The freshly aspirated ascitic fluid was diluted with an equal volume of 7% polyvinyl pyrrolidone (PVP) buffered with 0.02M tris/HCl pH 7.3, and the cells were sedimented by centrifuging at 2,000 r.p.m. in a bench centrifuge. The cells were then washed once in Ringers solution and resuspended in their original volume in PVP/tris buffer.

This washing procedure prevented the spontaneous aggregation of the cells which occurs rapidly in normal ascitic fluid; such aggregated cells did not respire well, therefore, washing was necessary to make the cells suitable for manometric studies. Moreover the high viscosity of the buffer, due to the large molecular weight of the PVP, helped to preserve the structural integrity of the ascites cells. The final concentration of cells in washed preparations varied between 1 and 2 x 10^8/ml when measured by counting an appropriate dilution of cell suspension in a haemocytometer. The Krebs 2 cells were used for manometric studies within an hour of aspiration.

Disintegrated preparations of Krebs 2 cells were made by sonicating a washed cell suspension in Ringers solution, using an MSE ultrasonic probe for 45 seconds after which 90% of the cells were disintegrated as assessed microscopically. These disintegrated cell preparations were still capable of oxidising succinate indicating that mitochondrial function had been preserved.

PREPARATION OF ISOLATED MITOCHONDRIA.

Mitochondria from mouse tissues.

Mitochondria were prepared freshly every day by the method of Lominski, Gemmell and Arbuthnott (1968). Porton white mice (35 to 45 g.) were killed by cervical dislocation and exsanguinated. Mouse liver was usually used as a source of mitochondria although mitochondria from other organs of the mouse, kidney and heart, were used in a few experiments. The methods employed were basically the same for all tissues. Three mouse livers were excised and placed in ice-cold suspending fluid; after removal of connective tissue by gentle teasing out, they were homogenised in a borosilicate glass grinder (Baird and Tatlock Ltd.) by applying 10 strokes of the matching glass plunger. The homogenate was suspended in the appropriate fluid in the proportions of 1 g. per 10 ml and first spun at 600 g. for 10 minutes; in order to remove nuclei and unbroken cells the sediment was discarded and the supernatant fluid spun at 8,500 g. for 10 minutes. This sediment was washed with 20 ml of suspending fluid, spun again at 8,500 g. for 10 minutes and was finally resuspended in 4 ml. Mitochondrial preparations were stored for 2 hours before use. The entire process was carried out at $4^{\circ}C$. Immediately prior to use in Warburg experiments the mitochondrial suspension was diluted 1:3 with suspending fluid. When the oxygen electrode was used mitochondrial samples were not diluted.

As a rule the same suspending fluid was used for the preparation of mitochondria, for subsequent dilution and assay and for dissolving all reagents. In most experiments this was 0.25M sucrose containing 0.023M sodium phosphate buffer (Henry 1948) pH 7.3. However, in some experiments mitochondria were prepared and assayed in 0.25M sucrose containing 0.033M tris/HCl.

Mitochondria from Krebs 2 Ascites tumour cells.

These were prepared by the method of Wu and Sauer (1967). The ascites tumour cells were harvested from 4 - 6 mice (7-9 days tumour). The ascitic fluid was immediately diluted in two volumes of ice cold sucrose (0.25M) solution containing EDTA (2mM), pH7.5 and the cells centrifuged at 600 g. for 5 minutes. The packed cells were washed twice by resuspending the cells in approximately 10 volumes of the same solution and centrifuged as above. For homogenisation, the packed tumour cells (approximately 5 ml) were mixed with sucrose-EDTA solution and 0.1 ml of 1M triethanolamine buffer, pH 7.4. The mixture was poured into a pre-cooled mortar (7 cm diameter); 3 g. of acid washed glass beads (Ballotini) were added and the mixture ground forcefully with a pestle for 6 minutes at 4°C. Then 25 ml of sucrose-EDTA solution was added to the mortar and the mixture ground for 20 seconds to give an even suspension. The mixture was poured into a 40 ml polyethylene tube and centrifuged at 600 g. for 10 minutes. The supernatant solution was decanted and stored at 4°C. The pellet was mixed with 2 ml of the sucrose-EDTA solution and 0.1 ml of 1M triethanolamine buffer, 2 g. of washed glass beads and ground for another 4 minutes. 25 ml of sucrose-EDTA solution was added and the mixture was centrifuged as previously. The two supernatant fluids were pooled and spun at 8,500 g. for 10 minutes at 4°C; the pellet

was washed twice with 25 ml sucrose-EDTA solution and finally suspended in 2 ml of this solution.

PREPARATION OF ERYTHROCYTE GHOSTS.

(Human 'O' Blood).

The packed red blood cells from 20 ml of blood were washed three times with phosphate buffered saline. The washed cells were transferred to a beaker in an ice bath and lysed by the rapid addition of 200 ml of ice cold 0.01M tris/HCl. The lysate was spun at 5,000 r.p.m. to remove any unlysed cells and the resulting fluid was spun at 38,000 g. for 30 minutes to deposit the erythrocyte ghosts. This pellet was then washed three times with 200 ml 0.01M tris/HCl and finally once with 200 ml distilled water saturated with carbon dioxide to remove most of the bound haemoglobin. Again the process was carried out at $4^{\circ}C$. After the final washing the pellet was white or pale pink and the supernatant fluid colourless. The erythrocyte ghosts were suspended in 2.5 ml 0.01M tris/HCl pH 7.3 and used immediately; occasionally ghost preparations were stored at $-20^{\circ}C$ until required.

ASSAY OF HAEMOLYTIC ACTIVITY OF STREPTOLYSIN S.

Doubling dilutions.

Serial doubling dilutions of Streptolysin S were made in 0.5 ml amounts of isotonic saline changing pipettes every third tube, 0.5 ml amounts of a 2% v/v suspension of washed, packed human group 'O' R.B.C. were added to each tube (3" x ½" or 4" x ½"). Tubes without haemolysin served as controls. Haemolytic activity was assessed visually, the dilution causing 50% haemolysis after 30 minutes incubation at 37°C. being accepted as containing 1 Minimal Haemolytic Dose (M.H.D.). This method was used for the determination of the approximate potency of Streptolysin S preparations.

Gradient dilutions.

This method was a modification of the method described by Bernheimer and Schwartz (1963), for measuring the haemolytic activity of staphylococcal alpha-toxin. Preparations of Streptolysin S were diluted in isotonic saline, (TABLE 7).

1 ml of a thrice washed suspension of human 'O' R.B.C. was added to each tube. The concentration of the red cell suspension used was adjusted so that the sample, after haemolysis with saponin and adding an equal volume of saline gave an absorbance at 545 mμ of 0.8 (read in a Pye Unicam SP600 spectrophotometer). Each series of tubes was incubated in a 37°C. water bath for 30 minutes and then centrifuged at 4,500 r.p.m. for 2 minutes in an MSE swing-out head centrifuge. A standard for 50% haemolysis was prepared by adding 1 ml of red blood cell suspension to 3 ml of distilled water. The haemoglobin in the supernatant fluid was assayed by reading the extinction value

at 545 mμ(E_{545}). The haemoglobin content in the supernatant fluid of each tube in the series which showed haemolysis was assayed in this way. The E_{545} for each dilution was plotted and the dilution of toxin haemolysing 50% of the red cells in the suspension was determined by interpolation (FIG. 7). A unit of haemolysin (HU) is defined as that amount which liberates 50% of the haemoglobin in the test red cell suspension under the conditions stated. Four haemolytic units determined in this way are approximately equivalent to 1 M.H.D. determined by serial doubling dilutions.

TABLE 7.

Preparation of Gradient dilutions.

Tube	Dilution Factor	Toxin (ml)	Saline (ml)
1	1.0	1.00	0
2	1.5	0.67	0.33
3	2.0	0.50	0.50
4	2.5	0.40	0.60
5	3.3	0.30	0.70
6	5.0	0.20	0.80
7	6.7	0.15	0.85
8	10.0	0.10	0.90
9	15.0	0.067	0.93
10	20.0	0.005	0.95

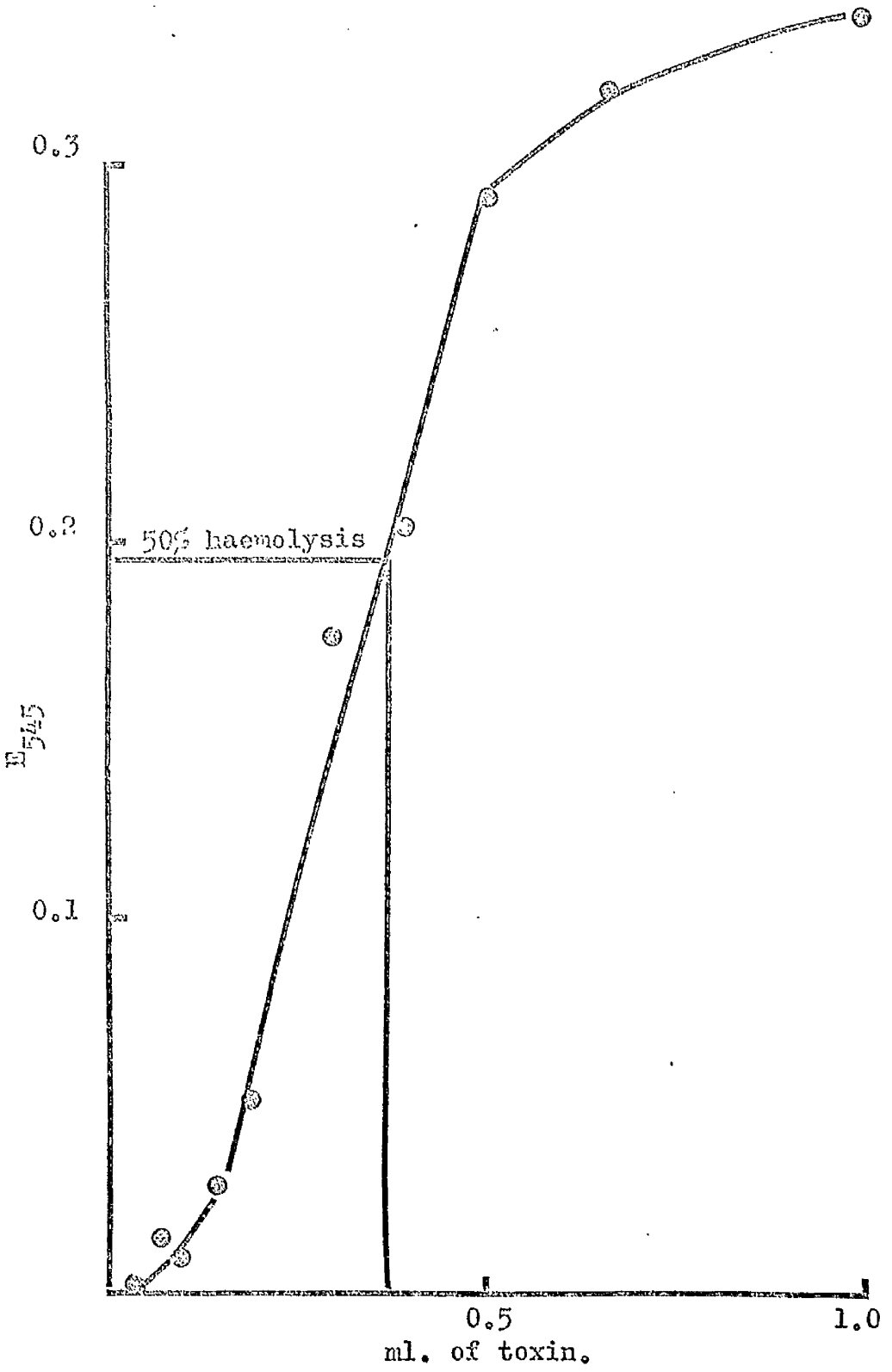


FIG. 7 . Titration of the haemolytic activity of SLS.
Starting dilution of toxin = 1/1000. Standard 50% haemolysis E_{545}=0.195
This is equivalent to adding 0.335 ml. 1/1000 toxin.
Titre = 1000/0.335 H.U./ml = 2,985 H.U./ml.

MEASUREMENT OF RESPIRATION.

The respiration of ascites tumour cells and isolated mitochondria was followed using two methods:-

(1) manometrically using the Warburg apparatus to measure the oxygen uptake.

(2) by using the oxygen electrode to measure the oxygen uptake.

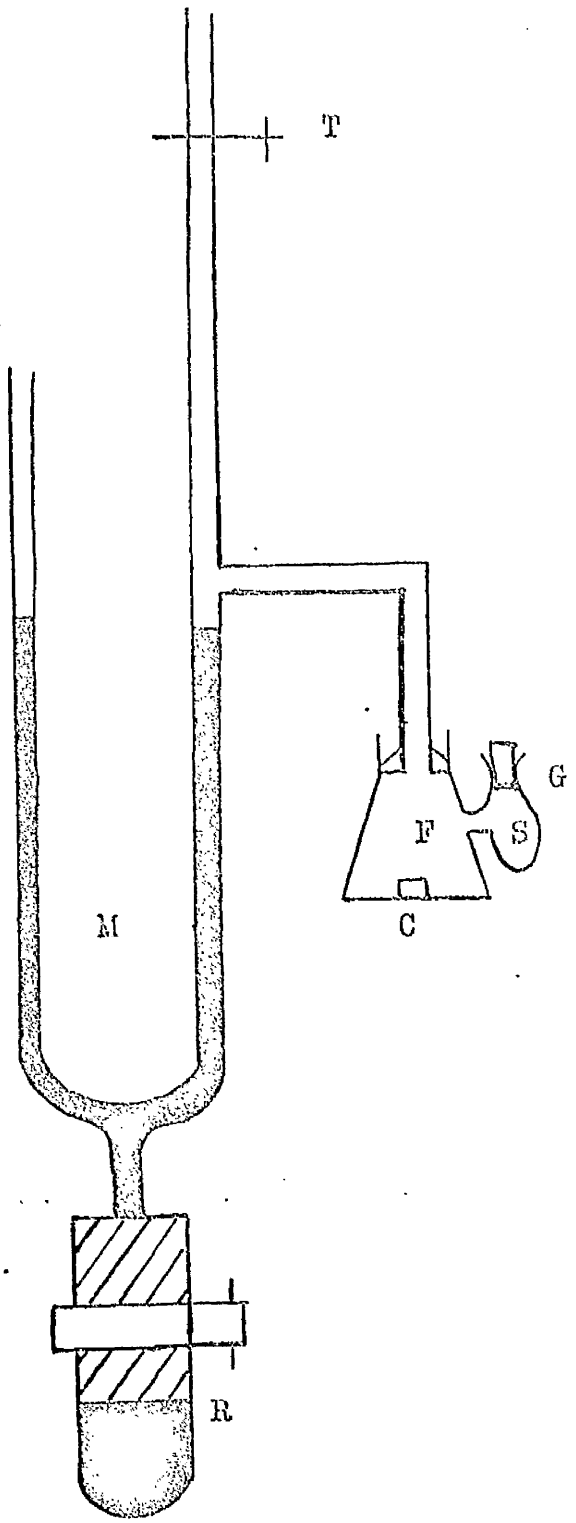
Most of the work was done using the Warburg apparatus because an oxygen electrode became available only in the later stages of the work.

Apparatus used.

(a) Manometric assays.

The Warburg apparatus used was manufactured by Townson and Mercer Ltd., Croydon, England and is illustrated in FIG. 8 . It consists of a detachable flask (F), having an approximate volume of 25 ml, equipped with either one or two side arms (S), attached to a manometer (K) containing a liquid of known density. The flask is immersed in a water bath at a constant temperature, and between readings is shaken to promote rapid gas exchange between the liquid and gas phases.

The manometer shown in FIG. 8 has an open and a closed end. A reference point on the closed side of the manometer (usually 150 mm) is chosen and the liquid in the closed arm of the manometer is always adjusted to this point before recording



F = Flask

S = Side arm

G = Side arm stopper with gas vent.

C = Centre well (for alkali)

M = Manometer

R = Fluid reservoir

T = Three-way stopcock

FIG. 8 . Warburg Apparatus. (Umbreit, Burris & Stauffer 1959).

pressure changes. During the consumption of oxygen by a biological system the level of fluid in the closed limb will rise. By adjusting the level in this arm once again to 150 mm, a reading of the amount of oxygen taken up can be calculated if one knows the gas volume of the flask, the volume of liquid in the flask, the temperature of operation, the gas being exchanged and the density of the fluid in the manometer. These factors are related by a constant for a matched flask and manometer. Flask constants were determined using the mercury method described by Umbreit et al (1959). If the uptake of oxygen by a biological system is accompanied by the formation of carbon dioxide, 20% NaOH must be placed in the centre well of the flask to absorb the carbon dioxide so that the change in oxygen tension can be recorded.

(b) Oxygen electrode.

The oxygen electrode is a fixed voltage polarographic probe which measures the partial pressure of oxygen. It has been made part of an integrated automated system capable of plotting oxygen uptake or evolution curves representing the activity of cells, tissues and homogenates suspended in physiological media. The system operates at approximately ten times the speed of conventional manometric techniques and useful information becomes available seconds after insertion of the probe into the reaction vessel. This provides significant information during the period usually required for equilibration time in the standard Warburg technique.

The Biological Oxygen Monitor consists of a stable oxygen electrode (sensor), an electronic amplifier, a bath stirrer assembly, and a constant temperature circulator. Although the change in oxygen tension can be read directly from the meter on the amplifier, in practice the signal is fed to a recorder which is operated at a speed compatible with the

Biological oxygen monitor: YSI model 53 (Yellow Springs Instrument Co. Inc.)
Recorder: Labograph E 428 (Metrohm, Switzerland.)
Constant temperature circulator: (Metrohm, Switzerland.)

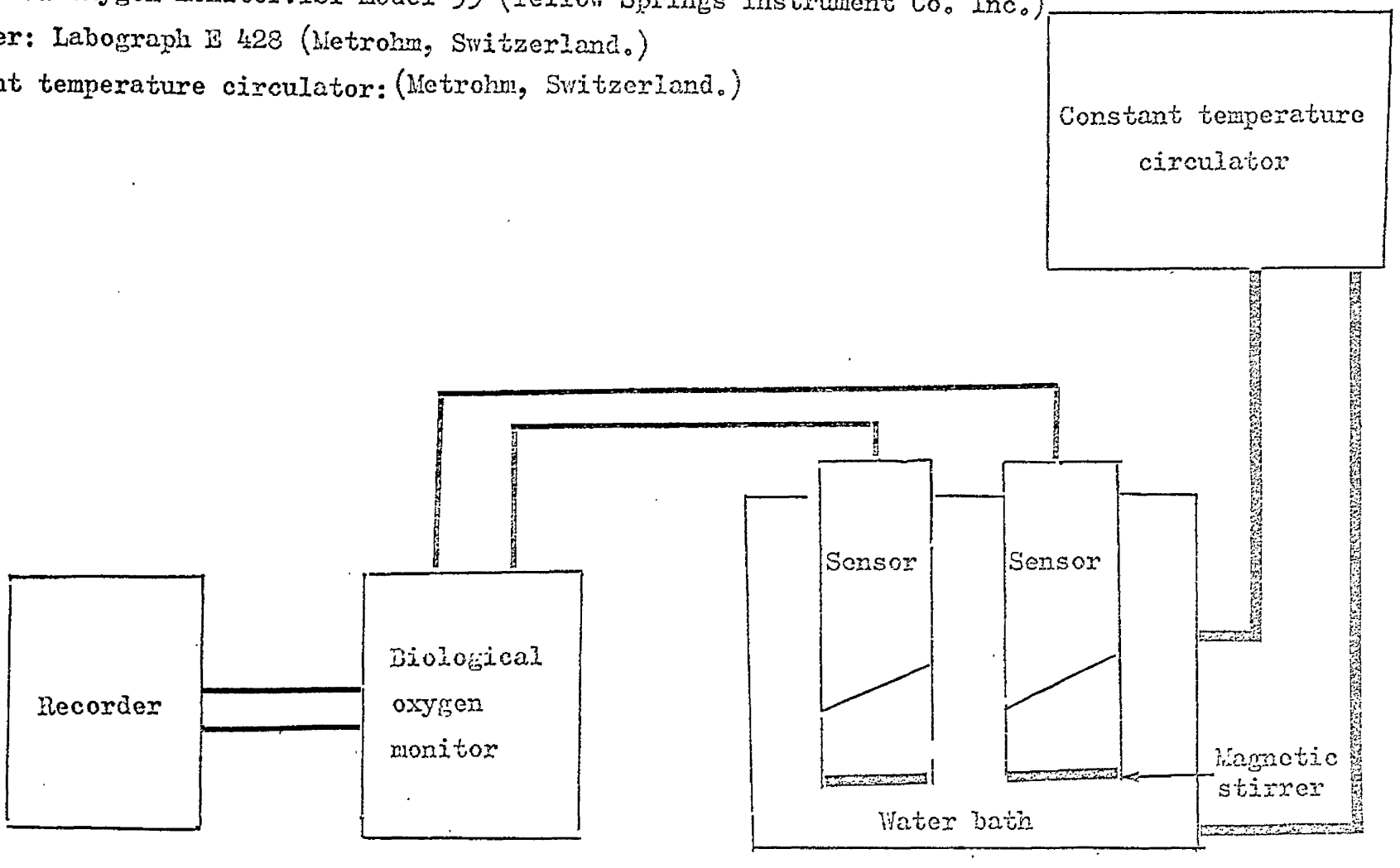


FIG. 9 . Block diagram of the biological oxygen monitor system.

reaction rate of the biological system. A block diagram of the system is given in FIG. 9 .

The oxygen sensor consists of a 0.025" diameter platinum cathode and a U-shaped silver anode encased in an epoxy block except for the exposed surfaces of the anode and cathode. A thin (0.001") teflon membrane stretched over the end of the sensor effectively isolates the sensor elements from the experimental solution but allows diffusion of oxygen into the sensor. When a polarising current of 0.8v is applied across the cell, oxygen will react with the cathode causing a current to flow through the cell. The current which flows is proportional to the amount of oxygen diffusing across the membrane.

The oxygen electrode is not an absolute device, it requires to be calibrated at a known barometric pressure in a solution with a known oxygen content. Although the oxygen electrode records oxygen tension (pO_2) rather than volumetric changes (as obtained by manometric methods), relatively simple calculations are required to transform the data into compatible volumetric values. This is illustrated in the following example:-

Assume a change of 82 to 64% saturation in 5 minutes, this means that 18% of the oxygen in solution was consumed in 5 minutes. Assume also that the reaction mixture consisted of 3 ml of Ringers solution at 37°C. If Ringers solution saturated with air at 37°C and ambient barometric pressure contains 5 µl oxygen/ml of solution, then a 3 ml sample will contain 15 µl oxygen. A change of 18% in saturation means that 15 x 18% = 2.7 µl oxygen was consumed in 5 minutes.

ASSAY OF ASCITES CELL RESPIRATION.

Glucose utilisation was estimated manometrically at 37°C. The contents of the Warburg flasks were as follows:-

Main well: 0.5 ml ascites cell suspension
1.7 ml PVP/tris HCl buffer pH 7.3
0.5 ml Streptolysin S or control fluid
Centre well: 0.2 ml 20% NaOH
Side arm: 0.3 ml 0.2M glucose
Total Volume = 3 ml.

After the required pre-incubation period (usually 30 minutes) with a shaking speed of 80 to 100 cycles per minute at 37°C the manometer was closed, a base reading taken, then the contents of the side arm were tipped into the main well and the change in pressure resulting from oxygen uptake read at 10 or 15 minute intervals. All Warburg assays were done in duplicate; if values in a pair differed by more than 5% the experiment was discarded. An impairment or stimulation of 10% or more was taken as significant.

Succinate oxidation was also estimated manometrically at 37°C. The contents of the Warburg flasks were as follows:-

Main well: 0.5 ml ascites cell suspension
1.7 ml PVP/tris HCl buffer pH 7.3
0.5 ml Streptolysin S or control fluid
Side arm: 0.3 ml 0.2M sodium succinate
Total Volume = 3 ml.

The method was as described above for glucose oxidation.

Glucose and succinate oxidation by disintegrated cell preparations was measured in the same way, substituting the disintegrated cell preparation for the ascites cell suspension.

The succinic oxidase action of the ascites cell mitochondria was assayed using the oxygen electrode and the method will be described later.

ASSAY OF ISOLATED MITOCHONDRIAL ENZYME ACTIVITY.

Succinic dehydrogenase was measured by the reduction of methylene blue by mitochondria employing sodium succinate as substrate in a Thunberg tube. The Thunberg tube had the following contents:-

Main vessel: 0.5 ml mitochondrial suspension
0.5 ml Streptolysin S or control fluid
1.4 ml sucrose phosphate buffer pH 7.3.

Side arm: 0.3 ml sodium succinate
0.3 ml 0.1 mM methylene blue dye.

The tubes were evacuated for 5 minutes with an Edward's High Vacuum Pump (Model 2SC 50) with constant shaking to remove air bubbles. The Thunberg tubes were then closed and the contents of the side arm tipped into the main well. The time taken to decolourise 90% of the dye (visually assessed) at 37°C was taken as a measure of dehydrogenase activity.

Succinic oxidase was measured either manometrically at 37°C or using the oxygen electrode.

(a) Warburg Assay

The contents of the Warburg flasks were as follows:-

Main well: 0.5 ml mitochondrial suspension
0.5 ml Streptolysin S or control fluid
1.7 ml sucrose phosphate buffer or
sucrose tris/HCl buffer both pH 7.3.

Side arm: 0.3 ml 0.2M sodium succinate
Total volume = 3 ml.

Following appropriate pre-incubation at 37°C the manometer was closed, a base reading was taken and the contents of the side arm were tipped into the main well. Readings of the change in pressure which resulted from oxygen uptake were taken at 10 or 15 minute intervals.

(b) Oxygen Electrode.

The contents of the cell were as follows:-

0.2 ml mitochondrial suspension
0.5 ml Streptolysin S or control fluid
2.0 ml sucrose phosphate buffer or
sucrose tris/HCl buffer both at pH 7.3.

The reaction mixture was stirred with the magnetic stirrer at 30°C for the required pre-incubation time (usually 15 minutes). The stirrer was stopped and 0.3 ml of 0.2M sodium succinate added, making the total reaction volume 3 ml. The sensor probe was inserted, the stirrer restarted immediately and the change in oxygen tension recorded automatically. A lower incubation temperature was used in these experiments in order to ensure that the rate of oxygen uptake was not too rapid to be recorded by the very sensitive electrode.

Cytochrome oxidase activity was measured in the same way as succinic oxidase activity except that paraphenylene diamine (PPHDI, Koch-Light Ltd.) was used as substrate (FIG. 10) by the method of Slater (1949). The mouse liver mitochondrial suspension oxidised this substrate in the absence of added cytochrome c.

Employing these methods the effect of Streptolysin S on the various parts of the succinic oxidase chain of mitochondria could be assessed. (FIG. 10).

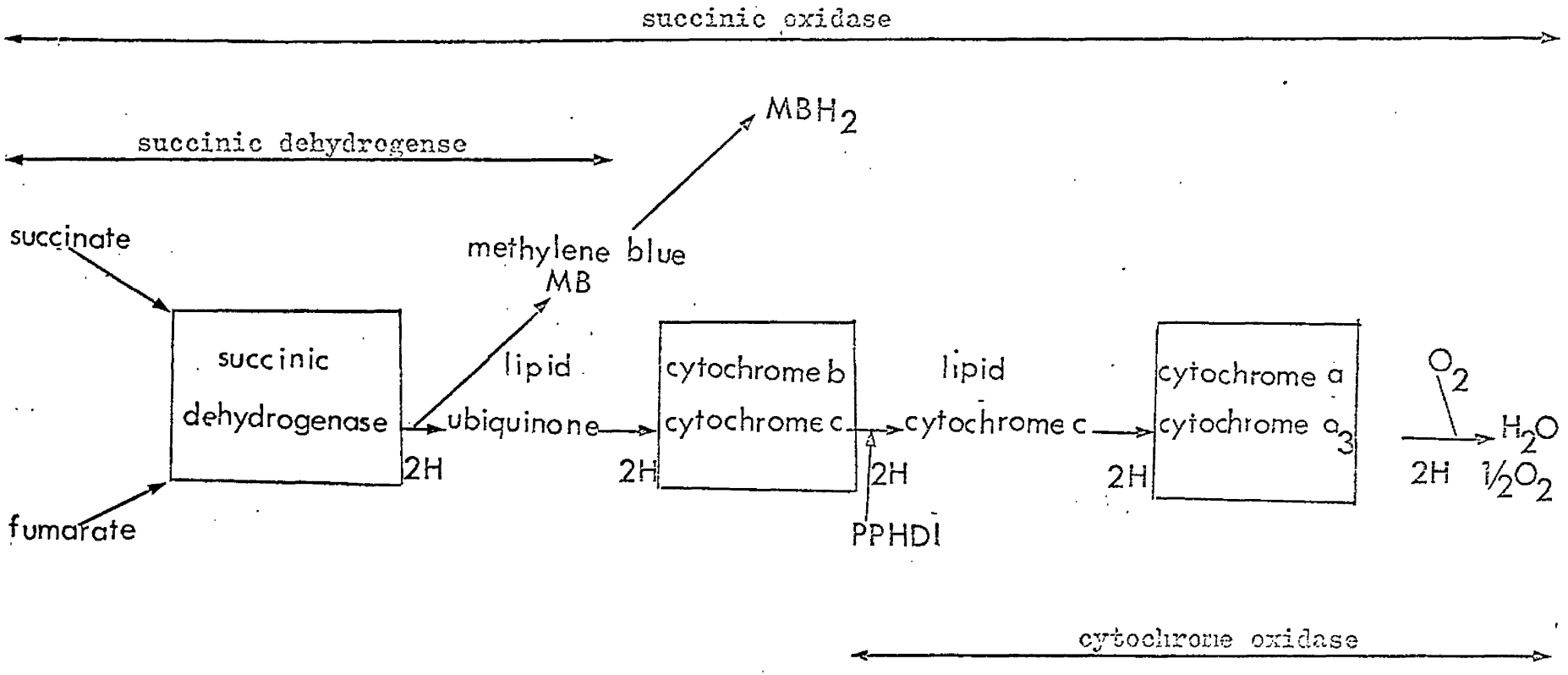


FIG. 10 . Model of the Respiratory Chain of Mitochondria.

