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EXPERIMENTAL SKELETAL TUBERCULOSIS IN THE GUINEAPIG

A METHOD FOR PRODUCING LOCAL LESIONS AND AN AUTORADIOGRAPHIC STUDY OF THEIR ACCESSIBILITY TO TRITIUM-LABELLED DIHYDROSTREPTOMYCIN

BY

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PART I

A method for producing experimental skeletal tuberculous lesions in guineapigs.

PART II

An autoradiographic study of the penetration of dihydrostreptomycin into experimental tuberculous lesions in the skeleton

PART I

I. INTRODUCTION AND PURPOSE

This investigation is a link in a research programme of skeletal infections, including skeletal tuberculosis. One of the purposes of the investigation was to devise a method for producing tuberculous lesions in the skeleton of guineapigs, *i.e.*, a method hitherto missing in the long-term study of slowly progressive tuberculous lesions in the skeleton. Such model experiments in laboratory animals are often necessary in the investigation of the course of the tissue reaction and other features of an infection, for example, as well as of the effect of therapy. It has hitherto not been possible to produce skeletal tuberculosis with a sufficiently low rate of progression. All attempts have failed either because the animals soon died from rapidly progressing systemic tuberculosis, or because the procedures induced skeletal lesions in far too small a percentage of the animals treated.

For such a method of the experimental induction of tuberculous lesions to be accepted as satisfactory in the long-term study of the individual foci it must satisfy the following requirements:

1) it should not result in premature death of the animals from systemic tuberculosis;

2) it must produce the tuberculous focus at a predetermined site; and

3) it must produce such lesions in a high percentage of the animals treated.

II. HISTORICAL

A search of the literature failed to reveal more than a few investigations in which skeletal tuberculosis had been induced. Müller (1886) produced foci in the tibia in goats by injecting tuberculous pus into the tibial artery. Friedlaender (1902) reported the regular development of tuberculous lesions in various parts of the skeleton of 5 goats following an injection of a mixture of tubercle bacilli and lycopodium into the femoral artery. Three of the animals died within 17 weeks and 2 were sacrificed after 31 weeks and 42 weeks, respectively.

Trudel (1933) reported the development of skeletal lesions in 75—85 % of rabbits following the injection of tubercle bacilli into the nutrient artery of the femur or the nutrient foramen of the femur. The duration of survival of the animals was not given.

Zappio and de Blasio (1935) succeeded in producing skeletal lesions in guinea-

pigs by depositing 1 mg of tubercle bacilli directly into the tibia, but the animals survived only 30—35 days.

Boquet and Laporte (1936), who used rabbits, claimed the development of skeletal lesions following intracutaneous injection of a special strain of tubercle bacilli, but the data they give are not sufficient to allow reproduction of the experiment.

Koch (1950) produced skeletal tuberculosis in unvaccinated rabbits by depositing a small volume — about the size of a pin's head — of human tubercle bacilli through a hole drilled in the distal part of the femur. Tuberculous lesions developed in the distal segment of the femur, in the knee and in the thigh of all 14 animals infected. Little is said about the duration or rate of survival of the animals, but at least one of them lived for 137 days after the inoculation.

Bastos-Mora and Portal (1953) injected tubercle bacilli directly into the femur of rabbits, in one third of which skeletal lesions afterwards developed. The duration of survival is not given. Several other workers in this field have also tried, but failed, to devise any satisfactory method for inducing local skeletal tuberculous lesions in rabbits, let alone in guineapigs. The lack of success was due either to early death of the animals from generalized tuberculosis or to the bacilli not obtaining a foothold in the bone marrow. Of particular interest in this connection is the work by Doan and Sabin (1927) and Mandelstamm (1933), in which histological examination showed that intravenous injection of tubercle bacilli had produced lesions in the bone marrow, but that these foci had healed (Doan and Sabin 1927) or had started to heal (Mandelstamm 1933) before the animal died from progressive tuberculosis of other organs.

In a search for factors of importance in the induction of local tuberculous lesions, it appeared but reasonable to peruse the literature on experimental, non-tuberculous osteomyelitis, which appears to resemble tuberculous osteomyelitis in origin and localisation.

The literature on human non-tuberculous osteomyelitis is enormous, but not that on experimental investigations of the disease in animals. Perusal of this part of the literature, which dates back to the end of the 19th century, when Rodet (1884) and Lexer (1894—98) published their first experiments, shows that difficulties have been encountered in producing non-tuberculous osteomyelitis in experimental animals. As in experimental skeletal tuberculosis, the animals often succumbed early (within a few days from septicaemia) and often before any demonstrable skeletal lesions had had time to develop.

Of the methods that have proved successful in the production of non-tuberculous osteomyelitis, *i.e.*, with a large percentage of takes and a relatively long survival of the animals, the following deserve mentioning.

1. Kuwahata (1930) induced Möller-Barlow's disease in young guineapigs in which he then produced osteomyelitis by injecting staphylococci intravenously.

2. Derizanov (1937 and 1938) sensitised rabbits by injecting horse serum subcutaneously. The sensitised animals were later given horse serum + staphylococci into the tibia with slowly progressing staphylococcal osteomyelitis as a

result. Csipak and Nemeth (1953) and Bashinskaya (1959) have since used this method with success.

4. Scheman et al. (1941 and 1943) injected first sodium morrhuate (which tends to produce necrosis and is also used as a sclerosant in the treatment of varicose veins) through a hole drilled in the tibia of rabbits, and half an hour later staphylococci through the same hole. This procedure invariably produced staphylococcal osteomyelitis. Unvaccinated animals survived at most 3 weeks, while animals pretreated with a "vaccination dose" survived longer.

5. On the basis of Letterer's theory (1948 and 1956) on the various stages of immunization Grundmann (1953) treated rabbits with staphylococcal vaccine and at various intervals after the vaccination he injected staphylococci intravenously and at the same time irradiated the tibia with a small dose of ultrasound, which served as a well localized and well dosed trauma. At a certain stage of immunization osteomyelitis of the tibia appeared, *i.e.*, at the site of the trauma.

III. PLAN OF INVESTIGATION

On the basis of preliminary experiments on more than 300 guineapigs it was decided to use unvaccinated animals (Series I), animals vaccinated with 0.3 mg BCG (Series II) and animals vaccinated with 40.5 mg tubercle bacilli (Series III), and the following experimental design:

1. Drilling of a hole through the outer side of distal part of the femur.

2. Insertion of pieces of Spongostan® impregnated with a suspension of tubercle bacilli through the hole into the marrow cavity.

3. Histological and bacteriological examination of both the operated femur and the unoperated femur as well as the lungs, liver and spleen of animals sacrificed 1 week to 6 months after insertion of the bacilli.

4. Examination of the histological picture of the bone marrow for any effect of the actual surgical trauma and of the deposition of the pieces of Spongostan in the marrow cavity.

5. Injection of a suspension of tubercle bacilli into the marrow cavity via the drilled hole to check the results reported by previous workers using this procedure.

IV. MATERIAL AND METHODS

A. EXPERIMENTAL ANIMALS

It was decided to use the guineapig, firstly because it has long been employed in tuberculosis research, and, secondly because the reactions of this animal to

human tubercle bacilli are so well known. In addition, the guineapig is easy to keep, it is readily infected with tubercle bacilli, and it is small enough to be used in series large enough to allow reliable statistical analysis of the findings.

The mouse, white rat and golden hamster are so small that the operation would have been technically difficult. Moreover, the histopathological picture of tuberculosis in the mouse differs essentially from that in the human being, in the guineapig and in several other experimental animals (Pagel 1940, Raleigh and Youmans 1948, Stewart 1950, Hurni et al. 1951, Grün and Klinner 1952, Lack 1956, Pathologie der Laboratoriumstiere 1958, Ippen 1959). Some authors claim that the guineapig is the animal of choice in experimental skeletal tuberculosis (Feldman and Hinshaw 1945, Steenken 1949). It would appear that the only animal that could have been used satisfactorily instead of guineapig is the rabbit.

In animal experiments it is of paramount importance that all procedures be standardized to avoid the introduction of unknown factors and to facilitate later reproduction of the experiments (Feldman and Hinshaw 1945, Raleigh and Youmans 1948, Hurni and Ragaz 1951, Spiess 1953).

In preliminary experiments not accounted for here the animals used had been obtained from A.B. Anticimex Breeding Station, near Stockholm. This station was, however, closed down before the present investigation for which the animals were purchased from the breeder who regularly supplies guineapigs to the Department of Bacteriology, Malmö General Hospital.

The animals weighed 200 gm. when purchased, this corresponds to an age of 8 weeks, so that the animals could be operated upon before the epiphyseal plate had fused (Robertson 1927, Thompson and Dubos 1938).

The animals were kept in the same stable. They were fed on hay, mainly timothy and in summer on fresh timothy grass. They were also given crushed oats and crushed barley supplemented in winter by cod liver oil, and mangels *ad lib.* instead of water. The animals used in the various experiments were selected irrespective of sex.

B. TUBERCLE BACILLI

Strain H 37 Rv from the Central Bacteriological Laboratory of Stockholm City, was used. This strain is passed through guineapigs twice a year. Between the animal passages it is recultured at least once a month on Löwenstein-Jensen medium.

The bacilli used for inoculation of the animals were cultured in the following way.

From test tubes containing Löwenstein-Jensen medium in which the bacilli were cultured, 10 platinum loopfuls of the culture were transferred to a dish. The colonies were homogenised with a glass rod and then suspended in 5 ml of physiological saline. This suspension was cultured further in test tubes containing 5 ml of liquid Dubos' medium. To each tube was added 0.5 ml of the suspension. The tubes were then covered — though not airtight — with a metal cap, and

allowed to stand for a week at 37° C, during which they were thoroughly shaken once a day.

At the same time 5 pieces of Spongostan (see page 17) were placed in each of a series of test tubes containing 5 ml of Dubos' medium. These tubes were also covered with a metal cap and kept at 37° C for a week, after which their contents were examined for growth. This was done to check that the pieces of Spongostan had not been contaminated. Preliminary experiments had shown that the pieces of Spongostan are liable to become infected when being cut out and handled.

After one week's incubation at 37° C the suspension was vigorously shaken, after which 1 ml of the culture was transferred to the above mentioned tubes containing Spongostan and culture medium. These tubes were re-incubated one week at 37° C during which they were thoroughly shaken once a day. Tubes in which contamination was suspected were rejected. On several occasions the purity of the cultures was checked by microscopic examination of stained smears.

When the pieces of Spongostan were treated in this way, embedded in paraffin, cut into microscopic sections and stained with Ziehl-Neelsen stain, they were seen to contain numerous tubercle bacilli in the pores of the Spongostan.

In experimental tuberculosis the rate and duration of survival of the animals, the course of the disease, and the histopathological picture vary with the size of the inoculum. The inoculum should therefore preferentially contain a predetermined number of bacilli (Feldman and Hinshaw 1945, Raleigh and Youmans 1948, Rosenthal 1957). In the method used in this investigation it was not possible to calculate the size of the inoculum, because the bacilli were situated in the pores of the Spongostan. This disadvantage was, however, not a serious one because it did not impair the usefulness of the method.

C. VACCINATION

A troublesome problem in experimental skeletal tuberculosis is the short duration of survival of the animals owing to massive spread of tubercle bacilli. This problem is serious because a typical skeletal lesion with reactive changes requires a fairly long time to develop. The duration of survival can be prolonged by previous vaccination with BCG (Bogen and Loomis 1935, Spiess 1953, Griesbach 1954, Rosenthal 1957).

The duration of survival of vaccinated animals varies with the interval between vaccination and superinfection. According to Bogen and Loomis (1935), in guineapigs vaccinated with doses between 0.0001 mg and 100 mg BCG resistance to tuberculosis begins to increase about 2 weeks after vaccination and is clearly increased after a further 2 weeks. In their animals the resistance was largely the same after 2 years. In the present experiments the animals were superinfected 2—3½ months after vaccination had been started (*i.e.*, 1—2 months after conclusion of vaccination).

According to some authors, immunological factors may also decide the site and

location of an infection (Lurie 1939 and 1942, Letterer 1948 and 1956, Csipak and Németh 1953, Grundmann 1953, Csipak et al. 1954, Ström 1955, Csipak 1956, Rosenthal 1957, Freerksen and Meissner 1960). These factors are commented upon in the Discussion (page 42).

In the present work the experimental animals were vaccinated for two reasons: to prolong the duration of survival and, secondly, if possible, to produce at the site of inoculation a reaction which, according to the authors referred to above, may be of importance for locating an infectious skeletal focus.

When purchased and again just before use the animals were examined with intracutaneous injection into the right flank of 0.1 ml of tuberculin diluted 1:100. All animals in which the reaction was equivocal were rejected. The experiments were carried out on three series of animals.

Series I consisted of unvaccinated animals. So that the animals in all three series could be operated upon at the same age the unvaccinated animals in this series were kept untreated in the stable until vaccination of the animals in series II and III had been concluded.

Series II was made up of animals vaccinated with 0.3 mg of BCG. The animals were vaccinated with 0.2 ml freeze-dried BCG vaccine, 0.5 mg bacilli/ml, wet weight, subcutaneously in the neck, three times at one week intervals. Six weeks to two months after the last vaccination the tuberculin test was performed and reactions characterised by a palpable thickening with erythema at least 10 mm in diameter after 2 and 3 days were said to be positive. All BCG vaccine was obtained from Statens BCG-laboratorium, Gothenburg.

Series III consisted of animals vaccinated with 40.5 mg bacilli. The animals were vaccinated in the way described below. They were first given an intraperitoneal injection of 1 ml and a subcutaneous injection of 0.5 ml BCG vaccine containing 20 mg bacilli/ml wet weight. This was followed one week later by administration of 0.15 ml of a paraffin oil suspension of killed bovine tubercle bacilli, strain K3, 10 mg bacilli/ml, wet weight, once a week for 7 weeks. The suspension was obtained from Statens Bakteriologiska Laboratorium, Stockholm. The injections were given *s.c.* at different sites in the neck and schoulders. A tuberculin test was done 1 month after the last injection of bacilli. The reaction was read after 2 and 3 days. All animals showed a positive reaction with thickening and reddening of the skin over an area of 1.5×1.5 to 3×3 cm. This area was characterised by a central pale necrotic area surrounded by a margin of intense reddening 2—4 mm wide. After a further few days the necrotic tissue was shed leaving behind a sore. This reaction is in agreement with the observations made by Wassermann (1962 and personal communication 1963).

D. DEPOSITION OF TUBERCLE BACILLI INTO MARROW CAVITY

Previous publications give but little information on the possible factors responsible for the location of infections in a bone and its marrow cavity. The present method of infection was therefore entirely empirical. No attempts were

made to simulate infection by a "normal" route, the purpose of the method being solely to produce a local lesion at a predetermined site in the skeleton.

1. *Choice of site of infection*

It was decided to use the lower part of the femur because it is readily accessible and because in human tuberculous and staphylococcal osteomyelitis this part of the skeleton is not uncommonly affected. This site satisfies some of the conditions which previous workers believe to be necessary for osteomyelitis to develop there, *e.g.*, the bone has red marrow and the epiphyseal plate is not fused (Randerath 1931, Thompson and Dubos 1939).

2. *Operation technique*

The operation was performed under intraperitoneal anaesthesia. 0.3 ml of Mebumal for veterinary use was mixed with 0.7 ml physiological saline. Of this mixture, 0.2 ml/100 g bodyweight was given intraperitoneally. 5—10 % of the animals died while under the anaesthesia. The hair on the lateral side of the left leg was cut off with scissors. The skin over the intended site of the incision was washed with a solution of benzalkonium chloride or the like. An incision 2—3 cm long was made through the skin in the longitudinal direction of the femur, after which the femur was readily approached between the muscles with only slight loss of blood. The edges of the wound were held apart with small elastic retractors. With a dental burr 1.2 mm in diameter an oblique hole was drilled in mediodistal direction at the junction between the distal metaphysis and the diaphysis. A piece of Spongostan containing tubercle bacilli was inserted through this hole. Blood and bacterial suspension extruded from the piece of Spongostan were swabbed up, after which the wound was closed. The operation required 5—10 minutes.

At operation attempts were made to deposit the bacilli in the distal metaphysis. But this was often difficult without involving the risk of penetrating the epiphyseal plate. The bacilli were therefore usually deposited at the junction between the metaphysis and the diaphysis.

3. *Spongostan*

Spongostan is a hardened porous gelatin spongy substance manufactured by Ferrosan, Malmö. It is used as a haemostatic in surgery. In the present investigation it was used as a carrier or receptacle for depositing the tubercle bacilli into the bone marrow cavity.

In an attempt to retain the bacilli at the site where they were deposited a search was made for a carrier substance that is fairly rapidly resorbed and that is practically inert. Various substances were considered such as polyvinylalcohol (resorbable) or polyvinyl sponge (not resorbable), both of which are practically inert (van Winkle 1960), methylcellulose (Hueper 1942 and 1944, Hueper et al. 1942) or hydroxyethylocellulose (Jullander 1960 and personal unpublished ex-

periments). Spongostan was, however, selected because it fills the aforementioned requirements and because its behaviour when deposited into living tissue is well known (Correll et al. 1945, Jenkins and Clarke 1945, Jenkins et al. 1946, Jenkins 1947, Bing 1947, MacDonald and Matthews 1947).

Previous authors have found sterile Spongostan deposited in various organs (liver, kidney, subcutis) of experimental animals to be resorbed within about 5 weeks. No experiments had been performed to study the rate of resorption in a medullary cavity. The Spongostan is said to be removed by macrophages and giant cells. The tissue reaction was judged as only mild, much milder than round suture silk, catgut and pieces of muscle, for example. It was reminiscent of that around a blood clot. When non-sterile Spongostan was placed in the tissues it was mainly polynuclear cells that appeared and then the sponge was resorbed much more rapidly and disappeared within one week.

