

University of Edinburgh.

AN INVESTIGATION OF THE MICROFLORA ASSOCIATED WITH
THE EARTHWORM.

by

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INTRODUCTION.

AN INVESTIGATION OF THE MICROFLORA ASSOCIATED WITHTHE EARTHWORM.INTRODUCTION.

It is widely accepted as an established fact that the activity of earthworms promotes soil fertility. While the mechanisms responsible for this general effect are only vaguely understood, it is reasonably certain that micro-organisms will participate in most of the processes that involve transformations of organic substances. The literature provides little information concerning the gut microflora of the earthworm or of the effect of earthworms on the micro-organisms of the soil. These problems have been investigated only by Bassalik (1913), Stöckli (1928), Dawson (1947) and Day (1950), and in no case did the work penetrate deeply into the subject.

The investigation to be described here had as its principal objective a study of the changes that occur in the microflora when soil passes through the alimentary tract of the worm. Information on this question is desirable for the reason that many tons of soil per acre may be passed through earthworms in the course of a year (Evans, 1948). Another objective was the investigation of any changes in the soil microflora that the worm might produce by other means, as for example by its secretion of slime.

The lack of previous experimental work made it necessary, as an initial step, to establish satisfactory methods/

methods of investigation. As it is not possible to examine the contents of the alimentary tract of the earthworm without contact with its coelomic fluid, it was also necessary to determine the nature of the microflora of the coelom.

In the limited time available, an attempt has been made to provide a preliminary investigation which may serve as a basis for future studies of these problems.

PRELIMINARY INVESTIGATIONS.

PRELIMINARY INVESTIGATION.INTRODUCTION.

The available literature dealing with the effect of earthworms on the microflora of the soil and changes which occur in this microflora during its passage through the worm gut fails to provide any information which can be used as a sound basis for further research. It became apparent that the initial stages of this investigation would have to be confined to the establishment of satisfactory techniques and a preliminary exploration of the nature of the microflora occurring in the worm gut.

The methods devised for the examination of the gut contents and the coelomic fluid and the results of microscopical examinations of these materials will be described first.

SPECIES OF LUMBRICIDAE EXAMINED.

During the preliminary investigations a large number of each of the following species were examined:

Allolobophora caliginosa

A. chlorotica

A. longa

Lumbricus rubellus

L. terrestris

Octoclasium cyaneum

The worms were identified by means of the key given by Bornebusch (1930). Following the preliminary investigations Lumbricus terrestris was used exclusively as its greater size facilitated dissection and the sampling of coelomic fluid and casts for quantitative bacteriological studies.

METHOD OF PREPARATION OF WORMS FOR EXAMINATION.

It was necessary to find a satisfactory method by which earthworms could be killed with the least possible damage to the fragile alimentary tract, and with a killing agent which would not be likely to effect the bacterial flora of the gut or the coelomic fluid. After trials with a number of killing agents the following procedure was adopted and used in all subsequent experiments.

The worm to be examined was removed from the soil/

soil, and washed in gently running cold water until the adherent slime and soil had been removed. The worm was then placed in a clean glass bottle in which a chloroform soaked pad was suspended from the airtight cover. As soon as it was observed that the worm had ceased all movement and could be considered dead, it was removed from the bottle. The worm was then washed in cold water again to remove the slime and castings which had been excreted during the death agony. The worm was then ready for the treatment indicated by the nature of the experiment to be conducted as will be outlined in the account of the experiment involved.

DETERMINATION OF THE RATE OF PASSAGE OF SOIL
THROUGH A WORM.

As the worms to be used in the experimental work came from different types of soil and from soils incubated for different periods it was apparent that the time lapse between the ingestion of a soil and its excretion as a cast must be determined. The following experiment was conducted in order to secure information on this question.

Two soils of different colour were selected. One was a black garden loam and the other a red clay. The air dried 3 mm. fraction of each of these soils was placed in a number of jars. When required a sample of each of the soils was brought to 60% of its water holding capacity and firmly packed. It was found that when worms which had been maintained in the black loam were transferred to the red clay, and vice versa, the gut contents consisted entirely of the new soil/

soil after a period of 48 hr. The difference in the colour of the two soils made it possible to observe when the castings consisted entirely of the newly ingested soil. Numerous observations were made and at no time did the period required to empty the earthworm gut of its previous contents exceed 48 hr.

On the basis of this information, a period of 48 hr. was always allowed to elapse from the time worms were first placed in a new soil, and the time the gut contents were first examined. When worm casts were required for bacterial counts, several passages of the worms were first made through a series of samples of the soil that was to be employed for the counts.

METHOD OF OBTAINING FRESH WORM CASTS.

Earlier workers (Jensen, 1898; Stöckli, 1928; Lunt and Jacobson, 1944; Dawson, 1947, and Day, 1950) failed to explain how earthworm casts were obtained for examination, or indicated that the casts were collected from the surface of the soil or a soil sample. Obviously, collecting casts from soil is unsatisfactory; the age of the cast is not definitely known, and the possibility of profound changes occurring in the microflora of the cast even within a few hours cannot be ignored. Further, this method makes it almost impossible to avoid including in the sample some of the soil on which the cast rests. It was decided that a more reliable method of sampling would have to be evolved.