Addition of Exogenous Mitochondrial Components.

In attempts to pinpoint the site of action of Streptolysin S on mitochondria, intermediates of the chain were added to toxin-treated mitochondria.

Effect of cytochrome c.

Either at zero time or during the assay of succinic oxidase or cytochrome oxidase, 0.3 ml of an aqueous solution of cytochrome c (crystalline, iron content 0.43% Koch-Light Ltd.) containing 2.4 mg/ml was added to both control and Streptolysin S treated mitochondria from the second side arm of the Warburg flask. Alternatively 0.1 ml of the same cytochrome c solution was injected into the oxygen electrode cell using a micro syringe.

Effect of ubiquinone.

5 mg/ml ubiquinone (Q_{10}, ex beef heart mitochondria, Koch-Light Ltd.) was dissolved in 1 ml ethanol and further diluted 1/5 in 0.25M sucrose phosphate buffer pH 7.3. Addition of this component was made only in Warburg experiments. Either at zero time or during the assay of succinic oxidase, 0.3 ml of this suspension was added from the second side arm of the reaction vessel to both control and toxin treated mitochondria. The addition of ubiquinone during succinate oxidation upset the gaseous equilibrium within the manometer due to evaporation of ethanol. This was corrected by opening the manometer stop-cock, re-setting the fluid level to 150 mm, and allowing 1 minute for re-equilibration. The rate of oxygen uptake thereafter was measured in the usual way.

GEL FILTRATION.

Sephadex is a modified dextran obtained by fermentation of sugar. It is manufactured in the form of minute beads by Pharmacia, Upsala, Sweden and swells when mixed with water or electrolyte solutions. When placed in a chromatographic column, it acts as a sieve for molecules of different sizes, since the porosity of the Sephadex gel is determined by the amount of cross linkage in the dextran network. If an aqueous solution of large and small molecules is placed on the surface of the gel bed, it starts to percolate through. As the solution is washed through the column with water, the small molecules that diffuse into the gel grains are slowed down. The larger molecules cannot enter the gel pores and move through the column faster and thus become separated from the smaller ones. Various grades of sephadex are available which exclude molecules of different sizes and ideal separation is achieved using the grade of Sephadex which will completely exclude the largest molecules and retain the smaller ones.

RNA-SLS was passed through columns of G 50, G 75, and G 100 Sephadex.

Preparation of columns.

The buffer used for the preparation and running of all columns was 0.25M sodium phosphate pH 6.0 containing 5% Glycerol and 0.1M KCl. An appropriate amount of the required grade of Sephadex to give a bed volume of 50 ml was weighed out, washed three times in buffer then left overnight at 4°C to swell. The Sephadex was then poured into a Quickfit glass column 2 cm in diameter (CR 32/30) placed at 4°C and washed

thoroughly with buffer. The end of the column was then attached via an LKB 8300 A uvicord 11 recording spectrophotometer to an LKB ultrorac 7000 fraction collector (LKB-PRODUKTER AB, Stockholm, Sweden).

Chromatography of RNA-Streptolysin S.

A sample of RNA-SLS was applied to the surface of the column, allowed to enter the gel, then washed through the column with buffer. The optical density of the column effluent was measured at 256 mμ, recorded by the uvicord and 2 ml fractions collected. These fractions were then screened for various activities. The exclusion volume of each column was determined by passing through a solution of blue dextran 2000 (Pharmacia) and measuring the amount of buffer collected from the application of the dye to its reaching the end of the column.

- o -

This description presents the basic methods used throughout the study. A number of experiments were carried out in which the aims dictated certain modifications. The design of experiments will therefore be referred to where necessary in the Results section.

RESULTS.

EFFECT OF STREPTOLYSIN S ON ISOLATED MITOCHONDRIA.

As mentioned in the Introduction to this Thesis, SLS is known to disrupt both the cell membrane and those membranes bounding intracellular organelles. Its attack on rabbit liver mitochondria has been reported to result in swelling and release of malic dehydrogenase (Weissmann et al 1963; Keiser et al 1964). Part of the object of this Thesis was to investigate whether the disruption of the mitochondrial membrane by SLS resulted in any alterations in respiratory function. Most recent studies on the cytotoxicity of this toxin have been made using the RNA form which is prepared in a chemically defined medium and is therefore almost free from other streptococcal products.

EFFECT OF STREPTOLYSIN S ON MITOCHONDRIAL SUCCINIC OXIDASE ACTIVITY.

The fundamental observation which emerged from early experiments was that RNA-SLS from strain C203S of Streptococcus pyogenes prepared as described on page 62. of the Material and Methods impaired the respiration of isolated mouse liver mitochondria employing succinate as substrate; such impairment was found using both the Warburg and Oxygen Electrode techniques (FIG. 11 a & b). Preparations from the SLS deficient mutant C203U did not impair mitochondrial respiration. Prior to describing in detail attempts to elucidate the mode of action of SLS preparations on mitochondria it is necessary to point out the inherent limitations in the methods, to present the general features of the inhibition and to establish the relationship between haemolytic activity and impairment of mitochondria.

Variation in Sensitivity of Mitochondrial Preparations.

It soon became clear that preparations of mitochondria varied from day to day in their susceptibility to the same amount of toxin. The extent of this variation, which could be up to two fold, is

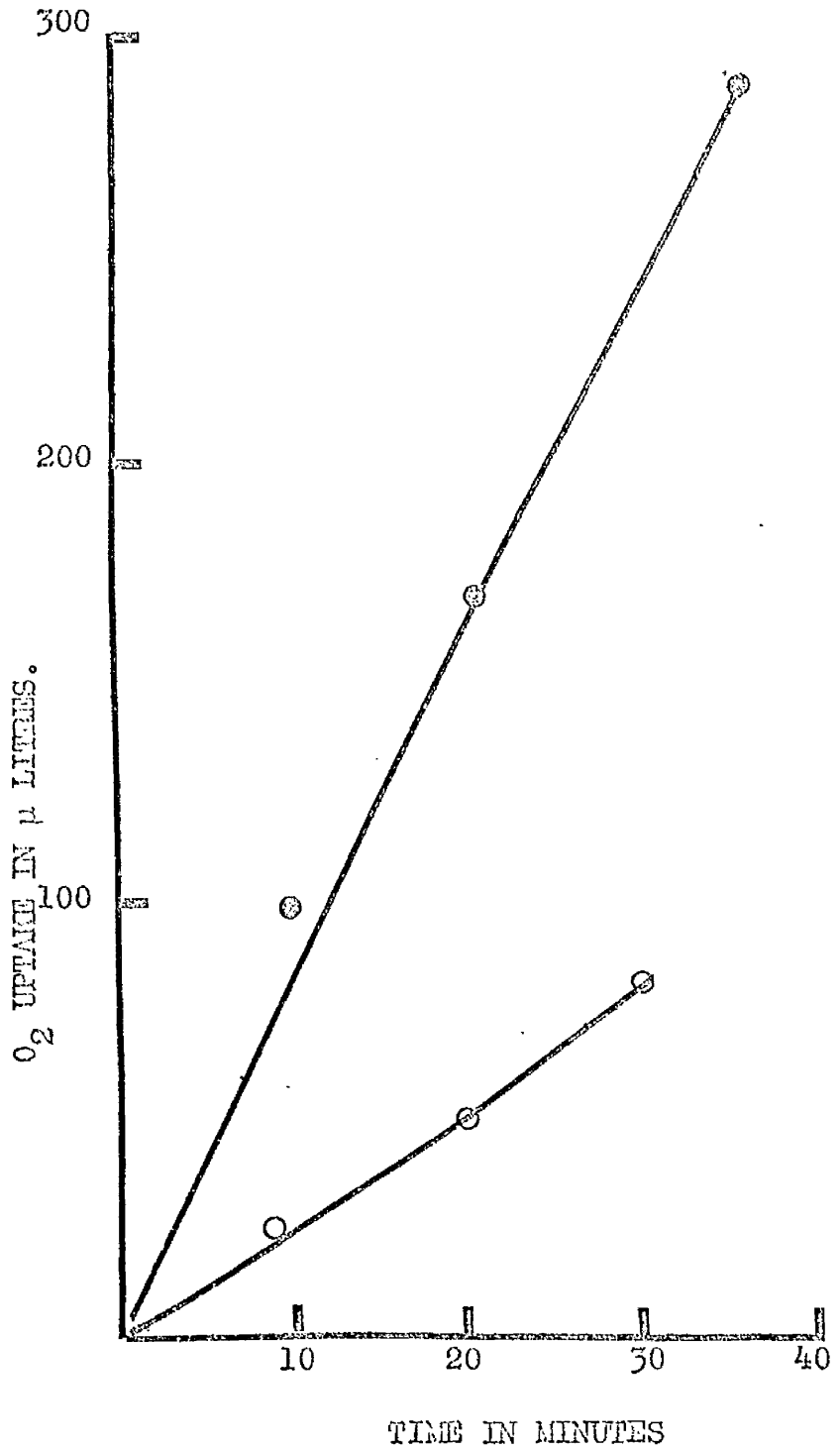


FIG-11.a. Impairment of succinic oxidase activity of mouse liver mitochondria treated with RNA-SLS at a final concentration of 109 HU/ml for 30 mins. as recorded by Warburg Manometry.

●—● respiration rate of control mitochondria
o —o respiration rate of toxin-treated mitochondria

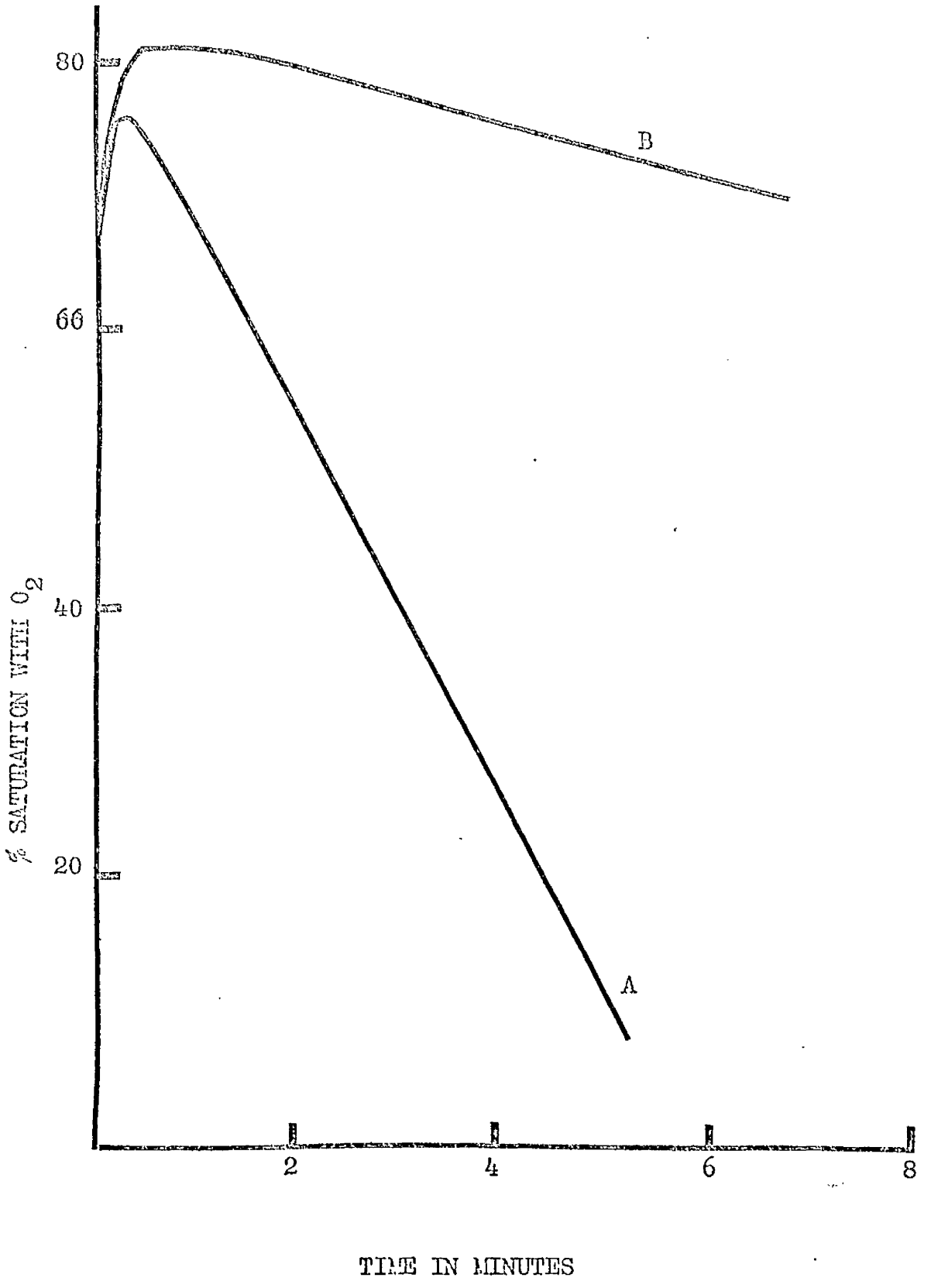


FIG 11.b. Impairment of succinic oxidase activity of mouse liver mitochondria treated with RNA-SLS at a final concentration of 109 H.U./ml for 15 mins. as recorded in the Oxygen Electrode.

A = respiration rate of control mitochondria
B = respiration rate of toxin-treated mitochondria

shown in TABLE 8. Similar observations have been reported for staphylococcal "succinic oxidase factor" (Gemmell 1968). This finding imposed certain restrictions on the work. Firstly, no standardised unit of mitochondrial impairment could be established and secondarily it was necessary to carry out comparative experiments on the same preparation of mitochondria on the same day. The variability of mitochondrial preparations made certain aspects of the work difficult to quantitate. However, it should be emphasised that the overall effects described in this section were consistently reproducible. Critical experiments were repeated at least three times.

Incubation Time.

Impairment of succinic oxidase was only observed when mitochondria were incubated with SLS prior to the addition of succinate. Addition of SLS to mitochondria which were actively metabolising succinate did not cause any alteration in the rate of oxygen uptake (FIG. 12). Initially an arbitrary incubation time of 30 minutes was used in Warburg experiments. The Oxygen Electrode allowed a detailed investigation of the influence of incubation time on the degree of impairment (TABLE 9). Over the first two minutes significant stimulation was observed followed by increasing impairment. The most rapid rise in the rate of impairment was between 2 and 5 minutes, thereafter it rose slowly to a maximum at 20 minutes. As a result of this finding a pre-incubation period of 15 minutes was allowed in all experiments using the oxygen electrode.

Effect of Altering the Concentration of SLS.

In order to determine whether the amount of impairment caused by SLS was proportional to the dose of toxin present, dilutions of toxin preparations were made and tested for activity on mitochondria. The variability of different mitochondrial preparations made it impossible to attribute an absolute impairment

TABLE 8.

Variation in the Sensitivity of Mitochondria.

Concentration of SLS+ (H.U./ml)	Date of Experiment	% Inhibition of Mitochondrial O_2 Uptake
167	1.11.67	45
167	28.11.67	71
167	29.11.67	60
167	25. 1.68	89
417	11. 4.69	76
417	14. 4.69	52
417	24. 4.69	74
417	5. 5.69	65
417	23. 5.69	32

All Experiments carried out by Warburg Manometry.

+ This represents the final concentration of SLS in the Warburg Flasks, and holds for all the results presented.

TABLE 9.

Effect of Incubation Time on Impairment of Succinic Oxidase.

Incubation Time in Minutes.	% Change in Mitochondrial O_2 Uptake.
0	16 stimulation
1	20 "
2	55 inhibition
5	64 "
10	71 "
20	78 "
30	74 "

A final concentration of SLS of 209 H.U./ml was present in each assay. All experiments carried out in Oxygen Electrode.

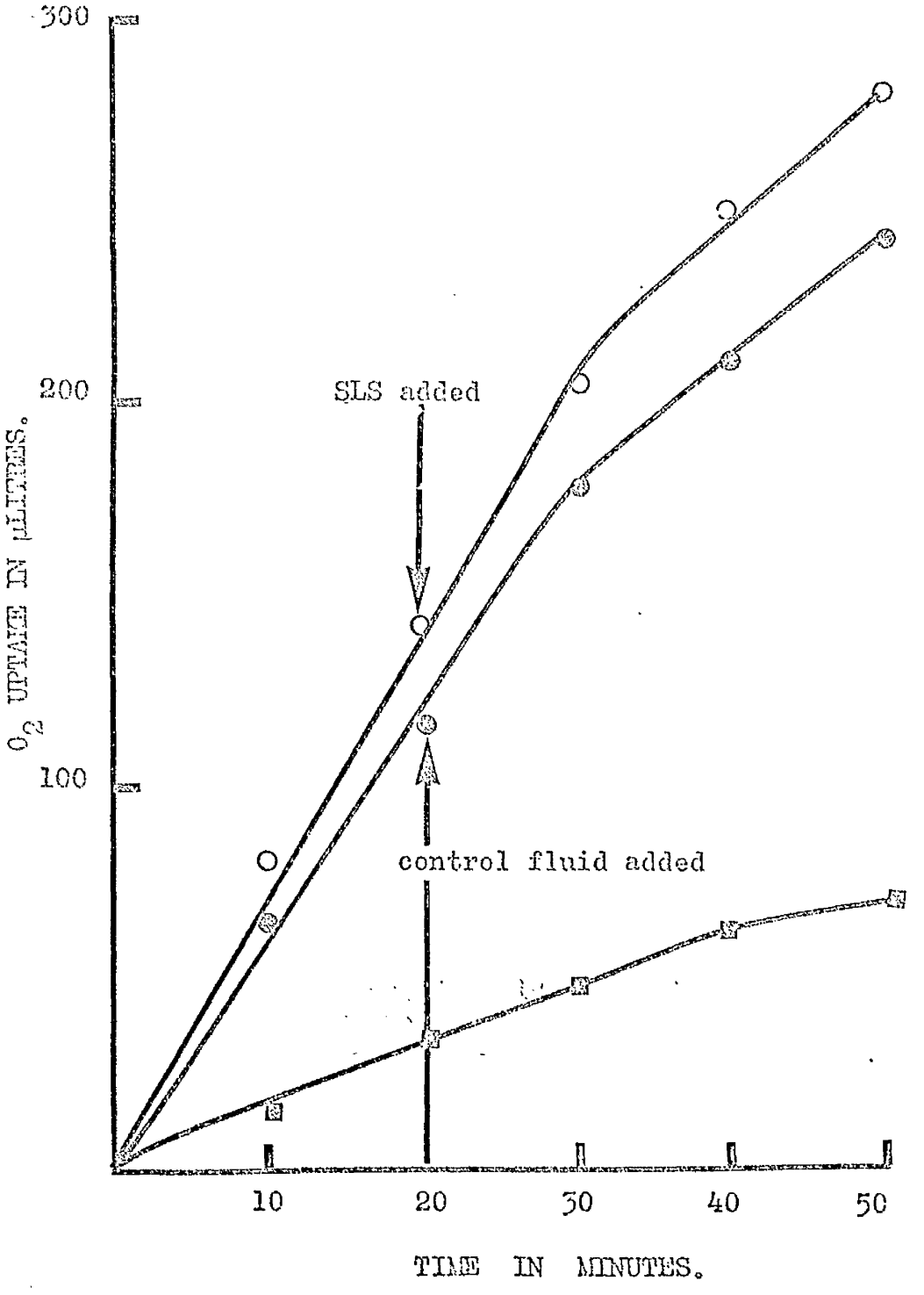


FIG. 12. The Effect of Adding SLS to mitochondria which are actively metabolising succinate. The final concentration of toxin in the Warburg flask was 56 H.U./ml.

■—■ Respiration rate of mitochondria treated with SLS for 30 minutes prior to the addition of succinate.

value to a given concentration of toxin. Nevertheless certain important points arose and the results of a typical experiment are given in FIG. 13. The percentage inhibitions of oxygen uptake recorded in this FIG. are expressed relative to the corresponding dilution of control fluid. This was necessary since the rate of oxygen uptake of mitochondria increased with increasing concentrations of control fluid. In view of this, the dilution of suspending medium or heated SLS used as controls throughout the work was always equivalent to that of the test material. The minimum amount of toxin required to cause observable impairment was a function of the mitochondrial preparation used. Above this threshold level, the percentage inhibition of oxygen uptake was dependent on the concentration of SLS present until a maximum was reached above which increases in the amount of toxin did not cause any significant rise in inhibition. This maximum level of inhibition also appeared to vary in different mitochondrial preparations but 100% inhibition was never observed even when very high doses of SLS (50,000 H.U./ml) were used. The maximum inhibition observed with most mitochondrial preparations was between 70 and 80%.

Effect of Different Buffers.

As mentioned previously (page 50) the properties of isolated mitochondria depend on the nature of the fluid in which they are suspended. In early experiments 0.25M sucrose buffered with 0.025M sodium phosphate pH 7.3 was used for mitochondrial preparation. In the course of the work it was pointed out that small amounts of phosphate may cause some mitochondrial swelling. Therefore, the results obtained using this buffer were compared with those obtained using 0.25M sucrose buffered with 0.055M tris/KpH 7.3 a generally accepted suspended medium for mitochondria. Results showed that the experimental observations using both suspending media were identical. Nevertheless, all recent experiments have been carried out in sucrose/tris buffer.

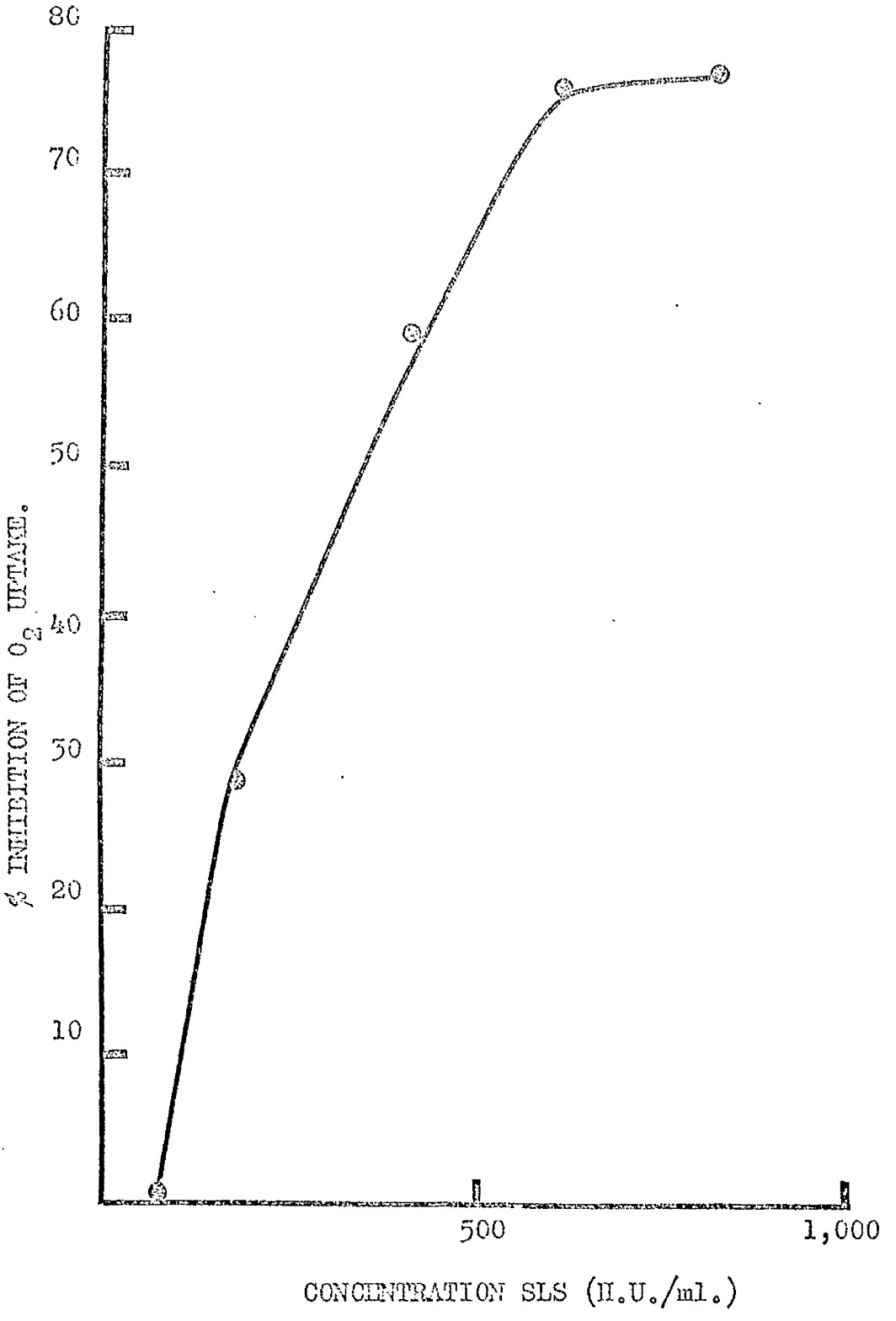


FIG.13 . Influence of SLS concentration on Impairment of mitochondrial succinic oxidase activity. The toxin and mitochondria were incubated together for 15 mins. prior to the addition of succinate and the experiment was carried out in the oxygen electrode.

Susceptibility of Mitochondria from Other Organs.

The action of RNA-SLS on the succinic oxidase activity of mouse kidney and heart mitochondria was also tested. Kidney mitochondria were prepared as described in the Materials and Methods (page 64) and suspended in a volume of buffer proportional to the weight of the kidneys (the ratio of kidney weight to final volume of mitochondrial suspension being the same as for liver). However, only half the amount of kidney mitochondrial suspension was required to obtain the same respiration rate as liver mitochondria. When equal quantities of SLS were tested on kidney and liver mitochondria respiring at the same rate the observed percentage impairment of succinic oxidase activity was almost the same in both preparations (TABLE 10). On the other hand, heart mitochondria appeared to be resistant to the action of SLS. All further experiments were carried out using mouse liver mitochondria unless specifically stated otherwise.

Action of SLS Prepared by Different Methods.

Serum-SLS, Albumin-SLS and Tween-SLS were prepared as described previously (page 61) from strain C203S. Such toxin preparations were tested on the succinic oxidase activity of mouse liver mitochondria using the Oxygen Electrode. Fifteen minutes interaction between toxin and mitochondria was allowed prior to the addition of succinate. The recorded values for percentage inhibition of oxygen uptake (TABLE 11) were calculated by comparison with the respiration rates of the controls in which mitochondria were treated with corresponding amounts of the suspending fluids used for toxin preparation. It can be seen from the results in TABLE 11. that the amount of mitochondrial impairment by the different toxin preparations did not correspond to the amount of haemolytic activity present.

TABLE 10.

Sensitivity of Mitochondria from other Organs.

Source of Mitochondria	Concentration of SLS (H.U./ml)	% Inhibition of Mitochondrial O_2 Uptake
liver	249	40
kidney	249	50
liver	167	81
heart	167	10

TABLE 11.

Effect of Various SLS Preparations on Succinic Oxidase.

Toxin Preparation	H.U./ml	% Inhibition of Mitochondrial O_2 Uptake
RNA-SLS	53	50
Tween 40-SLS	13	37
Tween 80-SLS	3	22
Albumin-SLS	11	49
Serum-SLS	213	50

All Experiments Carried Out in Oxygen Electrode.

These reults suggested either, that the nature of the carrier substance determines the degree of mitochondrial impairment or that a factor other than SLS was responsible for mitochondrial damage. In order to investigate this further the effect of different chemical and physical treatments on the haemolytic and mitochondrial damaging activities of RNA-SLS preparations were studied.

COMPARISON OF THE HAEMOLYTIC AND MITOCHONDRIAL DAMAGING EFFECTS OF STREPTOLYSIN S PREPARATIONS.

Effect of Heat.

The toxin was heated at varying temperatures for 30 minutes, titrated against human erythrocytes then tested on mitochondrial succinic activity (TABLE 12). At 40°C the haemolytic activity of the toxin was considerably reduced by this treatment but the inhibition of mitochondrial oxygen uptake was not significantly altered. At higher temperatures both activities were destroyed.

The temperature chosen to investigate the rate of heat inactivation of toxin was 60°C since both activities were significantly reduced but not completely destroyed by heating for 30 minutes at this temperature. The toxin was heated at 60°C for varying periods of time then tested for haemolytic and mitochondrial damaging activity. The results of this experiment are illustrated in FIG. 14. The mitochondrial inhibiting activity of the toxin was destroyed more slowly than the haemolytic activity but this may have been due to the fact that the amount of toxin remaining, although diminished, was still sufficient to cause maximum impairment of succinic oxidase activity. Once the toxin level fell below that giving maximum impairment, it could be seen to be inactivated by increased periods of heating.