With the aid of a trephine 3 mm in diameter cylinders were removed under sterile precautions from commercially available sheets of Spongostan about 1.5 cm thick. After removal and consequent expansion these cylinders were about 1 cm high and 3—4 mm in diameter.

As mentioned on page 15, these cylinders were placed in test tubes containing tubercle bacilli suspended in Dubos' liquid medium. Five cylinders were placed in each tube.

E. HISTOLOGICAL PREPARATIONS

In series I—III the entire operated left femur, the unoperated right femur and pieces of the lungs, liver and spleen were removed for histological examination. The necropsy specimens of the lungs, liver and spleen were excised from a predetermined part of each of the organs. The tissue specimens were placed immediately in 10 % formalin, and 1—4 weeks later they were sectioned and stained with haematoxylin-eosin and according to van Gieson and often also according to Ziehl-Neelsen.

The bones were decalcified with trichloracetic acid, or with formic acid, or nitric acid or by the method of Parengi. On one occasion the operated bone was decalcified electrolytically. Only in the electrolytically decalcified bone did staining acording to Ziehl-Neelsen show acid-fast rods. It would appear that the first four methods abolish or severely reduce the stainability of acid-fast rods according to Ziehl-Neelsen. Histological examination of bones decalcified by the four aforementioned routine methods showed a typical feature of tuberculosis but no acid-fast rods in slides stained according to Ziehl-Neelsen. That the animals really had tuberculosis was apparent from sections of the lungs, liver and spleen, which showed not only the typical picture, but also acid-fast rods when stained according to Ziehl-Neelsen. The diagnosis of tuberculosis was also confirmed by the positive results of culture of the bone marrow (page 41).

The examination of sections stained according to Ziehl-Neelsen was regarded only as a supplementary method, because the procedure is time-consuming and if the findings are negative, several sections should be examined. Examination of

sections stained by this method was therefore confined mainly to those cases in which the changes of minute foci in the liver and in the spleen, for example, were dubious. In these cases demonstration of acid-fast rods was useful.

F. CONTROL SERIES

1. *Surgical trauma*

In order to check the effect the surgical trauma *per se* had on the histological picture, 32 animals were subjected to sham operations, *i.e.*, the same operation as that performed on the experimental animals but without insertion of the Spongostan and bacteria.

The animals were sacrificed after various intervals, *viz.* 2, 4, 6 and 9 days, and 2, 3, 4, 5, 6, 7, 8 and 10 weeks. The legs operated upon were removed, decalcified, sectioned, stained and examined.

Results

In animals killed on *the 2nd day* after the operation a haematoma and bone and other tissue fragments were seen at the site of intervention. The haematoma extended through the drilled hole and into the medullary cavity. Some areas showed incipient cell proliferation from the endosteum.

By *the 4th day* a fair amount of granulation tissue had formed. This tissue contained bone fragments surrounded by numerous osteoclasts and osteoid trabeculae were beginning to appear.

By *the 6th day* the entire haematoma was invaded by granulation tissue and osteoid callus was fairly abundant. Periosteal callus was now seen in those areas around the drilled hole where periosteum had not been scraped off during the operation.

By *the 9th day* all, or almost all, of the granulation tissue in the medullary cavity had been replaced by endosteal callus in the form of a well developed trabecular system. In areas where the periosteum had not ben scraped off a similar periosteal callus had formed. The masses of callus extended along both the inside and the outside of the orifices of the burred hole.

After *2—3 weeks* the callus appeared to be fully developed. The osteoblast seams were no longer conspicuous, and red bone marrow had begun to extend between the trabeculae.

After *4 weeks* the resorption of the callus in the medullary cavity was so advanced that the most of the bone trabeculae had disappeared, and after *5 weeks* residual callus was seen in only one of the 4 preparations studied.

After *6, 7, 8 and 10 weeks* small, scattered rests of bone fragments were seen in the marrow, which was otherwise of normal appearance.

In a few preparations removed on the 4th day or later the preparations showed no sequelae after the operation trauma. This was probably because the reaction to the trauma in these cases was so small that it did not reach the middle of the marrow cavity, *i.e.*, that part of the marrow sectioned and examined.

2. *Resorption of Spongostan*

The following experiments were designed to assess the rate of resorption of Spongostan and the tissue reaction round the pieces of Spongostan deposited in the red bone marrow.

Forty-four guineapigs were operated upon in the way described above. A dry sterile piece of Spongostan of the same size as that used in the experiments with bacilli was inserted into a hole drilled in the femur and the wound was closed. The animals were killed after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 weeks (one animal died after 4½ weeks). The legs operated upon were removed, fixed in 10 % formalin, decalcified, sectioned and stained.

Results

After *1 week* granulation tissue made up of capillaries, fibroblasts and some polynuclear leucocytes and macrophages began to invade the Spongostan. A number of foreign body giant cells were seen in the periphery of the Spongostan.

In several areas normal bone marrow had grown into the Spongostan, but in some the normal bone marrow was separated from the Spongostan by a thin wall of granulation tissue.

The amount of callus and of Spongostan in the sections studied varied, probably because of differences in the position of the sections in the bone marrow.

After *2 weeks* the Spongostan was still invaded by the same cells as after 1 week. It is difficult to say whether any resorption had occurred because of the variation in the amount of Spongostan with the position of the sections studied.

The callus found in the marrow cavity was already sclerotic with thick, crowded trabeculae. Bone newformation was practically concluded and blood forming marrow had found its way between some of the trabeculae.

After *3 weeks* the resorption of Spongostan was well under way, the residual pieces of Spongostan were thin, less well defined and were sparser than after 1 week. The Spongostan was now enclosed in a thin layer of granulation tissue.

After *4 weeks* the spread of the Spongostan varied from a volume which together with the tissue reaction occupied half of the cross sectional area of the marrow cavity down to a few minute vesicular rests without any tissue reaction.

The tissue reaction was of the same type as after 3 weeks. When the amounts of residual Spongostan were large they were separated from the marrow by a thin wall of granulation tissue.

Bony callus was well preserved in 3 of the preparations, where it was of the same type as after 3 weeks, *i.e.*, sclerotic and with normal marrow between the trabeculae. In one preparation, the one with the largest amount of Spongostan, there was no bony callus at all.

After *5 weeks* the amount of callus had definitely decreased owing to resorption. This tendency to resorption was also noted in earlier preparations, but owing to the variation in the appearance of the sections with their position in the medullary cavity the decrease in the amount of callus could not be regarded as significant until after 5 weeks. Some of the preparations showed only minute pieces of Spongostan situated in the marrow without any surrounding tissue reaction.

The callus, which in the series after sham operation without insertion of Spongostan was practically all resorbed within 5 weeks, had in this series only been partly resorbed.

The last pieces of Spongostan were resorbed during *the 6th to 8th weeks.* A few preparations examined after this period showed small "scars" of connective tissue rich in cells and containing some minute rests of Spongostan and a few small pieces of necrotic bone. This picture represents one of the last stages of resorption of Spongostan before the histological picture of the red bone marrow again becomes normal. During this time the resorption of the bony callus became more rapid.

From *the 9th week* onwards none of the preparations contained any Spongostan and apart from a few small bone fragments, *i.e.*, rests of callus in some of the preparations, the changes had been replaced by normal red bone marrow.

In some preparations from animals killed soon after the operation only little or even no Spongostan could be detected. This was probably because the sections studied had missed the Spongostan. It was often necessary to study serial sections before any Spongostan could be found.

Comparison of the marrow after a sham operation without insertion of Spongostan and with Spongostan revealed some differences in the course of healing, but the picture of the site of the operation at the end of the study period after 10—12 weeks was the same, *i.e.*, normal red marrow with some small bone trabeculae which might have been rests of callus. The differences in the course of healing of the two types of operations were:

the callus formed persisted 1—2 weeks longer in the series in which Spongostan was inserted in the marrow cavity, and

the reaction in and round the Spongostan, which was missing in the first series. The Spongostan was, however, resorbed within about 8 weeks, after which the bone marrow was of normal appearance without any traces of cicatricious tissue.

This final picture was seen after 5—6 weeks in the series operated upon without Spongostan and after 6—9 weeks in the series subjected to the sham operation with insertion of Spongostan.

3. *Operation and injection of tubercle bacilli into the bone marrow*

In order to check the published results of injection of bacilli directly into the marrow cavity (pages 11—12), 16 unvaccinated guineapigs were operated upon in the way described. 0.05 ml of a suspension of tubercle bacilli prepared in the way described (pages 14—15) was injected through the drilled hole. Two animals were killed every other week. Five of the animals died during the experiment and thereby shortened the experimental period to 12 weeks. The legs operated upon were removed and examined histologically in the usual way.

Results

After *2 weeks.* No focus at site of operation. Several small foci scattered about in the marrow.

After *4 weeks.* One small focus at site of operation in both animals. Several small foci scattered about in the marrow.

After *6 weeks.* No focus at site of operation. Several small foci scattered about in the marrow.

After *8 weeks.* One small focus at site of operation in one of the animals. Several small foci scattered about in the marrow.

After *10 weeks.* A large abscess at the site of the operation in one of the animals. Several small foci scattered about in the marrow.

After *12 weeks.* No focus at site of operation. A few scattered foci in the marrow.

Of this series of 16 animals, an abscess was seen at the site of operation in only 1, and small foci in only 3. As in the main experiments described on pages 22—40 (Series I—III), small foci of epithelioid cells were also found out in the medullary cavity. The results are in agreement with those found by previous workers using this procedure, *i.e.*, progressive tuberculosis at the site of inoculation developed in only a few of the animals used.

Already in animals killed after 4—6 weeks necropsy revealed gross caseous foci in the lungs, liver and spleen as well as in the lymph nodes.

V. RESULTS OF PERSONAL EXPERIMENTS*)

The animal experiments described in this chapter were preceded by preparatory experiments on more than 200 guineapigs in which principally the same method was used and in which largely the same results were obtained as those described below. Since various technical details were modified during the elaboration of the method, only the results obtained in the last 3 experimental series with the final experimental design are described here.

Table 1 gives the intervals between the operation and sacrifice of the animals as well as the number of animals used for histological and bacteriological studies. None of the animals in this experiment died spontaneously.

In series I and III the experiments were stopped after 4 months and 3 months, respectively, because in preparatory experiments animals treated in the same way as in series I often died within 4 months, and in those treated in the same way as in series III the skeletal lesions usually healed within 3 months.

*)The evaluation of the histo-pathological preparations was supervised by professor Folke Linell, M.D., Department of Pathology, Malmö General Hospital, University of Lund, Malmö, Sweden.

TABLE 1

Survey of experimental animals in Series I—III

Interval between operation and sacrifice of animals	1 w	2 w	3 w	4 w	5 w	6 w	8 w	10 w	12 w	4 mths	5 mths	6 mths	
Series I													
Histological preparations	2	2	2	2	2	2	2	2	2	2	—	—	20
Culture of bacteria	—	1	1	1	1	—	1	—	1	—	—	—	6
Series II													
Histological preparations	2	3	3	2	3	3	2	2	2	2	2	1	27
Culture of bacteria	—	1	1	1	—	1	1	1	1	1	1	—	9
Series III													
Histological preparations	2	2	2	2	2	2	2	2	1	1	—	—	18
Culture of bacteria	—	1	1	1	—	1	1	1	—	—	—	—	6

A. LESIONS IN THE OPERATED FEMUR

1. *Development in series I*

After *1 week* the major part of the medullary cavity at the site of the operation was filled with non-tuberculous granulation tissue round a fairly large amount of Spongostan, which was undergoing resorption. Tissue fragments after the surgical trauma were also seen. Osteoid trabeculae were observed in various areas. No structures typical of tuberculosis were found at the site of operation.

The upper medullary cavity exhibited small areas with diffuse accummulations of macrophages, possibly a sign of incipient tuberculosis. These areas also contained single giant cells.

After *2 weeks* the area with granulation tissue at the site of the operation was the same size as after one week, but the tissue had now begun to become tuberculous and contained numerous epithelioid cells, macrophages and some giant cells of Langhan's type. A previously newformed trabecular system round the granulation tissue was being resorbed by the tuberculous tissue, and Howship's lacunae with clusters of osteoclasts were seen in several areas. The diffuse accumulations of macrophages in the medullary cavity had now assumed a tuberculous appearance and contained epithelioid cells. The bulk of the Spongostan had been resorbed, but small fragments were still seen in all preparations.

The lesions progressed and after *3 weeks* the foci at the site of operation had developed into productive tuberculous lesions with epithelioid cells and Langhan's cells. They now filled the entire cross sectional area of the medullary cavity. One focus showed signs of commencing central necrosis. Both osseous destruction and bone-newformation were seen in the periphery of the foci. The small foci scattered in the medullary cavity now consisted mainly of epithelioid cells and contained a large number of giant cells. One of the foci showed incipient central necrosis. Spongostan fragments were still seen in one preparation.

After *4 weeks* the necroses had increased in size, and the last pieces of residual Spongostan were seen. The small epithelioid cell foci in the medullary cavity persisted unchanged.

The first fully developed abscesses were seen after *5 weeks*. The foci had then increased in size and occupied one fourth to one third of the entire distal diaphyseal medullary cavity. The abscesses were made up of a central area of necrosis, which merged with a narrow border of epithelioid cells, which was in turn surrounded by a thin cuff of non-tuberculous tissue with *inter alia* fibrous streaks and in some areas also bone trabeculae. Largely the same picture was seen in all of the abscesses in series I, II and III.

The necroses continued to increase at the expense of the productive granulation tissue and the abscesses became larger, so that after *6 weeks* they occupied the distal half of the medullary cavity down to the metaphysis. Single, small, scattered epithelioid cell foci were still seen in the marrow.

From the *beginning of the 6th week to the end of the 4th month,* when the experiment was stopped, all of the preparations except two showed a largely uniform picture, namely a large abscess of principally the same structure as that described above. The abscesses varied in size, the largest occupying the entire diaphyseal medullary cavity; the smallest, only the distal fifth.

In two preparations (after 10 and 12 weeks), however, the site of the operation

showed only one small focus occupying about one fourth of the cross sectional area of the medullary cavity. Both lesions consisted largely of epithelioid cells and one of them showed a small necrosis.

Scattered small foci were seen in practically all preparations, and even in those animals in which no such foci were seen the presence of such lesions cannot be excluded because they may have been so small as to have been missed by the sections studied.

2. *Development in series II*

After *1 week* the medullary cavity at the site of the operation was filled with non-tuberculous granulation tissue. This tissue contained *inter alia* numerous polynuclear cells and small bits of bone undergoing absorption. Osteoid trabeculae had began to form. No fragments of Spongostan were found in any of the preparations.

Out in the medullary cavity were small scattered groups of macrophages and epithelioid cells and, in one preparation, also numerous Langhan's cells.

Already after *2 weeks* the distal part of the diaphyseal medullary cavity showed large abscesses. In the central necrosis were numerous polynuclear leucocytes and around the necrotic centre was a broad cuff of granulation tissue which had assumed a tuberculous appearance, and in several areas it contained bone fragments after the operation. Intense bone-newformation and bone-destruction were seen in several areas. The abscesses with the peripheral tissue reaction occupied the entire cross-sectional area of the medullary cavity. The abscesses were of largely the same structure as that in series I (page 24).

Small scattered clusters of epithelioid cells and macrophages, sometimes with Langhan's cells, were seen in the marrow of all preparations.

After *3 weeks* no Spongostan fragments were demonstrable and after *4 weeks* the central necroses in the abscesses had become more caseous, but otherwise the picture was largely unchanged.

After *5 weeks* the tuberculous abscesses at the site of the operation began to decrease in size owing to the reactive bone-newformation in the medullary cavity. It is difficult to estimate how much the reactive bone formation had progressed, because the extent of such formation in the medullary cavity varied with the level of the histological sections studied.

In later preparations the healing process could be followed up to the disappearance of the abscesses in preparations of animals killed after *4 months*.

The abscesses occupied a decreasing proportion of the cross sectional area of the medullary cavity, they sometimes increased in length, but were forced back towards the centre of the medullary cavity by normal bone marrow and spongy bone developing between the wall of the abscess and the inner side of compact bone. In some preparations, especially in those from animals killed after 10 weeks, the distal part of the medullary cavity was entirely filled with a sclerotic trabecular system.

After *4 months* the foci at the site of the operation had disappeared. Healing had advanced and the distal fourth of the diaphyseal medullary cavity was filled with sclerotic trabeculae. The trabecular interstices were filled with normal red bone marrow. The trabecular system successively decreased in size and after *5 to 6 months* only some sparse thick trabeculae persisted free in the medullary cavity. The last sign of tuberculosis at the site of operation, a small necrotic centre, possibly the remains of a once larger lesion, surrounded by tuberculous tissue diffusely outlined against surrounding bone marrow, was seen in a preparation from an animal killed after 5 months.

Small, scattered foci were seen in the bone marrow in practically all preparations from

the very beginning until the end of the 6th month after the operation. They varied in size but were never as large as those at the actual site of the operation, the smallest ones appearing to consist of only some tens of cells. These foci were seen in the diaphysis, metaphyses and epiphyses. Some of them showed signs of central necrosis but broadly speaking their appearance remained unchanged during the entire experimental period.

3. *Development in series III*

As in series I and II, after *1 week* the medullary cavity at the site of the operation was full of non-tuberculous granulation tissue with residual necrotic tissue and inflammatory cells. Osteoid trabeculae had begun to form and fairly numerous fragments of Spongostan were found in the preparations.

The marrow contained small scattered foci with *inter alia* epithelioid cells.

Already after *2 weeks* abscesses of the previously described structure (see page 24) were seen at the site of the operation. In one preparation the abscess extended down into the metaphysis. Small fragments of Spongostan were still seen.

Small, scattered clusters of epithelioid cell were observed in the marrow.

The abscesses persisted largely unchanged during the *3rd and 4th weeks* with certain variations from one animal to another. After 3 weeks the Spongostan had disappeared.

