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The University of Glasgow

EXPERIMENTAL ACTINOMYCOSIS

The pathogenic action of actinomycetes in mice

by

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Thesis submitted for the Degree of Ph.D. in the Faculty of Science

Department of Bacteriology

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P R E F A C E

This study was initiated by me and the design and conduct of the experiments was wholly in my hands. I prepared the cultures, made and recorded the necessary observations upon them, and inoculated the test animals. I made the observations required in connection with the progress of the infection in the animals, including the post-mortem examinations. For the histological part of the investigation my thanks are due to Dr. G.B.S. Roberts of the Pathology Department of the University of Glasgow, who advised on appropriate methods of processing, supervised the technical work connected therewith, and collaborated with me in interpreting the sections. I am grateful to my supervisors – first, Professor C.H. Browning and later, Professor J.W. Howie – and to Dr. I.R.W. Lominski for many stimulating discussions and for helpful suggestions; to Dr. Per Holm, Copenhagen, and to the Curator of the National Collection of Type Cultures for many of the strains used in the work; to Mr. David Colvin, and Mr. Frank Lonsdale, technicians, for help with some of the photography*; to Mr. Kerr for figure 42; to the technicians of the Bacteriology Department for media; and to the Rankin Fund for grants in aid of the work.

* The photo-micrographs (figs. 6-17, 21, 23, 25, 49, 51, 52) were taken by myself.

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

While engaged in routine bacteriological examination of hospital specimens, I isolated strains of actinomycetes from three clinical cases of human actinomycosis. In two other cases, morphologically similar organisms were seen in direct films but could not be cultured. The technical difficulty of isolating, growing, and keeping alive these cultures aroused my interest. A study of the literature soon made it clear that laboratory animals could not be used for experimental study of the disease. No paper examined showed an author with any degree of confidence that reproducible results would follow inoculation of cultures into animals. In fact, reproduction of an actinomycotic lesion in an experimental animal was evidently seldom accomplished. The few positive results recorded after inoculation usually represented a small proportion of a large number of inoculated animals. There was nothing comparable, for example, to the use of the guinea-pig for studying tuberculosis. Also, it was clear that the mouse had not been used as an experimental animal — an odd omission because the mouse is inexpensive and easy to handle and maintain in the laboratory. Most published work dealt with the inoculation of rabbits and guinea-pigs; but there

were references to the use of such animals as cows, pigs, rats, and kittens.

Negroni and Bonfiglioli (1937) alone used the mouse - but they inoculated only one animal. I decided to experiment with the mouse, and this thesis is based on the results of inoculating this animal with three different actinomycetes: *Actinomyces israelii*, *Actinomyces graminis*, and *Nocardia asteroides*.

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REVIEW OF THE LITERATURE

Taxonomy and nomenclature

A word must first be said briefly about taxonomy and nomenclature: otherwise the significance of the various names used by different authors quoted may be unduly confusing Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Hitchens, 1948) differentiates three genera of branched filamentous organisms:-

(1) Actinomyces. - Anaerobic or microaerophilic organisms characterised by production of filaments which, especially in culture, tend to break up into regular fragments.

(2) Nocardia. - Aerobic organisms often partially acid fast; many, though not all, are pathogens.

(3) Streptomyces. - Aerobic filaments which produce abundant aerial spores. All are saprophytes, many living free in soil and producing antibiotics.

Bergey's Manual refers to the species Actinomyces bovis (Harz) as a pathogen of cattle and to Actinomyces israeli* (Kruse) as a pathogen of man. Both are anaerobes or microaerophilic. An alternative taxonomy prefers to recognise only one species Actinomyces bovis with two types: human and bovine. Both forms appear in the literature. Actinomyces graminis

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* In agreement with the nomenclature of the National Collection of Type Cultures, the name A. israelii is used throughout this thesis.

(Bostroem) is an aerobic saprophyte which is badly named because its growth characters properly place it in the genus *Streptomyces*. Its importance is that it must be differentiated from pathogenic species. In this thesis it is referred to without qualification as *Actinomyces graminis* because this is the most commonly used name - though not the soundest taxonomically. In the work to be described here, organisms of each of these three genera were examined in culture and for pathogenic effects in animals.

Actinomycosis: the natural disease

Actinomycosis, the disease, may be broadly defined as an infection in the host resulting from invasion by any actinomycete. To-day, however, although this definition stands — at least for man — the term actinomycosis is generally taken to refer to the infection caused by the anaerobic *Actinomyces* genus. Infection by the aerobic members is referred to as nocardiosis. *Actinomyces* and *Nocardia* are both pathogenic for man and animals, although the infections are relatively uncommon.

Actinomycosis in man and animals, especially cattle, is a chronic granulomatous disease with suppuration. The individual lesion is a large swelling often with many fistulas draining pus. The lesion is a chronic suppurative one and the

abscesses have a multilocular character - the so-called honeycomb abscess. Within the pus, small yellow hard granules, known as "sulphur" granules may be seen by the naked eye. On microscopic examination these granules are found to consist of aggregations of much-branched Gram-positive filaments twined together with at the edge a radiate arrangement. Within the tissues of infected animals microscopic examination shows that these sulphur granules are surrounded by characteristic Gram-negative "clubs", whose nature is discussed later. (page 17). A tissue wall forms around the central organisms and clubs. The tissue-wall shows at its outermost limit a zone of fibrous tissue. Within this, there lies a cellular zone containing a great many polymorphs and, characteristically, many foam cells - macrophages laden with lipoid - which give the lining of each pocket a yellowish colour. See Cappell (1951); Anderson (1948).

Bollinger (1877) described the clinical condition of "lumpy jaw" in cattle. From material given to him by Bollinger, Harz (1878) described the occurrence and appearance of an organism he saw in the tissues and named it <u>Actinomyces bovis</u>. In 1878 Israel gave an account of the post-mortem appearance seen in two cases of human actinomycosis, and noted that tooth-extraction might lead

to cervico-facial actinomycosis. He described and drew sulphur granules (and even the clubs) present in the tissues; furthermore he again presented drawings and observations that had been made by Lagenbeck (1845), also from post-mortem material. Ponfick (1880) showed that the clinical conditions in man and animal appeared to be similar, and he suggested that there was a common infecting bacteria.

Wolf and Israel (1891) were the first to isolate in pure culture the causal organism of human and bovine actinomycosis; they called the bacterium A. bovis after Harz. (Kruse (1896) and Lachnar-Sandoval (1898), for the first time, used the synomym Actinomyces israeli for this organism isolated by Wolf and Israel). The organism isolated by Wolf and Israel was an anaerobe. At the same time Bostroem (1891) isolated from actinomycosis in cattle an aerobic organism which in morphology was similar to the organism of Wolf and Israel. The new aerobic organism was also - though wrongly - named by Bostroem Actinomyces bovis on the assumption that it was the same as that first described by Harz. It is now evident that Bostroem's aerobic organism was a contaminant and not the cause of the disease. Unfortunately, Bostroem's publication led to the creation of two schools of thought about the identity of the infecting agent in actinomycosis. Much of the early work is difficult to assess or follow for

this reason. The anaerobic organism isolated by Wolf and Israel is now accepted as the causal organism and I shall deal only with the literature based on this conclusion.

Morphology and cultural characters of *A. israelii*

The identification of *A. israelii* isolated from pathogenic and non-pathogenic material was and is chiefly based on a study of the morphology of the organism. Direct examination of natural material shows the presence or absence of 'sulphur' granules. Wet film preparations of slightly squashed granules, show in the centre an indistinct mass of branching hyaline hyphae and at the edge there is often a radial arrangement of hyphae with swollen club like ends. Films of the squashed granules stained by Gram's method will show either whole branching Gram-positive filaments massed in the centre of the granule and surrounded by Gram-negative clubs, or if the granule is very severely macerated no clubs will be seen only broken up pieces of filament. If sulphur granules are not present, films stained by Gram's method will show if either complete branched filaments or broken up remnants, or both these characteristic bacterial forms are present. Culture under reduced O_2 tension will confirm the presence of the actinomycete by yielding colonies of the anaerobic *A. israelii*. Examination of these

colonies by Gram-stained films will show the typical branching and broken up filaments. The information obtained by using these procedures has been considered sufficient for identifying A. israelii for clinical diagnostic purposes. Biochemical and serological methods are very time consuming and do not as yet help much in sub-dividing the species. The use of morphological criteria, both colonial and microscopic, for sub-division introduced great and unnecessary confusion into the early literature. The result was to raise doubt about what was the typical appearance of a colony of A. israelii.

The two strains of A. israelii originally isolated and described by Wolf and Israel (1891) from two human cases of Actinomycosis were anaerobic or microaerophilic. On the surface of agar cultures they gave small, rough, heaped-up colonies strongly adherent to the medium by means of a radiate fringe of hyphae. Microscopically, the organisms composing these colonies ranged from long branching filaments to short comma-like rods with olive-shaped swellings at the ends. This is the classical description of the A. israelii organisms, and cultures from human materials by all the workers in this field have yielded such colonial and morphological types. In addition to this classical Wolf-Israel type of colony the occurrence of a second type

of colony, which is smooth, entire, and not adherent to the medium has also been noted. Wright (1905) isolated two such strains, Colebrook (1920) three strains: these strains being isolated from human actinomycosis. Emmons (1938), Lentze (1938), Rosebury et al. (1944), and Holm (1948) also isolated strains giving smooth colonies from both pathogenic material and from mouths (healthy and otherwise): Magnusson (1928) isolated the classical Wolf-Israel organism from cattle suffering from lumpy jaw; from swine with actinomycosis, both colonial types of organism were isolated. Material from eleven cases of actinomycosis in swine gave both colonial types on culture; 71 cases gave only the classical type, and 99 cases the smooth colony. With the difference in colony, there was also a difference in the type of growth in liquid medium. The rough colony on plates when subcultured into broth tubes gave bread-crumb masses growing at the bottom of the tube with a clear supernatant fluid; on the other hand the smooth colony gave a diffuse turbidity throughout the medium with a fine deposit at the bottom of the tube. There was also a difference in microscopic morphology between the two types of growth. The bread-crumb masses were composed of long-branching filaments whereas the even turbidity showed regular, diphtheroid forms. This raised three questions: (1) were these forms two different

species? (2) was this merely the normal variation in colony type which is met with in a large number of bacteria? (3) was an identical organism responsible for the disease in man and cattle? Regarding the possibility of colonial variation, several workers Wolf and Israel (1891), Emmons (1938), Erikson (1940), Rosebury *et al.* (1944), Miller and Drake (1951), Morris (1951) have recorded that on continuous subculture the rough colonial type of *A. israelii* changed into the smooth type, and associated with this has been a corresponding change in the microscopic morphology from branching filamentous hyphae to the regular diphtheroid form. A particularly interesting paper on this aspect was that by Lentze (1938). He recorded the change of a rough colonial type of *A. israelii* into a smooth colony type; he described, and photographed under the microscope, "halo" colonies which he found developed in the process, before the smooth colony was formed. Lentze used the specific terms "R-Form" and "S-Form" to describe these colonial forms of *A. israelii*; Lentze stated that on continuous subculture this new S-Form of *A. israelii* was identical biochemically and serologically with the other S-Form previously isolated by him from natural material. He collected 40 strains of *A. israelii* from human lesions and found that these fell into the R-Form or the S-Form. Furthermore he showed that these R-Forms and S-Forms

differed biochemically (salicin being fermented by the R-Form and not by the S-Form.) and serologically, the S-Form having a more elaborate antigenic complex.

Erikson (1940) also found the halo-types of colony described by Lentze, but she was unable to obtain stable, consistent cultures and preferred to regard these forms as being only variations in the A. israelii colony. Erikson did not, however, approve of Lentze's terminology, because she considered that he had stretched too far the accepted meaning of the descriptive terms R and S.

Though A. israelii isolated from human sources shows either rough or smooth colonial forms, Erikson (1940) and Thompson (1950) showed the organism isolated from cattle usually was of the smooth colony type. Erikson (1940) compared culturally, biochemically, and serologically five strains of bovine origin with 15 strains of human origin. Four of the 15 human strains showed transient changes from rough to smooth colonies, the smooth forms going back to rough again after two passages -- one in broth and one on agar. By using direct slide microscopy (Ørskov, 1923) of growing cultures, Erikson found that the bovine strains did not produce aerial hyphae during the first 48 hours of growth, whereas many of the human strains did so. (It should be emphasised that Erikson's "aerial hyphae" were few, slender,

and transient — altogether different from those observed on Streptomyces). Furthermore, the colonial and microscopic morphology of the bovine strains was different from that of the human strains, in that they formed smooth colonies instead of rough colonies and bacillary forms instead of branching filaments. Sometimes on egg cultures 2-3 weeks old, bovine forms gave branching filaments. Biochemically the bovine strains did not ferment salicin and mannitol (Holm 1930) whereas the human strains did. Serologically the bovine strains did not react with antisera prepared against the human strains, and vice versa. Erikson was convinced that the two groups of organisms were different, though she was not dogmatic about their specificity for their particular hosts. It is mainly on Erikson's work that the classification of the genus Actinomyces in Bergey's Manual of Determinative Bacteriology is based.

Thompson (1950) also compared the morphology of strains of actinomycetes isolated from both human and animal sources. After seven days on hormone-agar, ten of eleven strains from bovine cases gave smooth colonies with a bacillary form. The eleventh strain, however, had the rough, pebbled adherent colony characteristic of the classic Wolf-Israel type, with the microscopic appearance of branching filaments. This eleventh strain was exactly similar to eight

strains of A. israelii recently isolated from cases of human actinomycosis. In addition there was an important cultural difference among the strains, whether of bovine or human origin when they were grown in liquid medium. The rough human strains and the one rough bovine strain grew in the form of bread-crumb masses in thioglycollate broth, whereas the smooth bovine strains grew diffusely throughout the medium.

Thompson records that after several months' culture there was no evidence of change from one colonial type to the other. Thompson considered that his results agreed with those of Erikson -- namely, that the infecting agents of human and bovine actinomycosis could be distinguished morphologically. Unlike Erikson, however, he considered that each type has a specific host. To explain the finding of a rough type of actinomycete in one cow, therefore, he suggested that this animal had been infected with a human strain! Morris (1951) carried out a cytological examination of strains of A. israelii, ten of bovine origin and two of human origin. He found — though he did not state which strains were involved -- that both rough and smooth colonies were present, and he stated that the rough colonies changed into smooth colonies. Both types of colony had exactly the same life cycle (involving haploid and diploid states), the

only difference being that in the smooth colonies the daughter cells separated immediately after cell-division, thus giving the separate bacillary forms and not the long, branching filamentous hyphae.

At the present moment, the position regarding the specificity of the infective agent in human and bovine infections is not clear, and work is apparently in progress at the present moment under the auspices of a committee not only to settle this point (See International Bulletin of Bacterial Nomenclature and Taxonomy: 1952, 2 49), but also to examine the characters of the actinomycetes.

Many workers have used serology in the attempt to produce a further means of sub-division of strains of A. israelii. The major difficulty, however, especially with the rough colony forms, whether grown in liquid or solid medium, has been the finding of spontaneous agglutination (Colebrook (1920), Lentze (1938), Ludwig and Hutchinson (1949)). Slack, Ludwig, Bird and Canby (1951) used ultra-sonic vibration for the preparation of A. israelii antigen, but concluded that the particles produced even by this method were too large and gave rise to large floccules in the control tube without serum. Holm (1930), Erikson (1940) have reported successful experiments using either formalin-treated cells or dried ground cells as antigen, but

even with these, antisera of disappointingly low titres have been produced. The labour of such work is impressive but its contribution to understanding cannot be said to be fully apparent.

Actinomycosis: the experimental infection

In reading the literature dealing with attempts at the experimental transmission of *A. israelii* to animals, it became clear to me that no two opinions agreed about what might be regarded as a positive result. As will be apparent from the details given later in my review of the literature, some workers regarded the production of tumours, however few, as being a positive transmission of actinomycosis. On the other hand, more exacting workers considered that the inoculated animals should die with the widespread development of actinomycotic abscesses - that is; a fatal progressive disease should develop; before an attempt at transmission was regarded as positive. Personally, I think that to adopt so exacting a criterion is to take an exaggerated view of the pathogenic possibilities of *Actinomyces* and to shut oneself off from the experimental study of the disease - experimental study, moreover, which could furnish useful information about the natural infection. Any swelling or lesion that arises in a laboratory animal as the result of

inoculation is worth regarding as a positive result, regardless of the ultimate fate of the lesion or the animal.

In the literature on experimental actinomycosis, great stress has been laid on the finding of sulphur granules with well developed clubs in animal lesions since this is a feature of the human disease; in fact demonstration of these granules in stained preparations can be used for laboratory diagnosis of the infection. Moreover, apart from their morphological appearance the clubs differ from the filaments in being acid-fast (resistant to 2% H_2SO_4). The actual mechanism of club-formation is host tissue has been another controversial point though not of paramount importance. Also, attempts have been made to produce clubs in A. israelii growing in pure cultures. Regarding the occurrence of 'clubs' at the periphery of the sulphur granules found in actinomycotic pus, two theories were extant. The simplest and at the time the most acceptable considered that the clubs were only very swollen thickened ends of bacterial filaments which arose as a protection for the internal mass of branching hyphae. The second theory considered that the clubs were a defensive mechanism, produced by the animal host, as a means of walling off the invading A. israelii. As evidence for this latter view, both Wright (1905) and Meyer (1934) showed that the inoculation intra-peritoneally of heat-killed broth

cultures into guinea-pigs and rabbits respectively, gave rise to club-development round the killed organisms in lesions produced in the animal host. The formation of clubs in natural occurring material from actinomycosis is not confined only to infections with A. israelii.

Lignieres and Spitz (1902) showed that, in actinobacillosis of cattle, pus containing granules was obtained from the ulcerating lesions, and at the periphery of these granules there were radiating club-like structures, inside of which was the causal organism Actinobacillus lignieresi. Similarly in botryomycosis of cattle due to Staphylococcus aureus infection, clubs were also seen at the periphery of the granules.

To differentiate between these three infections, microscopic examination of the granules, both by wet films and stained preparations, is essential. In granules from actinomycosis, long, branching, Gram-positive filaments are always seen.

Regarding the development of club-structures in pure cultures of A. israelii Wright (1905) and Naeslund (1931) stated that the addition of pleuritic fluid rich in fibrin or of serum to bouillon gave rise to club-formation in the filaments of A. israelii in pure culture, but that clubs were formed only where the filament was in contact

with the enriched medium. Bayne-Jones (1925) reported that, unlike Wright, he had seen club-formation in simple media free from serum and animal protein; in addition he observed the behaviour of isolated clubs in glucose broth when incubated as hanging-drop cultures. Two clubs showed growth of filaments which reached 50 to 100 μ in 10 days and then ceased; the growth took place at the thin end of the filament, there being no change in the club structure itself.

Gibson (1934) considered that clubs were really abnormal forms developed in old cultures in liquid media. Morris (1951), however, regarded club-like structures as pleomorphic forms developing in the life-cycle of *A. israelii*.

Attempts to produce actinomycosis in animals

Result of a Single intra-peritoneal injection

Wolf and Israel (1891) were the first to isolate and grow in pure culture the causal organism of human actinomycosis. In addition to carrying out a meticulous examination of the morphology, cultural requirements, and biochemical properties of the organism, the authors were the first to try to reproduce actinomycosis experimentally

in laboratory animals with pure cultures. They inoculated intra-peritoneally - using the technique of laparotomy - 14 rabbits, three guinea-pigs and one sheep, with the two strains of A. israelii isolated from their two cases of human actinomycosis. Pieces of agar containing colonies of A. israelii were planted deep in the peritoneal cavity of each animal. As a control experiment, one rabbit had pieces of sterile agar without any organisms placed in its peritoneum, and the reaction of this animal to the agar alone was observed. Wolf and Israel inoculated three other rabbits (with the same technique), but in these animals saline suspensions of A. israelii were employed as inoculum. A saline suspension of A. israelii was inoculated directly into the liver of the first rabbit; a mixed saline suspension of A. israelii and Staphylococcus aureus was injected into the liver of the second rabbit; and a mixture of A. israelii and lightly cooked egg yolk was placed in the peritoneum of the third rabbit.

All the experimental animals with the exception of four (two guinea-pigs and two rabbits) were killed from four to seven weeks after inoculation.

At post mortem the killed animals - except the sheep and the rabbit inoculated with sterile agar - showed

tumour growths over the peritoneal cavity. These tumours ranged in size from that of a millet seed to that of a plum, and were adherent to the abdominal wall, omentum, mesentery, and liver. The largest tumours were found in the animals inoculated with pieces of agar containing colonies of _A. israelii_; those animals inoculated with the suspensions of _A. israelii_ showed only scanty, tiny tumours on the peritoneal surfaces. The tumours were in fact abscesses containing cheesy pus. In the pus of the largest abscesses Wolf and Israel found definite small yellow 'sulphur' granules present. These granules — observed by wet-film and stained preparations — were composed of either masses of branching filamentous bacteria or of a mixture of short rod forms and broken up filaments. At the periphery of the granules, a ring of well developed clubs was usually present enclosing the entire mass of organisms. From these animal abscesses Wolf and Israel were able to isolate in pure culture the _A. israelii_ and compare it with the original strain used for animal inoculation.

The rabbit inoculated with the sterile pieces of agar showed no abscess development; some of the agar had disappeared, and the remaining portion was wrapped round with a few fibrin strands: the addition of sterile agar _per se_ did not lead to the development of actinomycotic

abscesses. Wolf and Israel experimentally transplanted the tumours found in one rabbit to another rabbit peritoneum but the resulting lesions in the second rabbit were no greater than those in the first; there was no increase in the invasive power of the A. israelii.

This work of Wolf and Israel was and is important both for the bacteriologist and the pathologist, as sections of these experimentally produced actinomycotic abscesses were for the first time shown to be identical histologically to sections of actinomycotic abscesses developing as a result of natural infection in man and cattle. Wolf and Israel were convinced that the organism they had isolated from human actinomycosis was the causal organism of both human and bovine actinomycosis and furthermore that they had successfully reproduced in experimental animals the characteristic actinomycotic abscess containing sulphur granules. Though their conclusions were correct, it must be emphasised that their animals did not develop progressive actinomycosis ending in the death of the animal as four of the inoculated animals were still alive and healthy seven to nine months after inoculation, although on palpation tumours (?actinomycotic abscesses) could be felt in their peritoneums.

After the work of Wolf and Israel, several workers

reported the isolation of anaerobic actinomycetes from human cases of actinomycosis but Wright's (1905) work is the best documented. Wright criticized the results of Wolf and Israel. Wright agreed that lesions histologically similar to those found in the human had been produced, but he argued that they did not represent the outcome of an active, progressive, infectious process. Wright himself inoculated 14 rabbits, 56 guinea-pigs, 13 "animals" (an unspecified number of rabbits and of guinea-pigs) one brown mouse, one kitten, and one calf. Broth cultures and agar cultures of 13 strains of <u>A. israelii</u> isolated from human and two from bovine actinomycosis were used as inoculum. Wright considered that his human strains belonged to the same species as the actinomycete described by Wolf and Israel (1891); furthermore, he was also of the opinion that the bovine and human strains of actinomycetes he himself had isolated belonged to one species as the organisms showed only minor cultural differences. Wright was not in favour of Wolf and Israel's method of placing pieces of agar with actinomyces in the peritoneum of animals; he preferred bouillon cultures. Wright stated: "That particles of agar provoke the formation of granulation tissue about them and become encapsulated so that tumour nodules are formed which obscure the effects produced by the micro-organism." Not

surprisingly perhaps the difference in method of inoculation was reflected in a difference in results between the experiments of Wright and those of Wolf and Israel. Of the 86 animals inoculated and killed for examination by Wright, only 24 guinea-pigs and four rabbits showed nodules from 1-10 mm in diameter developing over the peritoneum. These nodules gave the same general appearance on section as those seen by Wolf and Israel, but in some of Wright's lesions club formation was not found. Wright was interested in the influence of the time of cultivation of the culture used as inoculum in producing lesions. He considered that the largest and most marked abscesses developed when strains of organisms cultivated in the laboratory for only a 'short time' were used for inoculation. As proof Wright showed that a culture which he had maintained in the laboratory for two years produced no lesions when inoculated into animals. Wright himself stated that the results of his animal experiments with young cultures were by no means constant and that many inoculations were negative. Progressive actinomycosis was not obtained, furthermore no attempt was made to re-isolate the organism from the lesions. In addition to the use of organisms Wright tried the effect of transplanting "oat shells" along with organisms into the peritoneum. He also examined the effect of adding melted

butter to the inoculum and the use of silk setons placed in inoculated muscles, visceral tissue, and the tongue. These experiments were disappointing. Only in one rabbit, which was transfixed almost everywhere with setons impregnated with culture, were traces of infection found. Small nodules were found in the connective tissues when the rabbit was killed 41 days after inoculation. This was not a spectacular result from so much effort. One brown mouse tested did not develop infection after inoculation of a 2-week-old culture. The kitten inoculated subcutaneously and intra-peritoneally formed a subcutaneous abscess which opened and healed spontaneously. A calf was not infected after being inoculated in the jaw and by the intra-peritoneal route.

In his article Wright was very critical of the work of his contemporaries on the problem of actinomycosis. He criticised those who claimed to have isolated the causal organism, which often was a contaminant and had no resemblance to the Wolf-Israel strain. This stemmed from the Bostroem-Israel confusion already mentioned (p. 7). Wright also summarily dismissed the claims of workers of the 1891 period who attempted to reproduce actinomycotic lesions in animals by direct inoculation of pus. Their experimental animals often died from bacterial infections other than actinomycosis.

Musgrave, Clegg, and Polk (1908) were primarily interested in the miscellaneous conditions known as "Mycetoma", which arose from infection with aerobic actinomycetes. With strains of such organisms, animals were inoculated. The authors were unable to obtain cultures of the anaerobic _A. israelii_, so they used the observations made by Wright (1905) concerning the cultural character and animal pathogenicity of this organism as a yardstick for comparison with their results. This is a most interesting paper, as it carefully classifies in chronological order all the early literature dealing with the actinomyces group, including the first recognition of the clinical condition of actinomycosis, the early discussions on nomenclature and taxonomy, and the isolation by many authors of both aerobic and anaerobic actinomycetes. Many of the papers mentioned by Musgrave _et al._ are interesting historically but exact details are lacking.

Naeslund (1931) inoculated intra-peritoneally rabbits, guinea-pigs, pigs, and cattle with pure cultures of _A. israelii_ isolated from human actinomycosis. All animals inoculated failed to show infection except a one-month-old heifer which developed actinomycotic lesions at the site of injection. Three weeks after inoculation the lesion discharged sulphur granules with clubs. In this and in other

reports by Naeslund, it is notable that cattle are readily infected with material that is without effect in laboratory animals.

This monograph by Naeslund (1931) is very informative on general questions dealing with the problems of actinomycetes. He gives an extensive review of the early literature on the aetiological agent in actinomycosis, both human and animal, and on the question of the natural habitat of A. israelii and the theories of how infection is initiated. This includes a calm appraisal of the relative merits of the two sides in the early controversy on exogenous against endogenous infection. He discussed also the role of salivary concretions in setting up infection. These points are dealt with only lightly in this thesis and only in so far as they are related to experimental transmission of disease. For a full account of the questions, Naeslund's review may be consulted with confidence.

The most successful of the attempts at reproduction of actinomycotic lesions in animals was that reported by Hazen, Little, and Resnick (1952). By using hamsters for the first time as an experimental animal for actinomycosis, the authors were able to demonstrate that, after a single intra-peritoneal inoculation of 1 ml. of broth culture sediments of eight human strains of A. bovis (= A. israelii)

21 of 28 animals killed from four to six weeks later showed extensive abscess formation throughout the peritoneal cavity (the number of hamsters inoculated with each strain of A. bovis ranged from two to six.) In addition, in five of the hamsters the infection extended through the peritoneal wall with sinus formation. However, seven of the 28 animals killed showed no evidence of gross infection although "duplicate" animals inoculated at the same time with the same culture showed severe reaction. A. bovis was isolated from lesions observed in the 21 "positive" animals and the presence of granules with well marked club-formation was a constant feature. The animals were not kept under observation beyond six weeks. If the animals had been kept alive for a longer period, definite evidence of what happened to the lesions would have been obtained. Would these animals have died of progressive actinomycosis — a possibility in view of the experimental evidence, or would the lesions have regressed and the sinus infection cleared up? (Most of the work of the present thesis was done and reported to the Pathological Society in 1951 — i.e. before publication of the work of Hazen et al. described here.)

Result of repeated inoculation of *A. israelii*

Since inoculation of single doses of *A. israelii* did not give rise to constantly reproducible lesions in any experimental animal many workers used repeated inoculations, giving the injections after the first either by the same route or by a combination of different routes. This method however did not prove entirely successful for it did not attain the objective of a constantly reproducible lesion in experimental animals. However the results reported by various workers are worth discussing for they show some differences.

Naeslund (1929a) used pure cultures of *A. israelii* isolated from five human cases in an attempt to reproduce actinomycotic lesions in animals. Cattle and pigs were inoculated subcutaneously, guinea-pigs intra-peritoneally, and rabbits subcutaneously, intra-peritoneally, intravenously, and into the anterior chamber of the eye. The results in the pigs and guinea-pigs were negative. One of the rabbits killed two months after inoculation showed the development of "tight pea-sized" tumours, whose contents were viscous and pasty. Smears showed the presence of Gram-positive threads and rods, but the organism did not grow on culture.

Of the six rabbits inoculated in the anterior chamber of the eye, one showed, 14 days after the inoculation,

inflammation of the cornea. Examination of fluid aspirated from the eye showed a heavy growth of mycelium which gave a pure culture of the A. israelii. Similar fluid from the other five animals showed only short rods; and cultures from the eyes were negative for A. israelii. All the animals were killed 27 days after inoculation, when it was found that the rabbit showing acute inflammation of the cornea had an iris with thickening and infiltration with round cells and leucocytes. The other five animals had eyes without obvious pathological changes.

Naeslund regarded these results in rabbits as "negative"; but with cattle his results were described as "positive". Five cows were inoculated subcutaneously with young cultures growing vigorously in ascitic broth. A total of 20 injections was given; but it is not clear how many injections each cow received. After six months four cows showed no reaction. The fifth animal – an eight-month-old heifer – developed indurated swellings at two of the inoculation sites. The two swellings began to develop three weeks after inoculation; they increased in size during the next six months, and one grew into "a polyp the size of an egg" and exuded pus. The second tumour was smaller and gave rise to a discharging fistula, in the exudate of which could be seen the characteristic 'sulphur

granules' with clubs. The organism was isolated in pure culture. Naeslund reasonably considered this was "a live progressive actinomycosis" experimentally produced in cattle.

Mathieson, Harrison, Hammond, and Henrici (1935) inoculated five guinea-pigs, giving them each from two to five injections over periods of from 10 to 15 days up to six months. For one of the injections crushed granules from human actinomycotic pus was used, pure cultures of human strains A. bovis (= A. israelii) being used for the others. The inoculation routes were not identical for all animals. Thus one guinea-pig was inoculated only in the testicle; one only in the peritoneal cavity; and the other three received their inoculations by a combination of intra-testicular, intra-peritoneal, and subcutaneous routes.