TABLE 12.

Effect of Heating SLS Preparations.

Temperature of heating (30 minutes)	Concentration of SLS (H.U./ml)	% Inhibition of Mitochondrial O_2 Uptake
Unheated	700	53
40°C	267	47
60°C	67	30
70°C	1	0
80°C	1	0
100°C	1	0

All Experiments Carried Out by Warburg Manometry.

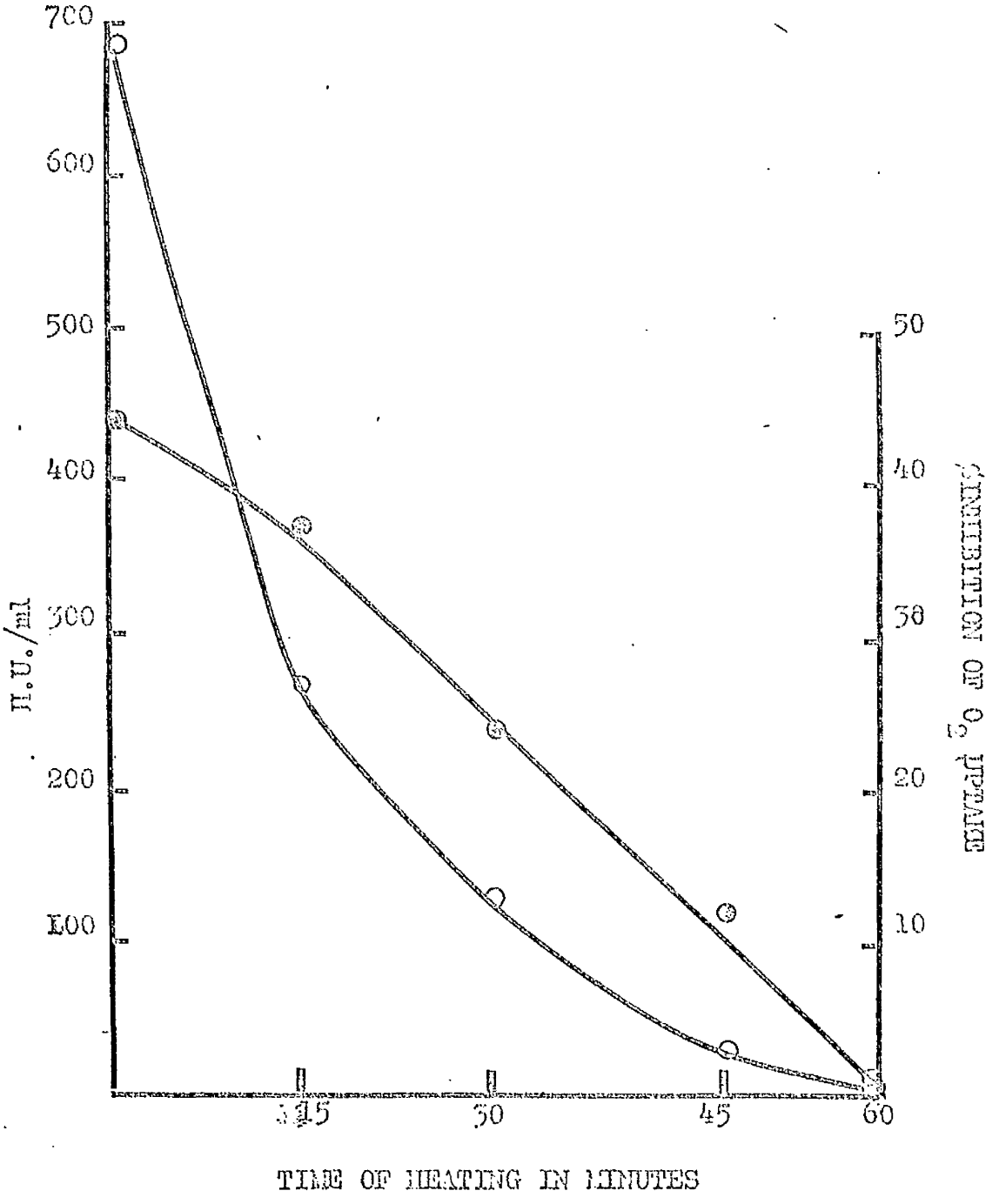


FIG. 14 . Effect of Heating SLS at 60°C on the two Biological activities.

O——O Haemolytic Activity
●——● Mitochondrial Damaging Activity.

Effect of Trypan Blue.

Trypan blue has been reported to inhibit the haemolytic, lethal and cytotoxic activities of SLS (Ito 1940; Rosendal & Bernheimer 1952; Taketo & Taketo 1966). In my experience if SLS is allowed to react with trypan blue at a concentration of 100 µg/ml and the mixture titrated in the usual way a haemolytic pattern of the type shown in the first line of TABLE 13. was seen. This indicated that the inhibition effect of trypan blue was reversed by dilution. On the other hand, if the concentration of trypan blue was maintained at 100 µg/ml in each tube the pattern in line 2 of TABLE 13 was observed. Trypan blue may act as a blocking agent or competitive inhibitor of SLS, the constant presence of the inhibitor being a requirement of inhibition.

In order to assess the effect of trypan blue on SLS impairment of mitochondrial succinic oxidase activity, Warburg experiments for the assay of succinic oxidase were set up as described in the Materials and Methods (page 76) and trypan blue was added to give a final concentration of 100 µg/ml. The rate of oxygen uptake of mitochondria was not adversely affected by the presence of this concentration of trypan blue nor did it affect the degree of impairment of succinic oxidase activity by SLS (TABLE 14). Thus at a concentration sufficient to cause marked inhibition of haemolysis trypan blue did not alter the capacity of the toxin to impair mitochondrial respiration.

Effect of Lecithin.

Lecithin is a well documented inhibitor of SLS (Hewitt & Todd 1939; Humphrey 1949; Cinader & Pillemer 1950; Stollerman et al 1950) but the quantities of lecithin reported to cause inhibition vary considerably. Humphrey (1949) found that 0.18 mg of lecithin was necessary to inhibit 1 H.U. of SLS. Using purer preparations of beef heart lecithin, Cinader & Pillemer (1950) found that only 1.4×10^{-3} mg. of lecithin neutralised 1 H.U. of SLS. Yet another value was reported by Stollerman et al (1950) who found that

TABLE 13.

Inhibition of Haemolysis by Trypan Blue.

Dilution of Toxin	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/250	1/500	1/1000	1/2000	1/400
Titration of Reaction Mixture in saline.	+	+	±	-	-	+	+	+	+	±	-	-
Titration of Reaction Mixture in saline containing 100 ug/ml Trypan Blue.	+	+	±	-	-	-	-	-	-	-	-	-
Titration of Toxin Control	+	+	+	+	+	+	+	+	+	±	-	

+ = Complete haemolysis. ± = 50% haemolysis. - = No haemolysis.

TABLE 14.

Effect of Trypan Blue on SLS.

Experiment number	Untreated SLS		SLS + Trypan Blue 100 ug/ml	
	Concentration SLS (H.U./ml)	% Inhibition of Mitochondrial O_2 Uptake	Concentration SLS (H.U./ml)	% Inhibition of Mitochondrial O_2 Uptake
1	440	47	107	51
2	250	76	3	81
3	333	52	53	58

All Experiments Carried Out by Warburg Manometry.

2.09×10^{-1} mg. of purified egg yolk lecithin inhibited 1 H.U. SLS. These discrepancies can be explained to some extent by the fact that these workers used different haemolytic units; therefore, the amounts of lecithin required to cause inhibition were not being compared with a standard amount of SLS. More recently lecithin has been used extensively to inhibit SLS and varying arbitrary concentrations have been used by different workers.

Preliminary experiments were carried out using lecithin prepared by the method of Hannahan, Turner and Jakyo (1951) from egg yolk. This preparation was kindly given to me by Mr. C.J. Smyth. The estimated purity of this preparation was 80% lecithin but three components were found to be present when it was analysed by thin layer chromatography. A 5 mg/ml dispersion of lecithin in distilled water was prepared by sonication and dilutions of this were made to give concentrations of 1 mg/ml and 2.5 mg/ml. Equal volumes of SLS and lecithin were mixed and allowed to interact for 30 minutes at 37°C. Aliquots of the mixture were then titrated for haemolytic activity and tested for impairment of mitochondrial succinic oxidase activity. The results of such experiments are given in TABLE 15.

In order to confirm that the observed effects were due to lecithin, a chromatographically pure preparation of L-alpha lecithin was purchased from General Biochemicals Ltd.. SLS was titrated against human erythrocytes by doubling dilutions using phosphate buffered saline containing 500µg lecithin/ml. as described by Koyama (1965). After making the toxin dilutions, 10 minutes incubation at room temperature was allowed prior to adding the erythrocytes. The same preparation of toxin titrated in the absence of lecithin had a titre of 5,000 H.U./ml but in the presence of lecithin this fell to 160 H.U./ml. Lecithin at the same final concentration of 500µg/ml was added to the reaction mixture when testing the mitochondrial damaging effect of SLS. Again 10 minutes

TABLE 15.

Effect of Lecithin on SLS.

Test Material	Concentration SLS (H.U./ml)	% Inhibition of Mitochondrial O_2 Uptake
SLS	5,000	65
SLS + lecithin (1mg/ml)	830	66
SLS	10,000	63
SLS + lecithin (2.5 mg/ml)	3,300	57
SLS + lecithin (5.0 mg/ml)	3,300	61

All Experiments Carried Out by Warburg Manometry.

interaction between the toxin and lecithin was allowed before the addition of mitochondria which was followed by 15 minutes incubation prior to the addition of succinate and measurement of mitochondrial oxygen uptake. Untreated toxin caused 55% inhibition of succinic oxidase activity and in the presence of lecithin 50% inhibition was observed. In order to establish whether the impairment of succinic oxidase observed in the presence of lecithin was perhaps due to residual toxin, a 1/30 dilution of the orginal toxin preparation was made containing 167 H.U./ml and tested on the same preparation of mitochondria. This amount of toxin caused no impairment of succinic oxidase activity. These results suggested that lecithin selectively blocked the haemolytic activity of RNA-SLS but did not interfere with the ability of the toxin to inhibit mitochondrial succinic oxidase activity.

The results from experiments with trypan blue and lecithin showed that the two biological activities of RNA-SLS preparations were not identical in all respects. This raised the possibility that each activity resulted from the action of separate entities in the toxin preparations.

EFFECT OF DNA-ASE ON MITOCHONDRIAL SUCCINIC OXIDASE ACTIVITY.

It was shown by Bernheimer and Ruffier (1951) that "resting" streptococci elaborated extracellular DNA-ase when suspended in a medium containing maltose, phosphate and magnesium ions. Since all these constituents are present in the suspending medium used to prepare RNA-SLS, these toxin preparations will contain DNA-ase. In order to test whether the observed inhibition of mitochondrial respiration was due to the action of SLS or contaminating amounts of DNA-ase the following experiment was carried out. "Resting" streptococci were prepared as described in the Materials and Methods

TABLE 16.

Effect of DNA-ase on Succinic Oxidase.

Test Material	Rate of Mitochondrial O_2 Uptake (µlitres/ml)	Effect
Complete Suspending Medium	2.75	
RNA-SLS (2560 H.U./ml)	1.35	40% inhibition
Suspending Medium without A.F.	1.09	
Streptococcal DNA-ase preparation (80 H.U./ml)	2.3	14% stimulation
Sucrose/PO_4 buffer	2.15	
Pancreatic DNA-ase. (200 ug/ml)	2.5	12% stimulation

This experiment was carried out using the Oxygen Electrode. Each cell contained 2.0ml sucrose/PO_4 buffer, 0.5ml test material, 0.2ml mitochondria and 0.3ml succinate.

and these were incubated for 3 hours at 37°C in an incomplete suspending medium lacking "Active factor". The resultant supernatant contained 80 H.U./ml compared to 2560 H.U./ml elaborated by the same cells when incubated in a complete suspending medium. The RNA-SLS caused 40% impairment of succinic oxidase while the supernatant from the cells incubated in incomplete suspending medium caused no significant alteration in the rate of oxygen uptake by the mitochondria. (TABLE 16).

Although streptococcal DNA-ase has been shown to be immunologically distinct from pancreatic DNA-ase (McCarty 1949) a preparation of this latter material with a known amount of DNA-ase activity was also tested on mitochondria. Pancreatic DNA-ase (Koch-Light DNA-ase 1 ex bovine pancreas, Activity 450 units/mg) was added to give a final concentration of 33 µg/ml (15 units/ml), and incubated with mitochondria for 15 minutes. Again such treatment did not cause any significant alteration in the respiration rate of the mitochondria.

It was, therefore, concluded that the observed effect was not due to a contaminating amount of DNA-ase on the RNA-SLS preparations. In an attempt to separate the mitochondrial damaging and haemolytic activities, RNA-SLS preparations were subjected to Gel Filtration on Sephadex.

GEL FILTRATION.

Separation of the haemolytic moiety from the bulk of the oligoribonucleotides in RNA-SLS preparations by gel filtration on G 75 Sephadex has been reported by Bernheimer (1967). He reported that the elution profile obtained was a peak containing a large amount of optically dense material (read at 256 mµ) which contained haemolytic material followed by a smaller peak of optically dense material which had no haemolytic activity. By calibrating this column with proteins of known molecular weight he

was able to calculate a molecular weight of 12,000 for SLS.

In an attempt to separate the haemolytic and mitochondrial damaging activities of RNA-SLS preparations the toxin was passed through G 75 Sephadex column. The column was prepared as described in the Materials and Methods (page 80). Samples of toxin were concentrated by per-evaporation prior to gel filtration in order to avoid the problem of excessive dilution of biological activity in the course of running the column.

A 2 ml sample of concentrated RNA-SLS containing 50,000 H.U. was passed through the column. The optical density of the effluent was monitored continuously at 256mμ and the elution profile obtained is illustrated in FIG. 15. The main peak possessed a marked shoulder suggesting the presence of two components which were incompletely resolved on this grade of Sephadex. This picture differs markedly from that obtained by Bernheimer (1967) but was consistently reproducible in three experiments.

Two ml. fractions were collected and screened for haemolytic activity at dilutions of 1/10, 1/100 and 1/1000 and those having a titre greater that 10 were titrated accurately. The haemolytic profile is shown in FIG. 15 and can be seen to be associated with the shoulder of the main peak. Because of technical limitations it was not possible to test individual fractions for impairment of mitochondrial succinic oxidase activity. So fractions from different regions of the elution profile were bulked as follows:

Fractions	12 to 15	bulked	to	form	Sample	1
"	16 to 18	"	"	"	"	2
"	19 to 22	"	"	"	"	3
"	24 to 30	"	"	"	"	4

These four samples were titrated for haemolytic activity and tested on mitochondria. The results obtained are given in TABLE 17.

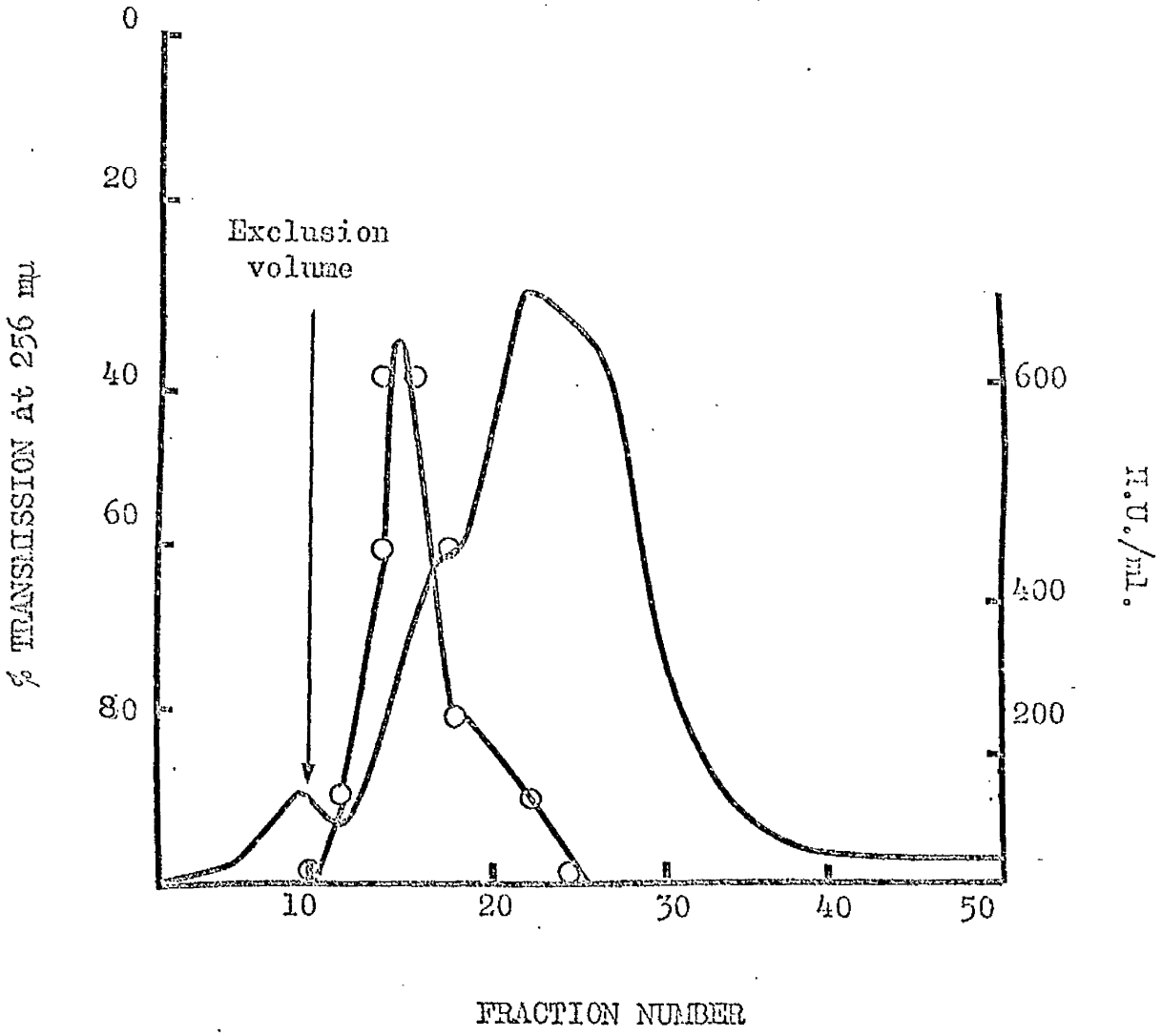


FIG. 15. Elution profile of SLS from G-75 Sephadex.

o --- o Haemolytic Activity.

TABLE 17.

Impairment of Succinic Oxidase by Fractions from G.75 Sephadex Column.

Sample	Titre (H.U./ml)	% Inhibition Of Mitochondrial O_2 Uptake
RNA-SLS[x]	12,500	82
1	830	48
2	585	36
3	290	20
4	0	0

x = Starting material prior to gel filtration.

TABLE 18.

Impairment of Succinic Oxidase by Fractions from G.50 Sephadex Column.

Sample	Titre (H.U./ml)	% Inhibition Of Mitochondrial O_2 Uptake
1	0	8
2	320	12
3	640	52
4	320	28
5	80	4
6	10	20 stimulation
7	0	12 "
8	0	8 "

All Experiments Carried Out Using The Oxygen Electrode.

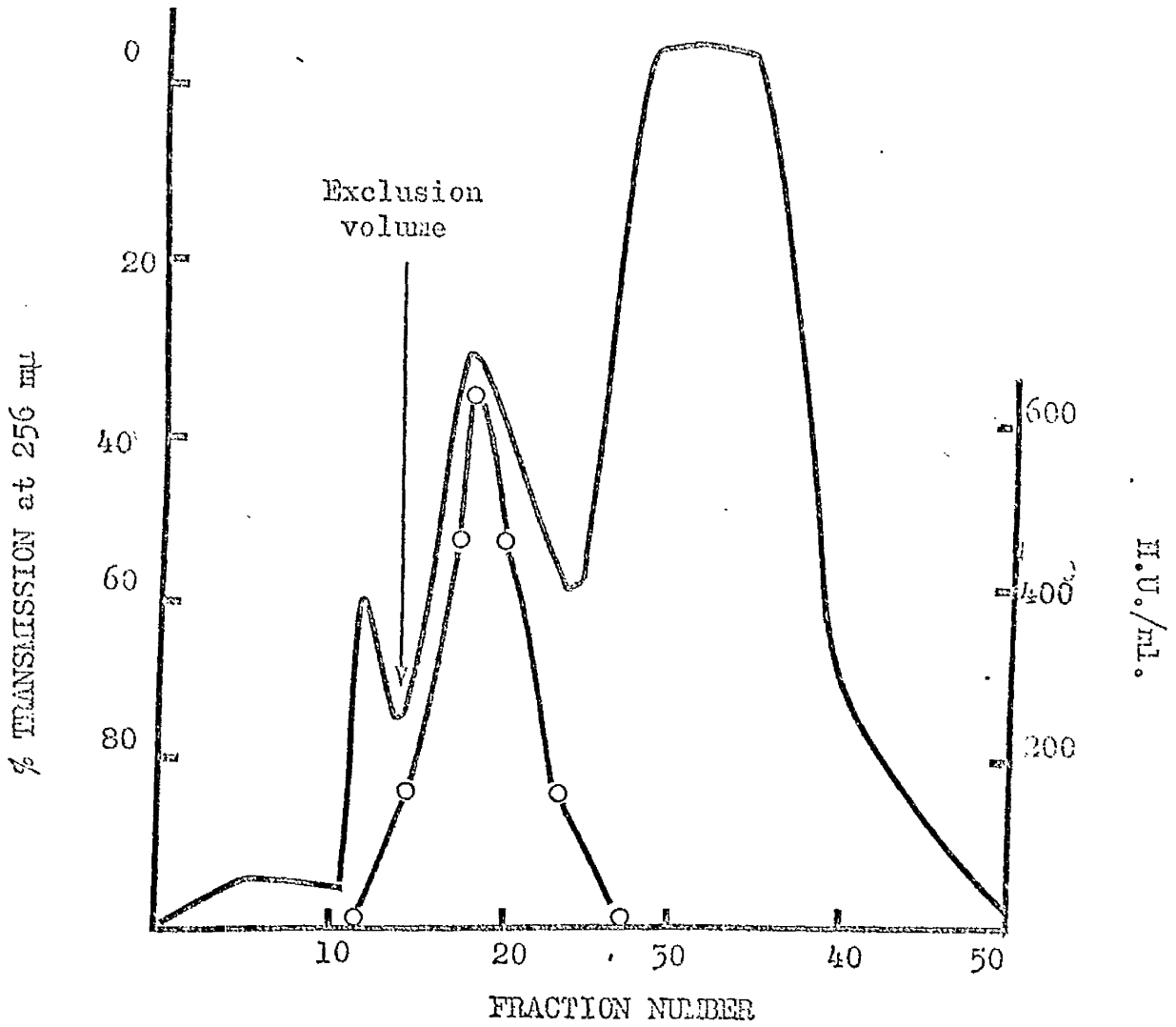


FIG. 16. Elution Profile of SLS from G-50 Sephadex.

o --- o Haemolytic Activity.

From these it can be seen that the impairment of mitochondrial succinic oxidase activity parallelled the haemolytic activity, both biological activities being associated with the shoulder of the main peak of absorbance at 256 mμ.

In an attempt to resolve the test material into three distinct peaks, it was passed through a G-50 Sephadex column. G 50 Sephadex was chosen because it possess a lower exclusion limit (10,000 Molecular Weight) than G 75 (50,000 Molecular Weight) and it was hoped that some of the higher molecular weight material in the RNA-SLS preparation would be excluded from this gel giving better resolution.

A 1 ml. sample of concentrated test material containing 58,824 H.U. was passed through a G 50 Sephadex column and three distinct peaks of optically dense material were found as illustrated in FIG. 16. The fractions collected were screened for haemolytic activity and those containing more than 10 H.U. were titrated accurately. The haemolytic profile is shown in FIG. 16 and it can be seen to be associated with peak 2. Eight samples were prepared by bulking fractions as follows:-

Fractions	11 to 13	bulked	to	form	Sample	1
"	14 to 16	"	"	"	"	2
"	17 to 19	"	"	"	"	3
"	20 to 21	"	"	"	"	4
"	22 to 24	"	"	"	"	5
"	25 to 30	"	"	"	"	6
"	31 to 36	"	"	"	"	7
"	37 to 42	"	"	"	"	8

When these were now tested on mitochondria it was found that impairment of succinic oxidase activity was also associated with the second peak. (TABLE 18).

These results clearly indicate that the haemolytic and mitochondrial damaging activities of RNA-SLS preparations cannot be resolved on the basis of molecular weight.

ABSORPTION OF STREPTOLYSIN S.

A further attempt was made to distinguish between the haemolytic and mitochondrial damaging activities of the RNA-SLS preparations by selective absorption of the activities on mitochondria and erythrocyte membranes. Elias, Heller and Ginsburg (1966) have shown that erythrocyte ghosts from human erythrocytes bind SLS irreversibly.

Human erythrocyte ghosts were prepared as described in the Materials and Methods (page 66). An equal volume of the resulting ghost preparation was incubated with SLS for 30 minutes at 37°C. The ghosts were removed by centrifuging at 18,000 rpm for 30 minutes. The resulting supernatant fluid was titrated for haemolytic activity and tested for impairment of mitochondrial succinic oxidase activity. This experiment was repeated several times using different ghost and SLS preparations (TABLE 19). It can be seen that both activities were significantly reduced by such treatment.

The converse experiment was carried out to investigate how interaction with mitochondria affected the two toxic activities. Mouse liver mitochondria were prepared as described for respiration experiments and an equal volume of mitochondria and SLS were incubated together at 37°C for 30 minutes; mitochondria were removed by centrifuging at 8,500 rpm for 10 minutes. The supernatant fluid from this reaction mixture was titrated for haemolytic activity and tested for impairment of succinic oxidase activity; both activities were diminished compared to the original toxin. The results of several such experiments are given in TABLE 20.

Possible explanations of the relationship between the two biological activities of RNA-SLS preparations will be discussed later.

- o -

TABLE 19.

Interaction between Erythrocyte Ghosts and SLS.

Experiment number	Concentration of SLS (H.U./ml)		% Inhibition of Mitochondrial O_2 Uptake	
	Before Absorption	After Absorption	Before Absorption	After Absorption
1	640	160	50	41
2	500	16	23	0
3	4,000	128	59	36
4	1,000	4	53	29

TABLE 20.

Interaction between Mitochondria and SLS.

Experiment number	Concentration of SLS (H.U./ml)		% Inhibition of Mitochondrial O_2 Uptake	
	Before Absorption	After Absorption	Before Absorption	After Absorption
1	640	160	50	18
2	500	128	23	16
3	4,000	16	59	24
4	1,000	4	53	0

All Experiments Carried Out by Warburg Manometry.

LOCUS OF ACTION OF STREPTOLYSIN S ON THE ELECTRON TRANSPORT CHAIN.

Results described thus far refer to the general features of impairment of mitochondrial succinic oxidase activity. It was considered important to extend this work and to attempt to pin point the site of action of SLS within the electron transport chain. A detailed knowledge of the interaction between SLS and components of the electron transport chain might well yield information relating to the mode of action of SLS at the molecular level. For reasons mentioned earlier, page 58 , it was necessary to locate the region of the succinic oxidase chain affected by SLS and to do this the action of the toxin on succinic dehydrogenase and cytochrome oxidase was assessed.

Succinic Dehydrogenase.

Using methylene blue as hydrogen acceptor to assay succinic dehydrogenase activity (page 76) it was found that in three experiments 167 H.U./ml of SLS caused no increase in the time taken to cause 90% decolourisation of the dye. Thus it was concluded that SLS had no detectable effect on succinic dehydrogenase activity of mitochondria.

Cytochrome Oxidase.

Most of the original experiments using cytochrome oxidase were carried out using serum-SLS prepared from strain NCTC 5163. It must be emphasised that the findings reported here have been fully confirmed using RNA-SLS and are essentially similar for both SLS preparations. In experiments the effect of SLS on succinic and cytochrome oxidase activities were compared (TABLE 21). Cytochrome oxidase was impaired by SLS and in each case the degree of impairment was less than that of succinic oxidase (on average cytochrome oxidase was 30% less impaired than succinic oxidase). These results suggested that SLS acted on the terminal

TABLE 21.

Impairment of Succinic and Cytochrome Oxidase by SLS.

Experiment number	Concentration of SLS (H.U./ml)	% Impairment of Succinic Oxidase	% Impairment of Cytochrome Oxidase
1^{w}	267^{s}	56	31
2^{w}	"	58	50
3^{w}	"	56	25
4^{w}	"	50	45
5^{w}	"	44	27
6^{e}	249^{r}	50	40
7^{e}	416^{r}	45	44
8^{e}	207^{r}	45	22

w = Experiment carried out by Warburg Manometry.

e = " " " in Oxygen Electrode.

s = Serum-SLS.

r = RNA-SLS.

part of the respiratory chain between cytochrome c and oxygen. In order to specify the site of action more precisely it was decided to attempt a restoration of normal function by adding constituents of the electron transport chain. The two components of the respiratory chain most likely to be affected by disorganisation of the mitochondrial membrane are the mobile components, ubiquinone and cytochrome c.