After *5—6 weeks* some of the foci had decreased in size owing to the formation of normal marrow between the compact bone and the wall of the abscess. It is difficult to say when this healing had started because of the differences between the individual preparations. It is possible that it had started already during the 3rd to 4th week.

The healing process advanced and in the preparation from one of the animals killed after *8 weeks* the abscess had largely healed and left behind only a small remnant while an abscess was seen in the preparation from the other animal.

After *10 weeks* the preparation from one animal showed no signs of tuberculosis while a large abscess occupying two thirds of the medullary cavity was found in the other. Sclerotic trabecular systems still persisted at the site of the operation after 8 and 10 weeks even when the abscesses had disappeared.

After *12 weeks* (one preparation) and *4 months* (one preparation) there were no signs of tuberculosis, and only a small rest of a trabecular system was found at the site of the operation.

From the 3rd week inclusive the small scattered clusters of epithelioid cells were sometimes missing and from the beginning of the 10 th week no such clusters were seen in any of the preparations.

4. *Comparison between the three series*

a. Lesions at the site of the operation

During the first week no tuberculous lesions developed in any of the series: all showed only a non-tuberculous reaction at the site of the operation (Fig. 1).

In series I a productive focus developed during the 2nd and the 3rd weeks (Figs. 2 and 3) and central necrosis began to develop after about 3 weeks (Fig. 4), to increase in size during the 4th week and to develop into a typical abscess after the 5th week with a largely necrotic centre surrounded by a thin wall. In series

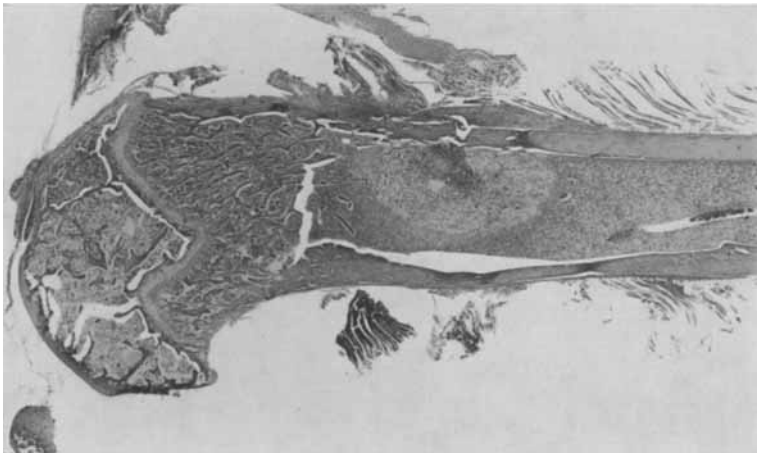

Fig. 1
Site of operation 1 week after operation. Series I. Htx-eosin. ×6.

I the abscesses then increased in size and after 6 weeks they occupied a large part of the medullary cavity (Figs. 5 and 6). In all preparations, except 2, large abscesses were then found throughout the experimental period, *i.e.*, until the end of the 4th month.

In series II the fully developed abscesses appeared already after 2 weeks (Figs. 7 and 8) and they began to decrease in size after about 5 weeks and to disappear after about 4 month (Figs. 9—13).

In series III the fully developed abscesses also appeared after 2 weeks (Fig. 14) and the healing of the lesions at the site of the operation appeared to progress in the same way as in series II (Fig. 15).

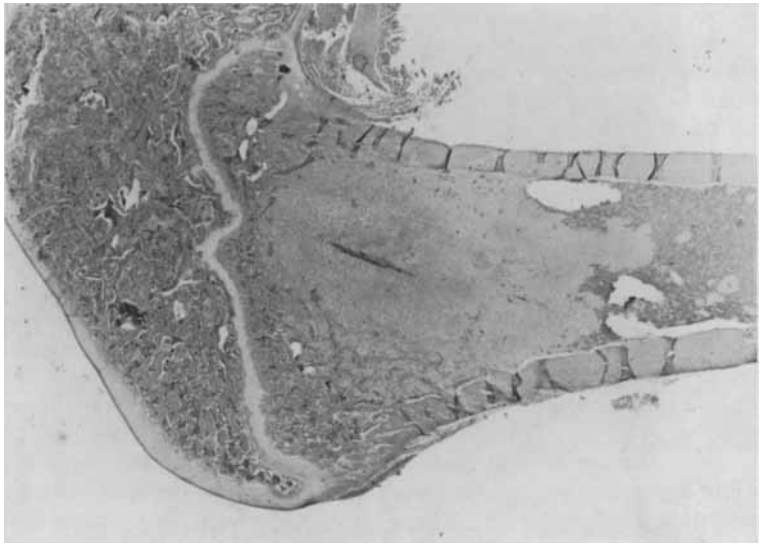

Fig. 2
Site of operation 2 weeks after operation. Series I. van Gieson. ×9.

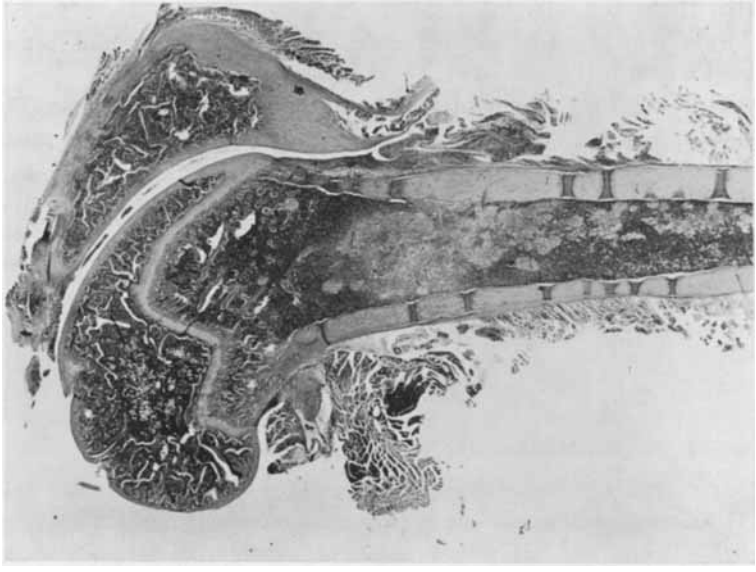

Fig. 3
Productive focus 3 weeks after operation. Series I. Htx-eosin. ×7.

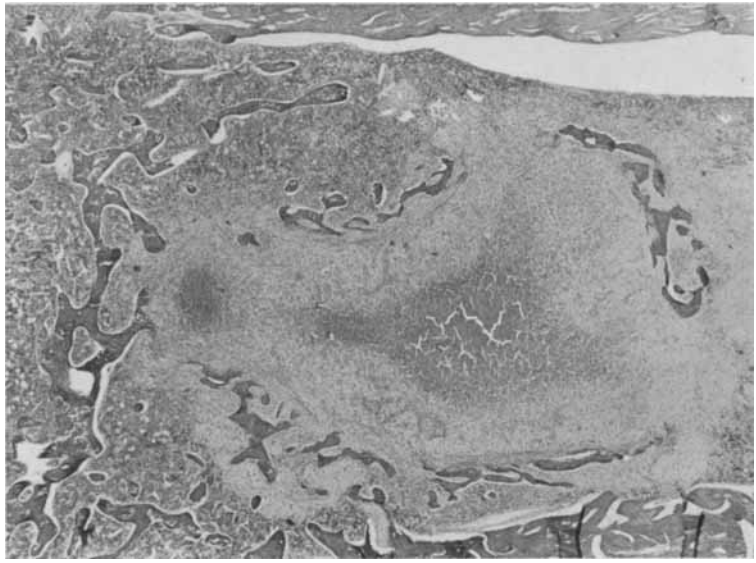

Fig. 4
Beginning central necrosis in lesion 3 weeks after operation. Series I. van Gieson. ×20.

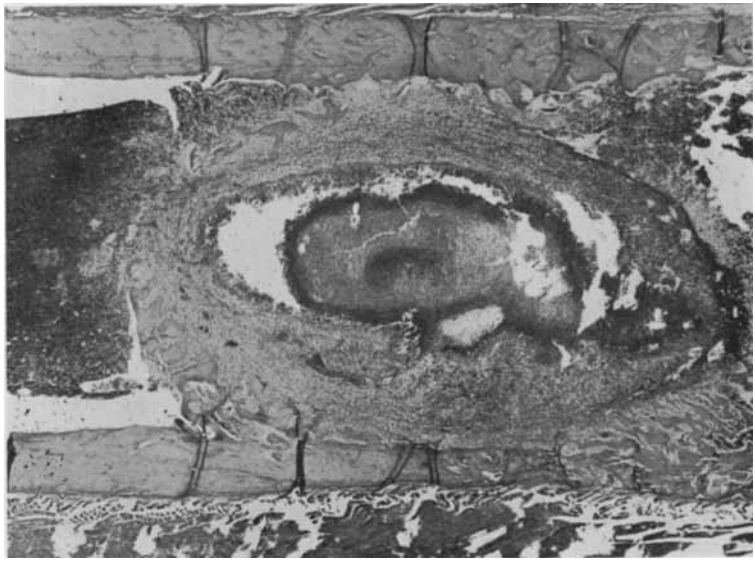

Fig. 5
Typical abscess. 6 weeks after operation. Series I. Htx-eosin. ×18.

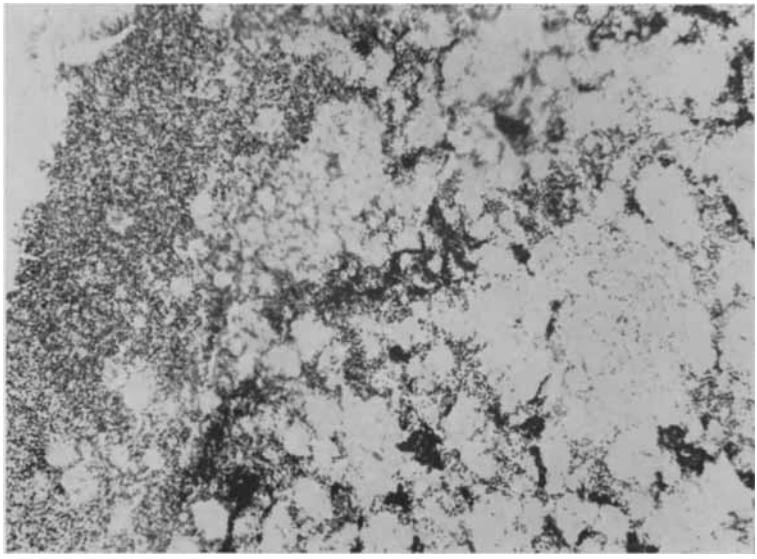

Fig. 6
Necrosis 6 weeks after operation. Series I. van Gieson. ×170.

Fig. 7
Central necroses surrounded by tuberculous, productive tissue and newformed bone, 2 weeks after operation. Series II. van Gieson. ×9.

Fig. 8
Abscess 3 weeks after operation. Series II. Htx-eosin. ×6.

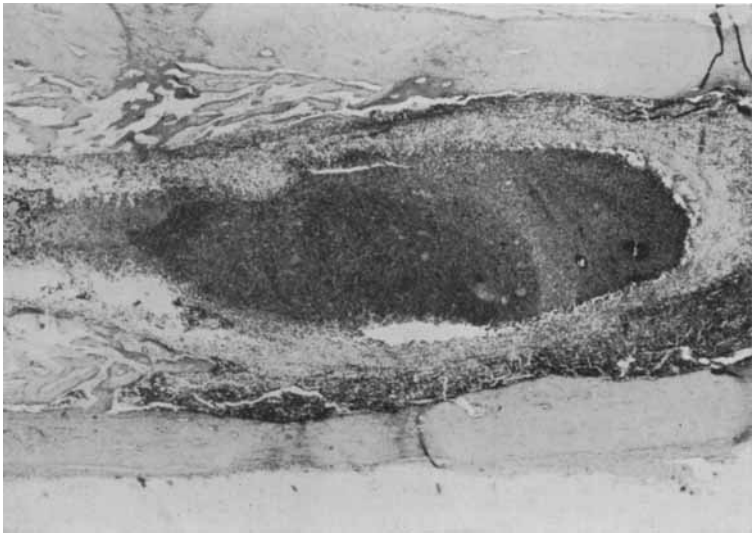

Fig. 9.
Abscess 6 weeks after operation. Series II. van Gieson. ×20.

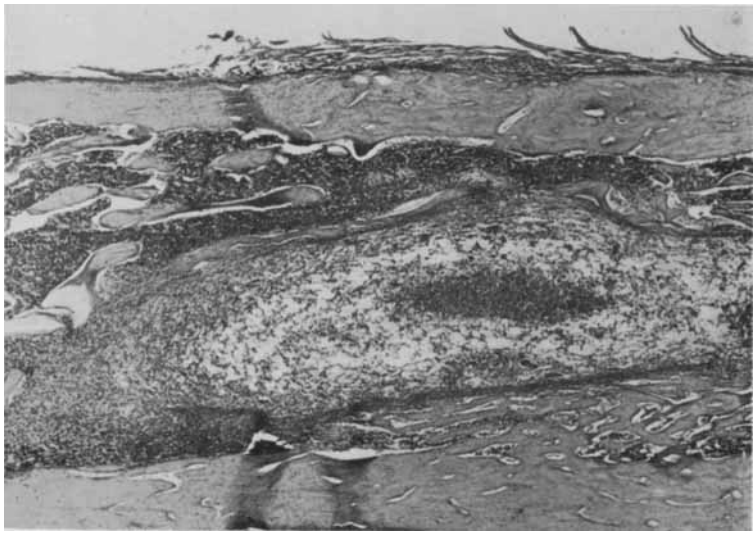

Fig. 10
Initial stage of healing of abscess 6 weeks after operation. Series II. Htx-eosin. ×20.

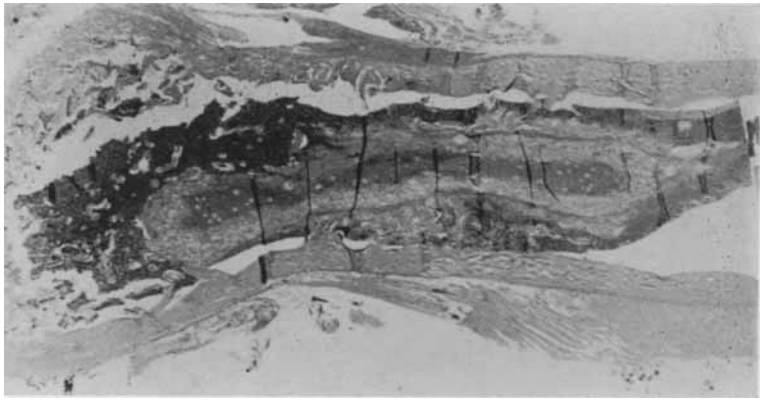

Fig. 11
Healing abscess 8 weeks after operation. Note the normal bone marrow between the wall of the abscess and the compact bone. Series II. van Gieson. ×9.

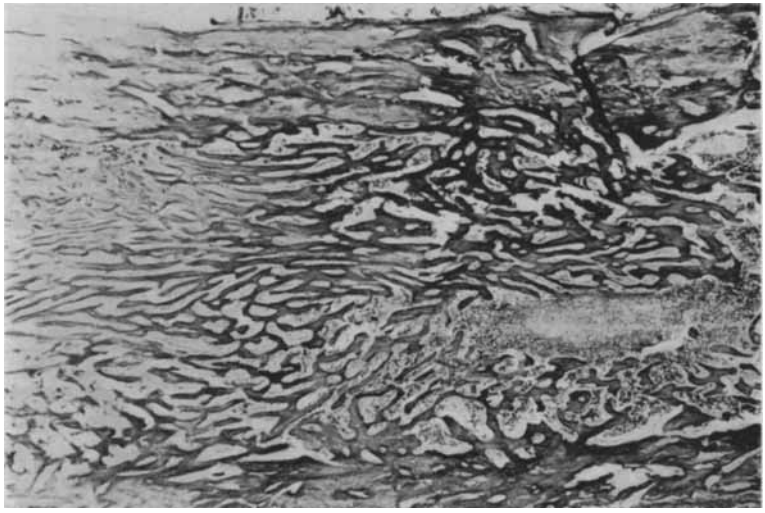

Fig. 12
Sclerotic bone in medullary cavity 10 weeks after operation. Observe that section passes through centre of medullary cavity. Series II. van Gieson. ×25.

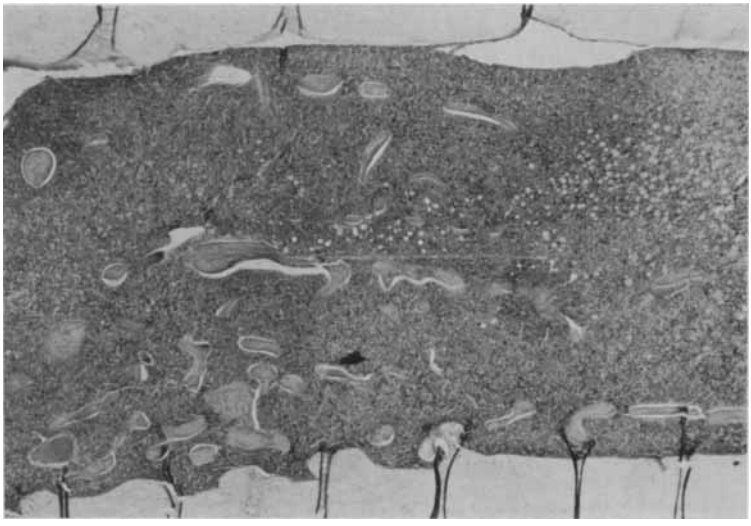

Fig. 13
Medullary cavity at site of operation 6 months after operation. Series II. van Gieson. ×20.