None of the guinea-pigs reacted to the first dose, but three that received later injections developed indurated lesions ranging in diameter from 1-2cm. at the inoculation site. When killed, these animals showed the presence of small nodules in the peritoneum -- one nodule in one guinea-pig showing granule formation. Another series of four guinea-pigs received multiple injections with pure cultures of A. bovis. Two of these animals received intra-peritoneal injections of the same strain of organism, one guinea-pig receiving two

and the other three injections. When killed seven days after the last injection, and about 30 days after the first, both animals were found to have lesions on omentum, liver, and diaphragm. Furthermore in the animal that received two intra-peritoneal injections, a striking appearance was observed in that the liver, omentum, and intestine were adherent to one another by a lesion containing granular pus. Section of this lesion revealed large abscesses containing small typical granules. In the other two guinea-pigs of this series, which received four and six injections respectively of two different strains of A. bovis by a combination of intra-testicular, subcutaneous, and intra-peritoneal routes, abscesses were developed on the testicle, liver, and omentum. In one of these animals the intra-testicular abscess was shown to contain typical granules with clubs. The other guinea-pig had only a small lesion on a testicle; granules were not found in pus from this lesion. No animal in either series developed a lesion after only one injection; at least three more were necessary to produce such a result. Mathieson et al. concluded from these experiments that repeated inoculation with A. bovis led to the sensitization of the animal host, and that this sensitization was and is an important factor in the aetiology of actinomycosis. Emmons (1935) was unable to obtain results comparable to those of

Mathieson *et al.* In his work, guinea-pigs (the number is not stated) were repeatedly inoculated intramuscularly with cultures of *A. bovis* (= *A. israelii*). As to the result Emmons states only that "with the intramuscular injections, the later inoculation produced local abscesses, which persisted longer than in animals inoculated for the first time". From these abscesses small fungous colonies ("part of the original inoculum perhaps") could be recovered after one week; these colonies however did not show club formation. Insufficient details about the animal experiments are given in this paper for them to be fully understood and adequately discussed. A brief mention is made however of some of the methods used — namely, the use of foreign bodies, the effect of varying the dose of organism, the use of different inoculation routes and the technique of repeated inoculation for increasing the invasiveness of the organism, but these methods over a period of months gave no progressive lesions. Similarly Negroni and Bonfiglioli (1937) were unsuccessful with repeated inoculation of rabbits. Three rabbits were each given two injections of pure cultures of *A. israelii* of human origin, each rabbit being inoculated by a different route, intra-peritoneally, intramuscularly, or intravenously. The dose of the second injection was ten times greater than that given as the first; 11 days elapsed

between the two injections. The rabbits were killed from six to 14 days after the second injection; on examination all three failed to show lesions. In a later paper, Emmons (1938) again stated that he was unable to produce progressive lesions in animals by repeated inoculation. In his later paper, however, he gave a fuller account of his experiments. Guinea-pigs simultaneously received two injections (1 ml. intra-peritoneally and 0.5 ml. subcutaneously) of pure cultures of A. bovis (= A. israelii); the cultures used were isolated from cases of human actinomycosis and also from human tonsils. These "double injections" were given over a period of from four days to two weeks, some animals receiving as many as eight double injections before being killed. At post-mortem examination only a few small abscesses were found on the peritoneum of some of the animals; disseminated actinomycosis did not develop. The organism in these lesions was viable even ten days after the last injection. Emmons's double injections did not lead, therefore, to the development of the lesions desired. Before receiving the inoculum of A. bovis some of the guinea-pigs had been injected 24-hours previously with Ca phosphate in an attempt to make them more susceptible to infection; this also was unsuccessful. Slack (1942) reported the most successful results of any that were obtained by

repeated inoculation. Using the spun sediments of broth cultures of A. israelii isolated from pyorrhoeal pus he repeatedly inoculated five rabbits intravenously at intervals of three weeks, increasing the dose at each successive injection. After from three to four injections, three of the five rabbits died with progressive actinomycosis. Of the two survivors, one animal was killed because of extreme emaciation; the other died of intercurrent infection. In four of the five rabbits, post-mortem examination showed lesions in the lungs, liver, spleen, and omentum. Even the kidneys showed scattered areas of necrosis, filaments of A. israelii being seen in one kidney. The histology of the lesions was similar to that of the human lesion: polymorphonuclear leucocytes, granulation tissue, macrophages, and giant cells being prominent. At the periphery of the liver lesions, foam cells with branching Gram-positive filaments inside them were also seen. In the lesions, granules with hyaline clubs were also formed. Slack's success was due, I think, to his being primarily interested in the possibility of immunisation by intravenous injections for which he employed massive doses of A. israelii under the belief that his strain was non-pathogenic or nearly so. This assumption is supported by noting the results of a preliminary experiment in which, using the same organism,

Slack repeatedly at three-week intervals inoculated a number of guinea-pigs intra-peritoneally, but with negative results. The difference between the inoculum used for the successful and the unsuccessful experiments was possibly important. At first, the strain of A. israelii used would grow only in deep tubes of agar, and this agar, containing the colonies of the organism, was crushed in saline and used for injection of the guinea-pigs -- without producing lesions. Later Slack was successful in growing his organism in broth cultures, and for his intravenous experiments in rabbits he used the pooled centrifuged sediment of 12 broth cultures. Using this same preparation he was now able to produce progressive lesions in a guinea-pig by inoculating it intra-peritoneally seven times. This positive result was in contrast to his previous negative experiments. The macroscopic and microscopic appearance of the lesions in the guinea-pig resembled those found in the three rabbits that gave positive results. One difference between the two series was the lack of peripheral-club development in the granules found in the guinea-pig, in contrast to the well developed clubs found in the sulphur granules in the rabbit.

Rosebury, Epps and Clark (1944) attempted to repeat Slack's experiments but reported markedly different results. They inoculated a total of 24 guinea-pigs and 16 rabbits,

of which only two guinea-pigs and one rabbit developed progressive actinomycosis. Nine strains of *A. israelii* were used for inoculation, of which three strains gave positive results, two from cases of human cervico-facial actinomycosis and one strain from human gingival scrapings. In this work the volume of the injecting dose of *A. israelii* was large. The spun sediments from glucose-broth cultures suspended in saline were used as inocula. At first the dose of organism given was 0.5 - 1 ml. for guinea-pigs and 1-2 ml. for rabbits, then these volumes were increased to a maximum of 3.5 ml. for guinea-pigs and 5.5 ml. for rabbits. The injections were given at three-week intervals, the number of these given to any one animal ranging from one to eight. At first the injections were given only intravenously or intra-peritoneally, but when no external signs of actinomycosis (chronic weight loss as described by Slack (1942)) were seen the organisms were inoculated intrapleurally, sublingually and subcutaneously. The most interesting feature of this work is that Rosebury *et al.* defined very precisely the criteria which they thought should be fulfilled before concluding that progressive actinomycosis was established. They required that visible signs of infection — for example, nodule formation or extensive fibrosis — should be present, and that lesions

should contain typical granules with or without club formation They conceded that if the granules were not present but if the organism could be cultured from the lesion, this would be accepted as a positive result. On this basis the authors found that only five animals developed active actinomycosis, three — two guinea-pigs and one rabbit — showing progressive disease and two rabbits localised actinomycosis. One of the two guinea-pigs with the progressive lesions had received two intra-pleural inoculations of A. israelii; the second injection consisting of a mixture of organisms and sterile salivary calculus. One of the rabbits showing signs of localised actinomycosis had also been injected once intravenously with a mixture of organisms and sterile calculus. The calculus appeared to be without enhancing effect on the pathogenicity of the organism as in similar experiments eight guinea-pigs and eight rabbits also received injections of organisms and sterile calculus intravenously, intrapleurally and sublingually without effect. Rosebury et al. concluded that in their experiments, on their criteria the repeated intravenous or intra-peritoneal inoculation of A. israelii did not lead to the development of progressive actinomycosis. Ten animals (five rabbits and five guinea-pigs) were inoculated subcutaneously, of which eight had already been inoculated by a combination of other routes;

thus two animals received injections only by the subcutaneous route. In this experiment nine animals showed reaction at the inoculation site. A moveable nodule of up to 2.5cm. developed under the skin over a period of ranging from 48 hours to two weeks. One rabbit and one guinea-pig which received two and three subcutaneous injections respectively developed in addition a chain of nodules in the skin giving an appearance which was very reminiscent of that seen in skin actinomycosis in man. The nodules contained cheesy pus and in one animal a granule without clubs was seen. All the lesions eventually disappeared either by evacuation or by resorption. The re-isolation of the organism from the nodules was successful in only two instances — namely, in lesions which developed four days after inoculation and were examined. If a longer interval elapsed between lesion formation and examination (three weeks) no organism could be isolated. In no animal did a progressive actinomycosis result from subcutaneous inoculation.

Miller and Drake (1951) attempted to confirm the work of Slack (1942) by using the technique of Rosebury _et al._ of repeated inoculation with both mouth strains and a known pathogenic isolate of _A. bovis_ (= _A. israelii_) from human actinomycosis. Miller and Drake isolated from tooth scrapings of a normal mouth a filamentous anaerobic

actinomycete which was compared with a known culture of <u>A. bovis</u> obtained from the American National Collection, in regard to morphology, cultural requirements, antigenicity, and animal pathogenicity. There were only minor differences morphologically and culturally between the strains. The authors state that they could come to no definite conclusion regarding the comparative pathogenicity of the two strains, their only evidence being the lack of reaction shown by the inoculated animals. Eight rabbits were repeatedly inoculated intravenously at monthly, weekly and daily intervals: six with the mouth strain, and two with the known <u>A. bovis</u> strain. The organisms were grown in 10ml. amounts of broth and Iml. of the final inoculum consisted of the total growth from this 10 ml. of broth culture; the density of the inoculum being greater than the No. 10 tube of the McFarland nephelometer series. Six of the rabbits developed local subcutaneous lesions on the marginal ear vein which eventually healed. In three cases the mouth strain was isolated from these lesions and shown to be identical with the original inoculated culture. Only one rabbit showed signs of actinomycosis (loss of weight) and when examined six months after receiving weekly inoculations of the mouth strain this rabbit showed a nodular lesion on the dorsal peritoneal lining. This nodule contained pus but no sulphur

granules and the organism was successfully re-isolated from the lesion. Five guinea-pigs received repeated intra-peritoneal inoculations, three being injected with the mouth strain and two with the known A. bovis strain. Of this series, two animals, each one of which received the different strain of organism, showed on post-mortem examination typical actinomycotic lesions on the peritoneal lining, on the mid and left ventral abdominal region. Although caseous material was present inside the lesions and Gram-stained films showed the presence of filaments and diphtheroids, no sulphur granules were present. In addition the authors found it impossible to re-isolate both strains of bacteria from the respective lesions as an anaerobic streptococcus was always present in the sub-cultures.

The poor results obtained by Miller and Drake are surprising in view of the massive dose of culture given to the animals. The rabbit which was injected intravenously with the mouth strain received, over 180 days by weekly injections, 88.7 ml. of the concentrated inoculum. As a result of this, three lesions only were found in the peritoneum. Indeed the figures of Miller and Drake tend to discount Emmons's and Mathieson et al's suggestion that sensitization could be used as a method of developing progressive actinomycosis in experimental animals. (The

question of sensitization is discussed on p. 71 of this thesis).

Attempts to produce experimental infection arising from discussions on the habitat of *A. israelii*

As already indicated the source and exact nature of the infecting organism of actinomycosis were both subjects of great argument. The investigations on these points not infrequently led to attempts to infect animals with cultures or material from natural sources. These experiments are in a category of their own and seem to require separate notice.

Followers of Bostroem's (1891) theory that an aerobic organism was the cause of actinomycosis were also of the opinion that the source of infection lay outside the body of the host. In some actinomycotic lesions Bostroem found pieces of grain or grass and he concluded that the causal organism normally led a saprophytic existence and that injury — for example, with a foreign body — was required before the organism was implanted in the host and could produce disease. Wolf and Israel (1891), however, considered it possible that their anaerobic organism could lead a saprophytic existence in the mouths of man and animals. Wright (1905) was also of this opinion and stated that

A. israelii could even be a commensal of the gastro-intestinal tract. Lord (1910), who agreed with Wright's view, made a series of animal experiments with material from teeth and tonsils but was unable to obtain pure cultures of the actinomycetes, which he observed in his material. Naeslund (1925a) showed conclusively that there are three types of actinomycete in the normal mouth: an anaerobic type which culturally and morphologically is identical with the Wolf-Israel (= *A. israelii*) strains; an aerobic type of actinomycete; and a third type which may grow either aerobically or anaerobically. In addition, Naeslund (1926) showed that in tartar-formation the actinomycetes, both aerobic and anaerobic, play an important part in building up the deposits. Anaerobic mouth strains inoculated into animals may produce lesions similar to those found in human actinomycosis. Lord (1910a) took the contents of carious teeth from 16 of 300 patients whom he examined. (It must be emphasised that these patients were not suffering from actinomycosis, but from other clinical conditions.) Lord divided the material from each sample into portions for culture, microscopy, and for animal inoculation. In each specimen of material examined by direct wet-film and Gram-stained preparations, branching filaments but not clubs were seen. All attempts at pure culture failed. The portion

of the material for animal inoculation was mixed with broth and injected into guinea-pigs each of which received only one intra-peritoneal injection. Nine guinea-pigs were inoculated with material from nine different cases but only five animals were completely investigated in the series. Six days after inoculation one guinea-pig died with peritonitis and at post-mortem examination it was observed that nodules had developed on the omentum. Four guinea-pigs were killed at intervals of 12 (two animals), 17, and 19 days. Three of the five guinea-pigs examined showed nodules over the peritoneum of 2-12mm. diameter. When these nodules were examined histologically the appearance of some of them was found to be the same as that seen in human actinomycosis: the centre contained branching masses of organisms with clubs at the edge and the mass was surrounded by zones of pus cells and fibrous tissues. In many nodules, however, organisms were not seen. As was to be expected, the lesions contained foreign bodies and other bacteria. Lord's results were rightly regarded as very interesting. It was striking at that time (1910) to learn that a single inoculation of carious tooth contents could lead in one guinea-pig to death with progressive actinomycosis and in three of five affected to large well developed abscesses. In the light of Naeslund's (1925) work on oral

bacteriology, it is virtually certain that Lord introduced both aerobic and anaerobic organisms into the peritoneum of the guinea-pigs that he inoculated. Lord (1910b), in continuation of his work inoculated 13 guinea-pigs intra-peritoneally; some with fluid expressed from tonsillar crypts and others with yellowish plugs of material from the tonsils, three animals died of generalised peritonitis 24 hours after inoculation. Of the ten survivors, four were killed 21 days after inoculation, three showed no lesions, the fourth guinea-pig had numerous adhesions over the peritoneum and also three small nodules, one of which contained pus. The remaining six animals were killed at intervals of 13, 15 (two animals) 17, 18 and 45 days; all showed the development of white omental tumours ranging in size from 1mm. to 0.5cm. On section these nodules were shown to contain colonies of branching filamentous Gram-positive organisms surrounded by pus cells. At the periphery of the colonial mass radial club-bearing filaments could be seen. Outside the pus cell layer was a layer of connective tissue. Again the histological appearance of these nodules resulting from the inoculation of tonsillar material was identical to that of the human actinomycotic lesion.

Naeslund (1925b) examined both microscopically and

culturally salivary calculi from ten human mouths. He isolated from these calculi three types of actinomycete similar to those which he previously described (1925a p. 43). With cultures of these organisms, using both the strict aerobic and anaerobic types, Naeslund inoculated 24 guinea-pigs and six rabbits intra-peritoneally. These inoculations were without effect. An unstated number of young cattle were inoculated with "actinomyces-containing" material in the sub-maxillary region, one cow being injected in the parotid gland. Again the animals showed no sign of infection but Naeslund stressed that although these experiments were inconclusive, an open mind should be kept about the potential pathogenicity of these actinomycetes in the salivary concretions. In a later publication Naeslund (1929b) reported positive results in animals inoculated with pure cultures of A. israelii isolated from salivary concretions. Cattle and guinea-pigs were repeatedly inoculated subcutaneously, the cattle receiving 15 and the guinea-pigs 10 injections.

Some of the cattle (numbers are not stated), developed at the inoculation-site tumours which in four months became "the size of a polyp" breaking down to discharge pus, which contained granules with clubs. This was a progressive actinomycotic infection in the cattle.

Four strains of actinomycetes were isolated by Lord and Trevett (1936) from human mouths. These strains grew both aerobically and anaerobically and therefore resembled the third group of organisms described by Naeslund (1925a p. 43).

Lord and Trevett inoculated animals with these strains. Seven guinea-pigs were inoculated intra-peritoneally with organisms suspended in bouillon. In addition, two guinea-pigs had pieces of sugar agar containing colonies of the organism placed in the peritoneum. Nine rabbits were inoculated intra-peritoneally, intravenously, intratesticularly, into the anterior chamber of the eye, and subcutaneously, again with broth cultures. One cow, a calf and a heifer were inoculated subcutaneously with 1-10 ml. of a heavy bouillon suspension made by emulsifying the surface growth of the organism from saliva agar. The results of all these experiments were negative. Only transient local abscesses developed. The naked-eye and histological appearance were not like those of an <u>A. israelii</u> lesion.

Emmons (1935) isolated six strains of microaerophilic actinomycetes from human mouths: one strain came from discoloured teeth which did not show dental caries; two strains were from two cases of dental caries; and three

strains from infected tonsils. The tonsillar actinomycetes grew rapidly and had coarser hyphae than the typical isolates of pathogenic A. bovis (= A. israelii). Emmons noted that on repeated subculture, however, the tonsillar strains resembled more closely cultures obtained from clinical actinomycosis. In regard to the animal pathogenicity he states that these tonsillar strains do not usually produce progressive lesions when inoculated into the common laboratory animals. From tonsils Emmons (1938) isolated also microaerophilic strains similar in cultural characters and in morphology to A. bovis (= A. israelii). With these tonsillar cultures and pure cultures of A. bovis from clinical actinomycosis, he inoculated guinea-pigs but did not produce lesions of actinomycosis (p. 34).

Sullivan and Goldsworthy (1940) also compared the cultural characters and effects in animals of anaerobic mouth strains with those produced by cultures of A. bovis (Wolf-Israel) (= A. israelii) isolated from clinical cases of human actinomycosis. From 100 periodontal pockets, i.e. of patients not suffering from actinomycosis, five anaerobic strains of actinomycetes were isolated. A similar strain was isolated after examination of material obtained from 24 cases of carious teeth. These six mouth strains were identical with five strains of actinomycetes isolated from

cervico-facial actinomycosis; they were alike morphologically, culturally, in their O_2 requirements, and in their biochemical fermentations. Sullivan and Goldsworthy concluded that micro-aerophilic/anaerobic A. bovis is a normal commensal of the mouth, and that their findings support the previous work of other authors. One point that these authors particularly noted in their work was how often patients with cervico-facial actinomycosis had a history of previous oral trauma. Regarding the pathogenicity of both "types" of actinomycete for guinea-pigs, little can be said. Cultures (the kind is not stated) of each of the two "types" of organism were inoculated subcutaneously into guinea-pigs. The only visible sign of a reaction was the development of a red swelling which soon disappeared leaving the animals quite normal. Large wooden splinters were impregnated with the mouth strain and the strain from the actinomycotic lesion; these splinters were placed subcutaneously in the guinea-pig tissue, again without effect. Sullivan and Goldsworthy discuss the meaning of the term pathogenicity as applied to A. bovis and state that in their opinion an organism is pathogenic only when its injection leads to progressive disease. On this basis therefore it would appear that both the strains of A. bovis used by them are non-pathogenic. Slack (1942) was interested in the source of infection in

human actinomycosis. He examined 100 pairs of tonsils and from the caseous plugs in the tonsillar crypts he isolated anaerobic actinomycetes in pure culture in 11% of cases. Similarly from 100 specimens of pyorrhoeal pus he isolated pure cultures of anaerobic actinomycetes from 12% of the cases. These strains of anaerobic actinomycetes both from the tonsils and from the pyorrhoeal gums were identical culturally and morphologically, but they were rather delicate and many of them died out on the fourth sub-culture. While examining these materials from mouths Slack isolated a strain of _A. israelii_ from a case of cervico-facial actinomycosis, the patient having had a history of dental extraction. This strain was used for comparison culturally and biochemically with one of the anaerobic oral strains - isolated from pyorrhoeal pus - which had been maintained in the laboratory for some time. Both strains were similar in their behaviour. It was with this oral strain isolated from pyorrhoeal pus that Slack as previously described (p. 34) showed the development of progressive actinomycosis in rabbits after repeated intravenous injection. The lesions that were found in the rabbits on _post mortem_ were identical histologically with those lesions found in human actinomycosis.

Rosebury _et al._ (1944) compared the cultural and pathogenic action of strains of _A. israelii_ isolated both

from cases of cervico-facial actinomycosis and gingival scrapings. Culturally, the strains were similar, but in the experimental production of actinomycosis, only one human strain and one mouth strain gave rise to progressive actinomycosis (p. 35) in guinea-pigs and rabbits when repeatedly inoculated. The other strains both of human and mouth origin had no pathogenic action on the experimental animals.

Thompson and Lovestedt (1951) isolated from 24 cases of oral lesions in patients (deep periodontal pockets, periapical abscesses, chronic lesions in the mandible) eleven strains of actinomycetes of which two strains were anaerobic; the other nine strains, however, grew both aerobically and anaerobically. All the strains were compared culturally with cultures of actinomycetes isolated from human and bovine actinomycosis. The two anaerobic strains were typical A. israelii forms, the other nine strains fell into the category of Naeslund's (1925a) group C (p. 43) classification of oral actinomycetes. In the cultural examination of these last actinomycetes, Thompson and Lovestedt also observed that these facultative strains gave on solid media R and S colonies. Each type of colony when subcultured gave the same variation into R and S. The authors consider that

this colonial variation and O_2 tolerance of these strains may give an answer to another problem found in the literature dealing with actinomycosis. Many workers have recorded that their classical strains of microaerophilic A. israelii on continuous sub-culture have changed into strains capable of growing in the presence of O_2. This finding has of course been hotly disputed. Thompson and Lovestedt consider that in such work actinomycetes similar to those described by them capable of growing both aerobically and anaerobically have probably been isolated and not a typical microaerophilic type. This would explain the change from anaerobic to aerobic conditions of growth. Unfortunately, the pathogenicity of these strains is dubious, although only meagre experimental details are given. White mice were inoculated intra-peritoneally with two strains; the mice were not affected. This is all that is said about the animal experiments; the number of mice, the type of culture, and time of examination after inoculation is not given.

Attempts to produce actinomycosis in animals by subcutaneous injection

Magnusson's (1928) experimental results are most interesting and are therefore discussed in detail. He

carried out a very comprehensive study of actinomycosis in cattle and swine, as well as of the two other animal conditions often confused with actinomycosis -- namely, actino-bacillosis and botryomycosis. His investigation of actinomycosis fell into two parts: the first was the isolation of the causal organism. From 61 cases of bovine actinomycosis, Magnusson isolated 54 pure cultures of an anaerobic actinomycete which was identical morphologically and culturally with the organism described by Wolf and Israel (1891) and Wright (1905). Secondly, Magnusson attempted to reproduce actinomycosis in cattle with a single subcutaneous injection. In this he was moderately successful from the numbers of animals inoculated and very successful in producing true progressive actinomycosis as is found naturally in cattle. Material from 32 cases of bovine actinomycosis was examined and cattle (an unspecified number) were inoculated either directly with the natural purulent material or with pure cultures of <u>Streptothrix Israeli</u> (= <u>A. israelii</u>) isolated from the pus. In eight cattle positive results were obtained. Though cattle were the main experimental animals used, Magnusson records that pigs, sheep, goats, horses, and all the small common experimental animals were also inoculated, both with pure cultures of <u>Strept. israelii</u> and with purulent material. As to result,

Magnusson states that "as a rule, no actinomycotic lesions were obtained". With pure cultures of Strept. israelii isolated from "lumpy jaw" in cattle, one bull and five heifers were inoculated subcutaneously with one single injection in the left shoulder. The inoculum was prepared from a broth culture and a broth suspension from a Loeffler slope of Strept. israelii, 2c.cm. being injected. The bull was killed three months later, and showed no actinomycotic lesions; four of the five heifers gave reactions at the inoculation site. There the skin became punctured with fistulas, around which "egg-shaped polyps" or "actinomycomas" developed. Seven months after inoculation, these had increased in size and contained pus with sulphur granules which showed well developed clubs. This lesion appearance was exactly similar to that seen in spontaneous actinomycosis in cattle. With pure cultures of Strept. israelii obtained from another case of bovine actinomycosis one cow, two calves, and two kids were again inoculated as in the previous experiment, subcutaneously in the neck. Only the cow showed any reaction to the inoculation and developed a swelling at the inoculation site six days later. This swelling in 12 days gave rise to a discharging fistula; the pus from which also contained 'sulphur' granules, with well developed clubs. From the

positively reacting animals in both series, the organism <u>Strept. israelii</u> was isolated in pure culture.

As with broth cultures, the direct inoculation of bovine actinomycotic pus into cattle gave rise to progressive lesions. The pus was first of all cultured both aerobically and anaerobically and only the anaerobic Wolf-Israel type of actinomycete was present there being no growth aerobically. Two series of cattle were inoculated with this actinomycotic pus; in one series the granules were crushed in the pus before injection, in the other series whole sulphur granules were inoculated into the cattle.

Five cattle (one bull, four cows) were inoculated subcutaneously into the right flank with a single injection of 2c.cm. of saline-pus mixture (crushed granules). Two animals (the bull and one cow) had abscess formation at the inoculation site; these abscesses healed after one month, and these animals showed no further signs of infection. The other three cows, during a period of ten months after inoculation, developed 'polyp' tumours at and in the area of the inoculation site. These 'polyps' which required three months to develop, were secondary to local abscesses which had originated directly after inoculation and had then disappeared. The 'polyp' tumours broke down to give extensive actinomycotic fistulas discharging pus containing

sulphur granules; again these granules on microscopic examination showed well developed clubs.

Of the five cattle (two cows and three calves) inoculated similarly in the neck but with pus-saline mixture containing whole sulphur granules, the three calves developed tumours in exactly the same manner as the animals inoculated with the crushed granules. The two cows developed only local abscesses at the inoculation site and when those animals were killed two months after inoculation, actinomycotic lesions were not seen. Again in this series of animals, it was possible to isolate <u>Strept. israelii</u> in pure culture from the discharging pus and Magnusson experimented in passaging both the actinomycotic pus and the pure cultures of <u>Strept. israelii</u> obtained from it, from animal to animal.

Having produced discharging fistulas in cows, Magnusson wondered if such animals would be susceptible or resistant to further infection. Material (pus) was evacuated from the polyps developed in the three cows after the direct inoculation of pus with the crushed and uncrushed sulphur granules. The pus was emulsified in saline and reinjected into the cattle subcutaneously in the right shoulder, a control uninoculated cow being also injected. It is not exactly clear if each cow was injected with its own pus, or pus from another animal, or with both specimens. All four

animals developed actinomycomas with pus containing sulphur granules five months after inoculation. From this experiment it would appear that the presence of an actinomycotic lesion does not inhibit the development of a new lesion after a fresh inoculation of actinomycotic material. Antibodies — if formed — do not seem to confer protection on the host.

In another series of experiments Magnusson directly inoculated three calves, one pig, one rabbit, one dog and one cow with pus subcutaneously. Only the three calves and the cow developed tumours at the injection site. From the pus obtained from these tumours, pure cultures of Strept. israelii were obtained, and these cultures were in turn inoculated subcutaneously into three other calves, two cows, and two kids. The kids showed local abscess development, but no granules or clubs were present. The three calves showed walnut-sized abscesses at the inoculation site, one calf only showing club-development in granules in the pus. Magnusson's passage experiments either with the pus or with cultures did not give results any more striking than those he obtained with direct inoculation. Magnusson in his discussion stresses that the development of the actinomycotic lesion in cattle requires time, ten-months at least, before the typical appearance is observed.

From this work, it would appear that cattle are

more susceptible than mice, rabbits, or guinea-pigs — for example — to the experimental transmission of Strept. israelii.

Attempts at enhancing the virulence of A. israelii for experimental animals

Since the results of inoculating pure cultures of A. israelii in animals were so unproductive, many authors tried to stimulate the development of actinomycotic lesions by artificial means.

Foreign Body - By incorporating foreign bodies along with the inoculum of A. israelii - Wright (1905) used melted butter, silk setons, and oat shells. Magnusson (1928) mixed fragments of horse hair, threads of wool and silk, and silicous marl with the inoculum. Naeslund (1931), incorporated cereal grains, straw, and awns with the organism. Emmons (1938) and Sullivan and Goldsworthy (1940) inserted wooden splinters impregnated with A. israelii into animals. All these experiments were fruitless. Negroni and Bonfiglioli (1937) mixed pieces of sterile rabbit kidney with human strains of A. israelii in broth cultures before inoculation and with this produced indurated abscesses in two rabbits which were inoculated subcutaneously. From these abscesses

the organism could be cultured but these abscesses spontaneously discharged and healed, the rabbits then showing no actinomycotic lesions.

Briefly, these experiments with inert materials yielded negative results whether the injections were made either singly or repeatedly into the peritoneum or into the subcutaneous tissue.

Surgical implantation - Wolf and Israel (1891) originally implanted in guinea-pig and rabbit peritoneums pieces of agar containing colonies of A. israelii. Wright (1905) considered that the success of Wolf and Israel in producing lesions in these animals was due to the use of the agar which acted as a foreign-body and thus protected the A. israelii from phagocytosis. Grooten (1934) failed to produce actinomycotic lesions in rabbits by inoculating them subcutaneously, intra-peritoneally, intracerebrally and intravenously with saline suspensions of A. israelii, isolated from three cases of human actinomycosis. Attempts were also made to enhance the pathogenicity of the bacteria by including tapioca and kieselguhr in the inocula; these were also unsuccessful. However by using the implantation technique Grooten was able to reproduce actinomycotic lesions in rabbits. Pieces of Veillon's medium (semi-solid agar)

0.05-1cm. in diameter containing colonies of A. israelii were placed in the abdominal cavities of four rabbits and the peritoneums closed by suture. The first rabbit was killed 24 days later and a nodule 1-2cm. in diameter was present on the omentum. On section the nodule showed little "grains" entirely composed of much branched Gram-positive filaments; clubs were not present. Two of the other rabbits were killed two and nine months respectively after inoculation and the third rabbit died of intercurrent infection five months after inoculation. On post-mortem examination all these three rabbits showed the development of tumours in the peritoneum. In each rabbit there were at least two pea-sized tumours as well as many pin-point lesions. The large lesion on section showed again the characteristic appearance of little "grains" lying in connective tissue. In the rabbit killed at nine months, well developed acid-fast clubs were present at the periphery of the mass of branching filaments, which made up the "grains". Again, clubs, though not so well developed, were also seen in the tumours found in the rabbit which died. Though tumours were produced in rabbits by this implantation technique, I do not think Grooten produced progressive actinomycosis. As she states herself, the agar probably protected the A. israelii from the polymorphs and the phagocytes; and thus Wright's (1905)

criticism of Wolf and Israel's (1891) work can also be applied to this work of Grooten (1934). Grooten also attempted to transmit A. israelii directly from one rabbit to another. From the tumour developed in the rabbit killed two months after inoculation, pure cultures of A. israelii were obtained; in addition, a piece of the tumour was cut into pieces, mixed with sterile kieselguhr, and placed in the peritoneum of a fresh rabbit, which was killed eight months after the implantation. On examination masses of small pin-point granules were found scattered over the peritoneum but films made from these little lesions stained badly by Gram's method, and on culture no organism could be isolated from them. Sections showed bits and pieces of branching filaments which were broken down and stained poorly. This attempt at passage was unsuccessful in that there was no increase either in the numbers or the size of the tumours; the finding was that these became smaller in size after the first passage. The organism in the material used for implantation was still viable as the positive culture result shows. Passage in the rabbit had not enhanced the pathogenicity of the strain of A. israelii for another rabbit; the organism appeared to have less effect on the host.

Lord and Trevett (1936) used laparotomy with

negative results. In the peritoneum of two guinea-pigs they placed pieces of sugar agar containing colonies of actinomycetes isolated from human mouths. But their mouth strains were not typical Wolf-Israel types in that they grew both aerobically and anaerobically and belonged therefore to the third group of Naeslund (1925) (p. 43).

Mucin - The use of mucin as an enhancing factor in bacterial pathogenicity was first recorded by Nungester, Wolf and Jourdonais (1932). These workers showed that when mucin suspensions of either pneumococci, β-haemolytic streptocci, and staphylococci were injected intra-peritoneally into white mice, the mice died more quickly than control animals inoculated with comparable numbers of bacteria in saline alone. In addition, Nungester et al. found that by using mucin it was possible to reduce the actual numbers of bacteria present in the mixture, without affecting the lethal action on mice. Olitski (1948) in his review stresses the importance of mucin in producing peritoneal infections in laboratory animals, as in the past the experimental work of many diseases has been handicapped by lack of susceptible small laboratory animals. By using mixtures of mucin and bacteria normally of low virulence for experimental animals, many workers have shown (Olitski 1948) that it is possible

to reproduce infection in animals with a wide variety of such bacteria — Haemophilus Staphylococcus, and Neisseria, for example. While the work described in this thesis was in progress Meyer and Verges (1950) published the results of inoculating Swiss white mice with a single intra-peritoneal injection of A. bovis (= A. israelii) suspended in 5% gastric mucin. Their results showed that the mucin had a definite enhancing effect on the virulence of the actinomycete for the host. Twelve human strains of A. bovis and five of bovine origin were used in this work. The surface growth of the bacteria on brain-heart-infusion agar was washed, suspended in saline, and standardised on a McFarland nephelometer; the suspension was now mixed with an equal volume of 5% gastric mucin, and 0.5 ml. amounts of this were injected once intra-peritoneally into Swiss white mice. For the first time in the literature, Meyer and Verges stress the necessity of using young animals in experimental actinomycosis; the mice used for these experiments were young and did not weigh more than 15 gm. The animals were killed from ten to 15 days after inoculation. 93 mice were inoculated with the human strains in mucin and of these 88 showed progressive infection in the sense that there was abscess formation in liver, spleen, diaphragm wall, kidney, inguinal nodes and omentum. When I studied this paper

closely, I found that of the 12 human strains used, ten — when mixed with mucin — had produced infection in all the mice inoculated; and two gave infection in 75% and 80% of the mice, respectively. Along with the mice given mucin and actinomyces, a series of control animals was inoculated with saline suspensions of the same 12 strains of A. bovis alone. The results showed that 15 of 47 mice so inoculated without mucin developed actinomycotic lesions over the peritoneum. In contrast to the result with mucin only two of the 12 strains of A. bovis in saline suspension produced macroscopic lesions in all the mice inoculated; four strains produced lesions in approximately half the number inoculated; and six strains had no effect on the mice.