Several/

Several methods were tested which proved unsatisfactory. Worms tend to defecate when placed in water. This was first considered as a method of obtaining a suspension which could be used quantitatively by determination of the dry weight of the cast after use in further dilutions. This was, of course, subject to the limitation that any bacteria present on the external surface of the worm would be incorporated in the sample and that the slime produced by the worm during its immersion in water would be incorporated with the sample giving a false weight of the cast. The possibility of obtaining a sample by dissection was considered but here again the introduction of extraneous matter in the form of coelomic fluid or portions of the worm gut could not be ignored. On a dry surface, worms failed to defecate sufficiently quickly to permit casts being collected in a short enough time to provide a sample representative of a fresh cast. After consideration of these methods and others, the following procedure was adopted.

A worm was washed in running cold water until clean and then passed through a series of washings in sterile water in the hope that as much as possible of the external microflora would be removed. The worm was then removed from the final wash water and its anus placed in contact with a sheet of sterile paper. The remainder of the worm was held in the air. The back of the blade of a sterile scalpel was placed about 1 cm. cephalad to the anus of the earthworm. When slight pressure was applied with the scalpel to the dorsum of the worm, the animal attempted to escape by a violent forward contraction. This movement produced a fresh cast. Several casts/

casts from different worms were obtained in this manner, collected on the blade of a sterile scalpel and placed in a sterile tared weighing bottle which contained 5 ml. of sterile water. A suspension was made of the sample in the usual manner and further dilutions prepared from it. The original weight of the sample was determined by drying the residual suspension at 100°, weighing and calculating the original weight of the casts. It was found that in general, the casts obtained from three earthworms in this manner approximated 0.05 to 0.1 g. dry weight of cast. It was considered that this method gave a representative sample of the final gut contents or a fresh worm cast.

DIRECT MICROSCOPICAL EXAMINATION OF WORM GUT CONTENTS.

Direct microscopical examination of the gut contents of the six species previously mentioned was made to determine if any obvious changes occurred in the microflora of the material passing through the gut. Worms were obtained from a variety of soils and examined as soon as possible after their removal from their natural habitat. Specimens of each species of worm were also placed in jars of soil to which various organic compounds had been added. These additions consisted of glucose, manure, dried milk and shredded cellulose. Worms of various species were removed from these soils periodically and their gut contents examined.

A standard procedure was adopted for the microscopical examination of the gut contents. After a worm had been/

been prepared for examination, as has previously been described, it was pinned to a sheet of paper placed over a cork board. A ventral incision was made along the entire length of the worm. Extreme care was taken not to puncture the gut wall. Samples of the gut contents were obtained from the oesophagus, gizzard and crop, foregut, midgut and hindgut by making a small incision in the gut wall and removing a sample with a sterile loop. A number of films were prepared from each sample.

After investigating several staining methods it was found that those of Gray and Thornton (1928), or Jones and Mellison (1948), were the most satisfactory for making comparisons with the soil in which the worm had been living.

Examinations of many hundreds of films failed to show any obvious difference between the microflora of the worm gut, and the microflora of the soil from which the worm had been removed. This applied to all the species of worm.

The nature of the gut contents of the worm makes it impossible to obtain a sample which can be used for a direct count of the numbers of bacteria present. It was found, however, that in every case the number of bacterial cells appearing in the films was much greater when the film was prepared from the gut contents than when the film was prepared from the contents of the oesophagus or the gizzard or crop. While direct evidence is lacking that the bacterial population in the worm gut increases as the soil moves through the gut posterior to the gizzard, the appearance of the films examined would indicate that such is the case.

DIRECT MICROSCOPICAL EXAMINATION
OF THE COELOMIC FLUID.

The nature and function of the coelomic fluid.

Lumbricidae is filled with a fluid which is in contact with the external environment by way of the nephridia and the dorsal pores. The physical characteristics of this coelomic fluid have been described by Lim Boon Keng (1895). The fluid varies in colour depending on its cellular contents. In most cases it has an opaque, milky-white appearance. Its concentration depends upon the environment of the worm. In worms obtained from moist or wet soil it tends to be thin and watery and occurs in large quantities. In worms obtained from dry conditions it is much reduced in quantity and tends to be thick and viscous. Lim Boon Keng describes the reaction of the fluid as alkaline but Robertson (1936) reports the coelomic fluid as varying between pH 6.5 and 6.9. The pH values of Robertson were confirmed in tests made during the present investigation. The coelomic fluid not only fills the coelomic cavity, but is in free contact with the intermuscular spaces and thus permeates the musculature of the earthworm.

The coelomic fluid has been described as containing many crystalline inclusions, chiefly crystals of CaCO_3 ; chloragogen cells; coelomic corpuscles and bacteroids.