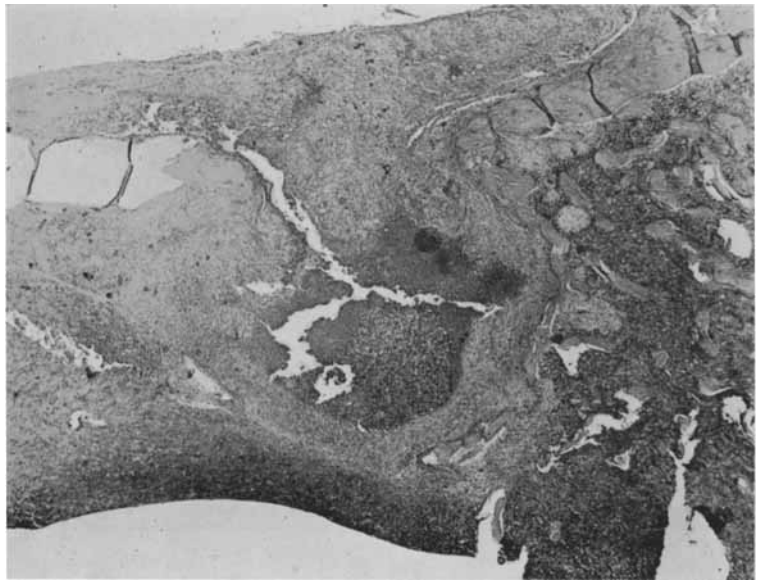

Fig. 14
Abscess 2 weeks after operation. Series III. van Gieson. ×20.

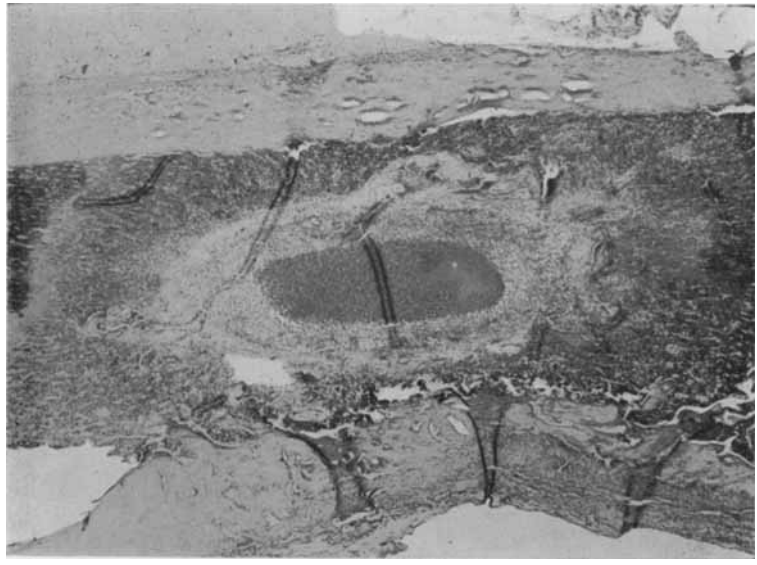

Fig. 15
Healing abscess 6 weeks after operation. Series III. van Gieson. ×20.

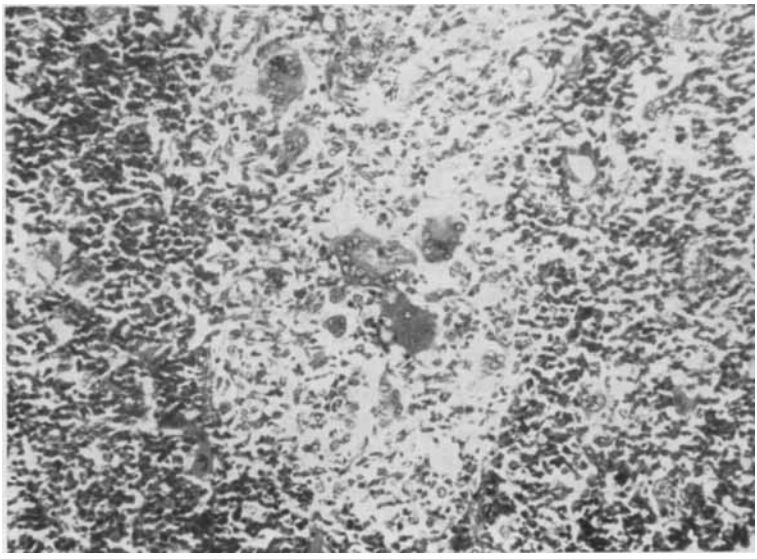

Fig. 16
Small focus in the bone marrow remote from the site of the operation. 3 weeks after operation. Htx-eosin. ×170.

b. Lesions in the medullary cavity outside the operative field

The small scattered foci seen remote from the site of application of bacilli (Fig. 16) appeared after about 1 week in all 3 series and in almost every preparation in series I and II, while in series III they were sometimes no longer demonstrable after 3 weeks and never from the 10th week on.

B. TUBERCULOUS LESIONS IN LUNGS, LIVER, SPLEEN AND THE UNOPERATED CONTRALATERAL FEMUR

1. *Development of pulmonary lesions*

In the evaluation of the results of this part of the investigation it should be borne in mind that only a piece of lung tissue, about one fifth of the left lung, was examined in every animal. Tuberculous changes differing in type from those described below may thus have occurred in those parts of the lungs that were not examined.

Series I

No histological signs of tuberculosis were seen during *the first 2 weeks*. After *the 3rd —4th weeks* the pulmonary parenchyma began to show evidence of histologically non-tuberculous, scattered, diffuse accumulations of macrophages, which might have been a sign of incipient tuberculosis. These diffuse accumulations of macrophages developed into epithelioid cell tubercles after *5—6 weeks* in some of the animals, but not until after *8 weeks* were any large areas of productive tuberculous granulation tissue seen and then only in one animal, while preparations from the other animal killed at that time showed areas with accumulations of macrophages. After *10 weeks* only diffuse accumulations of macrophages were seen. After *12 weeks and 4 months* epithelioid cellular foci were observed in all the preparations. One of the foci showed signs of incipient necrosis.

Thus no signs of tuberculosis were seen during the first 2 weeks. They began to appear during the 3rd week in the form of histologically non-tuberculous changes. During the 5th—10th weeks tuberculous changes appeared, *i.e.*, epithelioid cell foci, alternating with groups of macrophages in some of the 8 animals, but not until after 12 weeks and 4 months did all the animals (4) exhibit foci of epithelioid cells.

Series II

In this series the development of tuberculosis could not be followed clearly. During the entire experimental period some preparations showed no histological evidence of tuberculosis at all or only histologically non-tuberculous diffuse accumulations of macrophages or epithelioid cell foci.

After *1—8 weeks* diffuse accumulations of macrophages were found in six preparations, while signs of tuberculosis were missing in twelve. After *10 weeks* the first epithelioid cell foci appeared in two preparations. After *12 weeks, 4 months and 5 months* no signs of tuberculosis were found in three preparations, but diffuse accumulations of macrophages were observed in one preparation and epithelioid cells in two. Incipient necrosis was seen in only one preparation from an animal killed at the end of the 4th month. This preparation also contained epithelioid cells.

Series III

As in series II, during the entire experimental period preparations free from signs of tuberculosis alternated with preparations with diffuse accumulations of macrophages or epithelioid cell foci.

Already after *1—2 weeks* tuberculous changes were suspected in two preparations, but not in the other two. After *3—6 weeks* diffuse accumulations of macrophages were found in three preparations, but were missing in four. After *8 weeks* the first large tuberculous focus with necrosis was seen in one of the preparations but only diffuse accumulations of macrophages in the other. From the beginning of the *10th week to the end of the 4th month* inclusive one preparation showed groups of epithelioid cells, one, diffuse accumulation of macrophages; and two, showed no signs of tuberculosis.

Comparison between the three series

In series I no signs of tuberculosis were seen during the first 2 weeks, not even the histologically non-tuberculous accumulation of macrophages, but from then on signs of tuberculosis were seen in all preparations throughout the rest of the experimental period. In series II and III diffuse accumulations of macrophages, possibly the initial sign of tuberculosis, were seen already after 1 week, but from then onwards many of the preparations showed no signs of tuberculosis.

Compare also the results of bacterial culture of the pulmonary tissue (page 41).

2. *Development of lesions in the liver*

As in the examination of the lungs for tuberculosis, only relatively small pieces of the liver were removed for histological examination, so that changes other than those described below might have occurred in those parts of the liver that were not examined.

Series I

After *1 week* no signs of tuberculosis were seen, but after *2 weeks* both preparations showed single very small changes of non-tuberculous type with some macrophages, lymphocytes and central destruction. Acid-fast rods were found in one of these "foci". Also in preparations from animals killed at different intervals and in different series these small foci exhibited acid-fast rods, which were therefore regarded as a very early sign of tuberculosis. The foci increased in size and number, epithelioid cells and sometimes also Langhan's cells appeared so that after *4 weeks* small tuberculous foci were seen throughout the preparation. Some of the lesions showed incipient central necrosis. The now histologically tuberculous foci increased slowly in size and after *12 weeks* large and sometimes coalescent areas of tuberculous granulation tissue were seen over the entire area of the section. After *4 months* lesions of largely the same type were seen as after 12 weeks, they were, however, sparser than in the previous preparations, which may have been due to differences between the individual animals. But these preparations also showed small lesions of roughly the same type as those seen after two weeks consisting of some tens of macrophages and lymphocytes, often also a giant cell. These small lesions may have been due to secondary haematogenous spread of the bacilli.

Series II

Already after *1 week* the small foci mentioned in series I and consisting of some tens of macrophages and lymphocytes and often containing a giant cell began to appear.

After *2 weeks* the preparations showed not only such small foci but also a few larger lesions, some of which were partly necrotic. The lesions grew in size and after *3—4 weeks* numerous epithelioid cells were seen in several preparations.

In later preparations, *5 weeks—5 months* after operation, the individual variation was wide and the development of the tuberculosis was no longer uniform. Only in four preparations was there more advanced tuberculosis with large tuberculous lesions. In half of the remaining preparations there were only small lesions and in the other half also somewhat larger ones.

Series III

Already after *1 week*, as in series II, the aforementioned small foci began to appear. These small lesions were typical of this series. In sixteen of the eighteen preparations, *1 week—12 weeks* after operation, they were the main component. Single, larger productive foci were also seen in some of the preparations. The foci varied somewhat in number. One preparation showed not only numerous small foci but also several large ones.

Comparison between the three series

Series I showed no signs of tuberculosis during the first week, but in the 2nd week small foci began to appear. In series II and III the first small foci appeared after 1 week. Because of the wide variation of the different preparations it is not possible to compare the three series, though it appears as if the foci were densest and largest in series I, and least dense and smallest in series III.

3. *Development of lesions in the spleen*

The entire ventral half of the spleen was used for histological examination.

Series I

After *1 week* no tuberculous lesions were seen, but after *2 weeks* scattered small clusters of epithelioid cells and macrophages appeared in both preparations. The foci grew and after *3 weeks* there were numerous large productive lesions, some with incipient central necrosis. After *4 and 5 weeks* the appearance of the preparations was largely the same; all of them showed small to large epithelioid cell foci, some with central necroses. After *6, 8, 10, 12 weeks and 4 months* all the preparations contained a varying number of epithelioid cell foci.

Series II

After *1 week* one of the preparations showed no signs of tuberculosis, while the other one exhibited small groups of cells resembling macrophages or epithelioid cells. These "foci" were of the same appearance as those described in the section on liver tuberculosis (page 37). After *2 weeks* one of the preparations exhibited only equivocal evidence of tuberculosis, while another showed small epithelioid cells, and the remainder, a small group of macrophages and some giant cells as well as several giant cells scattered in the parenchyma, some of Langhan's type, — possibly early evidence of tuberculosis. The

foci grew and after *3 to 4 weeks* they assumed a tuberculous appearance, and numerous, small and large groups of epithelioid cells were found in all of the preparations. The foci appeared to be largest and most numerous at this time, after which they began to decrease in size and number. After *5 weeks* one of the preparations, like those examined after 3 and 4 weeks, contained numerous small and large foci. In the remaining two there were sparse epithelioid cell foci of varying size. After *6, 8, 10 and 12 weeks* and *4, 5 and 6 months* no histological signs of tuberculosis were seen in three of the preparations, from animals examined 6 weeks, 4 months and 5 months, respectively, after the operation. In one of the preparations from the animals killed after 6 months as well as in preparations from animals killed after 3—4 weeks there were numerous small and large groups of epithelioid cells. In all the remaining twelve preparations only small epithelioid cells were seen, usually sparse and scattered in the parenchyma.

Series III

No definite signs of tuberculosis were seen after *1 week*. In one preparation there were small accumulations of macrophages, which might have been an early sign of tuberculosis. After *2 weeks* there were no signs of tuberculosis. After *3 weeks* one preparation showed no signs of tuberculosis, while the other contained small sparse groups of epithelioid cells. After *4 weeks* there were no definite signs of tuberculosis — one of the preparations contained a small group of epithelioid-like cells but it was difficult to decide whether they were due to tuberculosis. After *5 and 6 weeks* all the preparations contained fairly sparse epithelioid cell foci. After *8 weeks* one of the preparations showed no signs of tuberculosis, while the other contained numerous large epithelioid cell foci. After *10 and 12 weeks* there were again no sign of tuberculosis in one preparation, while the others exhibited small scattered foci of epithelioid cells. In the last preparation from animals killed *4 months* after the operation there were large crowded foci of epithelioid cells occupying about half of the parenchyma.

Comparison between the three series

In series I and II the first tuberculous changes occurred at roughly the same time, *i.e.*, during the first — second week. From then on in series I foci were seen in all preparations throughout the rest of the experimental period, while in series II tuberculosis was missing in three preparations during the period 6th week—5th month. In series III the first tuberculous changes occurred 3 weeks after the operation, after which the course largely resembled that in series II, *i.e.*, tuberculous lesions were missing in some of the preparations, while productive foci of varying size were found in the remainder.

4. *Tuberculosis of the unoperated, contralateral femur*

Series I

There were no signs of tuberculosis after *1 week*. After *2 weeks*, however, the medullary cavity showed single, very small areas with what appeared to be macrophages. A giant cell was seen in one of the preparations. This might have been the first histological sign of tuberculosis. These small "foci" grew so that after *3 weeks* there were still very

small scattered miliary foci in the marrow. From the *4th week to the 4th month* all the preparations showed miliary foci. These foci varied in size from very small, consisting of some 10 cells or more, up to single foci occupying a third of the cross sectional area of the medullary cavity. These foci, however, never reached the size of those seen at the site of inoculation, no abscesses developed and there was no reactive connective tissue or bone newformation in their periphery. The foci were scattered in the red bone marrow and were seen in the diaphysis, metaphyses and epihyses as well as in the patella.

Series II

Already *1 week* after the operation one preparation showed some very small groups of cells of the type described in the liver preparations from animals examined after 1 week in series II. These foci might have been signs of incipient tuberculosis. During the *2nd—5th weeks* miliary foci were seen in five of eleven preparations and from the *6th week to the 6th month* miliary foci were found in seven of fourteen preparations. Evaluation of the negative preparations was difficult. Some of them contained very small groups of cells which might have been of the same type as those seen in preparations from animals killed 1 week after the operation. Since the bones were not serially sectioned, small foci may have been missed. Tuberculosis might thus have been present in some of these preparations. As in series I, foci were seen in all parts of the red marrow of the bone.

Series III

Already *1 week* after the inoculation miliary foci were seen in both preparations. After *2 weeks* one of the preparations showed definite miliary foci, while the other was difficult to evaluate. It might have contained one small focus. From the *3rd week to the 4th month*, when the experiment was stopped, tuberculosis was seen in eight of fourteen preparations, but not in the remaining six. As in series II, evaluation of the negative preparations was difficult. In this series, too, foci were seen in all parts of the red bone marrow.

Comparison between the three series

In series I there were no histological signs of tuberculosis after 1 week, but from then on miliary foci were seen in all of the preparations. At 1 week one of the preparations in series II and both of them in series III showed histological signs of tuberculosis. From then on signs of tuberculosis were missing in several of the preparations in these two series.

Moreover, in series I the foci were on the whole larger and more crowded than in series II and III.

C. CULTURE OF TISSUE FOR TUBERCLE BACILLI

Since the diagnosis of tuberculosis cannot be regarded as established without demonstration of tubercle bacilli in the tissue, tissue specimens were removed and

cultured for tubercle bacilli. In certain animals (Table 2) the diaphyseal marrow was removed from the operated femur and pieces from the lungs, liver and spleen were removed under sterile conditions and treated in the following way.

The pieces of the tissue were carefully homogenized in a mortar and treated for 10 minutes with 6 % sulphuric acid. The crushed pieces of tissue were then suspended in 100 ml physiologic saline and centrifuged for 20 minutes after which the fluid was pipetted off and the sediment was cultured. The sediment was cultured in 3 tubes containing solid Löwenstein-Jensen-medium and 1 containing Braun-Lebeck's medium. The rest of the sediment was filtered through a membrane and cultured on a plate with Löwenstein-Jensen-medium.

The results were read after 4—8 weeks by inspection of the culture on Löwenstein-Jensen-medium and the plates and by examination of smears from the Braun-Lebeck-medium stained according to Ziehl-Neelsen.

The results of culture of the various organs were in good agreement with those obtained by histological methods. It was checked by the niacin test that the strains isolated from the animals were of human type like strain H 37 Rv (Juhlin 1960).

TABLE 2

Results of culture for tubercle bacilli

Series	I (6 animals)						II (9 animals)									III (6 animals)					
Interval between operation and sacrifice in weeks	2	3	4	5	8	12	2	3	4	6	8	10	12	18	22	2	3	4	6	8	10
Bone marrow	+	+	+	+	+	+	+	+	+	—	C	+	—	—	C	+	+	+	—	+	+
Lung	+	+	+	+	+	+	+	+	+	+	—	+	—	—	—	—	+	—	—	—	—
Liver	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+	+	—	+
Spleen	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	—	+

+ = growth of Mycobacterium tuberculosis

— = no growth of Mycobacterium tuberculosis

C = contaminated

VI. DISCUSSION

A. FACTORS LOCATING AN INFECTION IN THE SKELETON

In previous investigations by other workers in this field the animals usually survived for only a short time and skeletal lesions occurred in only a few of them. Since the purpose of the present investigation was to devise a method for long-term studies, not only the literature on experimental skeletal tuberculosis but also that on experimental non-tuberculous infections of the skeleton was perused, it sounding reasonable to assume that some factors locating the infection in the skeleton may be the same in both types of disease.