Similarly with the bovine strains of A. bovis, mice inoculated with the organism suspended in mucin showed a higher proportion of progressive lesions; 17 of 21 inoculated as against three of 15 mice in the control series, which received the organism in saline only. Again when the results are examined, it is seen that of the five bovine strains used in the mucin series, three strains gave rise to lesion development in all mice inoculated, the two other strains giving lesions in 50% and 75% of the mice. In the saline series with these bovine strains, three strains had

no effect on the mice, one strain gave rise to lesions in 25% of the mice inoculated, the other strain gave lesions in 50% of the animals. Thus the mucin had increased the pathogenicity and invasiveness of both the human and bovine strains of the A. bovis to an equal extent.

In the lesions developed in the peritoneum after intra-peritoneal inoculation of the human strain of A. bovis sulphur granules and clumps of branched mycelia were found in the pus in all animals inoculated. In addition, it was possible from the lesions to isolate and grow A. bovis in pure culture. In their paper the authors say that some mice were left for 45 days after inoculation to determine whether the infection would kill the animals or become a chronic infection, but no indication is given of how many mice were left, and of the result. In addition it is stated that 16 deaths in the mice were due to the infection, but this is the only mention of mice dying; no other information about this point is given. One very interesting point in Meyer and Verges's work is the formation of actinomycotic abscesses actually inside the tissue of the spleen and kidney. The authors have shown definite invasion of these tissues by the organisms (compare Slack (1942) p. 35).

Geister and Meyer (1951) inoculated young albino mice intra-peritoneally with mucin suspensions of

A. bovis (= A. israelii), prepared and standardised by the method of Meyer and Verges (1950). Two strains of organism were used - one isolated from human actinomycosis, the other from a dental plaque. Geister and Meyer obtained better results than Meyer and Verges (1950) in that all the mice inoculated developed actinomycotic lesions over the peritoneal surfaces in the period five to 25 days after inoculation. (Precise details are not given about this work as to number of mice inoculated, time of killing, etc.) The lesions were first seen on the viscera five days after inoculation; the lesions increased and multiplied giving the appearence of a progressive infection till the 20th or 25th day after inoculation. The lesions were developed not only on the surface of the viscera, but also inside the tissue giving the appearance of little hard tubercles; it was possible to isolate the causal organism A. bovis from the surface abscesses. Twenty-five days after inoculation the infecting organism and the animal host appeared to become balanced so that in mice killed four months after inoculation a chronic infection seemed to be established in that the original lesions had regressed, becoming sterile and fibrous. The study of the infection in mice was not carried beyond four months. Geister and Meyer stated that histopathological examination of the experimentally produced

abscesses in the mouse tissue did not reveal "a definite picture" (of actinomycosis, I presume); only 15 sections through abscesses were examined, and the authors consider this may be a reason for the poor histological evidence. Despite this Geister and Meyer, in another small series of experiments, immediately started antibiotic (penicillin and aureomycin) therapy of mice injected with mucin suspensions of _A. bovis_, in an attempt to evaluate the usefulness of these drugs on the experimental infection. In both series of mice lesions were produced, and by a method of standardising the lesion counts in the treated and untreated mice, Geister and Meyer concluded that the antibiotics had effect in that the lesions in the treated mice were not so numerous. These authors admit the shortcomings in their methods of assay of infection and also the lack of histological examination in these treated animals. No mention is made of the action of mucin _per se_ on the antibiotic penetration of the host tissue.

Strauss and Kligman (1951) repeated the work of Meyer and Verges (1950) and Geister and Meyer (1951) but their results did not agree. Strauss and Kligman were interested in the pathogenicity-enhancing action of mucin on a wide range of pathogenic fungi when inoculated into mice; one species which they examined was _A. bovis_ (= _A. israelii_).

18 mice were inoculated intra-peritoneally with _A. bovis_ suspended in mucin; two mice only died at the end of ten days, there being no deaths in the control series inoculated with _A. bovis_ in saline. Two of the strains of _A. bovis_ were isolated from human infections and one was from a cow. The inocula, again prepared from agar cultures, were washed and centrifuged, and the packed organism mass was suspended in 5% gastric mucin. No indication is given of the number of bacteria suspended in the mucin. All that is stated is one volume of packed cells of _A. bovis_ was added to 50 volumes of mucin. This dilution of organisms may be the reason for the lack of lesion development, although Strauss and Kligman state that even in high concentrations (presumably with larger amounts of _A. bovis_) no abdominal lesions were seen in the mice. The authors put forward two possible speculations for the poorness of their results as compared to those of Meyer and Verges. Either the mice or the organisms showed strain differences in terms of increased resistance of host and lack of pathogenicity of organism, as compared to the experimental materials of Meyer and Verges. This is a possible explanation, but I think that the number of bacteria present in the inoculum may have been below the minimum required, even with the help of mucin as an adjuvant for increasing invasiveness. In addition Meyer and Verges

point out that older Swiss white mice are not suitable for inoculation with A. bovis as they appear to be resistant, and though Strauss and Kligman say that they used "young mice" for their inoculations, these animals may not have been young enough.

As this thesis was being typed Gale and Waldron (1955) reported successful development of actinomycotic lesions in mice following the simultaneous intra-peritoneal injections of 0.2 ml of a 2% suspension (V/V) of A. israelii into one flank and 0.8 ml. of broth (Difco brain-heart broth or Difco tb. broth) or mucin into the other flank. In the broth series 31 of 35 mice developed actinomycotic lesions over and in the viscera in a period of 21 days after inoculation. In contrast in the mucin series 41 of 66 mice inoculated developed actinomycotic lesions in the viscera — a lower proportion than in the broth series. The strains of A. israelii used did not appear to influence the development of the lesions as both rough and intermediate forms, both from lesions and from non-pathological (mouth) sources gave comparable results. Smooth strains of A. israelii however gave rise to fewer lesions or adhesions than the rough and intermediate strains. The actinomycotic lesions in the viscera were of two forms: either frank abscesses or small hard tubercles similar to those reported by

Geister and Meyer (1951). Histologically these abscesses were like those found in man. To culture the organism it was necessary to pool the organs, grind aseptically and then inoculate media; direct inoculation of media with the cut surfaces of organs was unsuccessful. In a further experiment, mice were inoculated, again by simultaneous single intra-peritoneal injections of organisms - one strain of A. israelii - with broth, mucin and saline respectively. The animals were killed daily for the first ten days, then at intervals of 16, 23 and 29 days. Comparison was made with the three adjuvants regarding site and numbers of both lesion and adhesion formation. Distribution of the lesions differed with the adjuvant used. The liver in the mucin series showed macroscopic and microscopic evidence of inflammation 48 hours after inoculation; adhesions were also formed in this time between liver lobes and diaphragm. With the broth and saline series lesion development was slower and was at first confined to the omental and mesenteric surfaces; then the liver was affected and adhesions were formed at these points. The saline series of mice gave the slowest development of lesions and the least progressive infection. In contrast to Geister and Meyer's (1951) findings there was no resolution of mouse actinomycotic lesions 23 days after inoculation. The mice were not observed by Gale and Waldron beyond 29 days. The

authors carried out similar experiments using guinea-pigs, but it was difficult to establish infection and they concluded therefore that the guinea-pig is not a suitable animal for this work. An explanation based on the differing absorptive and dispersal mechanisms of the animal host for dealing with the mucin, broth and saline is put forward by Gale and Waldron to explain the differences in lesion situation and size.

Relation of allergy to experimental infections with *Actinomyces israelii*

Mathieson and his co-workers ((1935) p. 31) were interested in the possibility that allergic reactions might develop both in man and experimental animals after injection with *A. israelii*.

In one of their experiments a six-month-old veal-broth culture shaken for six hours was used as antigen. Four guinea-pigs and four rabbits were inoculated intra-peritoneally with 1 ml. and 2 ml. respectively. Ten days later an intra-cutaneous injection of 0.5 ml. of the same antigen produced no response. With the same antigen, two normal individuals and one patient with pleural actinomycosis again gave no reaction. In a further experiment a Berkefield filtrate of a one-month-old veal-broth culture when injected intra-cutaneously into a normal and an actinomycotic patient

gave rise in both cases to a hyperaemic area, which persisted for 36 hours.

Finally a vaccine made by heating veal-broth cultures at 55°C. for two hours was injected (0.1 ml.) into the skin of five actinomycotic patients and 13 normal individuals. Only one of the five patients with actinomycosis gave a skin reaction, but 12 of 13 normal individuals gave a marked skin reaction with the 1/100 dilution of the antigen. This result was very surprising. Presumably, the veal-broth itself was not responsible for the skin reaction though no mention of controls appears in the paper.

Emmons (1938) also obtained no response in inoculated animals subsequently skin-tested with vaccines of _A. bovis (= A. israelii)_, and old tuberculin. Guinea-pigs (numbers are not stated) were inoculated intra-peritoneally with three strains of _A. bovis_, two of which were of tonsillar origin and the third isolated from bovine actinomycosis. After a time (again not stated) the guinea-pigs were inoculated intra-cutaneously with preparations of the strains and also with old tuberculin; the only reaction observed was slight erythema at the injection site. Emmons found no reaction in the animals skin-tested with either the homologous or heterologous strains of actinomycetes. The guinea-pigs were later inoculated intra-peritoneally with

0.5 ml. of undiluted old tuberculin; again the animals showed no reaction. Emmons's results were negative, and thus confirmed the findings of Mathieson *et al.*

Rosebury *et al.* (1944) also skin-tested their experimental animals to determine whether they had developed an allergic reaction after the repeated intravenous inoculation of *A. israelii*. Three rabbits were given four intravenous injections of *A. israelii* at three-week intervals, two of the rabbits being inoculated with a strain of *A. israelii* passaged through a guinea-pig (p. 37) and the third with a pathogenic strain of human origin. Two series of antigens were used, the first being saline suspensions prepared as for injections (p. 37); for the second antigen, the organisms were killed by heating them to 60°C for 30 min., after which they were suspended in saline containing 0.2% tricresol. A total of 13 strains of *A. israelii* — those used by Rosebury *et al.* for their other animal experiments — were employed as antigens. A control uninoculated rabbit was also injected with the antigen suspensions. All the animals gave negative reactions with materials used for preparing the antigens, the saline, tricresol solution, and broth.

Examination of the skin reactions of the skin-tested rabbits showed that the rabbits reacted to ten of the strains and that the previously-inoculated animals gave a more marked

reaction than the uninoculated control rabbit. These skin reactions appeared two days after inoculation; with the strains of A. israelii tested, the reactions ranged from areas of erythema and swelling to small indurated abscesses, from which the organisms could be isolated. The same reaction was observed with the heat-killed organisms. Unfortunately, this skin reaction did not indicate systemic infection in the rabbits, as was shown when the animals were killed. Only one showed signs of progressive actinomycotic infection in the lung and this rabbit had received further intravenous inoculations of culture after the skin-testing was finished. It was not possible to predict from these results that a rabbit giving a positive skin reaction would develop progressive actinomycosis. At all events, the facts so far available about allergy in actinomycosis are not helpful in suggesting an approach to the problem of regular production of actinomycotic lesions by inoculation of experimental animals.

Role of concomitant bacteria in natural lesions produced by Actinomyces israelii

Microscopic examination of actinomycotic pus usually shows the presence of a very rich mixed bacterial

flora. In addition to A. israelii a wide variety of Gram-positive and Gram-negative bacteria are normally present. The flora is most mixed and most in evidence if open discharging sinuses are formed, but such accompanying bacteria have also been found in closed actinomycotic lesions. Since the experimental inoculation of A. israelii alone into animals has yielded such uncertain results, interest has centred on the possibility of a virulence-enhancing role on the part of the other bacteria present in natural actinomycotic lesions. Many workers have suggested that the combined action of A. israelii and bacteria on the host leads to increased pathogenicity with subsequent spread of the actinomycete. How a natural actinomycotic lesion begins is speculative. Do the concomitant bacteria actively start infection and by the production of anaerobic conditions allow the A. israelii to grow and thus become a secondary pathogen? Or is there perhaps a combined synergistic action between A. israelii and other bacteria? Or is infection initiated by A. israelii and are the concomitant bacteria only secondary invaders? These questions however are still unanswered. Many workers, however, have made attempts to determine the most commonly occurring bacterial species in natural human actinomycotic lesions, and with these bacteria, either alone or mixed with A. israelii, the experimental reproduction of

actinomycotic lesions has been attempted. The results of such experiments have been negative. Nevertheless, the idea persists — notably in the papers of Holm (1948, 1950, 1951) — that these organisms may be important in the natural infection. For this reason it is necessary to examine the published facts on this matter.

The role of *Bacillus actinomycetem-comitans* in lesions

In material from cases of human actinomycosis one organism has regularly been found in association with *A. israelii*, namely, *Bacillus actinomycetem-comitans*. This organism — a facultative anaerobic Gram-negative coccobacillus — was originally isolated by Klinger (1912) from four of seven cases of human actinomycosis; the bacterium was present in large numbers only in the centre of the sulphur granules; it was not found free in the pus. Colebrook (1920) examined 30 cases of human actinomycosis and found *B.actinomycetem-comitans* along with *A. bovis* (= *A. israelii*) in 24 of them. Pure cultures of the concomitant bacterium were obtained in ten instances. Although the two organisms were found together it must be emphasised that Colebrook did not find a single case of

actinomycosis due to infection with B.actinomycetem-comitans alone. The actinomycete was present in the pus from all 30 cases - even in the pus of cases from which B.actinomycetem-comitans was absent. Actinomycotic pus from closed human lesions examined by Bayne-Jones (1925) was also shown to contain a small aerobic cocco-bacillus which conformed in morphology and biochemistry with the organism described by Klinger and by Colebrook. Bates (1933) examined specimens from 29 cases of human actinomycosis and stated that "A. bovis (= A. israelii) is always associated with other bacteria and in many cases where the organism would appear to be pure, closer examination of the periphery of the granule would show that B.actinomycetem-comitans is present". On the analogy of Fildes's (1929) hypothesis concerning the role of mixed infections in tetanus, which assumes that the oxygen tension is reduced by the associated bacteria, which helps the spores of Clostridium tetani to germinate and the organisms to multiply, Bates (1933) postulated a similar process in actinomycotic infections, the B.actinomycetem-comitans acting as an oxygen reducer.

Many other authors since Bates (1933) have reported on finding B.actinomycetem-comitans in association with A. israelii in actinomycotic lesions. Holm (1950) examined material from closed actinomycotic lesions in 650 human

cases - the largest number that has ever been described. In this series he found that in all cases with one exception, in which infection was solely due to Nocardia asteroides — B.actinomycetem-comitans was the organism most often accompanying A. israelii though there might also be a varied flora of such organisms as anaerobic streptococci, various bacilli and many other forms. A new anaerobic bacillus (resembling the influenza bacillus), which Holm termed the "corroding" bacillus was also found in this material. On his bacteriological findings that A. israelii was never found as the sole infecting bacterium but always in association with other organisms Holm formulated the hypothesis that actinomycosis is a multiple or combined infection due to the action of the actinomycete and the other bacteria on the host. Experimental animal proof of this has not been shown.

In a later publication Holm (1951) again dealt with his theory and discussed the importance of B.actinomycetem-comitans and his new corroding bacillus in maintaining actinomycotic-like lesions in two patients treated with penicillin. In the first case, which was one of cervico-facial actinomycosis, material was aspirated from the jaw and shown to contain, on anaerobic culture, A. israelii, B.actinomycetem-comitans and the corroding bacillus. After treatment for 28 days with 400,000 units of

penicillin a day, A. israelii could no longer be demonstrated in the pus or grown from it. The clinical condition of the patient deteriorated, however, and lesions persisted for several months in which only B.actinomycetem-comitans and the corroding bacillus could be seen and from which other organisms could not be isolated. This patient died 18 months after the development of the facial actinomycotic lesions of pneumonia and meningitis, no mention is made of any post-mortem findings and especially in relation to A. israelii. The second case, which originated as one of actinomycosis of the lung, gave material which yielded primary cultures of A. israelii and B.actinomycetem-comitans. The patient, after one month's treatment with penicillin, 400,000 units a day, also deteriorated in his clinical condition and had a discharging sinus which led to the development of a lesion in the lung with the production of granules in the sputum. These granules were found to be composed of masses of mixed bacteria, but A. israelii could not be isolated from them. Finally, the patient developed generalised eruptions over the surface of his body. The histology of the eruptions showed a picture resembling in all respects that found in actinomycosis except only that A. israelii was absent. Cultures of such tissue yielded growth only of B. actinomycetem-comitans and the corroding bacillus. Holm's

view was that penicillin treatment removed *A. israelii*, leaving only the *B.actinomycetem-comitans* and the corroding bacillus, and that these two organisms together could give rise to a condition clinically and histologically identical to that caused by *A. israelii*. But it must be noted that, in seeking to determine the presence or absence of *A. israelii* in material from penicillin-treated patients, Holm did not incorporate penicillinase in the culture medium. Instead, he stroked a penicillin-sensitive *Staphylococcus aureus* across the diameter of the petri-dish. If any penicillin was present in the inoculated material, Holm reasoned that growth of the *Staph. aureus* would fail. The *Staph. aureus* grew; hence Holm concluded that the material did not contain penicillin. Though these cases are interesting I think judgment must be reserved, as to the significance of the *B.actinomycetem-comitans* and the corroding bacillus in maintaining infection in the actinomycotic lesions. Speculations are interesting, but to prove a hypothesis, it must be substantiated by positive results from animal inoculations.

Thjotta and Sydnes (1951) reported a case with the typical history and signs of cervico-facial actinomycosis developing after extraction of a tooth. The pus however did not show granules, and smear preparations showed the

presence of only Gram-negative rods and cocci. Both aerobic and anaerobic cultures yielded an organism identical with B. actinomycetem-comitans. The authors stated that the bacterium was non-pathogenic for guinea-pigs — though experimental details are not given. The possibility that this organism they had isolated might be Actinobacillus ligniersi and not B.actinomycetem-comitans was carefully considered by Thjotta and Sydnes. Actinobacillus ligniersi is a pathogen both for man, cattle and swine. Culturally the bacterium will only grow under aerobic conditions; growth does not take place anaerobically, and experimentally it is pathogenic for guinea-pigs. Mainly on the basis of these two facts — as well as considering several minor biochemical differences — the authors were convinced that the organism they isolated was truly a species of B.actinomycetem-comitans and not an actinobacillus. This is the first record of B.actinomycetem-comitans being found as the main infecting pathogen in a condition that clinically appeared to be actinomycosis. This finding of course would appear to substantiate Holm's theory, but I cannot help thinking that, in Thjotta and Sydnes's case, the treatment of the patient with penicillin before the lesion was excised probably removed the A. israelii (which is sensitive to this antibiotic — although in the chronic

pockets the penicillin does not always penetrate) and left the penicillin-resistant B.actinomycetem-comitans.

Experiments have been attempted in an effort to assess the pathogenicity of B.actinomycetem-comitans for man and experimental animals. Klinger (1912) inoculated a mouse subcutaneously with the heavy growth of B.actinomycetem-comitans from an agar slope. There was no effect and he concluded that the organism was not pathogenic for small laboratory animals. Colebrook (1920) was interested in the possible pathogenicity of B.actinomycetem-comitans for animals and for man. Intracutaneous injections of "living suspensions" (not more clearly defined) of B.actinomycetem-comitans into one normal human did not cause any reactions but it is stated that vaccines of the organism in doses of over 40 millions have sometimes produced mild and febrile reactions.

Intravenous inoculation of a "culture" of the organism — "varying from 300 to 15,000 millions" — caused the death of two of three rabbits in 24 hours. Post-mortem examination of these animals showed only venous congestion of the lungs, kidneys and spleen. In contrast the intra-peritoneal and subcutaneous inoculation respectively of an unknown amount of B.actinomycetem-comitans into two rabbits gave negative results when the animals were killed

and examined after an unstated time. The intra-peritoneal inoculation of guinea-pigs with presumably similar material resulted in the death of two of the animals with diffuse peritonitis, and the development of white nodes on the omentum but these nodes did not contain granules. The time of death of these animals is not stated though it is recorded that a third guinea-pig died 30 days after intra-peritoneal inoculation with congestion of the tunica vaginalis but no peritonitis.

Colebrook made the important observation that in the animals that died after being injected with _B.actinomycetem-comitans_ there was not developed any lesion similar to that seen in natural actinomycosis. In addition guinea-pigs which were inoculated subcutaneously with _B.actinomycetem-comitans_ only developed an indurated inflammatory reaction, which did not go on to abscess formation.

The morphological and cultural characters of two freshly-isolated strains of _B.actinomycetem-comitans_ were described by Goldsworthy (1938). One strain of the organism was isolated from a closed actinomycotic abscess in a cervical lymphatic gland, the second strain being isolated from a supposedly pure culture of _A. bovis_ (= _A. israelii_) — which in turn had been obtained from a case of human

actinomycosis. His paper mentions but does not describe experiments which would suggest that this organism is of pathological importance. Unfortunately details are not given and the reader may only speculate whether this work produced evidence that the organism has pathogenic importance. If B.actinomycetem-comitans is the essential adjuvant in the aetiology of actinomycosis, injection of a mixture of A. israelii and B.actinomycetem-comitans might be expected regularly to produce actinomycotic lesions in animals, but this has not proved to be so. From two cases of human actinomycosis Naeslund (1931) isolated strains both of A. israelii and of B.actinomycetem-comitans. Using mixtures of these organisms he inoculated six guinea-pigs intra-peritoneally, and "several" cows subcutaneously; repeated subcutaneous inoculations were made into the cattle. In only one cow a hazel-nut-sized tumour appeared at the site of injection and this required three months to develop. Aspiration of this tumour yielded pus with well developed sulphur granules. On culture only A. israelii was grown; all attempts to isolate B.actinomycetem-comitans from this material failed. The tumour later broke down and drained through a fistula which healed after five months.

Naeslund (1931) carried out further experiments with mixtures of three strains of A. israelii and

B.actinomycetem-comitans, but these cultures of A. israelii were isolated from normal human mouths. In this series, one cow was inoculated subcutaneously and five guinea-pigs intra-peritoneally. Actinomycotic infection was not established in any of the animals.

Naeslund reasonably concluded that his experiments did not support the idea that B.actinomycetem-comitans was essential to initiate or was able to enhance the development of actinomycotic lesions.

Naeslund (1931) also attempted to isolate B.actinomycetem-comitans from human mouths. As A. israelii is present as a commensal in the mouth it seemed to him natural to expect that this might also be the habitat of B.actinomycetem-comitans, which has not been found free in nature. Naeslund was unable to demonstrate its presence in the mouth, however, and the habitat of B.actinomycetem-comitans remains unknown. Ignorance is seldom resolved as a result of pure speculation, however ingenious; but it seems worth giving a little space merely to record a suggestion made by Brown and Nunemaker (1942) in their memoir on "Rat Bite Fever". They think that B.actinomycetem-comitans may well be the L form of A. israelii.

Sullivan and Goldsworthy (1940), who inoculated

guinea-pigs subcutaneously with mixtures of _A. bovis_ (= _A. israelii_) — of both pathogenic and tonsillar origin — and _B.actinomycetem-comitans_, failed to produce lesions. Unfortunately, precise details about the number of animals inoculated and the form of the inocula are not given. The account of the experimental work is given only in the most general terms. Impregnation of a wooden splinter with the combined cultures of _A. bovis_ and _B.actinomycetem-comitans_ and placing the splinter in the subcutaneous tissue of guinea-pigs gave rise in only one instance to pus formation. The organisms could neither be seen in films nor cultured from the pus and when the pus was evacuated the lesion healed.

The relation of other bacterial species in experimental infections with _A. israelii_

Wolf and Israel (1891), at the same time as they made other animal experiments, inoculated one rabbit in the liver with a mixture of _A. israelii_ and _Staphylococcus aureus_, the actinomycete being taken from a culture growing on agar. When the rabbit was killed one month later the findings of a post-mortem examination were as follows. The abdominal wall was cicatrized, there was no lesion either on the peritoneum or liver, but the spleen showed five tumours

ranging in size from a pin-head to a pea. These tumours contained cheesy pus and microscopic examination (wet films) of this pus showed that sulphur granules with clearly marked clubs were present. No mention is made, however, of whether the Staph. aureus was also present in the pus. In the kidney, though actinomycotic abscesses were not present, two white scars with a doubtful redder zone around them were noted. This is the earliest published reference to involvement of the kidney in experimental actinomycotic infections. Wolf and Israel considered that these scars were due to the mass of the eliminated Staph. aureus being excreted from kidney after the passage from the liver. They apparently did not consider that the A. israelii itself could have any damaging effect on kidney tissue. The result obtained with inoculation of this bacterial mixture is not conspicuously different from that obtained by placing pure cultures of A. israelii in the rabbit peritoneum (p. 21).

Naeslund (1931) also inoculated "mixtures" (not more precisely defined) of A. israelii and Staph. aureus into guinea-pigs and rabbits; the cultures of Staph. aureus being originally isolated from spontaneous infections in both these animal species. The animals were first given a subcutaneous injection of actinomycetes — both the aerobic and anaerobic mouth species being used, then they

were given an inoculation of *Staph. aureus* straight through the original subcutaneous inoculation site where a small local lesion had developed. Thus the two organisms were mixed together. The result of this was an extension of the inflammatory and suppurative processes as compared with the results in control animals inoculated in the usual way with *A. israelii* and *Staph. aureus*.

Unfortunately Naeslund does not precisely state separately the results he obtained using (a) the aerobic and (b) the anaerobic strains of actinomycetes; both sets of results are treated as a whole. His conclusion was that although *Staph. aureus* may extend the extent of the inflammation of the actinomycotic process the organism does not have any noticeable effect on the formation or initiation of the actinomycotic lesion.

Emmons (1938) also inoculated guinea-pigs with mixtures of *A. bovis* (= *A. israelii*), *Staph. aureus*, *Streptococcus* and so forth. Details of the precise species of organism and routes of inoculation are not given in his paper. As to the results, Emmons merely states no progressive actinomycotic lesions were produced, but some type of lesion must have been formed as Emmons states that he found it impossible to re-isolate the *A. bovis* although the accompanying bacteria could be recovered. To explain his

failure to obtain progressive lesions or enhanced invasiveness by the different organisms he considers it possible that he used (a) the wrong dosage (i.e. numbers) of organisms as inoculum and (b) the wrong combination of bacterial species along with A. bovis. Sullivan and Goldsworthy (1940) also inoculated mixtures of A. bovis and Staph. aureus into guinea-pigs but with unsuccessful results.

What emerges from the present long and rather detailed review of the literature about attempts to produce experimental actinomycosis is that this may not be unduly difficult with cattle but is more likely to fail than to succeed with the usual laboratory animals. In preparation for work of the kind which I was about to embark upon it was necessary to analyse the available evidence in some detail because the few successes recorded are tantalisingly difficult to interpret ---- partly because of many unpardonable obscurities of text perpetrated by authors and allowed by editors, and partly because of the great lack of reproducibility in the results not only between one experimenter and another but also between the individual experiments of a single worker. Whatever the reason for failure it was clear to me that a systematic attempt should be made to establish reproducible successes -- with mice,

preferably -- to discover what was important for the regular production of experimental actinomycosis in small animals. The subsequent sections of the thesis describe the materials and methods used in such an attempt, the results, and what I think of their significance.

MATERIALS AND METHODS

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MATERIALS AND METHODS

This section gives details about the mice used as experimental animals, the sources of the strains of *A. israelii*, the methods of their isolation (two strains only) and culture, and the preparation of inocula for animal experiments.

Details about the routes of inoculation and the various time-ranges elapsing between inoculation and killing of the animals are given individually for each experiment in the section of the thesis dealing with results.

Mice

Pure-line CBA mice, which were originally obtained from the Department of Animal Genetics, Edinburgh University and bred in the Glasgow University Department of Bacteriology at the Western Infirmary, were used for all inoculation experiments. The mice were inoculated intra-peritoneally, intra-muscularly, intravenously, and subcutaneously. For ease and certainty in intravenous inoculation Swiss white mice were used as well as CBA mice for this procedure. Normal uninoculated mice of the same batch as the experimentally inoculated mice were killed in parallel for purposes of comparison.

TABLE I

Strains of *Actinomyces israelii* used

Strain	Source	Lesion from which isolated
104	Dr. Per Holm (State Serum Institute, Copenhagen).	Multiple abscess of cheek
54	"	"
161	"	Angle of right lower jaw
265	"	"
253	"	
170	"	Right pleural cavity
5195	National Collection of Type Cultures, London	Cervico-facial actinomycosis
5206	"	Pus from chronic sinus of neck
4956	"	Lung abscess
8047	"	"
Ralston	Western Infirmary of Glasgow (personal isolation)	Cervico-facial actinomycosis
Murray	"	"

Cultures of A. israelii

Twelve strains of A. israelii, all from human cases of actinomycosis, were used for the experimental inoculation of mice. Table I gives details of the origin of each of the strains.

Methods of Culture

The cultures of A. israelii were grown in Brewer's thioglycollate broth because the medium is easy to prepare and use and provides large amounts of culture quickly for inoculation purposes.

Brewer's thioglycollate medium was made and used as below:-

Digest Broth (Tryptic digest of horse heart)	100 ml.
Agar powder	0.05 g.
Glucose	0.3 g.

The broth was heated to dissolve the glucose and melt the agar; then it was cooled to room temperature and thioglycollic acid (0.1%) was added along with 1% of N/1 NaOH solution. The medium was dispensed in bottles ("medical flats") in 40-50 ml. amounts and sterilized in the autoclave at 15lb. pressure for 20 min. The final pH was 7.5

In the first batches of media, Methylene Blue, (1/500,000) was added,

as an indicator of anaerobiosis but in later batches this was omitted. In preparation of the digest broth base 0.5lb. of fat-free minced horse heart was added to 500 ml. of distilled water, which was made alkaline (pH 9-10) by the addition of 10-15 ml. of 10% NaOH. This mixture was heated at 80°C in a Koch steamer for 30 min. and cooled to 40°C, 10 ml. of $CHCl_3$ being added as preservative. The horse-heart mixture was left at room temperature overnight. In the morning, trypsin (2%) was added, the pH checked for alkalinity, and the medium placed in the incubator at 37°C for 18 - 24 hours with several shakings during the incubation period. At the end of 24 hours the mixture was removed from the incubator, and made acid to litmus by the addition of 10-15 ml. of 10% HCl to stop digestion.

The mixture was now placed in a cool Koch steamer and the temperature raised to 100°C, at which it was kept for 1½ hours. The mixture was filtered through Green's paper (No. 797) and made up to the original volume. NaCl (0.5%) was added, the reaction of the broth was adjusted to pH 8, and it was heated in the Koch steamer at 100°C for a further 20-30 minutes. Again the broth was filtered through Green's paper (No. 797) and the pH adjusted to 7.5. The broth was now distributed into bottles and autoclaved at 15 lb. pressure for 20 minutes. It was then ready for use.

