CAPSULATION IN *STAPHYLOCOCCUS AUREUS*

by

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"No scientist lives in isolation. What he is, is determined as much by his teachers and all the other influences of his cultural environment as by his innate individuality and his own efforts".

H.S.D. Garvon.

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PREFACE

The work described in this thesis began with the isolation of a capsulated, coagulase-positive staphylococcus. This organism is a contradiction in terms of common bacteriological experience: the presence of a capsule, like coagulase-production, is a major taxonomic criterion, but pathogenic staphylococci are not capsulated. This strain therefore poses a taxonomic problem: either it represents a rare species, or it is a rare form of a very common one.

Although this thesis contains a description of this bacteriological curiosity it is equally concerned with attempts to answer certain questions which this strain provokes. In particular, study of its taxonomic position, showing that it is a strain of Staphylococcus aureus, raises the question: why is this strain capsulated when modal strains of the species are not?

It is tempting and easy to assume that ordinary strains of Staph. aureus have simply lost the capacity to synthesise capsular material. The truth may be more complex however. Thus there are bacteria which lack a normal species characteristic, not because they are deficient in a particular respect but because they have an extra capacity.

For instance, some strains of Staph. aureus fail to clot plasma, not because they fail to produce coagulase but because they produce a coagulase-destroying enzyme; if they are tested under conditions which

limit the action of this enzyme they clot plasma as efficiently as other strains (Lominski, Smith and Morrison, 1953). Similarly, certain strains of streptococci fail to form chains, not because they have lost the capacity to do so, but because they produce an enzyme, absent from chaining cultures, which breaks up chains. When this enzyme is inhibited, chains appear in culture (Lominski, Cameron and Wyllie, 1958; Lominski and Gray, 1961).

In the light of these examples, and the concept of "antagonistic variants" which they illustrate, it is possible to frame an hypothesis explaining why ordinary strains of staphylococci are not capsulated. Stated briefly, this postulates that all pathogenic staphylococci produce capsular material; that ordinary strains do not have visible capsules because they either destroy capsular material or remove it from the cell surface, and that the capsulated strain has lost this power.

The experimental work described has been designed to test this hypothesis and falls into four sections: a detailed examination of the capsulated staphylococcus to determine how closely it is related to modal strains of Staph. aureus; a description of a "capsulo-stripping" enzyme and the coagulase-positive staphylococcus which produces it; a description of experiments made to induce the formation of capsules in laboratory strains of Staph. aureus by culturing them in antiserum to the capsulo-stripping enzyme; and a description of experiments designed to demonstrate that modal strains

of Staph. aureus can also remove capsular material from cells of the capsulated strain.

While all four sections are connected by and bear upon the same central theme they are considered sufficiently distinct to warrant separate discussion and summary; these have therefore been placed at the end of each section. A general discussion follows the last section.

INTRODUCTION:

PREVIOUS REPORTS OF CAPSULATION IN PATHOGENIC STAPHYLOCOCCI

"Micrococci present a capsule surrounding each ball, and binding them together into chains and groups. It appears as a glistening halo when they are unstained; but, after being stained and dried, it becomes invisible, and they present the appearance of being independent spheres".

Any review is selective, both in the papers presented and in the details abstracted from them. The quotation above, from Alexander Ogston's classic paper (Ogston, 1881), highlights the three main difficulties which have influenced the choice of papers for inclusion in this review and hindered their subsequent intergretation. These are: that the criteria by which pathogenic staphylococci have been classified have altered frequently since these organisms were first described; that there is considerable variation in the criteria by which different authors have judged that a capsule was present on individual strains of staphylococci, and that some reports in the literature contain only scant information.

Recognition of pathogenic staphylococci

The criteria used to define pathogenic staphylococci, i.e.

Staphylococcus aureus, have not remained static. It has been reported, for instance, that they can be distinguished by colonial colour (Rosenbach, 1884); by the fermentation of mannite and liquefaction of gelatin (Gordon, 1904, 1906; Mossel, 1962); by agglutination and precipitation reactions (Kolle and Otto, 1902; Julianelle and Weighard, 1935); by the production of certain patterns of haemolysins (Elek and Levy, 1950); by the production of free and bound coagulase (Daranyi, 1925, 1926, 1927; Duthie, 1954); by the production of phosphatase (Barber and Kuper, 1951) and by susceptibility to bacteriophage (Parker, 1962).

Not only the individual criteria, but the weight given to each has also altered. At present many bacteriologists accept that pathogenic staphylococci can be distinguished by the single property of coagulase production. In a recent classification Baird-Parker (1965) suggests, however, that a strain cannot be accepted as Staph. aureus unless it gives positive results in at least two of three tests: the production of phosphatase, fermentation of mannite, and production of free and bound coagulase. He considers that susceptibility to bacteriophage, production of a typical pattern of haemolysins and the ability to grow in normal human serum (Ekstedt and Nungester, 1955; Ekstedt, 1956 a, b; Fletcher, 1962) provide confirmatory evidence only.

Properties and recognition of capsulated bacteria

The demonstration of a bacterial capsule depends on properties

which are related not only to its presence but to its thickness. Although these properties appear together in capsulated strains and are together absent from non-capsulated variants, some of them may be shown for different reasons by strains which are not capsulated.

A well-developed capsule is visible with the light microscope in suitable preparations and especially in thin india ink suspensions (Duguid, 1951) as a distinct structure enveloping the cell, usually with a regular outline and morphologically distinct from the cell-wall. It is also often chemically distinct from the cell-wall (Salton, 1964). Unlike bacterial slime, the capsule remains attached to the cell when suspended or washed in distilled water (Duguid, 1951) and it is responsible for the phenomenon of "capsular swelling" (Roger, 1896; Neufeld, 1902) or the "specific capsular reaction" (Tomcsik, 1956) when the cells react with specific immune serum.

Capsulated strains may also be of increased virulence, resistant to lytic bacteriophage, and often form colonies of an unusual consistency described as "sticky" or "mucoid". These last three properties can also be shown by non-capsulated species for entirely different reasons; the fact that a strain possesses them does not necessarily prove that the strain is capsulated. Nevertheless, some authors have concluded that strains of pathogenic staphylococci were capsulated, even in the absence of a visible capsule, because the strain studied was of increased virulence, or produced colonies which were "sticky", "viscid" or "mucoid", or showed appearances interpreted

as a "capsular swelling reaction" after prolonged contact between cells and antiserum. In other reports there is unequivocal i.e. visible, evidence of capsulation but the taxonomic position of the strain discussed, as defined by modern criteria, is uncertain.

Although an attempt has been made to include in this review a reference to all the reports in the literature describing possible capsulated pathogenic staphylococci, not all have been discussed: it is considered pedantic to discuss reports in which the information recorded is so meagre that its significance cannot be correctly assessed. Sonnenschein (1927) for instance reported the isolation from a human nasal swab of a strain which he called "Staphylococcus mucosus", but he did not describe or characterise it further. This may have been a capsulated pathogenic staphylococcus but it is impossible to be certain and fruitless to speculate. Strains described by Ogston (1881), Nootzel (1896), Porter and Pelczar (1941), Barber (1964) and Fahlberg (1963) have not been discussed for similar reasons.

PREVIOUS REPORTS OF CAPSULATION IN STAPHYLOCOCCI

These can be grouped under five headings:

1. Those describing the phenomenon of pseudocapsulation.
2. Reports of capsules about the cells of viscid-colony variants of pathogenic staphylococci.

3. Reports of capsules demonstrated about pathogenic staphylococci by the use of special techniques.

4. Reports of pathogenic staphylococci with extra surface antigens.

5. Reports of three strains of staphylococci with large capsules.

This grouping is convenient but in some instances, arbitrary. For instance, strain "Smith", the prototype of the strains described in section 4, has been grouped with them although it is considered by some authors to have a distinct capsule (Mouse, 1960; Lenhert, Li, DeCouroy and Mudd, 1962; Koenig and Melly, 1965; Mudd, 1965; Mudd and DeCouroy, 1965; Kapral, 1966). The reports collected together in section 3 have little common ground but cannot reasonably be included in any of the other groups.

1. Pseudocapsulation

The term pseudocapsulation was used by Sall and his associates (Sall, Mudd and Tumbler, 1961; Sall, 1962) to describe a capsule-like structure, demonstrable by relief staining with India ink, which appeared round cells of a strain of Staph. aureus when grown on a gelatin medium containing high concentrations of lactose and mannitol. Under these conditions the strain produced large amounts of free coagulase but little gelatinase. The pseudocapsule precipitated with cadmium nitrate, which is known to precipitate

soluble coagulase (Duthie & Houghton, 1958) and was considered to be an accumulation of coagulase, unable to diffuse away into the surrounding medium because of the lack of gelatinase. It was not produced when a coagulase-negative variant of the same strain was grown under identical conditions.

2. Reports of capsulated viscid variants of pathogenic staphylococci

The papers in this group have common ground in that they deal with variant forms of staphylococci recognisable, and studied, because they produced colonies of an unusual viscid consistency.

Bigger, Boland and O'Meara (1927), working with a strain of Staphylococcus aureus which fermented mannite, liquefied gelatin and was haemolytic, grew the organisms for prolonged periods in broth with and without added glycerol and examined the variant colonies which appeared. These included colonies which were "sticky or viscid", and which, when touched with a platinum wire, "pulled out like glue or else lifted whole off the agar". No mention is made of the presence of capsules on these variants.

Variants of a similar colonial consistency were described and studied by Price and Kneeland (1954, 1956). Working with a coagulase-positive, mannite-fermenting strain, they passaged the organism on agar, in broth and once through a fertile hen's egg. After the final passage, a mucoid variant appeared, forming "watery but tenacious" colonies which were difficult to emulsify.

This remained stable for 18 to 20 subcultures before reverting to the parent type from which it could be again recovered by a single egg passage. It had the same biological properties as the parent and like it, was susceptible to 'phage lysis. Both were of equal and lowly virulence for mice by intraperitoneal injection, killing only when suspended in mucin. Price and Kneeland could not demonstrate capsules satisfactorily by "the usual, dry staining methods", but cocci of the viscid variant from young cultures gave an "unequivocal swelling reaction" when suspended in specific immune serum for 1 to 3 hours; the reaction with cocci from 24-hour cultures was less striking and appeared only after 18 hours contact with antiserum.

Examining the serological relation of the mucoid variant to the parent strain, Price and Kneeland found that antiserum to the mucoid variant produced rapid and complete swelling of homologous cells but only occasionally of cells of the parent strain. Antiserum to parent cells caused slight capsular swelling of mucoid organisms but none with homologous cells.

These observations were extended to 29 strains of staphylococci from human infections and ten from "normal conjunctivae" (Price and Kneeland, 1956). Thirty-two of those underwent "capsular swelling" when treated with antiserum to the mucoid variant. (Twenty-eight of these were from human infections; all produced alpha toxin and all but one were coagulase-positive). Egg passage was necessary to produce the phenomenon with 9 strains. Three of the 28 strains

from infections were mucoid, two on first isolation and one after egg passage. Six further strains of staphylococci, none of which produced coagulase or alpha toxin and all but one of which did not ferment mannite, did not undergo capsular swelling, even after passage three times through fertile eggs.

Price and Kneeland concluded that most strains of pathogenic staphylococci produced capsular material, that the amount produced varied from strain to strain, that it was antigenically similar in all strains, that it did not appear to be related quantitatively to virulence, and that non-pathogenic strains do not produce it.

Staphylococcal variants producing colonies of an unusual consistency and appearance were also described by Reimann (1957). Working with a coagulase-positive strain which fermented mannite and liquefied gelatin, he cultured it in nutrient broth at room temperature for 54 days. A subculture on agar incubated for a further 20 days included smooth yellow colonies with pale segments. When these segments were subcultured thick glistening pale yellow colonies appeared. These were confluent where crowded on the plate, tough or glairy in consistency and "pulled out like mucin". They bred true on subculture. The cocci forming them were smaller than those of the parent strain, "appeared at times to have capsules" and in stained preparations (the stain used is not stated) were seen to be enmeshed in a "stringy matrix, possibly of capsular material". No capsular swelling reaction was noted when cocci were suspended in homologous immune serum. Reimann isolated a second mucoid

variant from a 7-month old subculture of the same parent strain in broth. This also was stable on subculture. Cocci of this variant were often arranged in tetrads, "seemed to have less prominent capsules" than the first mucoid variant, but in stained smears were also enmeshed in pink staining material. Unlike the parent strain, and the first mucoid variant, this variant was coagulase-negative, did not ferment mannite and did not liquefy gelatin. Reimann considered that these mucoid forms were related to the sticky, glue-like colonies described by Bigger et al. (1927).

Wiley (1961, 1963 a, b, 1964, 1966) has extended the work of Price and Kneeland. Working with coagulase-positive strains able to ferment glucose and mannite anaerobically, he cultured them using the technique of Bigger et al. (1927) in a semi-synthetic broth containing 2 per cent glycerol. After incubation for 1 or 2 months, he isolated mucoid variants from 5 strains. He described these as gummy, the colonies lifting off the agar in a single mass when touched with a needle. Cells of these variants, taken from 6- to 8-hour cultures, and incubated overnight with immune serum and methylene blue, produced appearances which Wiley interpreted as a specific capsular reaction. All the mucoid variants were virulent when injected into 13-day-old fertile hens' eggs, in contrast to six strains of Staph. epidermidis, none of which was lethal even in high doses. Immune serum, human gamma globulin and commercial staphylococcal antitoxin protected

against lethal challenge but not if first absorbed with cells of the mucoid variant or concentrated soluble material from culture supernatants of it. Wiley did not compare the virulence of the mucoid variants and their parent strains because "non-encapsulated strains of Staph. aureus were not available" and it was very difficult to obtain cultures "in which none of the organisms exhibited a capsule", (Wiley, 1961).

Wiley (1963 a) has also shown that the serum of 80 per cent of healthy blood donors contained sufficient antibody to evoke a "specific capsular reaction" with mucoid strains and that almost half the strains of Staph. aureus isolated from healthy nasal carriers were "encapsulated", i.e. reacted with high titre immune serum to produce a "capsular reaction", (Wiley, 1963 a).

He has also shown by comparing viable counts with packed cell volumes, that strains highly virulent for fertile eggs had larger capsules; that they released up to ten times more capsular material in fluid cultures, and produced more coagulase than strains of low virulence (Wiley, 1963 b). In a further report (Wiley, 1964) he described virulence and encapsulation in hospital strains of Staph. aureus. 92.5 per cent of strains from wound infections were "encapsulated"; 48 per cent of these gave a positive "capsular reaction" with serum from acute or convalescent cases, but they were not significantly more virulent for fertile hen's eggs than strains carried by healthy hospital staff.

The surface material from the mucoid variants has been isolated and partially characterised (Wiley and Wonnacott, 1962). It contained four amino-acids, glucosamine, glycerophosphate and an unidentified phosphate ester. The amino-acid content was similar to that of the cell walls of the "Oxford" strain of Staph. aureus. According to Salton (1964) this is one of the few instances of chemical overlap between capsules and cell-walls in the same organism. More recently, Wiley (1966) injected mice intraperitoneally with partially-purified capsular material in doses of up to 1 mg. but was unable to demonstrate any antibody production by a variety of immunological techniques.

The phenomenon described by Price and Kneeland as "capsular swelling" and by Wiley as a "specific capsular reaction" has been re-examined by Mudd and DeCourcy (1965). Working with Wiley's mucoid strain, ordinary unselected strains of coagulase-positive staphylococci, and strain "Smith" (vide infra, page 19) these authors were unable to demonstrate capsules, except on strain "Smith", when cocci were examined in thin india ink suspensions. They confirmed that the phenomenon interpreted as capsular swelling was exhibited by Wiley's strain when cocci were allowed to react with specific immune serum; ordinary strains and strain "Smith" did not produce it. According to Mudd and DeCourcy (1965) the extracellular material produced by Wiley's strain was chemically and serologically distinct from the polysaccharide

antigen which forms the surface of strain "Smith". They considered that the phenomenon was not a true specific capsular reaction but was due to the extracellular precipitation of material round the cells, and it coined the term "extracellular peripheral precipitation reaction" (E.P.P.R.).

3. Reports of the demonstration of capsules on ordinary strains of pathogenic staphylococci by the use of special techniques

The reports considered under this heading form a heterogeneous group. They are considered together for two reasons: because each contains the tacit or explicit assumption that all or most pathogenic staphylococci produce capsules and need only be examined by special techniques for capsules to be seen, and because none can reasonably be included in any of the other sections.

Lyons (1937) studied 10 strains of Staph. pyogenes from human infections and one non-toxigenic strain from human skin. He examined cells either by suspending them directly in 15 per cent "collargol" or by using a positive capsule stain which he devised (Appendix i). He claimed that capsules were present on cells of toxigenic and non-toxigenic strains after 3 but not 20 hours incubation in glucose broth. Capsules were not formed in the presence of serum. They could be removed by mechanical shaking of cultures but were unaffected by heating at 100°C for 5 minutes. Young encapsulated cocci were more resistant to phagocytosis than cocci from old cultures and could be specifically opsonised with antiserum prepared in rabbits by the

injection of young, encapsulated cocci. Such an antiserum agglutinated both young and old cocci; antiserum prepared by injecting old cocci agglutinated them but did not agglutinate young cocci. Lyons concludes that staphylococci are encapsulated early in the growth phase and that the capsule is responsible for the invasiveness of the organism.

Spink (1939) attempted to repeat Lyons' findings, working with 30 pathogenic and 11 non-pathogenic strains of staphylococci. (The criteria of pathogenicity were not stated). He was unable to demonstrate capsules round cocci in young cultures, either by Lyons' original or modified positive staining method (Appendix ii), by the use of 15 per cent "collargol" or by the capsule staining methods of Hiss and Muir. In smears of young cultures stained by Lyons' modified method, he observed a capsule-like deposit of carbol fuchsin round the cells; similar deposits were seen round particles of animal charcoal smeared on a slide. Spink was also unable to confirm the resistance of young cocci to phagocytosis; staphylococci from 2-hour cultures were phagocytosed by human leucocytes in defibrinated blood as readily as cells from a 16-hour culture.

In 1934 Kaluzewski, working with 48 strains of staphylococci from human infections published findings similar to Lyons. The strains were cultured on an egg medium, in Lyons' broth, and in broth containing 10 per cent bovine serum. Kaluzewski was able to

demonstrate capsules using a special negative staining technique (Appendix iii), although capsules were not seen in smears stained by the methods of Hiss, Rebiger, Nicolle, Johne, Kauffmann or Mussolnikow.

In twenty-two (47 per cent) of the 48 strains examined he observed capsules, 0.8 - 1.1 μ thick, after three hours incubation on egg medium, or in Lyons' or serum broth; 9 per cent of the cells were not capsulated. Capsules were not demonstrated in 7- to 8-hour cultures in the same media. Seventeen of the capsulated strains were classified as Staph. aureus by colonial pigmentation; the remaining 5 were coagulase-negative albus strains. Attempts to produce capsules on two non-capsulating aureus strains by intraperitoneal passage in mice were successful with one strain, but only after six passages and one plating on egg medium.

Kaluzowski was unable to demonstrate any difference in virulence between capsulating and non-capsulating strains when injected intraperitoneally and intravenously into mice, but he tested only a small number of animals. Antiserum prepared in rabbits against capsulated cells from a 3-hour culture agglutinated homologous cells to high titre but consistently failed to produce a capsule swelling reaction.

Mesrobeanu and Taga (1961) studied two strains of Staph. aureus, "24" and "N". Both fermented mannite, produced coagulase, alpha

toxin and fibrinolysin, were resistant to penicillin and streptomycin, and were of 'phage-type 80/81. By intraperitoneal passage in guinea-pigs, previously prepared by injecting sterile broth to provoke a peritoneal leucocyte response, three variants were produced after seven and twelve passages. These were more virulent for guinea-pigs than the parent strains, but unlike the parent strains were sensitive to penicillin and streptomycin and were resistant to 'phages 80 and 81.

Cells of 3- to 5-hour agar cultures of the variants, suspended in India ink and examined by phase-contrast microscopy, were surrounded by "distinct capsules". Capsules were also visible when the same preparations were examined by "chromo-anoptral" phase-contrast microscopy, a new method (Ciurea et al., 1962) using "negative anoptral and positive polychromatic contrast". Capsules were not demonstrated on cells of the parent strain by phase-contrast microscopy, nor on cells of either parent or variant strains by the methods of Muir, Hiss or Lyons, when stained with methylene blue, or when examined in thin India ink suspensions by conventional light microscopy.

Mosrobeanu and Taga concluded that staphylococci, when exposed repeatedly to the action of leucocytes, gave rise to capsulated variants, and that these might be responsible for severe infections.

4. Reports of pathogenic staphylococci with extra surface antigens

In 1930 Dubos isolated a staphylococcus from a patient with

osteomyelitis. This strain, called "Smith" after the patient, was briefly described (Smith and Dubos, 1956) as coagulase-positive, pigmented and of phage type 44A/42E. It has been extensively used in experimental work in Dubos' laboratory and elsewhere in America because of its consistent and high virulence for mice.

Nearly 30 years later, Hunt and Moses (1958) showed that strain "Smith" dissociated on subculture to produce two colonial variants, but that only one of these was highly virulent for mice when injected intraperitoneally. The variants could be distinguished by culture in 0.15 per cent agar containing 1 per cent human or rabbit plasma ("plasma-soft agar"). In this medium the mouse-virulent variant grew in compact spherical colonies (Finkelstein and Sulkin, 1958; Hunt and Moses, 1958). The variants differed in other respects. The compact variant was bound coagulase-positive, agglutinated in antiserum to Cowan Group II staphylococci, and was lysed by 'phage 44A. The diffuse variant was bound coagulase-negative, did not react with antisera to Cowan Group I, II or III strains and was not lysed by routine typing 'phages. When grown in broth both variants showed spontaneous variation to produce a few cells of the other type. Analysis of the events following the injection of each into the mouse peritoneum showed that both were engulfed by leucocytes but the diffuse variant multiplied intracellularly to produce an overwhelming infection (Hunt and Moses, 1958).

Confirmation that the two variants were very similar was given by

Koenig (1962) and Koenig and Melly (1965) who showed that both produced the same amounts of free coagulase and alpha haemolysin; both produced delta (but not beta) lysin and both were sensitive to the same pattern of antibiotics. In contrast to Hunt and Moses, Koenig found that neither variant was susceptible to routine typing 'phages. He confirmed that the diffuse, bound coagulase-negative variant was highly virulent for mice and demonstrated, as did Rogers and Melly (1962) that this variant was resistant to phagocytosis in the early stages of intraperitoneal infection; the compact variant was readily ingested. Mice immunised with heat-killed cells of the diffuse variant were resistant to peritoneal challenge with it and phagocytosed injected cocci promptly and effectively. Vaccines containing cells of the compact variant, or ordinary strains of staphylococci were without effect. These results parallel those of S. Fisher who had earlier shown (S. Fisher, 1960) that culture supernatants of strain "Smith" contained a heat-stable, non-dialysable antigen which protected mice against intraperitoneal challenge with the homologous strain. Protection was maximal 4 to 48 days after injection of the antigen and could be conferred passively with specific antiserum raised in mice or goats.

Koenig concluded that the diffuse variant of strain "Smith" had antigenic properties and a resistance to phagocytosis which were not shared with the compact variant. Results of attempts to demonstrate capsules on cells of the diffuse variant using several different capsule stains were ambiguous; in some india ink suspensions both

variants "seemed to have capsules", but these observations were not reproducible (Koenig, 1962).

Strain "Smith" is not unique; similar strains have been described by Alami and Kelly (1959), Tompsett (1961), Morse (1962), Fisher, Devlin and Erlandson (1963) and Koenig and Melly (1965). Although they are said to be rare, these strains are possibly not as rare as suggested since their recognition depends on tests which are not usually carried out during the routine laboratory assessment of staphylococci, e.g. growth in plasma-soft agar, virulence tests in mice, and serological recognition of an extra surface antigen.

The eleven strains which have been described have certain characters in common (Koenig and Melly, 1965). All differ from ordinary coagulase-positive staphylococci by forming diffuse colonies in plasma-soft agar; all lack bound coagulase but produce soluble coagulase; none is consistently typable using routine 'phages; most, but not all, are more virulent for mice on intraperitoneal injection, and all are more resistant to phagocytosis in vitro. Four of them (Mudd, 1965) and strain "Smith" (Morse, 1960; Lenhart et al., 1962; Koenig and Melly, 1965; Mudd and DeCourcy, 1965; Mudd, 1965; Kapral, 1966) are thought to have visible capsules. The surface antigen responsible for resistance to phagocytosis is probably similar in all the strains: Koenig and Melly (1965) have shown that a vaccine containing heat-killed cells of the diffuse variant of strain "Smith" will protect mice against an otherwise lethal intraperitoneal

challenge with any of the "Smith-like" strains. Morse (1963) extracted surface antigen from strain "Smith" and five other diffuse strains with acid saline and showed that all the extracts gave a reaction of identity when diffused in agar against antiserum to strain "Smith". The conditions necessary for effective intraperitoneal phagocytosis of the diffuse strains are complex: all are phagocytosed in the presence of normal human serum which supplies a heat-labile and a heat-stable substance but are poorly phagocytosed in the presence of heated serum. Ordinary coagulase-positive staphylococci, and the compact variant of strain "Smith" are opsonised by either the heat-stable substance, or a heat-labile substance present in fresh human, rabbit and guinea-pig serum, which is probably complement (Li and Mudd, 1965).

Although "Smith-like" strains are said to be rare - Koenig and Melly (1965) found only one among 1833 strains of Staph. aureus isolated from human infections - many normal human sera contain an opsonising antibody which reacts with the surface antigen of the diffuse variant of strain "Smith" (Rogers and Melly, 1962; H. Fisher et al., 1963, 1964). Mudd (1965) has suggested that preparations of "Smith" capsular polysaccharide antigen which react with human sera in this way contain another antigen which is shared with ordinary staphylococci. Koenig and Melly (1965) believe, however, that many humans have had experience of "Smith" surface antigen or a similar substance. They follow Rogers (1962) in suggesting that ordinary strains of staphylococci may produce "Smith" surface antigen

when growing in vivo and that the "Smith-like" strains which have been recognised differ only in continuing to produce the extra surface antigen when subcultured in the laboratory.

The nature of the surface antigen on the diffuse strains has been studied by several workers.

Morse (1960, 1962) treated culture supernatants of strain "Smith" with ethanol after removal of protein and lipid and obtained a white amorphous material which he called "Smith surface antigen" (S.S.A.). This behaved as a homogeneous substance on ultracentrifugation and electrophoresis. On chemical analysis it was found to contain 70% carbohydrate, 30 to 35% of which was believed to be glucosamine with possibly another aminosugar present. Seven aminoacids were also present, but little nucleic acid and no muramic acid.

S.S.A. was not antigenic when injected into rabbits but absorbed agglutinins and opsonins for strain "Smith" from immune sera produced in rabbits by injecting whole organisms. Mice injected subcutaneously with S.S.A. were protected against intraperitoneal challenge with cells of strain "Smith" suspended in mucin, but not against challenge with organisms suspended in broth. The protective dose of S.S.A. showed upper and lower limits, the former possibly due to "immune paralysis". Perkins (1963) characterised the material as 2-deoxy-2-amino-glucuronic acid.

M. Fisher et al. (1963) studied the surface antigen of strain 05068, one of the mouse-virulent "Smith-like" strains. This antigen,

called staphylococcal polysaccharide antigen (S.P.A.) was prepared by phenol extraction of washed cells, or hydrolysis of washed cells with 0.1N acetic acid. They showed that this material was a potent protective antigen; the 50% protective dose (PD_{50}) for mice challenged intraperitoneally with 10^4 LD_{50} of homologous organisms was 0.005 µg. Like Morse's antigen S.P.A. showed the phenomenon of immune paralysis. Protection after injection was maximal from 2 to 70 days and correlated directly with the titre of antibody to S.P.A. S.P.A. was antigenic in mice, dogs and man, but not rabbits, guinea-pigs or monkeys.

Subsequently Haskell and Hanessian (1963, 1964) characterised S.P.A. as a polysaccharide, consisting of repeating units of 2-acetamino-2-deoxy-D-glucuronic acid and 2(N-acetyl-alanyl amino)-2-deoxy-D-glucuronic acid joined by a 1 - 4 linkage, probably beta in type.

5. Reports of three strains of staphylococci with large capsules

Staphylococci with large capsules are rare; only 3 strains have been described.

Gilbert (1931) isolated a gram-positive coccus from the pericardial and peritoneal fluids of a young man who died with ulcerative gonococcal endocarditis. This organism had the morphology of a staphylococcus except that it showed a well-defined capsule, about 1 µ thick, in india ink suspensions. It grew well on ordinary media to produce large, smooth, translucent and confluent colonies,

brilliant orange in colour. It fermented glucose but not mannite, and liquefied gelatin. Colonies on blood agar were surrounded by a zone of haemolysis. Coagulase production was not tested.

After the strain had been kept for a month in an ice-box it dissociated on subculture to yield a variant forming opaque cheese-like colonies composed of non-capsulated organisms. Dissociation was also noted in agar, broth and milk cultures more than 15 to 20 days old, and particularly in old broth cultures incubated at 37°C. The parent strain and non-capsulated variant differed greatly in their virulence for the guinea-pig: the intraperitoneal injection of 0.025 ml. of a 24-hour broth culture of the capsulated parent strain killed within 24 hours, whereas 5 ml. of a broth culture of the non-capsulated variant had no effect. The non-capsulated variant differed from ordinary strains of staphylococci by occasionally reverting to the capsulated type when injected into a guinea-pig.

Oosterlo (1936) cultured a gram-positive coccus from a specimen of pus, the source of which was not known, although it was probably human. Morphologically the organism was a typical staphylococcus except that it had a distinct and well-defined capsule, easily visible in india ink preparations. It grew well on ordinary media at 37°C and 22°C, producing orange, mucoid colonies, very slimy in consistency, which flowed over the surface of the agar on prolonged incubation. It fermented glucose, sucrose and lactose (but not mannite), liquefied gelatin and clotted milk. Colonies on blood agar lysed sheep, ox,

human and horse, but not rabbit erythrocytes. It coagulated plasma but more slowly than freshly-isolated strains. It was not lysed by the single strain of 'phage tested, and produced no 'phage lysis of 10 strains of pathogenic staphylococci when tested by cross-plating.

Mice were killed in 24 to 48 hours by the intraperitoneal injection of 0.2 ml. of a 24-hour broth culture. Oesterle produced non-capsulated variants by culturing the organism in sterile ox-bile for 18 to 22 days. The variants had "the biological properties, colour and appearance of the typical normal form of Staph. pyogenes aureus". Their virulence was not compared with that of the parent strain. Oesterle appeared to be in no doubt that the mucoid strain, except for its capsule, was a typical pathogenic staphylococcus.

Henriksen (1948) cultured a Gram-positive coccus from the throat of a patient with chronic rhino-pharyngitis. The organism had the morphology of a staphylococcus but dry india ink smears (Butt, Bonynge and Joyce, 1936) showed a distinct capsule. The coccus grew well on ordinary media, producing moist, mucoid, confluent colonies, thin and not viscid in consistency, which varied in colour from pure white to pale yellow. It fermented glucose, sucrose, lactose and mannite, liquefied gelatin and clotted milk. It was not haemolytic on blood agar. It coagulated plasma in 90 minutes. Mice were killed by the intraperitoneal injection of 0.5 ml., but not 0.05 ml. of a 24-hour broth culture. Unlike the strains described by Gilbert and Oesterle this strain remained stable on subculture and did not dissociate to

yield non-capsulated variants.