Restoration Experiments.

The addition of ubiquinone to a final concentration of 100 µg/ml failed to significantly restore the activity of succinic oxidase impairment by SLS. (TABLE 22). Since ubiquinone is involved only in carrying electrons between succinic dehydrogenase and the cytochrome c complex, it was tested on impaired succinic oxidase activity and not on cytochrome oxidase activity. Further more the incubation of mitochondria at a concentration of 100 µg ubiquinone/ml prior to the addition of SLS did not prevent impairment of succinic oxidase activity.

TABLE 22.

Addition of Ubiquinone to Impaired Succinic Oxidase.

Experiment number	Concentration of SLS (H.U./ml)	% Inhibition before adding ubiquinone	% Restoration after adding ubiquinone
1	100	42	9
2	100	55	9

All Experiments carried out by Warburg Manometry.

In contrast, strong evidence was obtained to indicate restoration of respiration of SLS impaired mitochondria following the addition of cytochrome c. A typical experiment showing the effect of the addition of cytochrome c at a concentration of 80 µg/ml to mitochondrial succinic oxidase and cytochrome oxidase activities impaired by SLS is shown in FIG. 17 a & b. The results of several such experiments on succinic oxidase activity are shown

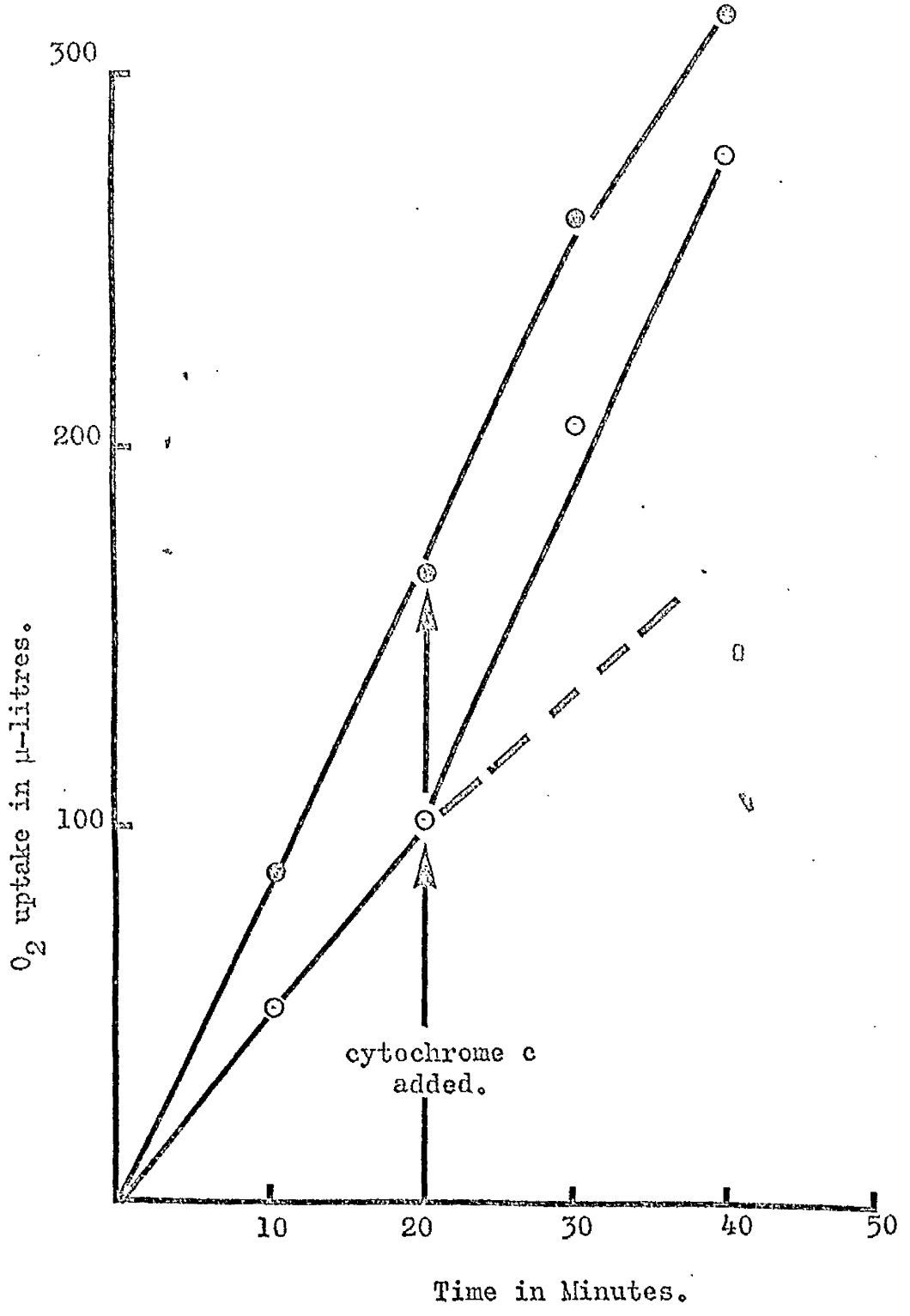


FIG.17a. Stimulation of succinic oxidase activity of mitochondria impaired by treatment with RNA-SLS (70H.U./ml.) for 30 mins. by the addition of cytochrome c (2.4 mg/ml.)

o — o Toxin treated mitochondria.

⊙— ⊙ Control mitochondria.

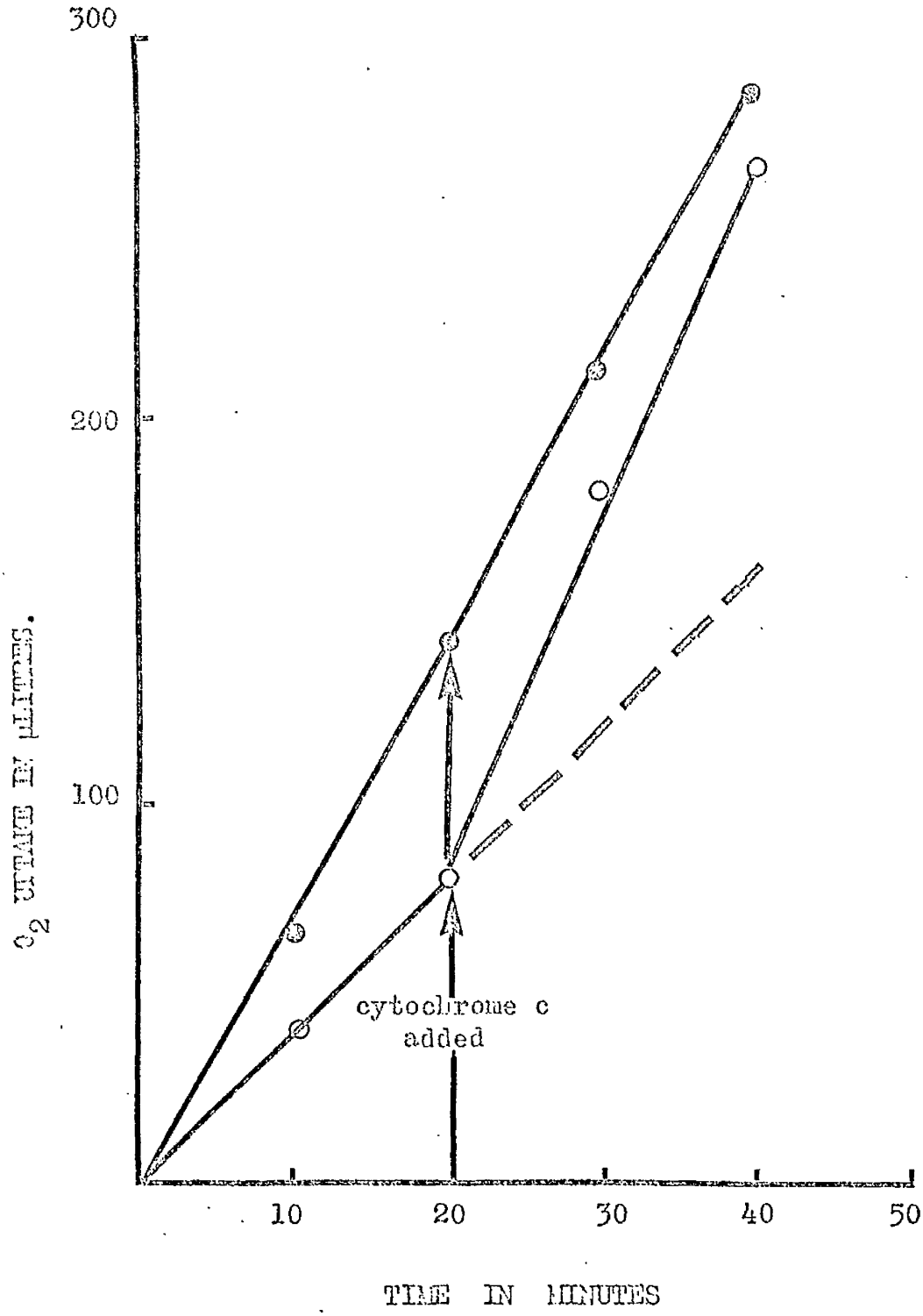


FIG.17 b. Stimulation of mitochondrial cytochrome oxidase activity impaired by treatment with RNA-SLS (533 H.U./ml) for 30 minutes by the addition of cytochrome c (2.4mg/ml).

o — o Toxin treated mitochondria
o— o Control mitochondria.

in TABLE 23. Although the percentage impairment and restoration varied from one experiment to another, in all experiments it can be seen that striking restoration was observed. The percentage restoration was determined by comparing the rate of oxygen uptake in controls with that of the test after the addition of cytochrome It is important to note that in many experiments the rate of oxygen uptake of restored mitochondria was equal to or higher than that of the controls. Thus the efficiency of cytochrome c restoration of succinic oxidase impaired activity is extremely high.

The effect of cytochrome c addition to cytochrome oxidase impairment was even more dramatic. The results of four similar experiments on cytochrome oxidase are shown in TABLE 24. Here the degree of stimulation following the addition of cytochrome c is even greater. Also it is important to note that incubation of mitochondria with cytochrome c at a concentration of 24 µg/ml prior to the addition of SLS completley abolished any observable inhibition of succinic oxidase or cytochrome oxidase activities.

These experiments indicate that ubiquinone, as expected, failed to restore succinic oxidase activity in mitochondria impaired by SLS. On the other hand the striking restoration of succinic oxidase and cytochrome oxidase activities by the addition of cytochrome c to toxin impaired mitochondria reinforces the opinion that SLS acts on cytochrome oxidase and that the observed impairment of succinic oxidase activity is a reflection of this. Mitochondrial damage by this toxin appears to cause dislocation of the electron transport chain at the cytochrome c locus and that this can be masked by the presence of exogenous cytochrome c, ab initio. This raised the question of whether such dislocation of the electron transport chain would result in leakage of cytochrome c from the mitochondria.

TABLE 23.

Restoration of Impaired Succinic Oxidase by Cytochrome c.

Experiment number	Concentration of SLS (H.U./ml)	% Impairment before adding Cytochrome c	% Restoration after adding Cytochrome c
1^{w}	100^{s}	58	86
2^{w}	100^{s}	50	102
3^{w}	28^{s}	23	100
4^{w}	208^{r}	38	90
5^{w}	"	35	75
6^{w}	"	52	84
7^{w}	"	65	100
8^{w}	"	76	65
9^{e}	"	81	100

TABLE 24.

Restoration of Impaired Cytochrome Oxidase by Cytochrome c.

Experiment number	Concentration of SLS (H.U./ml)	% Impairment before adding Cytochrome c	% Restoration after adding Cytochrome c
1^{w}	100^{s}	50	100
2^{w}	100^{s}	45	121
3^{w}	100^{s}	27	107
4^{e}	249^{r}	40	150
5^{e}	207^{r}	22	100

w=Experiment carried out by Warburg Manometry. e=Experiment carried out in Oxygen Electrode. s=Serum-SLS. r=RNA-SLS

Leakage Experiments.

In order to find if there was a detectable increase in the amount of cytochrome c in the supernatant fluid of isolated mitochondria treated with SLS the following experiment was carried out. Two reaction mixtures were set up as follows:-

Tube 1. 0.5 ml mitochondria + 1 ml. heated SLS (0 H.U./ml)

Tube 2. 0.5 ml mitochondria + 1 ml. SLS (6,667 H.U./ml)

After incubation at 37°C for 30 minutes, the mitochondria were removed by centrifuging at 8,500 rpm. The resulting supernatant fluids were titrated against human erythrocytes to detect residual SLS activity also protein concentrations were titrated by the Lowry method (Lowry, Rosenrogh, Farr and Randall 1951). There was no haemolytic activity in the supernatant of tube 1 and tube 2 contained only 40 H.U./ml compared to the 6,667 H.U./ml originally present. There was 0.984 mg. protein/ml in tube 1 and 1.250 mg protein/ml in tube 2 indicating that treatment of mitochondria with SLS results in leakage of protein. An absorption spectrum of the supernatants is illustrated in FIG. 18 . and from this it can be seen that there is a marked increase in material absorbing at 410 mμ in the supernatant fluid from mitochondria treated with SLS. Cytochrome c characteristically has an absorption maximum at 415 mμ and this strongly suggests that the leakage of protein from SLS-treated mitochondria can be partly accounted for in terms of cytochrome c. The succinic oxidase activity of mitochondria after such treatment was also tested and those treated with unheated SLS showed 47% impairment of oxygen uptake.

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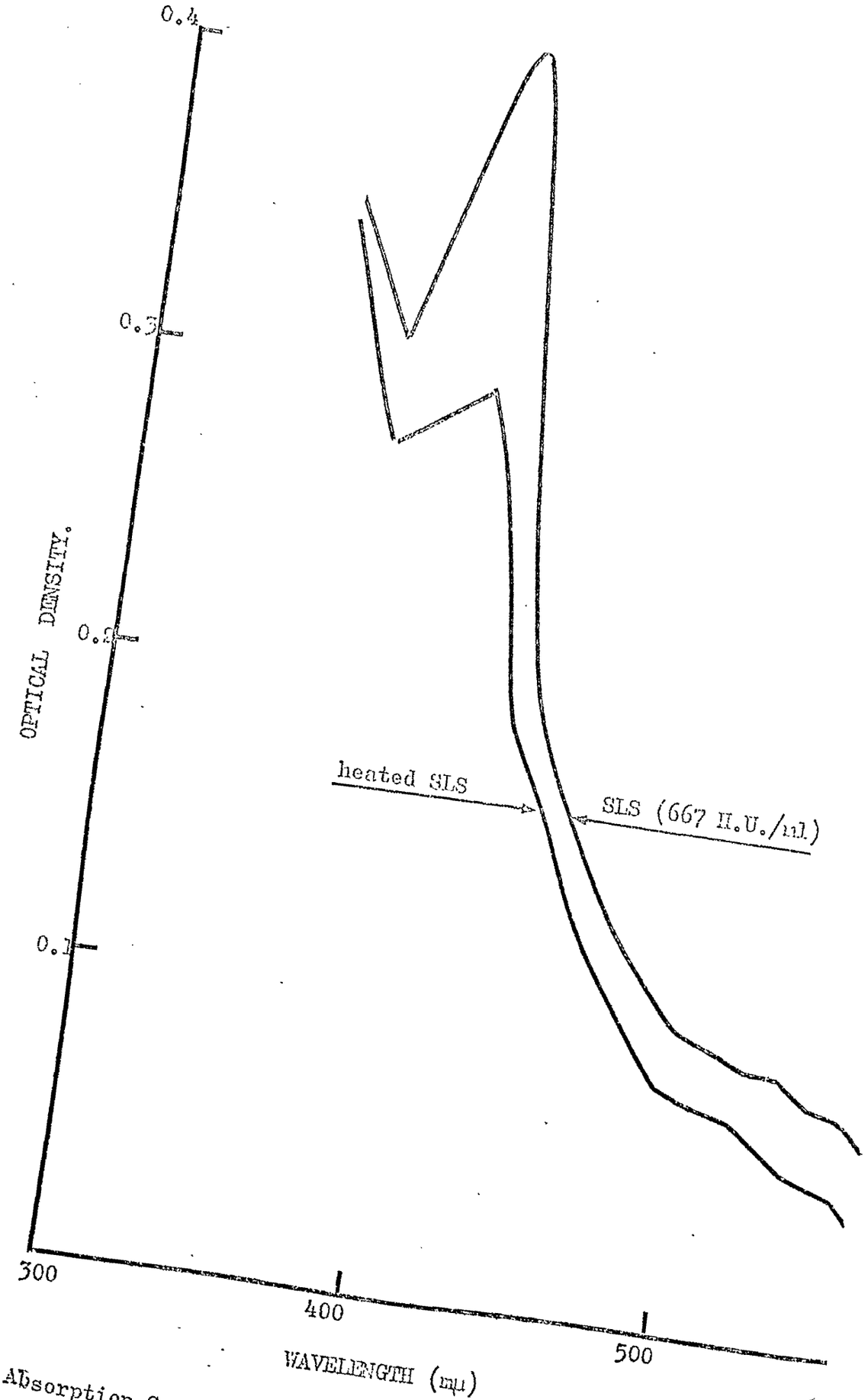

FIG. 18. Absorption Spectrum of Supernatant Fluid from Mitochondria treated with SLS.

EFFECT OF STREPTOLYSIN S ON KREBS 2 ASCITES TUMOUR CELLS.

The cytotoxicity of SLS for Ehrlich Ascites Tumour cells was first reported by Ginsburg (1959) when he observed that treatment of these cells with SLS or SLO resulted in swelling and loss of their ability to stain with vital stains such as trypan blue. Further work by Ginsburg and Grossowicz (1960) extended these findings and showed that Ehrlich Ascites Tumour cells injured by SLS or SLO were no longer able to proliferate in mice.

To my knowledge there has been no reports on the action of streptococcal toxins on Krebs 2 Ascites Tumour cells. Havas et al (1963) reported that living haemolytic streptococci had a cytotoxic effect on these cells. This group found that Krebs 2 cells incubated with Streptococcus pyogenes became enlarged, showed swelling, extensive granulation of the nucleus, emission of small and large blebs and cytoplasmic vacuolation. Time lapse photography, using phase contrast microscopy, revealed that these cytotoxic changes eventually resulted in sudden rupture of the cells. In the same paper it was stated briefly that purified SLS was also cytotoxic but no experimental details were given.

In view of these findings by previous workers it was decided to record the visible changes which occurred in Krebs 2 cells treated with SLS before investigating any resultant metabolic derangement.

PHASE CONTRAST MICROSCOPY OF KREBS 2 ASCITES TUMOUR CELLS TREATED WITH STREPTOLYSIN S.

Krebs 2 Ascites Tumour cells were aspirated from mice and washed as described in the Materials and Methods (page 63). Test tubes were set up containing 1 ml. PVP/tris buffer pH 7.3, 0.25 ml. SLS or control fluid and 0.25 ml. washed ascites cells. These tubes were then incubated at 37°C in a shaking water bath. Samples of the mixture were removed at varying times and examined by phase contrast microscopy.

The ascites cells treated with control fluid were highly refractile; the nuclei were clearly visible and a few cytoplasmic

vacuoles were always present. There were no visible changes in these cells after 30 minutes incubation at 37°C, and when injected into mice they produced tumours in 7 to 9 days. On the other hand in the presence of SLS (2,500 H.U.), marked changes in the morphology of the ascites cells were observed (FIG. 19 a & b, c & d After 10 minutes incubation, nuclei appeared to become irregular ar there was increased vacuolation of the cytoplasm. This became muc more marked after 20 minutes incubation when the entire cells were meshworks of small vacuoles and small blebs formed on outer membranes. At 30 minutes nuclei were hardly visible, many of the vacuoles appeared to have fused together to form large refractile areas occupying up to 50% of some cells. Also there were many more blebs round the outside of the cells at this time. SLS-treat Krebs 2 Ascites Tumour cells failed to cause tumours in mice even up to 2 weeks after injection when the experiment was terminated.

It is important to note however that, despite the fact that 30 minutes treatment with SLS resulted in irreparable damage to the ascites cells, 100% of the original haemolytic activity could be recovered from the supernatant fluid after removing the ascites cel by centrifugation.

These results clearly demonstrate that SLS is cytolytic for Krebs 2 Ascites Tumour cells, and the changes which were observed are consistent with the toxin acting on the cellular membranes causing gross disorganisation of the cells. It therefore, seemed relevant to determine what secondary metabolic changes occur within the cells as a result of the action of SLS.

FIG. 19. Phase contrast micrographs of Krebs 2 Ascites Tumour cells treated with Streptolysin S at a final concentration of 417 H.U./ml. (X 1,600).

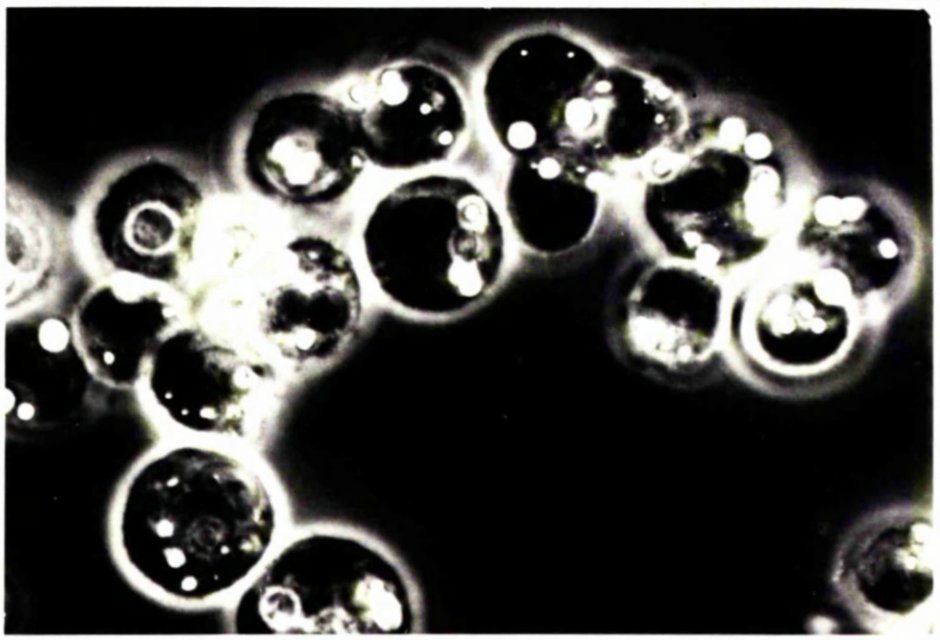

FIG. 19a. Cells incubated with control fluid for 20 minutes.

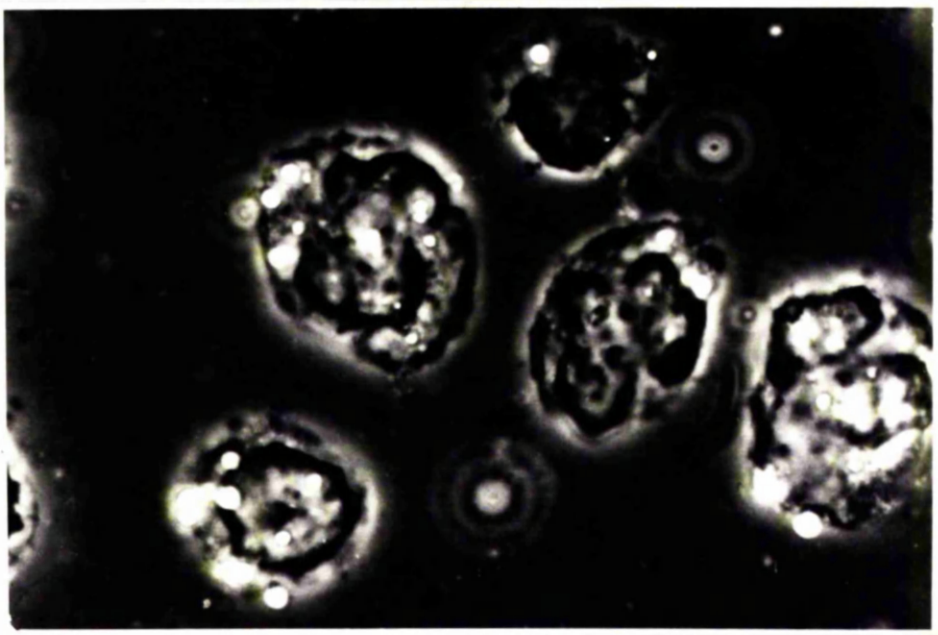

FIG. 19b. Cells treated with SLS for 1o minutes.

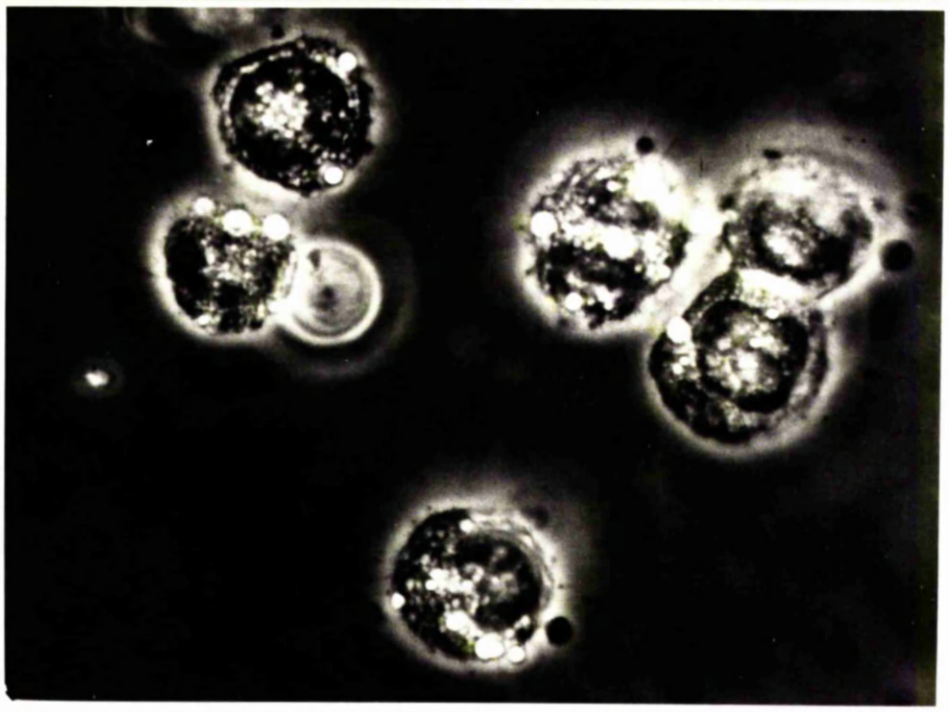

FIG. 19c. Cells treated with SLS for 20 minutes.

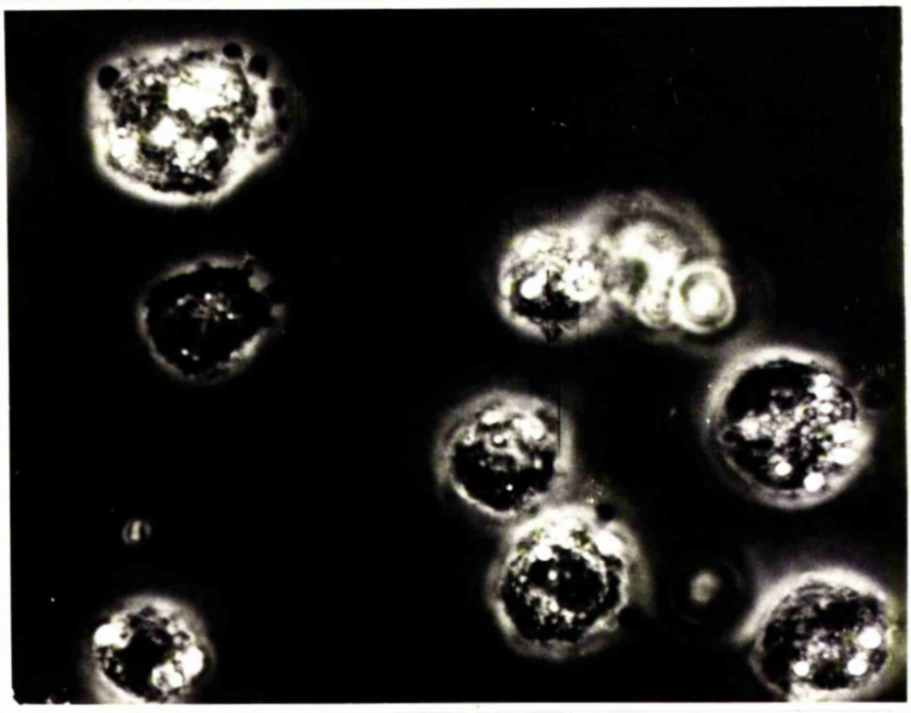

FIG. 19d. Cells treated with SLS for 30 minutes.

EFFECT OF STREPTOLYSIN S ON THE OXIDATION OF SUCCINATE BY KREBS 2 ASCITES TUMOUR CELLS.

Succinate was chosen as substrate for these studies because it is oxidised by an enzyme complex (succinic oxidase) which forms an integral part of the mitochondrion, shown in the first part of this Thesis, to be inhibited by SLS. Unlike other possible substrates metabolism of succinate does not require cytoplasmic enzymes or co-factors which might leak out of the cell following membrane damage. For these reasons it seemed likely that succinate oxidation offered a simple and meaningful system for study.