The methods used for isolation of two strains of *A. israelii* from pus

Strains 'Ralston' and 'Murray' (table I p. 92) were isolated from pus from human cases of cervico-facial actinomycosis. The pus from both cases contained well marked and typical sulphur granules. The pus was diluted with a large volume of sterile saline (about 20 ml. of saline to 1 ml. of pus) and then poured into a series of sterile petri dishes placed on a black background. The granules were more easily visible against the dark surround and could easily be fished out of the fluid with a platinum loop. From the pus from Ralston, 10 granules, and from that from Murray, five granules were collected in another sterile petri dish containing sterile saline. From this petri dish the granules were transferred through six petri dishes containing sterile saline to wash as much debris and concomitant bacteria from the surface as possible. Each granule in turn was then quickly passed through a dish of methylated spirit, the time of immersion not exceeding 30 seconds, and the granules were then passed through a second series of sterile saline washes to rinse off the spirit. After this washing process the granules were planted either singly or in pairs into a bottle with 40 ml. of thioglycollate broth or a digest-agar-deep

containing 10% glucose.

The thioglycollate broth was incubated in air at 37°C. After about five days the first sign of growth was indicated by an increase in the size of the granule; thereafter pieces separated from the granule and gave rise to smaller independent colonies.

Surprisingly, concomitant bacteria did not appear in the primary cultures of either of the two strains. Some of the granules which were sown in thioglycollate broth failed to grow. This could be attributed to 'decay' of the granule in the pus because it was found that in actinomycotic pus isolation of the organism was possible only if cultures were put up as soon as possible. From material allowed to stand for 24 hours before being cultured, viable actinomycetes could not be grown.

The glucose-agar-deep tubes were incubated at 37°C for from five to seven days in anaerobic jars in an atmosphere of 10% CO_2. Examination after this time showed cauliflower-like colonies developing in the depths of the tubes. Isolated colonies were fished out, filmed, and sown into thioglycollate broth.

Preparation of inocula for animal experiments

Cultures of *A. israelii* grown in 40 ml. of Brewer's

thioglycollate broth (incubated for six to ten days at 37°C.) were centrifuged and the bacterial sediment, washed three times in sterile saline, was resuspended in 10 ml. of sterile saline. The density of the suspension was usually about five times that of tube No. 10 of Brown's Opacity Tubes. The bacterial suspensions were prepared in the same way for all animal inoculations; the dose of organisms injected was either 0.5 or 1 ml. as detailed for individual experiments.

RESULTS

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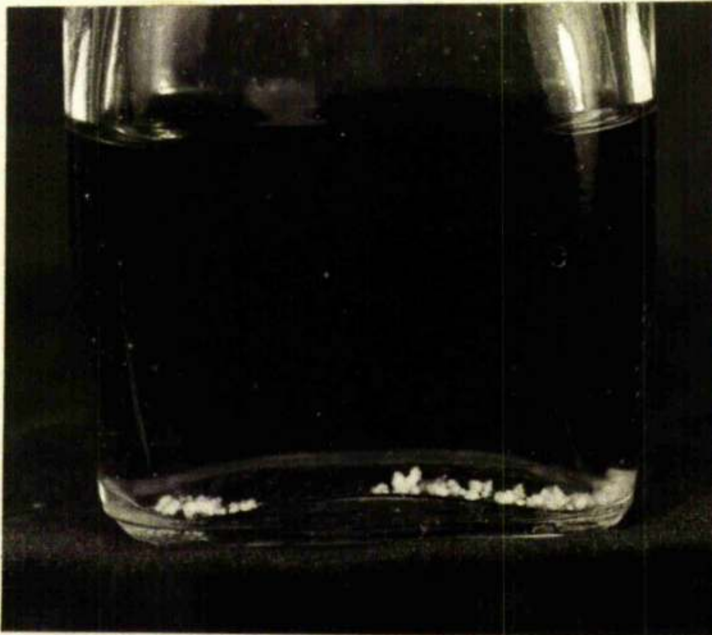
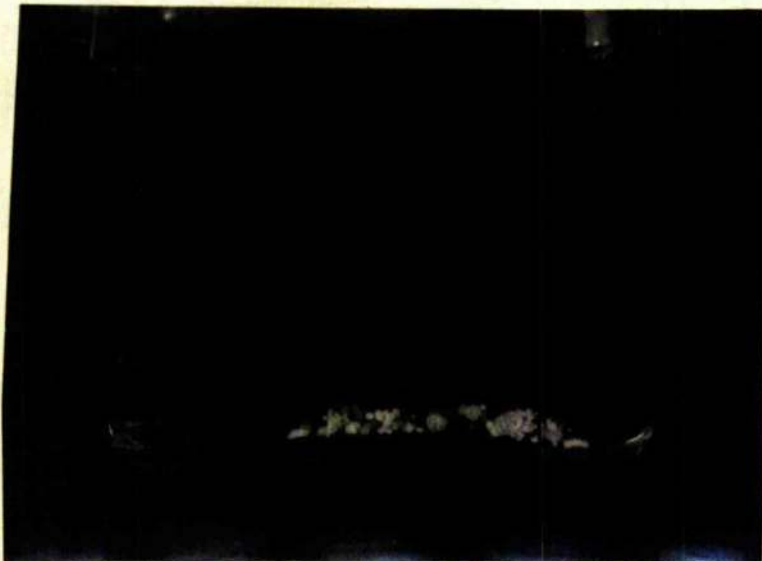
Figure 1



A. israelii strain 4956 in Brewer's thioglycollate broth showing rough cauliflower-like granules in the depth of the medium after five days at 37°C. x 2.

Figure 2



A. israelii strain 5206 showing a similar appearance to that in fig. 1, five days' growth at 37°C. x 2.

T A B L E   II

Characters of twelve strains of *Actinomyces israelii*

Strain of *A. israelii*	Character of growth in Brewer's thioglycollate broth	Microscopic Appearance
104	rough	branching filaments
54	"	"
170	"	"
5206	"	"
4956	"	"
8047	"	"
Murray	"	"
Ralston	originally rough then becoming intermediate	filaments and angled diphtheroids
161	intermediate	"
265	"	"
253	"	"
5195	smooth	diphtheroids (small and regular shape).

R E S U L T S

Observations on the morphology of the strains of A. israelii used for experimental inoculation of mice

Table I (p. 92) gives the source of the 12 strains of A. israelii used in this work.

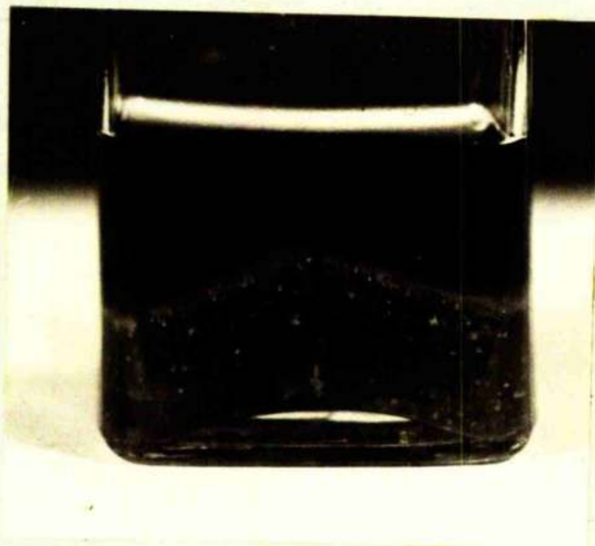
When first received all the cultures were grown in Brewer's thioglycollate broth, and it was noted that in this medium the strains showed differences between each other in their growth characteristics and microscopic appearances. The main points are summarised in table II.

Growth characteristics in Brewer's thioglycollate broth:-

The organisms showed three types of growth in Brewer's thioglycollate broth. The first type was that of small, rough, cauliflower-like granules which grew slowly during the first four to five days in the depth of the medium (figs. 1, 2). Then on further incubation for another four to six days, growth became more rapid, and the bottom surface of the bottle was covered with the small rough colonies. Seven strains, 104, 54, 170, 5206, 4956, 8047, and Murray showed this type of growth.

The second growth form in the same medium was an intermediate type. Small granules were seen after four days'

Figure 4



A. israelii strain 161. Intermediate form of growth at a later stage than in fig. 3. The small granules are suspended in a haze of growth and the line of demarcation of growth is clearly seen. 10 days at 37°C. (Natural size).

Figure 5

A. israelii strain 5195, showing smooth even turbidity of growth six days at 37°C. (Natural size).

Figure 3

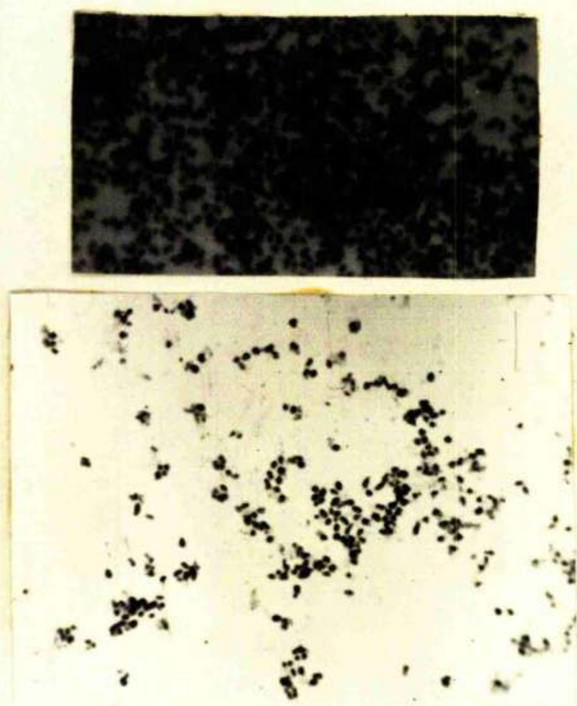
A. israelii strain Ralston showing intermediate form of growth in Brewer's thioglycollate broth: granules suspended in a fine haze of growth. Six days' incubation at 37°C. x 2.

incubation in the depth of the broth and round many of the granules at this time a fine halo of hazy growth was forming. This fine flocculent growth increased and coalesced on continued incubation. Fig. 3 shows the appearance of the culture after six days' incubation. A further stage in the development of this intermediate form of growth is seen in fig. 4 which shows the demarcation of the growth by a line formed well below the surface of the broth. Little compact granules can be seen in the light flocculent haze. This intermediate pattern of growth was shown by four strains (Ralston, 161, 265 and 253.)

The third type of growth (fig. 5) a smooth, evenly distributed turbidity - was shown by only one of the 12 strains (5195). - With this strain (5195) growth was more rapid; in two to three days an even turbidity was seen diffusing throughout the medium, further incubation of six days led to a heavier growth density, and part of this fell as a fine sediment to the bottom of the bottle. With this strain growth did not take place at the surface of the medium but stopped well below it, just as in the intermediate type.

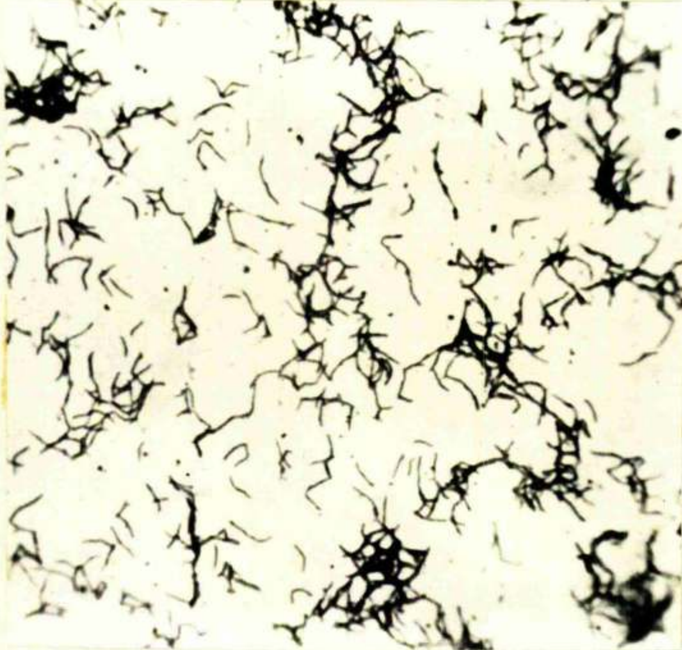
After one month's continuous subculturing in thioglycollate broth the strains giving the rough type of growth readily changed to the intermediate type, but not to the smooth.

Figure 14



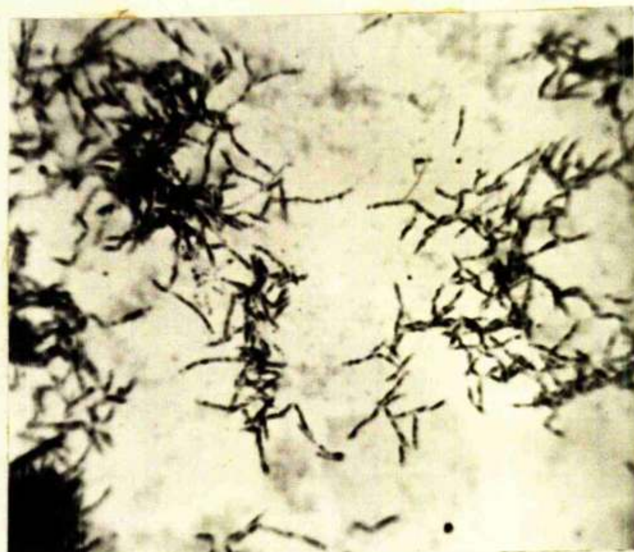
A. israelii Strain 5195. Film made from culture in Brewer's thioglycollate broth after five days at 37°C. Note the small regular diphtheroidal forms showing what appears to be bipolar staining. Gram's method x 1000.

Figure 13



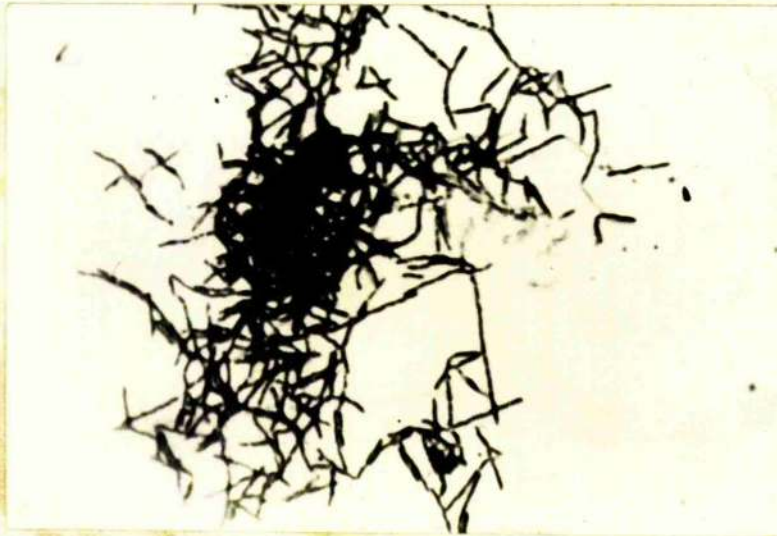
A. israelii strain Ralston. Film made from haze in Brewer's thioglycollate broth, (five days at 37°C.) Note the angular V and Y forms. Gram's method x 1000.

Figure 12



A. israelii strain Ralston. Film made from granule in the depth of Brewer's thioglycollate medium, (16 days at 37°C.). The branching, angular filaments can be seen. Note the 'spotty' appearance of filaments, due to age of culture, irregularly taking up Gram's stain. x 1000.

Figure 10

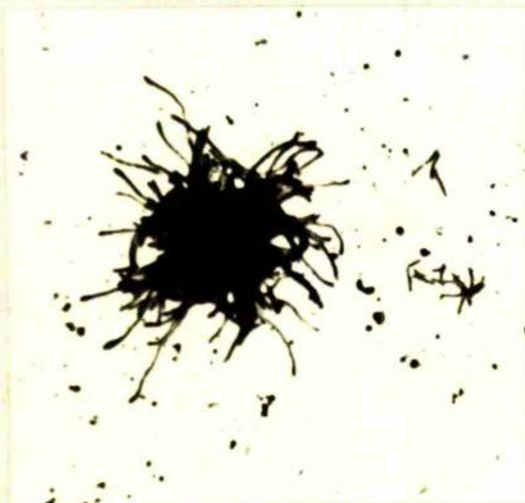


A. israelii strain 4956. Film from Brewer's medium again showing the long branching filaments (six days at 37°C.). Gram's method x 1000.

Figure 11

A. israelii strain 104. This figure again shows the angular morphology, (six days at 37°C. in Brewer's medium). Gram's stain x 1000.

Figure 8

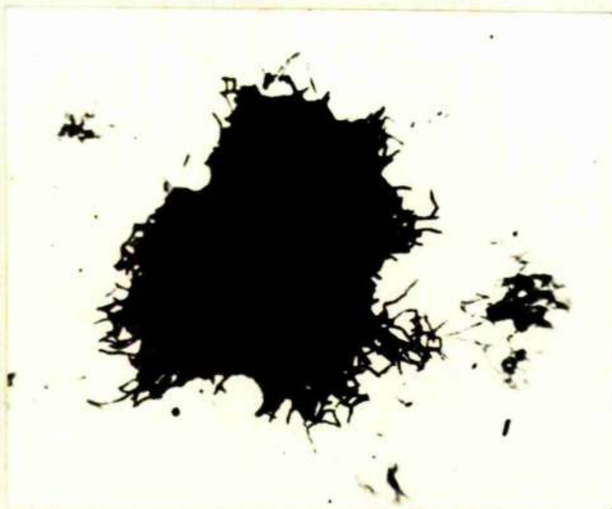


A. israelii strain 8047: film of culture in Brewer's thioglycollate broth (six days at 37°C.). Note the long filamentous threads taking up a radiate arrangement, the extreme ends of the filaments appear to be slightly thicker. Gram's method x 1000.

Figure 9

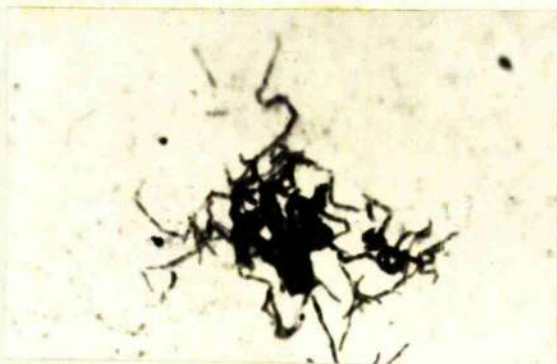
A. israelii strain 5206: film from Brewer's broth, (six days at 37°C.) showing angular diphtheroid forms. Note the spotted appearance. Gram's method x 1000.

Figure 6



A. israelii strain 54. A low power view of a granule growing in Brewer's thioglycollate medium (six days at 37°C.) to show the solid mass of filaments only at the very edge can radiating hyphae be seen. Gram's method x 50.

Figure 7

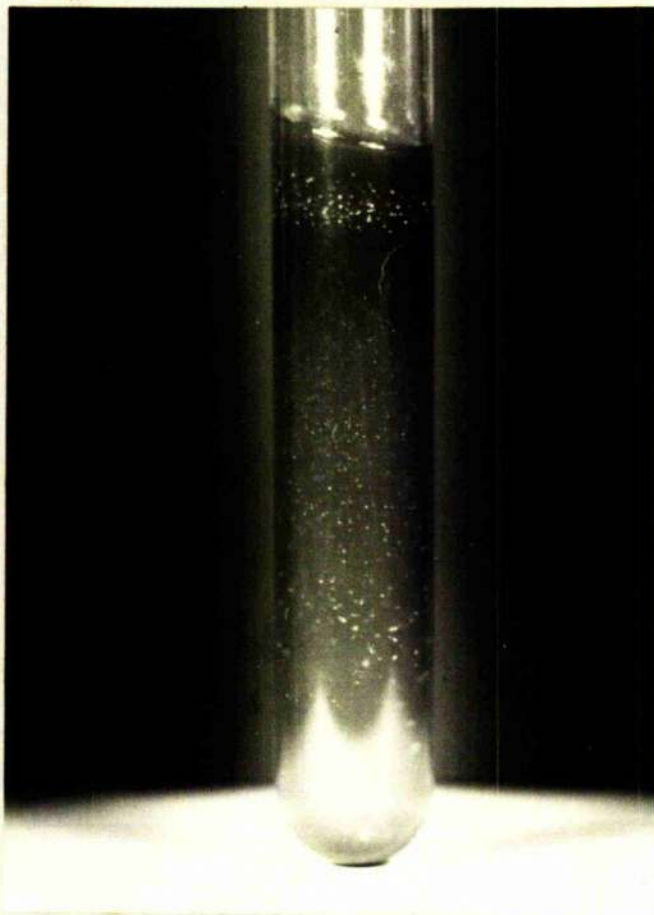


A. israelii strain 54 shows an oil-immersion view of the teased out granule similar to that shown in fig. 6. Gram's method x 1000.

Microscopic appearances of growth in Brewer's broth

The microscopic appearance of an organism in these cultures was linked with its growth character. One of the granular colonies examined under the low power of the microscope had the appearance of a solid mass of organisms and only at the periphery could the separate filaments be seen (fig. 6). Under oil-immersion Gram-stained films of rough cauliflower-like granules showed long branching Gram-positive filaments (figs. 7-11). The filaments often broke up in the making of the films and long diphtheroid-like angled Y and V forms were commonly seen as in figs. 9 and 11. Gram-stained films of the intermediate type of growth were made both from the granules and the fine growth haze. The granules showed branching filaments and the haze the V and Y forms (figs. 12-13) not very different from the appearances seen in films of the rough type of growth. A spotted appearance as in fig. 12 was often seen in the organisms especially in films made from old cultures (16 days). Films of the smooth evenly turbid growth characteristic of strain 5195 however showed that the organism had the appearance of a small diphtheroid and these sometimes showed marked bipolar staining (fig. 14). Occasionally in cultures which had been left in the 37°C incubator for one to two months, bizarre

Figure 18

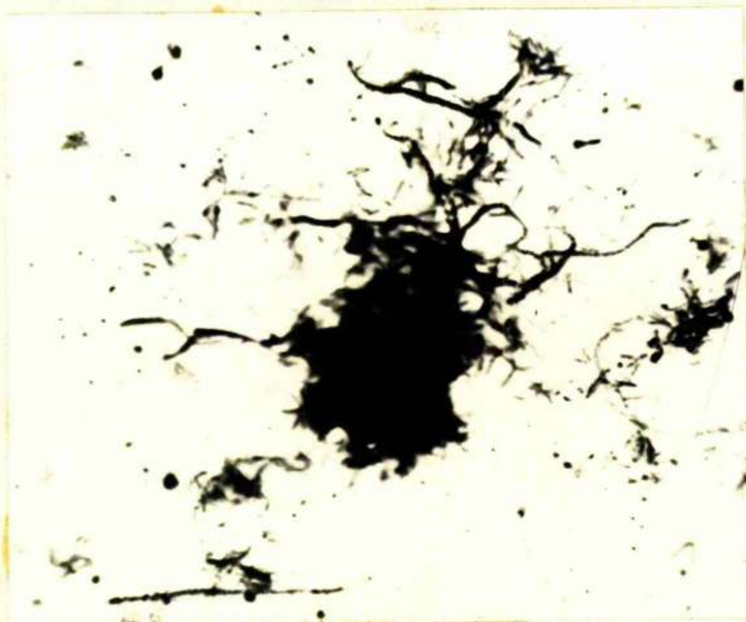


A. israelii strain Ralston. Culture in 10% glucose-digest-agar deep , incubated at 37°C. for five days in an atmosphere of 10% CO_2. Note the band of growth 0.5cm. below the surface of the agar. There is no growth in the top layer of medium. At the bottom of the tube can be seen single isolated colonies. x 2.

Figure 17

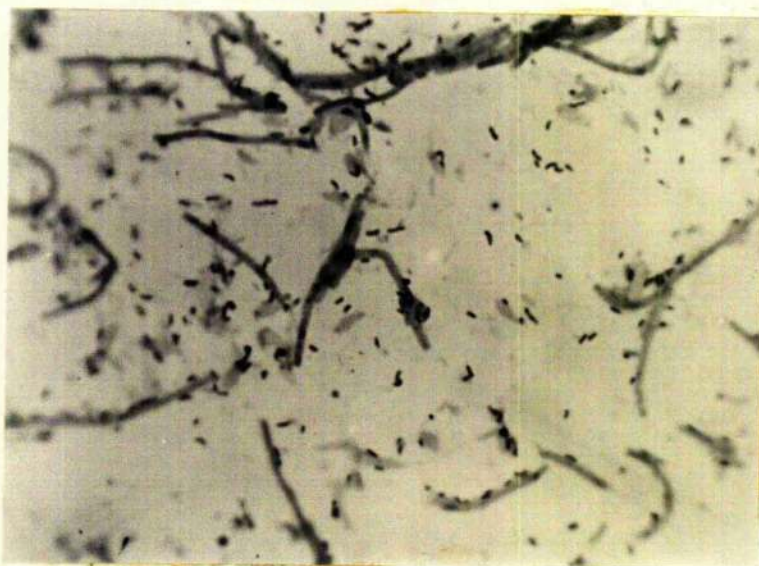
A. israelii strain 104. Film made from culture in Brewer's medium (two months' incubation at 37°C.) showing very large thickened forms, against the mass of lysed normal sized bacteria. Gram's method x 1000.

Figure 15



A. israelii strain 8047. Film made from a culture in Brewer's medium incubated for three weeks at 37°C. The very thick irregularly-staining bacterial forms can be seen against the background of transparent lysed bacteria. Gram's method x 1000.

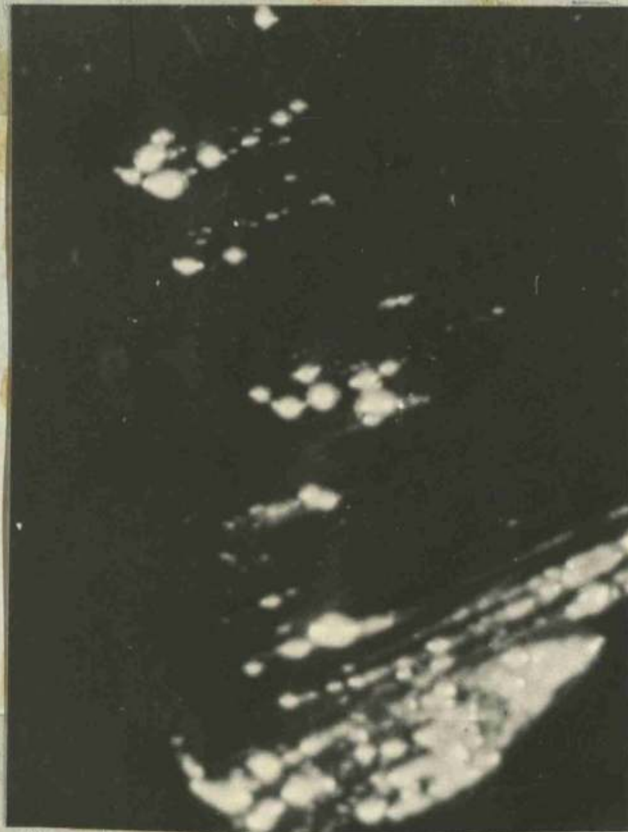
Figure 16



A. israelii strain Ralston. In this film again bizarre forms of irregular shapes are seen, (Four week-old Brewer's culture). Gram's method x 1000.

forms were seen in Gram-stained films (figs. 15-17). These forms were thicker and more irregular in appearance than the usual bacteria, and they varied in their reaction to the Gram stain - being Gram positive, variable and negative. Such old cultures were found not to grow in any form when they were subcultured, whether aerobically or anaerobically. They were not investigated. Though I have little information about the morphology of surface colonies of these strains used in this work I had some success from attempts at the production of isolated colonies. The anaerobic system used at first was a dessicator, with an HCl acid-marble-zinc complex, one strain produced colonies under these conditions, but better results were obtained with the McIntosh and Fildes jar (three strains). I found that in comparing solid media - a 10% glucose-digest-agar allowed colonies to develop, while a medium of digest-agar plus 10% oxalated horse blood gave no growth. This finding appeared to be confirmed when growth in agar-deeps was compared. Melted cooled glucose-digest-agar deeps (15 ml. amounts) were inoculated with strains of A. israelii both rough and intermediate forms; the tubes were then incubated at 37°C in an atmosphere of 10% CO_2 for five days. When examined small isolated colonies were seen in the depth of the medium (fig. 18) and these had the characteristic filamentous

Figure 22

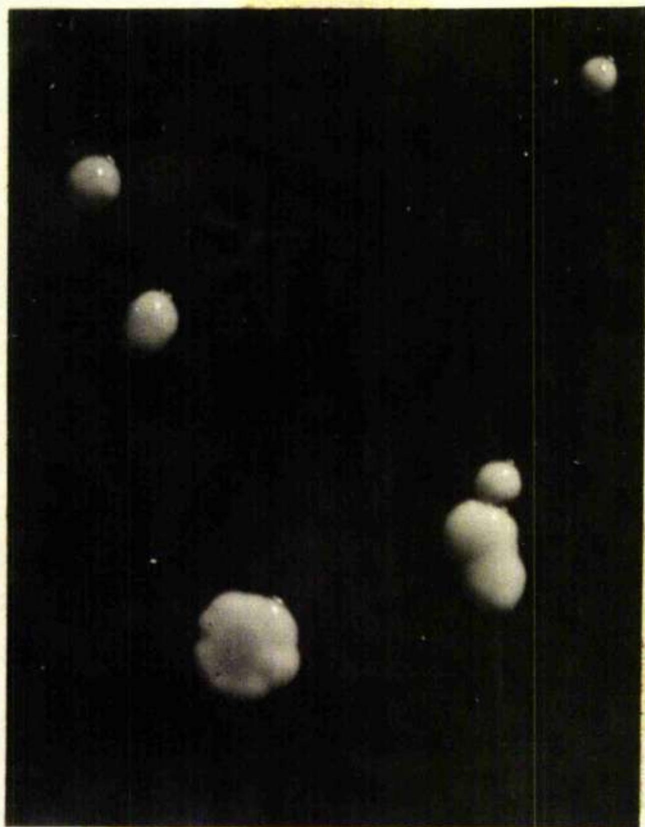


Colonies of A. israelii strain 5195 growing on the surface of 10% glucose-digest-agar, after five days' incubation at 37°C., under anaerobic conditions. The colonies are smooth, entire, and glistening, and are not adherent to the medium. x 2.

Figure 21

A. israelii strain 161. Film made from colony seen in figure 20. Note the massed angular diphtheroid forms, and the occasional very long branching filaments. Gram's method x 1000.

Figure 20



Colonies of A. israelii strain 161 growing on the surface of 10% glucose-digest-agar after five days' incubation at 37°C., under anaerobic conditions. The colony at the bottom left of the picture shows the slightly indented margin; the colonies are not adherent to the medium. x 4.

<u>Figure 19</u>

Roughish nodular colonies of <u>A. israelii</u> strain 8047 growing on the surface of 10% glucose-digest-agar after five days' incubation at 37°C., under anaerobic conditions. (Natural size).

diphtheroid appearance on Gram-stained films. A similar set of agar deep tubes containing 10% oxalated horse blood were inoculated with the same strains of organism, and were incubated under the same conditions. Examination of these tubes showed no growth, the medium was removed from the test tube and very carefully examined, but no growth was found.

The successful results of colony isolation on the surface of the 10% glucose-digest-agar showed that there were colonial differences among the strains, differences which could be linked with the appearance of the organisms when growing in Brewer's thioglycollate broth. The rough <u>A. israelii</u> strain 8047, incubated for five days on this medium gave a roughish, small colony (fig. 19) which in films gave the appearance of branching filaments. Under the same conditions intermediate strain 161 gave a slightly indented colony with a slight elevation (fig. 20). The entire colony had a delicate pink colour, which disappeared overnight on standing on the bench at room temperature, under aerobic conditions. Gram stained films showed long, angled diphtheroid forms, and long filaments (fig. 21).

The smooth strain 5195 gave small entire, colourless smooth colonies (fig. 22) films of which showed the appearance of short oval diphtheroidal forms. The surface colonies of all types were easily emulsified in

saline and they were not firmly adherent to the medium.

It must be emphasised that at no time was club-development seen in cultures of A. israelii either in liquid or on solid media. The nearest approach to anything of this kind was observed with strain 8047. Fig. 8 (p. 99) shows that this organism when grown in Brewer's medium had a radial arrangement of the hyphae, and the extreme tips of some of these appear to be slightly thicker than the rest of the filament.