Various suggestions have been offered for the location of the lesions in the skeleton, *e.g.*, trauma (Friedlaender 1902, Trudel 1933, Grundmann 1953), bacterial emboli (Friedlaender 1902, Mandelstamm 1933, Mayer et al. 1954, Tsuge and Tomochika 1955), vascular anatomy of the metaphysis (Lexer 1904, Siegmund 1948, Buchman 1959), paucity of "phagocyting elements" in the metaphysis (Hobo 1921), straining of the epiphyses with local haematoma as a result (Robertson 1927), C-avitaminosis (Kuwahata 1930), "chemical affinity" between bacteria and the body tissue (Rost 1913), vascular spasm (Baskinskaya 1959), "allergic" reactions (Derizanow 1937, Csipak et al. 1953 and 1954, Grundmann 1953, Csipak 1956), the presence of red bone marrow (Randerath 1931).

A critical analysis of the above-mentioned theories would fall beyond the scope of the present investigation. Therefore, suffice it here to give a few remarks on some pertinent factors in this experiment.

1. *Site of inoculation*

The lower femur was selected because it has some of the aforementioned anatomical structures considered of importance in the location of an infection to the skeleton. Besides this, the lower femur is not an uncommon site of tuberculous and non-tuberculous osteomyelitis in human beings.

2. *Trauma*

Several of the above-mentioned authors discuss the significance of the trauma. That trauma can contribute to location of the disease to the skeleton is probable. The bacteria or the host must, however, possess certain properties for an infection to occur at the site of the trauma, *e.g.*, a certain level of immunity of the host, a certain virulence of the bacteria. Thus trauma is only a contributory cause. This had been discussed especially by Grundmann (1953).

The local tissue destruction after a trauma is also probably of significance in

the location of the infection. Certain experiments (Derizanov 1937 and 1938, Scheman et al. 1941 and 1942, Csipak et al. 1953 and 1954, Csipak 1956, Baskinskaya 1959 and personal unpublished experiments) have shown that a skeletal focus can be produced if bacteria are injected into a local necrosis of the bone marrow.

3. *Tissue reaction around the Spongostan*

The reaction occurring around the Spongostan introduced into the bone marrow may also tend to locate the infection, it being a commonplace that infection round a foreign body is obstinate and that healing often promptly occurs after removal of the foreign body. Spongostan was, however, chosen because it is practically inert and therefore does not substantially interfere with the picture of the infection. Spongostan is also fairly readily resorbed — in series I—III already within 2—4 weeks.

4. *Depôt effect*

The fact that injection of bacteria into the actual bone marrow of the animals usually fails to produce foci at the site of injection may be explained by the assumption that a large proportion of the bacteria are rapidly transported away with the blood. Deposition of the Spongostan and tubercle bacilli in the bone in the way described prolongs the duration of contact between the local tissue and the tubercle bacilli and thereby increases the possibility of development of a local lesion.

5. *Immunological status of the experimental animals*

Experiments carried out by Lurie (1939), Spiess (1953), Spiess and Poppe (1954) and Ström (1955) suggest that tubercle bacilli are retained longer at the site of inoculation in vaccinated animals than in unvaccinated ones. The results obtained by Grundmann (1953) in experiments with staphylococci also suggest that the state of immunity of the animal may be of importance in the location of an infection.

In the present experiments, however, vaccination had no certain effect on location of the infection to the skeleton.

B. DURATION OF SURVIVAL

In numerous investigations referred to in the two monographs "Die BCG-schutzimpfung" (Griesbach 1954) and "BCG-vaccination" (Rosenthal 1957), BCG-vaccination has been found to prolong the survival of guineapigs, superinfected with tubercle bacilli. To achieve such prolongation, however, the

animals must be superinfected within a certain time after vaccination, the dose of bacilli must not be too large, and vaccination must be adequate. This again underlines the necessity of standardised experiments. The shorter survival of the unvaccinated animals is due to rapid development of tuberculosis in various extra-skeletal organs. In vaccinated animals the infection also occurs, but runs a much slower course. The histological and bacteriological findings in the present experiment were in accord with those described by previous workers.

It is, however, remarkable that none of the unvaccinated animals in the present investigation died spontaneously. This may be explained at least partly by the assumption that with the technique used a large proportion of the bacilli were not promptly transported away by the blood stream but were retained in the Spongostan. The properties of the bacilli and the strain of animals used may also have been of importance. In preliminary experiments (not described here), some of the unvaccinated animals died so that in the series the experiments were stopped after 4 months. As mentioned previously, the breed of animals was afterwards changed and the technique for culture of bacteria was modified.

Since none of the animals died, except a few in the preliminary experiments, the method used satisfies the first of the three requirements set forth on page 11.

C. TUBERCULOSIS OF THE LEG OPERATED UPON

In many, if not all, of the experimental animals a tuberculous lesion developed at the site of inoculation in the bone. It is not possible to say with certainty whether the animals killed after 1 week really would have developed lesions at the site of inoculation. Neither is it known with certainty whether those animals killed at the end of the experimental periods in series II and III really had had skeletal tuberculosis. But since it was possible to follow the growth and successive regression of the lesions in these two series and then to find that they were missing after a certain time, it would appear justified to assume that also those animals killed last had had such foci. This implies that the method described satisfied the last 2 requirements set forth in the introduction on page 11.

It is well known that superinfection of a vaccinated animal with virulent tubercle bacilli may cause initially larger tuberculous lesions at the site of inoculation than corresponding infection of an unvaccinated animal (Griesbach 1954, Rosenthal 1957). The lesions, however, tend to regress in vaccinated animals but to progress in unvaccinated ones. This tendency was also noted in the present investigation. In bone preparations in series I gross abscesses did not appear at the site of inoculation until 5 weeks after deposition of the bacilli, while in series II and III they appeared already within 2 weeks. In series I the abscesses persisted throughout the experimental period (4 months), while in series II and III they began to regress already after about 5 weeks. In series II these abscesses

healed completely within 4 months, and in series III healing also appeared to progress in the same way, though the end-result in this series was uncertain since the animals were sacrificed after 4 months instead of after 6 months as in series II. The experiments thus showed:

firstly, that the abscesses developed sooner in the vaccinated than in the unvaccinated animals, and

secondly, that the foci at the site of inoculation clearly tended to heal in the vaccinated animals, while no such tendency was observed in the unvaccinated animals during the experimental period.

In all three series the marrow in the operated leg showed miliary foci remote from the site of injection. They persisted throughout the experimental period in series I and II, but were no longer demonstrable after the 10th week in series III. This difference was probably due to the differences in the vaccination procedures.

D. TUBERCULOSIS OUTSIDE THE OPERATED LEG

The unoperated femur and tissue specimens from the lungs, liver and spleen were also studied histologically to estimate the spread and course of the infection elsewhere in the body. In these regions it was sometimes not possible histologically to demonstrate or exclude the possibility of tuberculous foci with certainty because the histological picture was not unequivocal. Sometimes the foci were seen at a very early stage, when they consisted of only some tens of cells and then had no specific appearance. These small foci were nevertheless regarded as being tuberculous because several of them contained acid-fast rods, cultur gave growth of tubercle bacilli and in the liver and spleen the small foci gradually developed into large tuberculous lesions.

Tubercle bacilli spread early to all of the organs examined. In the bone marrow, lungs, liver and spleen disseminated foci appeared earlier in the vaccinated than in the unvaccinated animals. In the latter such foci were first seen in animals examined 2 weeks after inoculation.

In the unvaccinated group tuberculous foci were seen from then on in all the organs from the animals killed during the experimental period (4 months). On the other hand, tuberculosis was often not demonstrable in the parts examined from specimens from the vaccinated animals. Though tuberculosis might very well have been demonstrable in unexamined parts of these organs the results showed that vaccination had clearly retarded the development of tuberculosis.

Since the purpose of the present investigation was to produce skeletal tuberculous lesions, the findings in the contralateral, unoperated femur were of particular interest. None of the foci produced by haematogenous spread of the bacilli to the unoperated femur developed into large productive foci, let alone abscesses, during the experimental period.

PART II

VII. INTRODUCTION AND PURPOSE

It is well known that skeletal infections, whether of tuberculous nature or not, are difficult to arrest or cure. Experience has taught that such infections respond only slowly to antibiotics and that recurrences are common. As far as tuberculosis is concerned, this also holds for lesions outside the skeleton and can be explained in part by the nature of the tubercle bacillus. But experience in the treatment of non-tuberculous osteomyelitis strongly suggests that also the actual site of the lesions in the skeleton retard or prevent healing.

The purpose of the investigation was to ascertain whether dihydrostreptomycin diffuses into the tuberculous abscesses described in Part I and if so, how it is distributed there.

Tuberculous skeletal lesions vary widely in structure and consist of areas of necrosis, specific and non-specific granulation tissue, necrotic and newformed bone, all in varying proportions. As the initial link in an experimental investigation of the ability of anti-tuberculous agents to diffuse into skeletal lesions, however, it was decided to avoid foci of complicated structure and instead study the tuberculous bone marrow abscesses of typical and simple structure, *i.e.*, of the type described in Part I and readily reproducible in animals.

VIII. PREVIOUS INVESTIGATIONS

A. INVESTIGATIONS WITH MICROBIOLOGICAL METHODS

Various researchers (Froyez and Froyez-Roederer 1949 and 1952, Felländer et al. 1952, Felländer 1955, Hevér and Riskó 1960 and Debeaumont 1966), who have used microbiological methods, have tried to determine the concentration of streptomycin in tuberculous pus from patients with skeletal tuberculosis. The results varied from patient to patient. In some cases the concentration was so high that it had a clearly anti-bacterial effect, in others no streptomycin at all could be demonstrated. No satisfactory explanation could be offered for this variation. Froyez and Froyez-Roederer (1949) discussed the possibility of the physico-chemical properties of the contents of the abscess being able to inhibit the antibacterial effect of streptomycin. Hevér and Riskó (1960) found that abscesses that had existed for only a few months contained a larger amount of streptomycin than old, chronic abscesses. The effect of a given antibiotic may

thus depend on the various properties of the abscess capsule and the varying composition of the debris in the necrotic lesion.

Katayama et al. (1954), who also used microbiological methods, determined the streptomycin content of tuberculous skeletal abscesses 1 hour and a half after injection of half a gram of streptomycin into a patient and found that at a blood concentration of 8.2 μg/ml the pus contained 2.7 μg/ml, the granulation tissue 0.53 μg/ml, the fibrous tissue 0.37 μg/ml and caseous necrosis 0.32 μg/ml.

B. INVESTIGATIONS WITH AUTORADIOGRAPHY

No detailed autoradiographic investigations have apparently been published on the distribution of anti-tuberculous drugs in tuberculous lesions.

In an investigation of the distribution of dihydrostreptomycin in healthy tissues in mice André (1956) also studied a single intramuscular tuberculous abscess in one mouse. Thirty minutes after injection of tritium-labelled dihydrostreptomycin the abscess was removed for autoradiography. No microautoradiograms were made, but according to André (1956) the published autoradiogram showed the highest concentration of dihydrostreptomycin in the abscess capsule. Inside the abscess the activity was highest in the periphery, and only low centrally.

Hanngren and André (1964) investigated the distribution of tritium-labelled dihydrostreptomycin in tuberculous lesions in the lungs of guineapigs by means of whole-organ autoradiograms. They found that "in the tuberculous foci of the lung the concentration was higher than in the normal lung tissue", where the concentration, however, did not reach that of the blood. Within the tuberculous lung cavities radioactivity was found only to a very little extent.

Hanngren (1959) examined a few abscesses in the lungs, chest and lymph nodes 15 and 30 minutes after injection of PAS labelled with ^{14}C and tritium. He, too, found the concentration to be highest in the abscess capsule and to fall rapidly towards the centre of the lesion. As in André's work (1956), he studied whole-organ autoradiograms but not microautoradiograms.

IX. CHOICE OF ANTIBIOTIC

Dihydrostreptomycin is no longer used clinically because it sometimes causes impairment of hearing. Streptomycin is now generally used instead. In the present investigation dihydrostreptomycin was nevertheless used for the following reasons.

1) Tritium-labelled dihydrostreptomycin is obtained by a catalytic hydration of streptomycin. The procedure is simple and in this way it is possible to obtain a final product with a high specific activity without any laborious purification. The method was devised and described by André (1956).

Tritium-labelled streptomycin cannot be obtained with this simple method but only by more complicated procedures, such as that described by Wilzbach (Wenzel and Schulze 1962, Advances in tracer methodology 1963). The products obtained by such procedures probably require extensive laborious purification. Since these methods for labelling streptomycin have apparently not been used before, it is not known what specific activity and what degree of purity of the final product can be obtained.

2) No substantial differences have been found between streptomycin and dihydrostreptomycin regarding their antibacterial effect or pharmacological properties such as the distribution of the two drugs in the body, and their excretion appear to be similar (Donovick and Rake 1947, Edison et al. 1948, Feldman et al. 1948, Hobson et al. 1948, Levin et al. 1948, Rake et al. 1948, Waksman 1949).

Certain small differences, such as the fact that dihydrostreptomycin can injure hearing and streptomycin the sense of balance, that dihydrostreptomycin is less inclined to produce allergic reactions (Hobson et al. 1948), is presumably irrelevant to the present study.

3) No convincing evidence has been produced that dihydrostreptomycin or streptomycin is broken down in the body (page 54).

X. SYNTHESIS OF RADIOACTIVE DIHYDRO-STREPTOMYCIN

A. CHOICE OF ISOTOPE

The beta particles of tritium have low energy, E max 0.018 MeV, and are therefore suitable for autoradiography where high resolution is desired. With a suitable autoradiographic technique the isotope can be located at the cellular level, which was desirable in the present investigation.

Labelling of dihydrostreptomycin with tritium is technically easy and gives a pure final product with high specific activity (page 50).

Tritium is also a much cheaper isotope than ^{14}C, which might also be used in such experiments.

B. PREPARATION OF RADIOACTIVE DIHYDROSTREPTOMYCIN

The radioactive dihydrostreptomycin was prepared at "Isotoptjänsten", AB Atomenergi in Studsvik, according to a modification of André's (1956) method.*)

250 mg of streptomycin HCl, 5 mg of platinum oxide, 0.5 ml dimethylformamide, and 0.075 ml distilled water were mixed in a 2 ml retort with a magnetic stirrer. The retort was connected to one of the limbs of a tritiating apparatus according to Wenzel and Schulze (1962, page 22, Fig. 13) in which 30 Ci tritium had been adsorbed on uranium in an ampoule connected to the other limb of the apparatus. The reagent solution was cooled with liquid nitrogen and the apparatus was evacuated to 0.01 mm Hg. After the mercury level had been raised in the gas buret and the manometer tube, the uranium ampoule was heated to about 420° C, at which the tritium evaporated and was collected in the buret. At the same time the reaction vessel was allowed to recover room temperature.

The volume of the tritium and the pressure were read on a calibrated scale, after which the gas was allowed to enter the reaction vessel via a three-way cock. The free volume of the reaction vessel was calculated from re-determination of the volume and pressure.

*) This modification was devised by Nils Walde, who also prepared the radioactive dihydrostreptomycin and composed this section.

The solution was stirred vigorously and the tritiumabsorption was followed by the decreasing pressure and volume. After the reaction had stopped the stirring was interrupted, the final pressure and the final volume were read, the reaction vessel was cooled as before and the remaining tritium gas was adsorbed on uranium. The mercury level was lowered so that the residual gases could be pumped out.

The reaction vessel was disconnected and the solution was filtered through Hyflo Supercel®. The filter and the vessel were washed with water to a final volume of about 30 ml. The water was removed by rotation evaporation at reduced pressure (20 mm Hg). The rest was dissolved in about 30 ml of water followed by re-evaporation. This was repeated twice. The residue was dissolved in the smallest possible amount of methanol, about 1.5 ml, after which 9 ml acetone was quickly added, with consequent precipitation of streptomycin and dihydrostreptomycin. The solvent was removed through a dipping filter. The precipitate was dried in a vacuum and the streptomycin mixture was dissolved and transferred to a 50 ml graduated flask.

C. ANALYSIS OF PREPARED DIHYDROSTREPTOMYCIN

The efficiency of the hydrogenation of streptomycin and the purity of the product were determined by the manufacturers according to the following description.*)

The efficiency of the streptomycin hydrogenation was determined by analysing the tritium-labelled product. The dihydrostreptomycin content was determined spectrophotometrically (Hiscox 1951) and the streptomycin content by an ultraviolet absorption method (Titus and Fried 1948). Two products were synthetized: the first contained 72.1 % dihydrostreptomycin and 27.9 % streptomycin; the second 81.4 % dihydrostreptomycin and 18.6 % streptomycin.

Paper chromatography was done according to Winsten and Eigen (1948). In the development of the chromatograms equal volumes of the following solutions were mixed and used immediately:

0.1 % diacethyl in water
20 % (w/v) KOH in water
2.5 % α-naphtol in alcohol.

This mixture stained both the dihydrostreptomycin and the streptomycin. The chromatogram was examined in a chromatograph scanner and the radioactivity was found only over the dihydrostreptomycin spots.

The specific activity of the product was determined with the aid of a liquid scintillation spectrometer. The activity of the first of the two products synthesized was 1.14 mCi/mg; that of the second, 0.14 mCi/mg. The low specific activity of the latter product was due to a technical mischance, which stopped the reaction too early.