This review reveals that the evidence for capsulation in pathogenic staphylococci is of three main types: the demonstration by special methods of capsule-like structures on strains which are unequivocally pathogenic staphylococci; the existence of a small group of pathogenic strains with extra surface antigens which, according to some authors, can be seen as capsules, and descriptions of three strains of staphylococci with unmistakable capsules but of doubtful taxonomic position. Further, more detailed assessment of individual reports, inevitably retrospective, is not likely to be fruitful, either because of doubt about the precise taxonomic position of the organism described, or because the evidence advanced for capsulation is equivocal.

It is clear, however, that these three classes of evidence contrast sharply with each other and with everyday laboratory experience which teaches that pathogenic staphylococci are never capsulated. It is against this background that the present work has been undertaken.

EXPERIMENTAL WORK

SECTIONS I - IV

DEFINITION OF TERMS

To avoid the repetition of detailed descriptions colloquial terms have been used in the experimental sections for certain media, preparations and methods which were employed repeatedly. The colloquial terms are defined in detail below.

Nutrient broth: Laboratory-produced horse-heart meat infusion extract broth.

Nutrient agar: Nutrient broth solidified by the addition of 1.2 per cent w/v "Oxoid" agar.

Blood agar: Nutrient agar containing 5 to 10 per cent v/v defibrinated horse blood .

Double strength agar: Nutrient broth solidified by the addition of 2.4 per cent w/v "Oxoid" agar.

Serum agar: Nutrient agar containing 10 to 20 per cent v/v human serum.

Salt agar: Nutrient agar containing 6 per cent w/v NaCl.

Saline: A sterile solution of 0.85 per cent w/v NaCl in distilled water.

Buffered saline: Equal volumes of saline (as defined above) and sterile isotonic phosphate buffer (Hendry, 1948).

Thiomersal: 1 per cent w/v sodium ethylmercurithiosalicylate (British Drug Houses) in distilled water. This was added to preparations to produce a final concentration of 1 in 10,000 w/v.

Shake cultures: Nutrient broth cultures incubated on an orbital shaker with a throw of 3cm at 110 rpm.

Static cultures: Nutrient broth cultures incubated without shaking.

Incubation: Except where stated incubation was carried out at 37°C.

Chemicals: The chemicals used were of B.D.H. "Analar" or B.D.H. "Laboratory" standard. Exceptions are indicated in the text.

pH: pH measurements were made with a Model 464 "Vibrot" pH meter (Electronic Instruments, Ltd., Richmond) fitted with a combined electrode and automatic temperature regulator.

SECTION I

THE CAPSULATED COCCUS

MATERIALS AND METHODS

Organism:

The capsulated strain was isolated in January, 1962, by Dr. D.D. Smith, from a human wound swab, along with a beta-haemolytic streptococcus. It is designated strain Morris. A derived non-capsulated variant, designated strain D, (see below, page 43) was also studied.

Media:

Growth was studied using nutrient broth, nutrient agar, blood agar, salt agar, serum agar, and double strength agar.

Morphology:

Colonial morphology was studied on nutrient agar and blood agar. Cell morphology was studied in Gram-stained smears, in smears stained with nigrosin and rose-bengal (Browning and Mackie, 1949) and in thin india ink suspensions (Duguid, 1951) after preliminary staining with rose-bengal. Details of these stains are given in the appendix. The average capsule thickness of cells was measured in thin india ink suspensions with a calibrated eyepiece graticule.

Physiological and biochemical characters:

The utilisation of glucose aerobically and anaerobically was tested by the method recommended by the Subcommittee on the taxonomy of staphylococci and micrococci (Subcommittee 1965) except that the anaerobic cultures were incubated in hydrogen in a McIntosh and Fildes jar. The production of acid from other carbohydrates, the production of acetoin, the final pH produced in glucose broth and the production of catalase and phosphatase were tested using the media and methods described by Baird-Parker (1963).

Coagulase production:

The production of bound coagulase was tested by the method of Cadness-Graves, Williams, Harper and Miles (1943) and soluble coagulase production by the method of Fisk (1940). Soluble coagulase production in shake cultures was also studied. Shake culture supernatants were concentrated by precipitating with 3 volumes of ethanol at 0^{0}C and redissolving the precipitate in buffered saline, pH 7.2, to one tenth of the volume of the original culture.

Unconcentrated and concentrated culture supernatants were tested for clotting activity by making serial doubling dilutions in 0.5 ml. volumes of "coagulase diluent", which contained 25 ml. of nutrient broth, 70 ml. of saline and 5 ml. of "Thiomersal". To these were added 0.5 ml. volumes of one of the following clotting mixtures: citrated human plasma, undiluted or diluted 1 in 10 with buffered saline, pH 7.2; the same preparations containing 40 units per ml. of heparin

("Pularin", Evans Medical Supplies, Ltd.), or a modified fibrinogen-activator mixture (Lominski et al., 1962) containing 1 per cent bovine fibrinogen (bovine plasma fraction I, Armour Pharmaceutical Co. Ltd., Eastbourne) and 0.1 per cent coagulase activator (Morrison, 1952) dissolved in coagulase diluent. The mixtures were examined for clotting after 24 hr. incubation and again after standing for 24 and 48 hr. at room temperature.

Haemolysin production:

Soluble haemolysin preparations were made from cultures grown in semisolid (0.4 per cent) nutrient agar incubated for 48 hr. in an atmosphere of 80 per cent air and 20 per cent gaseous CO_2 (Burnet, 1930). Fluid was expressed from the agar by freezing and thawing, centrifuged, and the cells discarded. Thiomersal was added. Similar preparations were concentrated by precipitating with 3 volumes of ethanol at $0^{\circ}C$ and redissolving the precipitate in buffered saline, pH 7.2, to one tenth of the original volume.

Titration of haemolytic activity:

Serial doubling dilutions of test preparations were made in 0.5 ml. volumes of "haemolysin diluent", containing 25 ml. nutrient broth, 74 ml. of saline and 1 ml. of "Thiomersal". To these were added 0.5 ml. of 2 per cent thrice washed rabbit, sheep or human erythrocytes suspended in saline. After incubation for 1 hr. in a waterbath the end-point was estimated visually; the tube showing 50 per cent haemolysis was

considered to contain 1 minimal haemolytic dose (MHD). The tubes containing sheep erythrocytes were then placed at 4°C and haemolysis estimated again 23 hr. later.

Haemolysin neutralisation:

Commercial staphylococcal alpha toxin (Burroughs Wellcome) and concentrated haemolysin preparations were titrated against rabbit erythrocytes. Serial doubling dilutions of commercial staphylococcal antitoxin (Burroughs Wellcome), starting at a dilution of 1 in 100, were made in 0.5 ml. volumes of haemolysin diluent. To these were added 0.2 ml. volumes of commercial toxin or concentrated haemolysin preparations, diluted to contain 20 MHD per ml; the mixtures were incubated for 1 hr. in a waterbath. Thereafter, 0.3 ml. volumes of 3 per cent thrice washed rabbit erythrocytes suspended in saline were added and incubation continued for a further hour. The end-point, that dilution or antitoxin inhibiting haemolysis completely, was estimated visually. At the same time the commercial toxin and concentrated haemolysin preparations were titrated against a 2 per cent suspension of rabbit erythrocytes to reconfirm their haemolysin content.

Susceptibility to bacteriophage:

Organisms were exposed to routine typing strains of bacteriophage by a standard technique (Williams and Rippon, 1952).

Isolation of bacteriophage from sewage:

Untreated sewage was centrifuged briefly at 3000 r.p.m. Equal volumes of the supernatant (usually 50 ml.) and nutrient broth were mixed, sufficient of a 4 per cent solution of $CaCl_2$ in sterile distilled water added to produce a final concentration of 200 µg. per ml. of $CaCl_2$, and the mixture seeded with 0.2 ml. of an overnight nutrient broth culture of strain Morris. Cultures were incubated overnight at 37° or 30°C, centrifuged and the supernatants filtered through sintered glass filters, average pore diameter 1.3 to 1.5 µ. The supernatants were examined for the presence of 'phage by spotting on to plates seeded with the test strains or by the agar layer technique (Adams, 1959). Supernatants which showed lytic activity were mixed with an equal amount of nutrient broth; $CaCl_2$ was added to the same final concentration, the cultures were reinoculated, incubated and re-examined as described.

Tests of antibiotic sensitivity:

Culture plates of sensitivity test agar ("Oxoid") containing 5 per cent defibrinated horse blood were flooded with an overnight nutrient broth culture and dried for 1 hr. in the incubator. Discs containing known amounts of antibiotic ("Multodisks", Oxoid) were placed on the surface, the plates incubated overnight and thereafter examined for inhibition of growth.

Animal experiments:

Swiss white mice, Porton strain, of 20 to 25 gm. weight and of either sex, were used to assess virulence. Laboratory-bred chinchilla rabbits, 2 to 3 kgm. in weight, of either sex, were used for the production of antisera.

Mouse virulence experiments:

Challenge doses of organisms were prepared by washing the cells from an overnight nutrient broth culture once in broth, and resuspending to the required density in broth. The number of organisms present was estimated by opacity using Brown's tubes, and by viable counts (Miles and Misra, 1938).

Virulence was determined by measuring the dose causing a 50 per cent kill in 10 days in each experimental group, which contained usually 10, but never less than 6, mice.

Production of antisera:

Overnight shake cultures in nutrient broth were centrifuged, the cells washed thrice, suspended in saline, killed by heating at 100°C for 5 min., and resuspended in saline to a concentration of 3×10^9 cells per ml. estimated by opacity using Brown's tubes. Rabbits were bled once from the marginal ear vein and injected intravenously on the first three days of each of three successive weeks with 0.5 ml. volumes of the heat-killed suspension. A fresh suspension was prepared at the beginning of each week. Ten days after the final dose the rabbits were bled from the marginal ear vein and the sera separated and

inactivated. The sera were titrated for agglutinins by preparing serial doubling dilutions in saline, adding an equal volume (usually 0.5 ml.) of a saline suspension of heat-killed cells containing 1 x 10^9 cells per ml., incubating for 1 hr. and standing the tests at room temperature for a further 23 hr. before reading. The highest dilution of serum causing agglutination was estimated visually.

Absorption of sera:

The absorbing strain was grown for 18 hr. in a nutrient broth shake culture, volume 100 ml. The cells were removed by centrifuging, washed thrice and resuspended in saline to a concentration of 4 x 10^{10} cells per ml., estimated by opacity using Brown's tubes. This suspension was heated at 100°C for 5 min. Volumes of 5 ml. of this suspension were centrifuged and the cells resuspended in an equal volume of serum diluted 1 in 2 or 1 in 4 with saline. The mixtures were incubated for 1 hr., centrifuged and the supernatant serum retested for agglutinins. Sera which still agglutinated cells of the homologous strain were reabsorbed.

Specific capsular reaction:

One loopful each of cells from an overnight broth culture, of antiserum and of 1 per cent aqueous methylene blue were mixed on a slide, a coverslip applied and the preparation examined microscopically under reduced light. Similar preparations in which the methylene blue was replaced with india ink were also examined.

Production of non-capsulated variants:

Two methods were used.

1) Serial subcultures of strain Morris were made daily on nutrient agar, to a total of 90 subcultures, and examined for variant colonies.

2) Several portions of nutrient broth, each of 100 ml. volume, were inoculated with material from a single colony of strain Morris cultured on nutrient agar. All the subcultures were incubated. One of them was marked and subcultured daily on to nutrient agar; the resulting cultures were examined for colonies of non-capsulated cocci in thin india ink suspensions. When these appeared, the other subcultures which had been incubated continuously without opening, were similarly examined.

Reversion of the non-capsulated variant:

Experiments were carried out to determine the virulence of the variant D for mice by injecting graded doses intraperitoneally. The same experimental conditions were used as for strain Morris (page 36).

Mice which died during the experimental period (10 days) and those surviving to the end of it were examined for reversion of the non-capsulated variant to the capsulated form. The peritoneal cavity was washed with sterile citrated saline, and a drop of heart blood removed from the left ventricle with a fine-pointed Pasteur pipette. Both samples were examined microscopically and after culture on blood agar for 24 hr.

SECTION I - RESULTS

Characters of strain Morris:

Morphology:

After 24 hr. incubation colonies of strain Morris on nutrient agar were 2 to 3 mm. in diameter, translucent, domed, with an entire edge (Figure I.1) and a mucoid but not sticky consistency, emulsifying easily in saline. Most cultures on ordinary agar were greyish-white in colour; on blood agar or carbohydrate-containing media colonies developed a greyish-yellow pigmentation which became more obvious when the cultures were allowed to stand at room temperature. After prolonged incubation on all solid media the colonies became watery and spread over the surface of the agar.

When cultured in nutrient broth for 24 hr. the strain produced a smooth even suspension, with no pellicle formation and a large, mucoid deposit which was easily resuspended.

Smears of overnight broth or nutrient agar cultures stained by Gram's method contained Gram-positive cocci, average diameter 1 μ, arranged singly, in pairs and small clumps; clumps were rarely seen in broth cultures. All the cells were surrounded by a distinct capsule, of average thickness 0.8 to 1.2 μ. (Figure I.2). Pairs and clumps of cells were enclosed in a common capsule. Loose slime was not seen. The capsule was evident about all cells, at all stages of the growth cycle, irrespective of the medium used for culture, or the

FIGURE I.1

18-hour nutrient agar culture

of strain Morris (x 1)

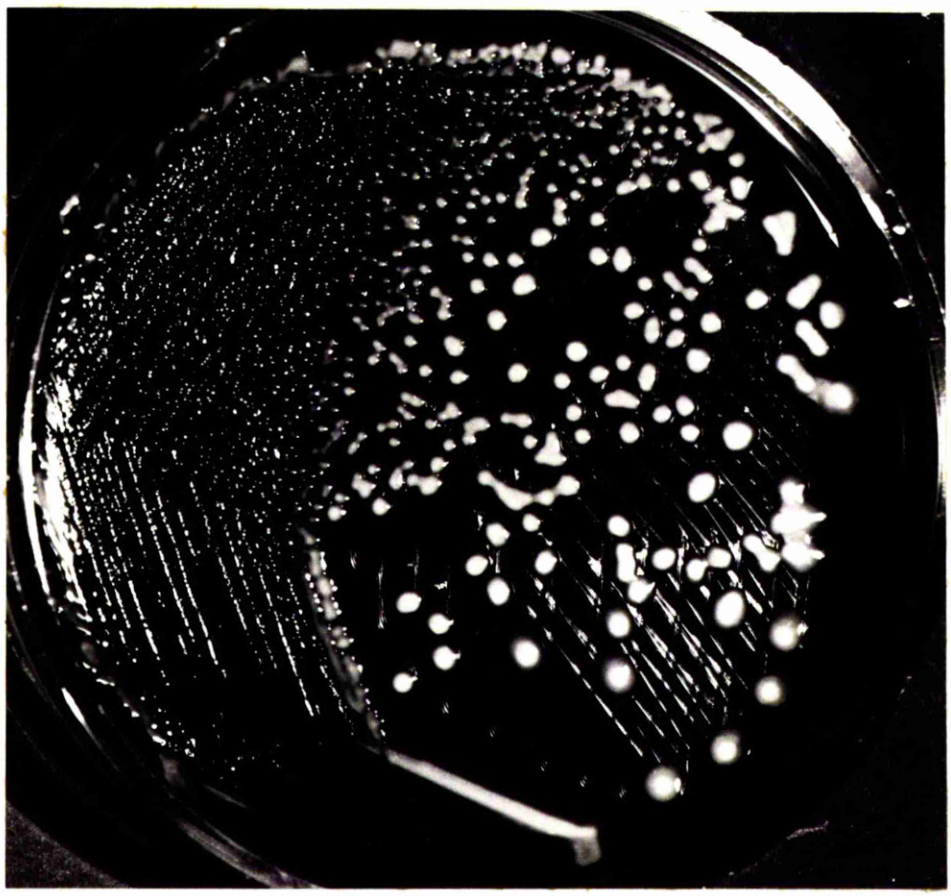
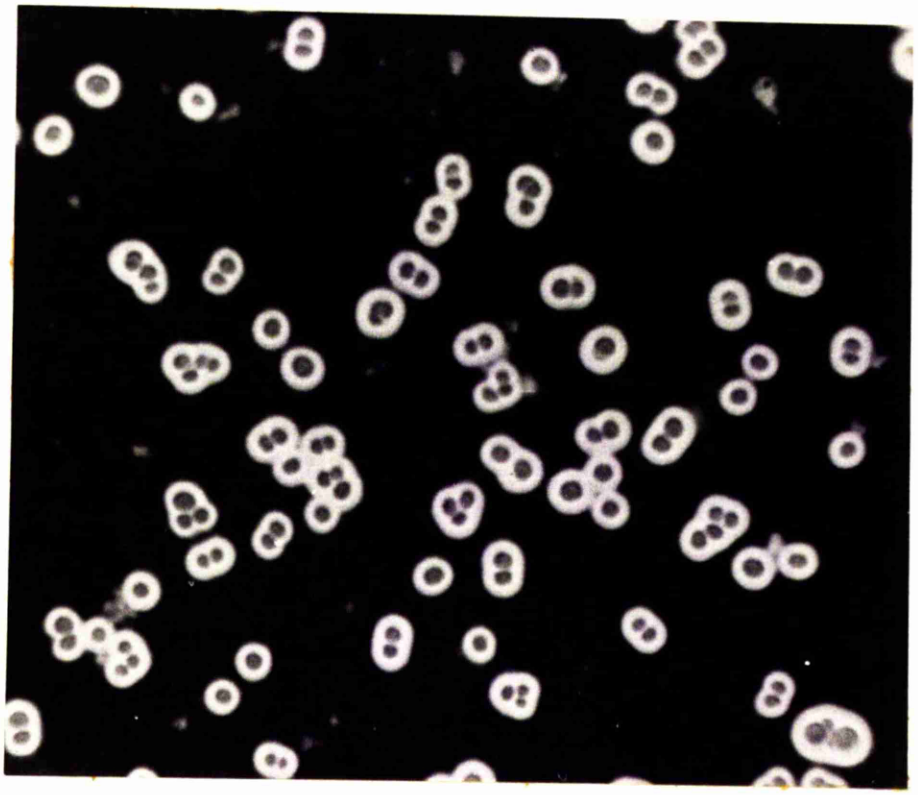


FIGURE I.2

Cells of strain Morris from a 24 hr. nutrient agar culture stained with rose-bengal and suspended in india ink (x 1500)



temperature of incubation.

Physiological and biochemical characters:

Strain Norris grew well at 37°C on all the culture media used. Growth took place at 22°C and 42°C but was less profuse. The strain was catalase positive. It utilised glucose aerobically and anaerobically with the production of acid, and fermented maltose with acid production. It did not attack mannite and lactose. It liquefied gelatin, produced phosphatase, acetoin and a final pH in glucose broth of 4.8. It grew in human serum and in the presence of 6 per cent NaCl.

Coagulase production:

The strain was bound-coagulase-negative. When first isolated it clotted dilute citrated human plasma in the Fisk test. Examination of shake culture supernatants showed that their clotting activity was maximal after 23 hr. incubation. The titre of concentrated 23 hr. shake culture supernatants tested against different clotting mixtures is shown in Table I.1.

Table I.1

The clotting activity of concentrated shake culture supernatants of strain Morris

Clotting mixtures:	Titre:
Human plasma	1:64
Human plasma with heparin	1:64
Diluted human plasma	1:64
Diluted human plasma with heparin	1:64
Bovine fibrinogen (1 per cent) with activator (0.1 per cent)	1:128

Haemolysin production.

The haemolytic activity of concentrated culture supernatants is shown in Table I.2. In neutralisation experiments the same amount of commercial antitoxin neutralised 6.25 MHD of commercial staphylococcal alpha toxin and 6.4 MHD of strain Morris rabbit erythrocyte lysin.

Table I.2.

The haemolytic activity of concentrated culture supernatants of strain Morris

Red cells tested:	Titre:	
	1 hr. at 37°C	after 23 hr. at 4°C
Rabbit	1:64	-
Sheep	1:2	1:2
Human	1:32	-

Susceptibility to bacteriophage:

Strain Morris was not lysed by routine typing 'phages applied at routine test dilution or 1000 RTD. Some sewage samples, seeded with strain Morris, incubated and filtered, produced large zones of bacteriolysis on surface or agar layer plates. Attempts to propagate the active agent from these areas by standard techniques were unsuccessful. This phenomenon is being further studied.

Animal virulence:

The viable count of suspensions of strain Morris varied from one half to one fifth of the total count measured by opacity, possibly because of clumping of cells in different cultures and the breaking up of the clumps while preparing suspensions for injection. Within these variations, the LD50 for mice challenged intraperitoneally was 5×10^6 colony-forming units.

Antibiotic sensitivity:

Strain Morris was found to be sensitive to penicillin, ampicillin, tetracycline, chloramphenicol, erythromycin, cloxacillin, streptomycin, cephaloridine, fucidin, lincomycin, soframycin and neomycin, and was resistant to sulphonamide, colistin and polymyxin B.

Specific capsular reaction:

Cells of strain Morris suspended in homologous antiserum with an agglutinin titre of 1:256 gave a rapid specific capsular reaction.

Isolation and characters of non-capsulated variants:

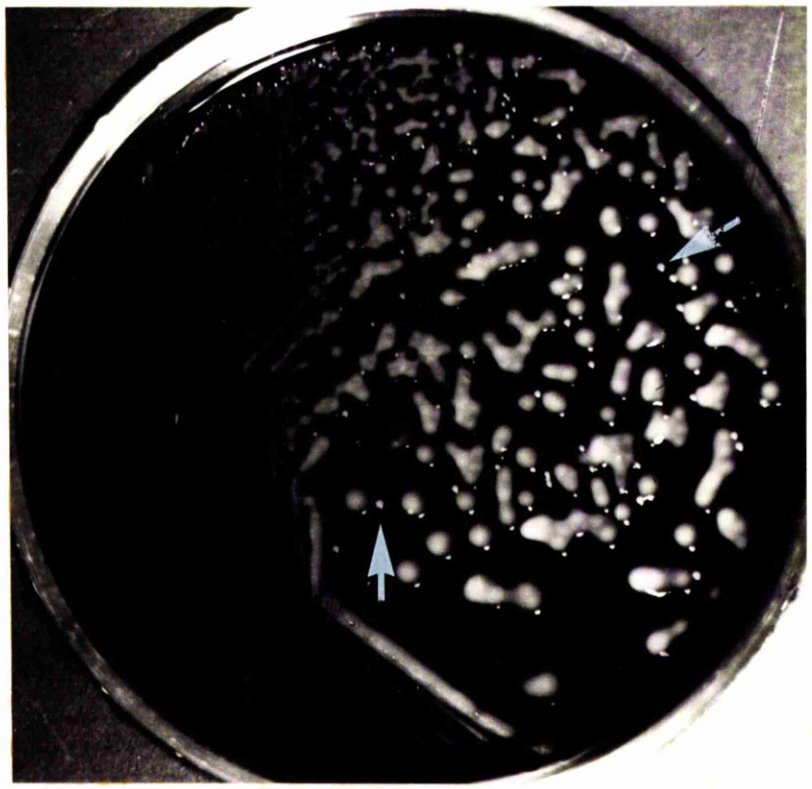
Attempts to isolate non-capsulated variants by serial daily subculture on nutrient agar were unsuccessful after 90 subcultures. However, subculture of 28-day-old static broth cultures contained numerous colonies, morphologically distinct from those of the parent strain, which were found to be composed of non-capsulated organisms. One variant, named strain D, was studied exclusively.

Apart from the absence of a capsule, strain D differed from strain Morris in three major respects: it was bound coagulase positive; it was susceptible to standard 'phages, typing at 1000 RTD as a group I strain, 'phage type 52/52A/80/81; and it was about one hundred times less virulent for mice on intraperitoneal injection, the LD 50 being about 6.5×10^8 C.F.U.

The LD 50 of strain D could not be assessed accurately. Using a dose of less than 1×10^8 c.f.u. none of the challenged animals died, and less than half died when the dose was increased to 4×10^8 c.f.u. With doses of 6.5×10^8 c.f.u. more than half of each challenged group died, but at this dose level, and the preceding one, examination of dead or moribund mice demonstrated in each instance the presence of capsulated cocci in either heart blood or peritoneal washings, indistinguishable from strain Morris on subsequent testing. (Figure I.3). Such organisms were not found in animals surviving challenge. The LD 50 of the variant D therefore lay at or above the dose level at which reversion to the capsulated parent type took place. The fact that this took place with doses in excess of 1×10^8 c.f.u.

FIGURE I.3

18 hr. blood agar culture of saline washings from the peritoneum of a mouse challenged with 6.5×10^8 c.f.u. of the non-capsulated variant D, showing colonies of the variant (arrowed) and of strain Morris (large colonies) (x 1)



suggests that reversion was due to mutation.

Other differences between strain D and strain Morris were noted. Colonies of the variant were smaller, 1 to 2 mm. in diameter after 24 hr. incubation on blood agar, opaque, grey in colour and non-mucoid or "cheesy" in consistency (Figure I.3). Strain D produced a granular growth in broth with a small surface pellicle.

In all other respects tested the variant behaved like the parent strain and was apparently identical in its physiological and biochemical characters, in its production of coagulase and haemolysins, and its sensitivity to antibiotics.

The serological relation of strain Morris and strain D:

The results of homologous and cross-agglutination tests with antisera raised in rabbits are shown below in Table I.3.

Table I.3

The serological relation between strain Morris and strain D

Antiserum to:	Agglutinin titre against:	
	Strain Morris	Strain D
Strain Morris	1:256	1:256
Strain D	1:2	1:500
Strain Morris absorbed with cells of Strain D	1:256	<1:2
Strain D absorbed with cells of Strain Morris	<1:2	<1:2

These results were interpreted as showing that antiserum to strain Morris contained antibody to both the capsule and to cellular antigens, whereas antiserum to the variant D contained antibody to cellular antigens only.

DISCUSSION

The examination of the capsulated strain Morris described in this section was undertaken to determine whether it represents a separate species of Staphylococcous or whether it is to be classified as a strain of Staphylococcous aureus.

Attempts to classify a single strain highlight one of the common problems of bacterial taxonomy. Although it is possible from the statistical analysis of a large number of strains to state the probability of any one strain reacting in a given way in a particular test, it is often difficult to place a test strain in an appropriate taxon if it does not comply exactly with the modal form. With strains of staphylococci the difficulty is enhanced because there is considerable disagreement about how many tests, or even which tests, should be employed to identify the modal form of Staph. aureus. This disagreement stems not only from the apparently close relationship of subgroups within this genus, but also in part at least from the unwillingness of medical bacteriologists to relinquish a classification giving weight to criteria of potential pathogenicity in favour of an Adansonian approach.

Despite these difficulties strain Morris is considered to be a strain of Staph. aureus. Apart from the ability to utilise glucose anaerobically, to produce phosphatase, and to grow in the presence of 6 per cent NaCl and in human serum, it possesses two characters which are usually accepted as typical of this species: production of

coagulase, and production of a rabbit erythrocyte lysin indistinguishable serologically from staphylococcal alpha toxin. In six respects it does not behave like Staph. aureus. It is frankly capsulated; it produces colonies with an unusual morphology and of unusual consistency; it is bound coagulase negative; it is not susceptible to standard 'phages; it is more virulent than most ordinary strains when injected intraperitoneally into mice, and it does not ferment mannite. Failure to ferment mannite need not preclude its placing in this species since carbohydrate fermentation by Staph. aureus is characteristically unreliable as a taxonomic criterion (Shaw, Stitt and Cowan, 1951; Baird-Parker, 1963).

The remaining features (abnormal colonial morphology; failure to react with plasma in the slide test; resistance to 'phage, and increased virulence for mice) can be related to the presence of the capsule. In this respect strain Morris is similar to strain Smith and the related "Smith-like" strains. The diffuse variants of these, with a serologically and possibly morphologically demonstrable extra layer on their surfaces, are also bound coagulase negative, are usually unaffected by standard 'phages, produce in some cases recognisably different colony types, and are usually more mouse-virulent than both their compact variants and ordinary strains. That the capsule of strain Morris is indeed responsible for its abnormal reactions is shown by the reactions of the derived non-capsulated variant, D, which is susceptible to 'phage, bound coagulase positive, much less virulent for

mice, and which produces colonies indistinguishable from those of ordinary strains of Staph. aureus.

Attempts to decide if strain Morris is similar to the three mucoid strains previously described, and if these should also be classified as Staph. aureus, are hampered by lack of evidence. Gilbert's (1931) strain fermented glucose but not mannite, liquefied gelatin, and was haemolytic on blood agar. (The red cell type was not specified). Coagulase production and susceptibility to 'phage were not tested. However, the assumption is implicit in Gilbert's report that the strain described is a "typical staphylococcus", i.e. Staph. aureus, despite the lack of evidence which would allow it to be so classified by modern criteria.

Oesterle (1936) states explicitly that the non-capsulated variant of his strain had "the biological properties of the typical normal form of Staph. pyogenes aureus". This view is based on the ability of the strain to ferment glucose, liquefy gelatin, and produce coagulase; although it lysed sheep, horse, ox and human erythrocytes, it did not affect rabbit erythrocytes and hence presumably did not produce alpha toxin. Henriksen (1948) describes his capsulated strain as Staph. aureus on the evidence that it fermented glucose and mannite, liquefied gelatin, and produced coagulase.

On balance, therefore, although the evidence is scanty, there are sufficient similarities between these strains, strain Morris and the ordinary form of Staph. aureus to suggest that they can be grouped together as a single species. Certainly there is not enough evidence

to demand that a separate species be created to accommodate them.

The mucoid strains differed in the ease with which they produced non-capsulated variants. Gilbert's strain, after storage in a refrigerator, dissociated to yield non-capsulated variants which were less virulent than the parent strain. Oesterle also produced non-capsulated variants (by prolonged culture in bile) but did not test their virulence. Henriksen's strain was unusual in that it remained stable for two years, although dissociation was only apparently sought by sub-culturing on solid media. This strain may therefore be similar to strain Morris which was apparently stable when examined grossly and microscopically during repeated serial subculture on solid media, but which produced non-capsulated variants when aged in broth.

As already noted, the non-capsulated variant of strain Morris reverted to the parent type when injected into mice. Reversion was only noted when challenge doses in excess of 1×10^8 c.f.u. were injected, suggesting that the change to the parent type was due to mutation. Oesterle (1936) and Henriksen (1948) do not state if their strains reverted to the parent form; Gilbert (1931) notes, however, that her strain "occasionally" reverted to the capsulated type when injected into a guinea pig.

The apparently low rate of interchange between capsulated and non-capsulated types shown by these strains contrasts with the apparent ease with which strain Smith and the related strains dissociate. The compact (non-capsulated) and diffuse (capsulated) types interchange readily, subcultures from a single colony of one

type showing several colonies of the other under ordinary cultural conditions (Hunt and Moses, 1958). From limited personal experience with one "Smith-like" strain in which the two variants could be recognised by colonial morphology, it appears that in single colonies one cell in approximately every 300 is of the opposite type (Scott, unpublished observations): a frequency of interchange which must be classed as an example of phase variation rather than mutation.

Rogers (1962) and Koenig and Melly (1965) have suggested that ordinary strains of pathogenic staphylococci, when growing in vivo, may produce a surface antigen similar to that of strain Smith, but lose the capacity to produce it when cultured on laboratory media. While there is as yet no direct experimental proof that this suggestion is correct, it has certain parallels in other bacterial species. Thus, strains of Pasteurella pestis, indistinguishable in the laboratory from avirulent strains, become capsulated and able to resist phagocytosis in the animal body (Burrows and Bacon, 1954). Protective surface antigens are rapidly lost when Bordetella pertussis is cultured in the laboratory, and Bacillus anthracis elaborates a powerful toxin in vivo but not in vitro (Smith, Keppie and Stanley, 1955). Louria and Kaminska (1963) have shown that immunisation of mice with live vaccines composed of ordinary coagulase-positive staphylococci may protect against challenge with the diffuse variant of strain Smith, and Pereira (1961) has shown that staphylococci lose agglutinogens when subcultured repeatedly in the laboratory.