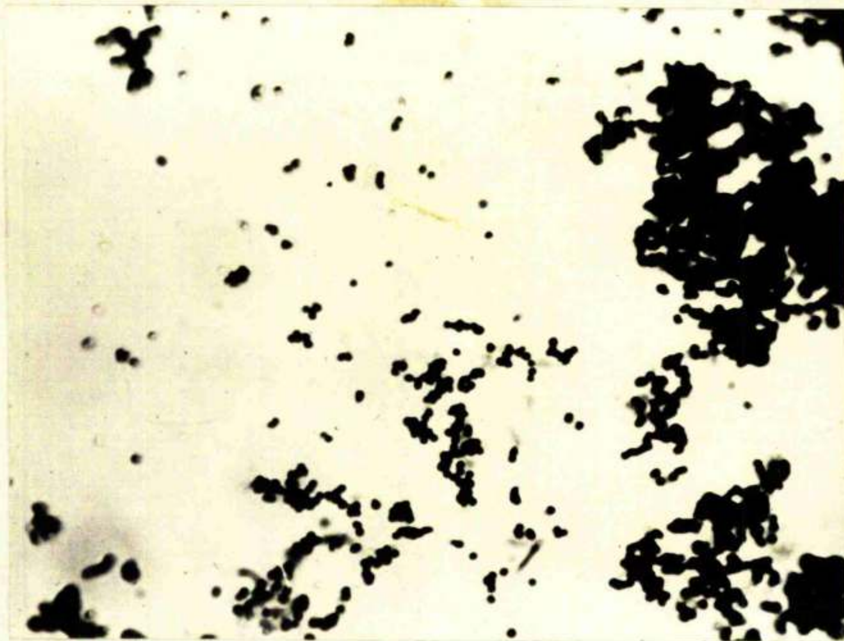
With constant sub-culturing in Brewer's broth, it was noted that all the strains of A. israelii except the smooth strain 5195, were undergoing a gradual change both in their growth characters and microscopic appearances. Gradually the rough and intermediate growth character in liquid medium was losing its distinctive appearance and assuming a smooth even turbidity like that found with strain 5195 (fig. 5 p. 98). Accompanying this change in form there was also a gradual change in microscopic appearance. Strains undergoing change lost their long filamentous and V and Y forms and assumed the appearance of short regular oval diphtheroids, sometimes with an appearance of bipolar staining. In short these strains became very similar in appearance to that of strain 5195 (fig. 14 p. 99). In addition these changing cultures grew quicker, for after

three days at 37°C, the whole medium showed a heavy even turbidity, similar to that observed with strain 5195 (fig. 5 p. 98). These changes appeared only after some six months' continuous sub-culturing in the same medium (Brewer's). Erikson (1940) has stated that to keep strains of A. israelii in the vigorous state, it is essential to alternate the medium in which they grow. Holm (personal communication) stated that he found all strains of anaerobic actinomycetes dissociated when subcultured continuously for a long time.

When the cultures had changed their growth character and microscopic appearance in the manner described, they were used for the intra-peritoneal inoculation of mice and the results were compared with those obtained with the rough and intermediate strains. This work is described on page 124.

At the same time as the cultures of A. israelii began to show changes in their morphology, it was observed that they were becoming less microaerophilic in fluid culture as judged by their growth nearer the surface of the broth. I found it difficult to grow my original strains of A. israelii on the surface of solid media; but when I was successful in growing isolated colonies on glucose-horse-heart, digest-agar plates I could do this only under anaerobic

Figure 23



Film made from a culture of A. israelii strain 161 growing in aerobic digest medium (second passage) incubated for five days at 37°C. Note the almost coccal-like appearance of the bacteria. Gram's method x 1000.

conditions of growth with or without the addition of CO_2. But with these changing strains of A. israelii especially strain 161 an attempt was made at growing the organism aerobically. When the Brewer's medium (inoculated with strain 161), showing growth at the interface of the medium and air was plated out aerobically on 10% glucose horse-heart-digest-agar plates, no growth occurred. I decided to try the process of serial transfer in aerobic liquid medium. Washed cells of strain 161 in the smooth phase were sown into medical-flat bottles (4), each containing 40 ml. amounts of digest broth (p. 92), without thioglycollic acid or any other reducing agent. As controls, bottles of digest broth containing thioglycollate (3 per transfer) were inoculated at the same time with these washed cells, on incubation at 37°C, smooth growth occurred in this as in the parent culture. After five days at 37°C good growth resulted in the digest broth sub-culture. The appearance of the growth however was not like that seen in the Brewer's medium. The bacteria formed a thin viscous slimy sediment which was confined to the bottom of the bottle. Films stained by Gram's method showed very short diphtheroid forms almost coccal in appearance (fig. 23). Strain 161 was subcultured through four consecutive passages in this aerobic digest broth; the cells from the previous

culture being spun and washed for each transfer. The second and third passage gave a slightly heavier sediment of organisms, but on the fourth passage, growth was scantier and on the fifth transfer no growth took place. Simultaneously with the inoculation of the aerobic digest medium, Brewer's thioglycollate medium was also inoculated with a portion of the aerobic washed cells, in this medium. Again growth was obtained on the second and third passage but not on the fourth or fifth transfer, the cells did not grow, even on prolonged incubation (21 days at 37°C).

With the second passage of strain 161 in the aerobic digest broth, five CBA mice were inoculated intra-peritoneally with washed cells; the result is described on page 147.

A N I M A L   E X P E R I M E N T S

A Time-Schedule for the Examination of Inoculated Mice

Critical examination of the literature on attempts at the production of experimental actinomycosis in laboratory animals revealed that experiments had not been made in which the inoculated animals had been killed and examined at regular intervals. I considered that this procedure alone could make it possible to trace the full effect of the inoculum on the animals. The mouse is a small, relatively cheap animal; therefore I decided to inoculate enough mice to be able to examine them - in the first instance, at least - at weekly intervals after inoculation. This proved a good plan, and in all series of experiments mice were killed after inoculation at intervals of 7, 14, 21, 28 and 48 days, and thereafter at intervals of from three to six months. Finally, in some experiments, animals were examined at periods of up to one and a half to two years. By adopting this procedure it was possible to observe the complete sequence of events in the production and disappearance of lesions after inoculation of mice with _A. israelii._ The picture presented by such a scheme is necessarily one built up as the result of recorded observations made at the post-mortem examinations of a great

TABLE III

Summarised results of inoculating mice intra-peritoneally with one dose of rough and intermediate forms of Actinomyces israelii

Strain	No. of mice inoculated	After inoculation: Number of mice				
		Dead in 7 days	Killed in 1-7 days	With actinomycotic lesions in 3 weeks	Without lesions in 3 weeks	Left alive after 3 weeks
104	18	6	1	4	0	7
253	6	0	1	2	0	3
Ralston	6	0	6	0	0	0
5206	8	1	0	4	0	3
4956	8	0	1	4	0	3
161	6	0	2	4	0	0
Murray	7	1	1	0	3	2
8047	10	0	1	0	1	8
265	16	0	0	0	3	13
Total	85	8	13	18	7	39

many mice. The appearances of the body cavities and of the liver, lungs, spleen, kidney, bowel and heart were regularly noted and often photographed. The organs were removed and fixed for histological examination, which was undertaken by Dr. G.B.S. Roberts of the Pathology Department.

RESULTS OF INTRA-PERITONEAL INOCULATION OF MICE WITH ROUGH AND INTERMEDIATE STRAINS OF *ACTINOMYCES ISRAELII*

At different times, 85 mice were inoculated intra-peritoneally with a single dose of 0.5 ml of a suspension of *A. israelii* in the rough or intermediate form. Various of the rough strains were used, and no difference in results was observed between them. The inoculum was prepared as described on p. 95. The results are summarised in table III. In the first week after inoculation eight mice died and 13 were killed in *extremis*. Of the remaining mice, 25 were killed and examined at weekly intervals up to three weeks for the development of actinomycotic lesions. Of these 25 mice, 18 inoculated with five different rough and intermediate strains of *A. israelii* showed actinomycotic lesions developing over the peritoneum. Seven of the 25 mice inoculated with three strains were negative for lesions. The remaining 39 mice were killed during a period of from one to 15 months from the date of inoculation, (table IV, p. 120).

and scanty actinomycotic lesions were found over the liver and omentum in eight of these mice. Four of the mice that died were found at post-mortem to have nephritis. The rest of the mice, 27 in number, were negative for actinomycosis and their organs seemed completely normal to the naked eye and on microscopic examination.

<u>A detailed study of the events and post-mortem findings after intra-peritoneal inoculation of mice with a single dose of *Actinomyces israelii* (rough and intermediate strains)</u>

A detailed account is now required of the course of events and of the post-mortem findings in the inoculated mice. This account includes details - as relevant - of the time-interval elapsing between inoculation and death, the distribution and arrangement of the macroscopic lesions, the results obtained on microscopic examination of stained films of the lesions, the histological appearance of lesions, and the tissue reactions in the inoculated mice.

<u>Immediate reaction to inoculation with *A. israelii*.</u> - All mice inoculated intra-peritoneally became very disturbed within 30 minutes of inoculation, showing symptoms of peritoneal irritation. The mice writhed and dragged themselves over the ground, finally lying in a prone position,

Figure 24

Typical front (above) and side (below) appearance of mice from 24 to 48 hours after intra-peritoneal inoculation of rough strains of A. israelii. Note the closed eyes, exudate on the eyelids, and ruffled appearance of the coat. x 2.

flat on their abdomens, with their fore and hind-legs fully stretched out. It was also noticeable that their rate of respiration was much increased. This prone position was held by the mice for a period of from four to six hours, after which time the animals gradually resumed the normal sitting position.

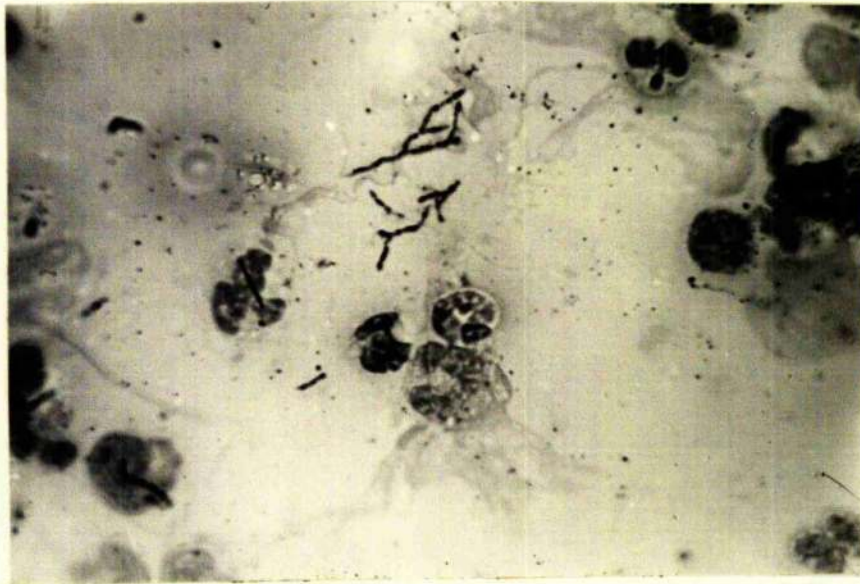
Reaction 24 hours after inoculation. - At this period all the inoculated mice appeared ill (fig. 24). They crouched with hunched backs; their respiration was very rapid and distressed; and a notable feature was the ruffled appearance of their coats. In many of the mice at this time, the eyes were closed (fig. 24) and showed blepharitis. A few of the mice also showed signs of enteritis. At the beginning of the experimental work, when these signs of illness appeared, I did not think that any of the mice could survive for another 24 hours. In consequence, I killed and examined them. In later experiments, however, I observed that many mice showing such forms of distress in the period immediately after inoculation recovered after another 24 hours and appeared normal. In such animals the eyes were again fully open and clear; the posture was normal; and although the coats were not so glossy as before inoculation they became much less ruffled in appearance.

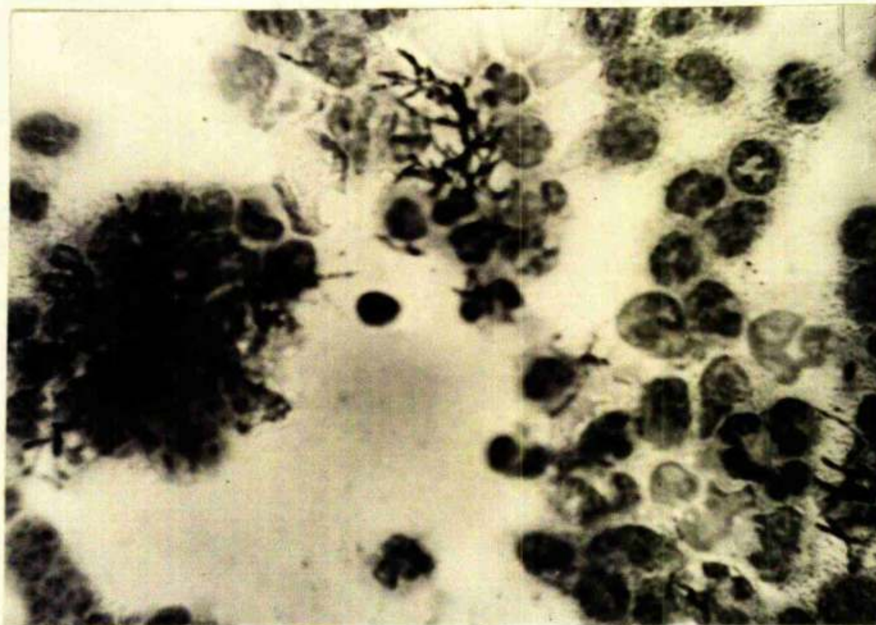
<u>Observations from 7-90 days after inoculation.</u> - The next observation of note was that in the period of from seven to 14 days after inoculation, the mice were thinner though still normal in their behaviour. The main reason for this was that from the twenty-first day after inoculation many of the mice began to have difficulty in stretching upward to their water-bottles. The skin over the abdominal area was tightened, and this restricted their upward movements. The drinking supply of the mice was accordingly changed so that they could reach it. The difficulty in stretching lasted for a period of from three to four weeks; thereafter the mice began to regain their full freedom of movement. As an accompaniment to this change, the mice began to gain in weight, and their coats returned to the normal glossy appearance. Three months after inoculation all the mice had an appearance comparable to that of a healthy, uninoculated, control animal. Some of these mice were kept for from one to two years after inoculation and showed no signs of illness attributable to inoculation. They showed no greater deterioration from increasing age than uninoculated mice.

Post-mortem appearances noted in mice

<u>24 hours after inoculation.</u> - In mice killed or dying at this time, a thin white film apparently of fibrin and cells was

<u>Figure 25</u>





Films made from fibrin and cellular exudate covering the liver and spleen of mice 48 hours after intra-peritoneal inoculation of rough forms of <u>A. israelii</u>. Note the branching filaments and the spotted appearance of the filaments in the upper picture. Gram's method x 1000.

found to cover the entire surface of the liver and spleen. This film could be stripped off and the underlying viscera, though a darker red than normal, otherwise appeared healthy to the naked eye. When the fibrin film was squashed on a glass slide and stained by Gram's method, microscopic examination revealed the presence of pus cells and of Gram-positive, branching filaments. Many of the filaments naturally had a broken appearance and showed the spotted appearance characteristic of the _Actinomycetaceae_ (fig. 25). Free fluid was found in the pleural and in the peritoneal cavity and the lungs showed haemorrhagic congestion. The capillaries on the peritoneal surfaces were also congested and in the mice showing signs of enteritis the bowel contained a jelly-like fluid which was coloured either red or a brilliant yellow. Gram-stained films of this material showed, in addition to other bacteria, short, broken-up, Gram-positive, branching filaments. These organisms, however, were not regarded as certainly being part of the original inoculum of _A. israelii_. Gram-stained films of the free fluid in the pleura and peritoneum showed only the presence of cocci, bacilli, and coliforms. The liver, spleen, and kidney - apart from the fibrinous exudate on the first two organs - appeared normal.

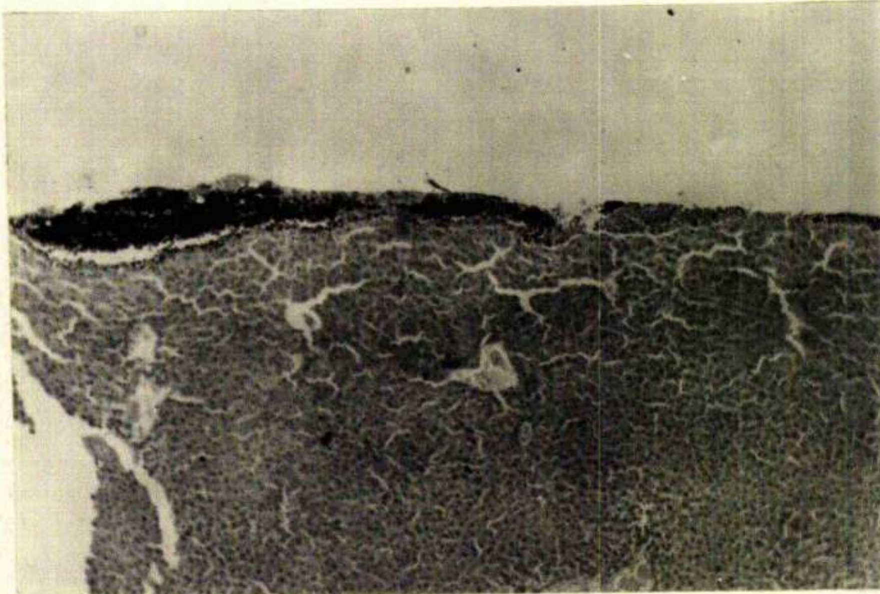
Figure 29

Peritoneum of mouse killed seven days after intra-peritoneal inoculation of A. israelii strain 104. The appearance is essentially similar to that of fig. 28. x 2.5.

Figure 28

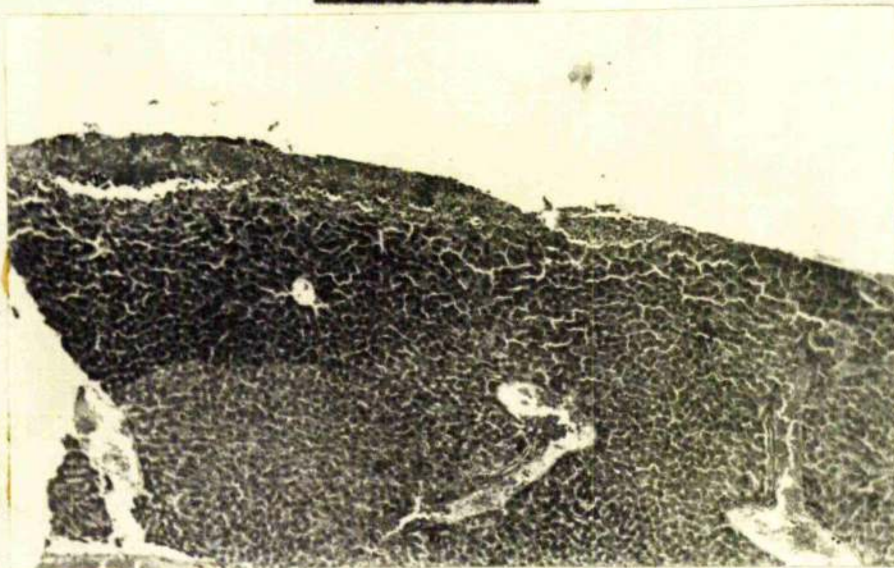
Peritoneum of a mouse killed seven days after intra-peritoneal inoculation of A. israelii strain 5206. Tiny actinomycotic abscesses can be seen over the bowel, on the liver, and at the edge of the spleen. (Natural size).

Figure 26



Section of a liver of a mouse killed 48 hours after intra-peritoneal inoculation with A. israelii strain Ralston. Masses of Gram-positive organisms are seen extending over almost the entire surface of liver. Gram's method x 50.

Figure 27



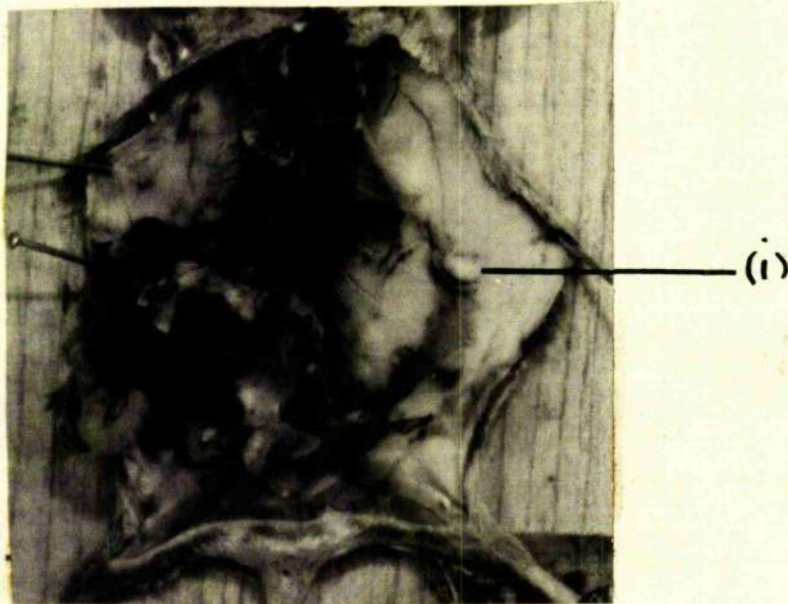
Section of same liver (as above) stained by haematoxylin and eosin x 50.

Histological appearance of viscera

24 hours after inoculation. - Sections of the liver and spleen of mice dying or killed 24 hours after inoculation showed the peritoneal surfaces of the organs were covered with an almost continuous layer of Gram-positive filaments (figs. 26, 27). In the tissue immediately below this layer the cells showed signs of necrosis. The kidney tissue was of normal appearance.

Findings in mice killed seven days after inoculation. - Mice killed at this time did not have free fluid in either the pleural or the peritoneal cavity. The continuous layer of fibrin seen over liver and spleen at 24 hours had disappeared; only localised patches of it were left on the diaphragmatic surface of the liver. Small white nodules, the size of pin-heads, were scattered over the upper surface of the liver along with the fibrin patches (figs. 28, 29). Similar nodules were also scattered over the peritoneal folds between the spleen and pancreas and on the omentum. A small nodule also developed where the injecting needle penetrated the anterior abdominal wall. These nodules were firm but when squashed were found to contain a little cream-coloured pus of which stained films showed the presence of Gram-positive branching filaments and short diphtheroid forms. The kidneys

Figure 30



Appearance of peritoneum of a mouse killed 14 days after intra-peritoneal inoculation with A. israelii strain 5206. The upper surface of the liver shows the diaphragm overlying superficial abscesses. The spleen and omentum are bound together by fibrin and the development of an abscess. The mark (i) points to a local abscess developed at the site of inoculation. (Natural size).

and lungs appeared normal but the liver immediately beneath the fibrin layer and small nodules had a mottled appearance; sometimes it was paler than normal. The spleen, though normal in colour and appearance, often showed enlargement. It must be emphasised that the small actinomycotic nodules were developed either on or very close to the surface of the viscera. They were not more than slightly embedded in the tissue of liver or spleen. Nodules were never found on the kidney. Sections through the nodules on liver and spleen showed in the centre a compact, irregular fretwork of Gram-positive branching organisms surrounded by a wide zone of polymorph leucocytes, outside which there was a layer of cells with a few fat-laden macrophages.

Findings in mice killed 14 days after inoculation. - By this time, the nodules developed over the peritoneal surface had increased both in size and number. (fig. 30). On the diaphragmatic surface of the liver there were usually some two or three flattened, coalesced nodules apparently attached on one side to the liver and on the other to the diaphragm. Only careful dissection and cutting of the diaphragm wall made it possible to photograph the liver in such a mouse; the remnants of the diaphragm wall are seen covering the lesions on the liver (fig. 30). At this stage, however, there was not

penetration of the diaphragm wall leading to nodule-formation in the pleura; the nodules developed only in the peritoneal cavity. They were found scattered over the omentum, down the edge of the spleen, and throughout the folds of the peritoneum. There was also extensive development of fibrin which emeshed the spleen and bound it to the pancreas and omentum. So intimately were these organs bound together that the fibrin bands had to be cut during dissection to permit a close examination of the nodules. Again, at the needle track a nodule had developed again; at this stage it was two or three times larger than that seen in the same situation in the mouse killed at seven days after inoculation. Only the lungs and kidneys were free from nodules and fibrin.

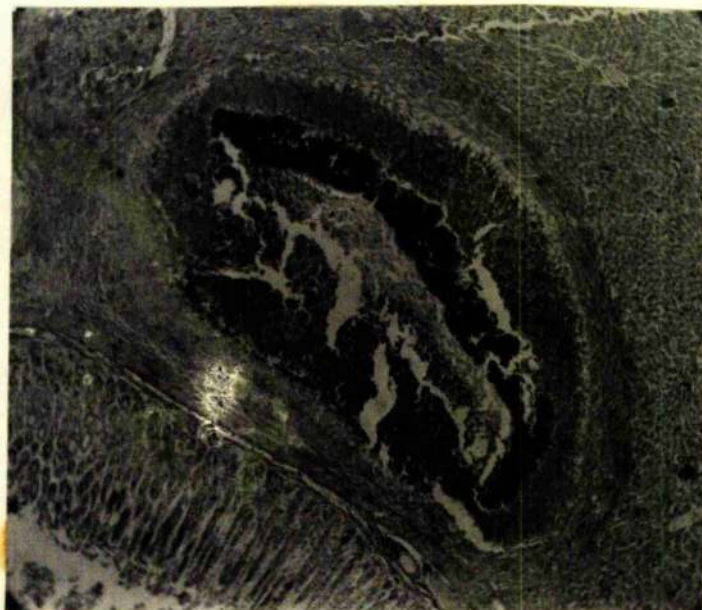
The nodules in the abdomen were firm to touch; when they were cut, cheesy, cream-coloured, odourless pus oozed from them. Wet-film preparations of the pus showed the presence of hyaline filaments, but sulphur granules were not seen. Films stained by Gram's method and by carbol-thionine showed the presence of abundant branching filaments and broken-up, short diphtheroid elements. In short, the nodules were abscesses containing the inoculated organism, A. israelii.

Sections of such abscesses revealed an increase in the ratio of organisms to necrotic tissue. The layer of polymorphonuclear cells around the organisms was now in turn

Figure 33

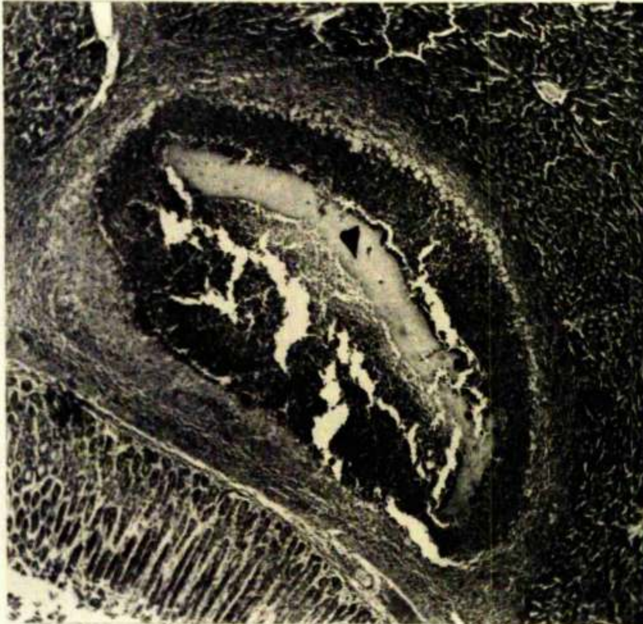
Post-mortem appearance in a mouse killed 21 days after intra-peritoneal inoculation of A. israelii strain 4956. Before dissection the whole peritoneum showed a mass of fibrin adhesions and abscess development. The end of the spleen can be seen in an abscess mass. The diaphragm wall lies on top of the liver. (Natural size).

Figure 32



Section stained by Gram's method showing the central mass of *A. israelii* inside an abscess produced experimentally in a mouse 14 days after intra-peritoneal inoculation with *A. israelii* strain 104. x 50.

Figure 31



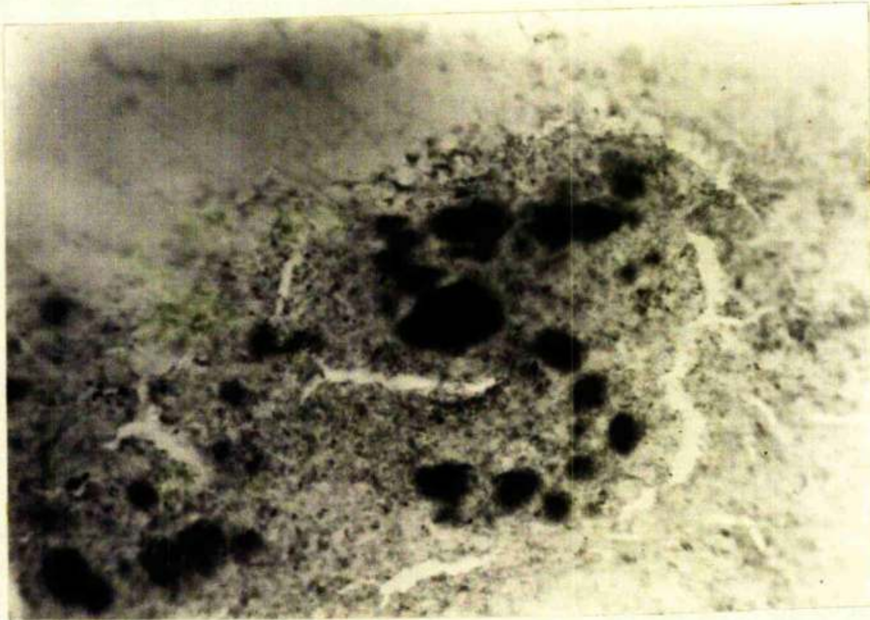
Abscess between bowel and spleen in a mouse 14 days after intra-peritoneal inoculation of A. israelii, strain 104. The central mass of organisms is surrounded by a collection of polymorphs and other cells. This in turn is surrounded by a fibrous capsule. Haematoxylin and eosin x 50.

surrounded by a layer of macrophage cells (figs. 31, 32).

Findings in mice killed 21 days after inoculation. - In mice killed at this period after inoculation, there was great enlargement of the abscesses distributed over the surface of the viscera in the peritoneum; in fact this was the period of maximum development of these experimental abscesses.

Accompanying this development of abscesses were numerous fibrin strands, which entangled the whole of the contents of the peritoneum. Indeed, when the peritoneum was opened the first thing to be seen was an involved mass of abscesses, viscera, and fibrin, which had to be carefully dissected before the extent of abscess-development could be assessed. The distribution of these lesions was similar to that found in mice killed at earlier stages of infection (fig. 33). The liver, the diaphragm, spleen, omentum and peritoneum all carried abscesses. At this time after inoculation in some mice the abscesses formed on the upper surface of liver had penetrated the diaphragm and had given rise to abscess-formation at the lung apex and, in one mouse, the heart muscle showed a small abscess on its surface. Very often the local abscess on the track of the inoculation needle became attached to the spleen by fibrin tissue; in fact, the spleen was often quite invisible, being covered with

Figure 34

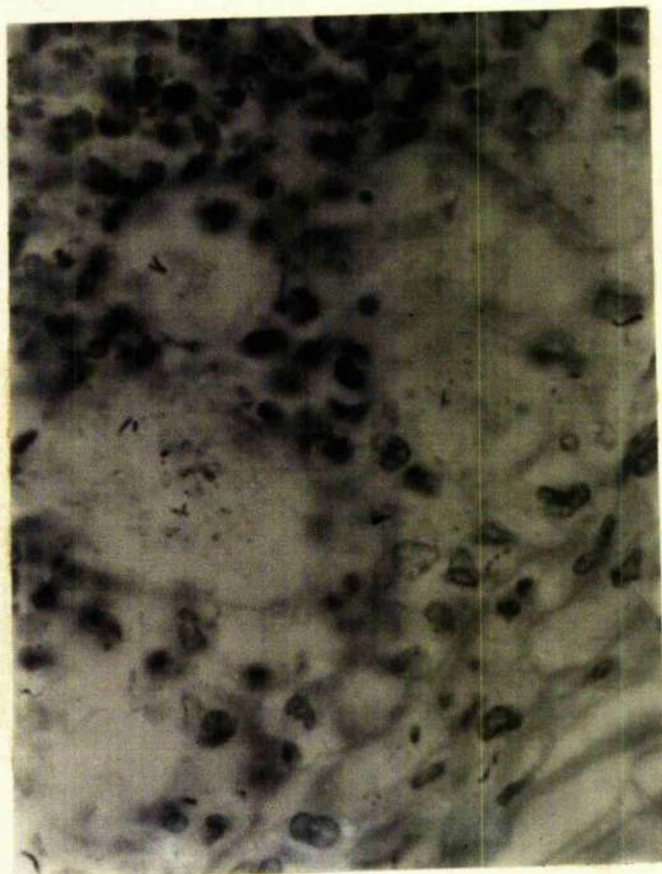


Low power view of organism masses in a thick film of pus from an experimentally produced actinomycotic nodule in a mouse peritoneum (intra-peritoneal inoculation of A. israelii strain 4956). No club formation is seen. Gram's method x 25.

abscesses and fibrin. Dissection revealed that these abscesses on liver, spleen, and peritoneum were relatively superficial; they did not penetrate into the visceral tissues. The only abscesses developed inside tissue were obtained by the accidental inoculation of A. israelii directly into the spleen of one mouse. The mouse so inoculated died seven days after inoculation and dissection showed the development of several lentil-sized abscesses inside the spleen tissue. There was also a small abscess developed on the needle track but other abscesses were not found in the peritoneum. Three weeks after inoculation the abscesses were soft and easily ruptured; inside there was creamy thick pus which showed the presence of very abundant organism masses (fig. 34) which consisted of branching filaments. Sulphur granules were not found in the pus and clubs also were naturally absent. The A. israelii organisms present in this pus were viable and isolates taken from the abscesses opened with sterile precautions yielded pure growth on culture of A. israelii identical with the original inoculum.