The purity and stability of the products obtained were checked also by the author with paper chromatography, which was done according to Ishida et al. (1953). A purified non-radioactive dihydrostreptomycin in water solution was

*) See footnote, page 51.

used as reference substance for all chromatograms*). Pieces of the paper strips were punched out for microbiological control, after which the development of the chromatograms was done with equal volumes of the following solutions:

0.1 % diacetyl in water

1 % α-naphtol in a solution of 8 % (w/v) NaOH in water.

The solutions were used immediately after they had been mixed (Chromatographic and Electrophoretic Techniques 1960). Finally the strips were examined in a chromatograph scanner.

The microbiologic control was carried out at the Bacteriological Institution, Malmö General Hospital, with the technique used for estimating the susceptibility to antibiotics (Eriksson 1960). A blood agar plate was covered with an even layer of staphylococcus albus, of a strain which is very susceptible to dihydrostreptomycin but resistant to other antibiotics. The punched-out pieces of paper, all of the same size, were placed on the blood agar surface, after which the plate was incubated at 37° C for 18 hours and then examined for inhibition zones around the pieces of paper.

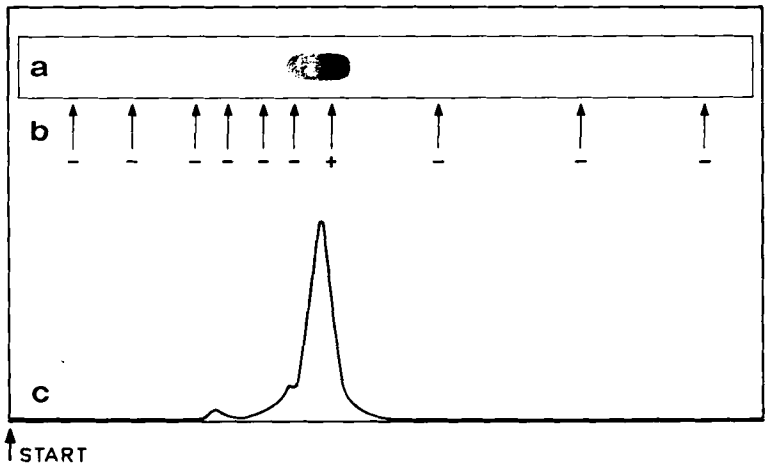

Fig. 17

Occurrence of colour reaction, antibiotic and radioactivity in paper chromatogram of the radioactive dihydrostreptomycin.

\+ occurrence of inhibition zone in microbiological tests
— absence of inhibition zone in microbiological tests
a. chromatogram with spot
b. arrows indicate segments of paper strip used for microbiological examination
c. curve of radioactivity in chromatogram

The colour reaction, the occurrence of radioactivity and the inhibition zones coincided in all the tests (Fig. 17). The degree of purity of the product obtained thus appeared to be high.

*) The purified dihydrostreptomycin was courteously supplied by Leo Pharmaceutical Products, Copenhagen.

XI. STABILITY OF DIHYDROSTREPTOMYCIN IN THE BODY

Dihydrostreptomycin and streptomycin appear not to be broken down or, if so, not appreciably, in the body. The bulk of dihydrostreptomycin given parenterally is excreted in the urine within 24 hours after the injection and no degradation products of the two substances have been found (Levin et al. 1948, Rake et al. 1948, Waksman 1949, André 1956, Goodman and Gilman 1965).

But since some researchers (Adcock and Hettig 1946, Giesen and Koelzer 1950) have found only about 60 % of streptomycin to be excreted in the urine, in the present investigation the excretion was checked, in cooperation with AB Kabi, in 8 volunteers. The urinary excretion of tritium-labelled dihydrostreptomycin in guineapigs was also studied.

1) Each of the volunteers received an injection of 1 "ampin" containing 1.030 grams of dihydrostreptomycin sulphate intramuscularly. Three of them excreted 0.84, 0.87 and 0.98 grams within the first 10 hours. Four others excreted 0.81, 0.89, 0.91 and 0.94 grams respectively in the first 24 hours. The eighth excreted 0.972, 0.016 and 0.006 grams during the first 3 days, thus all together 0.994 grams. It should be observed that, according to AB Kabi, injection of one "ampine" means injection of only 0.960—0.980 grams.

Urine analysis was done with the microbiological method used routinely by Kabi (Grove and Randall 1955). To check this method, the urine content of one of the patients was studied with a spectrophotometric (Hiscox 1951) and a colorimetric method (Monastero 1952). Good agreement was found between the results of the microbiological method and those of the other two methods.

2) Three guineapigs were given 1.5, 7 and 7 mCi tritium-labelled dihydrostreptomycin intracardially. The animals were killed 30 minutes, 1 hour and 4 hours later by intraperitoneal injection of mebumal and the urinary bladder was punctured immediately. Since the urine was very cloudy it was centrifuged and the clear supernatant was pipetted off and subjected to paper chromatography (page 52), after which the chromatogram was examined in a chromatograph scanner. A curve of the type shown in Fig. 18 was obtained for each of the animals.

The radioactivity was divided into two separate peaks. When stained and studied microbiologically (page 53) the colour reactions and the inhibition zones were found to correspond to these peaks, which argues strongly against any degradation of the dihydrostreptomycin.

To find out why dihydrostreptomycin in guineapig urine is divided into two fractions on paper chromatography, urine was taken from 2 guineapigs that had received an intracardial injection of 1 ml of an aqueous solution containing 10 mg non-radioactive dihydrostreptomycin/ml. The animals were killed 3 hours after

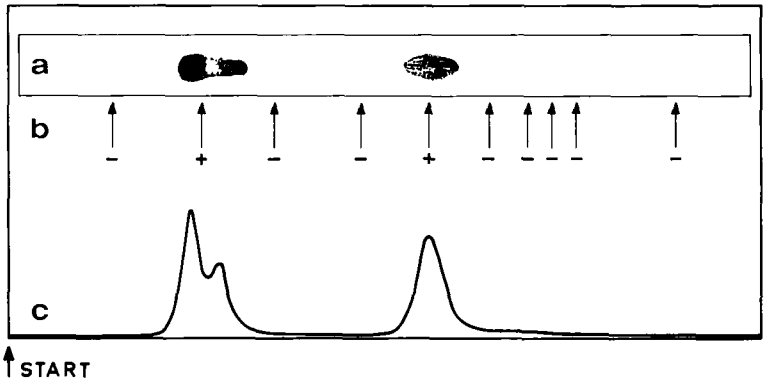

Fig. 18
Occurrence of colour reaction, antibiotic and radioactivity in paper chromatogram of guineapig urine.

\+ occurrence of inhibition zone in microbiological tests
— absence of inhibition zone in microbiological tests
a. chromatogram with spots
b. arrows indicate segments of paper strip used for microbiological examination
c. curve of radioactivity in chromatogram

the injection, the urinary bladder was punctured, and the urine was collected. A third of the urine was acidified to pH 1 with hydrochloric acid and a third was treated with β-glucuronidase (Ketodase®, manufactured by Warner-Chilkott). The urine was allowed to stand at room temperature over night, after which paper chromatography was done on both untreated, acidified and enzyme-treated urine. The untreated urine showed the usual distribution in two fractions, while in the acidified urine and the urine treated with enzyme the more rapidly migrating spot had disappeared while the slow one was seen at the same site as before.

Comment. The guineapig experiments revealed nothing suggesting that the dihydrostreptomycin is metabolised. Judging from the above investigation, the separation of the dihydrostreptomycin into two fractions on paper chromatography of the urine can be explained by part of the dihydrostreptomycin being bound to glucuronic acid and changing its rate of migration. No such separation occurred after acid hydrolysis or treatment of the urine with glucuronidase.

XII. AUTORADIOGRAPHIC TECHNIQUE

A. CHOICE OF AUTORADIOGRAPHIC EMULSION

Emulsion Ilford G 5 was used. It was supplied attached to glass slides 1″×3″. To decrease background blackening the thinnest available emulsion layer was selected, namely 10 μ.

Comment. Since both the specific activity and the amount of the tritium-labelled dihydrostreptomycin was smaller than expected (page 52) it was necessary to use such a sensitive film emulsion as Ilford G 5, even though the employment of such an emulsion results in high background blackening. According to the manufacturers the emulsion is sensitive to all charged particles of any energy.

Since it was thought that the background blackening with this emulsion might disturb identification of the dihydrostreptomycin at the cellular level, for microautoradiography use was also made of emulsion Ilford K 2, which is recommended by the manufacturers for tritium studies where high resolution is desired. Because of the relatively low sensitivity of this emulsion background blackening will be less. But the K 2 emulsion proved of no value in this experiment because of its low sensitivity.

Since the soft radiation of tritium only reaches about one μ into the film emulsion (Levi 1964), a thin emulsion layer could be used because it helps to decrease background blackening.

B. HISTOLOGICAL METHODS

In autoradiography of a substance such as dihydrostreptomycin, which is water-soluble, the ordinary histological technique cannot be used, because then the substance is dissolved from its original site in the tissue. Freeze-drying has therefore been recommended for such work by Holt et al. (1949), Harris et al. (1950), and Winteringham et al. (1950).

1. *Freeze-drying and sectioning of whole guineapig femur with tuberculous abscesses*

Since the guineapig femur could not be decalcified for the above reasons and since it has a thick and hard corticalis, which splits and markedly deforms histological sections cut in the usual way, a method devised by Ullberg (1954) for sectioning in a coldroom was used.

After injection of isotope the animal was killed (page 61), the femur with the tuberculous abscess was removed and frozen immediately in hexane and carbon

dioxide snow. It was then transferred to a coldroom with a temperature of —15° C and embedded in a mixture of carboxymethyl cellulose and water and frozen on a synthetic fibre block. The preparation was sectioned in the coldroom with a Leitz sliding microtome. The sections, which were about 15 μ thick, were caught on a strip of adhesive tape and allowed to dry in the coldroom for at least 12 hours.

In order to prevent condensed water from moistening the sections when they were taken out of the coldroom, they were placed in an airtight box with a Petri dish containing phosphorous pentoxide. They were then taken out of the coldroom while still in the box, which was not opened until it had assumed room temperature.

2. *Freeze-drying and sectioning of small marrow specimens with tuberculous abscesses*

The animals were killed and the femur with tuberculous abscesses was removed immediately. The proximal fourth of the bone was cut off with scissors. This caused longitudinal cracks in the corticalis down towards the distal end of the bone, and half of the corticalis could therefore be removed without any appreciable damage to the bone marrow. With a sharp pointed knife the bone marrow was then removed from the remaining corticalis, and divided into pieces, 4—5 mm long, and frozen immediately in a mixture of hexane and carbon dioxide snow. The interval between the death of the animal and the deposition of the pieces into the mixture was, as a rule, 2—3 minutes. The pieces were then freeze-dried for 1 week in a freeze dryer (Edward's Tissue Drier, T.D.2.). They were afterwards embedded in paraffin in the usual way and stored at -20° C until sectioned. Sectioning was done at room temperature, but to avoid the sections from coming into contact with water they were also caught on tape according to the method of Ullberg (1954).

Comment. Since the microscopical picture of the bone marrow and the tuberculous tissue was largely destroyed by sectioning and drying in the coldroom (page 56), this method could not be used for preparing microautoradiograms. Instead the method described above was used.

The specimens were freeze-dried for 1 week. This was presumably unnecessarily long (André 1956, Hanngren 1959) but was nevertheless used because the experimental conditions provided no possibility of finding out which time was the most suitable.

C. EXPOSURE, DEVELOPMENT AND FIXING OF WHOLE-FEMUR AUTORADIOGRAMS

The autoradiographic plates were exposed according to the technique described by *inter alia* Ullberg (1954) and André (1956). The freeze-dried taped sections

were stuck to the photographic emulsion in the darkroom. A piece of paper folded a few times, was placed on the tape in order to secure an even pressure against the emulsion. 3—4 plates with such paper were placed in a press between a pair of plywood plates and wrapped in opaque paper, after which a pair of paper clips were placed outside the package as a press. The films were then exposed in a freeze-box at -20 — -25° C. Before development the tape with the adherent section was carefully loosened from the emulsion, the film was developed 5 minutes in Gevaert X-ray developer G 230, rinsed for 1 minute in water and fixed 15 minutes in Gevaert X-ray fixer G 305, after which it was rinsed in water for 15 minutes and dried in the air.

D. EXPOSURE, DEVELOPMENT AND FIXING OF THE SMALL SPECIMEN-AUTORADIOGRAMS

Before the taped histological sections were fastened to the autoradiographic plates, the latter were dipped in a mixture of 12 % pure glycerin and 88 % absolute alcohol and then allowed to dry for 10—15 minutes (Hammarström et al. 1965).

In order to be able to apply several autoradiograms to the same plate, a piece of stiff paper the size of the autoradiographic plate was cut. An equally large piece of tape, adhesive on both sides, was fastened to the paper. Pieces of tape with the histological sections attached were trimmed and fastened to the collecting tape. The pieces of paper with the adherent tape and histological sections could then be readily fastened, in the dark, to the film emulsion treated with glycerin-alcohol (André 1956, Hanngren 1959). As in autoradiography of sections from whole guineapig femora, the sections and the photographic plates were placed in a press and exposed at an ambient temperature of -20 — -25° C. Before they were developed they were placed in xylol for 10—12 hours, which loosened the paper and the tape from the plate and section. They were then passed through an alcohol series, 2 minutes in absolute alcohol, 2 minutes in 96 % alcohol and 2 minutes in distilled water. They were developed 5 minutes in Gevaert X-ray developer G 230, rinsed for 1 minute in water, and fixed 5 minutes in Gevaert X-ray fixer G 305 and then placed for 15 minutes in distilled water.

Comment. In this investigation use was made of a technique, according to which the tape strip with the histological section is fastened to the photographic emulsion, after the exposure the tape is loosened but the histological section is left in position on the emulsion during development, fixation, staining and mounting. In this way a preparation is obtained where the histological section remains on the autoradiogram it has itself produced. Such a preparation gives the examiner the opportunity to identify the radioactive substance at the cellular level. One difficulty is that the section must not be dislocated from its original position,

if this identification is to be possible. The conventional method described by Pelc (1947) with stripping film where the section is fastened between the slide and the film emulsion could not be used for such a water-soluble substance as dihydrostreptomycin. Instead a method described by André (1956) and modified by Hammarström et al. (1965) was used. According to this modification the emulsion surface is pretreated with glycerin and absolute alcohol so that the section will adhere to it. If the film is not pretreated the section is apt to fall off when being developed, fixed and stained, but since the radiation energy of tritium is so weak the layer interposed between the film emulsion and the tissue layer must be very thin. Otherwise the radiation will be absorbed by the interposed layer. The alcohol evaporates and the glycerin is absorbed by the film emulsion, but the surface is then sticky and the section adheres to the surface of the emulsion.

E. STAINING AND MOUNTING

After the tape strips with whole femur sections were loosened from the film emulsion the sections were stained according to van Gieson and mounted on slides in Euparal. Attempts were made to stain the sections with haematoxylin-eosin but the bone marrow was stained so unevenly that this staining method could not be used.

The small bone marrow sections attached to the developed autoradiograms tended to fall off during the staining procedure. They were therefore held vertical during the staining procedure and the various solutions were slowly added with a pipett to one edge of the plate. The following staining method proved to give the best results: Mayer's haematoxylin 10 minutes, distilled water 10 minutes, 70 % alcohol 1 minute, 96 % alcohol 1 minute, absolute alcohol 1 minute and xylol 1 minute. After staining the sections were mounted in Euparal.

F. DISADVANTAGES OF THE METHODS

The methods described in the sections B—E have certain disadvantages which make themselves fell particularly in the study of bone marrow because of the structure of this tissue. The same methods was also tried on lung, liver and spleen tissue, but then none of these disadvantages proved so disturbing.

The finer histological structure of the bone marrow in the freeze-dried, stained whole-femur sections was distorted into a coarse network in which the individual

cells could barely be distinguished. The marrow in this sections cannot be studied under higher magnification than about 5—10 times.

A large part of the small, freeze-dried specimens loosen or become displaced from their original positions on the film emulsion during the procedures described on pages 58—59. In addition, the marrow often contracts in relation to the film emulsion, which can be observed on the narrow margin of blackening sometimes seen in the autoradiogram outside and around the histological section. The relation of the section to the rest of the autoradiogram shows that lateral displacement or diffusion of isotope cannot have caused this border. In the evaluation of the autoradiogram preparations or part of preparations must be used where no such contraction has occurred.

G. "HISTOCHEMOGRAMS"

Bone marrow, as well as other tissues, can sometimes blacken a photographic emulsion even in the absence of radioactive substances (Boyd and Board 1949, Boyd 1955).

To ascertain whether any such artefact had occurred on the autoradiograms, four guineapig femora with tuberculous abscesses were treated in exactly the same way as those used for whole-femur autoradiography the only difference being that the animals had not been given dihydrostreptomycin. The taped sections were placed on autoradiographic plates with Ilford G 5 emulsion. The plates were exposed for 6 months and developed and fixed. No blackening due to the tissue sections was observed.

Four tuberculous bone marrow abscesses were removed and treated in exactly the same way as those used for microautoradiography, and the animals had not been given dihydrostreptomycin. The taped sections were placed on plates with Ilford G 5 emulsion which were exposed for 3 months, after which the film and the tissue sections were treated in the way described on page 58. No blackening due to the tissue sections was seen here either.

XIII. STUDIES OF THE DISTRIBUTION OF DIHYDROSTREPTOMYCIN IN THE TUBERCULOUS ABSCESS

A. MATERIAL

In a series of unvaccinated guineapigs tubercle bacilli were inoculated into one of the femora, according to the method described in Part I. After 2—4 months, by which time abscesses had certainly developed in the bone marrow cavity, animals weighing about 500 grams were selected for injection of tritium-labelled dihydrostreptomycin. The experiment is described below.