If ordinary coagulase-positive staphylococci do elaborate extra surface antigens when growing *in vivo*, then this implies at least a threefold classification of staphylococci with respect to capsulation: the rare mucoid strains, consistently producing large amounts of capsular material *in vivo* and *in vitro*, and interchanging with the non-capsulated form at a very low rate; the "Smith-like" strains which also continue to produce capsular material *in vitro* but which revert more readily to the non-capsulated form; and ordinary strains of *Staph. aureus* which do not produce visible capsular material *in vitro* but may do so when growing in animal tissues. Further investigation is necessary to determine if these three groups form part of a continuous or discontinuous series.

SUMMARY

A capsulated staphylococcus is described. Apart from the presence of a large capsule, this has many of the properties of Staphylococcus aureus.

The capsulated strain differs from the modal form of Staph. aureus in four major respects. These can be explained by the presence of a thick capsule.

Non-capsulated variants, produced by prolonged culture in nutrient broth, are indistinguishable from ordinary strains of Staph. aureus. The variants reverted to the parent form after passage in mice.

The capsulated strain is similar in many respects to three strains of capsulated staphylococci which have been previously described. It is believed that these strains should also be regarded as unusual forms of Staph. aureus.

SECTION II

A CAPSULE-STRIPPING ENZYME AND THE STAPHYLOCOCCUS PRODUCING IT

MATERIALS AND METHODS

Organism:

The organism, named staphylococcus strain LS ("lytic staphylococcus") was discovered as a contaminant on a nutrient agar plate which had been heavily seeded with strain Morris and incubated overnight.

It was examined and characterised by the methods used for strain Morris (pages 31 to 34).

Preparation of active culture supernatants:

Shake cultures were prepared by warming 200 ml. of nutrient broth to 37°C, inoculating with 1 ml. of an overnight static nutrient broth culture of strain LS and incubating the resulting culture on an orbital shaker. Incubation was continued for 6½ hr; the culture was then centrifuged. The cells were discarded and "Thiomersal" added to the supernatant. These preparations are referred to as "enzyme". Some supernatants, prepared in the same way, were precipitated with 3 volumes of ethanol in the cold and the precipitate redissolved (to one tenth of the volume of the original culture) in buffered saline, pH 7.2; "Thiomersal" was added. These preparations are referred to as "concentrated enzyme".

Methods of demonstrating the effect of strain LS on the growth of strain Morris in mixed cultures on solid media:

Three methods were used: 1) overnight broth cultures of the two strains were mixed, plated out on nutrient agar and incubated; 2) nutrient agar plates were heavily seeded with strain Morris and then stab-inoculated with material from a colony of strain LS; 3) strain LS was inoculated on to nutrient agar as a single streak and the plate incubated overnight. The resulting growth was scraped off with a sterile slide, the plate exposed to chloroform vapour for 1 hr., and dried in an incubator for a further hour. It was then re-inoculated with a single streak of strain Morris at right angles to, and across, the site of growth of strain LS. The plate was incubated overnight and examined.

Method of demonstrating the effect of enzyme preparations of strain LS on the growth of strain Morris on solid media:

Nutrient agar plates were flooded with an overnight nutrient broth culture of strain Morris, the excess fluid removed, and the plates dried for 2 hr. in an incubator. Enzyme preparations were serially diluted in sterile saline. Volumes of 0.02 ml. of each dilution were placed on the surface of the inoculated plates; these were incubated for 18 hr. and examined.

Method of demonstrating the effect of strain LS on heat-killed cells of strain Morris on solid media:

Cells of strain Morris from an 18 hr. shake culture in nutrient broth were washed thrice, resuspended in saline and heated at 100°C for 5 min. They were then added to molten nutrient agar at 40°C to a concentration of 2×10^9 cells per ml. measured by opacity using Brown's tubes. The agar was poured immediately into Petri dishes, allowed to set, and dried for 1 hr. in an incubator. Thereafter the plates were either inoculated with strain LS or volumes of 0.02 ml. of an enzyme preparation, serially diluted with saline, were placed on the surface and allowed to dry in to the medium. The plates were then incubated overnight and examined.

Method of demonstrating the effect of enzyme preparations of strain LS on cells of strain Morris in suspension:

In preliminary experiments both crude and concentrated enzyme preparations caused a rapid fall in the turbidity of suspensions of live and heat-killed cells of strain Morris. Attempts were made to measure this fall with a spectrophotometer (Hilger and Watts, Model H.700). These were unsuccessful because with some very active preparations the fall was so rapid that it was difficult to obtain readings; the time necessary to transfer samples to cuvettes made it impossible to obtain readings which represented accurately the turbidity of the samples immediately after mixing; the continuous transfer of samples from cuvettes to test tubes in a waterbath and back again produced too much variation in the temperature of the samples, and the time necessary to "zero" and set the spectrophotometer allowed

sedimentation of the cells to begin and caused considerable variation in the readings given by control suspensions.

The following method, using a nephelometer (Evans Electroselenium, Ltd., Harlow, Essex; Model A) was therefore devised.

a) preparation of standard test cell suspensions:

An 18 hr. shake culture of strain Morris in nutrient broth was centrifuged, the cells washed thrice and resuspended in buffered saline, pH 7.5, to a concentration of 10^9 cells per ml. by opacity using Brown's tubes. These preparations are referred to as "live cell suspensions". "Heat-killed suspensions" were prepared in the same way except that the cells were heated at 100^0C for 5 min. before being suspended in buffered saline.

In some experiments buffered saline of different pH, within the range 6.0 to 9.0, was used. "Thiomersal" was added to some suspensions.

b) calibration of the nephelometer:

An 18 hr. shake culture of strain Morris in nutrient broth was centrifuged, the cells washed thrice and resuspended in sterile buffered saline, pH 7.5, to a concentration of 10^{10} cells per ml. by opacity. By tenfold dilution of this suspension with buffered saline a series of suspensions was prepared containing from 10 to 10^{10} cells per ml. The turbidity of each suspension was measured twice in the nephelometer; once with the nephelometer

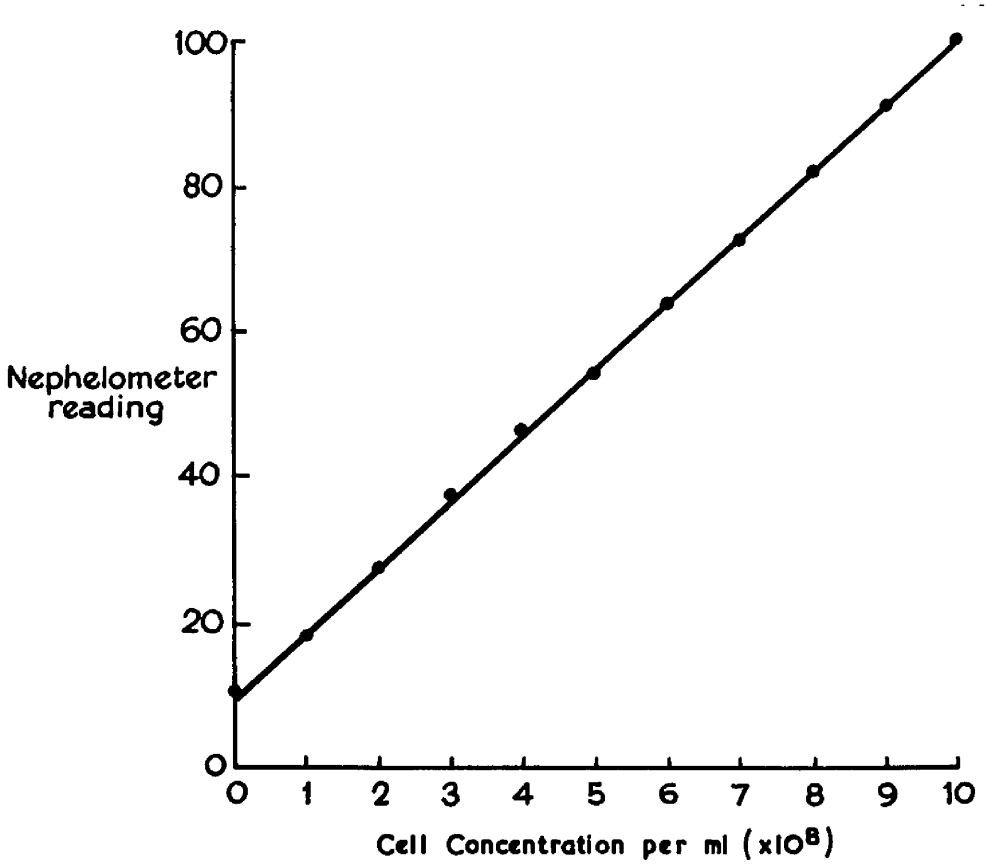
set to record zero when measuring buffered saline alone, and again with the nephelometer set to record 100 scale units when measuring the suspension containing 10^{10} cells per ml. The nephelometer was most sensitive over the range 10^{8} to 10^{9} cells per ml. It was decided to work within this range. The nephelometer was therefore re-calibrated. Ten suspensions containing between 10^{8} and 10^{9} cells per ml. were prepared by stepwise dilution, the nephelometer set to record 100 scale units when measuring the suspension containing 10^{9} cells per ml., and the turbidity of each suspension measured. Within the range 10^{8} to 10^{9} cells per ml. the turbidity recorded was directly proportional to the number of cells present (figure II.1).

c) assay of active supernatants of strain LS:

Volumes of 9.5 ml. of either live or heat-killed cell suspensions were placed in a series of matched tubes. To one tube was added 0.5 ml. of buffered saline, the tube stoppered and the contents mixed thoroughly. Using this suspension as a control, the nephelometer was set to record 100 scale units; the control tube was then incubated in a waterbath. Culture supernatants were examined for activity by adding 0.5 ml. volumes to 9.5 ml. volumes of cell suspension in matched tubes, mixing rapidly, measuring the turbidity of the mixture without altering the setting of the nephelometer and incubating the tubes in a waterbath. These manipulations were carried out as quickly as possible. During incubation, which was usually

FIGURE II.1

Calibration curve of the nephelometer (E.E.L., Model A) showing the response to suspensions of cells of strain Morris containing between 10^8 and 10^9 cells per ml



continued for 30 min., and in some experiments for an hou. or longer, the turbidity of control and test suspensions was measured every 5 min. after thorough mixing of the contents of each tube. A second control tube, containing 10 ml. of buffered saline was examined in the same way.

d) definition of activity:

The measured turbidity of control live and heat-killed cell suspensions, without enzyme, fell during incubation usually by 3 to 5 nephelometer scale units during 20 to 30 min. Neither the speed of this fall nor its extent were altered by altering the pH of the suspending buffer within the range 5.0 to 8.5, even when the time of incubation was extended to 80 min. This apparent decrease in the turbidity of control suspensions was found to be due to instrumental drift; the measured turbidity of a control glass "standard", exposed in the nephelometer for 1 hr., showed a similar decrease. Culture supernatants were considered to be active if, during 30 min. incubation, they caused any fall in the turbidity of live or heat-killed cell suspensions which was greater by at least 3 scale units than any fall in the turbidity of the control suspension under the same conditions. This fall, representing 3 per cent of the starting turbidity, is equivalent to the loss or destruction of 3×10^7 cells.

Measurement of the strength of active preparations:

Serial doubling dilutions of each test preparation were made in buffered saline, pH 7.5, and 0.5 ml. volumes of each dilution tested for activity against live and heat-killed suspensions during 30 min. incubation as described above.

Definition of activity:

Units of activity:

The highest dilution to show activity after 30 min. incubation was considered to contain 1 unit of enzyme.

Rate of clearance:

Clearance of both live and heat-killed cell suspensions by enzyme lasted for at least 10 min. in each experiment. The ratio: Fall in turbidity/Time during this period could be used to represent the activity of enzyme in any given experimental situation. This ratio is referred to as the "rate of clearance" and is defined as the average fall in turbidity per minute, in nephelometer scale units, during the first ten minutes incubation of cell suspension and test preparation.

Assessment of the relation between the culture age of strain LS and the enzyme activity of the culture supernatant:

A shake culture of strain LS was prepared by warming 200 ml. of nutrient broth to 37°C and inoculating with 1 ml. of an overnight static nutrient broth culture of strain LS. Immediately thereafter,

and at measured intervals during incubation for 24 hr., portions of 5 ml. were removed. The number of viable organisms present in each was counted by the technique of Miles and Misra (1938). Each sample was then centrifuged and the cells discarded. The pH of each supernatant was measured, and the activity of each assayed as described.

Testing the effect of pH on the activity of the enzyme:

Live and heat-killed cell suspensions were prepared in saline at a concentration of 2×10^9 cells per ml. To 5.0 ml. volumes of each of these in matched tubes was added 4.5 ml. of isotonic phosphate buffer of different pH, and 0.5 ml. of enzyme diluted in saline to contain 8 units per ml; the effect of enzyme on turbidity was measured as described.

Testing the effect of heat on the activity of the enzyme:

Concentrated enzyme, containing 32 units per ml., was diluted 1 in 4 with buffered saline pH 7.5. Matched test tubes were placed in a waterbath; to each of these was added 0.5 ml. of diluted supernatant. After heating for different intervals at different temperatures, pairs of tubes were removed and the contents tested for activity against live and heat-killed cell suspensions. Unheated diluted preparations served as controls.

Testing the effect of trypsin on the activity of the enzyme:

Concentrated enzyme containing 32 units per ml. was diluted 1 in 4 in buffered saline, pH 7.5, with and without 1 mg. crystalline pancreatic trypsin (British Drug Houses, Ltd.) per ml. The mixtures were incubated in a waterbath. At intervals, 0.5 ml. portions were removed and tested for activity. Phosphate buffer, pH 7.5, containing 1 mgm. trypsin per ml. served as a control.

Testing the effect of known chemicals on the activity of the enzyme:

The following substances were dissolved in saline to a concentration of M/20: iodoacetic acid; iodoacetamide; chloroacetamide; suramin ("Antrypol", Imperial Chemical Industries), cysteine hydrochloride (Roche Products, Ltd., Welwyn Garden City) and 2-3-dimercaptopropanol (British Anti-Lewisite, "B.A.L.": L. Light & Co., Colnbrook).

Serial doubling dilutions of each solution were prepared in buffered saline, pH 7.2, and 0.5 ml. volumes of each were added to 9.0 ml. volumes of either killed or live cell suspension. After mixing, 0.5 ml. volumes of concentrated enzyme diluted in saline to contain 8 units per ml. were added; the turbidity of the mixtures was measured immediately and at intervals during incubation in a waterbath. Two control preparations were made: a tube containing 9.0 ml. of cell suspension, 0.5 ml. of buffered saline and 0.5 ml. of enzyme; and a tube containing 9.0 ml. of cell suspension and 1.0 ml. of buffered saline. Experiments were also carried out in which 0.5 ml. volumes of enzyme and each dilution of enzyme

inhibitor were incubated for 30 min. before being added to the cell suspensions.

Method of testing the effect of repeatedly adding live cells of strain Morris to enzyme:

An 18 hr. shake culture of strain Morris in nutrient broth was centrifuged, the cells washed thrice and resuspended in two portions of buffered saline, pH 7.5, to concentrations of 5×10^{10} cells per ml. and 0.5×10^{9} cells per ml. respectively. The cell concentrations were measured by opacity using Brown's tubes. To 9.5 ml. of the suspension containing 0.5×10^{9} cells per ml. was added 0.5 ml. of enzyme preparation containing 8 units per ml; the turbidity of the mixture was measured immediately and at intervals of 5 min. during incubation in a waterbath. Incubation was continued until for 10 min. no further fall in turbidity took place. At this time 0.1 ml. of the suspension containing 5×10^{10} cells per ml. was added, the turbidity measured and the mixture re-incubated until, again, no further fall in turbidity took place during 10 min. observation. Four further additions of the dense cell suspension were made in this way, and their effects measured in the nepholometer.

Method of testing the effect of increasing concentration of enzyme on its reaction rate with live cell suspensions:

A live cell suspension of strain Morris was prepared in buffered saline, pH 7.5, to contain 2×10^{9} cells per ml. Eight,

increasing, volumes of concentrated enzyme containing from 10 to 80 units of activity and previously warmed to 37°C were placed in separate matched tubes, and enough warmed buffered saline, pH 7.5, added to bring the volume in each tube to 5 ml. To each tube was added 5 ml. of live cell suspension also warmed to 37°C. The turbidity of the mixtures was measured immediately after mixing, and at intervals during incubation in a waterbath for 1 hr.

To determine if enzyme lysis of live cells is due to an autolysin:

A suspension of live cells of strain Morris in buffered saline, pH 7.5, containing 1 x 10^9 cells per ml. by opacity was divided into two portions. One of these was heated at 100°C for 5 min. A volume of 9.5 ml. of the unheated live cell suspension was placed in one of three matched tubes and the same volume of the heat-killed suspension in the remaining tubes. To each of the three tubes was added 0.5 ml. of a concentrated enzyme preparation containing 8 units per ml. The mixtures were incubated in a waterbath until, during a period of 10 min., no further fall in turbidity took place. Both tubes containing heat-killed cells were centrifuged and the cells washed thrice in buffered saline, pH 7.5. The cells in one tube were resuspended in 9.5 ml. of buffered saline at the same pH, and to this was added 0.5 ml. of the enzyme preparation. The cells in the second tube were resuspended in the enzyme-lysed live cell preparation. The mixtures were reincubated for 30 min. and their turbidity measured at intervals of 5 min.

A second experiment was carried out to test the effect of enzyme-lysed live cells on the turbidity of heat-killed cells which had not first been exposed to enzyme. Suspensions of live and heat-killed cells of strain Morris were prepared in buffered saline to contain respectively 1 x 10^9 and 1 x 10^{10} cells per ml. by opacity. To a sample of 9.5 ml. of the live cell suspension was added 0.5 ml. of a concentrated enzyme preparation diluted to contain 8 units per ml; the mixture was incubated until, during a period of 10 min., no further fall in turbidity took place. To the mixture was then added 1.0 ml. of the heat-killed suspension. The tube was incubated and the turbidity measured at intervals. A control tube containing 8.5 ml. of buffered saline, 1 ml. of heat-killed suspension and 0.5 ml. of enzyme was similarly examined.

Method of testing the effect of increasing exposure to heat on the susceptibility of cells of strain Morris to enzyme:

a) the effect on whole cells:

Volumes of 10 ml. of a washed live cell suspension of strain Morris in buffered saline, pH 7.5, containing 1 x 10^9 cells per ml. by opacity were placed in sterile plugged test tubes and heated in a waterbath at 60°C. At measured intervals tubes were removed and cooled in running tapwater. The viability of the cells after heating was tested by transferring 0.5 ml. volumes from each tube to tubes of Robertson's meat medium and incubating the resulting

cultures for 1 week. The number of colony-forming units in the unheated suspension and in suspensions heated for 3 and 9 min. was estimated by the method of Miles and Misra (1938). The susceptibility to enzyme of the remaining cells was tested by adding to each tube 0.5 ml. of an enzyme preparation containing 8 units per ml. and measuring the turbidity of the mixtures during incubation for 80 min. After incubation two samples were removed from each tube, stained by Gram's method or with nigrosin and rose-bengal, and examined microscopically.

b) the effect on disrupted cells:

An 18 hr. nutrient broth shake culture of strain Morris, volume 200 ml., was centrifuged, the cells washed thrice, and resuspended in saline to a concentration of 4×10^{10} cells per ml. by opacity. The suspension was divided into two equal portions; one of these was heated at 100°C for 5 min. Both suspensions were shaken for 5 min. with sterile Chance No. 12 ballotini in a Braun disintegrator, cooled during working with gaseous CO_2. After this treatment the preparations were centrifuged for 5 min. at 2000 rpm to deposit coarse debris and intact cells; examination of Gram-stained smears of the supernatants at this stage revealed much Gram-negative debris but very few recognisable cocci. The disrupted cells were washed twice and resuspended to the same volume in sterile buffered saline, pH 7.5. The unheated preparation was divided into two equal portions; one of these was heated at 100°C for 5 min.

Volumes of 9.5 ml. of each of the three disrupted preparations were placed in matched tubes and to each was added 0.5 ml. of a concentrated enzyme preparation diluted to contain 8 units per ml. The turbidity of the mixtures was measured immediately and at intervals during incubation for 100 min, the nephelometer being set to record 100 scale units when measuring the turbidity of a control tube containing 0.5 ml. of buffered saline and 9.5 ml. of the disrupted cell preparation which had been heated after disruption.

Testing the effect of sucrose on the turbidity of suspensions of live and heat-killed cells of strain Morris:

Live and heat-killed suspensions were prepared in buffered saline, pH 7.5, to contain 1 x 10^{10} cells per ml. by opacity using Brown's tubes. A solution of sucrose, 100 per cent w/v, in buffered saline, pH 7.5, was diluted stepwise with buffered saline to give a range of dilutions containing from 10 to 100 per cent sucrose w/v. To 9.0 ml. volumes of each solution in matched tubes was added 1 ml. of either the live or heat-killed suspension. The contents were mixed, their turbidity and pH measured and compared with the turbidity and pH of the same number of cells in buffered saline without sucrose before and after incubation in a waterbath for 30 min.

At the end of this time a loopful of each mixture was removed, suspended in india ink, and examined microscopically. The mixtures

were then centrifuged at 5000 rpm for 20 min. and the supernatant fluid replaced by 10 ml. of buffered saline, pH 7.5. After resuspending the cells the turbidity of each suspension was measured again.

Testing the effect of 0.7M sucrose on the turbidity of different concentrations of live cells of strain Morris:

Washed live cells of strain Morris from an 18 hr. shake culture in nutrient broth were suspended in buffered saline, pH 7.5, to a concentration of 1×10^{10} cells per ml. by opacity. By dilution of this suspension with buffered saline and a buffered solution of sucrose (20 per cent w/v, 0.7M) suspensions were prepared containing increasing concentrations of cells within the range 2×10^{8} to 1×10^{9} cells per ml. The nephelometer was set to record 100 scale units when measuring the suspension in buffered saline containing 1×10^{9} cells per ml., and the turbidity of all the suspensions measured.

Testing the effect of enzyme on live cells of strain Morris in a buffered hypertonic solution of sucrose:

A suspension of live cells of strain Morris was prepared in buffered saline, pH 7.5, to contain 1×10^{10} cells per ml. by opacity using Brown's tubes. Volumes of 1 ml. of this were added to 8.5 ml. volumes of buffered saline, pH 7.5, and a 20 per cent w/v (0.7M) solution of sucrose in buffered saline at the same pH.

After mixing, 8 units of enzyme in a volume of 0.5 ml. were added to each tube, and the turbidity of both mixtures measured at intervals during incubation. Control tubes, in which enzyme was replaced by either buffered saline or buffered sucrose solution, were similarly examined.

RESULTS

Characters of staphylococcus strain LS:

Morphology:

Colonies of strain LS on nutrient agar after 24 hr. incubation were 1 mm. in diameter, opaque and grey in colour. On horse-blood agar colonies were surrounded by a distinct zone of complete haemolysis. In broth the strain produced granular growth with a small surface pellicle and a granular deposit which was easily resuspended.

In Gram-stained smears of broth cultures the cells appeared as Gram-positive cocci, average diameter 1 μ, arranged in small clumps.

Physiological and biochemical characters:

The strain was catalase-positive. It utilised glucose anaerobically and fermented sucrose, lactose and maltose but not mannite. It liquefied gelatin. It grew well on all media tested, in the temperature range 22 to 42°C; growth was most luxuriant at 37°C. It grew well in human serum and on agar containing 6 per cent w/v NaCl, produced phosphatase, acetoin, and a final pH in glucose broth of 4.9.

Coagulase production:

Strain LS clumped in the slide test for bound coagulase. It did not clot citrated human plasma in the Fisk test for soluble coagulase. However, concentrated culture supernatants at a dilution of 1 in 128

did clot a mixture of 1 per cent bovine fibrinogen and 0.1 per cent coagulase activator after incubation for 24 hr. A slight rise in titre, to 1 in 500, was observed when the test was allowed to stand at room temperature for a further 48 hr. The same preparations clotted undiluted citrated human plasma to a titre of 1 in 16, but did not clot a mixture of 1 per cent human fibrinogen and 0.1 per cent coagulase activator. Clotting activity was not affected by the presence of heparin at a concentration of 50 units per ml. in the reaction mixture, or by preparing test dilutions in saline buffered to pH 7.2.

Haemolysin production:

The activity of haemolysin preparations tested against the red cells of different animal species is shown in Table II.1 below:

Table II.1

The haemolytic activity of strain LS

Titre:	Red cells tested:		
	Rabbit	Sheep	Human
After incubation for 1 hr.	1:4	1:128	1:4
After incubation for a further 23 hr. at 4°C	Not tested	1:256	Not tested

Formolised sheep cells were used.

These results show that strain LS produces β, but not α or γ haemolysin.

Susceptibility to bacteriophage:

Strain LS was not susceptible to standard staphylococcal typing phages at either routine test dilution or 1000 times NTD.

The effect of strain LS on the growth of strain Morris in mixed cultures on solid media:

On nutrient agar plates bearing mixed cultures of strain LS and strain Morris, the growth of strain Morris adjacent to colonies of strain LS was inhibited and dissolution of strain Morris colonies was occasionally observed. On stab-inoculated plates zones of inhibition of the growth of strain Morris were visible round colonies of strain LS after 24 hr. incubation. These consisted of two areas: an inner ring of complete inhibition and beyond this a sharply bevelled edge. If the plates were re-incubated for 24 hr. or more the size of the inner zone increased, and a halo of lessened density became obvious outside it (Figure II.2). Cocci were not seen in smears taken from the inner zone. Smears taken from the bevelled edge or the halo contained capsulated and non-capsulated cocci.

The agent producing the zone of complete clearing did not reproduce the phenomenon when material from the zone was transferred to a second lawn plate of strain Morris, nor was lysis reproduced by transferring cells from the peripheral halo.

When strain Morris was streaked out on a chloroform-treated plate at right angles to the line of previous growth of strain LS, there was complete inhibition of its growth at the site of previous growth of strain LS (Figure II.3).

FIGURE II.2

Nutrient agar plate seeded with strain Morris, stab-inoculated with strain LS and incubated for 48 hours (x 1)

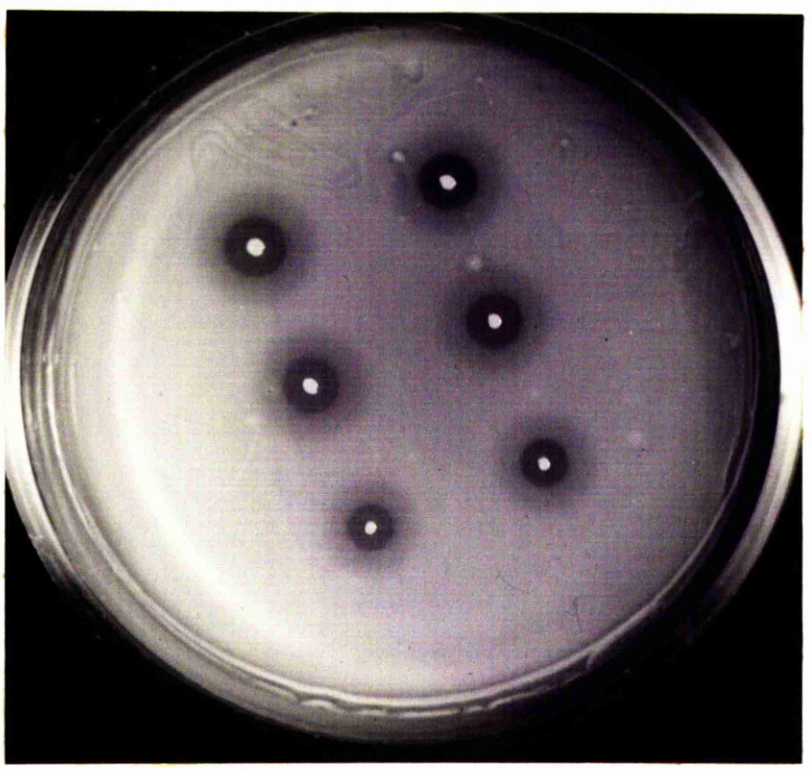
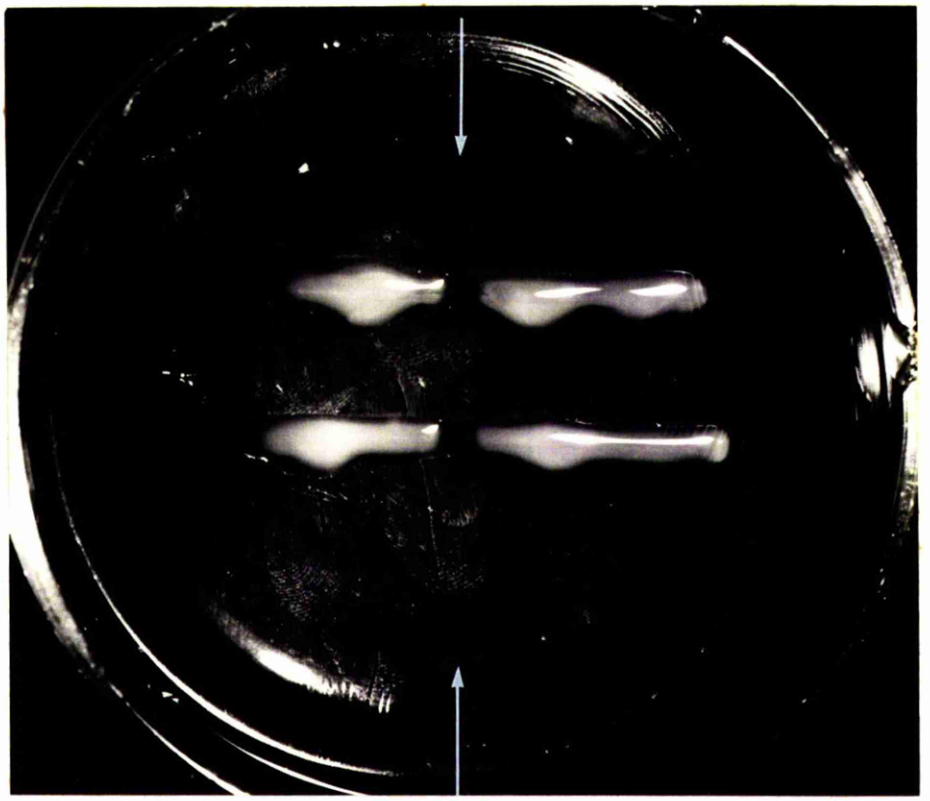


FIGURE II.3

Nutrient agar plate inoculated with strain LS (between the arrows) after incubation, removal of the growth, exposure to chloroform, cross-streaking with strain Morris, and further incubation. The growth of strain Morris is inhibited at the site of the original growth of strain LS. (x 1)



The effect of enzyme preparations on the growth of strain Morris on solid media:

Serial dilutions of enzyme preparations placed on plates heavily seeded with strain Morris produced visible zones of inhibition of growth after incubation for 18 hr. Concentrated enzyme preparations containing 32 units per ml. caused complete inhibition. Serial doubling dilution caused a stepwise reduction in the amount of inhibition until, at a concentration of 0.5 units of enzyme per ml., no effect was visible. Bacteriophage-like plaques were not seen.

These results and those noted in the preceding paragraph confirmed early impressions that the active substance produced by strain LS is a bacteriocine.