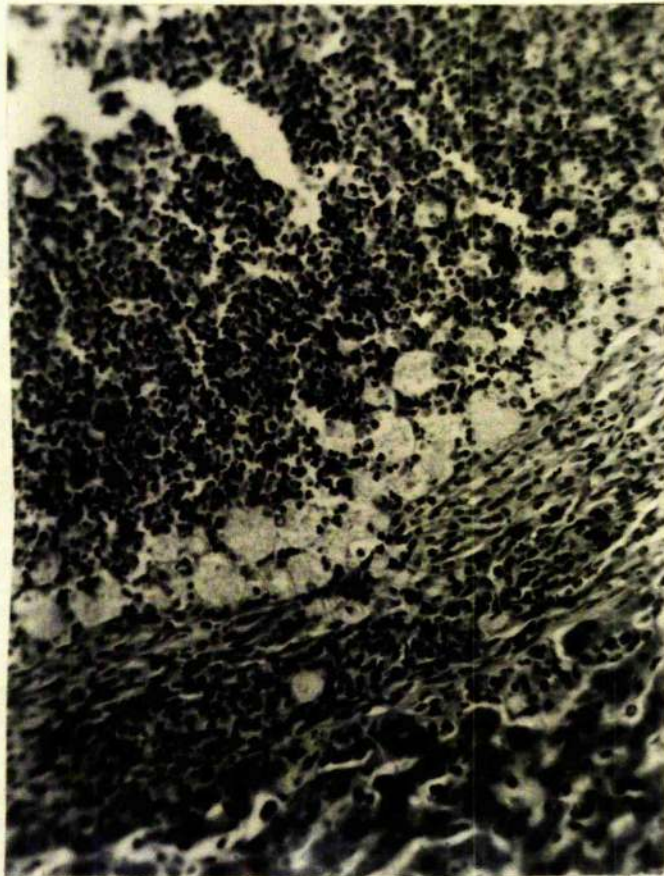
The abscesses when examined histologically again showed the features noted in the earlier experiments. In the centre of abscess there was as usual a mass of interweaving Gram-positive organisms. For the first time, however, the periphery of the mass had many filaments arranged in something

Figure 38



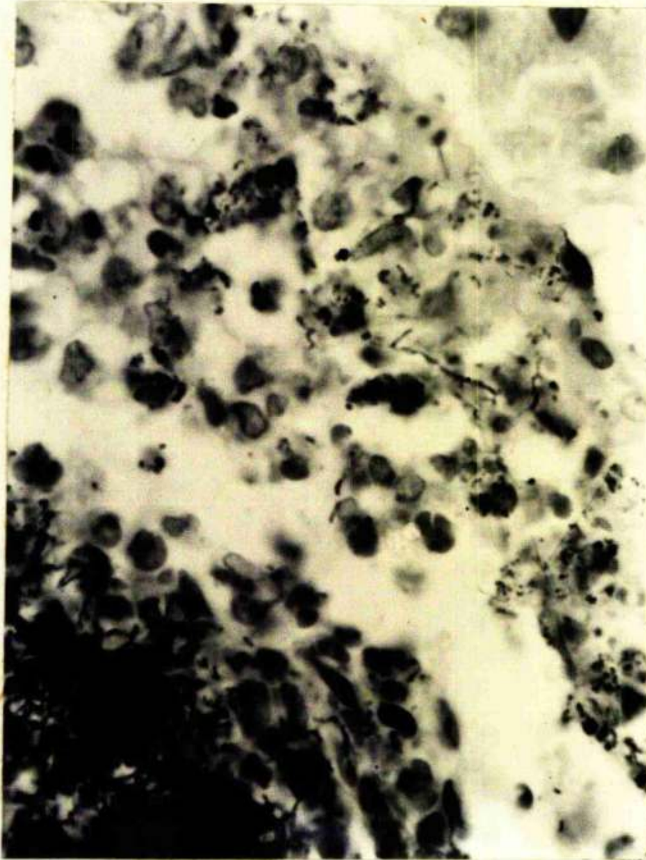
A high power view of fig. 37 (i) showing the macrophages at the periphery of lesion with small broken up mycelial filaments. Stained by Gram and carmalum x 700.

Figure 37



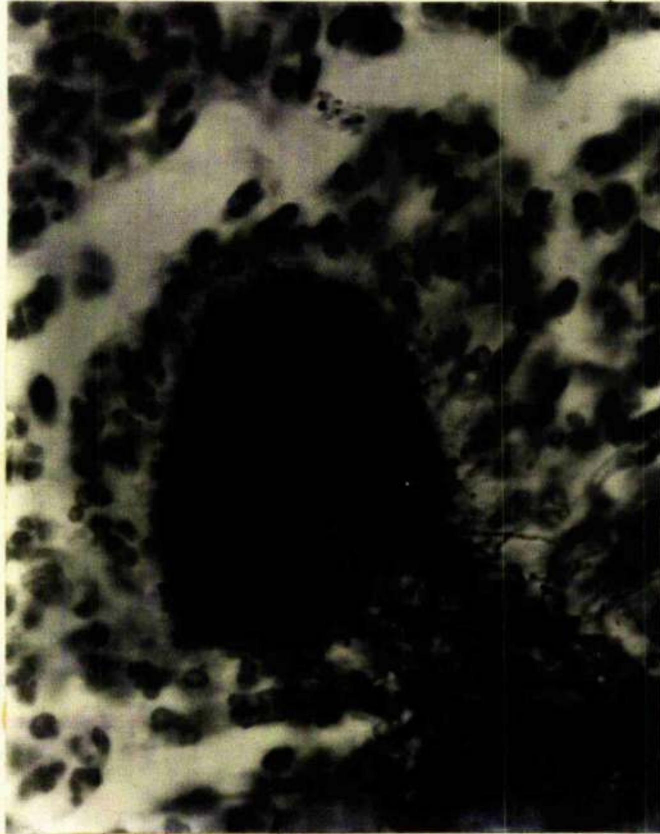
(i) Wall of abscess showing collection of large fat-containing macrophage cells external to the central collection of polymorphs and internal to a layer of fibrous granulation tissue. Haematoxylin and eosin x 250.

Figure 36



Edge of abscess of fig. 35, under a higher magnification, showing the branching felt-work of organisms. Gram's method x 1000.

Figure 35



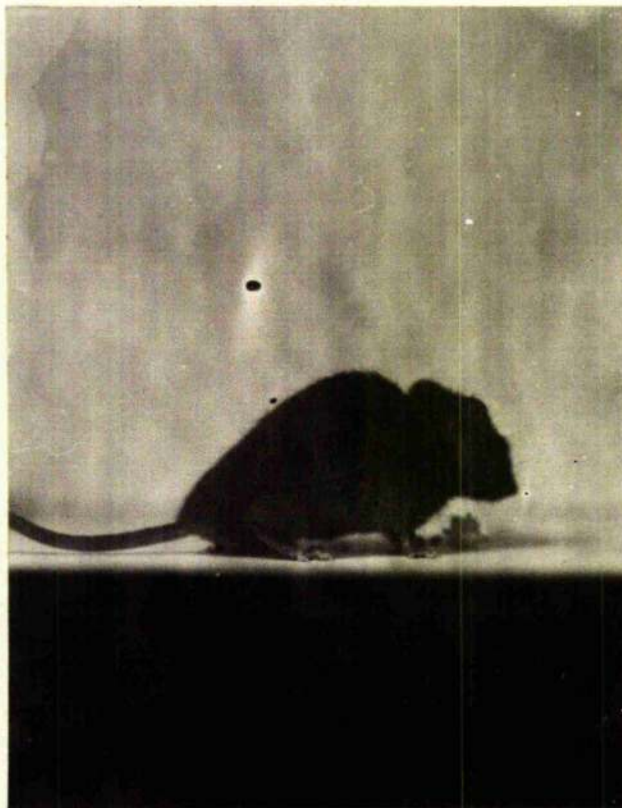
Section through an actinomycotic lesion formed three weeks after intra-peritoneal inoculation of a mouse with A. israelii strain 104. The central mass of organisms shows a radiate arrangement of filaments at the periphery, but there is not 'club' formation at the tips of the radiating filaments. Gram's method x 350.

like a radial fashion (figs. 35, 36). No acid-fast clubs were seen in these radiating hyphae, though some of the organism strands at the periphery were more acidophilic in that when stained with haematoxylin and eosin, these peripheral filaments had a greater affinity for the eosin than the hyphae in the centre of the abscess. In human actinomycotic abscesses also the filaments at the edge and those in the centre of the lesion, show a similar differentiation with this stain. A well marked layer of polymorphonuclear cells again surrounded the central mass of organisms. Outside the polymorphs was a thick layer of foam cells - macrophage cells containing fat - and at the periphery of the abscess was a layer of fibrous granulation tissue (figs. 37, 38). If there was much fibrinous exudate this layer could be extensive.

This period of 21 days after intra-peritoneal inoculation marked the maximum development of actinomycotic lesions; in some animals the widespread distribution of the lesions suggested that progressive actinomycosis had truly been produced in the mice. Histologically, the lesions in mice were identical with those recorded in human actinomycosis.

Again, the kidneys were of normal appearance both macroscopically and microscopically.

Figure 39



Appearance of a mouse three weeks after intra-peritoneal inoculation of A. israelii. The hunched posture is probably due to the development of fibrous tissue in the peritoneum. (Natural size).

While still alive nothing in the external appearance of the mice gave any indication as to whether widespread development of actinomycotic lesions was or was not taking place in their peritoneum. As previously mentioned the mice at this period had difficulty in stretching, the abdomen appearing to be constricted (fig. 39). Though this appearance was seen only in inoculated mice - being absent in uninoculated control animals - I did not regard it as a direct sign of active abscess formation but rather as a sequelae since post-mortem examination of such mice showed the development of fibrin bands with or without abscess formation.

Findings in mice killed 28 days after inoculation. - Mice which survived the period of maximum abscess formation showed, on post-mortem examination one week later, that abscesses were still present. There were, however, some differences in the size of these; one set of mice showed abscess formation equal in size to that found at the period of three weeks, whereas another set of mice showed that regression in size was begining to take place. It was not possible to link these differences with any particular strain of A. israelii, as mice inoculated with the same strain showed in themselves this variation in size.

Figure 41

Mouse killed between five and six weeks after intra-peritoneal inoculation of A. israelii. There is massive development of fibrous adhesions involving spleen, kidney, omentum and bowel. At this period abscesses are no longer seen in surviving mice. x 2.5.

Figure 40

Mouse peritoneum four weeks after intra-peritoneal inoculation of A. israelii strain 4956. Liver shows diffuse mottling due to hyperplasia of the reticulo-endothelial cells. Small pin-point abscesses are present on the edge of liver and spleen; fibrous tissue can be seen at the edge of the splenic lesion. x 4.

Findings in mice killed 48 days after inoculation. - In mice at this period, post-mortem examination revealed the presence of widespread fibrous adhesions which developed round bowel, spleen and omentum (figs. 40, 41) and occasionally large fibrous nodules were present in this area, but the actual abscess was much smaller in size than those seen at 21 days after inoculation. Only a pin-point of pus was obtained in cutting these nodules and films stained by Gram's method were either negative when examined for the presence of *A. israelii* or showed only broken, degenerate, short diphtheroid-like bodies. Histological sections of such fibrous nodules showed that the actual abscess was now small in size, the bulk of the nodule consisting of cellular granulation tissue.

The appearance of these experimentally inoculated mice on *post mortem* was compared with that of control uninoculated mice from the same batch killed at the same time intervals. No tissue changes and no abscess formation was found in such control mice.

Findings in mice surviving from 48 days onwards

Examination of the mice still surviving was carried out over a period extending from three months to one year

TABLE IV

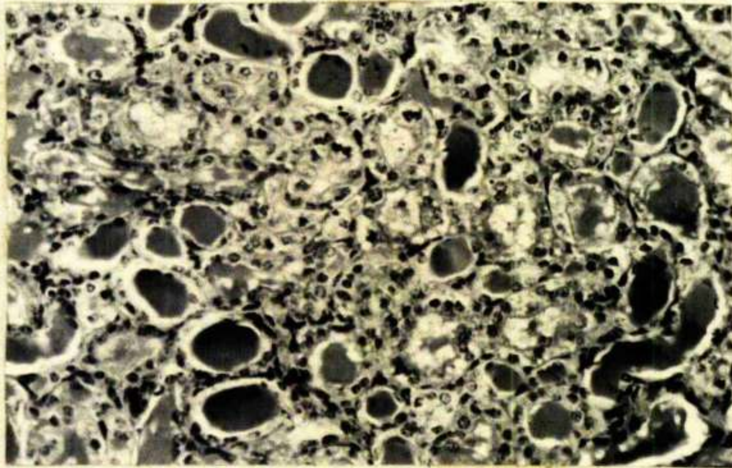
Summary of findings in mice surviving more than three weeks after a single intra-peritoneal inoculation of rough and intermediate forms of strains of Actinomyces israelii

Strain	No. of mice	No. of mice dying	No. of mice killed	Time from inoculation to death	Actinomycotic Lesions present (+)	Actinomycotic Lesions absent (-)	Other finding at death
4956	3	-	1	35 days	+		
		-	1	48 days	+		
		-	1	3 months		-	
Murray	2	-	1	6 months	+		
		-	1	2 years		-	
8047	8	-	1	28 days		-	
		-	1	46 days		-	
		-	1	3 months		-	
		1	-	6 months		-	nephritis
		2	-	9 months	not examined		
		-	2	1 year		- (2)	
104	7	-	1	28 days	+		
		-	1	45 days		-	
		1	-	48 days		-	
		-	1	3 months		-	
		-	2	6 months		- (2)	
		-	1	247 days		-	
253	3	-	1	28 days	+		
		-	1	48 days		-	
		1		145 days		-	nephritis
5206	3	-	1	35 days	+		
		-	1	44 days		-	
		-	1	3 months		-	
265	13	-	1	26 days	+		
		1	2	3 months	+ (1)	- (2)	
		1	-	6 months		-	nephritis
		1	-	9 months		-	nephritis
		-	1	9 months		-	
			3	1 year		- (3)	
			3	14 months		- (3)	

from the date of inoculation. The results are summarized in table IV. In these animals the actinomycotic lesions and fibrous adhesions completely disappeared after three months. The experimental actinomycotic lesions formed would thus appear to be self-healing and not progressive. This conclusion naturally rests on the assumption that the mice allowed to survive developed actinomycotic abscesses comparable to those already found in the animals killed and examined.

Though progressive actinomycotic lesions did not develop in mice remaining alive three months after inoculation, eight mice died in the period six months after inoculation and, at post-mortem examination in four animals the kidneys revealed an interesting abnormality. In colour, the kidneys were pale, almost blanched, and showed a rough warty surface. They were very friable and quickly broke up when touched with a scalpel. On histological examination this kidney tissue showed the presence of a type of tubular nephritis which has not been recorded, so far as I can find, as a natural disease of mice, or as a sequel to inoculation with A. israelii. The other organs in these mice were of normal appearance, both on macroscopic and microscopic examination. The renal lesions are discussed in the next section.

Figure 42



Section of kidney of a mouse dying nine months after intra-peritoneal inoculation of A. israelii strain 265. Many of the tubules are filled with dense acidophilic colloid material. Haematoxylin and eosin x 200.

HISTOLOGICAL APPEARANCES OF VISCERA

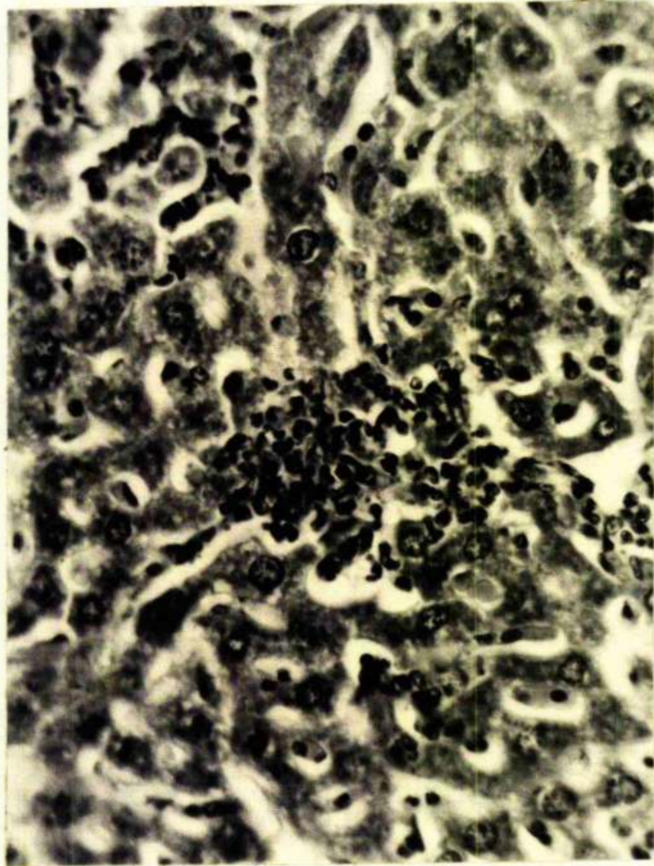
Tissue changes observed in mouse kidney

The kidney tissue of four mice dying from three to nine months after intra-peritoneal inoculation of A. israelii (table IV) showed extensive tubular damage on histological examination (fig. 42). The glomeruli, however, were relatively unaffected, although in many instances the cells lining Bowman's capsule were hypertrophied. Many of the tubules were dilated and contained plugs of colloid material, and the cells lining these tubules were flat and atrophied. This lesion appeared to be a form of tubular nephritis.

Careful examination of sections of these damaged kidneys did not reveal the presence of any bacteria, particularly of A. israelii. It must be emphasised that in my experiments this organism was never found in the kidney tissue, not even at the stage of maximum development of the actinomycotic lesions of the peritoneal surface, at the period of from three to four weeks after inoculation.

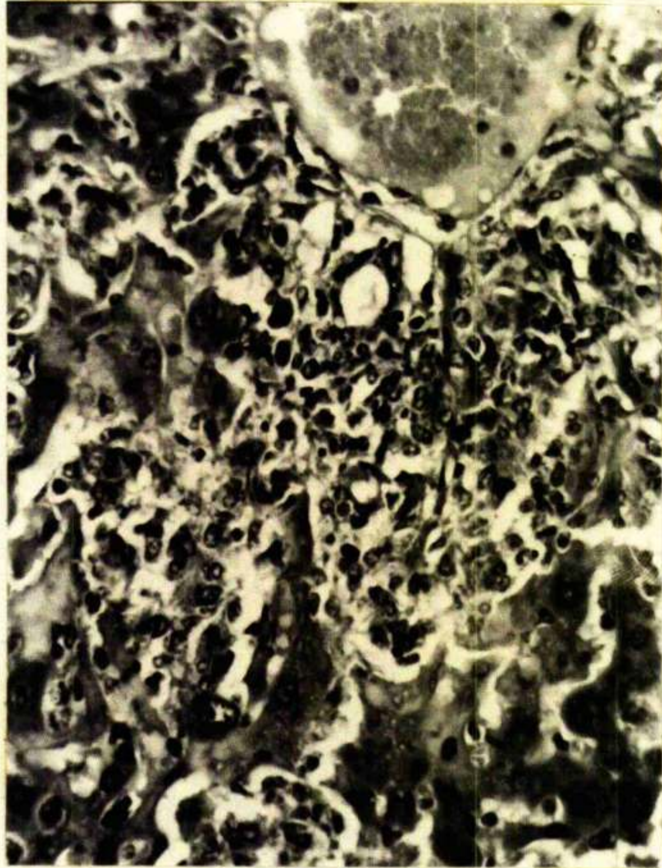
In these mice with damaged kidneys, the other organs - spleen, liver, lungs and bowel - showed no abnormality. Their appearance was comparable to that of the tissues in an uninoculated control animal.

Figure 45



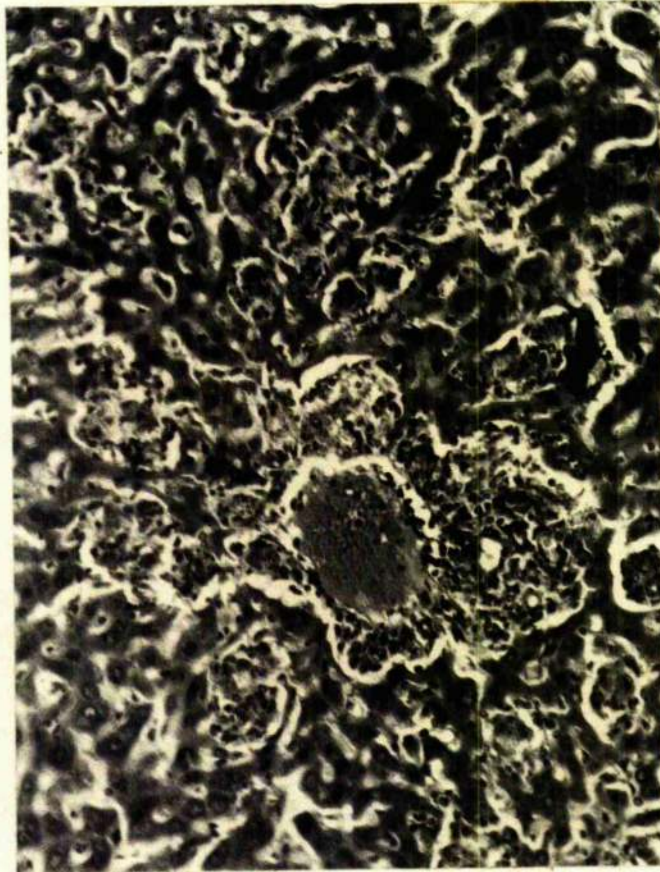
Section of liver of a mouse four weeks after intra-peritoneal inoculation with A. israelii strain 265 showing a later stage in the liver reaction the involution of the cellular reaction. Haematoxylin and eosin X 500.

Figure 44



High power view of fig. 43 to show details of
an area of reticulo-endothelial hyperplasia. x 380.

Figure 43



Section of a liver of a mouse killed seven days after intra-peritoneal inoculation of A. israelii strain 265. Around a central vein and between the columns of liver cells there is hyperplasia of cells of the reticulo-endothelial system. Haematoxylin and eosin x 180.

Cellular Changes in Liver and Spleen

Intra-peritoneal inoculation of A. israelii into mice gave rise not only to actinomycotic nodules but also, to a lesser degree, to changes in the cellular tissue of both the liver and spleen. These changes were visible not only in histological sections but also to the eye (figs. 43 -45). These tissue reactions were particularly noticeable in the period from seven to 14 days after inoculation. On post-mortem examination, as has already been noted (p. 113), the liver was observed to have a patchy mottled appearance; in some cases it was even slightly paler in colour than a normal liver. Examination of histological sections of the liver at this period showed early foci of cellular hyperplasia which were first observed around the central veins of the liver and later between the sinusoids. The cellular hyperplasia consisted of spindle-shaped cells and their distribution suggested that they were derived from the reticulo-endothelial system. These cells occasionally contained large numbers of ingested organisms, but this was not a general finding, for organisms were not found in many of the livers examined. The cellular hyperplasia appeared seven days after inoculation and reached its maximum development in fourteen days. After this time

the hyperplasia steadily diminished in amount and the liver returned to its normal appearance. In mice killed during a period of from three months to two years after inoculation the liver had a completely normal histological appearance.

A similar cellular change was observed in the tissue of the spleen. Macroscopically the spleens of mice killed from seven to 14 days after inoculation were enlarged, being from two to three times greater than that of a normal mouse. On the surface of the spleen slight mottling was observed. On histological section the lymphoid tissue surrounding the malphigian nodes was seen to be reduced in amount and there was a diffuse hyperplasia of the cells of the splenic pulp. These cells, which have large, pale, oval nuclei and pale-staining cytoplasm, were similar to those seen in the liver. This type of cellular reaction was seen throughout the spleen but was most marked in some areas of the cortex where there were focal aggregations of these endothelial cells. The development of this change in the spleen, like that in the liver, was at its maximum 14 days after inoculation. Thereafter the spleen gradually returned to normal, and in mice killed from three months to two years after inoculation the organ appeared normal in every respect.

TABLE V

Results of the inoculation of mice intra-peritoneally with smooth cultures (short diphtheroid forms) of *Actinomyces israelii*

Strain of A. israelii	No. of mice inoculated	Period after inoculation: 1-7 days		1-3 weeks: Actinomycotic Lesions		No. of mice surviving beyond 3 weeks
		No. dead	No. killed	+	-	
5195	20	3	1	3	4	9
161	4	2	0	0	1	1
104	4	1	0	0	1	2
253	4	0	0	0	1	3
265	4	1	0	0	1	2
$11F_2$ (passage strain)	4	1	0	0	1	2
Total	40	8	1	3	9	19

INTRA-PERITONEAL INOCULATION OF MICE WITH ACTINOMYCES ISRAELII IN THE SMOOTH FORM

Regular examination of the A. israelii strains kept in culture revealed that their morphology was undergoing a profound change. The long filamentous forms disappeared and were gradually replaced by short diphtheroid forms; but the cultures retained their anaerobic character (p. 102). There was such a gradual transition in the morphology that the question of external contamination seemed to be ruled out; moreover, the N.C.T.C.* strain 5195, had a short diphtheroid morphology and smooth, even type of growth in broth when I first received it. With this strain and the other cultures showing short diphtheroid forms a total of 40 CBA mice were inoculated intra-peritoneally. The results are summarized in table V.

Results of Inoculation. - After inoculation the mice did not show such marked signs of distress as were observed in animals earlier inoculated with rough and intermediate cultures. There appeared to be some initial discomfort but this effect disappeared in from one to two hours. In the twenty-four hour period after inoculation few mice showed

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* National Collection of Type Cultures (London).

T A B L E   VI

Summarizing the findings in mice killed later than 21 days after intra-peritoneal inoculation with *Actinomyces israelii* (smooth diphtheroid form)

Diphtheroid strain of *A. israelii*	No. of mice observed	No. of mice dead	No. of mice killed	Time since inoculation	Actinomycotic or renal lesions (+ present; - absent)
161	1	0	1	28 days	-
104	2	0	1	28 days	-
		0	1	45 days	-
253	3	0	1	28 days	-
			1	3 months	-
			1	21 months	-
265	2	0	1	28 days	-
			1	3 months	-
11F	2	0	1	28 days	-
			1	415 days	-
5195	9	0	1	1 month	-
			2	3 months	-(2)
		1	0	6 months	* not examined
		0	1	6 months	-
		0	1	10 months	-
		0	1	1 year	-
		0	2	$1\frac{1}{2}$ years	-(2)

* Autolysed and could not be examined.

signs either of blepharitis or of enteritis, — signs which had been noted in mice earlier inoculated with the <u>A. israelii</u> filamentous forms. Of the 40 mice inoculated intra-peritoneally, eight died and one was killed seven days after inoculation. All of these animals, at post-mortem examination, showed fluid in the peritoneum and sometimes in the pleura. Over the liver and spleen surface there were small white patches of fibrin, and both organs had a very mottled appearance. These findings were very similar to those earlier observed at the same period after inoculation of the filamentous forms of <u>A. israelii</u>. Of the eight mice killed from one to three weeks after inoculation, three of four inoculated with strain 5195 alone showed lesions. These were developed scantily over the surface of the viscera and a small local lesion in the inoculation track was also found. These lesions or abscesses contained pus, and films stained by Gram's method showed the presence of abundant Gram-positive, short diphtheroid forms. The 19 mice which survived longer than three weeks were killed during the period of from one month to two years after inoculation. (Summarized in table <u>VI</u>). All these animals gave negative findings at post-mortem examination. In particular the renal lesion already described in four mice long after inoculation with rough and intermediate filamentous forms,

(table IV p. 120) was not found in any of the 19 long-term survivors of inoculation of smooth cultures. Comparison of the results with this series of mice with those of the mice inoculated with the rough and intermediate filamentous forms led to four conclusions:-

1) There was a difference in the size and number of abscesses formed. These were both smaller and fewer in mice inoculated with the smooth strains than in mice given the rough filamentous strains. The distribution of abscesses however was similar in both sets of mice.

2) There was relatively little fibrinous exudate in mice inoculated with the smooth cultures as judged by the examination of animals killed or dying within three weeks of inoculation.

3) Fibrous adhesions did not develop in the peritoneal cavities of long-term survivors of the inoculation of smooth cultures.

4) Renal lesions were not found in long-term survivors of the inoculation of smooth cultures.

Microscopically there was a difference between the abscesses developing after inoculation with the rough and intermediate filamentous and the smooth diphtheroid forms of *A. israelii.*

In the abscesses seen in mice inoculated with strain 5195 there was a central zone of amorphous necrotic material surrounded by a zone of macrophages. An irregular mass of diphtheroid organisms was seen, but there was no evidence of an attempt at "organisation". Some of the bacteria could be seen inside the smaller macrophages. There was no layer of lipophagic macrophages (foam cells) around these abscesses as were seen in the lesions resulting from inoculation of the rough and intermediate filamentous cultures of *A. israelii.*

Cellular hyperplasia of liver and spleen were observed in mice inoculated with the smooth cultures as with the rough cultures especially in the period immediately after inoculation.

THE ACTION OF AN AEROBIC CORYNEBACTERIUM ON MICE

As a corollary to the experiments in which mice were inoculated with the smooth, short, diphtheroid forms of A. israelii (p. 124) an experiment was set up in which an aerobic corynebacterium, originally isolated from a dead guinea-pig, was inoculated intra-peritoneally into mice.

The organism was grown aerobically and anaerobically in 40 ml. amounts of horse-heart digest broth (prepared as described on p. 92) and incubated at 37°C for two days. The resulting growth was centrifuged, washed, and inoculated intra-peritoneally into 10 mice — five being inoculated with the aerobically-grown organisms and five with the organisms grown in the anaerobic medium. These animals were killed during a period of from one to six months after inoculation but none of them showed any lesion or abnormality.

THE EFFECT OF INOCULATING MICE INTRAMUSCULARLY WITH ACTINOMYCES ISRAELII

Because a search of the literature did not reveal information regarding the course of events in mice after intramuscular inoculation of A. israelii, an experiment was set up to investigate this point. Judging from the results of the intra-peritoneal inoculation I believed that some type of lesion would develop and my interest centred on the question whether such a lesion would be only local or whether with deep implantation of culture in muscle tissue infection might spread through the whole body of the inoculated mouse. In addition, I hoped that "sulphur-granule" formation with well developed clubs might be stimulated after inoculation into compact tissue.