Krebs 2 Ascites Tumour cells showed increased oxygen uptake on addition of exogenous succinate, indicating that they are permeable to this substrate. It was noticed in early experiments that unwashed Krebs 2 Ascites Tumour cells aggregated during Warburg manometry. Thus the washing procedure described on page 63. of the Materials and Methods was devised to render the cells suitable for this technique. When measuring the oxidation of succinate by these cells no NaOH was put in the centre well of the Warburg flasks since carbon dioxide is not evolved in the oxidation of succinate or other subsequent intermediates of the Tri-carboxylic acid cycle. The endogenous rate of oxygen uptake was always subtracted from the rate in the presence of exogenous substrate before expressing the results.

When Krebs 2 Ascites Tumour cells were treated with SLS for 30 minutes prior to the addition of sodium succinate there was a marked increase in the rate of oxygen uptake by these cells compared to cells treated with suspending medium (FIG. 20). That this was not a non-specific effect was shown by the fact that SLS preparations when heated for 15 minutes at 100°C lost both their haemolytic activity and the ability to stimulate the rate of oxygen uptake by ascites cells (TABLE 25). SLS had no effect on actively respiring cells, a minimum period of contact of 10 minutes between washed cells and toxin prior to the addition of succinate

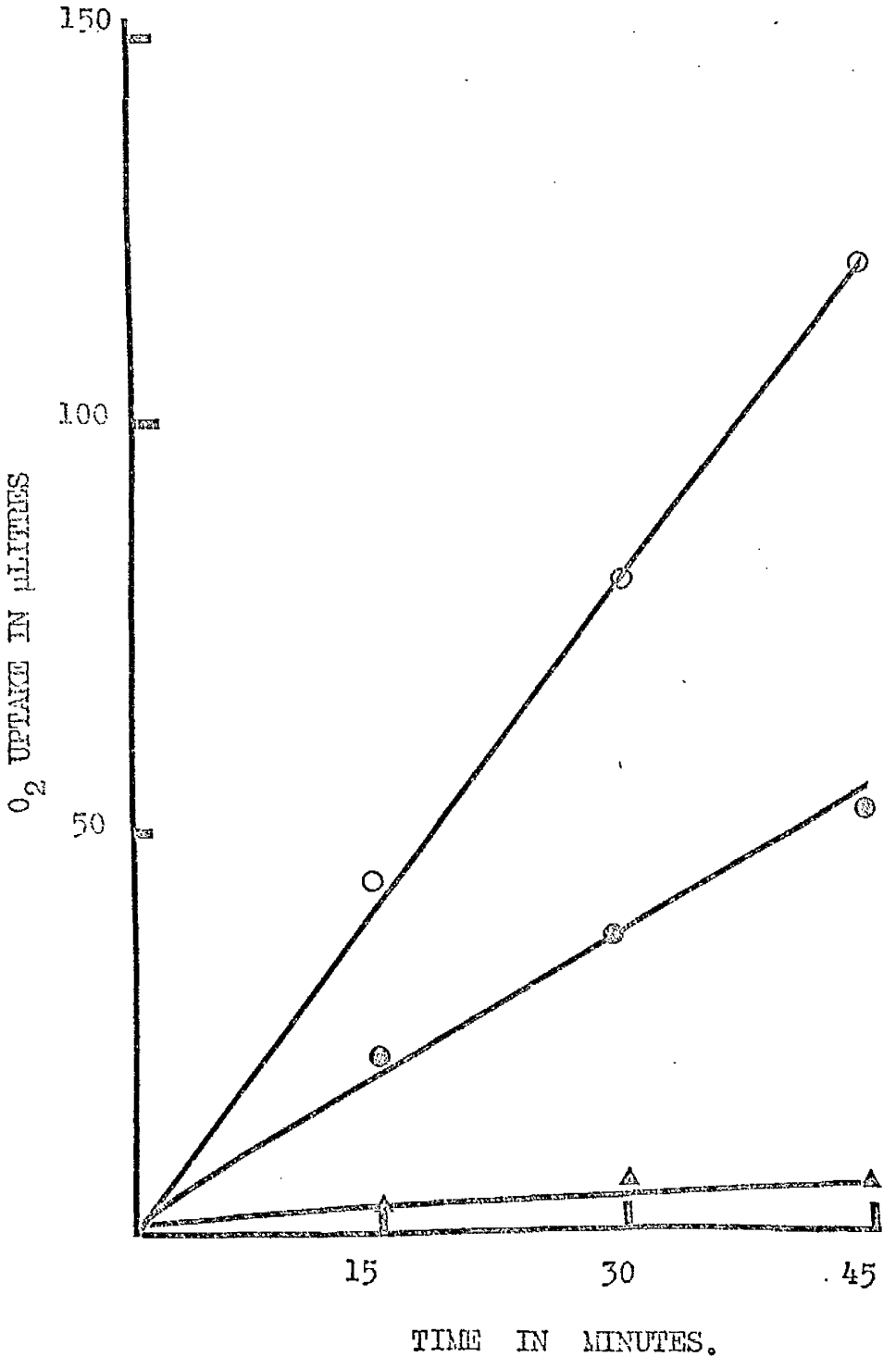

FIG. 20. Increased oxygen uptake by ascites cells utilising succinate following 30 minutes treatment with SLS at a final concentration of 2,300 H.U./ml.

O—O Toxin-treated cell suspension.
●—● Control cell suspension.
▲—▲ Endogenous respiration.

TABLE 25.

Effect of Heating SLS.

Sample.	H.U./ml. in Warburg Flask.	Change in ascites cell O_2 Uptake.
RNA-SLS	801	116% stimulation
RNA-SLS heated at 100° for 15 minutes.	0	15% inhibition

TABLE 26.

Effect of Age on Sensitivity of Ascites Cells to SLS.

Age of Cells in days.	% Stimulation of O_2 Uptake.
6	88
7	151
8	156
9	116
10	173

A final concentration of 193 H.U. SLS/ml. was present in each assay. All experiments using ascites cells were recorded by Warburg Manometry.

was necessary to induce stimulation. Pre-incubation for longer than 10 minutes did not significantly influence the degree of stimulation obtained.(FIG. 21).

The Ascites Tumour cell preparations varied in their susceptibility to SLS, indeed in 3 out of 13 experiments, no stimulation was observed, even when very high concentrations of SLS were employed. The reason for this variation is not known but it is not simply due to minor differences in the age of the cells. (TABLE 26). Because of this variability in response, a detailed study of the effect of varying concentrations of SLS was not practical. However, in preparations of sensitive cells, 200 H.U./ml. or more of SLS were required to cause observable stimulation.

Treatment of Krebs 2 Ascites Tumour cells with SLS for 30 minutes has been shown previously to cause irreversible lethal changes in the cells. That the effect on succinate oxidation is also irreversible was also shown by the fact that cells pre-treated with 1,000 H.U./ml. of SLS for 30 minutes and washed free of toxin prior to the assay exhibited 98% stimulation of oxygen uptake.

It has been shown previously in this Thesis that SLS impairs the succinic oxidase activity of isolated mouse liver mitochondria. However, the results with Krebs 2 Ascites Tumour cells, namely stimulation of oxygen uptake, suggests that the oxidative capacity of the mitochondria remains intact and is indeed increased. Therefore, SLS probably does not penetrate to the mitochondria of these cells. Indeed the fact that SLS does not bind to Krebs 2 Ascites Tumour cells suggests that the metabolic disturbance described above probably results from the action of the toxin on the cell membrane in such a way as to cause an increase in its permeability to succinate.

In order to confirm this hypothesis it was necessary to examine the effect of SLS on the succinic oxidase activity of Krebs 2 Ascites

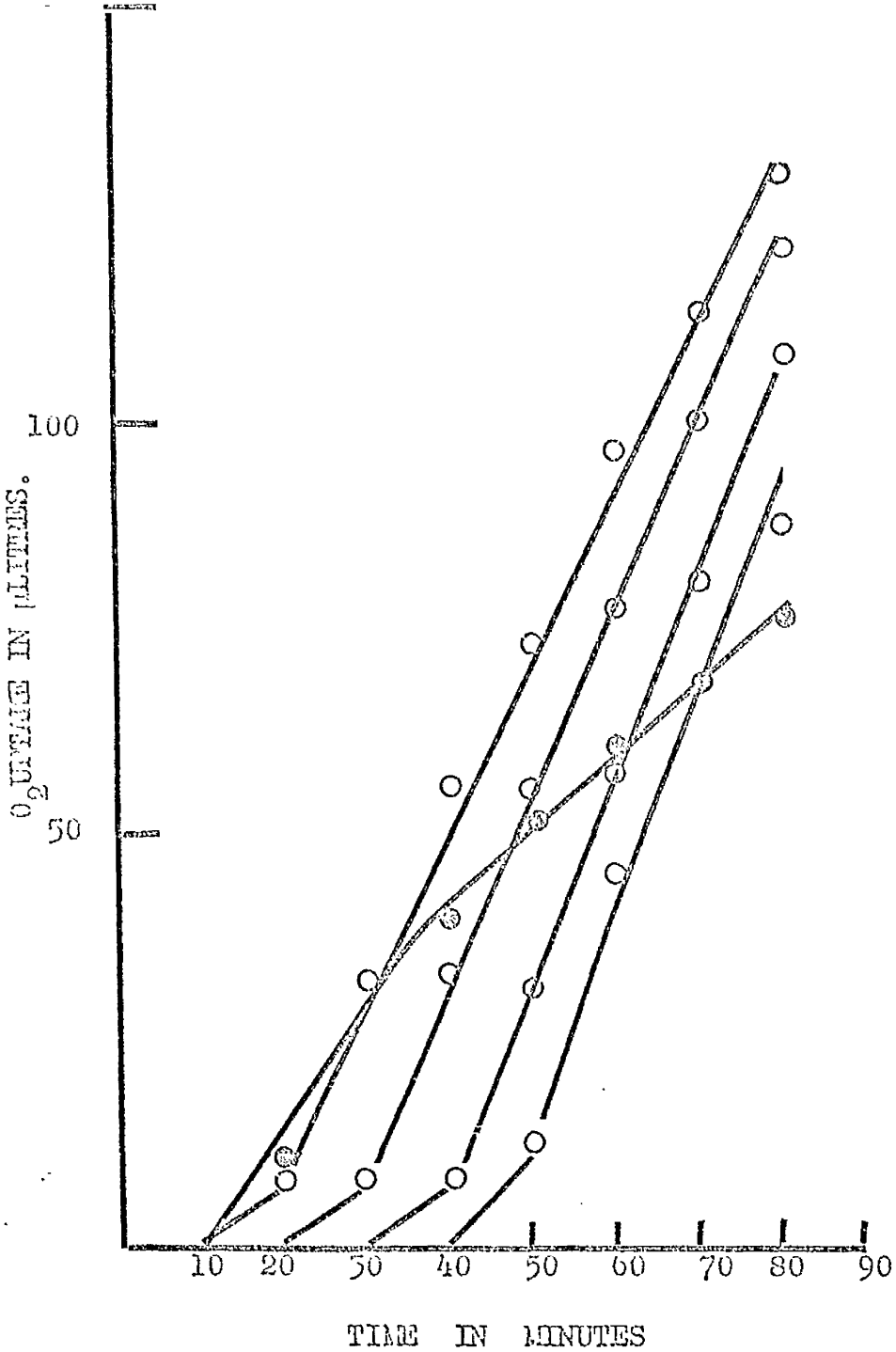

FIG. 21. Increased oxygen uptake by ascites cells utilising succinate following varying periods of treatment with SLS at a final concentration of 801 H.U./ml.

o — o Toxin-treated cell suspensions
● — ● Control cell suspension.

Tumour cell mitochondria. Initially, disintegrated cell preparations, prepared as described on page 64 . of the Materials and Methods, were employed. Cells were disrupted by sonication sufficient to destroy the limiting cell membrane without damaging the mitochondria; this treatment exposed these intracellular organelles to SLS and in these preparations the rate of oxygen uptake was impaired by SLS (FIG. 22). Further confirmation that the succinic oxidase activity of Ascites cell mitochondria was impaired by SLS was made when isolated mitochondria were prepared from these cells by the method of Wu and Sauer (1967) and tested with SLS, (FIG. 23). From FIG. 23 it can be seen that these mitochondria were indeed impaired by the toxin.

These findings support the idea that the secondary metabolic changes which occur in Krebs 2 Ascites Tumour cells following treatment with SLS result, not from an action on intracellular organelles, but from the disorganisation of the limiting cell membrane.

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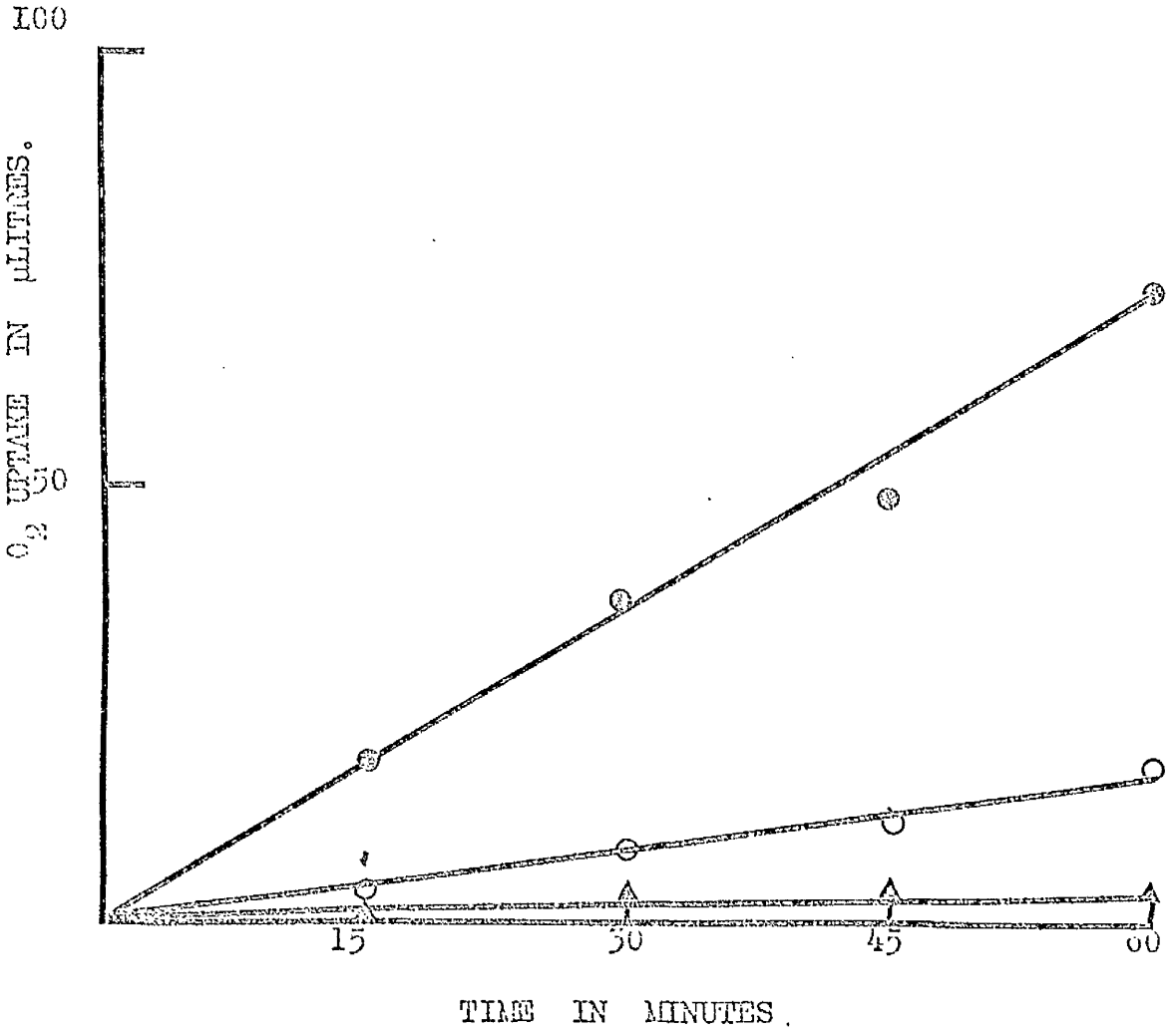

FIG. 22. Impairment of succinate oxidation by ultrasonically disintigrated ascites cells by 30 minutes treatment with SLS at a final concentration of 1,600 H.U./ml.

o — o Toxin-treated cell suspension
● — ● Control cell suspension.
▲ — ▲ Endogenous respiration.

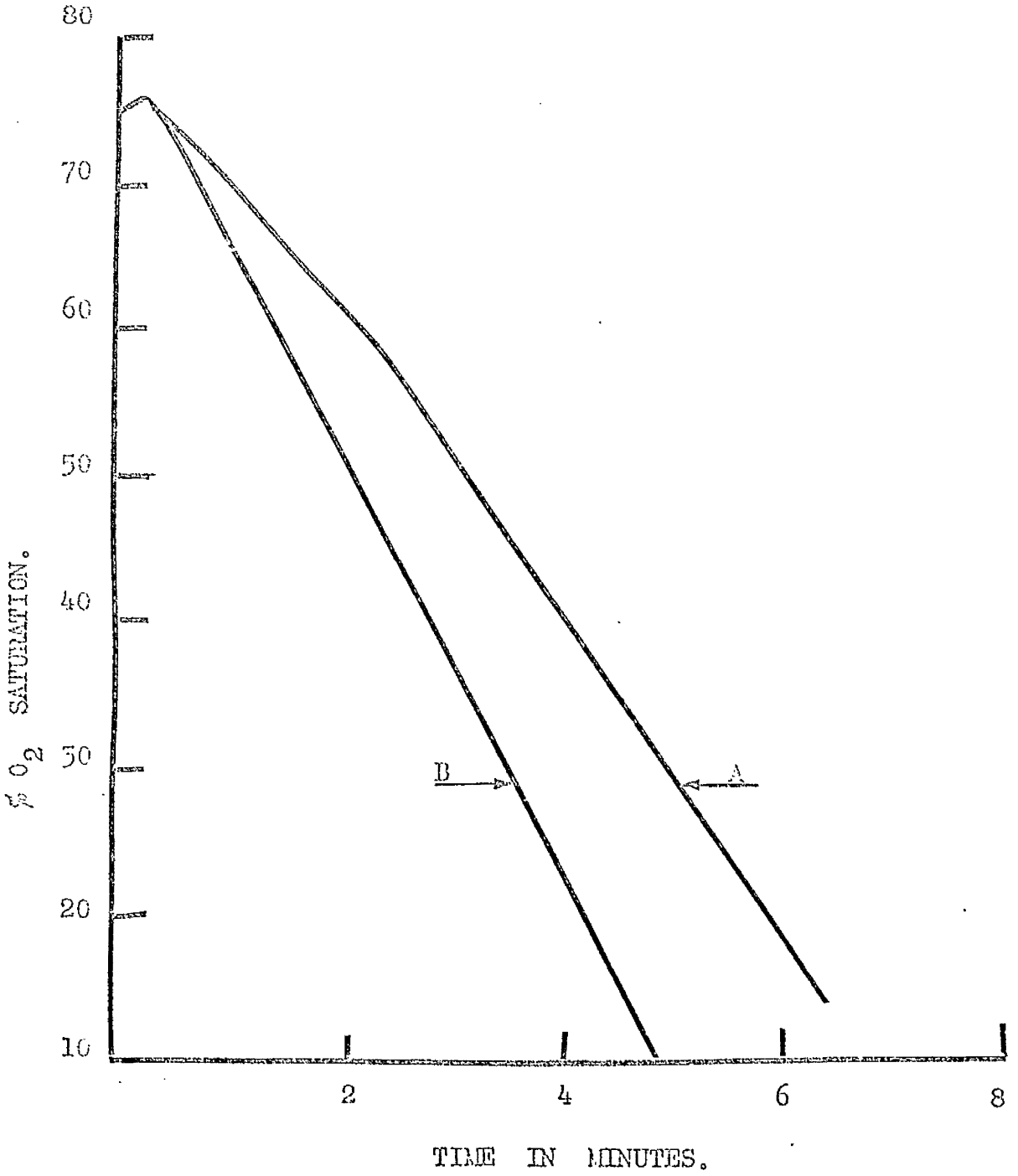

FIG. 23. Impairment of succinic oxidase activity of ascites cell mitochondria treated for 15 minutes with SLS at a final concentration of 417 H.U./ml.

A = Toxin-treated mitochondria.
B = Control mitochondria.
This experiment was recorded in the Oxygen Electrode.

EFFECT OF STAPHYLOCOCCAL ALPHA - TOXIN ON KREBS 2 ASCITES TUMOUR CELLS.

As mentioned previously (page 46) staphylococcal alpha-toxin has been reported to cause gross morphological damage to Ehrlich Ascites tumour cells (Nadoff et al 1962). Nadoff reported extensive internal disorganisation, the most striking changes being the appearance of large blebs round the perimeter of the cells. These appeared to be swellings of the outer cell membrane and were on occasions as large as the cell itself. These workers also showed that the cytopathic changes in these cells caused by the action of this toxin resulted in the rapid release of free amino acids followed by the release of trichloroacetic acid-precipitable S^{35}-L-methionine indicating that larger molecular weight polypeptides were also escaping from the cells. Such gross alterations in structure rendered the cells incapable of proliferating in mice.

Before comparing the effect on metabolism of Krebs 2 cells of staphylococcal alpha-toxin with that of SLS, the visible cytopathic changes caused were viewed under the phase contrast microscope to find out whether they resembled those described by Nadoff for Ehrlich ascites tumour cells.

The staphylococcal alpha-toxin used in this study was kindly prepared for me by Miss Christine McNiven from Staphylococcus aureus (strain Wood 46 NCTC 7121) by the method of McNiven and Arbuthnott (1968). These toxin preparations had a potency of approximately 8,000 H.U./ml. when titrated against rabbit erythrocytes by the method of Bernheimer and Schwartz (1963).

PHASE CONTRAST MICROSCOPY OF KREBS 2 ASCITES TUMOUR CELLS TREATED WITH STAPHYLOCOCCAL-ALPHA TOXIN.

Two reaction mixtures were set up:-

(1). contained 1 ml. PVP/tris buffer pH 7.3, 0.25 ml. washed Krebs 2 Ascites Tumour cells and 0.25 ml. staphylococcal alpha-toxin. The final concentration of alpha-toxin present was 120 H.U./ml.

(2). contained 1 ml. PVP/tris buffer pH 7.3, 0.25 ml. washed Krebs 2 Ascites Tumour cells and 0.25 ml. boiled toxin. Boiling totally inactivated the alpha-toxin and this reaction mixture served as control. Samples of these mixtures were taken at 10 minute intervals while incubating in a shaking water bath at 37°C and viewed by phase contrast microscopy.

The Krebs 2 Ascites Tumour cells which were treated with heated toxin remained unaltered during 30 minutes of incubation, their appearance was the same as that described for the ascites cells treated with SLS control fluid and is illustrated in FIG. 24 a. By contrast, after being in contact with active alpha-toxin for 10 minutes, the outer membrane of the cells appeared more irregular and blebs could be seen on a few cells; the cytoplasm of these cells was less dense than that of the controls. (FIG. 24 b). The formation of blebs was much more marked after the cells had been in contact with active alpha-toxin for 20 minutes and some cells appeared to be undergoing lysis. (FIG. 24 c). Cells which were treated with alpha-toxin for 30 minutes showed marked aggregation and the cytoplasm of several cells seemed to have fused together although some nuclei in these cells remained visible. Blebbing was even more extensive at this time and blebs were larger than those seen on cells which had been treated with toxin for shorter periods (FIG. 24 d).

After 30 minutes treatment with alpha-toxin the cells were no longer able to proliferate when injected into mice and when the cells

FIG. 24. Phase contrast micrographs of Krebs 2 Ascites Tumour cells treated with staphylococcal alpha-toxin at a final concentration of 120 H.U./ml. (X 1,600).

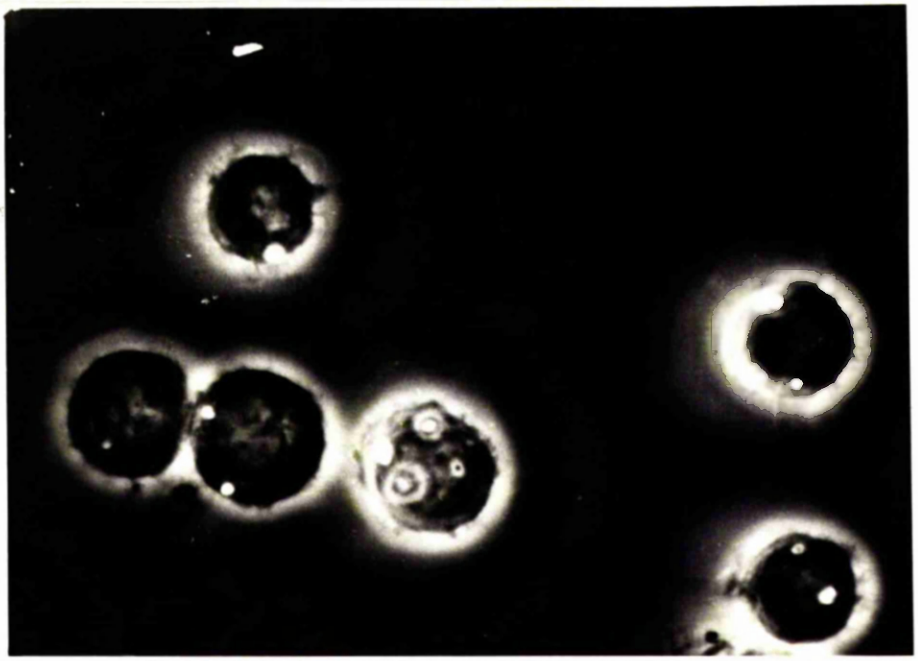

FIG. 24a. Cells incubated with control fluid for 20 minutes.

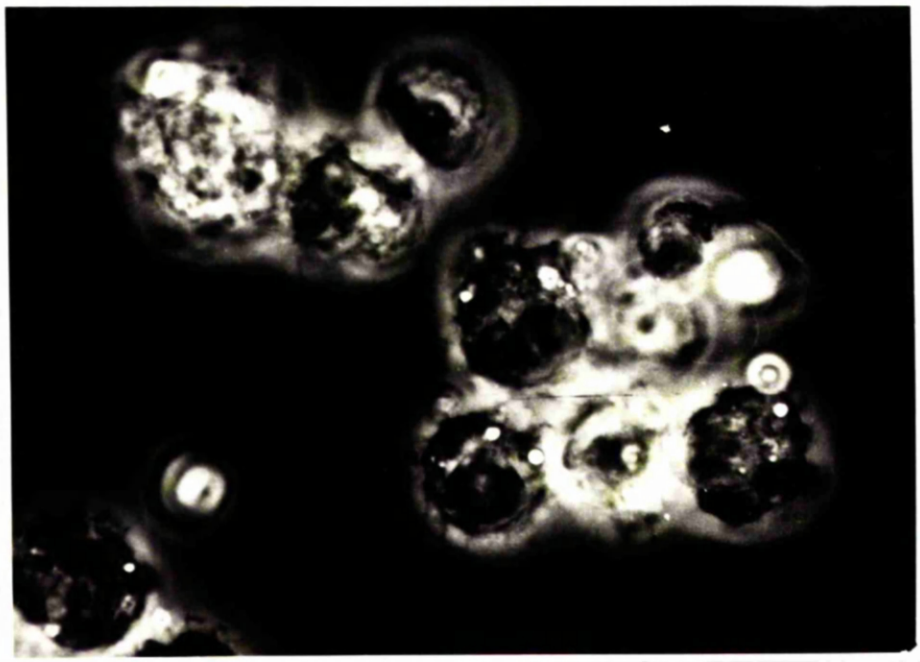

FIG. 24b. Cells treated with α-toxin for 10 minutes.

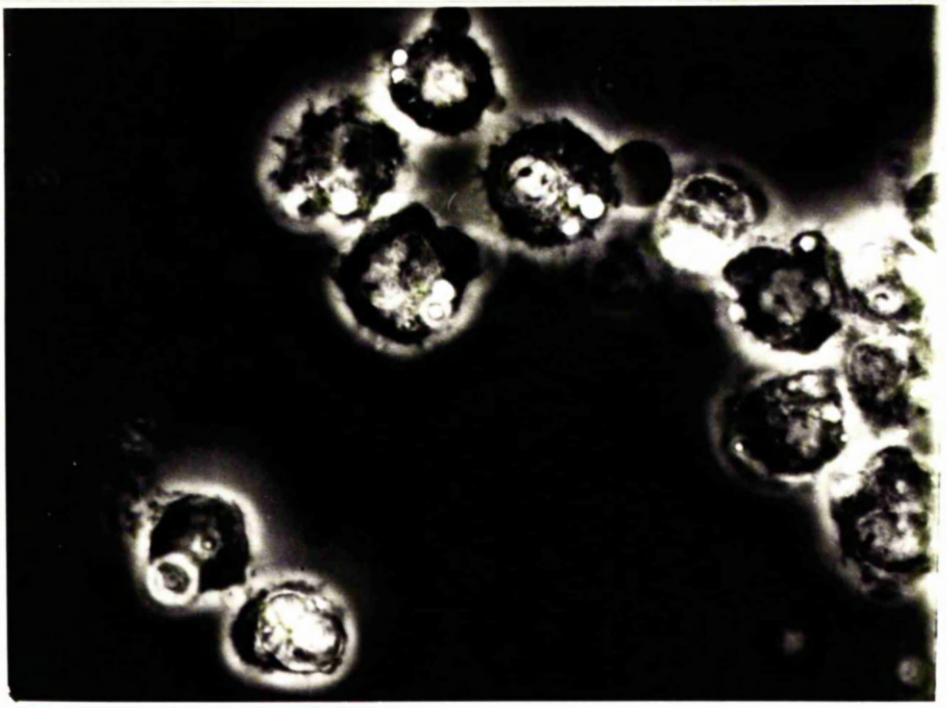

FIG. 24c. Cells treated with α-toxin for 20 minutes.