The effect of strain LS on heat-killed cells of strain Morris on solid media:

Colonies of strain LS growing on the surface of nutrient agar containing heat-killed cells of strain Morris, or concentrated enzyme preparations placed on the surface of the same medium, cleared the opacity due to the heat-killed cells in the medium. Serial dilution of enzyme preparations caused a stepwise reduction in the size of the cleared areas; enzyme preparations containing 0.5 units of enzyme per ml. or less had no effect.

The effect of enzyme on suspensions of cells of strain Morris:

Enzyme preparations affected suspensions of both heat-killed and live cells of strain Morris, but in different ways.

a) The effect on heat-killed cells:

Enzyme preparations added to suspensions of heat-killed cells produced a rapid and linear fall in turbidity for a short time (10-20 min.); thereafter the rate of clearance decreased rapidly and ceased abruptly at a final turbidity of about 70 per cent of the starting level. Continued incubation did not produce any further significant fall. Results of a typical experiment are listed in Table II.2 and shown graphically in Figure II.4.

Striking appearances were seen when the heat-killed suspensions were examined microscopically after their turbidity ceased to fall: many Gram-positive cocci were present but most or all of the capsular material surrounding them had disappeared (Figure II.5). It will be seen that heated cells exposed to enzyme stain more deeply at the periphery than in the centre. No experimentally-based evidence to explain this can be advanced but it is believed to follow modification of the cell by the action of enzyme.

Table II.2

The effect of enzyme on the measurable turbidity of suspensions of live and heat-killed cells of strain Morris

Incubation time (min)	Control	Turbidity (Nephelometer scale units)	
	Live cells in buffer	Live cells + enzyme	Heat-killed cells + enzyme
0	98	92	104
5	97	86	92
10	97	82	85
20	97	72	78
30	96	61	76
35	...	...	...
40	96	51	76
45	96	46	75
50	96	42	74
55	...	...	...
60	96	38	74

FIGURE II.4

The effect of enzyme on the turbidity of suspensions of live and heat-killed cells of strain Morris

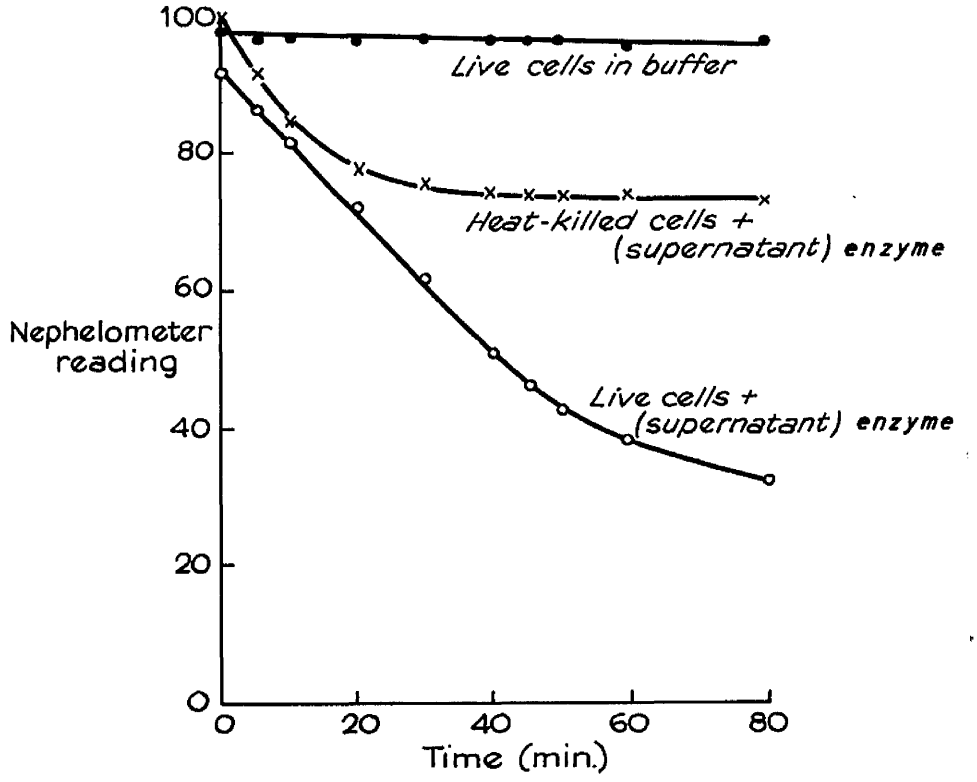
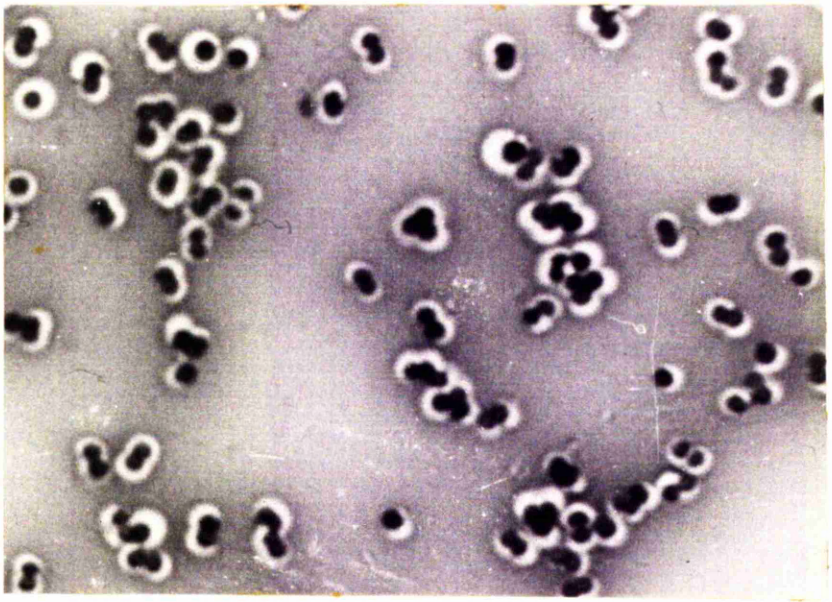


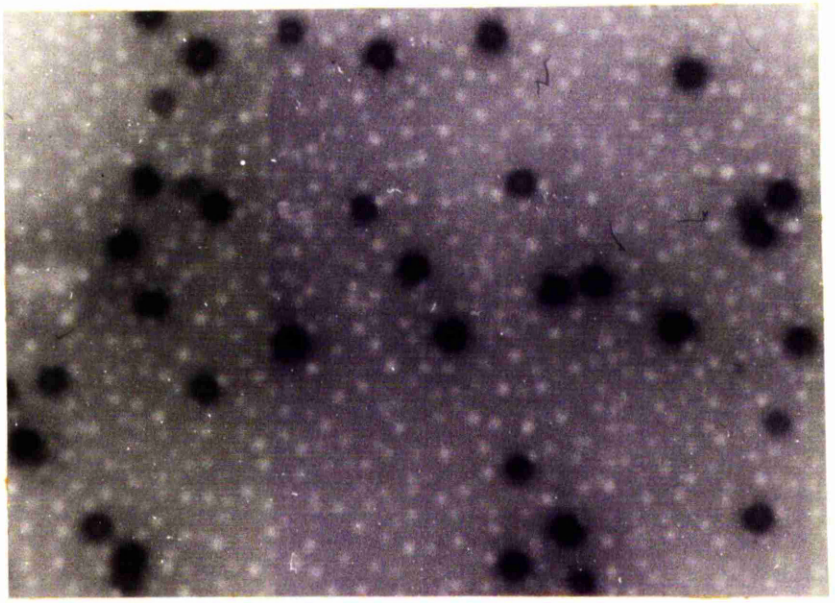
FIGURE II.5

The effect of enzyme on the morphology of heat-killed cells of strain Morris

(a) heat-killed cells in saline stained with nigrosin and rose-bengal (x 1500)



(b) heat-killed cells exposed to enzyme and stained with nigrosin and rose-bengal (x 1700)



b) the effect on live cells:

Enzyme preparations added to suspensions of live cells also produced a rapid and linear fall in turbidity which did not, however, cease after 10 to 20 min. but continued for 40 to 50 min. The rate of clearance thereafter gradually declined and finally ceased when the turbidity had fallen to 30 to 35 per cent of its original value. Results of a typical experiment are listed in Table II.2 and shown graphically in Figure II.4. The divergence of the turbidity curve of live cells from that of heat-killed cells when both were exposed to enzyme was reflected in the microscopic appearances. Live cell suspensions examined after enzyme activity had ceased contained very few recognisable cocci, the specimens consisting almost wholly of Gram-negative debris. The few cocci which were present were Gram-positive, capsulated and viable, subculture of the deposit yielding colonies with the morphology and characters of strain Morris. The cells forming these were not resistant to the action of enzyme and probably represented that part of the bacterial population which had not been attacked.

Enzyme preparations therefore produced different effects when acting on heat-killed and live cell suspensions. They acted on heat-killed cells to remove all or most of the visible capsular material from their surfaces and on live cells to lyse them.

The relation between the culture age and enzyme activity of shake cultures of strain LS:

In nutrient broth shake cultures, volume 200 ml., activity was first detected 5 hr. after inoculation; it increased to a peak between 7 and 8 hr. and declined thereafter to a steady level. The increase in activity was roughly parallel to, although later in time than, the exponential phase of growth. Peak activity coincided with the lowest pH reached by the culture. Figure II.6 shows the number of colony-forming units per ml. and the amount of enzyme in units per ml. of the culture at different times.

The effect of pH, heat, trypsin and chemicals on the activity of enzyme:

The activity of enzyme increased with increasing pH, reaching a maximum at pH 7.7. Further increase reduced enzyme activity. The rate of clearance of standard cell suspensions by a constant amount of enzyme at different pH levels is shown in Figure II.7.

The activity of preparations containing 8 units of enzyme per ml. was abolished by heating to 60°C for 3 but not 2 min. and was abolished by incubation with trypsin for 30 min.

Enzyme activity was inhibited by M/200 iodoacetamide; M/400 to M/6400 "Suramin"; M/200 to M/6400 cysteine hydrochloride, and M/200 to M/25600 2-3-dimercaptopropanol. These substances were equally inhibitory whether added to the test cell suspensions before the enzyme, or incubated with enzyme before being added to the test cells, and inhibited equally the action of enzyme on live and heat-killed cells.

FIGURE II.6

The relation between culture age and enzyme activity of shake culture supernatants of strain LS

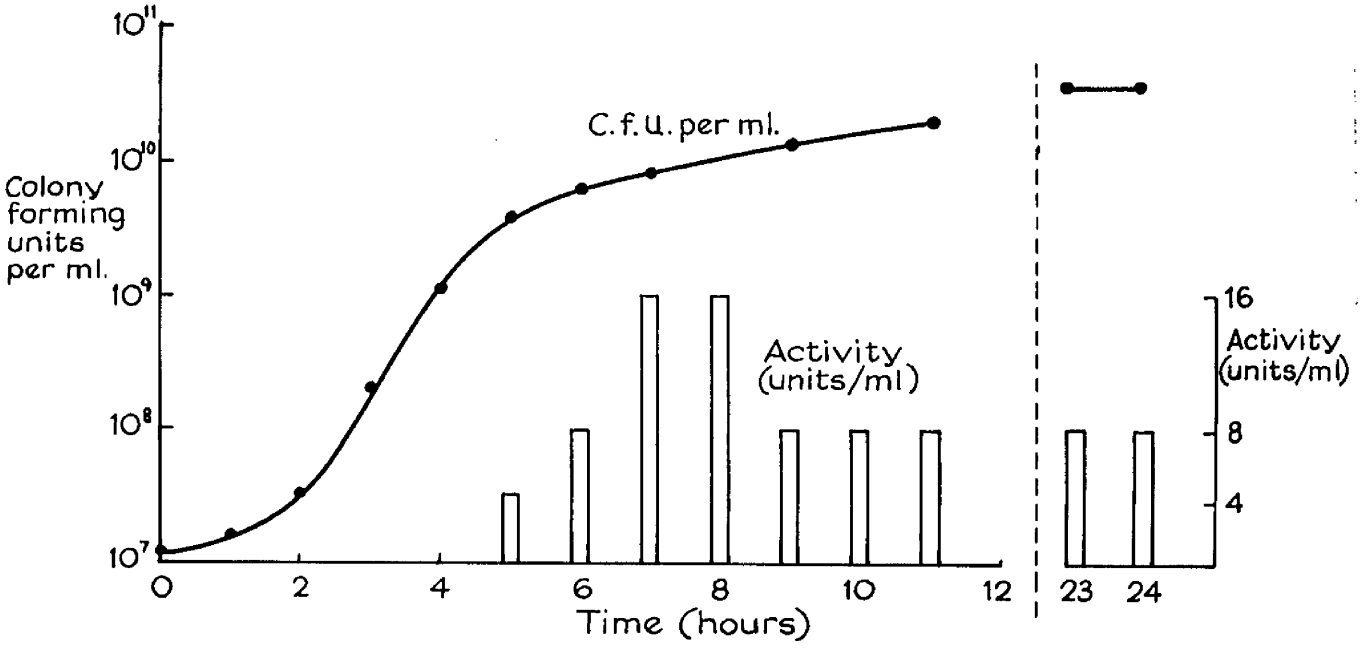
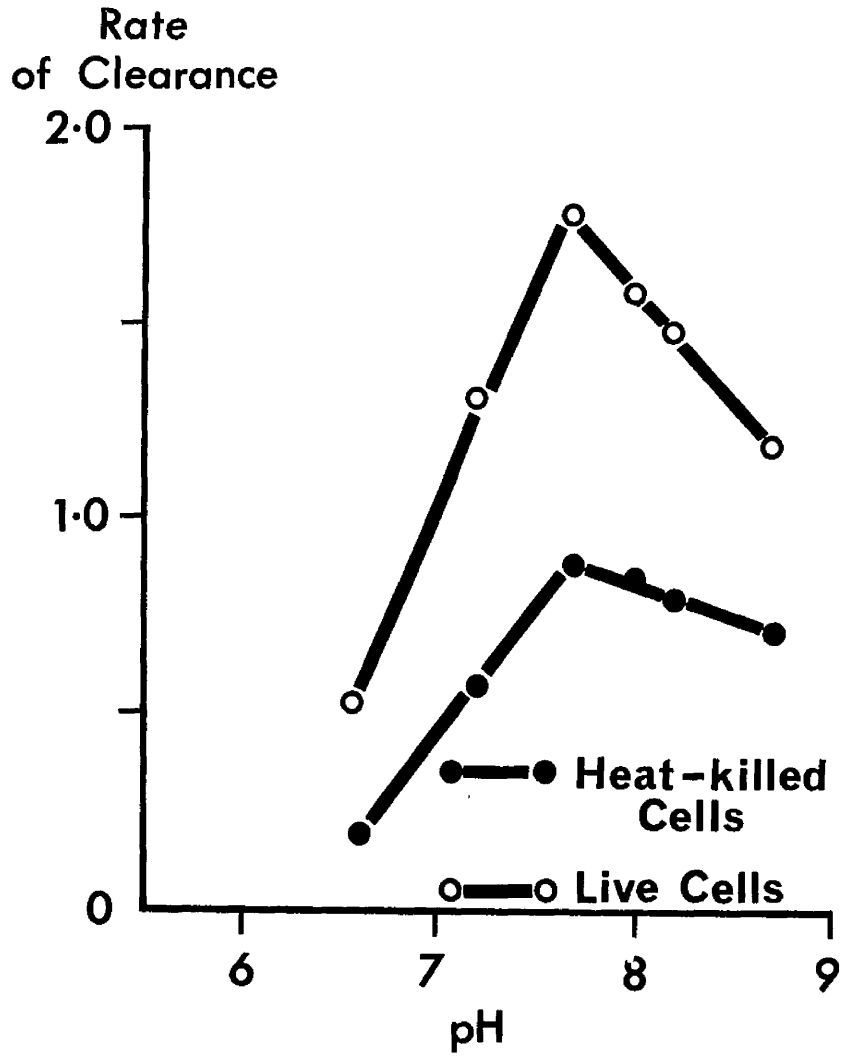


FIGURE II.7

The effect of pH on the rate of clearance of standard live and heat-killed suspensions by enzyme at a concentration of 0.8 units per ml.



The rate of clearance is the ratio fall in turbidity/time during the first ten minutes of each experiment; e.g. at pH 7.7 the rate of clearance of a standard live cell suspension is 1.76. This is equivalent to a fall in turbidity of 17.6 nephelometer units during the first ten minutes incubation and represents the destruction of 2×10^9 cells.

The effect of repeated addition of live cells of strain Morris on the activity of enzyme:

The rate of clearance of live cell suspensions by enzyme at a starting concentration of 0.8 units per ml. was little affected by the repeated addition of fresh cells to the reaction mixture (Figure II.8). After 5 additions of fresh cells, i.e. after the enzyme had acted on 2.5×10^{10} cells, about 5 times the number present in the first reaction mixture, the rate of clearance was still about half that of the first reaction. These findings provide clear evidence that the bacteriolytic material did not react stoichometrically with susceptible cells, and that it was therefore an enzyme.

The effect of enzyme concentration on the rate of clearance of standard cell suspensions:

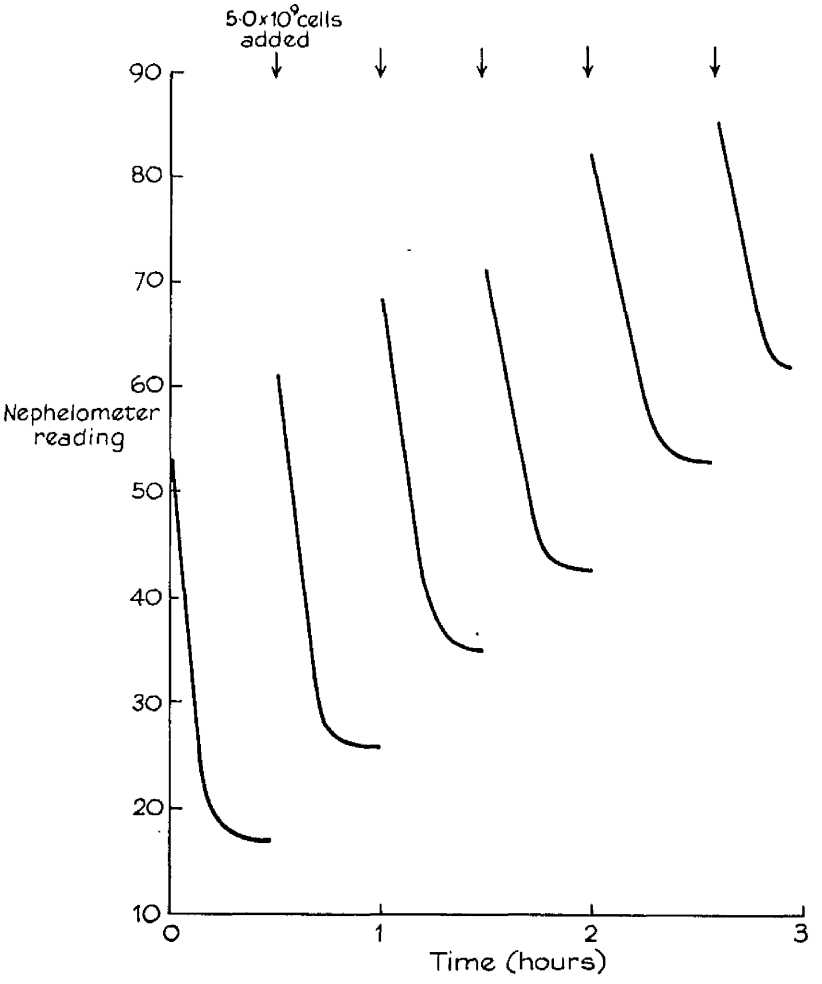
Increasing the concentration of enzyme increased the rate of clearance of standard live cell suspensions; the rate of clearance was directly proportional to the amount of enzyme present within the range 2 to 7 units per ml. (Figure II.9). Greater concentrations of enzyme cleared the suspensions too rapidly to permit accurate measurement of the rate.

Increasing concentrations of enzyme also increased the rate but not the extent of clearance of standard heat-killed cell suspensions.

Failure to produce lysis of heat-killed, enzyme-treated washed cells by exposure to enzyme-lysed live cells:

FIGURE II.8

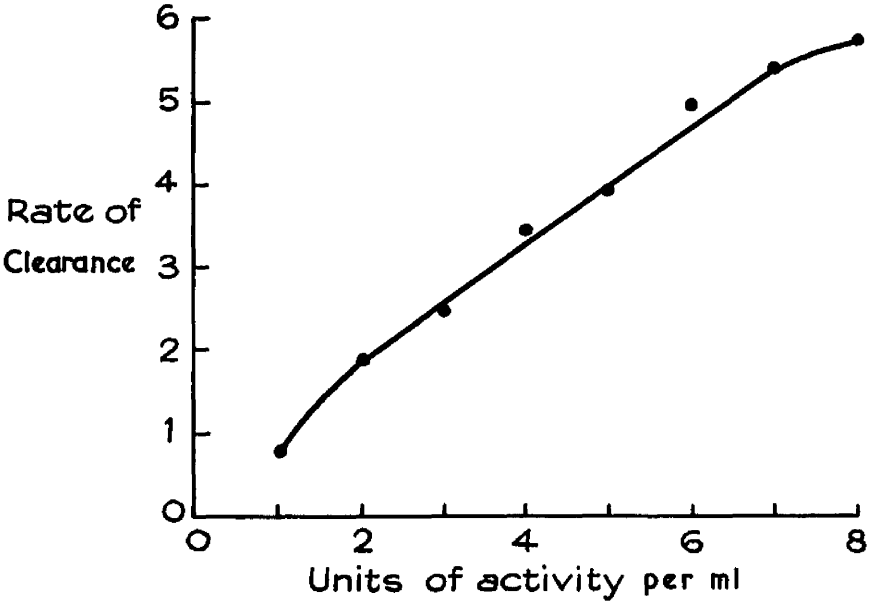
The effect of the repeated addition of live cells of strain Morris on the activity of a constant amount of enzyme



(The increase in turbidity measured after each fresh addition of cells represents the sum of their turbidity and the residual turbidity of the reaction mixture at the end of the previous run)

FIGURE II.9

The effect of enzyme concentration on the rate of clearance of live cell suspensions of strain Morris



The turbidity of heat-killed cells which had been exposed to enzyme and washed did not alter when the cells were resuspended in live cells which had been lysed by enzyme, or when they were exposed to fresh enzyme in buffered saline (Figure II.10). The turbidity of heat-killed cells which had not previously been exposed to enzyme fell when they were suspended in a preparation of live cells lysed by enzyme at a concentration of 0.8 units per ml. The fall was no greater, however, than the fall in turbidity of control cells exposed to the same concentration of enzyme (Figure II.11).

These results demonstrate that the lysis of live cells is due to exogenous active material produced by strain LS and not to the action of an endogenous autolysin present in live cells of strain Morris, and that the resistance of heated cells to lysis is not due to heat-inactivation of such an autolysin.

The effects on active culture supernatants of strain LS of pH, heat, trypsin, known enzyme inhibitors, and the repeated addition of substrate in the form of live cells are discussed at the end of this experimental section. They were considered to be compatible with the existence of a single enzyme, acting on heat-killed cells to remove most or all of the visible capsular material from the cell surface, and on live cells to lyse them. Three possible reasons were advanced to explain these different effects; these are also discussed in detail at the end of this section. The most likely explanation seemed to be that a single enzyme, in acting on live

FIGURE II.10

The effect of enzyme-lysed live cells, and of fresh enzyme, on enzyme treated heat-killed cells.

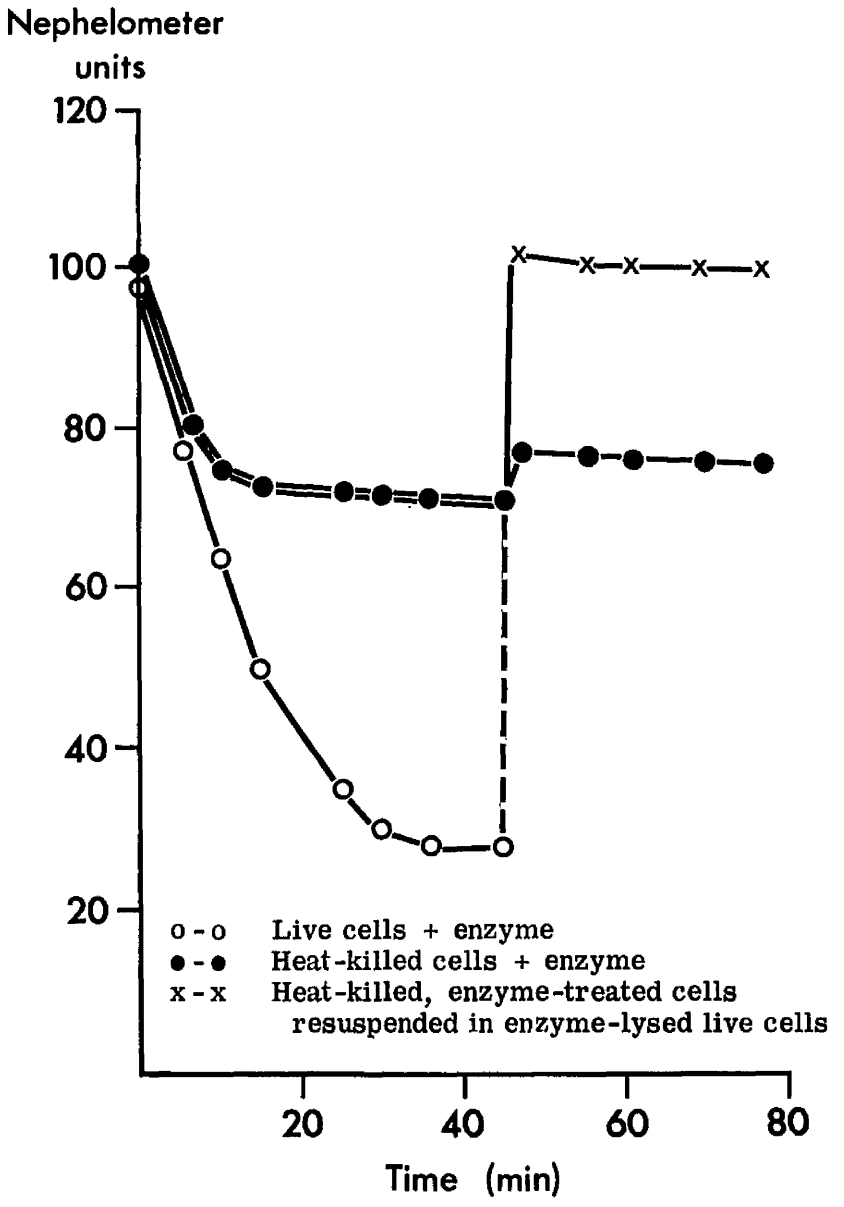
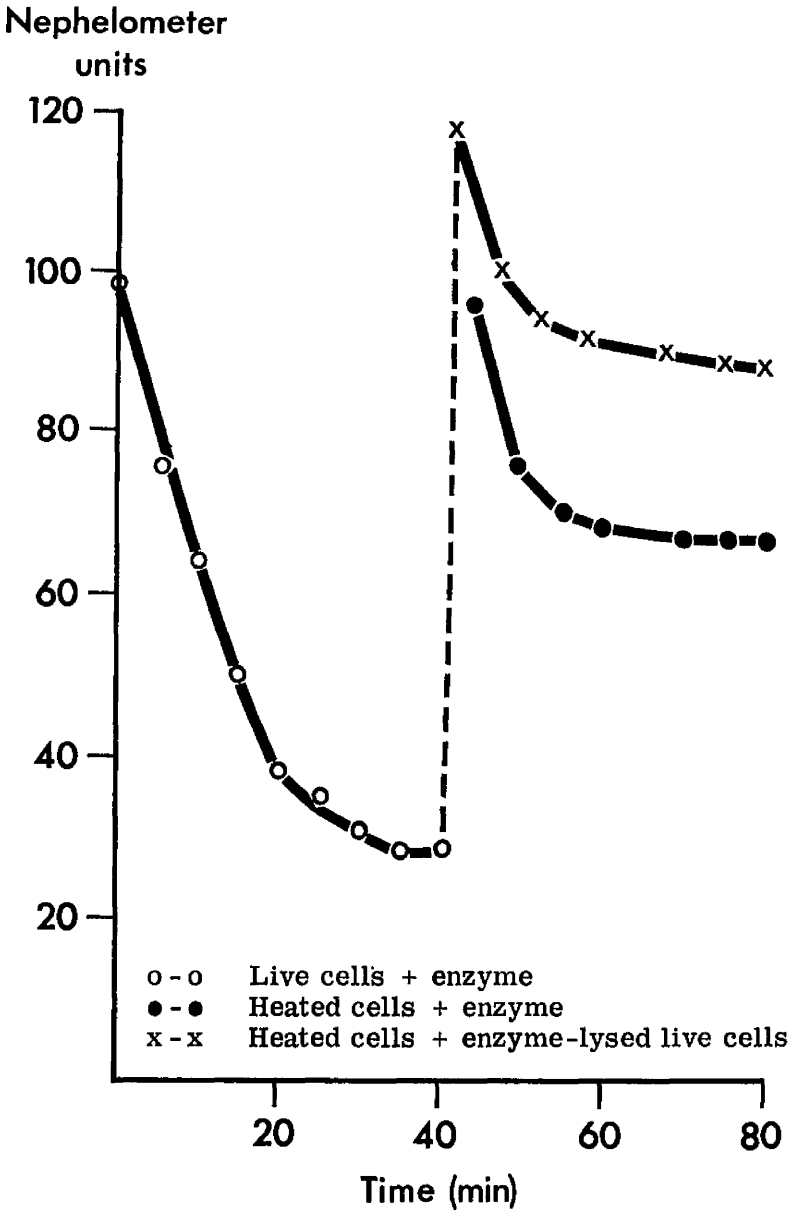


FIGURE II.11

The effect of enzyme-lysed live cells and enzyme alone on heat-killed cells.



cells, destroyed the integrity of the cell-wall so that osmotic rupture of the cells took place. Heating the cells, on the other hand, was believed to denature the cell-wall or some component of it in such a way that enzymic removal of the capsule was still possible but osmotic rupture of the cells was prevented.

It was reasoned that, if this postulate were correct, it might be possible to demonstrate three phenomena: that the longer a live cell suspension was heated, the more resistant it would become to enzyme-induced lysis; that if denaturation of the cell-wall produced the resistance to enzyme of whole heat-killed cells then heated disrupted cells would also be more resistant to the action of enzyme than similar but unheated preparations; and that, by using hypertonic stabilisers, e.g. sucrose, the lysis of live cells by enzyme would be delayed. The following experiments were therefore carried out.

The effect of increasing exposure to heat on the susceptibility of cells of strain Morris to enzyme:

a) the effect on whole cells:

Under the conditions of test suspensions of live cells were sterilised by heating for 21 but not 18 min. at 60°C. The effect of heating on the number of colony-forming units per ml. in suspensions heated for shorter periods is shown below in Table II.3. Most of the cells were killed after 3 min. at 60°C.