If clubs developed, this would be in contrast to the results of intra-peritoneal inoculation. For the present experiments three intermediate strains of A. israelii were used: strain Ralston, strain 265, and strain 253. With each strain six mice were inoculated in the thigh muscles of the hind leg. The inoculum was prepared as already described on p. 95 , the amount injected was 0.5 ml. After inoculation, the mice were observed to record their reactions; then they were killed at regular intervals to observe the

TABLE VII

Result of intramuscular inoculation of mice with Actinomyces israelii

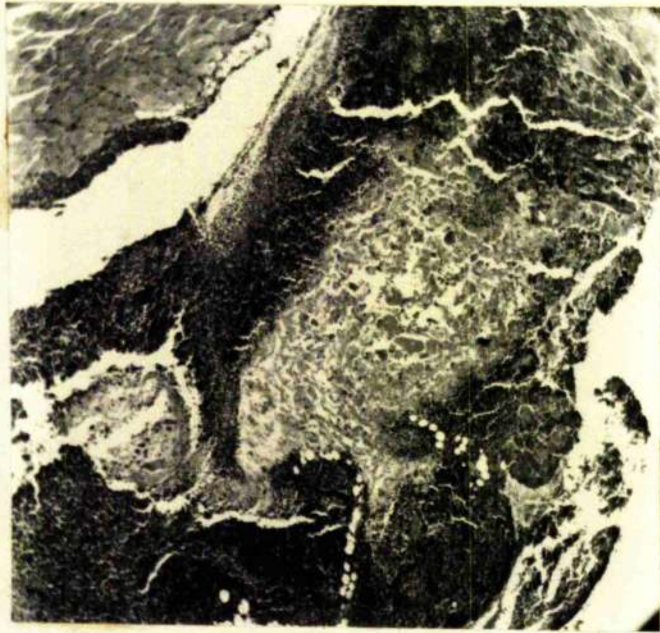
Strain	No. of mice	No. mice killed	Time after inoculation	Development of muscle abscess (present; +)	(absent) -
Ralston	6	1	4 days	+	
		1	7 days	+	
		1	14 days		-
		1	21 days		-
		1 died	48 days	Generalised development of lesions in peritoneum.	
		1	3 months		-
253	6	1	7 days	+	
		1	14 days	+	
		1	21 days	+	
		1	3 months		-
		1	6 months		-
		1	9 months		-
265	6	1	7 days	+	
		1	14 days	+	
		1	21 days	+	
		1	3 months		-
		1	6 months		-
		1	9 months		-

development of any lesions (table VII). Post-mortem examinations were made both of the area of the inoculated leg muscle and of the peritoneum.

Twenty-four hours after inoculation, all the mice showed signs of tenderness and oedema at the injection site. This tenderness increased and the mice did not use the inoculated leg as normal mice would do; the animals showed no other sign of discomfort after inoculation. There was no systemic upset comparable to that after intra-peritoneal inoculation. It was noted that an abscess was beginning to develop in the leg muscle of each mouse in from 48 to 72 hours after inoculation, and this was confirmed on post-mortem examination. The leg muscle of one mouse inoculated with strain Ralston was opened four days after inoculation; it showed oedematous tissue surrounding a small abscess which contained soft yellow pus. In these mice the development of the abscess was observed during a period of 28 days, and in each mouse the process of lesion formation was exactly similar. Although three different strains of A. israelii were used for inoculation, the following description of abscess development is typical for each mouse, and for each strain of actinomycete used.

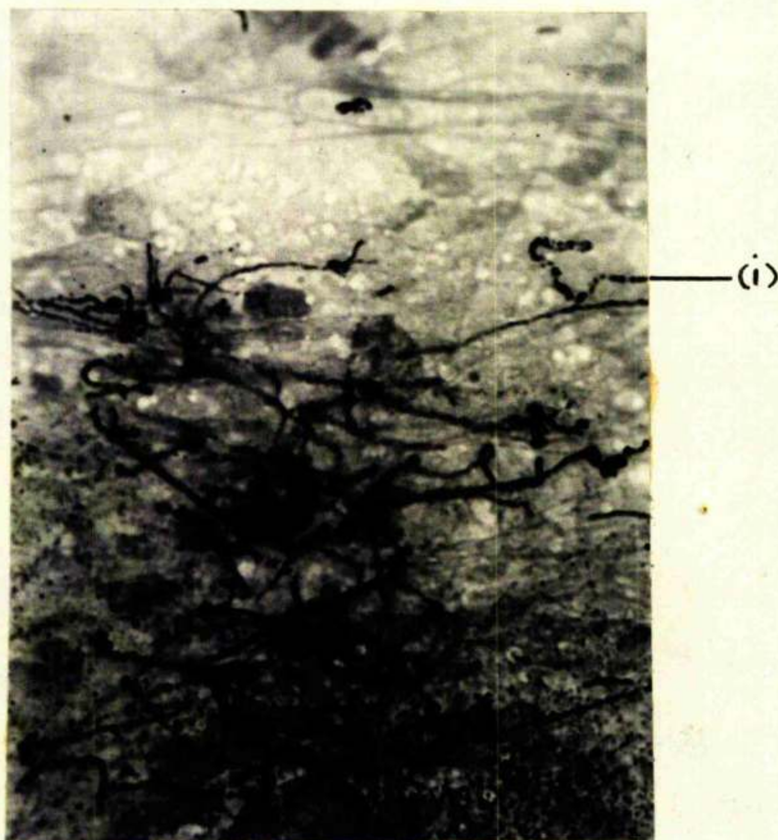
Between the fourteenth and twenty-first day after inoculation the abscess was at its maximum; it varied in

Figure 47



Section through a muscle abscess showing cellular necrosis surrounded by acute inflammatory reaction. This abscess is four days old. A. israelii strain Ralston was used for intramuscular inoculation. Haematoxylin and eosin. X 25.

Figure 46



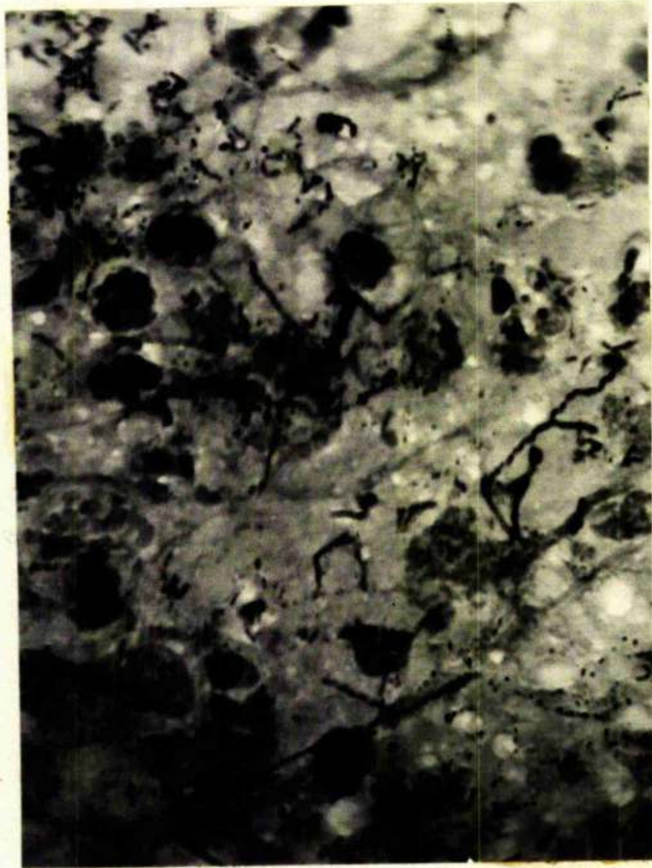
Film of pus from an intramuscular abscess which developed in mouse hind leg four days after inoculation with A. israelii strain Ralston. Gram-positive filaments showing branching can be seen; some of the filaments (i) are breaking up. Gram's method x 1000.

size from that of a pea to that of a bean. The abscess was soft, no fibrous thickening developed around the pus. Naked-eye and microscopic examination did not reveal sulphur granules. When the smears were stained by Gram's method, either Gram-positive branching filaments (fig. 46) or short diphtheroid forms were seen. Clubs were not seen in any smears of the pus. Histological examination of the intramuscular abscess (fig. 47) showed only cellular necrosis with masses of organisms, some of which were intercellular. Clubs were not seen in the histological sections of the abscesses. From the twenty-first day after inoculation the abscess began to regress, and in three months from the date of inoculation a lesion could no longer be detected in the muscle tissue. Improvement was obvious also from the manner in which the mice began to use their hind legs normally; the disappearance of the lesions was confirmed on mice killed and examined _post mortem_. Mice examined six and nine months after inoculation did not show lesions on post-mortem examination. In short, typical actinomycotic lesions did not develop either locally or in the viscera. The kidney especially was examined but this organ appeared to be completely normal. It will be seen (table VII p. 130) that the local lesions had completely disappeared in from 14-21 days after inoculation. One

interesting finding was that one mouse inoculated with the Ralston strain died 48 days after intramuscular injection with widespread actinomycotic lesions in the peritoneum over the liver, spleen, and omentum; the appearance of the mouse at post-mortem examination was exactly similar to that recorded in mice after intra-peritoneal inoculation of A. israelii. This was the only mouse of those inoculated intramuscularly that showed this type of spread, and conclusions cannot be drawn as to the significance of this result, though speculation might be made of the possibility of blood-borne infection.

It was found, in short, that intramuscular inoculation of A. israelii into mice gave rise to only a local self-healing abscess. This abscess contained pus in which branching filaments could be seen and grown in viable culture; but sulphur granules and clubs were not formed. The host reaction was similar to that found after subcutaneous inoculation, which is next described.

Figure 49



Film of pus from subcutaneous abscess showing branching filaments. Gram's method x 1000.

Figure 48

Mouse five days after subcutaneous inoculation of A. israelii strain Ralston, showing the development of an abscess on the surface of the abdominal wall below the skin which has been carefully removed. The abscess is confined to the surface, penetration into the peritoneal cavity has not taken place. (Natural size).

OBSERVATIONS ON MICE SUBCUTANEOUSLY INOCULATED WITH *ACTINOMYCES ISRAELII*

Four CBA mice were inoculated subcutaneously on the abdominal surface with 0.5 ml. of an intermediate suspension of *A. israelii* strain Ralston prepared as previously described (p. 95). In from 24 to 48 hours after inoculation a swelling the size of a lentil developed at the site of inoculation. The swelling gradually increased in size to that of a small pea and when one mouse was killed five days after inoculation (fig. 48) a nodule was found under the skin and on the surface of the abdominal wall. This nodule was soft and contained yellowish, cheesy pus. Smears from this material stained by Gram's method and by carbol thionine showed the presence of branching filaments (fig. 49). When the abdominal wall was incised and the peritoneal cavity exposed examination revealed that the actinomycete had not spread through the abdominal wall; the contents of the peritoneum were normal. In a second mouse killed eight days after inoculation the subcutaneous abscess was larger than that formed in the first mouse, but the abscess was similar in content and again there was no spread of the organism through the peritoneal wall into the peritoneal

cavity. In the remaining two mice of this series, the abscesses formed in both animals at the inoculation site spontaneously discharged on the ninth day and had completely healed by the fifteenth day. The third mouse was killed when the subcutaneous lesion had healed; examination of the other parts of the body did not reveal abnormalities. The last and fourth mouse was left alive for one month after the healing process was complete before being killed. The tissues of this animal appeared normal both in macroscopic appearance and on histological examination. Thus it can be seen that subcutaneous inoculation of _A. israelii_ results in the development of only a localised small abscess which does not spread through the animal body. This local lesion discharges spontaneously and heals and the other tissues of the inoculated mouse retain an appearance of normality.

INTRAVENOUS INOCULATION OF MICE WITH ACTINOMYCES ISRAELII

When the intravenous inoculation of CBA mice was attempted difficulty was experienced in seeing and injecting the tail vein. For greater ease of manipulation Swiss white mice were used, as their tail veins are very prominent and clearly visible. Six Swiss white mice were injected intravenously with 0.2ml. of washed sediment of an intermediate culture of A. israelii, strain Ralston (p. 95). These mice were observed and killed at intervals during a period of 42 days.

In the 24-hour period after inoculation, the animals appeared ill; the tip of the nose was markedly swollen and the eyes were covered with a film of mucus. This swelling of the nose persisted during the 42 days of observation; it gradually diminished in extent but the nose did not regain normal size.

In the mouse killed seven days after inoculation there was no change either macroscopically or microscopically. In the three mice killed at 12, 15, and 18 days after inoculation small pin-point abscesses were found in the liver and spleen and, in one mouse, in the lung; the liver surface again showed a mottled appearance but these were

the only abnormal findings. Histologically the tissue reactions were the same as those described in the livers of mice after intra-peritoneal inoculation.

The relative absence of lesions was more notable than those that were observed. These permitted the conclusion, however, that blood-borne spread of _A. israelii_ was a possibility. In the mice killed at 21 and 42 days after inoculation post-mortem examination gave only negative findings, which were confirmed as negative by histological examination.

INOCULATION OF MICE WITH STRAINS OF ACTINOMYCES ISRAELII PASSAGED THROUGH MICE

As has been seen in the literature the passage of A. israelii through successive animals has not increased the pathogenic action of the parasite for the host as regards the production of a progressive systemic disease, which kills the host; only localised abscess processes which eventually heal up have been produced in cattle and guinea-pigs Magnusson (1928), Naeslund (1931).

An experiment was set up, using for mouse inoculation cultures of A. israelii which were isolated from actinomycotic lesions experimentally produced in mice. This was an attempt to see if passage through the mouse would enhance the pathogenicity of A. israelii and if by using this technique it would eventually be possible to produce a progressive actinomycotic disease in the mouse. For this work, two cultures of A. israelii strain Ralston and 253 isolated from experimental mouse lesions were used.

Their source was as follows.

In the experiment on the effect of intramuscular inoculation of mice with A. israelii strain Ralston, one animal was killed four days after inoculation. The mouse was dissected with sterile precautions, and the abscess

which had developed in the muscle was incised and a portion of the pus removed. A Gram-stained film of this pus showed as has been previously described, Gram-positive branching filaments (fig. 46 p. 131). With this material, a wide variety of media was inoculated; bottles containing 40 ml. amounts of Brewer's thioglycollate broth (five), Robertson's meat medium, 10% glucose - horse-heart, digest-agar plates, and 10% horse blood meat-extract-agar plates. The agar plates were incubated both aerobically and anaerobically. By using this range of media it was possible to determine with reasonable certainty the complete bacterial flora both anaerobic and aerobic, present in the experimentally produced lesion. Growth was obtained only in the Brewer's thioglycollate medium (all five bottles) and microscopic examination revealed the presence of the actinomycete alone. No other bacterium grew either aerobically or anaerobically after seven days' incubation at 37°C. From this medium showing growth, subcultures were again made on the agar plates and Robertson's meat medium, to control whether contaminating bacteria missed perhaps on microscopic examination were present; these sub-cultures again were negative for bacterial growth even after seven days' incubation at 37°C. Only <u>A. israelii</u> was isolated from the primary culture

of the A. israelii strain Ralston obtained in the Brewer's thioglycollate medium. Two consecutive subcultures into Brewer's medium were carried out before the organism was used for animal inoculation. The growth of the organism in the Brewer's medium was characteristic; it resembled the growth of the A. israelii strain Ralston as originally described (fig. 3 p. 98) i.e. little granules composed of the branching organisms developed, and round these a fine haze of growth appeared. Microscopically, Gram-stained films showed branching filaments breaking up into V and Y angled diphtheroid forms.

Using cultural methods similar to those described for the re-isolation of the actinomycete from the intra-muscular abscess, A. israelii strain 253 was re-isolated from a perisplenic abscess which had developed three weeks after intra-peritoneal inoculation of a mouse with the organism. Pure cultures of strain 253 were obtained in Brewer's thioglycollate broth also. With each re-isolated strain of A. israelii, 12 CBA mice were inoculated intra-peritoneally with 0.5 ml. of washed bacterial suspensions which were prepared as previously described (p. 95). Concurrently with these experiments, 12 CBA mice of the same batch were inoculated intra-peritoneally with washed suspensions of A. israelii strain 104. The

TABLE VIII

Result of intra-peritoneal inoculation of mice with strains of Actinomyces israelii isolated from mouse peritoneal lesions

Strain of A.israelii	Isolated from mouse lesion	No. of mice inoculated I.P.	Deaths=D Killed=K	Time	Post-mortem appearance
Ralston	Intra-muscular abscess on mouse leg	12	4 (D)	72 hours	Fluid in pleura
			1 (D)	15 days	Abscess formation
			2 (K)	3 months	No abnormality
			2 (K)	6 months	Nephritis (1)
			1 (D)	101 days	Nephritis
			2 (K)	247 days	No abnormality
253	Peri-splenic abscess in mouse	12	5 (D)	48 hours	Fluid in pleura
			5 (D)	72 hours	Fluid in pleura
			1 (K)	3 months	No abnormality
			1 (K)	6 months	No abnormality
104	Control strain (not passaged through mice).	12	1 (D)	48 hours	Fluid in pleura (White patches of fibrin on viscera)
			4 (D)	72 hours	"
			1 (K)	15 days	Abscess formation
			1 (K)	45 days	"
			1 (D)	48 days	Abscess at base of right lung
			1 (K)	3 months	No abnormality
			2 (K)	6 months	No abnormality
			1 (K)	247 days	No abnormality

results obtained by inoculating this strain of actinomycete into mice were already known (table III p. 107), and these animals thus acted as a control series in comparing the reaction of the mice to the passaged strains. Immediately after the inoculation of the passaged strains Ralston and 253, the mice became distressed and ill. Their reactions were similar to those previously observed in mice after intra-peritoneal inoculation A. israelii (p. 108): their coats were ruffled, their eyes closed, and their posture hunched. With A. israelii strain 253, 10 of a total of 12 mice died in the 72-hour period after inoculation (table VIII). In the previous experiments intra-peritoneal inoculation of the A. israelii strain 253 into mice gave no deaths in the six animals inoculated (table III p. 107) — thus with this passaged strain 253 of A. israelii a death rate of 80% in the first three days was obtained. With the passaged strain Ralston, four died of the 12 mice inoculated (table VIII). In an earlier experiment summarized in table III p. 107 the mice were killed 24 hours after inoculation because they appeared moribund at that time.

The mice inoculated with the unpassaged strain 104 gave a death rate of five out of 12 mice in the first 72 hours, (table VIII) — a result of the same order as in the previous finding that six died of 18 mice inoculated

(table <u>III</u> p. 107). The higher death rate resulting from inoculation of <u>A. israelii</u> passaged strain 253 might therefore be due to the inoculum and not to any increased susceptibility of the mice used in the later experiment.

In the mice dying at 72 hours after inoculation with the passaged strains Ralston and 253, post-mortem examination showed again that fluid was present in the pleura and peritoneum and there was a fibrin deposit on the viscera: the appearance of these mice was identical to that found in the previous intra-peritoneal inoculation experiments. Histological examination of the visceral tissues gave identical findings.

The mice which did not die in the period one to three weeks after inoculation were left and killed at intervals of three and six months onwards. This course was adopted to see if the passaged strains would give rise to progressive lesions. Though abscesses would probably be developed on the mice peritoneal surfaces - judging from the basis of the previous experimental findings - it was hoped that these would be more extensive. There might even be actual involvement of the visceral tissue giving well marked changes and this could lead to the death of the mice with either disseminated actinomycosis or tissue damage.

Only two mice survived the intra-peritoneal

inoculation of passaged strain 253. These animals gradually regained their normal healthy appearance after inoculation and when they were killed at three and six months respectively, no abnormality could be seen either on post-mortem or histological examination, (table VIII p. 140).

Seven of a total of 12 mice survived the intra-peritoneal inoculation of the passaged strain Ralston. Two of these mice were killed at three months and two at six months after inoculation. No macroscopic actinomycotic lesions were seen and in one of the mice killed at six months histological examination of the kidney showed the development of nephritic lesions, similar to the lesions previously described (p. 121). Finally one other mouse in this series died 101 days after inoculation, again with the finding of nephritis, there being no other lesion present either macroscopically or microscopically. The last two mice were killed 247 days after inoculation and showed a normal appearance on post-mortem and histological examination.

In the surviving mice inoculated with the control unpassaged strain 104 A. israelii, the pattern of actinomycotic lesion development on the peritoneum and lesion healing was shown as has been previously described (p. 120).

From this experiment it would seem that the inoculation of mice with strains of _A. israelii_ isolated in pure culture, from experimentally produced mouse actinomycotic lesions, did not lead to the development of progressive actinomycosis and indeed did not even lead to more widespread and longer persisting abscess formation. The only finding was that with _A. israelii_ strain 253, the initial death rate increased, perhaps hinting at an increased toxicity of the strain for the mouse, but this increase, if such, is not permanent and has had no permanent damaging effect on the mouse tissue, as the two surviving mice in this series had completely normal appearance both on post-mortem and histological examination.

With strain Ralston similarly there was no extension of abscess formation or tissue damage. The finding of the kidney lesion is probably not related to the passaging of the organism, since this nephritic lesion has sporadically appeared in previous experiments.

The cultures were vigorously growing when used for inoculation and from this point lack of lesion development can not be due to the impaired vitality of the organism.

EXTRACTION EXPERIMENT WITH *ACTINOMYCES ISRAELII*

In an attempt to discover if the cells of *A. israelii* contained a toxic substance - an endotoxin perhaps - which might be responsible for the nephritic lesion found in mice six to nine months after intra-peritoneal inoculation with the bacteria, the following simple extraction experiment was set up.

A. israelii strain 104 (a new freeze-dried culture was opened for this work; the morphology of the organism was filamentous) was grown in Brewer's thioglycollate broth for six days at 37°C.

The culture was centrifuged, and the resulting sediment of organisms was washed six times with saline. After washing, the dried sediment was ground in a mortar with 2ml. of sterile distilled water and a little sterile sand. The suspension was ground carefully till a creamy suspension was obtained; Gram-stained films of this showed broken irregularly staining organisms. Ten ml. of sterile distilled water were added to the ground suspension and the mixture was decanted into a sterile universal container. The container was totally immersed in the water bath at 65°C for one hour (time was allowed for the container to reach the temperature before the ground bacteria were added to it).

The complete operation was carried out as quickly as possible. After heating, the cooled suspension was then frozen in the refrigerator for 24 hours. After this period the suspension was allowed to thaw at room temperature, when this had happened the suspension was again centrifuged. The supernatant fluid was aspirated, and the ground bacterial sediment suspended in saline.

With the supernatant fluid two CBA mice were inoculated intra-peritoneally; similarly two other CBA mice were also inoculated with the ground sediment intra-peritoneally; the amount injected in each case being 1 ml.

Reaction to inoculation. - No mouse showed any evidence of discomfort either immediately after inoculation or within a period of three weeks. Two mice, one of each pair, were killed at nine months after inoculation; and the remaining two were killed fourteen months after inoculation. The mice inoculated with the ground sediment showed on post-mortem examination no reaction either microscopically or macroscopically. The other two mice which had received the supernatant fluid, showed kidney damage; the animal killed at nine months, showed small focal areas of damage, while the mouse killed at fourteen months showed the type

of nephritic lesion (fig. 42 p. 121) which has been previously described. There was no external sign of oedema or adiposity, for example, to indicate the probability of kidney damage. On the other hand the mice were thin and rather emaciated in appearance. A tentative suggestion could be made on the evidence of this experiment: namely, that a water-soluble toxic component might have been extracted from the cells of A. israelii, because such renal lesions have not appeared spontaneously in control uninoculated mice. Nine months is a long time between the ~~incubation~~ inoculation and the appearance of the kidney lesion, but the mice were not examined at shorter intervals, and it is possible that the hypothetical material extracted may not be immediately toxic to the mouse. This observation will require to be repeated with adequate numbers of mice.

INOCULATION OF MICE WITH THE "AEROBIC" STRAIN 161 OF ACTINOMYCES ISRAELII

The second passage strain of A. israelii 161, which was becoming adapted to aerobic conditions of growth, was used to inoculate four CBA mice intra-peritoneally. The organisms of a culture in aerobic digest broth were centrifuged and the sediment was washed twice with sterile saline and suspended into ten ml. of saline: one ml. was injected intra-peritoneally. The individual mice were killed at 14 and 21 days and at three and seven months after inoculation. On post-mortem examination all the mice had a normal appearance and this was confirmed on histological examination. Adaptation to the aerobic form of growth was evidently correlated with loss of virulence.

INOCULATION OF MICE WITH HEAT-KILLED SUSPENSIONS OF ACTINOMYCES ISRAELII (ROUGH AND INTERMEDIATE FORMS)

A small series of experiments was carried out in which CBA mice were inoculated intra-peritoneally with strains of A. israelii (rough and intermediate) which had been killed by heating; different temperatures and differing periods of exposure being employed.

The primary aim of these experiments was to observe the effect of these heat-killed organisms on the mouse kidney: would a lesion develop comparable to that found previously with living organisms? Secondly, would abscess formation still occur in the mouse peritoneum following inoculation of heat-killed organisms? In this connection, Wright (1905) and Meyer (1934), both reported abscess formation in animals inoculated with heat-killed A. israelii. Moreover, according to these authors, such abscesses on section revealed the presence of club-structures (p. 17). Lastly, would the hyperplasia of liver which was a marked result of inoculation with living organisms, still develop, although the inoculum contained only killed organisms?

The strains of A. israelii, Ralston, 104, 4956, 54, 253, 161, 265, were grown in Brewer's thioglycollate

TABLE IX. Showing the temperatures and time of heating for suspensions of *Actinomyces israelii*

Method of Heating	Temperature or pressure	Time of heating (Min.)	*A. israelii*	Mice: No. inoculated	No. dead in 72 hrs.	No. dead in days	Surviv -ors	No. with Abscess formation	Site of Abscess
H_2O Bath	65°C.	60	Ralston	6	0	0	6	0	
"	"	"	104	5	0	0	5	0	
"	"	120	Ralston	5	0	0	5	3	Liver spleen
"	"	"	104	5	0	0	5	3	Spleen needle-tract
"	80°C.	60	4956	5	2	0	3	0	
"	"	"	104	5	0	0	5	0	
"	"	"	54*	5	0	0	5	0	
"	"	"	235*	5	0	1 in 52 days	4	0	
Autoclave	12 lb. per sq. in.	15	161	6	1	0	5	0	
"	"	"	104	6	1	0	5	1	Liver
"	"	"	265	6	0	1 in 12 days	5	1	Spleen

*ground sediment

broth for seven days at 37°C, and saline suspensions were prepared as previously described on p. 95 , the density of the suspension being again the same. Two of the sediments (from strains Nos. 235 and 54) were ground thoroughly in a mortar before heating in an attempt to see if such grinding would liberate any endotoxin. Two methods of heating were used, the suspensions were either heated in the water bath or autoclaved. Table XX shows details of the method of heating, the temperature, the time of exposure, the strains of _A. israelii_, and the number of mice inoculated with each heat-killed suspension.

When suspensions of _A. israelii_ were heated in the water-bath, the suspensions after preparation were transferred into sterile containers, previously immersed in the water-bath, and thus already at the required temperature. After heating and autoclaving, the suspensions of _A. israelii_ were allowed to cool to room temperature and then were subcultured into bottles of Brewer's thioglycollate broth and meat extract broth -- four bottles of each medium at least being inoculated from each suspension. These subcultures were incubated at 37°C. for at least 21 days. No growth was ever obtained either aerobically or anaerobically in subcultures made from heat-killed _A. israelii_ suspensions. Till the result of the subcultures were known, the

suspensions were frozen in the refrigerator -- this was usually over a period of seven days, then the frozen suspensions were allowed to thaw at room temperature, and were then used for mouse inoculation.

Films of these heat-killed A. israelii suspensions, when stained by Gram's method showed a Gram-negative background of broken up organisms which showed an irregular spotted appearance, an appearance which has already been found in old cultures of A. israelii.

A total of 59 mice were inoculated with heat-killed organisms. The procedure of examination was as before. The mice were killed at intervals of roughly seven, 14, 21, and 28 days, of three and six months, and in a few cases after a year, from the time of inoculation.

No mouse after inoculation of heat-killed organisms, showed immediate signs of discomfort or distress. This lack of reaction is in contrast to the disturbance found in mice following inoculation with living cells. The mice appeared normal; their coats and eyes remaining as they were prior to inoculation. Four mice, however, died during the 72 hours after inoculation. These animals on post-mortem examination showed the presence of turbid free fluid in the peritoneum and pleura. In addition, a fine film of whitish-gray fibrin was present on the liver and

spleen, especially the liver. Underneath the fibrin, the liver had a mottled appearance. The macroscopic appearance of these animals was very similar to that observed in mice which died 72 hours after inoculation of living organisms. Histological examination of the liver showed again the development of hyperplasia.

One mouse died 12 days after inoculation and both this mouse and seven of 21 mice killed seven to 14 days after inoculation showed abscess formation in the peritoneum. These abscesses were single and small, and were localised either on the liver, spleen, or omentum, or, in one case, at the needle tract. There was not the extensive proliferation of abscesses found with living organisms in this same period of time. These abscesses contained a very small quantity of white pus; films made from this and stained by Gram's method showed only cellular debris; individual organisms could not with certainty, be distinguished. Sections of the abscesses showed no massing of organisms; there was only necrotic material, then an irregular mass of variable Gram-positive organisms, which did not show any sign of multiplication. Polymorphonuclear cells did not develop round the organism masses. In the 13 mice which did not show abscess formation during the period there was however the development of small white patches of

fibrin scattered over the upper surface of the liver and very occasionally on the spleen. Squashed preparations of this fibrin stained by Gram's method showed no specific bacterial forms to be present. The remaining 34 mice were examined at regular intervals up to a year - one mouse died 52 days after inoculation - but no abnormality was detected in any animal either on post-mortem or histological examination, the kidney tissue especially was shown to be normal.

In addition, the grinding of the bacteria as carried out in strains Nos. 54 and 235 seemed not to have any effect on the mouse.

CONTROL AND OTHER EXPERIMENTS

Along with the experiments using living A. israelii organisms for animal inoculation, experiments were carried out using as inoculum: thioglycollate broth (uninoculated); washed sediments of old cultures (nine-months) of A. israelii; the supernatant thioglycollate broth of six-day old cultures of A. israelii; and a mixture of sediment of old organisms in the fresh supernatant of a young culture.

I. Four mice were inoculated intra-peritoneally with 1 ml. of Brewer's thioglycollate broth medium. The mice were killed 14 days, three, six and nine months after inoculation. No abnormality was detected either macroscopically or microscopically.

II. The washed sediments of two nine-month-old cultures of two strains of A. israelii were each inoculated in 1 ml. amounts into four CBA mice. The mice were killed at 15 and 40 days and at six and nine months. The only tissue change observed was in the liver of mice killed at 15 days, a slight surface reaction of hyperplasia developed. The other animals were normal.

III. The supernatant fluid of five strains of A. israelii growing for six days in Brewer's thioglycollate medium at

37^{0}C. was inoculated into two mice per strain of organism - 1 ml. being inoculated. The mice were killed at seven and 14 days after inoculation. No abnormality was found macroscopically or microscopically.

IV. The sediment of a six weeks old strain of A. israelii in Brewer's thioglycollate broth was suspended without washing in the supernatant broth from a six-day old culture of A. israelii. Ten mice were injected intra-peritoneally with 1 ml. of the suspension. The animals were killed at seven, 14, 21 and 28 days and at three, six and nine months and one year after inoculation. One mouse killed 14 days after inoculation showed a small abscess on the pancreas, but no macroscopic lesion developed in any other mouse. In the one affected mouse the liver tissue showed again a reaction of hyperplasia at the surface. One mouse in this experiment died seven months after inoculation with a nephritic lesion; one other mouse lived for a year after inoculation, and was normal on examination.