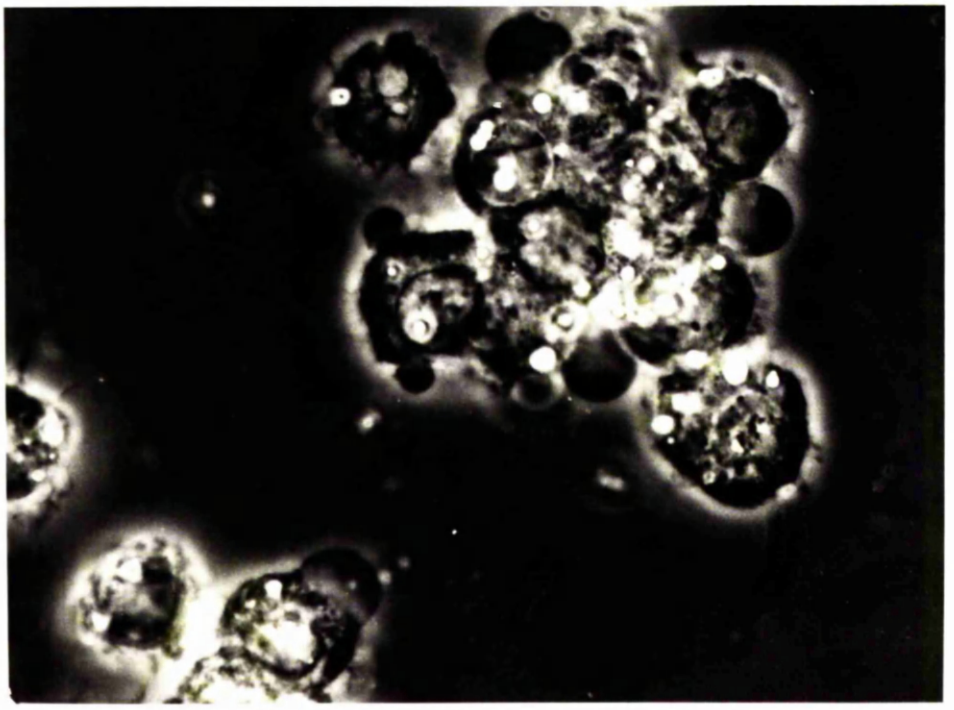

FIG. 24d. Cells treated with α-toxin for 30 minutes.

were removed by centrifugation, only a small proportion of the added haemolytic activity remained in the supernatant fluid.

EFFECT OF STAPHYLOCOCCAL-ALPHA TOXIN ON THE OXIDATION OF "" SUCCINATE BY KREBS 2 ASCITES TUMOUR CELLS.

Purified staphylococcal alpha-toxin had an immediate effect on the respiration of Krebs 2 Ascites Tumour cells when succinate was present as substrate. In each of 12 experiments the addition of alpha-toxin caused a marked increase in the rate of oxygen uptake by the cells (FIG. 25). The degree of stimulation observed was not directly proportional to the concentration of alpha-toxin. Above a threshold level of 6 to 12 H.U./ml. maximum stimulation was observed (FIG. 26).; the extent of which was fairly constant, until high concentrations of alpha-toxin were reached (greater than 200 H.U./ml.). At such high concentrations the degree of stimulation was reduced in some experiments. The fact that the damage to the ascites cells was irreversible was demonstrated by treating a suspension of cells with alpha-toxin for 30 minutes and washing them free of toxin prior to assaying succinate oxidation. There was 145% stimulation of oxygen uptake by the cells following such treatment.

In order to establish that these effects were specific for staphylococcal alpha-toxin, Krebs 2 cells were treated with an amount of alpha-toxin which had been just neutralised with an appropriate amount of commercially prepared alpha antitoxin. (FIG. 27). Neutralisation of the alpha-toxin also resulted in neutralisation of the stimulation effect. In view of the recent work of Bernheimer, Avigad and Grushoff (1968) it was necessary to establish if traces of staphylococcal delta-toxin were responsible for the effect. The presence of normal, inactivated horse serum, in amounts known to inactivate the haemolytic activity of delta-toxin,

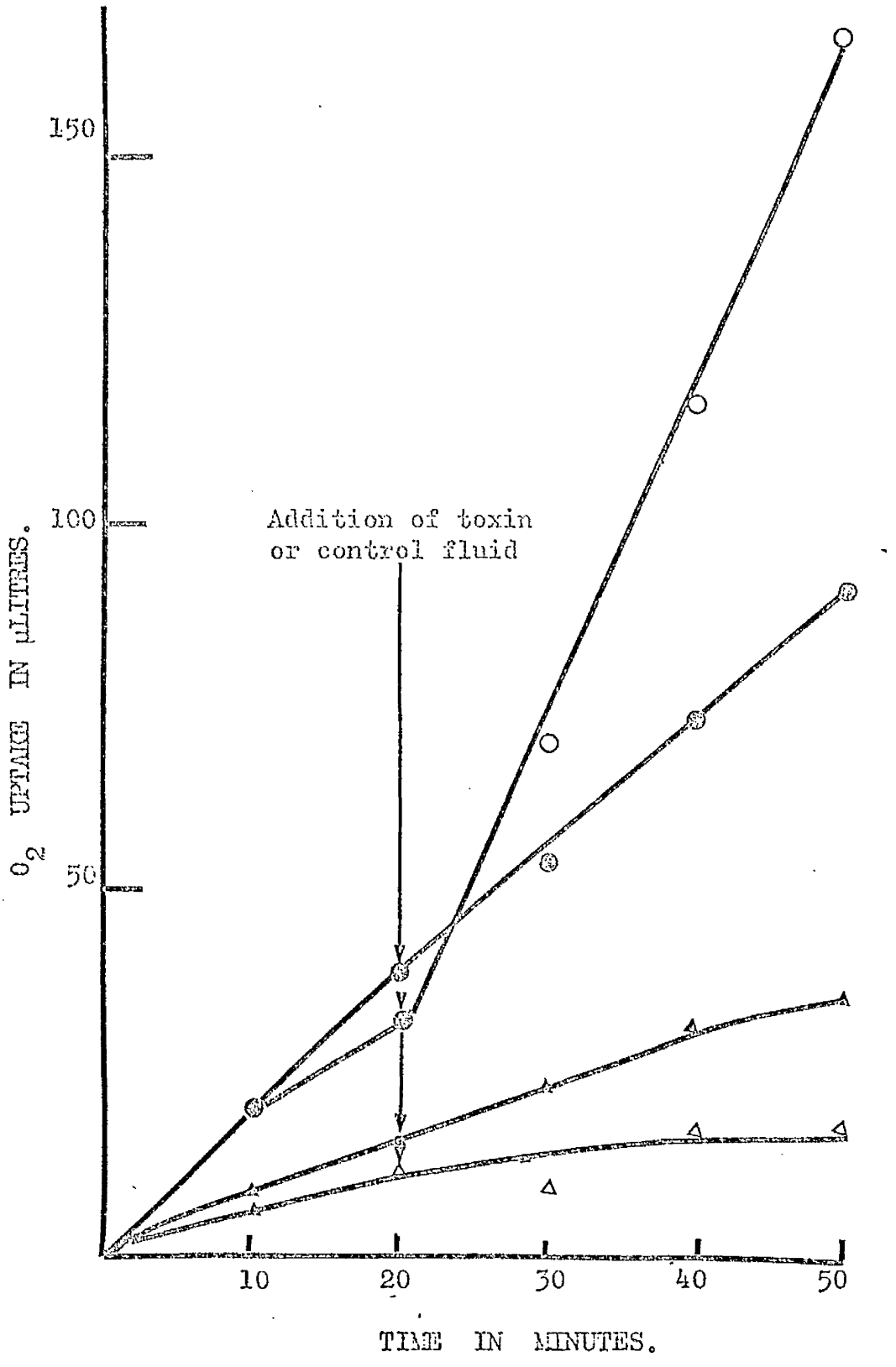

FIG.25. Immediate increase in the rate of oxygen uptake by ascites cells utilising succinate on the addition of staphylococcal alpha-toxin at a final concentration of 133H.U./ml. The endogenous respiration of these cells was inhibited by the toxin.

o — o Toxin-treated cell suspension
● — ● Control cell suspension
△ — △ Endogenous respiration with toxin
▲ — ▲ Control endogenous respiration

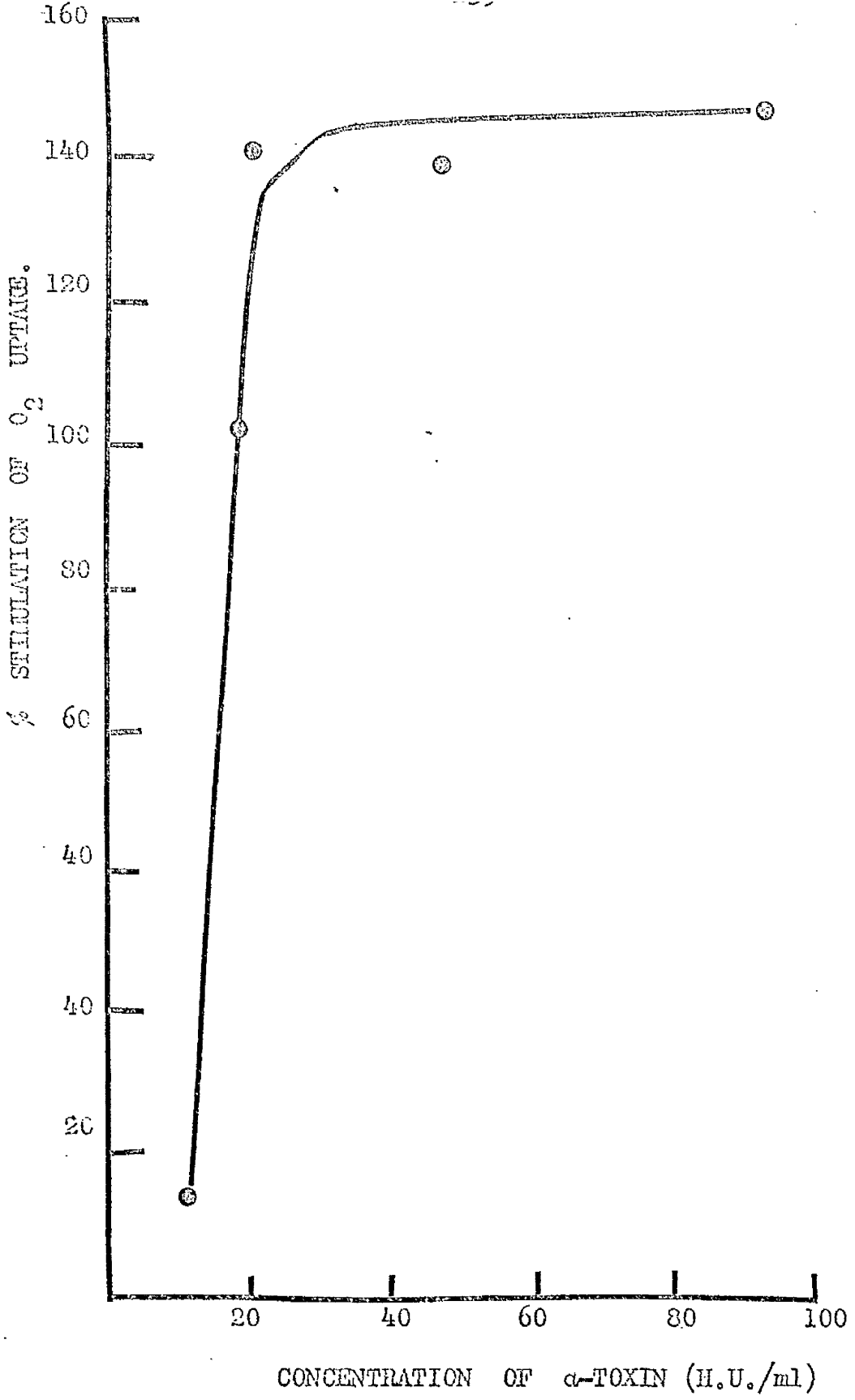

FIG. 26. Effect of the concentration of alpha-toxin present on the stimulation of succinate oxidation by ascites cells. The cells and toxin and cells were incubated together for 30 minutes prior to the addition of succinate.

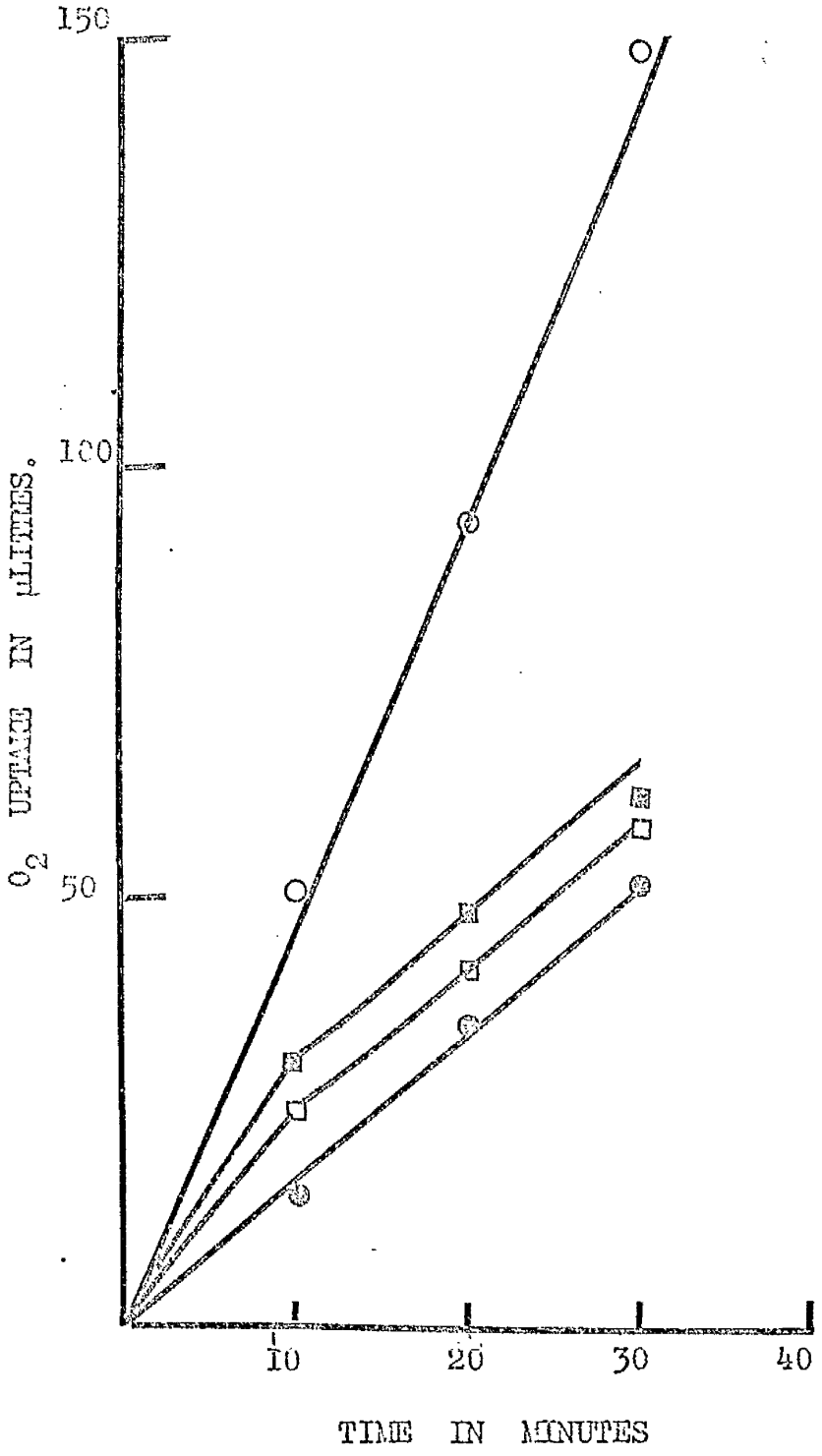

FIG. 27. Neutralisation of staphylococcal alpha-toxin with specific antiserum. The ascites cells were treated with toxin (133 H.U./ml or neutralised toxin for 30 minutes prior to the addition of succinate.

o — o Toxin-treated cell suspension.

o — o Control cell suspension.

□ — □ Cell suspension treated with neutralised toxin

■ — ■ Control cell suspension with antiserum.

had no effect on stimulation. Also staphylococcal delta-toxin was prepared from a strain of Staphylococcus aureus isolated from a case of toxic epidermal necrolysis (Arbuthnott, Gemmell, Kent & Lyell 1969) which produced delta-toxin and no detectable alpha-toxin. Partially purified delta-toxin, prepared by the first stage of the method of Wiseman & Caird (1968), and containing 160 H.U./ml. was used. The addition of this toxin to the Krebs 2 cells did not cause stimulation. These observations would seem to exclude the possibility of the effect being due to small contaminating amounts of delta-toxin in the alpha-toxin preparation and strongly suggests that the ability to stimulate respiration was a specific property of the staphylococcal alpha-toxin.

Interaction between alpha-toxin and Krebs 2 cells also resulted in a loss in the haemolytic activity of toxin preparation, the drop in haemolytic titre with increasing time of contact between cells and toxin being shown in FIG. 28. However, at this point the possibility that alpha-toxin was being inactivated by a product leaking from damaged cells could not be excluded. A crude washed membrane fraction was therefore prepared by ultrasonically disrupting 5×10^8 Krebs 2 cells, removing the cell debris by centrifuging at 2,000 r.p.m. for 10 minutes and harvesting the membrane fraction by centrifuging at 18,000 r.p.m. for 30 minutes followed by washing in PVP/tris buffer. After 30 minutes' interaction between the membrane fraction and 900 H.U. of alpha-toxin, an 80% drop in haemolytic activity was observed. Thus, while enzymes released from damaged cells may well be capable of degrading toxic protein, this experiment suggests that alpha toxin treated cells can bind to the cell membrane with resulting inactivation.

It seems likely that this observed increase in the rate of succinate oxidation by Krebs 2 Ascites Tumour cells following treatment with staphylococcal alpha-toxin may be the result of

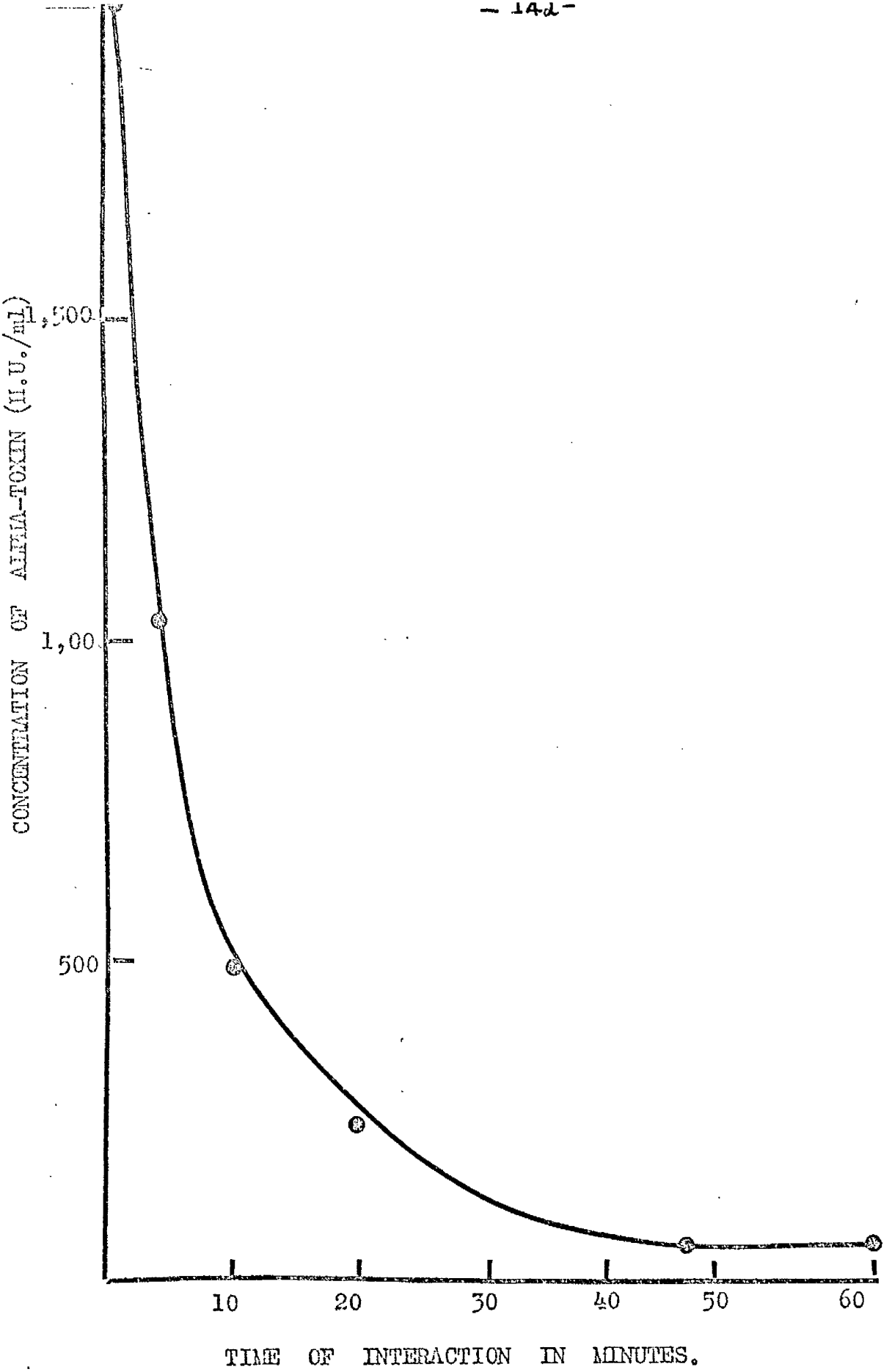

FIG. 28. Inactivation os staphylococcal alpha-toxin by ascites cells. The α-toxin titre of control incubation mixtures in which α-toxin was mixed with PVP/tris buffer showed no drop in haemolytic activity.

extra availability of this substrate to the mitochondria following an increase in the permeability of the cell membrane. Unlike SLS this toxin; would not be expected from previous work of Lominski et al (1964), to have a direct action on the ascites cell mitochondria. Indeed, in contrast to the effect observed with SLS, staphylococcal alpha-toxin did not alter the rate of oxygen uptake of disintegrated cell preparations utilising succinate (FIG. 29).

A study of the comparative effects of SLS and staphylococcal alpha-toxin on the respiration of Krebs 2 Ascites Tumour cells formed the basis of a paper to be published in the near future (Symington and Arbuthnott 1969).

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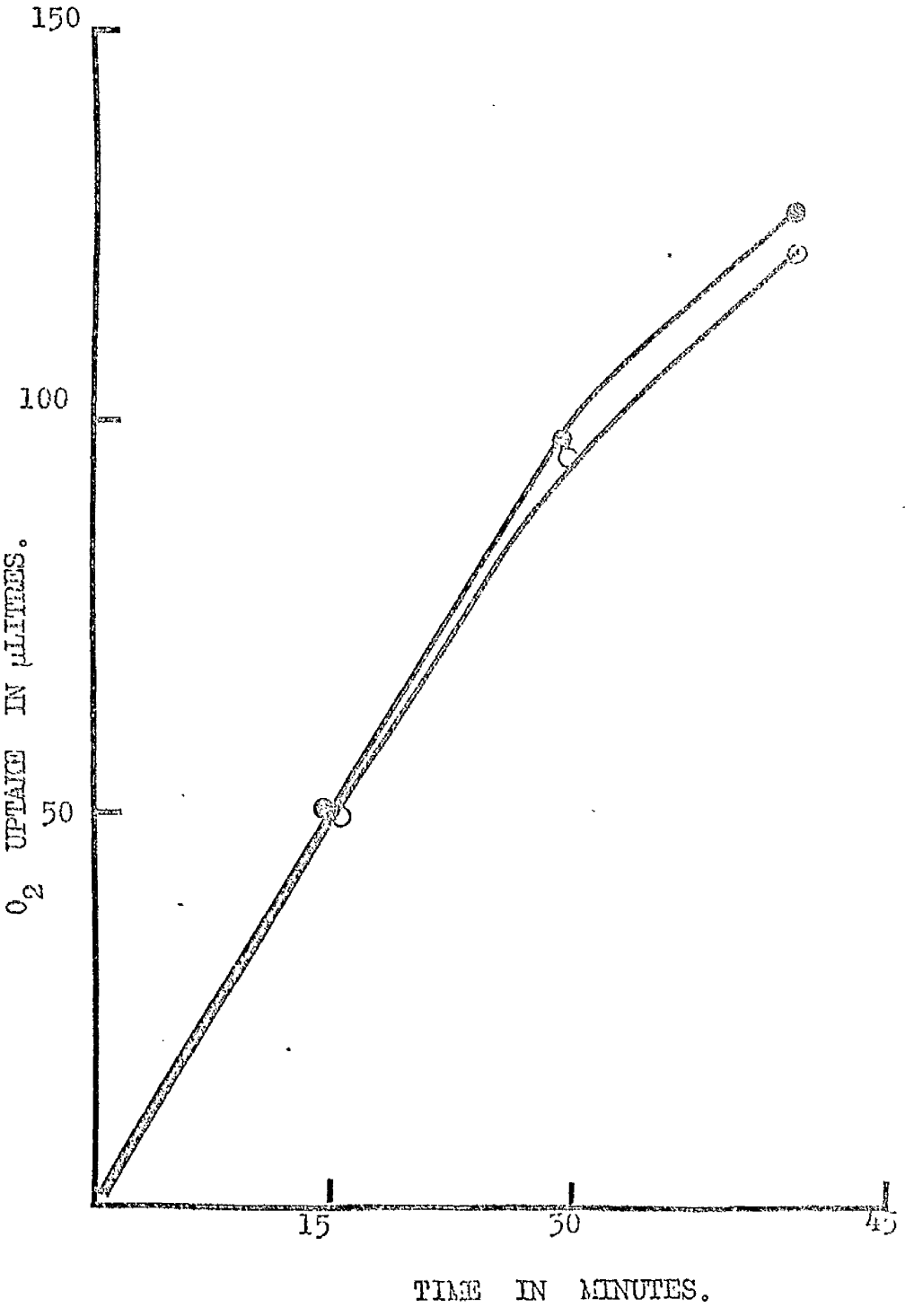

FIG. 29. Effect on oxygen uptake by ultrasonically disintegrated ascites cells following 30 minutes treatment with diphtheria toxin at a final concentration of 416 H.U./ml.

o — o Toxin-treated cell suspension
● — ● Control cell suspension.

DISCUSSION.

The results presented in this Thesis provide useful, new information about certain properties of SLS and also extend the work of other workers. In this discussion I shall attempt to relate my work to that of previous workers and to suggest its contribution to a further understanding of the role of this extracellular toxin in the pathogenesis of Streptococcus pyogenes.

ACTION OF STREPTOLYSIN S ON MITOCHONDRIAL FUNCTION.

The Assay System.

The finding that preparations of SLS impair isolated, mammalian mitochondrial function is novel and shows that the action of this cytolytic agent can result in disruption of an essential cellular function, namely respiration. The observed variation in sensitivity of different mouse liver mitochondrial preparations to the same toxin preparation may result from a number of factors. These might include, variation in sensitivity of individual animals, minor differences in preparation technique from day to day and differences in the metabolic state of the liver. In addition SLS may interact with contaminating amounts of membranous cell debris (e.g. endoplasmic reticulum, lysosomes or cell membrane) which electron micrographs have shown to be present in the mitochondrial preparations used.

The relative differences in sensitivity of the haemolytic and mitochondrial assays of SLS preparations may be due to three factors.

(1.) Differences in the total surface area of membrane present in the two systems.

(2.) Differences in the number and nature of the SLS combining sites in the two systems.

(3.) Intrinsic differences in membrane stability resulting from differences in lipid and protein composition e.g. the striking difference in cholesterol content between erythrocyte and mitochondrial membranes. (Rouser, Nelson, Fleishner & Simon 1968).

Essentially the difficulty in relating different assay procedures for various cytolytic toxins stems from the lack of direct techniques for measuring the interaction between these toxins and their substrates. This necessitates the detection of their action by indirect means e.g. haemolysis represents the end result of a series of events which culminates in the release of haemoglobin. For these reasons results from such indirect assays must be interpreted with caution.

The minimum amount of SLS required to cause impairment of mitochondrial succinic oxidase activity is much higher than that required to cause haemolysis under standard conditions. It is difficult to state an absolute value for the amount of toxin required to impair mitochondrial activity. However, taking into account the majority of experiments, 100 H.U./ml consistently caused impairment. From the data of Koyama and Egami (1963), who concluded that the specific activity of SLS was 2,000,000 H.U./mg, this would indicate that approximately 0.05 µg of toxin is sufficient to cause mitochondrial impairment.

The Identity of the Mitochondrial Damaging Factor in SLS Preparations.