Table II.3

The effect of heat on the viable count of suspensions of live cells

Suspension heated for:	Colony-forming units per ml:	Surviving viable c.f.u. (percentage of unheated suspension):
0 min.	0.27×10^9	100
3 min.	2.5×10^3	0.001
9 min.	1.0×10^3	0.0005

The effect of heat on the enzyme-susceptibility of whole cells is shown in Figure II.12. Cells heated for 21 min. or more at 60°C responded to enzyme in a manner exactly similar to standard heat-killed suspensions heated at 100°C for 5 min; i.e. as already described, their turbidity fell rapidly and linearly at first but the fall ended sooner and at a higher level than the fall produced by enzyme acting on live unheated cells. With increasing exposure to heat the duration and extent of the fall in turbidity decreased and the number of cocci seen on microscopic examination increased.

b) the effect of heat on disrupted cells:

When exposed to enzyme the turbidity of both heated and unheated suspensions of disrupted cells decreased (Figure II.13). However, the disrupted preparations showed the same type of response to enzyme as the whole cells from which they were derived, the fall in turbidity of the heated preparations lasting for a shorter time and ending at a higher level than the fall in turbidity of

FIGURE II.12

The effect of increasing exposure to heat on the susceptibility of whole cells of strain Morris to enzyme.

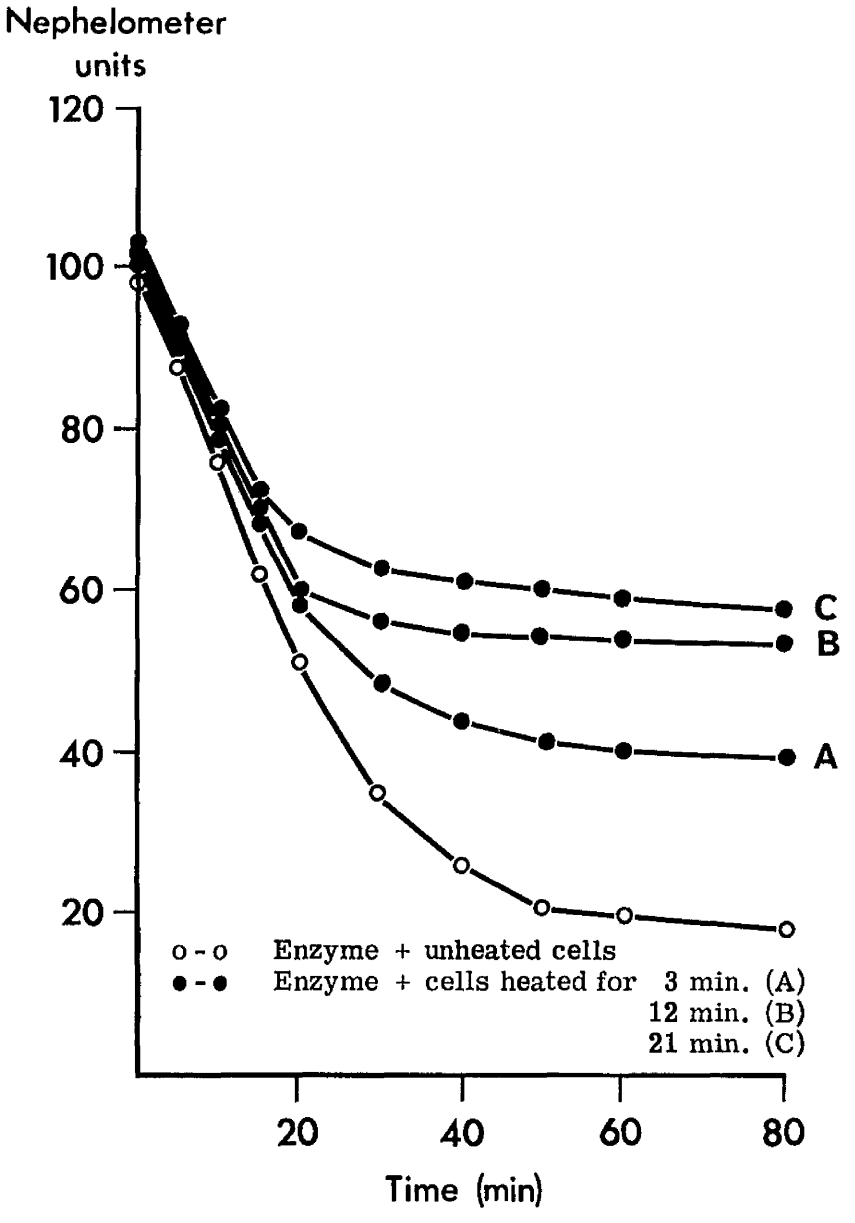
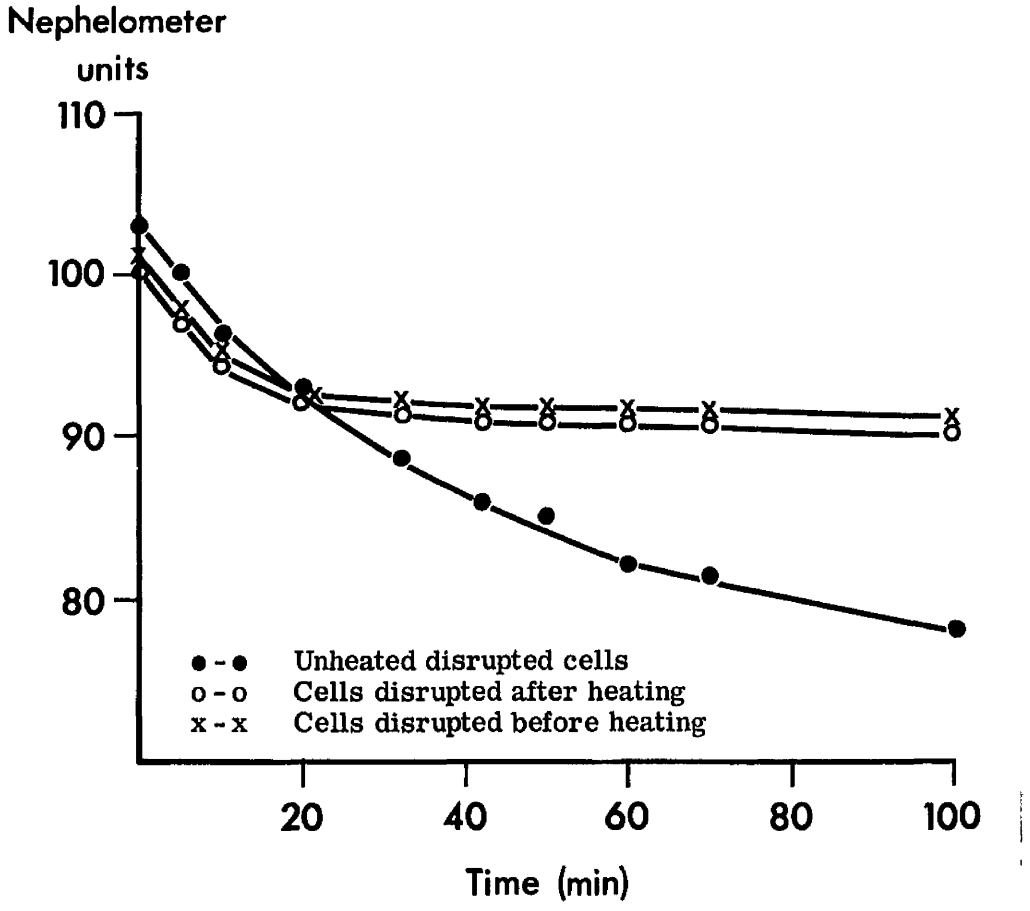


FIGURE II.13

The effect of heat on the susceptibility of disrupted cells of strain Morris to enzyme.



unheated preparations. The heated preparations were more resistant to enzyme whether the cells were heated before or after disruption.

These findings, and those in the preceding paragraph, support the postulate that the resistance to enzyme of heated cells is due to heat denaturation or masking of enzyme substrate in the cell-wall.

The effect of sucrose on the turbidity of suspensions of live and heat-killed cells of strain Morris:

Suspending live and heat-killed cells of strain Morris in hypertonic sucrose solutions produced an unexpected and unusual decrease in their turbidity compared with that of the same number of cells suspended in buffered saline, (Figure II.14); e.g. in a solution containing 20 per cent sucrose w/v (about 0.7 M sucrose) the measured turbidity of live cells was only 70 per cent of that of the same number of cells suspended in buffered saline. Sucrose did not affect the pH of the suspending medium.

When the suspensions of cells in sucrose were centrifuged and the cell deposit resuspended in buffered saline, the expected turbidity was not fully restored. Figure II.15 shows the difference between the turbidity of a constant number of cells suspended in buffered sucrose solutions, their turbidity when resuspended in buffered saline, and the turbidity in buffered saline of the same number of cells which had not been exposed to sucrose. The difference increased with increasing concentrations of sucrose above 20 per cent w/v.

FIGURE II.14

The effect of sucrose on the measurable turbidity of a constant number of live and heat-killed cells of strain Morris suspended in buffer.

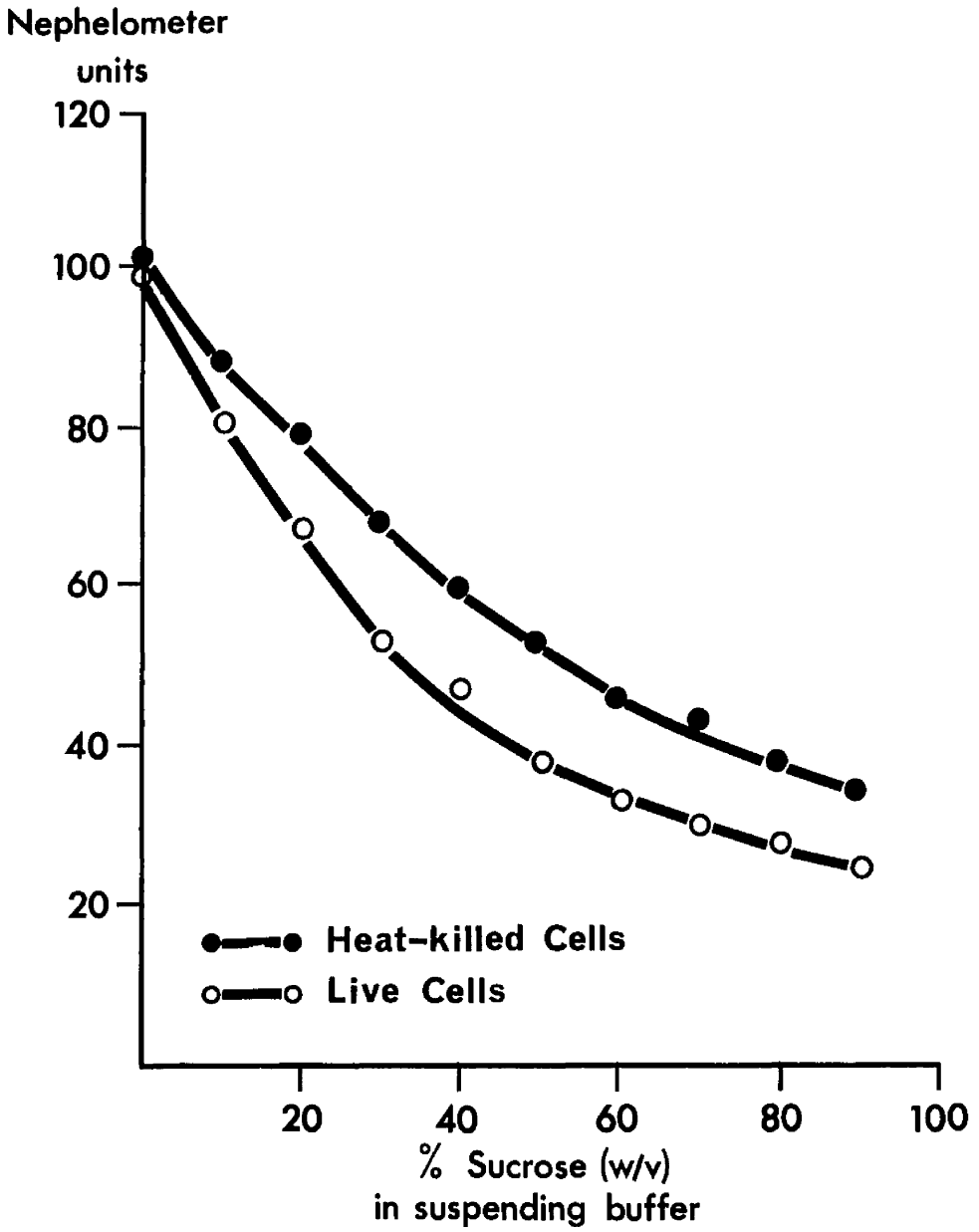
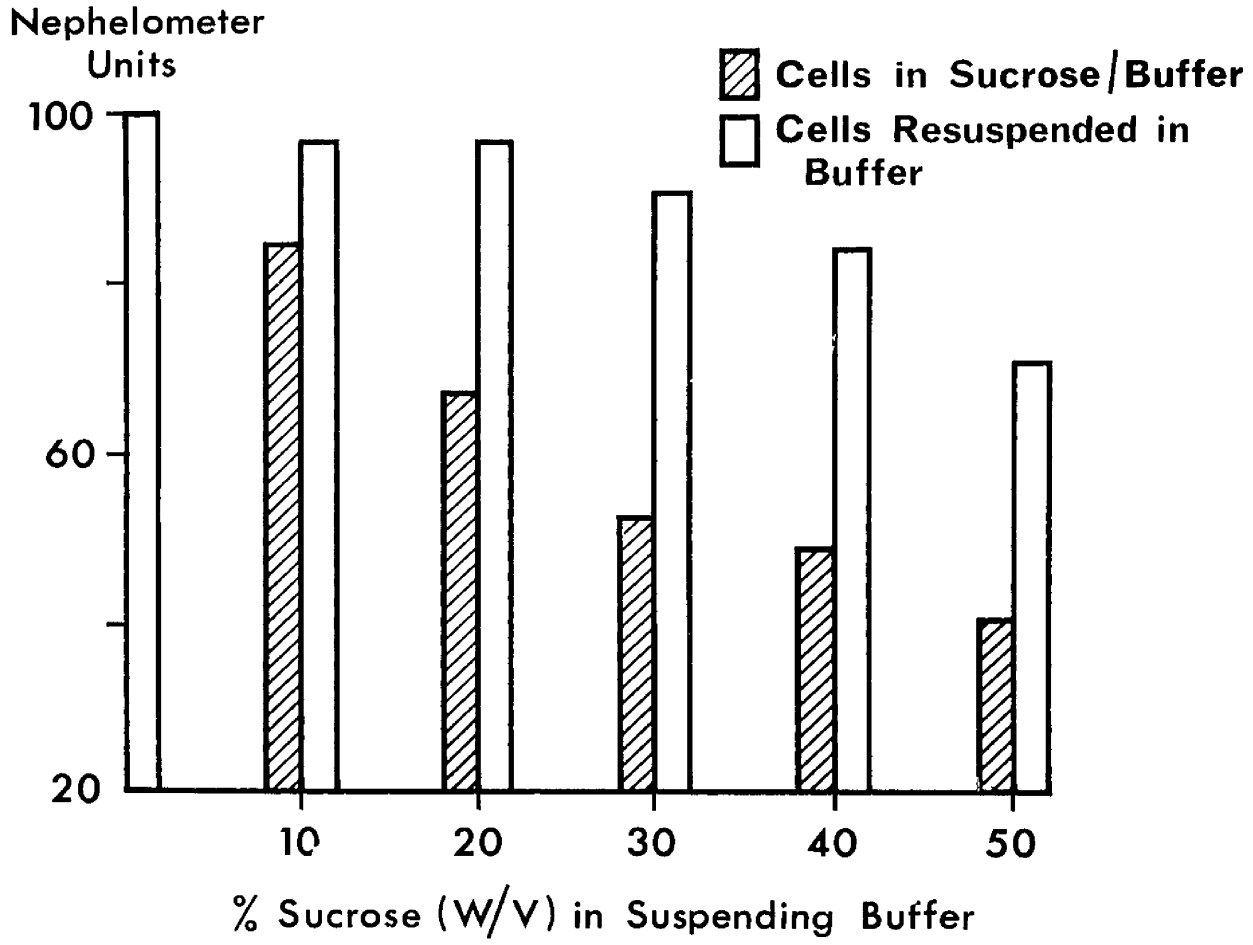


FIGURE II.15

The effect of sucrose on the turbidity of live cells of strain Morris compared with the turbidity of the same cells resuspended in buffered saline.



The loss in turbidity was reversible with concentrations below 20 per cent w/v.

These results suggest that the loss of turbidity caused by sucrose in the suspending buffer was due to destruction of cells, possibly by plasmolysis, or to an alteration of their optical density, possibly by abstraction of water, or to both these effects. However, when suspensions of live cells in buffer containing increasing concentrations of sucrose were examined microscopically no perceptible difference in capsular thickness or constant alteration in cell diameter were seen.

The effect of 0.7 M sucrose on the turbidity of different concentrations of live cells of strain Morris:

Table II.4 shows the turbidity of different concentrations of live cells in buffered saline, and the turbidity of the same number of cells suspended in a buffered solution of sucrose 20 per cent w/v, 0.7 M.

Table II.4

The effect of 0.7 M sucrose on the turbidity of live cells of strain Morris

Cell concentration per ml. ($\times 10^8$)	Turbidity (nephelometer units) in:	
	Buffered saline	Buffered sucrose
10	100	59
8	82	49
6	64	42
4	45	31
2	32	22

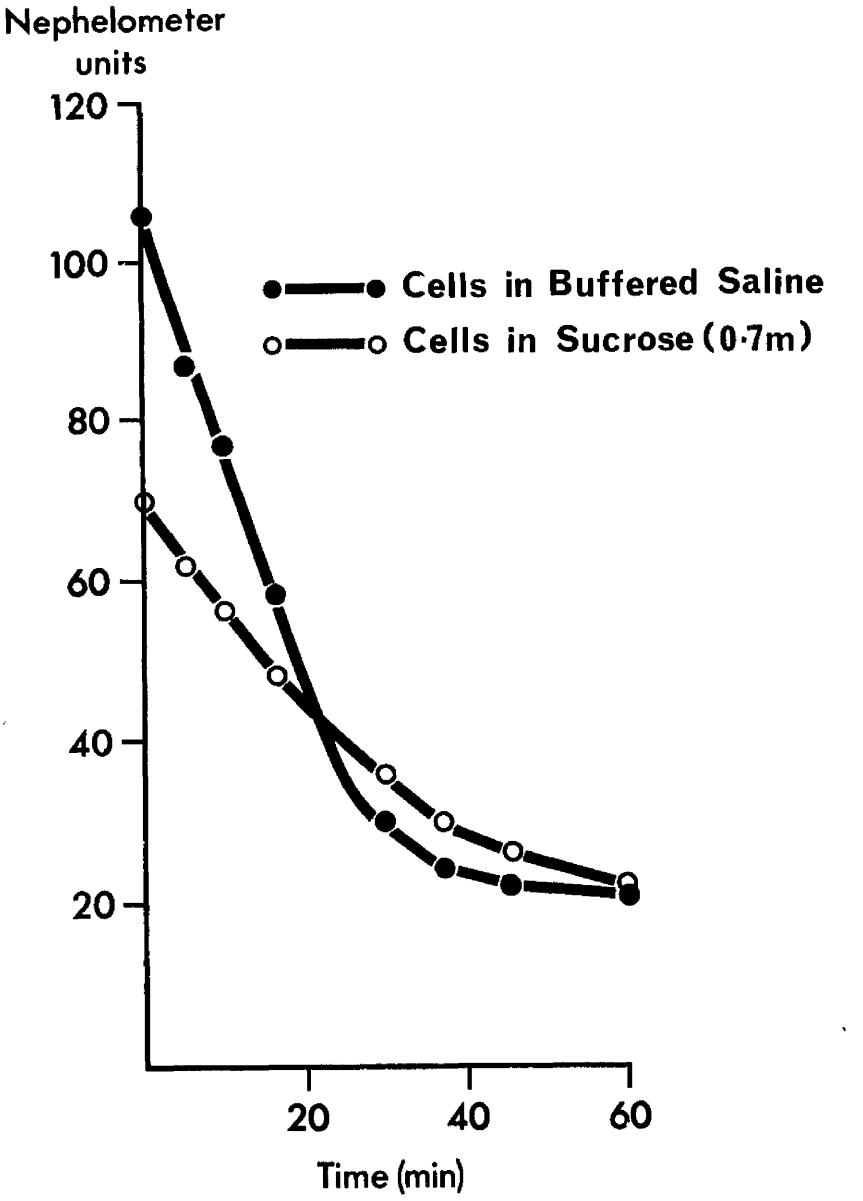
The effect of sucrose on the clearance of live cells of strain Morris by enzyme:

The effect of enzyme on live cells of strain Morris suspended in a buffered hypertonic (20 per cent w/v, 0.7 M) solution of sucrose is shown in Figure II.16 and compared with the effect of enzyme on the same concentration of cells suspended in buffered saline. As expected, when the cells were suspended in the buffered sucrose solution their turbidity fell, in this experiment to 66 per cent of the expected level. When enzyme was added the turbidity of both preparations fell but the rates of clearance differed, that of cells in buffered saline being 2.5 and of cells in buffered sucrose 0.8 - a threefold difference.

By interpolation from the results shown in Table II.4 the slower

FIGURE II.16

The effect of enzyme on live cells of strain Morris in a buffered hypertonic (0.7 M) solution of sucrose.



rate of clearance of cells in sucrose is seen to be a true slowing of the rate of lysis. Thus, after 30 min. incubation the turbidity of cells exposed to enzyme in buffered saline was 30 nephelometer units, equivalent to a concentration of 2×10^8 cells per ml. At the same time, the turbidity of cells suspended in sucrose was 38 nephelometer units, equivalent to a concentration of cells in sucrose of 5 to 6×10^8 cells per ml.

The concentration of sucrose used in this experiment, 20 per cent w/v, 0.7 M, is sufficient to balance the known internal osmotic pressure of staphylococci of about 20 atmospheres (Mitchell and Moyle, 1957). This experiment therefore supports the postulate that live cells exposed to enzyme undergo osmotic lysis.

DISCUSSION

To substantiate the working hypothesis on which this thesis is based, both the capsulated strain Morris and the organism strain LS described in this section must be strains of Staphylococcus aureus.

The taxonomic position of strain Morris has already been discussed. It is believed that strain LS is also a strain of Staph. aureus, for the following reasons: it utilised glucose anaerobically, produced phosphatase, grew in human serum and in the presence of 6 per cent NaCl; it clumped in plasma in the Cadness-Graves test for bound coagulase, and although it did not clot plasma in the conventional Fisk test for soluble coagulase, concentrated culture supernatants clotted both citrated human plasma with heparin and a heparinised mixture of bovine fibrinogen and coagulase activator.

In two respects only did strain LS not comply with the modal form of Staph. aureus. It did not ferment mannite; the unreliability of carbohydrate fermentation as a taxonomic criterion in this genus is well known (see page 47). It was not lysed by standard staphylococcal typing 'phages, but neither are a quarter of otherwise typical strains of Staph. aureus (Parker, 1962). Taxonomically, these differences are not important and become even less so when balanced against the fact that this strain produced bound and soluble coagulase.

Accepting that strain LS is a strain of Staph. aureus, its relevance to the present work lies in its action on cells of the capsulated strain. Strain LS was first noticed, and was studied, because it destroyed the growth of strain Morris on a lawn plate. (It has been found (see section IV) that staphylococci which do this are rare). Further study of this bacteriolytic effect has shown that it was not due to bacteriophage: lytic culture supernatants did not produce discrete plaques; they acted on heat-killed cells; they began to lyse live cells immediately without any latent period, and during the lysis of live cells the active substance did not reproduce itself.

It followed that the bacteriolytic material in culture supernatants of strain LS must be an enzyme. That it was indeed so was shown by the following facts: lytic activity depended on pH, and had a definite optimum at pH 7.7; it was abolished by heating to 60°C and by incubation with trypsin (evidence that the active material was a protein), and it was inhibited by the known enzyme inhibitors - iodoacetamide, "Suramin", and 2-3-dimercaptopropanol. (Surprisingly, it was also inhibited by cysteine hydrochloride); lytic activity was directly related to the amount of culture supernatant present, and active supernatants did not react stoichometrically with susceptible cells.

The enzyme affected suspensions of heat-killed and live cells but in different ways. In both it caused an immediate, rapid and linear fall in turbidity, but after the first 10 to 20 minutes of the

reaction the turbidity curves of the two preparations diverged: the turbidity of heated cells stopped falling when it had reached about 70 per cent of its starting level and further incubation with or without adding fresh enzyme produced no further change. The turbidity of live cell suspensions, on the other hand, continued to fall rapidly and did not stop until the turbidity had reached about 30 per cent of its starting level.

The differences recorded in the nephelometer were accompanied by striking differences in the microscopic appearances of the suspensions examined after enzyme activity had stopped. Heat-killed suspensions contained many cocci but few of them were capsulated; by contrast, live cell suspensions contained very small numbers of viable and morphologically normal capsulated cocci.

Three possible reasons were advanced to explain this difference in effect. They were:

1) that active culture supernatants of strain LS contain two enzymes, one of which decapsulates the cells while the other lyses them.

This explanation was considered to be unlikely because both effects of active culture supernatants, i.e. on live and heat-killed cells, were equally affected by pH (the optimum for both actions was 7.7), by the same enzyme inhibitors at the same concentrations, and were simultaneously destroyed by heat. It is still possible that this explanation is the correct one, but if it is

the two enzymes show a similar response to pH, heat and inhibitors.

2) that active culture supernatants of strain LS contain a single enzyme which decapsulates both live and heat-killed cells and that bacteriolysis of live cells is due, not to material produced by strain LS, but to the action of an endogenous autolysin present in cells of strain Norris. Heated cells do not lyse because this autolysin has been destroyed. The following results suggested that this was not the correct explanation:

a) the repeated addition of fresh live cells to a fixed amount of enzyme caused a gradual stepwise reduction in the rate of clearance; if an autolysin were responsible for bacteriolysis of live cells, the speed of lysis should have increased: in fact no such increase was noted.

b) the turbidity of heat-killed cells exposed to enzyme and then resuspended in an enzyme-lysed preparation of live cells did not alter. If a heat-labile autolysin were responsible for lysis the turbidity of the heat-killed cells should have fallen rapidly (cf. the action of pneumococcal autolysin (Dubos, 1937)).

3) It was finally postulated that active supernatants of strain LS contain a single enzyme which decapsulates live cells and in so doing destroys the integrity of the cells so that they undergo osmotic rupture. Heat-killed cells are also decapsulated but

osmotic rupture is prevented because heating denatures the cell-wall or some component of it.

This postulate was submitted to proof by attempting to demonstrate three phenomena which should follow if it is correct:

a) that the longer live cells were heated the more resistant they became to enzyme-produced lysis. It was found that the resistance of live cells to lysis did indeed increase when they were heated for increasing periods (page 80).

b) that if heating caused the resistance to lysis of whole cells, disrupted cells should also become more resistant to the action of enzyme when heated. Again it was found that heated disrupted cells were more resistant to enzyme action than unheated preparations (page 80).

c) that if lysis of live cells was due to osmotic rupture following the action of enzyme, it should be possible to delay this by the use of osmotic stabilisers, e.g. sucrose.

Difficulties were met when attempting to test this last supposition because suspensions of cells in buffered sucrose solutions were found to be less turbid than the same number of cells suspended in buffered saline. However, the rate of clearance of live cells by enzyme was reduced by adding sucrose to the suspending buffer to a concentration sufficient to balance the known internal osmotic pressure of staphylococci. It is believed therefore that

the different effect of the enzyme produced by strain LS on live and heat-killed cells of strain Morris reflects a difference in the osmotic stability of each cell type after they have been acted upon by enzyme, live cells being affected to an extent that they undergo osmotic lysis.

The experimental results described and discussed in this section are interpreted as demonstrating that a single enzyme produced by strain LS acts on live cells to destroy their osmotic integrity and on heat-killed cells to remove most or all of the capsular material from their surfaces.

If, as is suggested, a single enzyme is responsible for both effects then it differs in one important respect from other capsule-destroying enzymes which have been described; its action on live cells results in cell death.

The enzymes derived from saprophytic bacteria which hydrolyse the capsular polysaccharide of pneumococci (Dubos and Avery, 1931) destroy the capsular material without affecting the viability of the cells or their ability subsequently to re-form capsular material. Similarly, hyaluronidase removes the hyaluronic acid capsule of streptococci, and trypsin their "microcapsular" M antigens, without killing the cells or destroying their ability to resynthesise the polymers when the enzyme is removed (McLean, 1941; Morison, 1941; Lancefield, 1943). The 'phage-induced capsular depolymerases released by certain enterobacteria (Adams and Park, 1956; Sutherland and Wilkinson, 1964, 1965) have similar properties; although the

underlying cell is necessary for the production of the enzyme it is not attacked by it.

The capsule-destroying enzyme produced by strain L8 differs from these in that it kills live cells. While there is no direct experimentally-based proof of its site of action, the morphology of heat-killed cells exposed to it and the fact that live cells attacked by it undergo osmotic rupture suggest that the enzyme removes the capsule by removing also surface components of the cell wall, and not by depolymerising the capsular material or by destroying the point at which the capsular polymer is attached to the cell-wall. This suggestion is of course susceptible to experimental proof by qualitative and quantitative studies of the capsular material of strain Morris and the material released from heat-killed cells by enzyme action.

The bacteriolytic effect of the enzyme also deserves comment. Many bacteria produce soluble bacteriolytic substances acting either upon themselves, on other strains of the same species, or on distinct genera. Staphylolysins, produced by and acting upon staphylococci, have been frequently described; some of them have been reviewed by Elek (1959). They can be grouped into four distinct types:
1) Staphylococcal "lysozymes". These, like lysozyme itself, (Fleming, 1922) act upon Micrococcus lysodeikticus (Staphylococcus aforementans), and on heat-killed or disrupted, but not live, cells of Staph. aureus (Richmond, 1959; Lominski, Morrison and Smith, 1953; Kashiba, Nuzu, Tanaka, Nozu and Amano, 1959; Jay, 1966).

2) Staphylococcal autolysins released by autolysing coagulase-positive staphylococci. These act also on M. lysodeikticus and on heat-killed staphylococci, or on staphylococci stressed by exposure to ultraviolet light or acetone (Welsch, 1949, 1950; Welsch and Salmon, 1949, 1950; Ralston, Baer, Lieberman and Krueger, 1955; Ralston, Lieberman, Baer and Krouger, 1957), although like lysozyme they do not attack live staphylococci.

3) "Virolysin", a staphylolytic enzyme released by 'phage-infected cocci. This attacks live staphylococci if 'phage is present but alone acts only upon heat-killed staphylococci (Ralston et al., 1955, 1957).

4) "Lysostaphin", an enzyme produced by a coagulase-negative strain of staphylococcus (Schindler and Schuhardt, 1964). Unlike the other three types of staphylolysin "lysostaphin" lyses live staphylococci more readily than heat-killed.

The staphylolytic enzyme produced by strain LS most closely resembles "lysostaphin". Both act more readily and completely upon live than on heat-killed staphylococci and have similar pH optima (7.5), but they differ in that strain LS is coagulase-positive and strain K-6-M which produces "lysostaphin" is coagulase-negative. The effect of "lysostaphin" upon capsulated staphylococci is not as yet known.

The claim that "lysostaphin" is a single enzyme has recently been challenged by Browder, Zygmunt, Young and Tavormina (1965). They showed that preparations of "lysostaphin" contained two enzymes: a peptidase which lysed live staphylococci and released N-terminal

glycine and alanine from cell-walls of Staph. aureus; and a hexosaminidase, devoid of lytic activity, which acted upon the glucosaminyl-muramic acid linkage in staphylococcal cell-walls, in this differing from lysozyme and related enzymes which cleave the alternate bond in the carbohydrate backbone (Salton and Ghuysen, 1960). Browder and his colleagues considered that lysis of live staphylococci was due to the action of the peptidase on the peptide cross-linkages which provide much of the cell-wall rigidity.