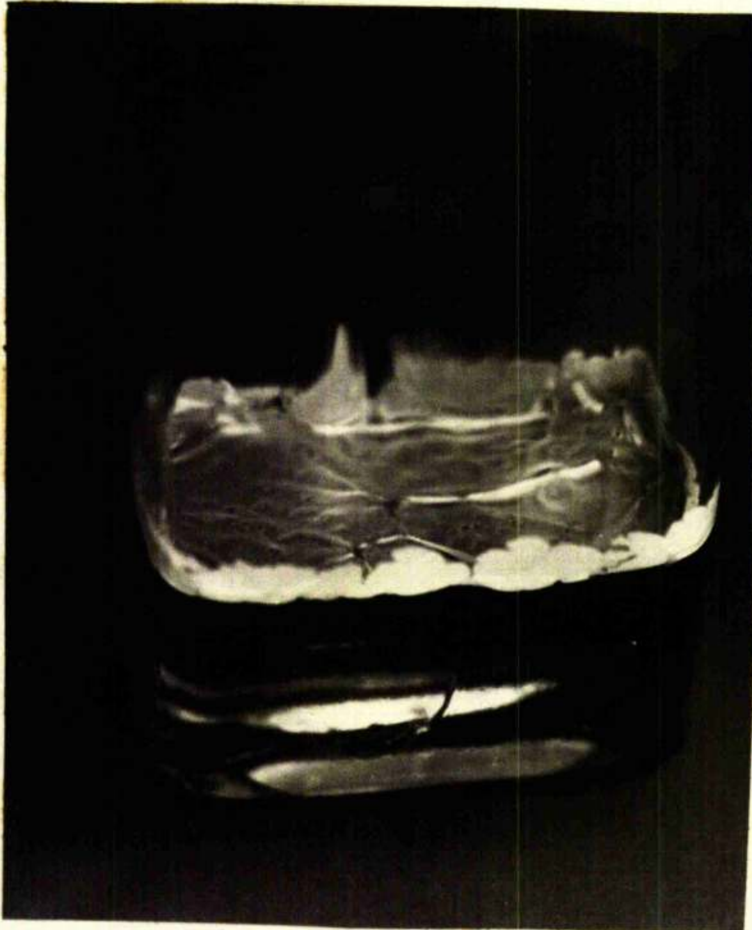
EXPERIMENTS WITH *NOCARDIA ASTEROIDES* AND *ACTINOMYCES GRAMINIS*

In addition to the experiments in which mice were experimentally infected with *A. israelii*, a small number of mice were also inoculated intra-peritoneally with *Nocardia asteroides* and *Actinomyces graminis*. According to published work both these organisms on inoculation into mice may or may not produce abscesses. Although Eppinger first recorded in 1891 the isolation of an aerobic acid-fast actinomycete – *N. asteroides* – from a fatal case of meningitis, infection with this organism in man and animal is comparatively rare; a fact which is evident when comparison is made between the reported incidence of nocardiosis and actinomycosis. The literature dealing with *N. asteroides* falls therefore into two categories; the first part deals with the recording of cases of human nocardial infection – this usually includes a full description of the clinical and pathological appearances observed, and also the isolation of the organism (Davis and Garcia, 1923; Goldsworthy, 1937; Kirby and McNaught, 1946; Tucker and Hirsch, 1949); the literature in the second category covers the experimental production of nocardiosis in laboratory animals; as with *A. israelii*, the results recorded of such experiments are contradictory. Gordon and Hagan (1936) found their strains of *N. asteroides* pathogenic for rabbits; Goldsworthy (1937), Ginsberg and

Little (1948) found only minor lesions developing in rabbits, Drake and Henrici (1943), Tucker and Hirsch (1949) found N. asteroides had no effect when inoculated intra-peritoneally into rabbits. Ginsberg and Little (1948) found N. asteroides was non-pathogenic for mice; Tucker and Hirsch (1949) produced lesions on the liver of one mouse while Runyon (1951) in contrast produced a 60% mortality in mice by using massive doses of N. asteroides (14mgm.) for intra-peritoneal inoculation.

Reports of the occurrence of A. graminis - now regarded as being identical with the organism originally isolated by Bostroem (1891) - in pathological literature are very scanty. Lynch and Holt (1945) isolated it from a case of clinical atypical pneumonia. Kyles quoted by Browning and Mackie (1949) also isolated it from sputum and pleural exudate from one case. Kessel and Goolden (1938) under the term A. hominis describe two strains isolated from cases of actinomycosis. These authors moreover showed that this organism was of low pathogenicity or non-pathogenic for mice and guinea-pigs. Although limited information was available about the experimental injection of mice with these two organisms I was specially interested in the possibility of a late nephritic lesion developing in mice after inoculation

Figure 50



Nocardia asteroides strain 6761, growing on broth, forming a smooth entire pellicle on the surface; four days' incubation at 37°C. x 4.

of these aerobic genera; also I wished to compare the over-all reaction (if any) of the mouse to these aerobic genera with that resulting from inoculation of A. israelii. These experiments are briefly outlined.

INOCULATION OF MICE WITH NOCARDIA ASTEROIDES

Two strains of N. asteroides (Nos. 6761 and 3258 from the National Collection of Type Cultures) were used for the intra-peritoneal and intra-venous inoculation of CBA and Swiss white mice respectively.

Both strains when grown on the surface of agar gave rough creamy-yellow colonies which were very adherent to the medium, and very difficult to remove. In liquid medium – meat-extract-broth – however good growth which could easily be harvested occurred. Strain 6761, grew in broth, as a creamy pellicle extending over the entire surface of the broth, leaving the underlying fluid clear (fig. 50).

Strain 3258 showed the start of pellicle formation, but this fell to the bottom of the bottle and gave rise later to cotton-wool-balls of fluff. Microscopic examination of films made from both organisms in broth and stained by Gram's method showed the appearance of long, angled,

T A B L E X

Showing the numbers and time interval of abscess formation in mice inoculated with Nocardia asteroides

Strain of N. asteroides	No. of mice inoculated	No. of mice dead in 72 hours	Survivors (beyond 72 hours)							
			No. dying	Time in days	Abscess formation +	Abscess formation -	No. killed	Time in days	Abscess formation +	Abscess formation -
6761	13	0	1	10	+		1	45	+	
			1	17	+		1	77	+	
			1	21	+		2	120	+(1)	-(1)
			2	25	+		1	147		-
			1	31	+					
			1	37	+					
			1	50	not examined					
3258	13	2	1	120	+		1	58		-
							1	105		-
							1	107		-
							2	180		-
							1	150		-
							2	365		-
							1	400		-
							1	291		-

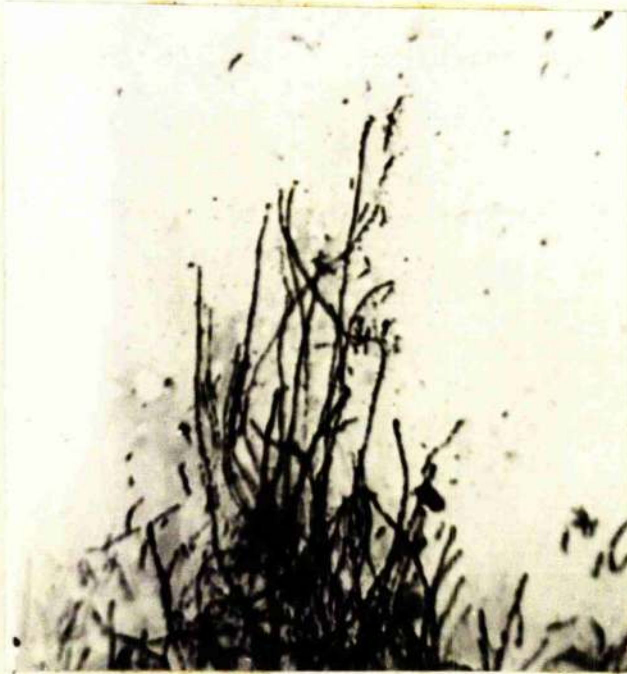
diphtheroid Gram positive forms - strain 3258 especially showed longer filaments with pronounced branching; both strains were partially acid-fast (1% H_2SO_4).

For the inoculum, both strains of N. asteroides were grown in 40 ml. amounts of meat-extract broth and incubated at 37°C. for 4-6 days. The cultures were centrifuged and the organism sediments were well washed three times in saline, then resuspended to give a density two times that used in the A. israelii series of experiments. With the suspension of each organism 13 CBA mice were inoculated intra-peritoneally; the dose being 1 ml.

From Table X it would appear that the mice were more susceptible to infection with strain 6761, as seven mice in this series died within the period of 37 days after inoculation with widespread abscess formation in the peritoneum; only two of the remaining five mice when killed between 45 and 147 days after inoculation did not show abscess formation. With strain 3258, two mice died during the 72 hours immediately following inoculation and only one died with abscess formation 120 days after inoculation. The ten surviving mice killed from 58-400 days after inoculation showed no abscess formation and were normal on post-mortem examination.

Mice inoculated with strain 6761 showed the

Figure 51



Pus from peritoneal abscesses in the mouse after inoculation with N. asteroides. Note the very extensive branching. Gram's method x 1000.

development of extensive abscess formation on the peritoneum, the liver, spleen, omentum all showing lesion development. Furthermore, the abscesses were not confined to the visceral surfaces as with A. israelii but were actually formed inside the visceral tissue. The kidney especially was a focus of infection either one or both showed abscess development. The abscesses were at first, in mice dying ten days after inoculation, pin-point to lentil sized, and in mice dying at 42 days, the abscesses were comparable in size to those found in mice at 21 days after intra-peritoneal inoculation of A. israelii. One macroscopic difference noted was that with the nocardial abscesses there was little development of fibrous tissue in the peritoneum, the viscera lay quite free in the peritoneum and were not bound together, as had been the result with A. israelii.

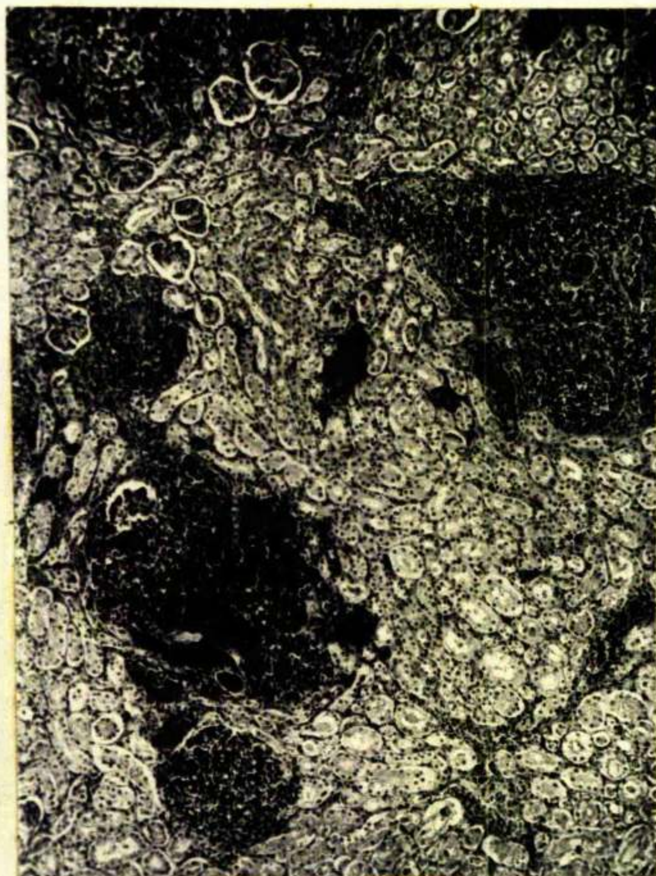
These nocardial abscesses contained whitish-cream coloured smooth pus and films made from this stained by Gram's method, showed very extensive branching Gram-positive filaments (fig. 51). The N. asteroides in the abscesses was still viable as successful cultures were obtained from an abscess in a mouse killed 45 days after inoculation. This re-isolated strain of Nocardia gave on culture colonies with a deep orange-pigment, this pigment development was more strongly marked than in the parent culture. Films

Figure 52

N. asteroides, strain 6761. Film made from passaged culture growing on digest agar. Note the very short thick forms, with little branching. This is in contrast to fig. 51. Gram's method x 1000.

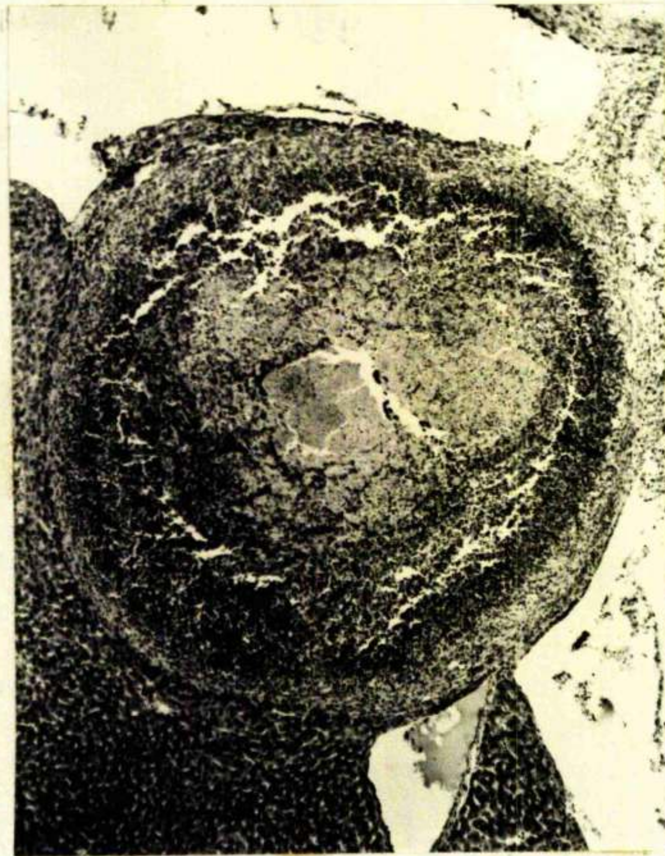
made from colonies on agar showed the morphology of the organism to be as in fig. 52, branching was not so pronounced. The two mice that died 72 hours following intra-peritoneal inoculation of strain 3258, showed on _post mortem_, a little free fluid present in the peritoneum, but there was no fibrin developed over the surface of the viscera, tiny pin-point nodules were dotted over the bowel serous surfaces, and these when squashed and examined by Gram's method, showed the presence of long much branched Gram-positive filaments. The mice inoculated with strain 3258 and left for a year or longer after inoculation showed, when they were killed and examined, a normal appearance. There was never at any time, the development of a nephritic lesion comparable to that developed in the _A. israelii_ series of experimental animals.

Figure 54



Section through a kidney of a mouse which died 21 days after an intra-peritoneal inoculation with N. asteroides, strain 6761. Note the large collections of organisms, around some of these, abscesses are forming. No macrophages are seen at the periphery of these lesions. Haematoxylin and eosin x 100.

Figure 53



Section through abscess in liver tissue of a mouse which died 45 days after intra-peritoneal inoculation with N. asteroides, strain 6761. There is a central mass of necrotic tissue and this is surrounded by a wide zone of polymorphs. No macrophages are present at periphery of lesion. Haematoxylin and eosin x 50.

HISTOLOGICAL APPEARANCES IN MOUSE TISSUES AFTER INOCULATION OF N. ASTEROIDES

In sections of spleen, kidney, and liver of mice (figs. 53, 54) there are present multiple large abscesses inside the visceral tissue. These abscesses have developed round large clumps of organisms, which have an irregular arrangement, these do not show any radiate arrangement of hyphae at the periphery as was seen with A. israelii. Surrounding this mass of organisms is a large collection of polymorphonuclear cells, but no fat-laden macrophages ever develop in this lesion. Fibrous tissue was not formed round the abscesses; these develop through the viscera giving widespread tissue destruction. The liver did not at any time show the surface reaction of hyperplasia which was such a noticeable feature in the early period in mice following inoculation with A. israelii.

INTRAVENOUS INOCULATION OF SWISS WHITE MICE WITH N. ASTEROIDES

Eight Swiss white mice were inoculated intravenously, four animals receiving N. asteroides strain 6761, and four N. asteroides strain 3258. The suspension was prepared as for the intra-peritoneal inoculation series, the dose being 0.25 ml.

Two of the animals inoculated with strain 6761 died 48 hours after the inoculation. Post-mortem examination showed a little free fluid present in the pleura. Histologically and macroscopically the tissues were normal. The other two mice in this series were killed at three and six months after inoculation. These animals also showed no abnormality. Of the mice inoculated with strain 3258, one mouse died three months after inoculation with pin-point abscesses in both the kidneys. The other three mice were killed at two, three, and seven months after inoculation; all were normal in appearance.

Figure 55

Cellophane round, with colonies of A. Graminis on surface. This has been removed from the surface of the agar plate. x 2.

INTRA-PERITONEAL INOCULATION OF CBA MICE WITH ACTINOMYCES GRAMINIS

This actinomycete belongs to the genus Streptomyces; and it produces the characteristic conidia over the surface of the colony. Culturally, the organism is aerobic, and grows easily on the surface of solid media - either 10% glucose-digest-agar or 10% glucose-meat-extract agar. The colonies are at first grey in colour, leathery in consistency, and are very adherent to the medium. This appearance is seen after 48-72 hours' incubation at 37°C.; on incubation for another 48 hours, the colonies usually develop a white bloom due to the development of conidia over the entire surface. Impression preparations of the conidia, stained by Gram's method, show small oval Gram-positive bodies resembling cocci, the colonies are difficult to squash to make films for microscopic examination. Such films show the presence of long Gram-positive branching filaments. Since the colonies were so firmly adherent to the medium it was impossible to obtain sufficient organisms to prepare suspensions for inoculation. Use was therefore made of a cellophane technique in growing A. graminis (fig. 55) for inoculation of mice. Autoclaved rounds of cellophane were placed on the surface of 10%

glucose-digest-agar medium in petri plates; these were then incubated at 37°C. for 24-48 hours to check sterility before being inoculated with A. graminis strain No. 4728 of the N.C.T.C.* on the surface of the cellophane. Colonies of A. graminis showing conidia formation were obtained on the cellophane surface after 4-5 days' incubation at 37°C. These colonies on cellophane were identical with colonies formed directly on the surface of the agar in a control series. It was a simple operation to scrape the colonies of A. graminis off the cellophane — with sterile precautions — and collect them in 2 ml. of sterile saline. These colonies were now ground in a tissue grinder to give a uniform suspension. This suspension of A. graminis, thus prepared, contained not only filaments but also conidia. For animal inoculation the density of the suspension of A. graminis was made equal to that of the A. israelii series. A total of eight CBA mice were inoculated intra-peritoneally with 0.5 ml. of this suspension.

Another eight CBA mice were also inoculated intra-peritoneally with a saline suspension of A. graminis prepared from organisms grown in broth. In broth A. graminis grows submerged in the form of cotton-wool-balls, which on microscopic examination were composed of branching Gram-positive filaments. As the organism is growing in the

* National Collection of Type Cultures (London)

depths of the broth, conidia production does not occur. To prepare the suspensions, 40 ml. amounts of digest-broth, inoculated with A. graminis were grown for five days at 37°C. The broth cultures were then centrifuged, the sediments were well washed and suspended in saline, and lightly ground in the tissue grinder. This ground suspension was then made equal in density to that of the A. israelii used in the previous experiments (p. 95). Mice inoculated with both suspensions were killed and examined at seven, 14, 21, and 28 days and at three, six, and nine months. Two mice died in 48 hours after inoculation with the A. graminis suspension containing conidia. These mice underwent partial putrefaction before post-mortem examination. Such examination as could be made showed that fluid was present in the pleura. No other abnormal finding was recorded. These were the only deaths recorded in this experiment. The remaining 14 mice were killed, as stated above, and these animals showed no abnormality on post-mortem and microscopic examination. This strain of A. graminis has little pathogenic effect, if any, on mice.

D I S C U S S I O N

As may be seen in the review of the literature, many workers have explored a great diversity of approaches in order to solve the problem of reproducing actinomycosis in laboratory animals. From all these efforts there has not as yet emerged any single, definite formula for success. Slack (1942) was the first to produce true progressive actinomycosis in rabbits by repeated intravenous inoculation. His results have not yet been confirmed. The second and most recent success was that of Hazen _et al._ (1952) with hamsters which received a single intra-peritoneal injection of _A. israelii_, these authors produced in six weeks, in 21 of 28 animals inoculated, extensive abscess formation with the development of sinuses discharging pus and sulphur granules; but even about this work a reservation must be made since seven animals when killed showed no sign of infection. It would appear that even the hamster is not universally sensitive to infection with _A. israelii_. Meyer and Verges (1951) by using mucin as an adjuvant produced actinomycotic lesions in mice with a single intra-peritoneal injection, 17 of 21 mice inoculated developing progressive lesions as compared to three of 15 mice inoculated with saline suspensions of the organisms without mucin. These animals

were killed at from 10-15 days after inoculation, but it would be interesting to know if such animals, when left for a further period, would die as a result of the infection or if these lesions would heal spontaneously. Geister and Meyer (1951) also reported that mice inoculated with mixtures of _A. israelii_ and mucin developed actinomycotic abscesses, but four months after inoculation these lesions were found to be sterile and fibrous, a chronic infection and not a progressive disease had resulted. These authors did not observe their animals beyond this four month period. One criticism to be made about recorded attempts to produce experimental actinomycosis in animals has been the relatively haphazard conduct of the experiments described. No previous author appears to have set up a systematic experiment killing his animals over a definite and lengthy period of time. This is essential, as I think, and it is quite possible that valuable information has been lost through each author's taking a random period for killing all or most of his animals, and thus missing lesion-development. Though Meyer and Verges (1951) were successful in producing actinomycotic lesions in mice with mucin, Strauss and Kligman (1951) could not confirm this result, and this may be due to the fact that the second workers used a different density of suspension of _A. israelii_ and also because they waited for the animals to die at ten days after inoculation: they did not kill them and examine them. This is

yet another example of the difference in approach to the experimental problem.

Surveying the literature as a whole, I am impressed with the fact that production of actinomycotic lesions in guinea-pigs, rabbits, and even cattle seems to be so much apparently a matter of random chance. Successful production of lesions in animals does not appear to depend on the origin of the strains of A. israelii used for the experimental work: it does not seem to matter whether they are derived from human or animal pathogenic sources, or even from the normal healthy mouth. This makes me wonder if perhaps there is not a natural underlying susceptibility in some individual animals towards A. israelii. If the analogy of human experience may be used: many people indeed have teeth extracted, and the great majority of these wounds heal normally; but a small proportion of such patients develop facial actinomycosis. This problem of what initiates infection has not been solved. Are such people naturally susceptible to infection with A. israelii? What are the factors disposing towards infection? In examination of actinomycotic material a wide range of bacterial species have been found to accompany the A. israelii. Holm (1950,51) is a very strong advocate for the co-action of B. actinomycetem-comitans in initiating infection and even in continuing the disease process after the A. israelii has been removed. This theory has not yet been experimentally proved to

be true. It may be that susceptible hosts lack a particular factor responsible for inhibiting the active metabolism of A. israelii in the tissues. At least there is a fundamental influence, so far not defined, which governs the spread of actinomycosis in both man and animal. It is remarkable that in such people suffering from natural infections, there is such extensive proliferation of organisms and of host tissue.

In the work which I attempted and have described in this thesis, a systematic examination of the mice has shown that in some animals injected with some strains of A. israelii it has been possible to watch the development and regression of actinomycotic lesions. I should perhaps amplify this statement and point out that only a few strains of A. israelii gave rise consistently in mice to actinomycotic lesions. These actinomycotic lesions produced in mice were histologically similar to those found in natural human disease. The morbid histology of the experimental disease was definitely the same as that of the natural disease although the experimental infection was not true progressive actinomycosis. The spontaneous self-healing of the lesions produced was surprising in view of the extensive abscess formation in the peritoneum, though these were found only on the surface of the tissues and organs and not inside, as were the lesions reported by

Meyer and Verges (1951), Geister and Meyer (1951) and Gale and Waldron (1955). This may be the reason for the lack of progression of the disease; no extensive tissue damage occurred.

In my work, 18 of 25 mice inoculated with saline suspensions of A. israelii developed actinomycotic abscesses in the peritoneum 14 days after a single intra-peritoneal injection and these were at their maximum three weeks after inoculation. This result may be compared with a finding of lesions in three of 15 mice inoculated in the same way of Meyer and Verges (1951) and compares not unfavourably with the results reported by Hazen et al. who found abscesses in 21 of 28 hamsters inoculated. In my experiments the abscesses disappeared in from 4-6 weeks after inoculation leaving the mouse apparently normal.

An unexpected finding was that of a kidney lesion developing in six mice six to nine months after inoculation. This kidney lesion though appearing in only a few animals was not due to any parasitic infection such as Klossiella nor has it been previously described in any paper that I have been able to find. The lesion developed only in mice inoculated with young living cultures of A. israelii. The lesion was slow to develop and may well be due to damage caused by the active elimination of the A. israelii from the

peritoneum; either from mechanical damage or from sensitization to break-down products of the A. israelii. These are only speculations and no concrete evidence has been found to support them. In one experiment in which distilled-water extracts were prepared and 1 ml. inoculations made intra-peritoneally into mice, these nephritic lesions appeared. Was this chance or was there some active principle in the extract which produced this lesion? The time factor involved seems long, the quantity of material injected was about the same as that of the living cells of A. israelii injected. The finding of this lesion is interesting, and I think that more work on this line might be valuable.

Cultures of A. israelii killed by heat had no effect on mice when inoculated intra-peritoneally; few abscesses were formed and these were non-specific in character.

In common with other workers, I found that A. israelii in culture is not stable. It gradually underwent a morphological transition from filamentous to diphtheroid forms and there was also an increased tolerance for O_2. In regard to the changing morphology of A. israelii under continued subculture it would appear that associated with this diphtheroid form there is also a

change in the pathogenicity of the organism as measured by lesion formation in mice. Three strains of A. israelii 161, 104, and 253, which in their normal branched form gave rise to extensive abscess formation in the mouse peritoneum, when changed into the diphtheroid form did not produce lesions, but hyperplasia of the liver tissue was again observed. This hyperplasia did not develop in mice inoculated with an anaerobic diphtheroid of guinea-pig origin which was originally pathogenic for that animal. This change in morphology from the branched filamentous form to the diphtheroid form leads to speculation if such a change will occur naturally in the normal habitat of the organism, which is in the body of man or cattle. I have been struck, as have other workers - Meyer and Verges (1951), for example - in routine examination of blood cultures and other specimens, how often anaerobic diphtheroids are found. The significance of such organisms is not understood, and I have noted that in certain cases, growth in Brewer's medium results in the formation of little granular aggregations of bacteria, which at first glance may resemble very strongly the appearance of a rough colony of A. israelii. Meyer and Verges showed that such anaerobic diphtheroids when inoculated intra-peritoneally with mucin into mice gave rise with only two strains to abscess formation and that

these abscesses were different macroscopically and microscopically from the lesions produced by A. israelii. I think that it should be always borne in mind that anaerobic diphtheroids found in many bacterial pathological specimens may well be "involution" forms of A. israelii.

Club formation in culture medium was never observed. This finding is in contrast to that of Bayne-Jones (1925) who reported that he found club formation in simple medium free from serum and animal protein. In addition he observed the behaviour of isolated clubs in hanging drop cultures. Two of these showed growth, which took place at the thin end of the filament, the club itself did not actually participate in this proceeding. I saw no pleomorphic forms as envisaged by Morris (1951) as part of the life cycle of A. israelii. The only peculiar forms observed in the present work were those seen in old cultures, and these forms were not viable on subculture.

The increased O_2 tolerance of certain strains of A. israelii as shown by growth in aerobic medium had previously been reported by Rosebury et al. (1944). These workers made serial passages of eight strains of A. israelii, four from the mouth and four isolated from cases of human actinomycosis in (a) an aerobic atmosphere, (b) aerobic atmosphere with 5% CO_2 (c) an anaerobic

atmosphere and (d) an anaerobic atmosphere containing 5% CO_2. The organisms were grown both in streak cultures on agar and in pour plates, being serially transferred every fifth day. The *A. israelii* strains isolated from pathogenic lesions would grow only under anaerobic conditions, but one mouth strain grew on air on the surface of agar for six consecutive passages, but at the end of this period it was not viable. It may well be that this mouth strain of *A. israelii* of Rosebury *et al.* belonged to Naeslund's (1925) third group of actinomycetes and even to the new species of *A. naeslundi* proposed by Thompson and Lovestedt (1951), which can grow both aerobically and anaerobically. If this was so however the strain of *A. israelii* should still have been viable even after the sixth passage. Erikson (1940) has stated that strains of *A. israelii* isolated from pathogenic material will survive two consecutive passages in glucose broth before dying out. The strains of *A. israelii* used in this work were all originally derived from human cases of actinomycosis and not from mouths so the change in O_2 requirements can not be explained on this basis. I think now that the *A. israelii* species form a very variable group of organisms and some fundamental investigations into their metabolism would be enlightening from (a) classification

of the species (b) the pathogenicity of the species.

Nocardia asteroides, though only two strains were used, appeared similarly to show differences in animal pathogenicity, one strain 6761 being definitely pathogenic for the mouse, the other strain being non-pathogenic. The abscess produced by *N. asteroides* in the mouse is quite clearly differentiated from that of *A. israelii* on histological examination. Moreover, the abscesses form inside the tissues of the viscera and not on the surface as with *A. israelii*. Following intra-peritoneal inoculation of *N. asteroides* no hyperplasia developed in the liver tissue. This hyperplasia was a marked feature with *A. israelii*, and both living and heat-killed organisms stimulated the reaction. Nephritic lesions did not develop in any of the mice surviving after inoculation with *N. asteroides*.

A. graminis was non-pathogenic for mice.

The results of mouse inoculation with the three genera of actinomycetes indicates a difference in pathogenicity for laboratory animals which supports their differentiation upon other grounds.

S U M M A R Y

1. Hitherto, laboratory investigations into the disease actinomycosis have been almost wholly restricted to cultural and serological studies of the causal organism, *Actinomyces israelii*. The reason for this limitation is lack of a method of inducing a satisfactory infection in laboratory animals.

2. The literature on actinomycosis contains many references to unsuccessful attempts to infect laboratory animals. There are also accounts of methods of infection which succeeded only irregularly or depended upon methods which introduced such a degree of artificiality that the resulting infections were of little value for any comparative purposes - for example, the testing of therapeutic substances that might be used for the treatment of actinomycosis in man or domestic animals. A critical review of the relevant literature is presented.

3. By inoculating mice with cultures of *A. israelii* grown in thioglycollate broth I found that lesions developed in the inoculated animals with sufficient regularity to suggest the usefulness of further investigation of the disease so produced in mice. Success in establishing a useful experimental infection

in mice probably depended on three influences: (1) the use of fluid cultures grown for a relatively short period (4 - 5 days), which gave high yields of infective material; (2) the use of "rough" strains of *A. israelii*; and (3) awareness that non-fatal lesions developed in the inoculated animals.

4. A comparative study of the lesions so produced in mice and of those found in natural infections of the human subject showed many striking points of similarity between the two.

5. Histological studies of organs of infected mice revealed the presence of abscesses in liver and spleen but not in other organs. The abscesses showed branching filaments of *Actinomyces* arranged in a radial disposition, giving a picture closely akin to that of the "sulphur granule" typical of the lesions found in natural infection of man. In the mouse, clubs at the edge of the lesion were not observed; so that the picture approximated more closely to that found in man than to that observed in bovines.

By killing animals at different times after inoculation I found that the abscesses underwent spontaneous regression from about 6 - 8 weeks after inoculation.

6. In fluid culture A. israelii may grow either in the rough form with long branching, a characteristic deposit, and clear supernatant or in the smooth form with diphtheroid morphology and even turbidity throughout the medium. The lesions in mice already described were produced by inoculation of the rough form. When inoculated into mice, smooth cultures gave rise to macroscopic lesions which superficially appeared to be similar to those produced by the organism in the rough filamentous form, but a clear difference in the tissue reaction was shown by histological examination. With smooth cultures nothing like a sulphur granule was seen, the diphtheroids did not give rise to branching filaments in the animal and large lipophagic macrophages were not seen.

7. Among the mice which recovered from the initial actinomycotic infection but died at 6 - 9 months after inoculation, some showed tubular nephritis, which is a type of lesion not previously described in mice. Other survivors of the initial infection died at about 12 - 18 months after inoculation; these did not show the presence of tubular nephritis. Attempts were made on a limited scale to produce tubular nephritis in mice by endotoxins of A. israelii. The results, though

inconclusive, were suggestive; they are given and discussed in the thesis.

8. Experiments were also carried out to compare the action of two other members of the order Actinomycetales with that of A. israelii upon inoculation into mice. Nocardia asteroides, an aerobic acid-fast branching and relatively uncommon pathogen of man and animals produced a fatal infection in the mouse. Abscesses formed in liver, spleen and kidney. Contrary to what was found with A. israelii the kidney became a focal point of the experimental infection which was invariably fatal within two months. The type of lesion and tissue reactions were entirely different from those produced by A. israelii. Actinomyces graminis, the aerobic saprophyte commonly found in grasses, had no effect upon mice, whether the inoculum employed was in the sporing or non-sporing phase.

9. The experimental infections thus produced with A. israelii in mice are reproducible if the necessary care is taken to observe the details of method emphasised in this thesis as important. The findings assist in establishing the status of the organism as an undoubted pathogen but of low virulence. The experimental infection has obvious uses in assessing the

value of chemotherapy and for studying the pathology of the disease. In this connection the renal lesions would appear to merit particular attention.

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