Almost all previous descriptions of the cytolytic effects of SLS have been made using SLS prepared in washed cell suspensions using yeast RNA-core as carrier. Up to the present the use of such preparations has been justified on the basis that the preparation method is fairly specific for SLS and only one other streptococcal product, namely DNA-ase, has been detected in these preparations. However the possibility that other as yet unidentified products may be present has been recognised.

"There do not exist satisfactory methods for obtaining SLS in pure form, and some of the biological effects ascribed to it may prove to be due to other products of streptococcal growth." (Bernheimer 1969). In one instance the cytolytic effect of highly purified RNA-SLS has been examined (Taketo & Taketo 1966) and the purified product was both haemolytic and cytotoxic for Ehrlich Ascites Tumour cells.

In the present work, using St. pyogenes strain C203S and RNA-core for production, all preparations of toxin which were inhibitory for mitochondria were also haemolytic. Moreover the SLS-deficient mutant C203U kindly supplied by Dr. Alan Bernheimer, when cultured under identical conditions, failed to impair mitochondrial respiration. Also no evidence was obtained that contaminating amounts of DNA-ase were responsible for inhibition. On the other hand, it appeared that when carriers other than RNA-core (albumin, serum, Tween 40 and 80) were used, the ratio of haemolytic to mitochondrial inhibiting activity was not constant.

On the basis of the above results, it was considered that the two activities were probably manifestations of the SLS moiety or a closely related product. The fact that the haemolytic and mitochondrial damaging activities of SLS preparations from various carriers differed from RNA-SLS and from one another was not altogether unexpected. It is conceivable that the nature of the carrier substance attached to the SLS polypeptide chain might well influence the affinity of the polypeptide for membranes of different composition.

In order to investigate further the relationship between the haemolytic and mitochondrial damaging activity of SLS, toxin preparations were subjected to gel filtration on G-75 Sephadex. Comparison of the elution pattern in FIG 15 with that of Bernheimer shown in FIG 30 reveals a marked difference between the two preparations. This dissimilarity probably is due to differences in the oligoribonucleotides present in the RNA-core used for toxin preparation. The resistant "core" resulting from the action of RNA-ase on yeast RNA may not be homogenous and it seems likely that perhaps only the terminal base sequence of a particular nucleotide would determine its suitability to act as a carrier for the toxic polypeptide.

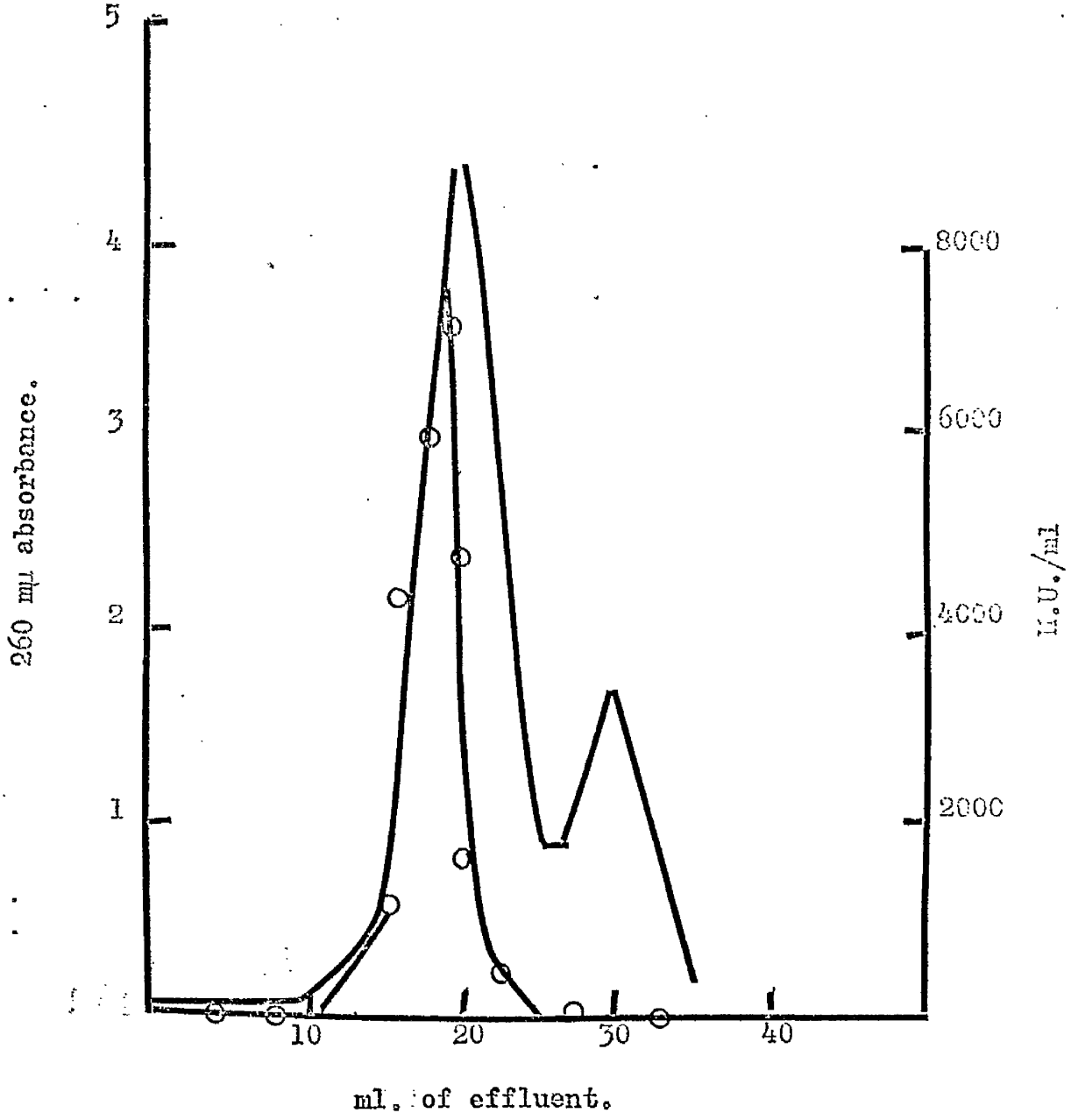

FIG. 30. Distribution of SLS hemolytic activity (o — o) and absorption of light at 260 mμ ($\overset{A}{—}$) in Sephadex G-75 effluent. From Bernheimer (1967) J.Bact 93, 2024.

The most important finding using G-75 Sephadex was that both the haemolytic and mitochondrial damaging activities were located in the same fractions and that the fractions having high haemolytic activity were also the most active in impairing mitochondrial succinic oxidase activity. Considerably better resolution of RNA-SLS from the residual inactive oligoribonucleotide was achieved using G-50 Sephadex. Again the two biological activities ran parallel to each other. On the basis of gel-filtration, therefore, it appeared that the haemolytic and mitochondrial damaging activities were either identical or had very similar molecular weights. Very recently, since the writing of this Thesis was begun, Taylor (1969) has reported the isolation of a lymphocyte transforming factor from RNA-SLS preparations by DEAE cellulose chromatography. This factor was distinct from the component responsible for haemolysis and release of lysosomal enzymes. It is intended to apply this method of Taylor to further investigate the identity of the haemolytic and mitochondrial damaging activities of SLS.

Collateral attempts were also made to selectively absorb or inhibit either the haemolytic or mitochondrial damaging activities of SLS preparations. From the work of Koyama (1965) and Elias, Heller and Ginsburg (1966) SLS was known to bind irreversibly to erythrocyte ghosts with a complete loss of haemolytic activity. I have found that both toxic activities were abolished by mixing SLS with erythrocyte ghosts or with mitochondria. This finding again supports the idea that the haemolytic and mitochondrial damaging activities are properties of the same or closely related moieties. It also indicates that the toxic agent is either bound to a receptor in membranes or that interaction with membranes results in configurational changes and inactivation.

The two biological activities of SLS preparations also ran parallel in heating experiments showing similar sensitivity to inactivation at 60°C. Preliminary results obtained employing trypsin and chymotrypsin to digest SLS (not included in the results

section) suggested that toxic activity against both erythrocytes and mitochondria, was destroyed by chymotrypsin but not by trypsin.

Dissociation of haemolytic and mitochondrial damaging activity was achieved in inhibition experiments using lecithin and trypan blue. Both are well known inhibitors of SLS (page 38). Indeed it has been suggested that the former, which is a principle constituent of mammalian cell membranes, is, in fact, the receptor for SLS. In both cases these substances caused inhibition of haemolytic activity but not of mitochondrial damaging activity of SLS preparations. The intrinsic differences between the haemolytic and mitochondrial assay systems emphasise the danger of placing too great an importance on these results. For instance, the interaction between SLS and either lecithin or trypan blue might well be influenced by the presence of mitochondria. However, accepting that the results do indicate selective inhibition against haemolytic damaging activity, two explanations can be put forward:-

(1.) That the haemolytic and mitochondrial damaging activities of SLS preparations are indeed due to distinct toxic products and that the results obtained with the mutant C203U, by gel filtration, heating experiments and enzymic digestion reflect the difficulties in resolving two closely related activities.

(2.) That the two biological activities reside in a single moiety having two active sites, one site being responsible for haemolysis and the other for impairment of mitochondrial succinic oxidase activity. These sites differ in their sensitivity to lecithin and trypan blue.

Not enough evidence exists at present to distinguish between these two possibilities. In my view, the work of this Thesis taken overall, supports the identity of the haemolytic and mitochondrial damaging factors and that these are properties of an

entity known as RNA-SLS. RNA-SLS, as prepared by most workers, is itself probably a heterogenous population of molecular species containing a characteristic polypeptide linked to oligoribonucleotides of varying length and base composition. The final resolution of this complex problem awaits the application of refined methods of separation allied to a critical evaluation of AJL the reported cytolytic activities of this important toxin. Thus far, such an investigation has not been carried out.

The Mode of Action of SLS on Mitochondria.

Having examined the general features of the impairment of mitochondrial succinic oxidase activity by SLS, it was considered necessary to extend the work and attempt to characterise, as far as possible, the nature of the damage to mitochondrial respiration. By locating the region of the electron transport chain which was damaged by SLS, it was hoped to gain more information about the mode of action of SLS within the mitochondrion.

The results of assaying different regions of the succinic oxidase chain, namely succinic dehydrogenase and cytochrome oxidase (FIG 10) showed that the former was resistant to SLS attack while cytochrome oxidase activity was impaired. These prelimanary observations clearly indicated that SLS caused functional impairment of respiration within the cytochrome oxidase portion of the electron transport system, i.e. between cytochrome c and O_2. However, the observation that, on average, the impairment of cytochrome oxidase, as assayed using paraphenylene diamine (PPHDI), was 30% less than that caused by the same toxin on the succinic oxidase (whole chain) activity of the same mitochondrial preparation remained to be explained. This observed difference in impairment could be interpreted in two ways:-

(1.) Differences in the efficiences of electron transfer at the cytochrome c locus of the intact, whole-chain, succinic oxidase and at the PPHDI-cytochrome c link of the cytochrome oxidase chain could explain this finding.

(2.) Alternatively the possibility existed that the whole chain succinic oxidase was being affected at more than one point.

"Restoration" and "protection" experiments using the two mobile components of the electron transport chain, ubiquinone and cytochrome c, clarified this problem. The results in FIGS 17 a and b and TABLES 23 and 24 show that impairment of both succinic and cytochrome oxidases can be completely restored by the addition of exogenous cytochrome c; no appearance of toxin impairment remained after the addition of cytochrome c. Conclusive evidence has, therefore been obtained which indicates that SLS causes the dislocation of the electron transport chain at the cytochrome c locus. It is also important to note that treatment of mitochondria with SLS resulted in the leakage of protein including a product having an absorption maximum at 410 mμ; this was probably cytochrome c. The fact that cytochrome c itself was not the substrate for SLS was shown by treating cytochrome c with SLS for 30 minutes, such material was still capable of restoring toxin impaired mitochondrial activity. It must be emphasised that these experiments pin-point the secondary functional lesion within the mitochondrion, resulting from damage by SLS but do not provide evidence for the primary site of action.

Cytochrome c is a prism shaped molecule having a molecular weight of 1200 (Levin 1962). It readily combines with lipids and phospholipids, forming complexes known as lipocytochrome c which are soluble in lipid solvents. In the mitochondrion, cytochrome c acts as an oxidation-reduction component in the electron transfer process but is not a permanent constituent of any of the four complexes (TABLE 6). It is thought to be present as a mobile molecule in a lipid milieu shuttling electrons between complexes III and IV (Widmer & Crane 1958; Ambe & Crane 1959). Therefore, exogenous cytochrome c may either provide a by-pass around the SLS-affected areas or replace cytochrome c leaked from the mitochondria as a

result of altered permeability. The presumptive identity of cytochrome c in the leaked products from toxin-treated mitochondria suggests that restoration of electron transport function by the addition of exogenous cytochrome c could be accounted for in part, at least, by a re-equilibration of the molar balance of respiratory carriers within the mitochondrion. However the fact that the addition of cytochrome c stimulates the cytochrome oxidase activity of toxin-impaired mitochondria to a level up to 50% above that of controls suggests that a by-pass mechanism utilising the added exogenous cytochrome c was also operating. Both mechanisms may therefore contribute to the observed effect of adding exogenous cytochrome c. Whatever the mechanism, it seems highly unlikely that the addition of exogenous cytochrome c reverses the action of SLS on the mitochondrion. It is far more likely that the presence of cytochrome c masks the secondary effects which are being measured in the assay system used.

The complete resistance of actively respiring mitochondria to toxin attack, suggests either that there is a configurational change in the mitochondrial membrane during electron transport which involves the masking of the SLS binding sites, or that when the respiratory chain is functioning, cytochrome c is more tightly bound. Furthermore, the oxidation reduction state of cytochrome c could also influence its leakage from the mitochondrion.

The results obtained thus far provide evidence of the locus of the electron transport chain affected by SLS but do not conclusively indicate whether the primary action of the toxin is on the mitochondrial limiting membrane or on the internal spatial arrangement of essential components in the cristae. As will be mentioned later this problem may be clarified by studying the effect of SLS on isolated fragments of the respiratory chain. A diagramatic representation of the possible changes resulting from the action of SLS on mitochondria are illustrated in FIG 31.

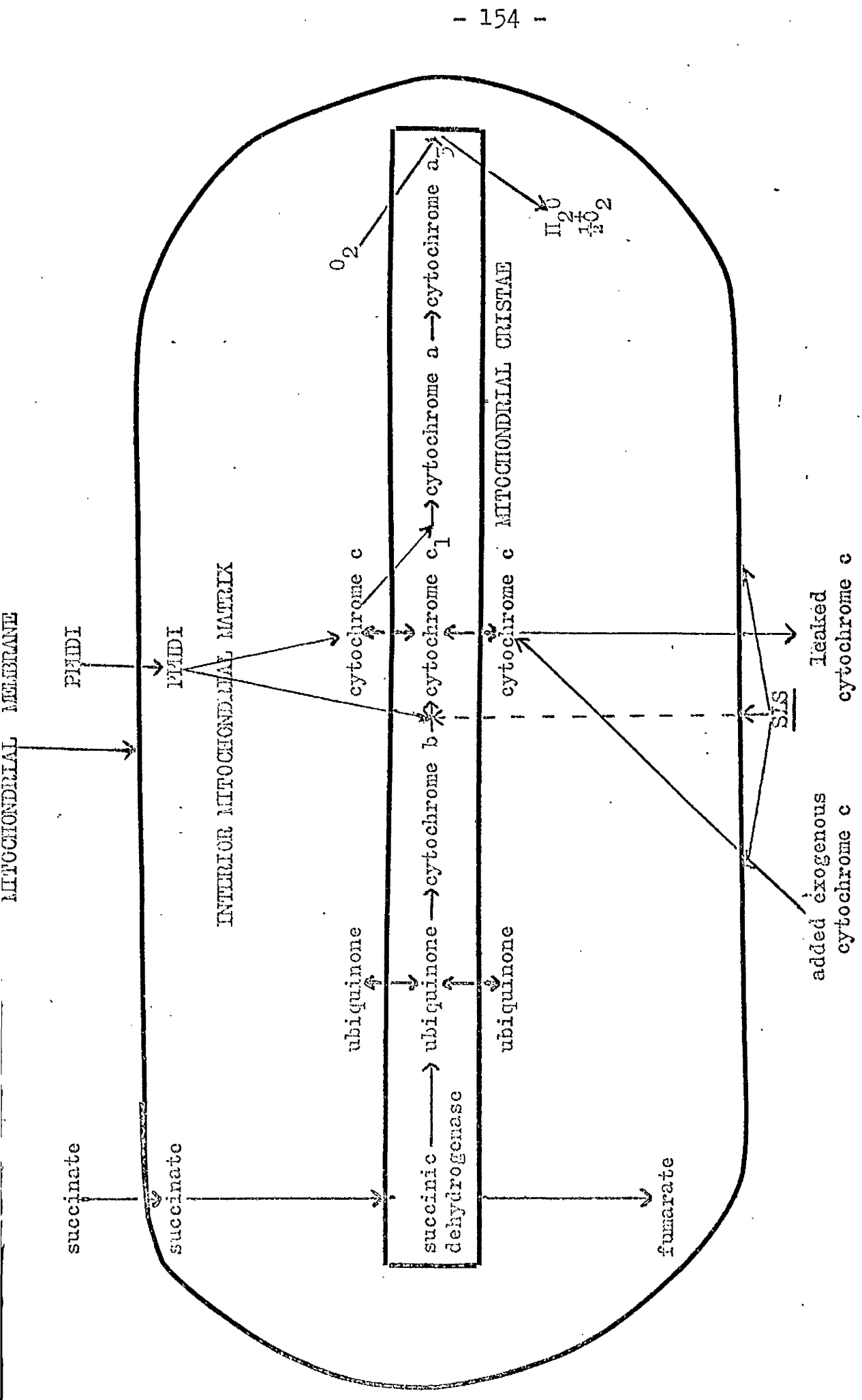

FIG 31. Diagramatic Summary of the Mode of Action of Streptolysin S.

General Significance to Previous Work.

The finding that SLS impairs mitochondrial respiration represents a significant extension of the work of Keiser et al (1964). These authors found that SLS caused swelling and release of protein which suggests that the toxin causes a certain degree of internal disorganisation within the mitochondrion. As mentioned previously (page 56) any disturbance in the delicate spatial arrangement of the electron transport carriers might be expected to result in impairment of respiratory function.

Initially, in the course of the present work, I failed to detect mitochondrial swelling; this was probably due to the high concentration of magnesium ions present (2×10^{-2} M) in the undialysed preparations of toxin which were used. When dialysed toxin was used, swelling similar to that described by Keiser et al (1964) was observed (FIG 32). The fact that very brief contact between toxin and mitochondria (page 87) resulted in a stimulation in the oxygen uptake by mitochondria rather that impairment might be explained by increased permeability to succinate. It is significant to note that the maximum rate of swelling took place during the first five minutes of contact and it seems feasible that this could result in an influx of exogenous substrate into the mitochondrion.

The inhibition of oxygen uptake which followed longer periods of incubation could logically follow if there was a subsequent leakage of essential electron transport carriers to osmotically balance the initial influx of exogenous material. Furthermore, the eventual penetration of SLS to the interior of the mitochondrion with resultant structural disorganisation of the cristae cannot be excluded.

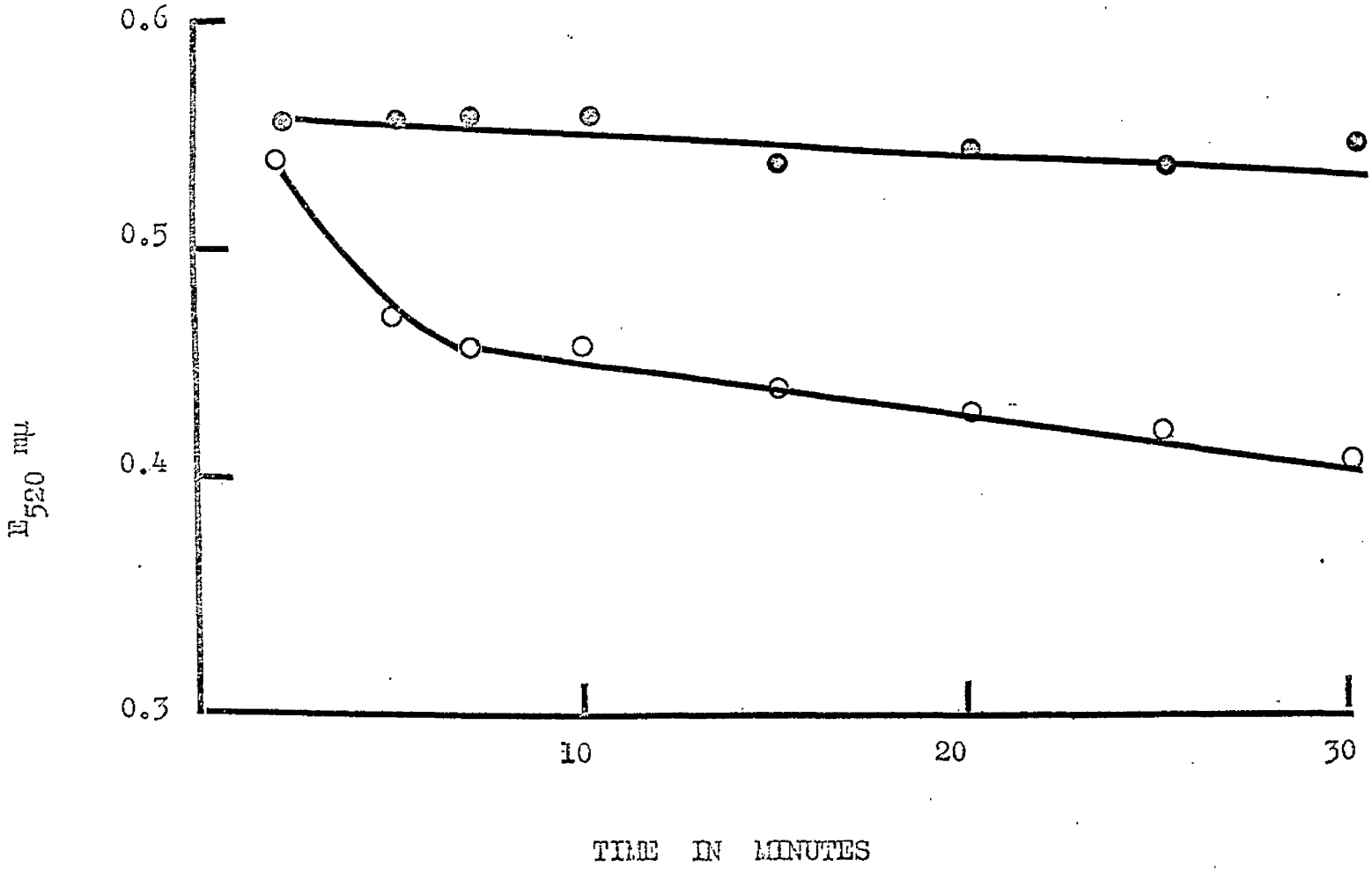

FIG. 32. Swelling of mitochondria treated with dialysed RNA-SLS (41 H.U./ml)

⊙—⊙ Control mitochondrial suspension.
○—○ Toxin-treated mitochondrial suspension.

Certain other bacterial extracellular toxins have also been shown to impair mitochondrial function, e.g. Clostridium welchii alpha-toxin (Edwards & Ball 1954; McFarlane & Datta 1954), staphylococcal succinic oxidase factor (Lominski et al 1964) and Pasteurella pestis murine toxin (Ajl, Woebke and Ruste 1964). With plague murine toxin, it was demonstrated that the species specificity of this agent existed at the sub-cellular level. By studying the effects of this toxin on the absorption spectrum of mitochondria to see if any alterations in the absorption peaks corresponding to specific cytochrome components were revealed, Ajl's group showed that the addition of toxin caused the oxidation of cytochrome a, b and c. This finding suggested that the toxin exerted its inhibitory effect on mitochondrial respiration by acting on the electron transport chain in the region between $NADH_2$ or succinate and cytochrome b.

Further investigation of the mode of action of this toxin have been made using isolated enzyme complexes derived from beef heart mitochondria by members of D.E. Green's laboratory, Wisconsin U.S.A. and Kadis, Monti and Ajl (1966) were able to show that, when plague murine toxin was incubated with purified $NADH_2$ - cytochrome c reductase, significant inhibition of activity was obtained. Similarly $NADH_2$ - ubiquinone reductase is also inhibited by this toxin (Hatefi, Haavik & Griffiths 1962). Although the precise manner in which the toxin inhibits $NADH_2$ - ubiquinone reductase has not yet been elucidated, it is provisionally suggested that plague murine toxin acts on the electron transport system by interfering with the enzymic activity of $NADH_2$-ubiquinone reductase; this prevents the reduction of ubiquinone and therefore impairs an essential pre-requisite for electron transport in the mitochondrion.

This approach, which has yielded important results with respect to plague murine toxin, could provide the answer as to whether or not SLS acts directly on the enzymic activity of QH_2 - cytochrome c reductase complex (111) or the cytochrome oxidase complex (1V), and may also give insight into the mode of action of various bacterial

toxins at the subcellular level. The use of toxins in this way may also elucidate some of the complex mechanisms occurring within the mitochondrion. Biochemists may find bacterial toxins useful tools in the study of mitochondrial membrane structure, which, in turn, may help to elucidate the role of the membrane in respiratory function.

This study has added to the understanding of the changes which occur in mitochondria as a result of SLS attack; a discussion of the implications of this finding in terms of the pathogenicity of Group A streptococci will be presented later (page 171). It has however, emerged that the measurement of secondary changes is of limited value. Further understanding of toxin-membrane interactions remains absolutely essential if the cytolytic mechanism of SLS is to be fully understood.

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ACTION OF STREPTOLYSIN S ON CELLULAR METABOLISM.

As mentioned in the introduction (page 45), the object of this aspect was to determine the metabolic changes which occur within the cell as a result of the cytotoxic action of SLS. It was hoped that such investigations might throw some light on the role of this toxin in the pathogenesis of the streptococcus. The reasons for choosing Krebs 2 Ascites Tumour cells as a model for this study are explained on page 58 . It was also felt that these studies might contribute to a further understanding of the oncolytic activity of Group A streptococci and the role of SLS in this.

Morphological Changes caused by SLS.

Phase contrast microscopy of Krebs 2 Ascites Tumour cells showed that gross disorganisation occurred in these cells following treatment with SLS; many of the observed changes could be explained by membrane damage. The appearance of numerous blebs round the edge of SLS-treated cells indicated that the cell surface and probably the cell membrane was dramatically affected. Cell damage was also characterised by the appearance of gross cytoplasmic disorganisation and nuclear irregularity. In view of the fact that such cytopathic effects occurred without detectable absorption of SLS, it seemed likely that these were secondary results of disorganisation of the limiting membrane.

Effect of SLS on Succinate Oxidation.

To my knowledge the findings relating to the effects of SLS and staphylococcal alpha-toxin on succinate oxidation are novel and contribute useful knowledge about the cytolytic effects of these toxins.

The increased rate of oxygen uptake shown by SLS-treated Krebs 2 cells utilising exogenous succinate suggested that, despite the apparent extensive cellular damage, the mitochondria of such cells remained functional. The simplest explanation of the increased

rate of oxygen uptake was that SLS caused an alteration in the permeability of the cell membrane which in turn removed a permeability barrier to succinate; the resulting influx of succinate would lead to a substantial increase in the intracellular succinate concentration with consequent increased availability of substrate to the still functioning mitochondria. Such an explanation probably represents an oversimplification but serves as a useful working hypothesis.

Effect of Staphylococcal alpha-toxin on Succinate Oxidation.

Staphylococcal alpha-toxin has been shown by Kadoff et al (1962) to alter the permeability of Ehrlich Ascites Tumour cells. Also it is known from the work of Lominski et al (1964 & 1968) that staphylococcal alpha-toxin does not damage mitochondrial function. This toxin, therefore, provided a valuable comparative tool for testing the effect of altered cell permeability on succinate oxidation. The appearance of blebs on ascites cells was even more marked following alpha-toxin treatment; this further indicated that the cell surface was substantially affected by alpha-toxin. The clear-cut finding that treatment of Krebs 2 Ascites Tumour cells with this cytolytic agent also resulted in increased rate of oxygen uptake provided further evidence for the hypothesis that the observed effects were secondary results of toxin-membrane interactions resulting from the breakdown of normal permeability. Work is currently underway in collaboration with Dr. P.G. Toner to examine the effects of both SLS and staphylococcal alpha-toxin on the fine structure of Krebs 2 cells using electron microscopy.

Comparative Effects of SLS and Staphylococcal alpha-toxin.

Predictably, although the overall effect on the metabolism of succinate by Krebs 2 cells was the same following treatment with SLS or staphylococcal alpha-toxin, several differences were noted which indicated that the site and mode of action of these two cytolytic agents on the cell membrane differed. Staphylococcal

alpha-toxin acted much more rapidly than SLS, causing an immediate increase in the rate of oxygen uptake even in cells which were metabolising exogenous succinate. It is worth stressing here that both the limiting cell and mitochondrial membranes appear to be resistant to SLS-attack if the cell or organelle is actively respiring.

Comparatively, Krebs 2 Ascites Tumour cells are less sensitive to staphylococcal alpha-toxin than to SLS; 200 H.U. of SLS (=0.025 µg of toxin) caused stimulation, whereas 20 H.U. (=2 µg) of alpha-toxin were required to cause increased oxygen uptake. Furthermore, the interaction between staphylococcal alpha-toxin and Krebs 2 cells involved binding or inactivation of the toxic protein while the haemolytic activity of SLS was not altered in this way.