1. *Whole-femur autoradiography*

Seven animals were each given 3 mCi of tritium-labelled dihydrostreptomycin, corresponding to 6 μCi per gram bodyweight, intracardially. Before the injection non-radioactive dihydrostreptomycin was added as carrier substance, so that the total amount of dihydrostreptomycin was about 11 mg dihydrostreptomycin per animal. The animals were killed by a blow against the neck one ½ hour, two 1 hour, two 2 hours and two 4 hours after the injection. The femur with the tuberculous abscess was removed immediately and placed in a mixture of hexane and carbon dioxide snow. The bone was then sectioned and autoradiograms prepared in the way described on pages 56—59. The plates were exposed for about 6 months.

Comment. Since autoradiography of whole bones was thought to require a longer exposure time than microautoradiography, the animals received twice the dose given to those used for microautoradiography. The dose of 11 mg dihydrostreptomycin per animal corresponds roughly to a dose of 1.5 gram for human beings. It would have been desirable to use only labelled dihydrostreptomycin and then in a dose corresponding to 2 grams for human beings in order to reduce the exposure time to a minimum, but this was not possible because the radioactive substance would then not have been enough for the investigation.

2. *Microautoradiography*

Thirteen animals were used for microautoradiography of the tuberculous abscesses. Each was given 1.5 mCi tritium-labelled dihydrostreptomycin intracardially, *i.e.*, 3 μCi per gram bodyweight. Also here non-radioactive dihydrostreptomycin was added so that the dose should correspond to about 11 mg dihydrostreptomycin per animal. The animals were killed by a blow against the neck, four animals ½ hour, three 1 hour, three 2 hours, two 4 hours and one 5 hours after the injection. The tuberculous abscesses were then removed, freeze-dried and sectioned by the method described on pages 57—59. The plates were exposed for about 3 months.

B. RESULTS

Half an hour after the injection the whole-femur autoradiograms showed a high concentration of radioactivity in the periphery of the tuberculous abscess and less in its centre (Fig. 19).

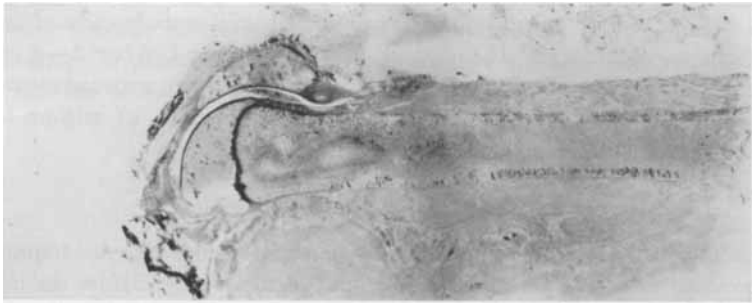

Fig. 19
Whole-femur autoradiogram half an hour after injection of dihydrostreptomycin. ×2.

The microautoradiograms showed a marked concentration in the peripheral part of the central necrosis (Figs. 20 and 21), where the blackening of the autoradiogram was much stronger than over the surrounding normal marrow, the outermost non-necrotic layer of the abscess, and over the innermost part of the necrosis.

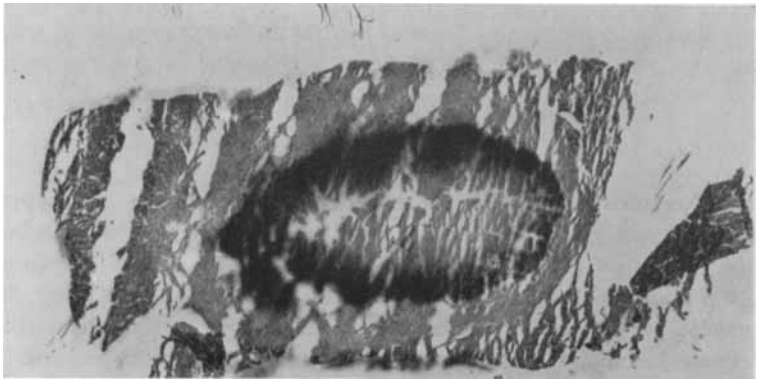

Fig. 20
Microautoradiogram half an hour after injection of dihydrostreptomycin. ×15.

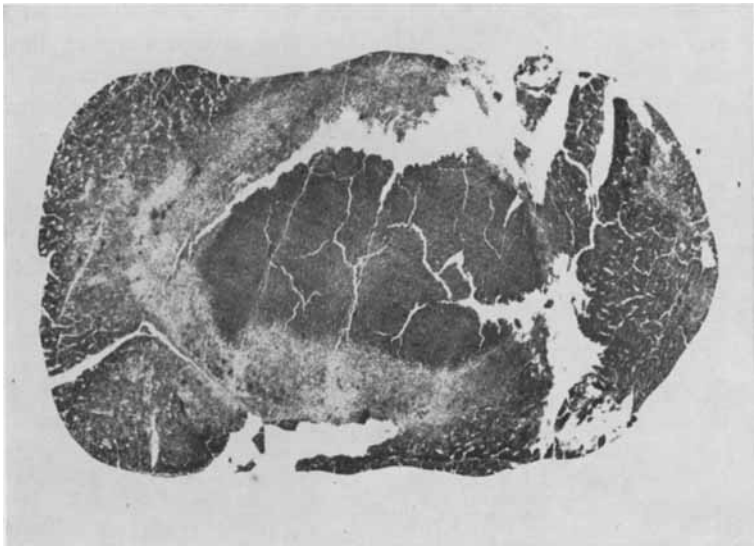

Fig. 21
Histological section of same lesion as shown in Fig. 20. Htx-eosin. ×15.

After *1 hour* the blackening on the whole-femur autoradiograms over the abscess was relatively even stronger than after half an hour compared with that over normal marrow and musculature. It was, however, weaker than over the periosteum and epiphyseal line (Fig. 22).

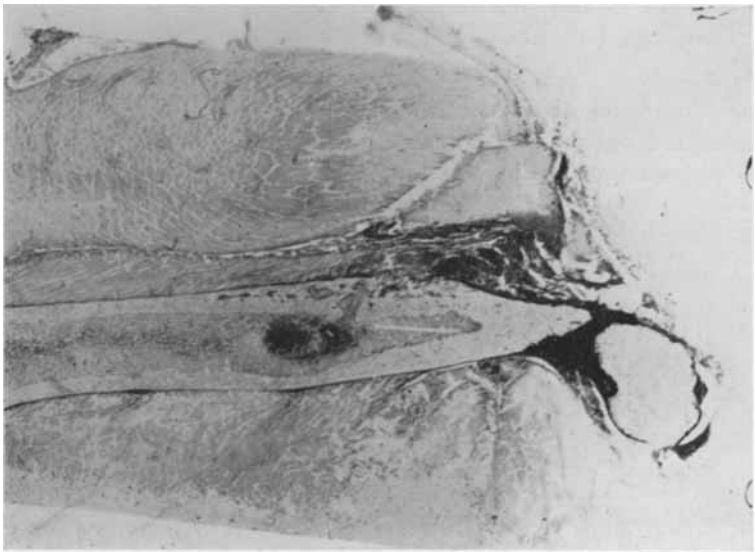

Fig. 22
Whole-femur autoradiogram 1 hour after injection of dihydrostreptomycin. ×3.

On the microautoradiograms the blackening over the necrosis was scattered in clusters over the nuclear remnants (Fig. 23). This pattern was seen during the entire course of the investigation, even in the last preparation removed 5 hours after the injection. It was not possible to decide with certainty what sort of tissue

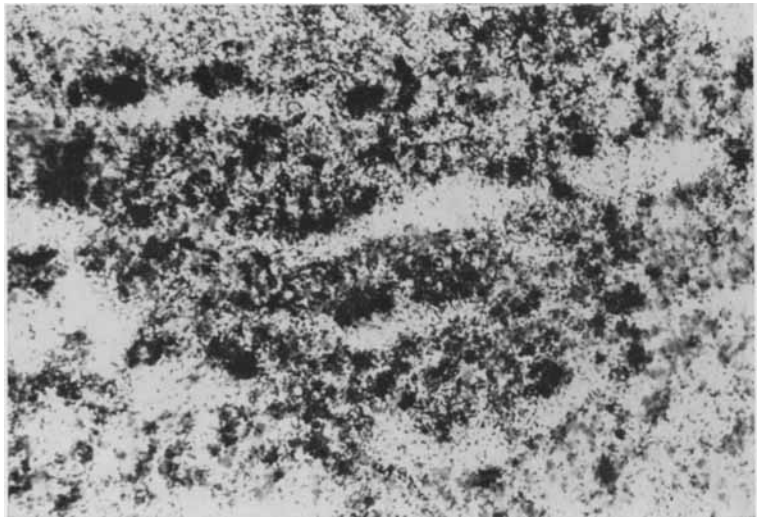

Fig. 23
Blackening over necrosis 1 hour after injection of dihydrostreptomycin. ×260.

was thus marked, because of the blurredness of the sections. But it was always clearly confined to necrotic areas and there it was found only over parts with nuclear rests and not in intermediate areas without nuclear remnants (Figs. 30—37).

After *2 hours* the blackening over the abscess was still much stronger than over the normal marrow (Fig. 24). It was stronger over the necrotic centre than over the peripheral parts of the abscess (Figs. 25 and 26).

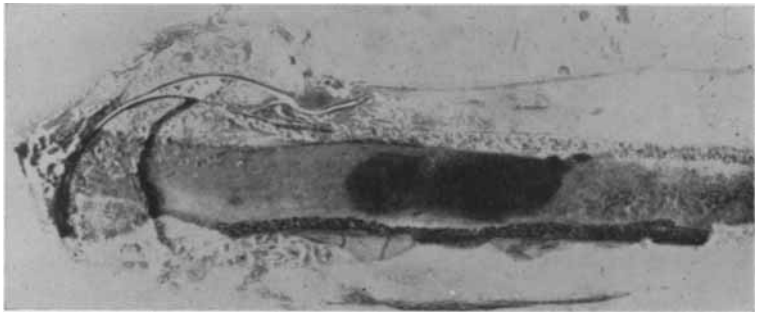

Fig. 24
Whole-femur autoradiogram 2 hours after injection of dihydrostreptomycin. ×3.

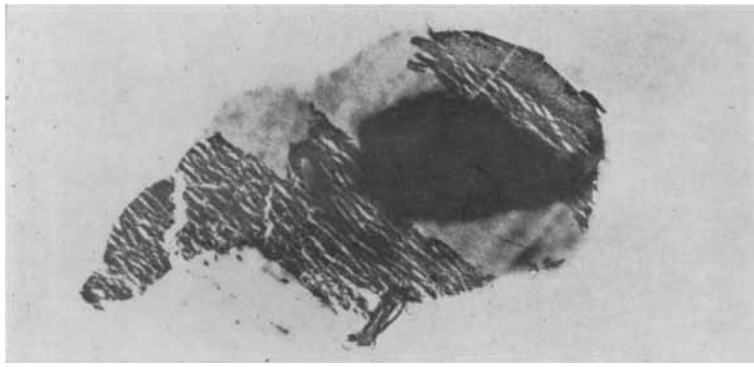

Fig. 25
Microautoradiogram 2 hours after injection of dihydrostreptomycin. ×15.

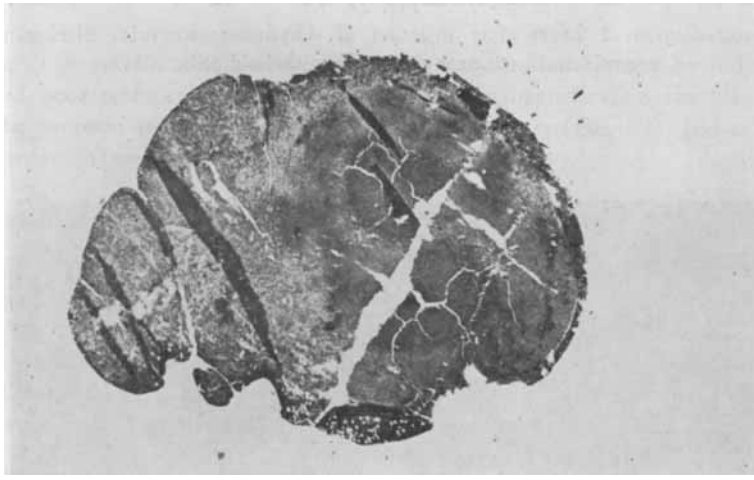

Fig. 26
Histological section of same lesion as illustrated in Fig. 25. Htx-eosin. ×15.

This was still more obvious under higher magnification (Figs. 27 and 28) where it was seen that the blackening over the granulation tissue layer outside the necrosis was weaker than over the necrotic area.

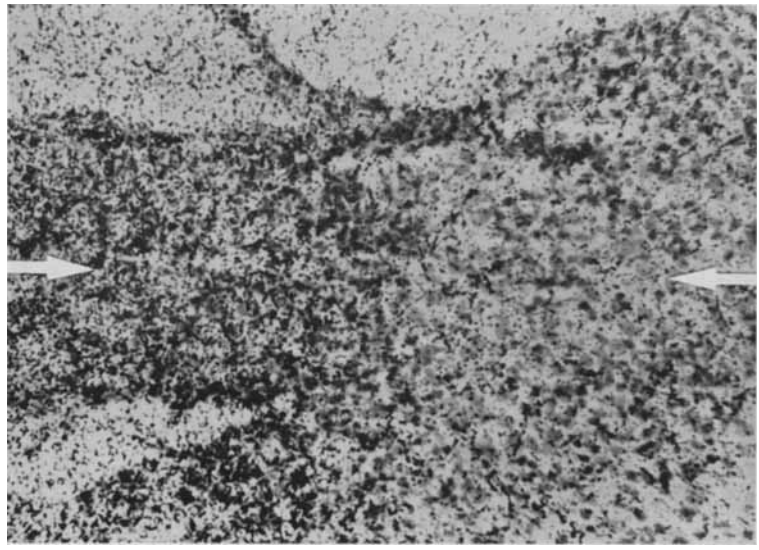

Fig. 27
Microautoradiogram 2 hours after injection of dihydrostreptomycin. Blackening over necrosis much stronger than over epithelioid cells. ×300.

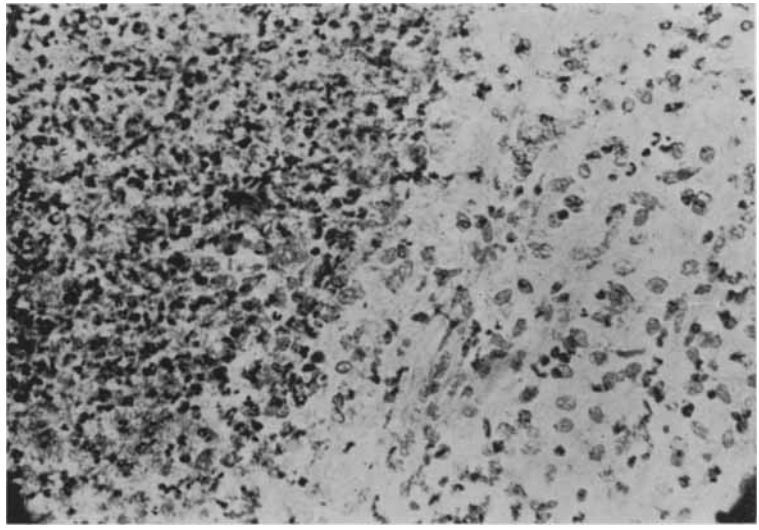

Fig. 28
Histological section showing border between epithelioid cell layer and necrosis, as in Fig. 27. Htx-eosin. ×300.

After *4 hours* the blackening over the abscess was still clearly stronger than over surrounding normal marrow and musculature, but appeared to be weaker than over the epiphyseal line and joint cartilage (Fig. 29).

Fig. 29
Whole-femur autoradiogram 4 hours after injection of dihydrostreptomycin. ×2.

After *5 hours* the whole microautoradiograms could no longer be judged because of poor technical quality, but under higher magnification the blackening over the necrotic area was still seen as scattered clusters (Fig. 30), and as before it was more diffusely spread over normal marrow (Fig. 31).

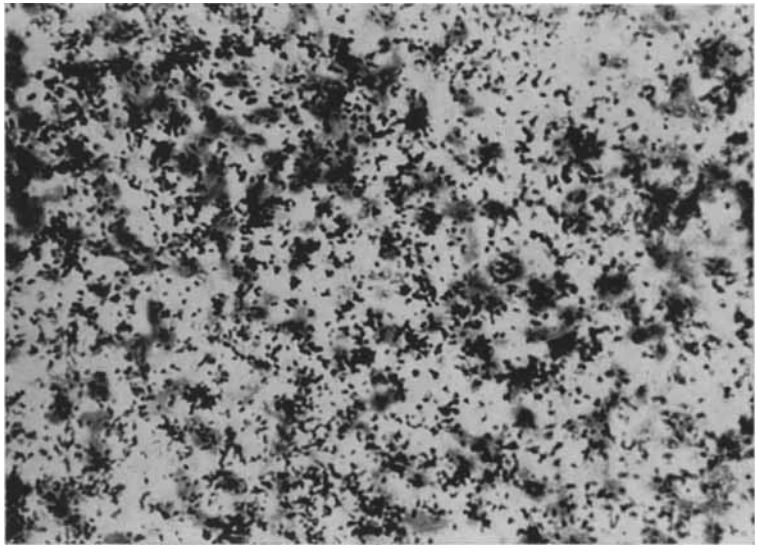

Fig. 30
Microautoradiogram of necrosis 5 hours after injection of dihydrostreptomycin. ×670.