These findings and the similarities between "lysostaphin" and the enzyme produced by strain LS have some bearing on the demonstration that live cells of strain Morris exposed to the latter undergo osmotic rupture. As has been noted, attempts to demonstrate that staphylolytic cultures of strain LS contain two enzymes have so far been unsuccessful. In the light of the findings of Browder and his colleagues further attempts will have to be made to determine if the different effects of strain LS on live and heat-killed cells are indeed, as is suggested here, due to the effect of heat on the susceptibility of the cells to a single enzyme and not to the existence of two enzymes with similar physical characters but distinct modes of action.

SUMMARY

A strain of Staphylococcus aureus is described. It produces a non-particulate soluble staphylolysin which has many of the characters of an enzyme.

The enzyme acts on heat-killed cells of strain Morris to remove most or all of their visible capsular material, and on live cells to lyse them.

An explanation for these different effects is advanced and the relation of the enzyme to other capsule-destroying enzymes and other staphylolysins is discussed.

SECTION III

THE FORMATION OF CAPSULES BY NON-CAPSULATED STAPHYLOCOCCI GROWN IN ANTISERUM TO THE CAPSULE-STRIPPING ENZYME

MATERIALS AND METHODS

Organisms:

Eleven strains of staphylococci were taken at random from strains sent to the Phage Typing Laboratory, Western Infirmary, Glasgow. Retrospective investigation showed that they came from 5 different hospitals, 11 different patients, and at least 8 different clinical conditions. All were coagulase-positive when tested by the method of Fisk (1940) and Cadness-Graves et al. (1943). All produced phosphatase and gelatinase, fermented mannite, and were able to grow in human serum and in the presence of 6 per cent w/v NaCl. None was capsulated when examined in smears stained with nigrosin and rose-bengal, or by the dry india ink method of Butt, Bonynge and Joyce (1936), or in thin india ink suspensions after staining with rose-bengal. The clinical source and 'phage-type of each strain is shown in Table III.1. Staphylococcus strain 7 (the non-capsulated variant of strain Morris, page 43) and strain L8 (page 53) were also used.

Method of producing antiserum to the capsule-stripping enzyme:

Enzyme preparations containing 32 units of activity per ml. were

alum-precipitated by the method of Froom (1943).

Table III.1

The clinical source and phage-type of 13 laboratory strains of Staphylococcus aureus

Strain	Source	Phage type
D	Non-capsulated variant of strain Morris	52/52A/80/81
LS	Plate contaminant	Not typable
0957	Wound Swab	Not typable
1346	Lung	B
1348	Sputum	52/52A/80/81
1356	Wound swab	52/52A/79/80/7/42B/81/C
1357	Osteomyelitis	81
1361	Ear swab	55
1377	Nasal swab	A
1379	Septic finger	52/52A/79/80/42B/B
1381	Pleural fluid	80/81
1383	Sputum	Not typable
1387	Ear swab	53

Laboratory-bred chinchilla rabbits of either sex, weighing between 1.2 and 2.5 kg. were bled from the marginal ear vein and the next day injected with 10 ml. of alum-precipitated enzyme, (equivalent to 80 units), intramuscularly into each thigh in two equal portions.

Ten days later the rabbits were bled from the marginal ear vein, and 14 days after the first injection of enzyme were again injected intramuscularly with 10 ml. of alum-precipitated enzyme. Twenty-seven days after the first injection the rabbits were bled again and the next day received a third injection of enzyme. Ten to 14 days after this injection the rabbits were bled again. Serum was separated and heated at 56°C for 30 min. on the same day that the blood samples were withdrawn. The sera were stored at 4°C.

Some rabbits received a fourth injection of alum-precipitated enzyme after a lapse of three months. This was followed 10 to 14 days later by removal of blood samples.

Assay of sera for enzyme-neutralising activity:

Serial doubling dilutions of each serum were prepared in 0.5 ml. volumes of sterile saline. To each was added 8 units of enzyme in a volume of 0.5 ml. The mixtures were incubated in a waterbath for 1 hr; 0.5 ml. volumes of each mixture were transferred to matched tubes and tested for enzyme activity against volumes of 9.5 ml. of standard live and heat-killed suspensions of cells of strain Morris. Control tubes containing 1 ml. of saline or 1 ml. of enzyme alone were also examined. The end-point of each test was considered to be the highest dilution of serum causing complete inhibition of enzyme activity during 30 min. incubation with the test suspension.

Neutralisation of anti-enzyme serum with enzyme:

Serum inhibiting the capsule-stripping enzyme to a titre of 1 in 32 was diluted 1 in 4 with sterile saline. To 1 ml. volumes was added 8 units of enzyme in a volume of 0.25 ml. or 0.25 ml. of sterile saline. The mixtures were incubated for 1 hr. (No visible precipitate was formed). The mixtures were then tested for enzyme activity by incubation for 30 min. with standard suspensions of live cells of strain Norris (see page 56, Section II), and for their ability to neutralise capsule-stripping enzyme as described above.

Absorption of antisera:

An 18 hr. nutrient broth shake culture of the absorbing strain was centrifuged, the cells washed thrice and resuspended in saline at a concentration of 4×10^{10} cells per ml. by opacity. Volumes of 5 ml. of this suspension were centrifuged at 17,000 rpm for 5 min. and the supernatant fluid replaced by anti-enzyme serum diluted with sterile saline. In most experiments the serum was diluted 1 in 2 and in some 1 in 4. The cells were resuspended and the mixtures incubated for 1 hr. in a waterbath. They were centrifuged and filtered through sterile membrane filters ("Oxoid", Oxo Ltd., London).

Before and after absorption the sera were tested for their ability to agglutinate the absorbing strain by mixing on a slide two loopfuls of diluted serum and two loopfuls of the suspension used for absorption. The slides were rocked at room temperature for 5 min. and examined with

the naked eye for agglutination. Sera which agglutinated the test strain after absorption were re-absorbed.

Demonstrating the effect in culture of enzyme-neutralising serum on non-capsulated strains of Staphylococcus aureus.

Enzyme-neutralising serum from rabbits immunised with alum-precipitated capsule-stripping enzyme was diluted 1 in 3 with equal volumes of sterile isotonic phosphate buffer, pH 7.2, and sterile nutrient broth. Cells from an overnight nutrient broth culture of the test strain were washed and suspended in broth to a concentration of 1×10^7 cells per ml. Volumes of 1 ml. of this suspension were added to 1 ml. volumes of antiserum-buffer-broth mixture and the cultures incubated in a waterbath. At measured intervals during incubation, which was continued for 24 hr. a loopful of the cultures was removed and examined microscopically after staining with either nigrosin and rose-bengal or by the dry india ink method of Butt, Bonynge and Joyce (1936). (Details of these staining methods are given in the Appendix). In some experiments cultures were examined in this way, and in thin wet india ink suspensions, after centrifugation.

Control cultures in which anti-enzyme serum was replaced by serum from unimmunised rabbits, by nutrient broth, by anti-enzyme serum absorbed with the test strain, by anti-enzyme serum diluted from 1 in 4 to 1 in 64, or by anti-enzyme serum neutralised by previous incubation with enzyme, were also examined.

In similar experiments in which strain D, the non-capsulated variant of strain Norris, was cultured in anti-enzyme serum a further control was set up in which anti-enzyme serum was replaced by a specific agglutinating serum prepared in rabbits (see Section I, page 36).

Experiments were also carried out with heat-killed cells of strain D as follows:

The cells from an overnight nutrient broth shake culture were washed, resuspended in saline and heated at 100°C. for 5 min. The cells were resuspended in broth to give a series of suspensions containing between 1 x 10^8 and 1 x 10^{10} cocci per ml. by opacity. Volumes of 0.1 ml. of each suspension were added to 1 ml. samples of either enzyme-neutralising serum diluted 1 in 3 with broth and phosphate buffer, pH 7.0, or a specific agglutinating serum diluted 1 in 4 with equal volumes of broth and buffer. The mixtures were incubated in a waterbath and examined as described above. Control suspensions containing broth or serum from unimmunised rabbits instead of anti-enzyme serum, or specific agglutinating serum, were similarly examined.

The effect of repeated washing on the capsules formed by staphylococci grown in enzyme-neutralising antiserum:

Cultures of the test strains were prepared in antiserum-buffer-broth mixtures as described. After incubation for 2 to 4 hr. smears of the culture were made, stained with nigrosin and rose-bengal, and

the culture washed in sterile distilled water. After resuspending in broth, smears were made and stained. Washing was repeated four times, the cells being examined microscopically in stained smears after each washing.

Demonstrating the effect of strain LS enzyme on staphylococci grown in enzyme-inhibiting serum:

Cultures of the test strains were prepared in antiserum-buffer-broth mixtures as described; after incubation for 2 to 4 hr. the cultures were centrifuged and the cells resuspended in 0.25 ml. of broth diluted 1 in 2 with phosphate buffer, pH 7.5. The suspensions were heated at 100°C in a waterbath for 2 min., cooled in running tapwater, a loopful smeared on a slide and stained with nigrosin and rose-bengal. The culture was mixed with an equal volume of enzyme diluted with buffer, pH 7.5, to contain 4 units per ml. The mixtures were incubated in a waterbath, and at measured intervals samples examined microscopically after staining with nigrosin and rose-bengal.

As described later, cells of strain D and of other coagulase-positive strains of staphylococci cultured in antiserum to the capsule-stripping enzyme developed structures morphologically resembling capsules. Experiments were made with fluorescein-labelled anti-rabbit globulin, and by agar-gel diffusion to determine if these structures were antigenically similar to the capsular material produced by strain Morris.

Fluorescent antibody studies:

Preparation of chicken anti-rabbit globulin:

Rabbit gamma globulin was precipitated from serum with 50 per cent w/v ammonium sulphate, redissolved in saline and dialysed against saline. The solution was injected intravenously into chickens on each of three successive days, each dose containing 20 mgm. of protein. Three further courses of injections were given 2, 4 and 6 months after the first. After each course the chickens were bled, the serum separated and examined for antibody to rabbit gamma globulin by agar gel diffusion against the solution used for injection. Serum withdrawn after the final course of injections was conjugated with fluorescein-isothiocyanate by the method of Chadwick and Fothergill (1962).

Test:

Cells of the test strain were cultured in antiserum to the capsule-stripping enzyme diluted in buffered broth, as described. During incubation samples from each culture were examined microscopically after staining with nigrosin and rose-bengal. When capsules were seen to be present round the cocci in the culture (usually after 2 to 4 hr. incubation) the cultures were centrifuged and the cells smeared on slides. The smears were allowed to dry at room temperature, were fixed by heating briefly, and washed for 10 min. by rocking at room temperature in a Coplin jar containing 0.1 M phosphate buffer, pH 7.2. The slides were

placed in a moist chamber and the smears exposed to fluorescein-labelled anti-rabbit globulin or to antiserum to strain Morris prepared and absorbed with the test strain as described in Section I, page 36. After 30 min. the slides were washed again. The smears exposed to fluorescein-labelled anti-rabbit globulin were mounted in buffer and examined without further treatment. The remainder were exposed for 30 min. to fluorescein-labelled anti-rabbit globulin, washed and mounted in buffer for examination.

Control cultures of the test strain in broth, in serum from unimmunised rabbits, and suspensions of heat-killed cells of the test strain at a concentration of 1 x 10^{10} cells per ml. in antiserum to the capsule-stripping enzyme, were similarly treated.

The preparations were examined with an oil immersion lens using a Wild M20 microscope fitted with a high-pressure mercury-vapour lamp, and incorporating UG1 and GG13 exciter and barrier filters.

Gel-diffusion studies:

Preparation of capsular material from strain Morris:

An overnight nutrient broth culture of strain Morris, volume 100 ml. was centrifuged, the cells washed thrice in saline and resuspended in 50 ml. of N/16 HCl. The suspension was heated at 56°C for 30 min. and the cells removed by centrifugation. The supernatant was brought to pH 7.2 with 2N NaOH, dialysed for

48 hr. against distilled water and precipitated with 3 volumes of redistilled ethanol without added electrolyte. The faint precipitate which appeared was discarded after centrifuging. The supernatant was treated with solid sodium acetate until a heavy flocculent precipitate appeared; this was removed by centrifugation, washed thrice in acetone and dried over P_2O_5. Before use the capsular material was redissolved in saline at a concentration of 200 µgm. per ml. w/v.

Preparation of capsular material from the test strains:

Test strains were cultured for 3 hr. in 1 ml. amounts of antiserum to the capsule-stripping enzyme diluted 1 in 3 with buffered broth as described. The cultures were centrifuged, the cells resuspended in buffered saline, pH 7.5, heated for 3 min. in a waterbath at 100°C and cooled in running tapwater. To each suspension was added 0.25 ml. of enzyme diluted with buffer, pH 7.5, to contain 4 units per ml. The mixtures were incubated for 1 hr., centrifuged, and the supernatant fluid heated at 60°C in a waterbath to destroy enzyme activity.

Control cultures of the test strain in broth and in serum from unimmunised rabbits were similarly treated.

Wells 8 mm. in diameter were cut in plates containing 1.5 per cent w/v agar dissolved in saline containing 1 in 10,000 "Thiomersal". The supernatant fluids from cultures of the test strains in anti-enzyme serum before and after treatment

with enzyme were placed in the wells and allowed to diffuse against antiserum to strain Morris absorbed with the test strain. As a control, a preparation of Morris capsular material dissolved in saline to a concentration of 200 µg. per ml. (w/v) was included in each test. The plates were incubated for 18 hr. then sealed and stored at 4°C. They were examined daily for 3 weeks before being discarded.

Neutralisation of capsule-stripping enzyme by rabbit sera:

Sera from rabbits injected with alum-precipitated capsule-stripping enzyme neutralised the action of enzyme on both live and heat-killed cells, i.e. after incubation with serum the enzyme did not decapsulate live or heat-killed cells and did not lyse live cells. The immunised rabbits differed in the speed and extent of their antibody response: in general their sera did not neutralise enzyme under the conditions of test until each had received three injections of enzyme (equivalent to a total dose of 240 units or 15 ml. of the most active culture supernatants of strain LS).

The enzyme-neutralising titres of the sera from three rabbits before, during and after immunisation are shown in Table III.2.

Table III.2

The enzyme-neutralising activity of sera from rabbits injected with alum-precipitated capsule-stripping enzyme

Rabbit number:	Neutralising titre of serum: Number of injections:				
	None	1	2	3	4
202	<1/2	<1/2	<1/2	1/16	1/32
221	<1/2	<1/2	1/2	1/16	1/32
222	<1/2	<1/2	<1/2	1/8	1/16

These titres represent considerable neutralising potential. Thus, under the conditions of test 0.5 ml. of the final serum from rabbit number 202, when diluted 1 in 32, neutralised 8 units of enzyme; 1 ml. of the same serum was thus able to neutralise 512 units, the amount present in 32 ml. of the most active supernatants of strain LS.

The formation of capsules by coagulase-positive staphylococci cultured in antiserum to the capsule-stripping enzyme:

A. Capsule formation by strain D (the derived non-capsulated variant of strain Morris):

After 30 to 60 min. incubation in antiserum-buffer-broth mixtures small unstained blebs were visible outside but in contact with individual cocci. Small unstained areas were also present in the centre of clumps of cocci.

After 60 to 90 min. incubation smears of the cultures contained many cocci outside of which were unstained semilunar areas, formed apparently by coalescence or enlargement of the unstained blebs seen in younger cultures. In smears made from 2 hr. cultures many of the cocci or clumps of cocci were enclosed by distinct and often wide unstained zones morphologically resembling capsules. These surrounded single cocci, pairs and clumps although not always completely; many of the zones showed indentations, often level with and on the same axis as the common

cell-wall between two cocci (Figure III.1).

After 5 hr. incubation the number of cocci in the cultures had increased considerably. Nearly all the cells or cell clumps were surrounded by wide capsules; the outer edges of most of these were irregular and breaks in them were still to be seen (Figure III.2).

During incubation for the next 15 hr. the number of capsulated cocci and the size of individual capsules decreased as the total number of non-capsulated cocci increased.

Capsules were also seen in smears of the cultures stained by the method of Butt, Bonynge and Joyce (1936) (Figure III.4).

The appearance of capsulated cocci correlated directly with the presence in the cultures of serum able to neutralise the capsule-stripping enzyme. Capsules were not seen in the following preparations:

a. Cultures containing serum from unimmunised rabbits (Figures III.3 and III.5);

b. Cultures from which enzyme-neutralising serum was omitted;

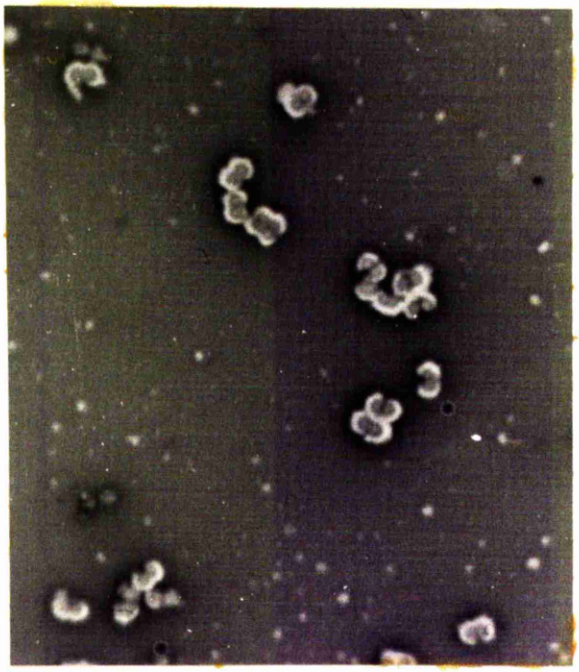
c. Cultures containing anti-enzyme serum which had been neutralised by previous incubation with enzyme;

d. Cultures containing enzyme-neutralising serum at a non-neutralising dilution, e.g. 1 in 64;

e. Suspensions of heat-killed cells of strain D incubated in enzyme-neutralising serum;

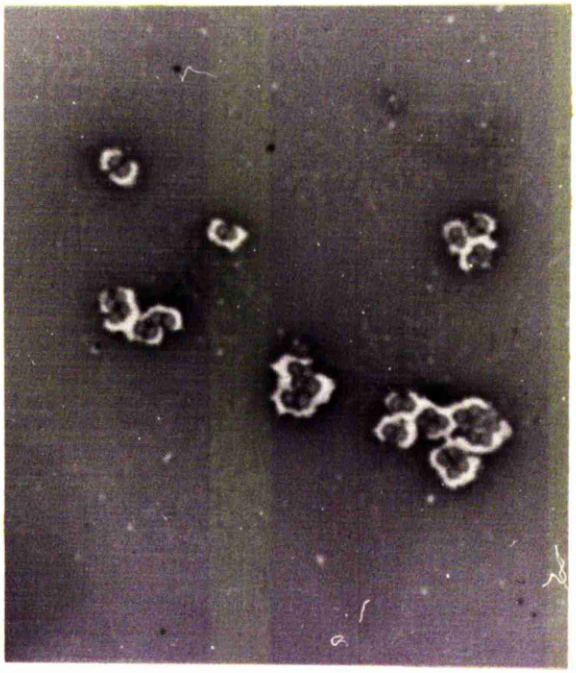
FIGURES III.1-3

Capsule formation by strain D (the noncapsulated variant of strain Morris) cultured in antiserum to the capsule-stripping enzyme



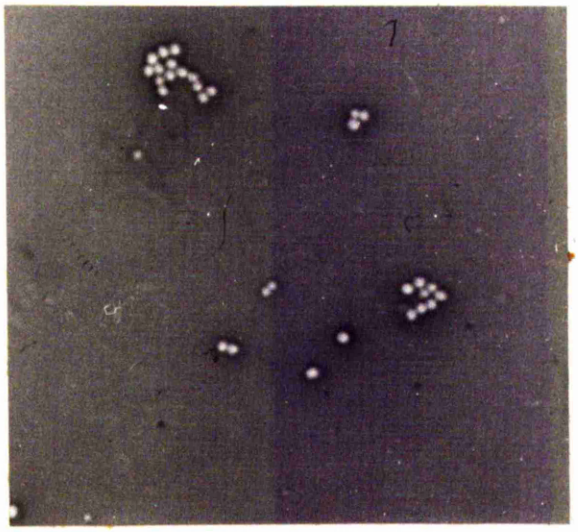
III.1

after 2 hr. incubation (nigrosin and rose-bengal, x 1500)



III.2

after 5 hr. incubation (nigrosin and rose-bengal, x 1500)



III.3

Control culture after 4 hr. incubation in serum from an unimmunised rabbit (nigrosin and rose-bengal, x 1500)

FIGURE III.4

Capsule formation by strain D cultured for 3 hr. in antiserum to the capsule-stripping enzyme and stained by the method of Butt, Bonynge and Joyce (1936).

(x 1500)

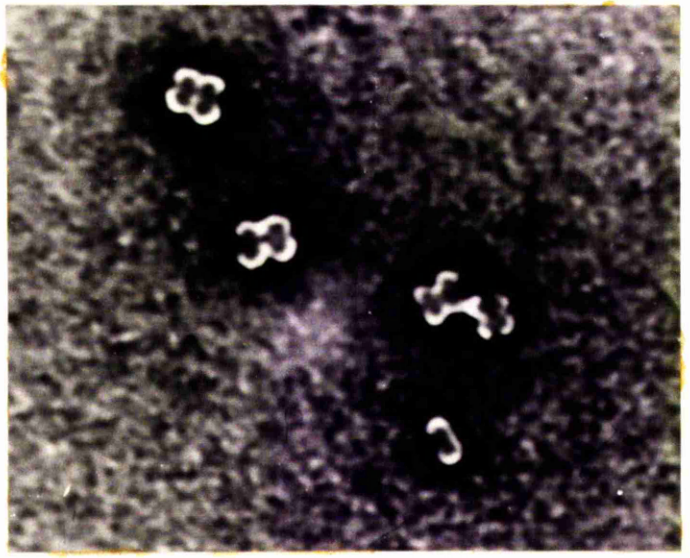
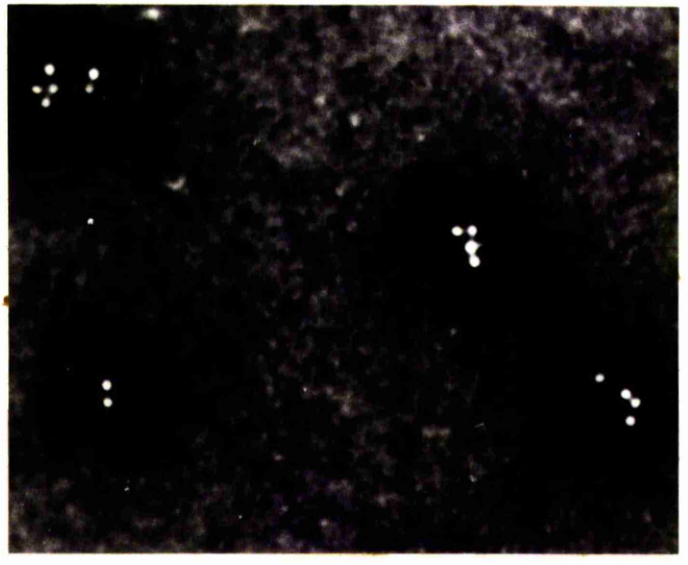


FIGURE III.5

Cells of strain D cultured for 3 hr. in serum from a non-immunised rabbit and stained by the method of Butt, Bonynge and Joyce (1936).

(x 1500)



f. Suspensions of live or heat-killed cells of strain D incubated in a specific agglutinating serum.

These results show that the development of capsules by strain D is a specific response of live cells to the presence of enzyme-neutralising serum.

Surprisingly, capsules were not seen when the same cultures were examined microscopically suspended in wet india ink films. However, many of the cells were enmeshed in clumps of unstained material, usually irregular in outline, and most of the remainder were surrounded by a narrow clear zone, distinctly but not measurably wider than the diffraction halo normally seen about all cells in such suspensions.

The speed with which capsules appeared, the size to which they developed, and the speed with which they disappeared from cultures were not affected by first absorbing the enzyme-neutralising serum with cells of strain D but were modified either by lowering the concentration of enzyme-neutralising serum present in the cultures, or by increasing the number of cells added to each culture at the beginning of incubation. For instance, using serum with an enzyme-neutralising titre of 1 in 16, capsules appeared at the same time, developed to the same degree and persisted for the same time whether the serum was present in the culture diluted 1 in 2, 1 in 4 or 1 in 8. When the serum was diluted 1 in 16 or 1 in 32, fewer cells developed capsules, the capsules took longer to appear, were smaller when fully developed, and disappeared more rapidly when incubation was

continued. Capsulated cocci were not seen in cultures containing serum diluted 1 in 64. Similarly, in cultures containing enzyme-neutralising serum diluted 1 in 3 but inoculated with 1×10^{10} cocci per ml. or more (i.e. one thousand times more then the number usually added), few cocci became capsulated, the capsules were smaller, took longer to appear and disappeared more rapidly from the cultures.

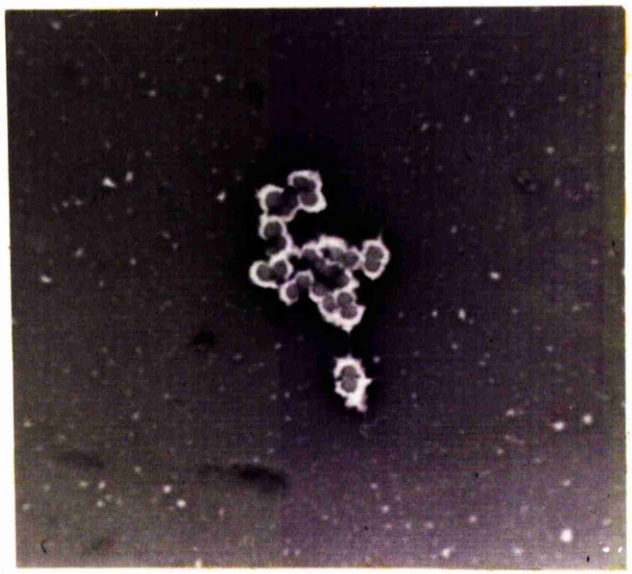
B. Capsule formation by strain LS:

As with strain D, capsules appeared about cells of strain LS when cultured in antiserum to the "capsule-stripping" enzyme. Certain differences were noticed, however:

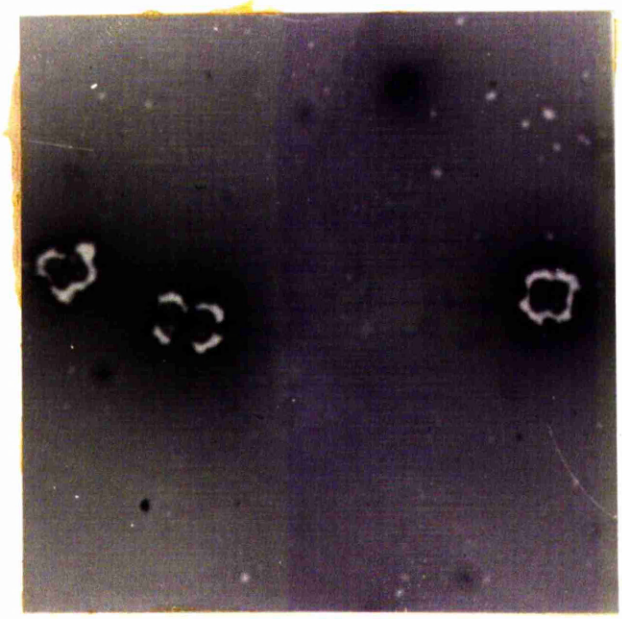
1. Blobs of unstained material were slower to appear at the periphery of the cells and in general were not visible until incubation had been continued for 90 min., not after 30 to 60 min. as with strain D.
2. The speed with which capsules developed thereafter and their maximum thickness did not differ from strain D but the capsules were less regular in outline; like the capsules about cells of strain D they showed indentations along the axis of common cell-walls.
3. The formation of capsules was accompanied by the aggregation of the cells into large clumps containing from 20 to 30 or more cocci, enclosed in a common capsule, Figure III.6. Although cell clumps appeared in cultures of strain D in

FIGURES III.6 and 7.

Capsule formation by coagulase-positive staphylococci cultured in antiserum to the capsule-stripping enzyme



III.6 Strain LS, after 4 hr. incubation (nigrosin and rose-bengal, x 1500)



III.7 Strain 0957, after 3 hr. incubation (nigrosin and rose-bengal, x 1500)

broth-antiserum mixtures and in cultures of strain LS in broth, they did not reach this size. Neither the clumping of cells of strain LS, nor the appearance of capsules was affected by absorbing the antiserum with homologous cells before culture.

4. The most striking difference was in the length of time for which capsules persisted in the two cultures. After 18 hr. incubation many cells of strain D were still capsulated. By contrast, cultures of strain LS after 12 hr. contained few capsulated cells and the capsules which remained were small; capsulated cells were not seen in cultures of strain LS incubated for 18 hr. or longer.

C. The formation of capsules by other coagulase-positive strains of staphylococci:

Like strain D and strain LS, coagulase-positive staphylococci from human infections developed capsules when cultured in antiserum to the capsule-stripping enzyme. The appearances of one strain, 0957, are shown in Figure III.7.

When inocula of the same size were cultured in the same volume of a single enzyme-neutralising serum diluted with buffered broth five variables were noted in the response of the strains: either capsules did not develop at all during 24 hr. incubation, or in the case of those strains which did develop capsules, there were differences in the speed with which capsules first appeared; the size to which they developed; the number of cells in each culture

becoming capsulated, and the length of time for which capsules persisted on continued incubation.

The response to enzyme-neutralising serum of 11 coagulase-positive strains from human infections was compared by grading the degree of capsulation of each at measured intervals during incubation, as follows:

\- : appearances the same as those in control cultures in buffered broth without antiserum;

\+ : small semilunar unstained areas or small unstained blebs at the periphery of less than half the cocci examined;

\++ : semilunar unstained areas, larger than in (+) above, at the periphery of more than half the cocci examined;

\+++ : wide capsules about most of the cocci or groups of cocci examined.

Using this method of grading, the response to enzyme-neutralising serum of 11 coagulase-positive staphylococci from human infections, and of strains D and LS were assessed during the first 5 hr. of culture and after 18 hr. The results are shown in Table III.3.

Of a total of 13 strains tested, strains D, LS and 7 of the 11 strains from human infections developed capsules when cultured in antiserum to the capsule-stripping enzyme. The ability to produce capsules was not apparently related to the phage-type of the strains. The speed with which capsules appeared, the extent to which they

developed and their persistence on continued incubation also varied.

Thus, cultures of seven strains (strains D, LS and 5 human strains) contained capsulated cocci after 1 hr. incubation; all of these except strain LS and strain 1357 showed maximal capsulation after 2 hr., persisting for the next three hours, and capsulated cocci were still present in smears made from 18 hr. cultures. As already noted capsulated cocci were not seen in 18 hr. cultures of strain LS. Some cocci of strain 1357 were capsulated after 1 hr., and most after 2 hr., but the capsules rapidly disappeared and capsulated cocci were not present in 5 hr. cultures.

Two of the human strains showed a different pattern of response to antiserum. Strain 1348 did not produce capsules until incubation had been continued for 3 hr; when capsules did appear they were small and although more than half the cells examined were capsulated the capsules did not persist for 18 hr. The development of capsules by strain 1346 was also delayed, capsulated cells appearing only after 2 hr. incubation; thereafter the capsules developed quickly to a maximum and some capsulated cocci were still to be seen in 18 hr. cultures.