Effect of SLS on Isolated Ascites Cell Mitochondria.

Although SLS did not appear to penetrate sufficiently to cause impairment of mitochondrial function in the whole-cell model system, isolated mitochondria prepared from such cells resembled liver mitochondria in that their succinic oxidase activity was impaired by SLS. These experiments reinforce the view that SLS fails to penetrate to the interior of intact Krebs 2 cells. Alternatively the failure of SLS to impair mitochondrial function in the whole cells might be explained if these organelles were under cellular control e.g. if they were actively metabolising endogenous substrate. As has been shown previously in this Thesis, actively metabolising liver mitochondria are resistant to SLS attack.

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Any alteration in the permeability of the ascites cell membrane as a result of the action of SLS, as well as increasing the permeability to exogenous materials, would also result in diffusion out of intracellular constituents. If this included leakage of soluble, cytoplasmic enzymes from the cell, an upset in the delicate balance required for the operation of certain metabolic pathways such as the Embden-Meyerhoff Pathway would occur. This might be

reflected in the rate of metabolism of intermediates of this pathway. Indeed, staphylococcal alpha-toxin, acting on KB - tissue culture cells, has been shown to cause a decrease in glucose utilisation and a corresponding drop in lactic acid production (Korbeck & Jeljaszewicz 1964).

Effect of SLS on Glucose Oxidation.

Although not presented in the results, some experiments were carried out in which the effect of SLS on the metabolism of glucose was studied. These proved inconclusive for reasons discussed below but are considered to be relevant to this aspect, and the main findings will therefore be mentioned briefly at this point.

Ascites cells are known to metabolise glucose by means of the Embden-Meyerhoff Pathway, the Tri-carboxylic cycle and the Pentose-Phosphate cycle. Most of the enzymes catalysing the various steps of these pathways are soluble in the cytoplasm. It was, therefore, thought that the action of SLS or staphylococcal alpha-toxin would result in a decrease of glucose utilisation by these cells. In preliminary experiments carried out as described on page 75 of the Materials and Methods, results indicated that both SLS and staphylococcal alpha-toxin immediately inhibited any glucose oxidation by these cells and any measurable endogenous metabolism was also immediately halted by the addition of either of these toxins. Considerable technical difficulties were encountered in the use of this assay system. For instance when exogenous glucose was added as substrate to control cell suspensions in Warburg experiments, an appreciable increase in oxygen uptake above the endogenous level was not consistently observed. Such inconsistent results, using glucose as substrate for tumour cells have been encountered by many workers and several possible explanations exist to account for this. The most important is probably the Crabtree Effect (Crabtree 1929). In this phenomenon, there is inhibition of respiration following the addition of glucose to many types of glucose metabolising cells. It is now recognised as a generalised effect and has been reviewed by Ibsen (1961) who made specific reference to the phenomenon in Ehrlich Ascites Tumour cells.

This is summarised as follows :-

After the addition of glucose to cells a short-lived (20 to 120 seconds) stimulation of respiration is followed by an inhibitory period which reaches a constant level equal to approximately 30% of the endogenous rate and lasts until all the glucose has been utilised. About 25 seconds after the glucose disappears the cells are released from such inhibition and consume oxygen at a rate which can be as great as the pre-inhibitory endogenous respiration rate.

These observations were made using an oxygen electrode. In my experiments the Crabtree Effect would be complete before the first manometric reading was taken at 15 minutes. Thus it is extremely difficult to interpret the contribution of this phenomenon to the overall picture which was observed. But it is important to note that in 5 out of 7 experiments carried out using glucose as substrate, increased oxygen uptake above the endogenous level was observed.

Clearly it would be necessary to measure glucose utilisation more directly in order to obtain conclusive results. Since the experiments mentioned here were carried out during the terminal stages of the work for this Thesis, it is hoped to extend them further in the near future using alternative assay systems. Despite these limitations significant indication that SLS and staphylococcal alpha-toxin do cause disruption of cytoplasmic enzymic function does emerge from the findings that (a.) both toxins immediately impaired respiration in those experiments which increased oxygen uptake occurred on the addition of glucose and (b.) that the endogenous oxygen uptake by Krebs 2 Ascites Tumour cells was abolished.

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Extrapolation from the work with SLS preparations on Krebs 2 Ascites Tumour cells to the possible effects of this toxin during infection in vivo, must remain speculative at this stage. However, it emerges that SLS is a potent cytolytic toxin capable of disrupting the normal permeability control of cells at low concentrations. Also this agent has the capacity of profoundly impairing the

fundamental energy producing system of the cell namely the mitochondrion. Any agent having such properties would be expected to play an important role in damaging the cells and tissues of the host. Admittedly, doubts exist as to whether SLS can actually penetrate into the cell sufficiently to paralyse mitochondrial function. It must be emphasised however, that in pathogenesis SLS may act synergistically with other streptococcal products and in those cases where streptococci succeed in penetrating the cell interior, SLS elaborated intracellularly would almost certainly exert its toxic action on mitochondria.

MODE OF ACTION OF STREPTOLYSIN S.

The observations of the effect of SLS and staphylococcal alpha-toxin on cellular metabolism are in agreement with the cell membrane being the point of attack of these two cytolytic agents. Their action upsets the permeability of the cell membrane which results, among other changes, in a fatal disruption of metabolism.

The limitations mentioned previously, of having to measure the action of cytolytic toxins indirectly makes it difficult to assess the order in which the observed changes occur. They also severely limit the detailed study of toxin membrane-interaction. However, certain results indicate that the action of SLS on the cell and mitochondrial membrane is fundamentally different.

The active polypeptide portion of the SLS molecule consists of only about 28 amino acids, yet this molecule is capable of disrupting the cellular membranes of a wide variety of cell types. This suggests that there is a common receptor for SLS on all sensitive cell membranes. On account of differences in sensitivity and conditions of assay systems, a direct comparison of the affinity of different cell species for RNA-SLS seems rather difficult. The inhibition of the haemolytic and cytolytic activity by lecithin and other phospholipids has raised the possibility that these compounds may

constitute the membrane binding site for SLS. At this point, however, it should be pointed out that in the opinion of the author, it is unlikely that lecithin alone represents this universal substrate since its absence from the membranes of bacteria is well documented; yet bacterial protoplasts have been reported to be lysed by SLS. Elias et al (1966) found that SLS was also inhibited by amounts of cholesterol equivalent to those present in the erythrocyte membrane and suggested that the erythrocyte membrane SLS-binding site is a combination of cholesterol and phospholipid. Bernheimer (1969) reported that he was unable to repeat the cholesterol inhibition of SLS. In agreement with Elias et al (1966) and Taketo and Taketo (1966), I have found that the affinity of Ascites Tumour cells for RNA-SLS, as measured by absorption of haemolytic activity, seems to be considerably less than that of erythrocytes. Furthermore, the mitochondrial membrane SLS-binding site may differ from than on either erythrocytes or Krebs 2 Ascites Tumour cells since lecithin did not interfere with the toxin mitochondrion interaction. Taketo and Taketo have suggested that the differences in affinity of ascites cells and erythrocytes for RNA-SLS may be due to the amount of receptor phospholipids present in the two membranes or to the presence of some unknown masking substance (s).

Clearly there is much to be gained by studying the effect of this haemolysin on various membrane systems, but such a study will be of limited value until there is a direct assay system available for measuring the toxin-membrane interactions. At present the artificial lipid spherules used by Bangham, Standish and Dawson (1965) and Freer, Arbuthnott and Bernheimer (1968) to study toxin-membrane interactions probably constitue one of the most useful model systems because their constituent bilayers have certain physical properties which closely resemble those of cell membranes. A knowledge of the mechanism of spherule lysis by cytolytic toxins may provide useful information concerning in vivo activity of these agents. Moreover, an understanding of the interaction between toxic proteins and orientated lipid may be relevant to the general problem of protein-lipid interactions involved in biological

membranes. Knowledge of this aspect is, in turn, dependent on advances in knowledge of the detailed structure of all biological membranes which is at present, highly controversial and lacking in agreement.

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ONCOLYTIC ACTIVITY OF HAEMOLYTIC STREPTOCOCCI:- THE ROLE OF STREPTOLYSIN S.

In 1868 Busch attempted to treat a sarcoma patient by deliberately inducing an attack of erysipelas and found a rapid decrease in the size of the tumour. Coley (1891) also reported that an attack of erysipelas in an inoperable sarcoma patient caused complete disappearance of the tumour. Since these early "clinico-experiments" a great deal of work has been carried out throughout the world to investigate the anticancer (oncolytic) activity of haemolytic streptococci.

Up to the present time twelve different types of transplantable animal tumours including carcinomas, sarcomas, leukaemias and ascites hepatomas have been reported to be sensitive to living haemolytic streptococci. No other bacterial species has been demonstrated to possess this anticancer activity. Not even all species of haemolytic streptococci have this ability. Only strains which can synthesise SLS act on a wide range of transplantable animal tumours. (Koshimra , Nishida, Bando, Shoin, Minami and Kadono 1965).

Detailed studies of the cytolytic action of SLS-forming streptococci on Ehrlich Ascites cells have been reported in a comprehensive series of thirty one papers by Okamoto and his co-workers in Japan. Their findings are summarised in a review article by Okamoto, Shoin, Koshimura and Shimizu (1967). Although these workers showed that only streptococci which were capable of synthesising SLS had oncolytic activity and that brief incubation of

a suspended mixture of washed, live, haemolytic streptococci and Ehrlich Ascites cells in phosphate buffered Ringers solution at 37°C resulted in the production of SLS (Koshimura, Shimizu, Nasusaki, Ohta and Kishi 1958), they reported that pre-formed SLS was without obvious effect on the cancer cells (Okamoto, Koshimura, Shoin, Hirata, Murasawa, Bando & Shimizu 1958). This finding is in complete contrast to results reported by Ginsburg (1959), Ginsburg and Grossowicz (1960) and Taketo and Taketo (1966) and those reported in this Thesis, that SLS is highly toxic for Ehrlich Ascites cells and Krebs 2 Ascites cells. The reported antitumour activity of Streptococcus pyogenes and some of its products are summarised in TABLE 27.

There are striking similarities between the reported effects of living haemolytic streptococci and SLS on cancer cells. The phase contrast micrographs in this Thesis illustrating the visible cytopathic changes resulting from the action of SLS on Krebs 2 Ascites cells show very similar changes to those in the paper of Havas et al (1963), illustrating the action of the live organisms on these cells. Shimizu, Nishida, Bando, Koshimura, Hayshi and Kobayishi (1964) demonstrated the release of nucleic acid material from Ehrlich Ascites cells incubated with streptococci, similar leakage was reported following SLS treatment of such cells by Taketo and Taketo (1966).

These results suggest that the SLS moiety is involved in the anticancer activity of haemolytic streptococci. The Japanese workers hold that SLS exerts its effect while in the intracellular form (i.e. ICH-S see page 36) and have proposed that SLS may be formed by a secondary process during interaction between the live streptococci and the RNA which is released together with other intracellular constituents from destroyed tumour cells. In early experiments these workers added penicillin to their streptococci/ tumour cell mixtures, prior to inoculation in order to prevent the mice dying from streptococcal septicaemia. In more recent

TABLE 27.

Antitumour Activity of Streptococcus pyogenes and its Products.

Materials Tested.	Antitumour Effect.
Living haemolytic streptococci virulent	+
" " " avirulent	+
Cell-free extracts (acetone powder) of cocci	+
Phage - lysate of cocci	+
Heat - killed cocci	-
Formalin killed cocci	-
Culture supernatant	-
SLS preparations	±
SLO preparations	±
Varidase (streptodornase + streptokinase)	-
RNA-ase, DNA-ase, hyaluronidase, trypsin proteinase, pepsin, erepsin, diastase, hemicellulase, invertase, β-glucosidase, -glucuronidase, papain and lipase.	-

+ = Abolishes ability of Ehrlich Ascites Tumour Cells to proliferate in mice.

- = Do not abolish ability of Ehrlich Ascites Tumour Cells to proliferate in mice.

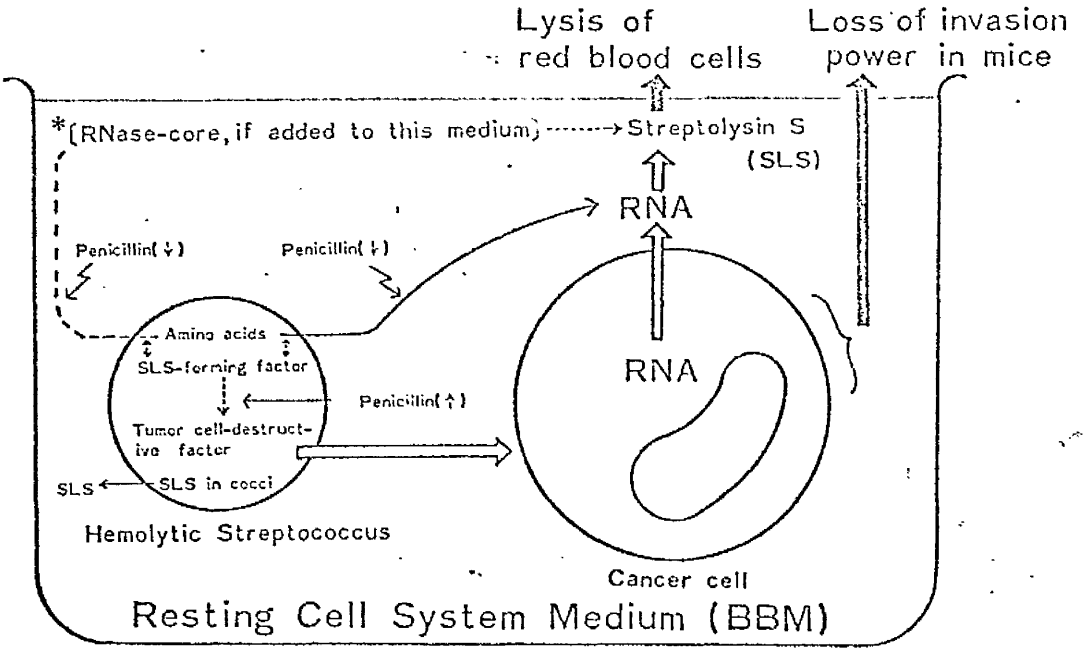
± = Both the above activities have been reported by different workers.

Modified from Okamota, H., Shoin, S., Koshimura, S. and Shimizu (1967). Jap. J. Microbiol 11, 323.

experiments they have shown that this antibiotic had a differential effect on the SLS-forming ability and the antitumour activity of streptococci. They found when streptococci were incubated in Bernheimer's basal medium (Bernheimer 1949) that (1.) the addition of RNA-ase core (0.1%) resulted in a decrease in the anticancer activity and an increase in SLS formation and (2.) the addition of penicillin (40,000 units/ml) had the converse effect (Okamoto, Shoin, Koshimura and Shimizu 1965). From these results this group have suggested the existence of a counterpoise between SLS-forming and anticancer activities contained in resting streptococci. This hypothesis is illustrated in FIG 33. Subsequently these workers found that, by preincubating cocci in Bernheimer's basal medium containing 2 to 4 x 10^4 units of penicillin at 37°C for 20 minutes followed by heating for 30 minutes at 45°C yielded a streptococcal product which had high anticancer activity but no SLS activity.

The role of SLS in the oncolytic action of streptococci therefore remains controversial. Although the largest amount of work has been done by Okamoto and his co-workers, their finding that SLS does not damage cancer cells has been disproved by three independent groups of workers (Ginsburg & Grossowicz 1959; 1960; Taketo & Taketo 1966 and Symington & Arbuthnott 1969). Of great significance is the preliminary report by Taketo and Taketo (1966) that certain agents which inactivate the ability of the cocci to form extracellular SLS complexes and cell-bound haemolysin but not intracellular SLS deprive the cocci of their in vitro oncolytic activity.

The identification of the factor within the streptococcus responsible for its anticancer activity may conceivably provide a new means of treating certain forms of cancer. The possibility that SLS is this factor is supported by the work of this Thesis. The lack of antigenicity of SLS makes the possibility of its use as an anticarcinogen seem feasible but its cytotoxicity for normal cells would severely limit its application. However, it might be possible to



Probable effect of penicillin on anticancer activity of resting S-streptococci.
(↓): Inhibition
(↑): Promotion
* Addition of RNase-core to this suspending mixture results in an enhanced SLS production, but a decreased anticancer activity of the streptococci.

FIG. 33. From Okamoto, Shoin, Koshimura, and Shimizu. (1967) Jap. J. Microbiol. 11, 323.

use SLS in the treatment of tumours if its spread to normal tissues could be prevented.

The disruption of cellular metabolism by SLS reported in this Thesis, provides a possible new approach to the study of its role in the oncolytic activity of streptococci.

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BRIEF EVALUATION OF THE ROLE OF STREPTOLYSIN S IN THE PATHOGENICITY OF GROUP A STREPTOCOCCI.

This Thesis presents a comprehensive review of the products elaborated by Group A streptococci and may help in the understanding of the pathogenicity of these organisms. The multiplicity of factors and their contribution to the pathogenicity of the organism are summarised in FIG 34 and TABLE 28. Such a complex toxic armoury endows the organism with great powers of adaptability. The present study clearly shows that SLS is an important by-product elaborated by the organism. It is admitted, however, that streptococcal disease almost certainly does not result from the monotoxic action of this agent. At the present state of knowledge and with the techniques available, it is impossible to predict with certainty the role of individual streptococcal products *in vivo*. However, this Thesis does emphasise the toxic activity of SLS *in vitro* and has added to the detailed knowledge of the toxic armoury of the Group A streptococcus.

The results presented clearly indicate that SLS can damage both the limiting cell membrane and the mitochondrial membrane. In the Krebs 2 Ascites Tumour cell system, however, the toxin does not appear to gain access to the intracellular organelles as a result of its own action on the cell membrane. *In vivo*, it may be that SLS gains access to the interior of the cell as a result of membrane damage caused by SLO. At present it would be extremely difficult, if not

impossible, to distinguish which of the measurable secondary cytolytic effects are due to SLO or SLS if both were present and acting on the same cell. Moreover, the fact that RNA-SLS is synthesised during the oncolytic attack of whole, viable streptococci on Ascites Tumour cells (Koshimura et al 1958), suggests that SLS may be elaborated inside the cells of the host i.e. the streptococci may gain access to the host cells by the synergistic action of other toxic products and the SLS moiety may then be transferred from the cell-bound form to the RNA of the host cell. Such intracellularly-elaborated SLS might then be able to act directly on the mitochondria of the cell and cause impairment of essential cellular respiration.

The fact that the action of SLS can result in the impairment of basic functions common to all cells partially explains the diversity of streptococcal infections.

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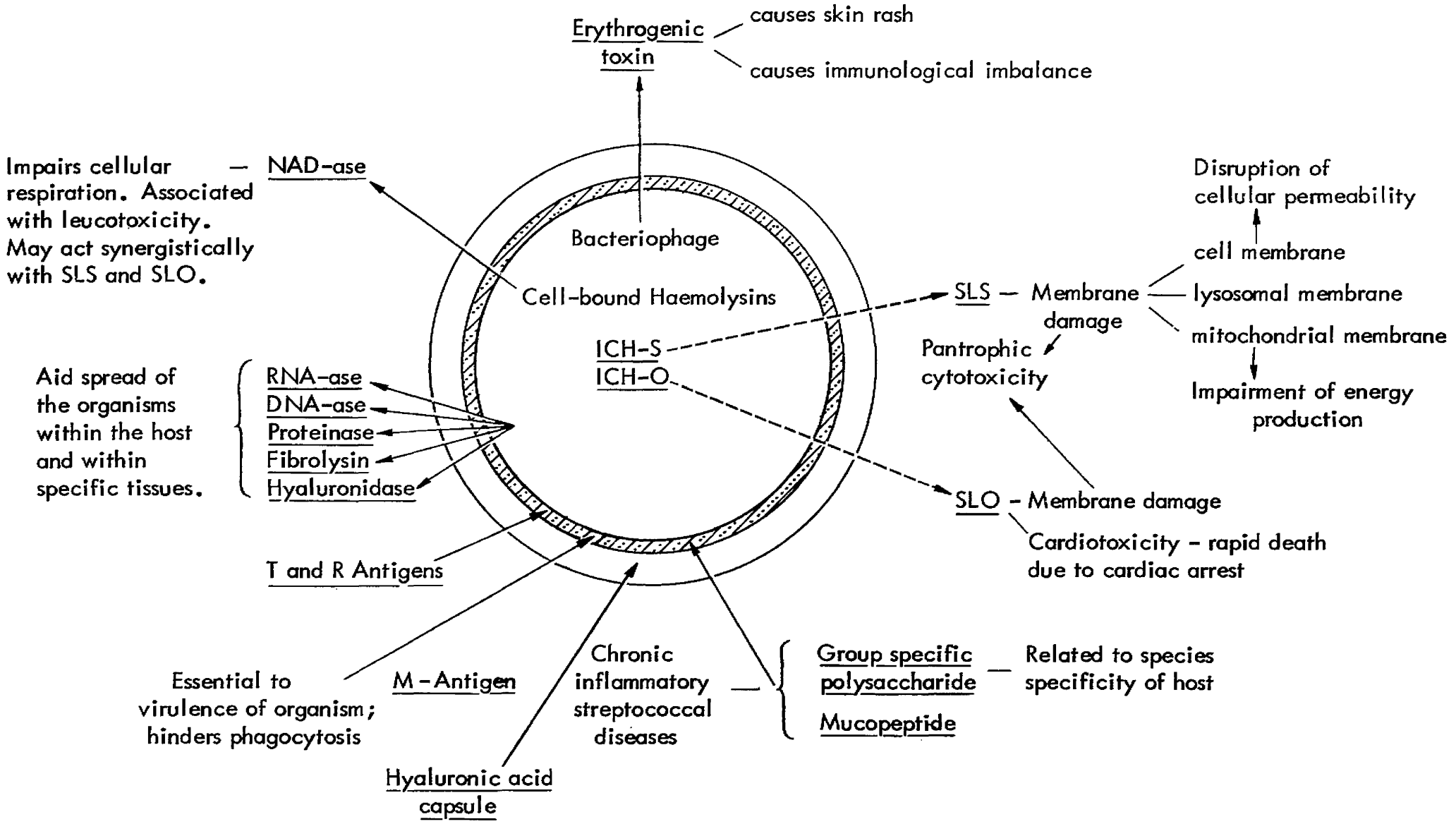
Erythrogenic toxin
causes skin rash
causes immunological imbalance
Impairs cellular respiration. Associated with leucotoxicity. May act synergistically with SLS and SLO.
NAD-ase
Bacteriophage
Cell-bound Haemolysins
Disruption of cellular permeability
cell membrane
SLS – Membrane damage
lysosomal membrane
mitochondrial membrane
ICH-S
ICH-O
Pantrophic cytotoxicity
Impairment of energy production
Aid spread of the organisms within the host and within specific tissues.
RNA-ase
DNA-ase
Proteinase
Fibrolysin
Hyaluronidase
SLO – Membrane damage
Cardiotoxicity – rapid death due to cardiac arrest
T and R Antigens
Essential to virulence of organism; hinders phagocytosis
M-Antigen
Chronic inflammatory streptococcal diseases
Group specific polysaccharide
Related to species specificity of host
Mucopeptide
Hyaluronic acid capsule

TABLE 28.

Contribution of Various Products to the Pathogenicity of Group A Streptococci.

VIRULENCE OF ORGANISM	associated with M-antigen which hinders phagocytosis.
SPREAD OF ORGANISM WITHIN HOST TISSUES	large number of contributary factors, Proteinase, Streptokinase, Hyaluronidase, DNA-ase, NAD-ase.
PERSISTENCE OF ORGANISM WITHIN HOST	Complex of Group specific polysaccharide and cell wall-mucopeptide associated with chronic inflammatory streptococcal disease.
PANTROPHIC CYTOTOXICITY	SLS and SLO cause widespread membrane damage resulting in disruption of cellular permeability. Cellular energy production impaired by SLS and NAD-ase. Overall cytotoxicity is possibly the result of the synergistic action of SLO, SLS, NAD-ase, DNA-ase and RNA-ase.
IMMUNOLOGICAL INBALANCE	Erythrogenic toxin induces hypersensitivity. Cellular antigen similar to heart antigen - results in auto-immunity, contributory factor rheumatoid arthritis. SLO - also cardiotoxic.

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SUMMARY

Streptolysin S (SLS) is one of the several extracellular factors elaborated by the Group A streptococcus. It is a powerful cytolytic toxin which is capable of acting on a wide variety of cell types by virtue of its ability to disrupt biological membranes.

The aim of this Thesis was to determine whether metabolic changes occur within the cell as a result of the cytotoxic action of SLS. Knowledge of the effect of SLS on essential, energy-producing, cellular metabolism could help in the elucidation of its role in the pathogenesis of Group A streptococci. Two aspects of cellular metabolism were studied.

(1) The action of SLS on the functioning of the respiratory and energy-producing centre of the cell, the mitochondrion.

(2) The action of SLS on the respiration of whole cells using Krebs 2 Ascites Tumour cells as models.

Treatment of isolated mouse liver mitochondria resulted in impairment of their succinic and cytochrome oxidase activities. The toxin had no effect on actively respiring mitochondria; a period of interaction between SLS and the mitochondria, prior to the addition of substrate, was required before inhibition was detected.

The addition of exogenous, soluble, cytochrome c to SLS-impaired mitochondria restored their succinic oxidase activity to that of untreated controls. The cytochrome oxidase activity of SLS-treated mitochondria was increased by up to 50% above the controls following the addition of cytochrome c. The presence of exogenous cytochrome c during the interaction between SLS and mitochondria masked the impairment of both succinic and cytochrome oxidase.

SLS caused swelling of mitochondria and leakage of protein including a product having an adsorption maximum at 410 mμ which was possibly cytochrome c.

The results presented in this Thesis have extended significantly knowledge of the mode of action of SLS on mitochondria. Conclusive evidence has been obtained which indicates that SLS causes dislocation of the electron transport chain at the cytochrome c locus. It is recognised that the observed results pin-point a secondary functional lesion within the mitochondrion resulting from damage by SLS but do not provide conclusive evidence for the primary site of action. Possible explanations of the sequence of events occurring in mitochondrial disruption by SLS are discussed.

In its extracellular form, the biologically active polypeptide of SLS requires to be stabilised by attachment of an inert carrier substance. A variety of carriers have been used by different workers for the preparation of SLS. In this Thesis an RNA-ase resistant RNA-core was the carrier used, this was incubated with washed cells of Streptococcus pyogenes strain C203S to prepare the toxin according to the method of Bernheimer (1949). The resultant product was designated RNA-SLS.

Toxin prepared in this was was both haemolytic and caused disruption of mitochondrial function. Both of these biological activities in toxin preparations were destroyed by heating at 60°C. When the SLS-deficient mutant C203U was incubated with RNA-core, the resultant supernatant fluid caused neither mitocondrial damage nor haemolysis. Toxin preparations incubated with either erythrocyte ghosts or mitochondria no longer possessed either of the biological activities. Furthermore, it was not possible to physically separate the two activities by

gel filtration on different grades of Sephadex.

Lecithin and Trypan blue inhibited the haemolytic activity of RNA-SLS preparations but did not affect their ability to damage mitochondria. Taken overall, the work of this Thesis suggests that the haemolytic and mitochondrial damaging activities are properties of the entity known as RNA-SLS but may be located at distinct sites within the SLS molecule.

Phase contrast microscopy of Krebs 2 Ascites Tumour cells treated with SLS was carried out and showed that this toxin caused gross disorganisation of the cells. Many of the observed changes could be explained by membrane damage. SLS is known to attack the limiting cell membrane causing alterations in cellular permeability. The effect of such membrane damage on the respiration of Krebs 2 Ascites Tumour cells was investigated.

When exogenous succinate was present as substrate, Krebs 2 Ascites Tumour cells which had been previously treated with SLS, exhibited an increased rate of oxygen uptake. However, the addition of toxin to cells which were actively metabolising succinate did not cause any alteration in the rate of oxygen uptake. On the other hand, the oxidation of succinate by mitochondria isolated from Krebs 2 Ascites Tumour cells was impaired by SLS. Thus it appears that the toxin fails to penetrate to the mitochondria of intact Ascites cells. Therefore, the alteration in membrane permeability caused by SLS appears to result in extra-availability of succinate to the still-functioning mitochondria and a consequent overall increase in the rate of cellulose oxygen uptake. The fact that staphylococcal alpha-toxin, which is known to act on limiting cell membranes but not on mitochondrial membranes, had the same effect on cellular succinate oxidation supports this hypothesis. Both SLS and staphylococcal alpha-toxin also caused inhibition of endogenous

oxygen uptake and any detectable glucose oxidation.

It is recognised that there is a discrepancy between the fact that SLS is capable of damaging isolated mitochondria and its inability to penetrate to these organelles when they are within the cell. The possibilities of SLS acting synergistically with other streptococcal products or being elaborated intracellularly during disease are discussed.

From the results presented in this Thesis further knowledge of the action of SLS *in vitro* has been gained. It is clear that the action of SLS, in addition to causing morphological damage, may also result in the disruption of essential cellular metabolism which, in turn, could result in cell death. The role of SLS in the anticancer activity and pathogenicity of the Group A streptococcus is discussed.