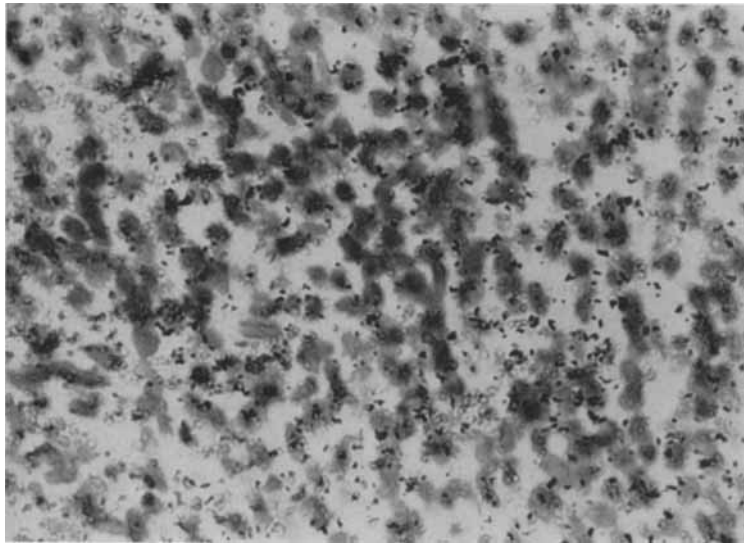

Fig. 31
Microautoradiogram of normal marrow 5 hours after injection of dihydrostreptomycin. ×670.

The blackening over the necrosis was localised especially to areas with nuclear remnants and was much weaker over intermediate areas (Figs. 32—37).

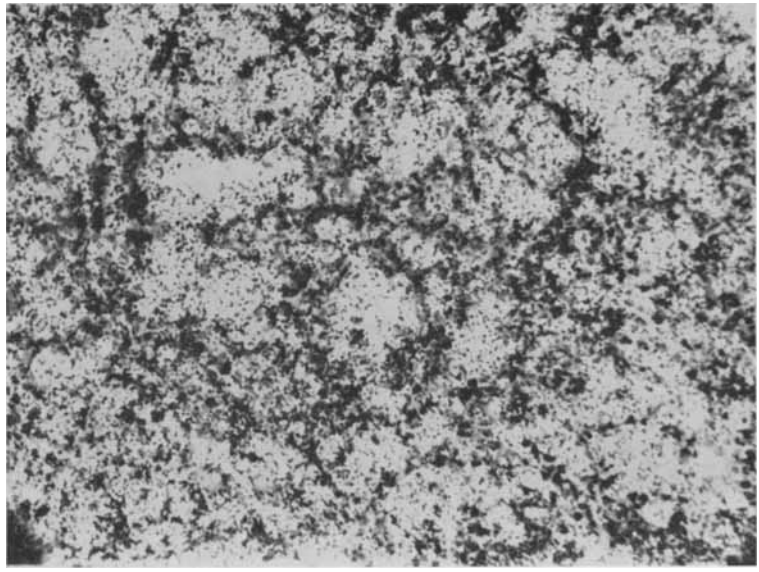

Fig. 32
Microautoradiogram of necrosis 5 hours after injection of dihydrostreptomycin. Blackening strongest over areas with nuclear remnants and weakest over intermediate areas. ×260.

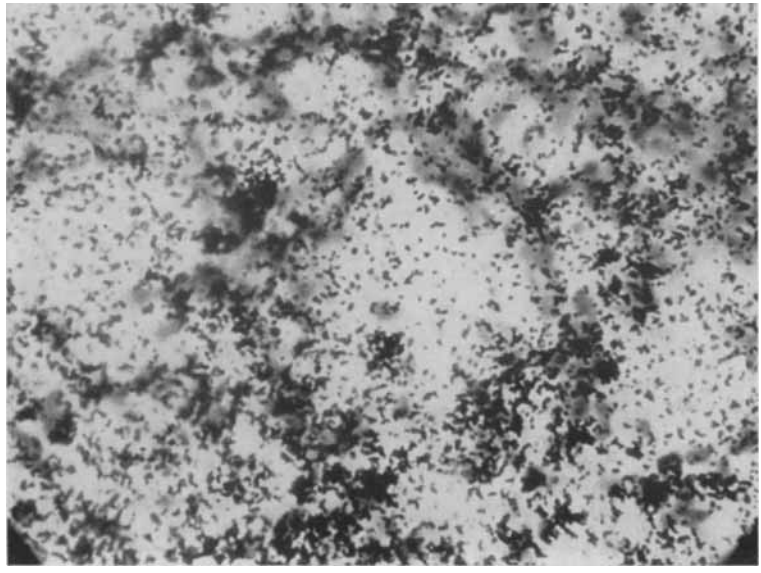

Fig. 33
Detail of area in Fig. 32. ×670.

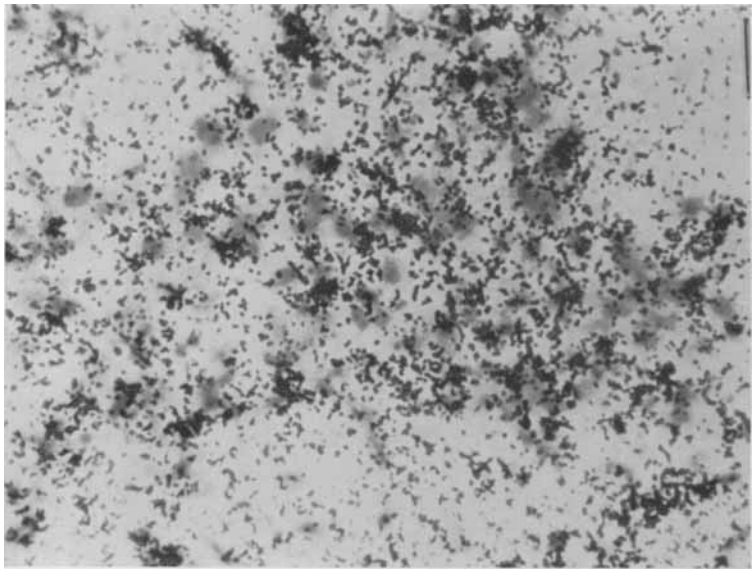

Fig. 34
Microautoradiogram of necrosis 5 hours after injection of dihydrostreptomycin. Microscope focused on silver granules. ×670.

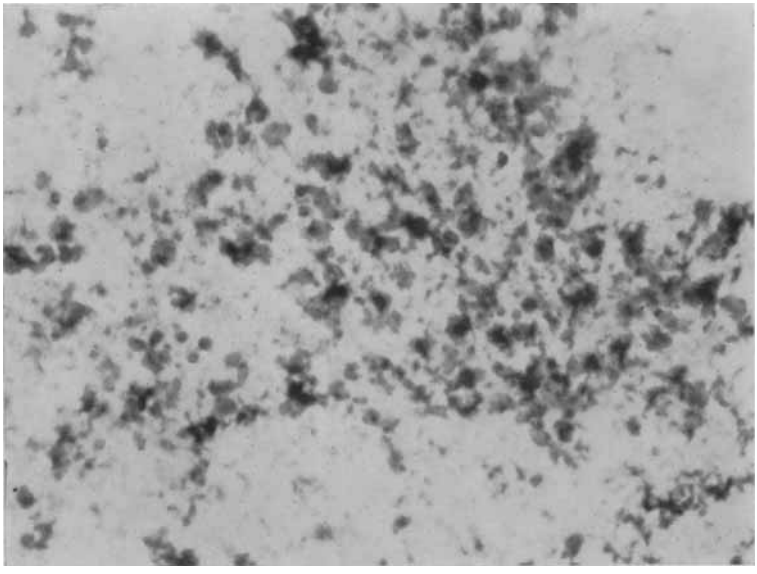

Fig. 35
Same area as in Fig. 34. Microscope focused on nuclear remnants. ×670.

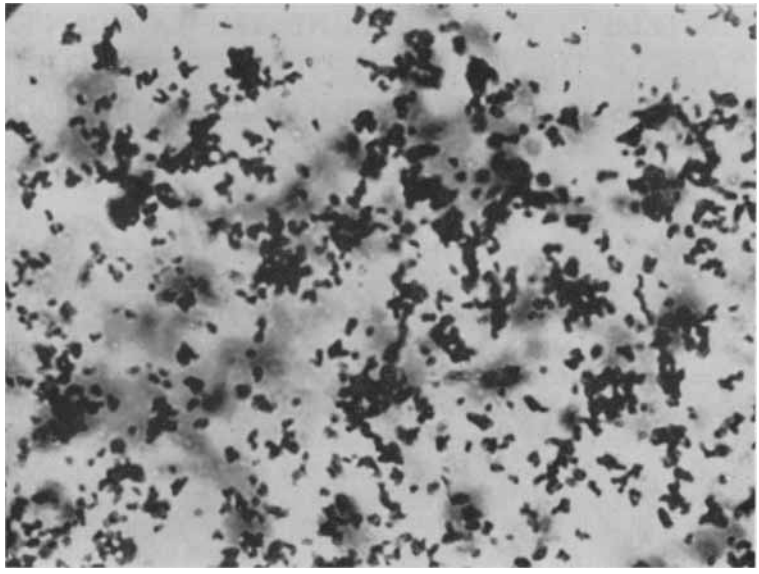

Fig. 36.
Microautoradiogram of necrosis 5 hours after injection of dihydrostreptomycin. Microscope focused on silver granules. ×1300.

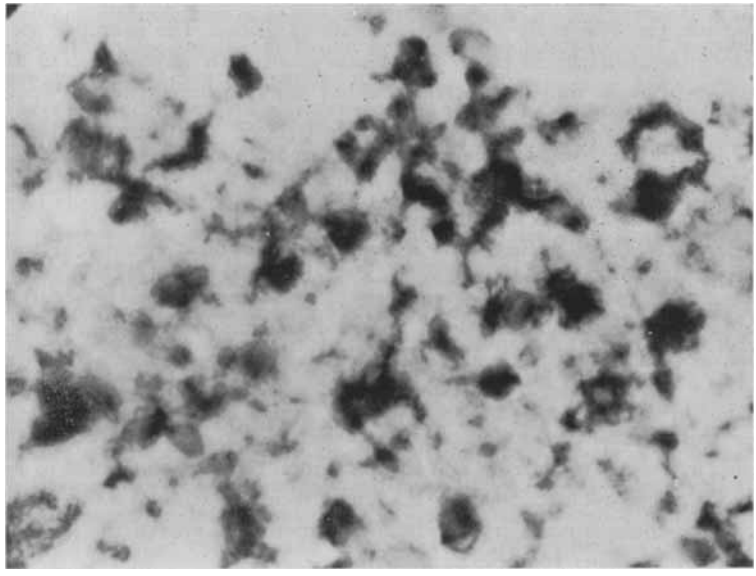

Fig. 37
Same area as in Fig. 36. Microscopic focused on nuclear remnants. ×1300.

XIV. ATTEMPTS TO DETERMINE THE CONCENTRATION OF DIHYDROSTREPTOMYCIN IN THE ABSCESSES WITH MICROBIOLOGICAL METHODS

The marked blackening over the necrotic area in the abscesses suggested a high concentration of dihydrostreptomycin there. Since earlier workers in this field found no degradation products of dihydrostreptomycin or streptomycin in the bodies of animals or human beings and since no such products were found in the present investigation (page 54), there appeared to be no reason to assume that the above mentioned blackening was to any substantial extent due to radioactive degradation products. Exclusion of this possibility, however, would require determination of the amount of dihydrostreptomycin in the abscesses and comparison of the values found with the amount of radioactive substance.

The following experiment was carried out in an attempt to check whether the marked blackening was really due to an accumulation of dihydrostreptomycin in the necrotic region of the abscesses.

The material consisted of 10 guineapigs. Of these, 8 had artificially induced tuberculous abscesses in the femur, while the remaining 2 were intact. Five of these 8 animals and the 2 intact ones were each given 11 mg of non-radioactive dihydrostreptomycin intracardially. All 10 animals were killed 3 hours after the beginning of the experiment. The femoral abscesses and pieces of macroscopically healthy marrow were removed from the infected animals and bits of healthy marrow from the 2 animals without tuberculous lesions. The necropsy specimens were placed immediately on blood agar plates sown with staphylococcus albus of a strain susceptible to dihydrostreptomycin but not to other antibiotics (page 53). Specimens consisting of necrotic material only, were removed from 2 of the abscesses in animals that had received dihydrostreptomycin and placed on separate blood agar plates. The plates were incubated at 37° C for 18 hours and examined for inhibition zones around the pieces of tissue.

All the pieces from animals that had received dihydrostreptomycin were surrounded by distinct inhibition zones 2—5 mm broad. But owing to differences in shape and size of the bits of tissue it was not possible to say whether or not the breadth of their inhibition zones varied with the type of tissue. No inhibition zones were seen around pieces of tissue from the animals that had not received dihydrostreptomycin.

After this preliminary experiment an attempt was made to elute dihydrostreptomycin from the marrow abscesses and intact marrow in order to estimate the dihydrostreptomycin content of the eluate. But the attempt was unsuccessful because the technique necessary for such an experiment proved too advanced for the laboratory facilities available at that time.

Comment. The experiment demonstrated the presence of antibiotic in the necropsy specimens, but was uninformative regarding any differences between

the concentration of the antibiotic in the bits of tissue. This experiment shows that further investigation is necessary to check whether the radioactive substance in the necrotic region of the abscesses consists entirely or partly of active and/or inactive dihydrostreptomycin and/or degradation products.

XV. DISCUSSION

A. WHOLE-FEMUR AUTORADIOGRAMS

Since the middle of the tuberculous abscess is necrotic and does not contain any blood vessels, one might expect the concentration of dihydrostreptomycin to be lower there than in surrouding tissues. Research on patients has, however, shown that streptomycin can diffuse into necrotic parts of the tuberculous abscesses in the skeleton (page 48). But the difference between these investigations and the experimental ones discussed below are so large that no comparisons can be made with our present knowledge of the problem.

André (1956) found the activity to be highest in the capsule rapidly to decrease towards the centre of the abscess. This seems to suggest that dihydrostreptomycin diffuses into a necrotic abscess only to a small extent. Hanngren's (1959) observations on the diffusion of para-aminosalicylic acid in tuberculous abscesses appears to argue in the same direction.

In the present investigation, however, the dihydrostreptomycin seems to have a special and unexpected affinity for the necrotic centre of the abscess. On the other hand, the blackening over both the outermost non-specific granulation tissue layer and the inner specific tissue layer was only slightly stronger than that over normal marrow. The difference in the results can probably be explained by the fact that André (1956) stopped his investigation already half an hour after the injection of the radioactive dihydrostreptomycin, while in the present investigation the experiment was continued up to 5 hours after the injection. Differences in the size of the abscesses and their structure as well as the composition of the necrotic material may perhaps also be of importance.

The autoradiograms illustrated in Figs. 19 and 20 show the distribution of the blackening in abscesses half an hour after the injection. The blackening is seen in the periphery of the abscesses as in André's (1956) autoradiogram. In the preparations removed later, however, the radioactive substance had diffused into the entire necrotic centre, and the concentration there was much higher than in surrounding normal marrow up to 4 hours after the injection. The dihydrostreptomycin thus appears to have concentrated in the actual necrotic substance.

B. MICROAUTORADIOGRAMS

The microautoradiograms showed that the blackening over the necrotic parts was invariably concentrated over parts of cells or remnants of cells that had taken on nuclear stain. It was not possible to decide exactly over what sort of tissue this labelling was located because the tissue was necrotic, which makes identification difficult and because the preparation was somewhat blurred. But this labelling pattern was not seen over areas void of nuclear remnants (Figs. 30—37). In many of the preparations the blackening over the necrosis was so intense that it obscured the tissue. But since sections had also been taken simultaneously for histological preparations, it was known that the blackening had occurred over the necrotic areas.

No explanation can be offered for this pattern of distribution of the radioactivity. The radioactive substance obviously diffused into the necrotic tissue and concentrated there. Whether the substance really consisted of active dihydrostreptomycin or entirely or partly of inactive dihydrostreptomycin bound to one of the components of necrotic substance, as suggested by Froyez and Froyez-Roederer (1952) (page 48), or a degradation product, is not known.

If the dihydrostreptomycin (or streptomycin as in Froyez' investigation) is bound to any of the components of the necrosis and thereby inactivated, this may explain the accumulation in the autoradiogram as well as the discrepancies between the results obtained when the streptomycin content in tuberculous pus was determined by microbiological methods.

The high activity found by André (1956) over the abscess capsule could not be verified by microautoradiography in this investigation. It was found that the blackening was considerably weaker over both the normal bone marrow and the non-specific and specific granulation tissue of the abscess than over the necrosis. The blackening was mainly diffusely spread over all three layers. In some of the preparations the degree of blackening was the same over all three, while in others the blackening was slightly stronger over the specific tissue layer than over the other two. A possible explanation why André (1956) believed to find a higher activity over the abscess capsule may be that the fine histological details in his autoradiogram were difficult to interpret and that the activity was in reality confined to the outer layer of the necrosis, *i.e.*, the layer immediately inside the wall of the abscess.

SUMMARY

Part I

A method is described for inducing a progressing local tuberculous lesion of the skeleton of the guineapig.

According to this method, a piece of Spongostan impregnated with a suspension of human tubercle bacilli is inserted in the marrow cavity through a hole drilled in the distal part of the femur of unvaccinated and vaccinated guineapigs.

Vaccination proved to influence the development and healing of the lesions induced. In the unvaccinated animals productive foci appeared after 2 weeks and these foci developed after about 5 weeks into abscesses, which persisted until the end of the experiment (4 months). In the two series of vaccinated animals abscesses appeared already after 2 weeks. After 5 weeks they began to diminish and all of them healed within 4 months.

The method filled three requirements set up:
no premature deaths, almost regular development of a lesion and production of the lesion at a predetermined site.

Part II

Tritium-labelled dihydrostreptomycin was injected into guineapigs with tuberculous skeletal abscesses. The penetration of the dihydrostreptomycin into, and its distribution in, the abscesses was studied on autoradiograms made with a freeze-drying technique. It was found that the dihydrostreptomycin penetrated the whole abscess and was especially concentrated in the necrosis, where the concentration was considerably greater than in the wall of the abscess and in the normal bone marrow.

On microautoradiograms it was seen that the dihydrostreptomycin was concentrated to cell remnants that had taken on nuclear stain.

The possibility that the blackening of the autoradiograms or parts of them was due to radioactive metabolites of dihydrostreptomycin is discussed.

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