These results substantiate the hypothesis that the presence of visible staphylococcal capsules represents a predominance of capsular synthesis over destruction. The fact that different strains responded in different ways and at different times to a single

Table III.3

Strain	Source	Phage type	Response to anti-enzyme serum (capsulation) Hours 0	1	2	3	5	18
D	Strain Morris	52/52A/80/81	-	+	+++	+++	+++	++
LS	Plate contaminant	Not typable	-	+	++	+++	+++	-
0957	Wound swab	Not typable	-	+	+++	+++	+++	++
1346	Lung	B	-	-	++	+++	+++	+
1348	Sputum	52/52A/80/81	-	-	-	+	++	-
1356	Wound swab	52/52A/79/80/7/42E/81/C	-	+	+++	+++	+++	+
1357	Osteomyelitis	81	-	+	+++	+	-	-
1361	Ear swab	55	-	-	-	-	-	-
1377	Nasal swab	A	-	-	-	-	-	-
1379	Septic finger	52/52A/79/80/42E/B	-	+	+++	+++	++	+
1381	Pleural fluid	80/81	-	-	-	-	-	-
1383	Sputum	Not typable	-	-	-	-	-	-
1387	Ear swab	53	-	+	+++	+++	+++	+

(i) - = Appearances the same as those of control cultures in broth alone.
(ii) + = Semilunar unstained areas at the periphery of less than half the cells examined.
(iii) ++ = Unstained semilunar areas, larger than in (ii) above, at the periphery of more than half the cells examined.
(iv) +++ = Numerous wide capsules about most cells or groups of cells examined.

antiserum can also be interpreted as demonstrating that individual strains differ in the speed with which they synthesise capsular material, or in the amount of capsule-destroying enzyme which they produce.

The staining reactions of the capsules formed by strain LS and the laboratory strains from human infections were the same as those formed by strain D: i.e. capsules were seen in smears stained with nigrosin and rose-bengal, or by the dry india ink method of Butt, Bonynge and Joyce (1936) but definite capsules were not seen when cocci from the same cultures were suspended in thin, wet india ink suspensions. As with strain D, however, some cocci in thin india ink suspensions were enmeshed in irregular clumps of unstained material and the remainder were surrounded by a narrow unstained zone, wider than the normal diffraction halo.

The effect of repeated washing and of capsule-stripping enzyme on the capsules formed by staphylococci grown with enzyme-inhibiting serum:

Unlike the capsules of strain Morris the capsules formed by strain D, strain LS and 7 of 11 laboratory strains of coagulase-positive staphylococci from human infections were removed by washing in distilled water. The size of the capsules as seen in smears stained with nigrosin and rose-bengal was diminished by washing the cells once; three or four washes were necessary to remove visible capsular material completely.

Incubation of heated capsulated cells with capsule-stripping enzyme for 30 to 60 min. also removed visible capsules from them.

Fluorescent antibody studies:

Table III.4 shows the results of culturing staphylococci in antiserum to the capsule-stripping enzyme and exposing them to fluorescein-conjugated anti-rabbit globulin with or without first treating them with antiserum to strain Morris.

Capsulated cocci exposed first to antiserum to strain Morris and then to fluorescein-labelled anti-rabbit globulin were fluorescent; the intensity of fluorescence was greatest with strain Morris, less with strain D and less still with strain 0957. The intensity of fluorescence was not affected by absorbing the antiserum to strain Morris with the test strain, except in the case of strain Morris itself, when absorption removed fluorescence.

Capsulated cocci exposed directly to fluorescein-labelled anti-rabbit globulin did not fluoresce.

Table III.4

The fluorescence of staphylococci cultured in antiserum to the capsule-stripping enzyme and exposed to antiserum to strain Morris (AMS) and/or fluorescein-conjugated chicken anti-rabbit globulin (FARG).

Test strain:	Exposed to:	Degree of fluorescence:
Morris	AMS and FARG	Strong
	AMS absorbed with strain Morris cells, and FARG	None
	FARG	None
D	AMS and FARG	Moderate
	AMS absorbed with strain D cells, and FARG	Moderate
	AMS absorbed with strain Morris cells, and FARG	None
	FARG	None
0957	AMS and FARG	Weak
	AMS absorbed with strain 0957 cells, and FARG	Weak
	AMS absorbed with strain Morris cells, and FARG	None
	FARG	None

Agar-gel diffusion studies:

A broad single band of precipitate developed immediately outside the well containing Morris capsular material when diffused against antiserum to strain Morris in agar. This precipitate, believed to represent the interaction of Morris capsular material and homologous antibody, did not appear if the antiserum was absorbed with cells of strain Morris or with the preparation of capsular material; absorption of the antiserum with cells of strain D did not affect its development.

No precipitate was seen when the supernatants of cultures of strain D and strain 0957 in antiserum to the capsule-stripping enzyme were allowed to react with antiserum to strain Morris nor did a precipitate develop when fluid obtained by treating the cells from these cultures with capsule-stripping enzyme was examined in the same way.

DISCUSSION

When cultured in antiserum to the capsule-stripping enzyme, 9 of 13 coagulase-positive staphylococci examined developed capsules. In smears stained with nigrosin and rose-bengal, or by the method of Butt, Bonynge and Joyce (1936), the morphological evidence of capsulation was often striking but capsules were not seen when the same cultures were examined suspended in thin india ink films.

Capsules appeared only in cultures containing enzyme-neutralising antiserum. They did not appear when the same strains were cultured in serum from unimmunised rabbits, in non-inhibitory dilutions of anti-enzyme serum, or in antiserum absorbed with capsule-stripping enzyme, nor were they seen when strain D (the non-capsulated variant of strain Morris) was cultured in a specific agglutinating serum. Live cells were necessary for capsules to appear: they did not appear when cells of strain D, killed by heating, were incubated in anti-enzyme serum for up to 24 hours.

For these reasons it is believed that the capsules represent a specific response on the part of live cells to antiserum neutralising the capsule-stripping enzyme. While there is no direct experimental evidence to explain why they are visible in dried smears but not in fluid suspensions of india ink, their irregularity in stained smears and the finding that they can be removed from the cells by repeated

washing in distilled water suggest that their physical characters may be more akin to those of loose slime than to those of true bacterial capsules.

The differentiation of bacterial capsules from loose slime and slime layers is based largely upon morphological criteria which are arbitrary to the extent that they can be affected greatly by the exact conditions under which cells are prepared for examination. Thus, if the polymer is seen to be aggregated about the cells in a regular fashion and remains attached to them when the cells are washed or suspended in distilled water, it is referred to as a capsule. Loose slime, on the other hand, although often aggregated about the cells if cultures on solid media are examined, is irregular in outline and arrangement and disperses if the cells are suspended in fluids. Slime layers occupy an intermediate position. Although it has been claimed that capsules and slime differ morphologically and biochemically (Kleineberger-Nobel, 1948) it is clear that in some instances the morphological difference must reflect differences in physical characters rather than chemical composition. Wilkinson, Duguid and Edmunds (1954), for instance, described a strain of Aerobacter aerogenes which produced both capsules and slime, and a variant which produced slime only; all three polymers were, however, serologically identical.

It is possible therefore that the capsules formed by staphylococci in response to enzyme-neutralising antiserum have physical characters more akin to those of loose slime or slime

layers than true capsules, remaining aggregated about the cocci and visible in dried stained smears, but dispersing or being infiltrated by carbon particles when the cells were suspended for examination in wet india ink films.

Fluorescent antibody studies showed that the capsules formed by strain D were antigenically similar to those of the parent strain Morris, i.e. cells of strain D cultured in antiserum to the capsule-stripping enzyme and treated with anti-capsular antibody (prepared by absorbing anti-Morris serum with cells of strain D) fluoresced strongly when exposed subsequently to fluorescein-conjugated anti-rabbit globulin. The same culture exposed first to anti-Morris serum absorbed with strain Morris cells did not fluoresce although it still contained agglutinins for strain D. In similar experiments with other coagulase-positive staphylococci, capsules also developed about the cells but they did not fluoresce when examined in a similar way, i.e. the capsular material was antigenically distinct.

The hypothesis on which this work is based postulates that ordinary staphylococci are not capsulated because they either destroy capsular material or prevent its aggregation as a visible structure at the cell surface, and as a corollary, that inhibition of the anti-capsular mechanism will result in capsules appearing about the cells. While it may be valid to extend the hypothesis and assume that the capsule-destroying enzyme of most strains is serologically identical, it is not necessarily valid to assume that

its inhibition will result in different strains producing serologically identical material.

Recent experiments with Shigella flexneri (Simmons, 1967) have shown that rough mutants of different serotypes cannot complete the formation of the specific O-antigen side-chain because they lack the enzyme UDP-galactose-4-epimerase. If the enzyme deficiency is by-passed by supplying the necessary sugar the mutants complete the O-antigen, which naturally differs from mutant to mutant depending on the serotype from which each was originally derived. In this instance, reversal of a single enzyme defect leads to the production of a number of different antigens.

Even if a single or serologically identical enzyme is responsible for preventing the formation of visible capsules by different strains of staphylococci, its inhibition need not therefore result in complete serological identity of the capsular material formed. If this reasoning is correct, it might explain the observed differences in fluorescence seen when staphylococcal capsules produced by culture in enzyme-neutralising serum were exposed to antiserum to strain Morris capsules and fluorescein-conjugated anti-rabbit globulin.

Four strains of staphylococci failed to develop capsules when cultured in enzyme-neutralising antiserum. Their failure may reflect a serological difference in their capsule-destroying enzyme, so that it was not inhibited by antiserum to the enzyme produced by strain L3. On the other hand it may reflect the fact that these

strains are intrinsically unable to synthesise capsular material.

Evidence has already been presented and discussed for the view that there are at least three kinds of capsulated staphylococci (Section I, pages 49 to 51): the rare mucoid strains producing large amounts of capsular material *in vivo* and *in vitro*; the "Smith-like" strains which produce capsular material *in vitro* but which readily produce non-capsulated variants indistinguishable from modal pathogenic strains; and (in the majority) ordinary pathogenic strains which, it has been suggested (Rogers, 1962), may produce capsular material *in vivo* only. The demonstration that some of last group can form capsules *in vitro* under certain conditions make it more likely that these three types represent parts of a continuous and not discontinuous series. The four coagulase-positive strains which failed to form capsules may represent one extreme of such a series.

SUMMARY

Nine of 13 coagulase-positive staphylococci examined developed capsules when cultured in antiserum to the capsule-stripping enzyme.

The capsules differed in some respects from those of the naturally-capsulated strain Morris.

The capsules formed by strain D (the non-capsulated variant of Strain Morris) were serologically similar to those of the parent strain.

Four strains did not form capsules when cultured in enzyme-neutralising serum. They may produce a serologically distinct capsule-destroying enzyme or be intrinsically unable to synthesise capsular material.

SECTION IV

THE EFFECT OF NON-CAPSULATED COAGULASE-POSITIVE STAPHYLOCOCCI ON THE CAPSULES OF STRAIN MORRIS IN CULTURES AND SUSPENSIONS

MATERIALS AND METHODS

Strain LS, described in Section II (pages 69 to 74) produces an enzyme, one action of which is to decapsulate heat-killed cells of strain Morris. Other coagulase-positive staphylococci were examined to determine if any had the same decapsulating action.

Organisms:

Five hundred strains of staphylococci from human infections were taken at random from those sent for examination to the Phage-typing Laboratory at the Western Infirmary, Glasgow. All were coagulase-positive when tested by either or both the methods of Fisk (1940) and Cadness-Graves, Williams, Harper and Miles (1943).

Three methods were used in series to determine if the strains were able to decapsulate cells of strain Morris. In the first place, because the decapsulating strain LS inhibited the growth of strain Morris on solid media, all the strains were tested for their ability to inhibit strain Morris on solid media. Seven strains which did inhibit the growth of strain Morris (referred to as inhibitory strains), six coagulase-positive non-inhibitory strains,

and strain D (the non-capsulated variant of strain Morris) were examined in more detail by the second and third methods for their effect on the capsules of strain Morris in suspensions and fluid cultures.

Method I: To demonstrate inhibition by test strains on solid media:

Lawn plates of strain Morris were stab-inoculated with the test strains, incubated and examined for inhibition, as follows: Nutrient agar plates, dried in an incubator, were flooded with an overnight broth culture of strain Morris. (In preliminary experiments the concentration of strain Morris cells in the culture was varied within the range 10^9 to 10^4 cells per ml. by opacity; a concentration of 10^6 cells per ml. was finally used). The excess fluid was removed, the plates dried for 1 hr. in an incubator and stab-inoculated with material from an overnight broth culture of each test strain. At first, the concentration of test strain cells inoculated was also varied by applying cells from overnight broth cultures resuspended in broth at concentrations between 10^{10} and 10^4 cells per ml. Undiluted overnight broth cultures, containing approximately 10^7 cells per ml., were finally used.

The inoculated plates were incubated for three days and examined daily.

Method 2: To determine the effect of culture supernatants on the turbidity and capsules of suspensions of cells of strain Morris:

Culture supernatants of strain LS decapsulated heat-killed cells of strain Morris and reduced the turbidity of live-cell suspensions. Culture supernatants of the test strains were examined for similar effects as follows:

To 9 ml. volumes of standard live and heat-killed cell suspensions of strain Morris (suspended in phosphate buffer, pH 6.5 and 7.5) were added volumes of 1 ml. of different culture supernatants of the test strains. The turbidity of the suspensions was compared at measured intervals during incubation with that of control suspensions in which saline replaced the culture supernatants. After incubation for 24 hr. all the suspensions were centrifuged and the cell deposit examined microscopically in thin india ink films and after staining with Gram's stain.

In some experiments "Thiomersal" at a concentration of 1 in 10,000 w/v, or cysteine hydrochloride at a concentration of 1 x 10^{-6}M were added.

The following preparations were tested:

(a) Duplicate cultures of the test strains prepared by inoculating 1 ml. of an overnight broth culture into 100 ml. of nutrient broth warmed to 37°C. One culture was incubated on an orbital shaker, the other incubated without shaking. At measured intervals (hourly for the first 8 hr., 4-hourly from 8 to 24 hr., and daily

thereafter up to 7 days) samples were removed, centrifuged and tested as described above.

(b) Attempts were made to induce the formation of capsule-stripping enzyme by growing the test strains in the same way in broth containing live or heat-killed cells of strain Morris added at an initial concentration of 1 x 10^9 cells per ml. by opacity, or capsular material from strain Morris (prepared as described in Section III, page 103) at a concentration of 10 µgm. per ml.

(c) Duplicate cultures, incubated with and without shaking, and including or omitting live or heat-killed cells of strain Morris or strain Morris capsular material, prepared in nutrient broth containing 10 per cent w/v sterile human serum.

(d) Shake cultures in nutrient broth, prepared as described in paragraph (a) above, incubated for 6 hr. and stored at $4^{\circ}C$; samples of the supernatant fluid were tested daily.

(e) The cells from shake cultures in nutrient broth, volume 500 ml., were washed thrice, suspended in 50 ml. of sterile buffered saline, pH 7.2, and disintegrated by shaking with Chance No. 12 ballotini in a Braun disintegrator cooled with gaseous CO_2. The cell "sap" and cell-walls were separated by centrifugation before testing.

(f) Supernatants from cultures of the test strains, prepared as described above, were concentrated by precipitation with either 50 per cent w/v ammonium sulphate at room temperature, or with 3 volumes of ethanol at $4^{\circ}C$, redissolving the precipitate in buffered saline, pH 7.2, to one tenth of the volume of culture treated.

Preparations concentrated by ammonium sulphate were dialysed overnight against buffered saline, pH 7.2.

(g) Cultures of the test strains grown in semisolid (0.4 per cent w/v) nutrient agar incubated for 48 hr. in an atmosphere of 80 per cent air and 20 per cent CO_2 (Burnet, 1930). Fluid expressed from the agar by freezing and thawing once was centrifuged and the cells discarded.

Attempts were also made to demonstrate capsule-stripping enzyme in culture supernatants of the test strains by increasing the concentration of supernatant and decreasing the concentration of capsulated cells exposed to them, as follows:

To 8 ml. of 18-hour broth culture supernatants of the test strains, mixed with 2 ml. of phosphate buffer at pH 6.5 or 7.5, was added 0.04 ml. of a saline suspension of heat-killed cells of strain Morris containing 4×10^{10} cells per ml. by opacity, (giving a final concentration of 1.6×10^{8} cells per ml., about one-sixth of the cell concentration first used, suspended in eight times more culture supernatant). "Thiomersal" was added to a final concentration of 1 in 10,000 w/v. The mixtures were incubated and their turbidity compared with that of control suspensions containing the same culture supernatants heated at $100^{\circ}C$ for 5 min. After 24 hr. incubation the suspensions were centrifuged and the cell deposit examined in thin india ink films and after staining with Gram's stain.

Preparations which lowered the turbidity of heat-killed cells of strain Morris or altered the number of capsulated cells present after 24 hr. incubation were retested in the presence of antiserum able to neutralise the capsule-stripping enzyme of strain LS. A single antiserum with an enzyme-neutralising titre of 1 in 32 was used throughout; this was added to the test suspensions to give final concentration of 1 in 16 or 1 in 128. Serum from unimmunised rabbits served as a control.

Method 3: To demonstrate the effect of live cultures of the test strains on the capsules of strain Morris:

Overnight broth cultures of each test strain and of strain Morris were centrifuged, the cells washed thrice and resuspended in buffered saline, pH 7.2, at a concentration of 10^{10} cells per ml. by opacity. Half of each suspension was heated in a waterbath at $100^{o}C$ for 5 min. The suspensions were diluted tenfold in sterile broth. The live or heat-killed suspensions of strain Morris cells were mixed with equal volumes (1 ml.) of the live suspensions of the test strains and the mixtures incubated; the heat-killed suspensions of the test strains served as controls. At measured intervals during incubation, which was continued for 24 hr., samples were removed and examined microscopically in thin indin ink suspensions after staining with rose-bengal.

Similar experiments were carried out in which antiserum to strain LS capsule-stripping enzyme (inhibitory titre 1 in 32) was added to

the mixtures at concentrations of from 1 in 4 to 1 in 64. Serum from unimmunised rabbits served as a control.

Inhibition of strain Morris on solid media:

Seven (1.4 per cent) of the five hundred coagulase-positive staphylococci examined inhibited the growth of strain Morris when cultured with it on nutrient agar (Figure IV.1). The diameter of the zones of inhibition produced varied from strain to strain and was affected by the size of the inoculum of strain Morris used to seed the plates (Table IV.1). Within the range 10^4 to 10^9 cells per ml. the zone sizes were not affected by the size of the inoculum of the test strains.

The zones of inhibition produced by the seven strains differed from that produced by strain LS under identical conditions in that the latter caused complete inhibition after 24 hr. incubation and, in addition, on continued incubation a halo of lessened density became apparent in the bacterial growth outside it (Figure II.2, page 71). This secondary halo did not develop outside the zone produced by the seven other inhibitory strains, even after 5 or 6 days' incubation. As with strain LS, the inhibitory effect of these strains was not due to bacteriophage: it could be reproduced by transferring cells from the inhibitory colonies but not with material from the zones of inhibition.

FIGURE IV.1

The inhibitory effect of seven coagulase-positive staphylococci on the growth of strain Morris on nutrient agar after 48 hr. incubation. (x 1)

Table IV.1

Inhibition of strain Morris by seven coagulase-positive staphylococci

Strain No:	Phage type: RTD	Phage type: 1000 RTD	Diameter in mm. of inhibitory zones in the presence of Morris cells at a concentration of (per ml.): 10^9	10^8	10^7	10^6	10^5
6223	not typable	30w/71w	NIL	NIL	1	4	4
6230	not typable	30w/71w	NIL	NIL	1	6	6
6236	47/53/54	-	NIL	NIL	NIL	3.5	3.5
6253	not typable	30w/71w	NIL	NIL	1	4.5	5.0
6265	30	-	NIL	NIL	1	6	6
6266	not typable	54+	NIL	NIL	NIL	2	2
6307	not typable	30w/71w	NIL	NIL	1.5	7	7.5

The effect of culture supernatants of inhibitory and non-inhibitory strains on the turbidity, Gram reaction and capsules of strain Morris cells:

None of the culture preparations tested reduced the turbidity of standard suspensions of live cells.

Culture supernatants of the seven inhibitory strains grown in shake cultures for 6 hr. and stored at 4°C for 2 days before testing (the method used by Ralston, Lieberman, Baer and Krouger (1957) to prepare staphylococcal "autolysin") reduced slightly the turbidity

of heat-killed cells at a concentration of 1 x 10^9 cells per ml. during incubation at pH 6.5 and 7.5 for 8 hr. Continued incubation did not greatly increase their effect. Table IV.2 shows the results of one experiment at pH 6.5.

When the cell deposit from these suspensions was examined after 24 hr. incubation the morphology of the cells differed depending on the pH. At pH 6.5 most of the cocci present were still capsulated, but Gram-negative. At pH 7.5 the cocci were also Gram-negative but few capsulated cocci were present in suspensions incubated with supernatants from five of the seven strains. Of the two remaining strains one (strain 6236) decapsulated less than half the cells added at pH 7.5 and the other (strain 6266) did not decapsulate any.

Similar results, shown in Table IV.3, were obtained when larger amounts of 18-hour broth culture supernatants of the same strains, and of the non-inhibitory strains, were incubated with smaller numbers of heat-killed cells of strain Morris.

As shown in Table IV.3, culture supernatants of 5 of 7 inhibitory strains, 5 of 6 non-inhibitory strains and strain D decapsulated heat-killed cells of strain Morris at pH 7.5; all the strains tested altered the Gram reaction of heat-killed cells at pH 6.5.

Decapsulation, but not the alteration in turbidity or Gram reaction, was prevented by antiserum able to neutralise strain LS capsulo-stripping enzyme, at a final dilution of 1 in 16. Anti-

Table IV.2

The effect of supernatants from 6 hr. nutrient broth shake cultures (stored at 4°C for 2 days) on the turbidity of heat-killed cells of strain Morris in buffered saline, pH 6.5.

Time:	Nephelometer reading: Strain No:							
	Control	6223	6230	6236	6258	6265	6266	6307
0 min.	100	94	92	94	94	94	94	94
10 min.	97	92	88	91	90	90	92	89
25 min.	93	90	88	88	89	88	91	89
2 hr.	88	84	84	84	84	83	86	84
3½ hr.	88	84	80	83	81	82	84	81
7 hr.	88	82	75	82	75	77	84	76
24 hr.	86	76	62	74	64	65	76	64

Table IV.3

The effect of 18-hour broth culture supernatants of coagulase-positive staphylococci on the capsules and Gram-reaction of heat-killed cells of strain Morris.

Strain No.	pH	% capsulated cells after 18 hours incubation	Gram reaction	Effect on decapsulation of enzyme-neutralising serum
D	6.5	90-100	negative	----
	7.5	5	negative	Inhibited by antiserum
Morris	6.5	90-100	negative	----
	7.5	100	negative	----
Inhibitory strains				
6223, 6230, 6258, 6265, 6307	6.5	90-100	negative	----
	7.5	5	negative	Inhibited by antiserum
6236	6.5	90-100	negative	----
	7.5	50-60	negative	Inhibited by antiserum
6266	6.5	100	negative	----
	7.5	100	negative	----
Non-inhibitory strains				
1, 2, 3, 4, 5	6.5	90-100	negative	----
	7.5	5	negative	----
6	6.5	90-100	negative	----
	7.5	90-100	negative	----

enzyme serum at a non-neutralising dilution (i.e. 1 in 128) and serum from unimmunised rabbits did not prevent decapsulation.

Decapsulation of strain Morris cells in growing cultures of the test strains:

Strains, culture supernatants of which decapsulated heat-killed cells of strain Morris as described above, also decapsulated heat-killed cells when grown with them. In cultures to which heat-killed cells had been added approximately equal numbers of capsulated and non-capsulated cells were present immediately before incubation (Figure IV.2). The same cultures after 4 hr. incubation contained fewer capsulated cocci; after 8 hr. incubation most of the capsulated cocci had disappeared; those remaining were surrounded by thin and irregular capsules (Figure IV.3).

Many of the films examined contained small unstained blebs, seemingly detached pieces of capsular material (Figure IV.3). As with cell-free culture supernatants, strain 6266, strain 6 and strain Morris itself did not decapsulate heat-killed cells.

Decapsulation did not take place in any of the cultures containing live cells of strain Morris, or heat-killed cells of the test strains. Decapsulation by live cultures was abolished by adding to the cultures antiserum able to neutralise strain LS capsule-stripping enzyme in concentrations of from 1 in 4 to 1 in 16; antiserum at lower concentrations and serum from

FIGURE IV.2

Mixed culture of heat-killed cells of strain Morris (capsulated) and live cells of strain 6307 (noncapsulated) before incubation.
(India ink and rose-bengal x 1700)

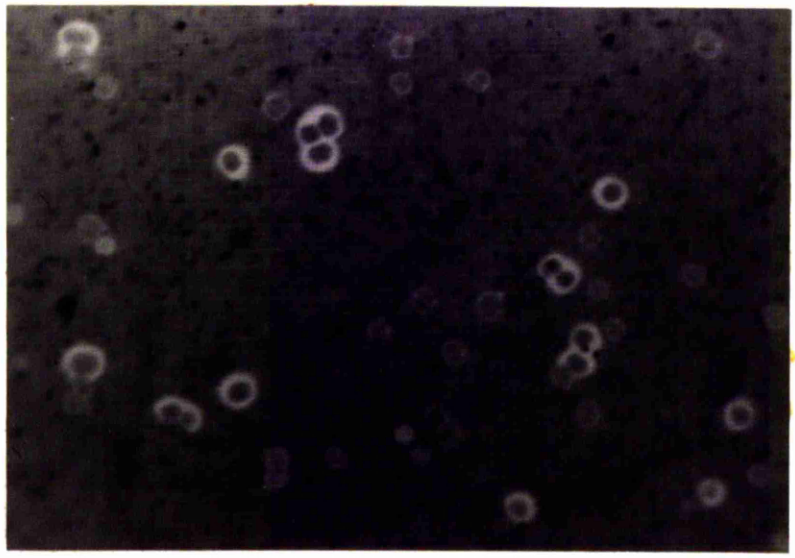
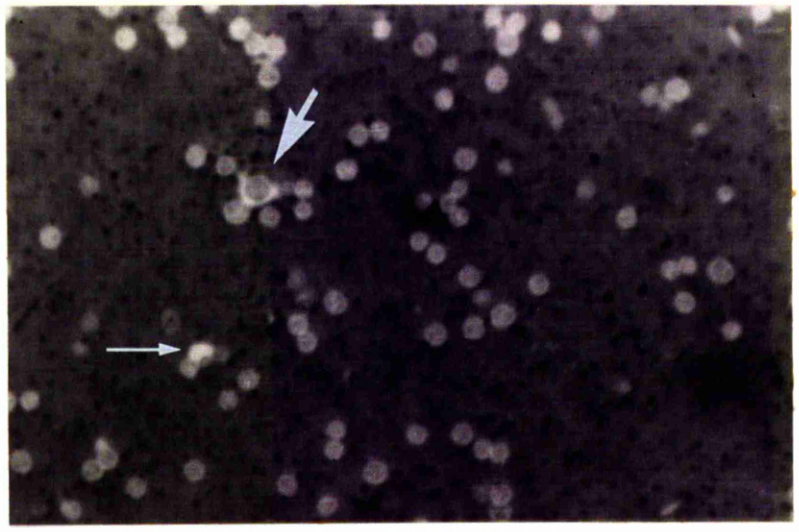


FIGURE IV.3

The same culture after incubation for 8 hours.
(India ink and rose-bengal x 1700)



A cell with a thin irregular capsule (large arrow) and a detached piece of capsular material (small arrow) are present. (The unstained area about the remaining cells is the diffraction halo normally seen in wet india ink preparations).

unimmunised rabbits were without effect.

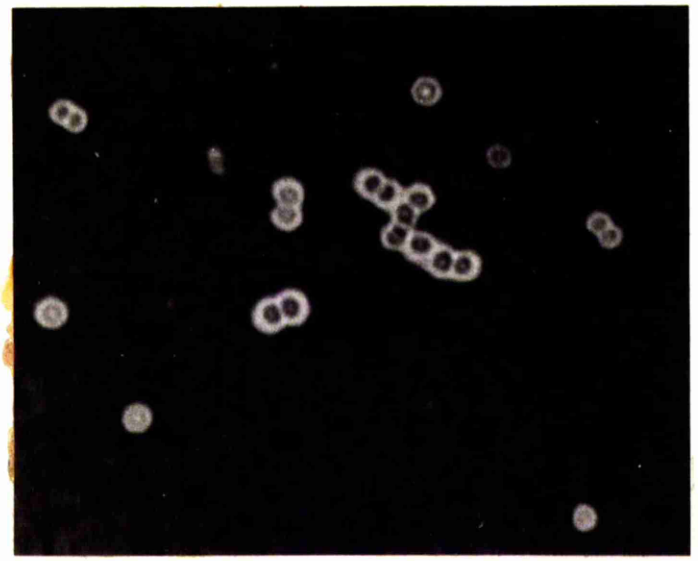
The morphology of the inhibitory and non-inhibitory strains:

The colonial morphology of all the inhibitory and non-inhibitory strains was that of modal strains of Staphylococcus aureus. The finding that strain 6266 and strain 6, like strain Morris itself, consistently failed to decapsulate heat-killed cells required explanation. Possible reasons for their failure are discussed later; one explanation is that they fail to produce capsule-stripping enzyme, and are ipso facto capsulated. Examination revealed that strain 6266, like strain Morris, was heavily capsulated (Figure IV.4) although it did not produce colonies of an obviously mucoid consistency. None of the other strains was capsulated.

The results presented in this Section show that live cultures and cell-free culture supernatants of non-capsulated coagulase-positive staphylococci were able to decapsulate heat-killed cells of strain Morris. Decapsulation was prevented by serum neutralising the capsule-stripping enzyme produced by strain LS. Cells decapsulated by culture supernatants were also rendered Gram-negative; this effect was not prevented by anti-enzyme serum.

FIGURE IV.4

24-hour blood agar culture of strain 6266 suspended in india ink after staining with rose-bengal (x 1500)



DISCUSSION

Twelve (*circa* 85 per cent) of 14 coagulase-positive staphylococci tested decapsulated heat-killed cells of strain Morris added to both culture supernatants and live cultures. Unlike strain LS none of these strains was shown to produce large amounts of capsule-stripping enzyme, but decapsulation by them was inhibited by serum which also neutralised strain LS capsule-stripping enzyme.

In general, these results substantiate the hypothesis that ordinary coagulase-positive staphylococci are not capsulated because they produce a capsule-destroying enzyme which destroys or removes capsular material so that it cannot form a visible aggregate at the cell surface. In particular, the demonstration that culture supernatants and live cultures of strain D (the non-capsulated variant of strain Morris) were able to decapsulate strain Morris cells and that culture supernatants of strain Morris itself had no such effect, offers a possible explanation for the absence of visible capsules from cells of the variant.

Two of the coagulase-positive strains examined (strains 6266 and 6), like strain Morris itself, were unable to decapsulate heat-killed cells. There are, *ex hypothesi*, three explanations for their failure: that they cannot form both capsule-destroying enzyme and capsular material; that they form a capsule-destroying enzyme which cannot destroy strain Morris capsules, or that they

are intrinsically unable to form any capsule-destroying enzyme. It is believed that the last explanation is valid for strain Morris which is capsulated and unable to decapsulate homologous cells. The retrospective recognition of capsules about cells of strain 6266, confirms the validity of this argument in a striking manner.

The failure of some coagulase-positive strains to form capsules when cultured in antiserum to the capsule-stripping enzyme (discussed in Section III, pages 122 to 123) has been interpreted as implying that staphylococci can be placed in a continuous series according to the amount of capsular material which they produce. The finding of non-capsulated strains which produce large amounts of capsule-stripping enzyme (strain LS) or which are unable (strain 6) or only partly able (strain 6236) to decapsulate capsulated cells may imply a second series of staphylococci, graded according to the amount of capsule-destroying enzyme which they produce. This suggestion is strengthened by the finding (Section III, page 113) that strains which do form capsules when cultured in a fixed amount of enzyme-neutralising serum lose their capsules at different times, suggesting that the amount of capsule-destroying enzyme which they produce or the speed with which they produce it, differs from strain to strain.

The coagulase-positive staphylococci described in this section acted on strain Morris in three ways. Seven (1.4 per cent) of 500

strains examined inhibited the growth of strain Morris when cultured with it on solid media. Culture supernatants of all of these strains, of all of 6 non-inhibitory strains tested and of strain D altered the Gram reaction of heat-killed cells of strain Morris at pH 6.5 and 7.5, and supernatants of 5 of the inhibitory strains, 5 of the non-inhibitory strains and of strain D decapsulated heat-killed cells.

These three actions were clearly distinct. All the inhibitory and non-inhibitory strains tested altered the Gram reaction of heat-killed cells but only some decapsulated them. Decapsulation and alteration of the Gram reaction took place at pH 7.5 and the latter also at pH 6.5 but only decapsulation was inhibited by anti-enzyme serum. The fact that this difference did not depend on pH is exemplified by the non-inhibitory strain 6 which did not decapsulate heat-killed cells at either pH but did alter their Gram reaction.

The activity of cell-free culture supernatants on the turbidity and Gram reaction of heat-killed cells of strain Morris is reminiscent of (and believed to be due to) a staphylococcal autolysin similar to that described by Ralston, Lieberman, Baer and Krouger (1957). Those workers found that supernatants of 6-hour shake cultures stored at $4^{\circ}C$ before examination contained an enzyme with a pH optimum of 6.5 which acted upon heat-killed or stressed staphylococci to reduce their turbidity and which was unusual in

that although its speed of action was related logarithmically to the amount of enzyme present, only a constant percentage of cells was attacked by it. The active culture supernatants described in this section had a similar action in that although they reduced the turbidity of heat-killed cells of strain Morris during incubation for 8 hr. continued incubation did not increase their effect.

It is likely, therefore, that the three actions were due to separate substances: a growth inhibitor demonstrable on solid media; a decapsulating enzyme active at pH 7.5 and alone inhibited by antiserum inhibiting strain L9 enzyme, and autolysin, active at pH 6.5 and 7.5, responsible for altering the Gram reaction of heat-killed cells.

SUMMARY

Twelve (circa 85 per cent) of 14 coagulase-positive staphylococci tested decapsulated heat-killed cells of strain Morris added to cell-free culture supernatants or live growing cultures. Unlike strain L8 none produced large amounts of capsule-stripping enzyme but decapsulation was prevented by serum able to neutraline strain L8 enzyme.

Of the two strains which did not decapsulate heat-killed cells one was itself capsulated.

Seven (1.4 per cent) of 500 coagulase-positive staphylococci examined inhibited the growth of strain Morris when cultured with it on solid media. The ability to inhibit was not directly associated with the ability of the strains to decapsulate heat-killed cells.

GENERAL DISCUSSION

The rarity of frankly capsulated strains of Staphylococcus aureus can be explained by postulating that non-capsulated modal strains are incapable of synthesising capsular material. This statement is final and experimentally sterile. The finding of a non-capsulated strain (strain LS) producing an enzyme which removes the capsules from cells of the naturally capsulated strain Morris suggested an alternative and experimentally fruitful hypothesis: that non-capsulated strains of Staph. aureus are able to synthesise capsular material but that they do not have visible capsules because this material is destroyed, or removed from the cell surface.

Two assumptions are implicit in this hypothesis. First, that non-capsulated modal strains can remove or destroy existing capsular material; and second, that non-capsulated modal strains will form visible capsules if their capsule-destroying mechanism is inhibited.

Experiments have shown (Section IV) that non-capsulated staphylococci are able to destroy or remove existing capsular material. Other experiments (Section III) confirmed the second assumption; non-capsulated modal strains do form capsules when cultured in antiserum which neutralises the capsule-stripping enzyme of strain LS.

Not all the strains tested formed capsules when grown in enzyme-neutralising serum. The strains which failed to do so may either be genetically unable to synthesise capsular material, or else the enzyme responsible for their being non-capsulated is antigenically distinct from that produced by strain LS.

Not all the strains tested were able to remove existing capsular material. The failure of one strain (strain 6266) to do so adds weight to the validity of the hypothesis because this strain is itself capsulated and, like strain Morris, can be considered to be devoid of a capsule-stripping enzyme. The other non-capsulated strain which failed to decapsulate strain Morris cells (strain 6) may produce an enzyme which does not attack the capsule of strain Morris; it may, of course, produce neither enzyme nor capsular material.

These results do not invalidate the hypothesis but they do imply that the truth is less inclusive and that the hypothesis be modified. It can be restated as follows: Most strains of Staphylococcus aureus are not capsulated because, although they produce capsular material, most of them also produce an enzyme which prevents the formation of visible capsules by destroying or removing capsular material.

Evidence has already been presented and discussed for the view that staphylococci can be arranged in a series depending on the amount of capsular material which they synthesise, and that a second series exists in which strains can be ranked according to

their production of capsule-destroying enzyme. If synthesis and destruction of capsular material can vary independently, each strain will belong to one of four qualitative types and produce both substances, either substance or neither.

Strain Morris and strain 6266 represent one type and can be considered to produce capsular material but not capsule-destroying enzyme. There should exist strains (not as yet isolated) which produce capsule-destroying enzyme but not capsular material. In practice, because of the limits imposed by conventional light microscopy, only two cell types will be recognised - capsulated and non-capsulated. In the former synthesis exceeds destruction and in the latter lags behind.

The common non-capsulated type should embrace strains in which synthesis only so far exceeds destruction that submicroscopic amounts of capsular material are present at the cell surface. It is possible that these strains are already being identified by detailed serological typing methods, such as that elaborated by Oeding (1952, 1960) and that some of the surface antigens which he describes are in fact capsular antigens.

It will be recalled that the capsulated strain Morris is about one hundred times more virulent for mice than the derived non-capsulated variant. Unless the loss of the capsule is associated with the loss of another substance or property conferring virulence, it is justifiable to link increased virulence with the presence of a

capsule, at least in this experimental model.

The fact that pathogenic staphylococci are not capsulated when isolated in the laboratory does not mean that capsules are irrelevant to staphylococcal virulence. As Dubos has pointed out "It seems possible that the staphylococci that initiate the disease process may become profoundly altered in some of their characteristics during sequestration in an abscess" (Dubos, 1956). The same loss of characteristics may well take place when staphylococci from any infection are cultured on laboratory media, which reproduce crudely, if at all, the environment at the site of an infection.

This work has been concerned with the phenomenon of capsulation in staphylococci. Despite the recognised association between bacterial capsules and virulence no attempt has as yet been made to explore the effects of staphylococcal capsular antigens on the initiation or course of infection in man and animals. It is believed, however, that the hypothesis constructed and tested here allows a fresh examination of the basis of virulence and pathogenicity in Staphylococcus aureus.

APPENDIX

SOME METHODS OF DEMONSTRATING BACTERIAL CAPSULES

Staining methods for demonstrating bacterial capsules are legion; their very number indicates that no single method is entirely satisfactory. Duguid (1951) has reviewed the most satisfactory methods.

The details of seven methods are given below. Four of them formed the basis for reports that pathogenic staphylococci are capsulated; the remaining three have been used during the present work.

Lyons' capsule stains

(1) Original method (Lyons, 1937)

a. Broth cultures:

Smears are dried in the incubator for 30 minutes and never flamed. The fixed slide is covered with undiluted carbol fuchsin and the excess decanted after 5 seconds so that only a thin layer of stain remains, but this should not be allowed to dry. After 1 minute the slide is quickly rinsed in running water and covered with an aqueous solution of 2 per cent potassium hydroxide for 10 seconds. This is decanted and without rinsing Loeffler's alkaline methylene blue is added. After 10 seconds the slide is rinsed in water and blotted dry.

The cocci stain blue and the capsules pink. The capsule is most readily seen when the smear is examined in a reduced light. The staining is usually best at the margins of the smear; it fades on keeping.

b. Pus and blood smears

Slides are prepared and stained with carbol fuchsin as above. The slide is quickly rinsed in running water and then in "Lysol" diluted 1 in 20 with distilled water. It is washed again in water and stained for 10 seconds with crystal violet. The "Lysol" rinsings decolourise the precipitated serum and must be adapted to the thickness of the smear.

The cocci stain blue and the capsules pinkish-purple.

(ii) Modified method (Spink, 1939)

Smears of broth cultures are dried in the incubator without flaming. The smears are stained for 1 to 3 minutes with carbol fuchsin solution (see below); the stain is decanted and the smear covered for 10 seconds with 2 per cent aqueous potassium hydroxide. This is decanted, the smear blotted dry and stained with Loeffler's alkaline methylene blue for 10 seconds. The smear is rinsed in water and blotted dry.

Carbol fuchsin solution: 0.025 gm. of basic fuchsin is added to 3 ml. of dehydrated alcohol and 22 ml. of 5 per cent phenol in distilled water, shaked well, and filtered before use.

Kaluzewski's capsule stains (Kaluzewski, 1954)

(iii) Method I:

A loopful of condensation water from an egg medium slope culture is placed at the edge of a clean, grease-free slide. One drop of india ink is added and smeared as for a blood film with the edge of another slide. The smear is dried at room temperature and stained for 1 minute with Victoria 4R, then rinsed and dried.

(iv) Method II:

A thin even film is made on a grease-free slide, dried in air and fixed by heating. It is stained for 1½ minutes with dilute carbol fuchsin (Gram counterstain), washed and dried. Two drops of nigrosin solution (see below) diluted 1 in 5 with distilled water are placed at the edge of a second slide. The smear is warmed gently (to ensure that the nigrosin dries immediately) and the nigrosin-carrying slide moved over it at an angle of 45^{o}.

Organisms stain red, the capsule is pink or unstained and the background is dark grey.

Nigrosin solution: 6 gm. of nigrosin are dissolved in 60 ml. of distilled water, stirred and boiled for 15 minutes, and filtered while still hot. The volume is adjusted to 50 ml. and the stain boiled again for 10 minutes, filtering again while hot. One ml. of 40 per cent formaldehyde is added. The stain is diluted 1 in 5 with distilled water before use, and should be kept tightly stoppered.

(v) Wet india ink method (Duguid, 1951)

Duguid's description of this method is as follows: "The ink should be dense, homogeneous and free from large particles or clumps of particles. Thin ink may be improved if concentrated somewhat by evaporation. A large loopful of the undiluted ink is placed on a clean slide. Into this is mixed either a small portion of a colony or a small loopful of the centrifuged deposit of a liquid culture; a large number of bacteria must be added but the ink should be diluted as little as possible. A clean coverglass is placed on the drop and pressed down with a pad of blotting paper. Several attempts may be necessary to achieve a film of the proper thickness. The ink film should be about the same thickness as the capsulated bacteria so that these are just lightly gripped between the slide and coverglass. The capsule appears as a clear light zone between the refractile cell outline and the dark ink background".

Greater contrast between the cells and background has been achieved in the present work by first staining the organisms with rose-bengal. To avoid diluting the ink, this is best accomplished by adding the rose-bengal directly to the ink to give a final concentration of 2 to 3 per cent w/v.

(vi) Dry india ink method (Butt, Bonynge and Joyce, 1936)

"Pelikan" india ink is spun to remove coarse particles. A thin suspension of organisms from an agar culture is made in a drop of 6 per cent aqueous dextrose solution. A second drop of india

ink is mixed with this and spread with the edge of a second slide as for a blood film. The smear is dried in air, stained with alcoholic methylene blue, washed, and dried in air without heating.

(vii) Nigrosin and rose-bengal stain (Browning and Mackie, 1949)

One loopful of exudate, fluid culture or broth suspension of a small portion of a colony is mixed with one loopful of 1 per cent aqueous rose-bengal and 1 per cent aqueous nigrosin, spread as thinly as possible, and allowed to dry.

Cocci stain pink and the background grey-pink. The capsules are seen as clear unstained haloes.

REFERENCES

Adams, M.H. (1959) "Bacteriophages", 1st ed., p. 450, New York. Interscience Publishers, Inc.

Adams, M.H. and Park, B.H. (1956) Virology, 2, 711: An enzyme produced by a phage-host cell system. II. The properties of the polysaccharide depolymerase.

Alami, S.Y. and Kelly, F.C. (1959) J.Bact., 78, 539: Demonstration of staphylococcoal free coagulase and clumping factor in soft agar.

Baird-Parker, A.C. (1963) J.gen.Microbiol., 30, 409: A classification of micrococci and staphylococci based on physiological and biochemical tests.

Baird-Parker, A.C. (1965) J.gen.Microbiol., 38, 363: A classification of staphylococci and micrococci from world-wide sources.

Barber, Celia (1964) J.Hyg.Camb., 62, 121: The site of specific substances in capsulated organisms.

Barber, M. and Kuper, S.W.A. (1951) J.Path.Bact., 63, 65: Identification of Staphylococcus pyogenes by the phosphatase reaction.

Bigger, J.W., Boland, C.R. and O'Meara, R.A.Q. (1927), J.Path.Bact., 30, 260: Variant colonies of Staphylococcus aureus.

Browder, H.P., Zygmunt, W.A., Young, J.R. and Tavormina, P.A. (1965) Biochim.biophys.Res.Comm., 19, 383: Lysostaphin: enzymic mode of action.

Browning, C.H. and Mackie, T.J. (1949) "Textbook of Bacteriology", 1st ed., p. 785. London. Oxford University Press.

Burnet, F.M. (1930) J.Path.Bact., 33, 1: The production of staphylococcoal toxin.

Burrows, T.W. and Bacon, G.A. (1954) Brit. J.exp.Path., 35, 134: The basis of virulence in Pasteurella pestis. Comparative behaviour of virulent and avirulent strains in vivo.

Butt, E.M., Bonynge, C.W. and Joyce, R.L. (1936) J.Infect.Dis., 58, 5: The demonstration of capsules about haemolytic streptococci with india ink or azo blue.

Cadness-Graves, B., Williams, R., Harper, G.J. and Miles, A.A. (1943) Lancet, i, 736: Slide test for coagulase-positive staphylococci.

Chadwick, C.S. and Fothergill, J.E. (1962) In "Fluorescent protein tracing", ed. R.C. Nairn. 1st ed. Edinburgh. E. and S. Livingstone.

Ciurea, C.C., Zaharinde, C., Ebinger, C. and Ieremia, T. (1962) : quoted by Mesrobeanu and Tago (1961).

Daranyi, J.von (1925) Arch.Hyg.Berl., 96, 182: Qualitative Untersuchung der Luftbakterien.

Daranyi, J.von (1926) Zbl.Bakt., I. Abt. Orig., 99, 74: Pathogenität und Enteilung der Staphylokokken.

Daranyi, J.von (1927) Wien.Med.Wschr., 77, 149: Uber die Virulenz der Staphylokokken.

Dubos, R.J. (1937) J.exp.Med., 66, 101: Mechanism of the lysis of pneumococci by freezing and thawing, bile, and other agents.

Dubos, R.J. (1956) Ann.N.Y.Acad.Sci., 65, 243: The unknowns of staphylococcal infection.

Dubos, R.J. and Avery, O.T. (1931) J.exp.Med., 54, 51: Decomposition of the capsular polysaccharide of pneumococcus Type III by a bacterial enzyme.

Duguid, J.P. (1951) J.Path.Bact., 63, 673: The demonstration of bacterial capsules and slime.

Duthie, E.S. (1954) J.gen.Microbiol., 10, 427: Evidence for two forms of staphylococcal coagulase.

Duthie, E.S. and Haughton, G. (1958) Biochem.J., 70, 125: Purification of free staphylococcal coagulase.

Ekstadt, R.D. (1956a) Ann.N.Y.Acad.Sci., 65, 119: The effect of coagulase on the antibacterial activity of normal human serum against selected strains of Micrococcus pyogenes.

Ekstedt, R.D. (1956b) J.Bact., 72, 157: Further studies on the antibacterial activity of human serum on Micrococcus pyogenes and its inhibition by coagulase.

Ekstedt, R.D. and Nungester, W.J. (1955) Proc.Soc.exp.Biol., N.Y., 89, 90: Coagulase in reversing antibacterial activity of normal human serum on Micrococcus pyogenes.

Elek, S.D. and Levy, E. (1950) J.Path.Bact., 61, 541: Distribution of haemolysins in pathogenic and non-pathogenic staphylococci.

Elek, S.D. (1959) "Staphylococcus pyogenes and its relation to disease". Edinburgh. E. and S. Livingstone.

Fahlberg, W.J. (1963) Bact.Proc., p. 77: The relationship between coagulases and capsular material in Staphylococcus aureus.

Finkelstein, R.A. and Sulkin, S.E. (1958) J.Bact., 75, 339: Characteristics of coagulase-positive and negative staphylococci in serum-soft agar.

Fisher, M.W., Devlin, H.B. and Erlandson, A.L. (1963) Nature (Lond.), 199, 1704: A new staphylococcal antigen. Its preparation and immunizing activity against experimental infections.

Fisher, S. (1960) Aust.J.exp.Biol.Med.Sci., 38, 479: A heat-stable protective staphylococcal antigen.

Fisk, A. (1940) Brit.J.exp.Path., 21, 311: The technique of the coagulase test for staphylococci.

Fleming, A. (1922) Proc.roy.soc.B., 93, 306: On a remarkable bacteriolytic element found in tissues and secretions.

Fletcher, S. (1962) J.Path.Bact., 84, 327: The growth of staphylococci in human serum.

Gilbert, Isabelle (1931) J.Bact., 21, 157: Dissociation in an encapsulated staphylococcus.

Gordon, M.H. (1904) Rep.loc.Gov.Board, Rep.med.off., 33, 388: Report of some characters by which various streptococci and staphylococci may be differentiated and identified.

Gordon, M.H. (1906) 34th Annual Report of the Local Government Board 1904-5, H.M.S.O., London, p. 387: Report on bacterial test whereby particles shed from the skin may be detected in the air.

Haskell, T.H. and Hannessian, S. (1963) Nature (Lond.), 199, 1075: 2-amino-2-deoxy-D-glucuronic acid, the main constituent of SPA (Staphylococcal Polysaccharide Antigen).

Haskell, T.H. and Hanessian, S. (1964) Biochim.biophys.Acta, 83, 35: The purification and characterisation of a new active immunising polysaccharide prepared from Staphylococcus aureus.

Hendry, E.B. (1948) Edinb.Med.J., 55, 142: The preparation of iso-osmotic phosphate buffers.

Henriksen, S.D. (1948) Acta.path.microbiol.scand., 25, 485: Some unusual mucoid organisms.

Hunt, G.A. and Moses, A.J. (1958) Science, 129, 1574: Acute infections of mice with Smith strain of Staphylococcus aureus.

Jay, J.M. (1966) J.Bact., 91, 1804: Production of lysozyme by staphylococci and its correlation with three other extracellular factors.

Julianelle, L.A. and Weighard, C.W. (1935) J.exp.Med., 62, 11: The immunological specificity of staphylococci.

Kaluzewski, S. (1954) Med.dosw.Mikrobiol., 6, 419: Observations on the capsules of E. coli and staphylococcus.

Kapral, F.A. (1966) J. Bact., 92, 1188: The clumping of Staphylococcus aureus in the peritoneal cavity of mice.

Kashiba, S., Nuzu, K., Tanaka, S., Nozu, H. and Amano, T. (1959) Biken's J., 2, 50: Lysozyme and index of pathogenic staphylococci.

Kleineberger-Nobel, E. (1948) J.Hyg.Lond., 46, 345: Capsules and mucoid envelopes of bacteria.

Koenig, M.G. (1962) Yale.J.Biol.Med., 34, 537: Factors relating to the virulence of staphylococci. I. Comparative studies of two colonial variants.

Koenig, M.G., Melly, M.A. and Rogers, D.E. (1962) J.exp.Med., 116, 589: Factors relating to the virulence of staphylococci. II. Observations on four mouse-pathogenic strains.

Koenig, M.G. and Melly, M.A. (1965) Ann.N.Y.Acad.Sci., 128, 231: The importance of surface antigens in staphylococcal virulence.

Kolle, W. and Otto, R. (1902) Z.Hyg.Infekt.-Kr., 41, 369: Der Differencirung der Staphylokokken mittelst der Agglutination.

Lancefield, R.C. (1943) J.exp.Med., 78, 465: Studies on the antigenic composition of Group A haemolytic streptococci. I. Effect of proteolytic enzymes on streptococcal cells.

Lominski, I., Morrison, R.B. and Smith, D.D. (1953) Atti del VI Cong. Int. Microbiol., I, 728: Rough and smooth variants of Staphylococcus aureus.

Lominski, I., Cameron, J. and Wyllie, G. (1958) Nature (Lond.), 181, 1477: Chaining and unchaining Streptococcus faecalis - a hypothesis of the mechanism of cell separation.

Lominski, I. and Gray, S. (1961) Nature (Lond.), 192, 683: Inhibition of lysozyme by "Suramin".

Lominski, I., Smith, D.D., Scott, A.C., Arbuthnott, J.P., Gray, S., Muir, D., Turner, G.H. and Hedges, C.K. (1962) Lancet, i, 1315: Immunisation against experimental staphylococcal infections with coagulase-rich preparations.

Louria, D.B. and Kaminska, T. (1963) J.Lab.Clin.Med., 62, 535: Immunity in experimental staphylococcal infection.

Lyons, C. (1937) Brit.J.exp.Path., 18, 411: Antibacterial immunity to Staphylococcus pyogenes.

McLean, D. (1941) J.Path.Bact., 53, 13: The capsulation of streptococci and its relation to diffusion factor (hyaluronidase).

Mesrobeanu, I. and Taga, S. (1961) Arch.roum.Path.exp., 20, 591: Formations capsulaire chez les varientes des staphylocoques soumis a l'influence des leucocytes in vivo

Miles, A.A. and Misra, S.S. (1938) J.Hyg.Camb., 30, 732: Estimation of the bacteriedal power of the blood.

Mitchell, P.A. and Moyle, J. (1957) J.gen.Microbiol., 16, 184: Autolytic release and osmotic properties of "protoplasts" from Staphylococcus aureus.

Morison, J.E. (1941) J.Path.Bact., 53, 1: The physiology of capsulated streptococci.

Morrison, R.B. (1952) Ph.D. Thesis. University of Glasgow.

Morse, S.I. (1960) Nature (Lond.) 186, 102: Isolation of a phagocytosis-inhibiting substance from culture filtrates of an encapsulated Staphylococcus aureus.

Morse, S.I. (1962a) J.exp.Med., 155, 295: Isolation and properties of a surface antigen of Staphylococcus aureus.

Morse, S.I. (1962b) J.exp.Med., 116, 229: Studies on the chemistry and immunochemistry of cell walls of Staphylococcus aureus.

Morse, S.I. (1963) Nature (Lond.), 200, 1126: Distribution of a surface antigen of Staphylococcus aureus.

Mossel, D.A. (1962) J.Bact., 84, 1140: Attempt in classification of catalase-positive staphylococci and micrococci.

Mudd, S. (1965) Ann.N.Y.Acad.Sci., 128, 249: Discussion.

Mudd, S. and DeCourcy, S.J. (1965) J.Bact., 89, 874: Interaction of viscid material of Staphylococcus aureus with specific immune serum.

Neufeld, F. (1902) Z.Hyg.Infekt.-Kr., 40, 54: Ueber die Agglutination der Pneumokokken und über die Theorieen der Agglutination.

Noetzel, W. (1896) Fortschr.Med., 14, 41: Uber den Nachweis von Kapseln an Mikroorganismen.

Oeding, P. (1952) Acta.path.microbiol.scand., Suppl. 93, 356: Serological typing of staphylococci.

Oeding, P. (1960) Bact.Rev., 24, 374: Antigenic properties of Staphylococcus aureus.

Oesterle, P. (1936) Zbl.Bakt., I.Abt.Orig., 136, 221: Die Verschleimung des Staphylococcus pyogenes aureus; Beitrag zur Biologie der Aureusstämme.

Ogston, A. (1881) Brit.med.J., i, 369: Report upon the microorganisms in surgical diseases.

Parker, M.T. (1962) J.appl.Bact., 25, 389: Phage-typing and the epidemiology of Staphylococcus aureus infection.

Pereira, A.T. (1961) J.Path.Bact., 81, 151: Antigenic loss variation in Staphylococcus aureus.

Perkins, H.R. (1963) Biochem.J., 86, 475: A polymer containing glucose and aminohexuronic acid isolated from the cell-walls of Micrococcus lysodeikticus.

Porter, J.R. and Pelczar, M.J. (1941) J.Bact., 41, 173: Nutrition of Staph. aureus: the influence of biotin, bios IIB and vitamin A on the growth of several strains.

Price, K.M. and Knoeland, Y. (1954) J.Bact., 67, 472: a mucoid form of Micrococcus pyogenes var. aureus showing capsular swelling with specific immune serum.

Price, K.M. and Knoeland, Y. (1956) J.Bact., 71, 229: Further studies of the phenomenon of capsular swelling of Micrococcus pyogenes var. aureus in the presence of immune serum.

Proom, H. (1943) J.Path.Bact., 55, 419: Preparation of precipitating serum for the identification of animal species.

Ralston, D.J., Baer, B.S., Lieberman, M. and Krouger, A.P. (1955) Proc.Soc.exp.Biol.,N.Y., 89, 502: Virolysin, a virus-induced lysin from staphylococcal phage lysates.

Ralston, D.J., Lieberman, M., Baer, B.S. and Krouger, A.P. (1957) J.gen.Physiol., 40, 791: Staphylococcal virolysin, a phage-induced lysin. Its differentiation from the autolysin of normal cells.

Reimann, H.A. (1957) Proc.Soc.exp.Biol.,N.Y., 96, 411: Transformation and culture phase dissociation of Staphylococcus (Micrococcus pyogenes).

Richmond, M.H. (1959) Biochim.biophys.Acta., 31, 564: Lytic enzymes of Staph. aureus 524.

Roger, H. (1896) Rev.gen.Sci., 7, 770: Les infections non bacteriennes. Recherches sur l'Oidio-Mycose.

Rogers, D.E. (1962) J.A.M.A., 181, 38: Staphylococci and man.

Rogers, D.E. and Melly, M.A. (1962) Yale J.Bio.Med., 34, 560: Observations on the immunology of pathogenic staphylococci.

Rosenbach, F.J. (1884) "Mikroorganismen bei den Wundinfektions-krankheiten des Menschen". Weisbaden. J.F.Bergmann.

Sall, T., Rudd, S. and Taubler, J. (1961) J.exp.Med., 113, 693: Concerning the surfaces of cells of Staphylococcus aureus. I. A pseudocapsulation phenomenon under certain experimental conditions.

Sall, T. (1962) J.Bact., 83, 1238: Interrelationship of extracellular enzymes and pseudocapsulation in a strain of Staphylococcus aureus.

Salton, M.R.J. (1964) "The Bacterial Cell Wall", 1st ed., p. 9. Amsterdam. Elsevier.

Salton, M.R.J. and Ghuysen, J.M. (1960) Biochim.biophys.Acta., 45, 355: Acetylhexosamine compounds enzymically released from Micrococcus lysodeikticus cell walls. III. The structure of di- and tetra-saccharides released from cell walls by lysozyme and Streptomyces F_1 enzyme.

Schindler, C.A. and Schuhardt, V.T. (1964) Proc.Nat.Acad.Sci., 51, 414: Lysostaphin: a new bacteriolytic agent for the staphylococcus.

Schindler, C.A. and Schuhardt, V.T. (1965) Biochim.biophys.Acta, 97, 242: Purification and properties of lysostaphin, a lytic agent for Staphylococcus aureus.

Shaw, C., Stitt, J.M. and Cowan, S.T. (1951) J.gen.Microbiol., 5, 1010: Staphylococci and their classification.

Simmons, D.A.R. (1967) Biochem.J. (in press): Smooth to rough mutation in Shigella flexneri.

Smith, H., Keppie, J. and Stanley, J.L. (1955) Brit.J.exp.Path., 36, 460: The chemical basis of virulence of Bacillus anthracis. V. The specific toxin produced by B. anthracis in vivo.

Smith, I.M. and Dubos, R.J. (1956) J.exp.Med., 103, 87: The behaviour of virulent and avirulent staphylococci in the tissues of normal mice.

Sonnenschein, C. (1927) Zbl.Bakt., I.Abt.Orig., 104, 365: Die Mucosus-Form des Pyocyaneum-Bakteriums, Bacterium pyocyaneum mucosum.

Spink, W.W. (1939) Proc.soc.exp.biol., N.Y., 40, 549: Attempts to demonstrate surface antigens of staphylococci and specific phagocytosis.

Subcommittee (1965) Int.Bull.Bact.Nomenel., 15, 109: Separation of staphylococci from micrococci. Recommendations of the Subcommittee on Taxonomy of staphylococci and micrococci.

Sutherland, I.W. and Wilkinson, J.F. (1964) J.gen.Microbiol., 34, xii: Exopolysaccharide depolymerase produced by phages in association with Escherichia coli K 12.

Sutherland, I.W. and Wilkinson, J.F. (1965) J.gen.Microbiol., 39, 373: Depolymerases for bacterial exopolysaccharides obtained from phage-infected bacteria.

Tomcsik, J. (1956): In "Bacterial Anatomy", p. 48, 6th Symposium of the Society for General Microbiology. London. Society for General Microbiology.

Tompsett, R. (1961): In "Antimicrobial Agents and Chemotherapy", ed. M.Finland and M.S.Savage. Detroit. Amer.Soc. Microbiol. p. 67: Relation of clumping factor produced by staphylococci to their phagocytosis and intracellular survival.

Welsch, M. (1949) C.R.Soc.Biol.Paris, 143, 721: Production et activité bacteriolytique de l'autolysine staphylococcique.

Welsch, M. (1950) C.R.Soc.Biol.Paris, 144, 1253: Sels dérivés du phosphore et autolyse de Staphylococcus aureus.

Welsch, M. and Salmon, J. (1949) J.gen.Microbiol., 3, xxvii: Bacteriolytic properties of actinomycetes and staphylococci.

Welsch, M. and Salmon, J. (1950) Ann. Inst.Past., 79, 802: Quelques aspects de la staphylolyse.

Wiley, B.B. (1961) Canad.J.Microbiol., 7, 933: A new virulence test for Staphylococcus aureus and its application to encapsulated strains.

Wiley, B.B. (1963a) Canad.J.Microbiol., 9, 27: The incidence of encapsulated staphylococci and anticapsular antibodies in normal humans.

Wiley, B.B. (1963b) Bact.Proc., p. 76: The relationship between capsular synthesis and virulence in Staphylococcus aureus.

Wiley, B.B. (1964) Bact.Proc., p. 76: Virulence and encapsulation in hospital strains of Staphylococcus aureus.

Wiley, B.B. (1966) Bact.Proc., p. 69: The detection of staphylococcal anticapsular antibodies in mice.

Wiley, B.B. and Wonnacott, J. (1961) J.Bact., 83, 1169: Isolation and partial characterisation of a capsular material from Staphylococcus aureus.

Wilkinson, J.F., Duguid, J.P. and Edmunds, P.N. (1954) J.gen. Microbiol., 11, 59: The distribution of polysaccharide production in Aerobacter and Escherichia strains and its relation to antigenic character.

Williams, R.E.O. and Rippon, J.E. (1952) J.Hyg.Camb., 50, 320: Bacteriophage typing of Staphylococcus aureus.