The Lumbricidae are capable of ejecting large quantities of coelomic fluid through their dorsal pores. The worms seem to be constantly covered with a slimy layer of coelomic fluid. Any irritation of the external body wall produces/

duces an immediate increase in the excretion of coelomic fluid. This effect may be produced by chemical or mechanical means, sudden changes in temperature, or by changes in the humidity of the environment. Stephenson (1930) suggests that the excretion of the fluid is of use in keeping the surface of the body moist, and in a suitable condition for the respiratory exchanges of the worm, thus protecting it from adverse conditions. The fluid also acts as a lubricant to aid the movement of the worm.

The coelomic fluid is rich in amoebocytes.

Lim Boon Keng believed that the presence of the coelomic fluid on the external surface of the worm prevented the invasion of the worm by bacteria present in the worm's environment. It was reported by Keng that when anthrax bacilli were injected into the coelomic cavity they were rapidly ingested by the amoebocytes.

BACTERIODS.

The occurrence of small, red shaped bodies in the connective tissue, intermuscular spaces and in the coelom of many members of the Oligochaeta has been reported. These inclusions have been named bacteroids. They appear to be of universal occurrence in members of the Lumbricidae. They have been described by Cuénot (1898), and Knop (1926), as being irregular in size, sharp angled or rounded, rod-like forms which show transitions to granules.

Cerfontaine (1890) considered these structures, which he named "corpuscules bacilliformes", to be bacteria. His conclusion was derived from microscopical observations. He also considered that their presence might explain the rapid decomposition of dead earthworms.

Lim Boon Keng (1895) reported the isolation of
a/

a number of types of bacteria from the coelomic fluid of Lumbricus terrestris. He found that three types of organisms occurred in large numbers. The organisms isolated were mostly bacilli but micrococci and spirilla also occurred. The organism which he found in greatest numbers resembled the bacteroids morphologically although he did not designate it specifically as such. He considered that these organisms might have a symbiotic relationship with the earthworm, as they did not appear to be subject to phagocytosis.

Cuénot (1848), after a microscopical study of worm tissues and the coelomic fluid, agreed with Cerfontaine (1890), that the "corpuscules bacilliformes" were bacteria. Cuénot was the first to designate these structures as bacteroids.

Willem and Minne (1900) believed these bacteroids to be crystals of uric acid. They found in certain peritoneal cells masses of little, prismatic crystals which varied in size and in some cases had the shape of lozenges or hexagons. Willem and Minne considered these structures to be identical with those described by Cerfontaine and Cuénot. They considered that Cuénot had failed to observe the essentially crystalline nature of the majority of the structures, and had concentrated his observations on those forms which assumed the appearance of bacteria. Willem and Minne based their conclusion that these structures were crystals of uric acid on the evidence of microscopical studies conducted with polarized light, and of a series of macro- and microchemical tests.

Butschowitz (1931) reports that Schneider (1902), by using staining methods reached the conclusion that the bacteroids/

bacteroids were either bacteria as described by Cuenot or were possibly a form of 'trophochondria'.

Maziarski (1901,1903), in investigations of the structure of the nephridia in earthworms states that the inner wall of the ampulla is covered by a layer of rodlets, which by culture show themselves to be bacteria.

The presence of bacteria in the ampulla of the nephridia of Lumbricus terrestris was investigated by Knop (1926) by microscopical examination of stained sections. He concluded that the structures in the nephridia were bacterial cells and suggested a method by which they were transferred to successive generations of earthworms through the cocoons. His work is reviewed and discussed by Buchner (1930).

Knop (1926) distinguished between the organisms which occurred in the nephridia and the bacteroids, as described by Cuenot (1898) and Willem and Minne (1900), which occurred in the connective tissue, intermuscular spaces and in the coelom. He concluded from a variety of staining reactions that the bacteroids were crystals of unknown end-products of metabolism.

Pandazis (1931) in studies of several Oligochaeta claimed to have isolated the organism occurring in the nephridia. He considered that the bacteria served a symbiotic function by providing an enzymatic system which split the albumin excreted with the urinary fluid. Part of the albumin fraction was then reabsorbed by the parietal cells of the nephridia.

Butschowitz (1931) repeated and enlarged upon the methods used by Willem and Minne (1900), in a study of the bacteroids of Lumbricus terrestris. He concluded that the bacteroids of the connective tissue of earthworms were crystals of/

of protein.

Gianfurari and Cantoni (1936) reported that a Gram-negative, non-motile, capsulated rod resembling both the Friedlander group, and the non-motile types of the paracolon group was constantly present in the coelomic fluid of Lumbricus terrestris. They did not, however, attempt to relate this organism directly as the bacteroid.

It was considered advisable to examine the coelomic fluid to determine if a bacterial flora existed in the fluid. This examination was indicated for the following reasons. The work previously reported had failed to indicate conclusively whether the bacteroids present in the coelomic fluid were bacteria or crystalline inclusions. If a specific flora was present in the nephridia, then, as the coelomic fluid was in constant contact with the nephridial canals, this flora might be expected to occur in the coelomic fluid. The coelomic fluid was in constant contact with the external environment through the nephridial canals and dorsal pores, thus many chances occurred for the infection of the coelom by soil organisms. If a bacterial flora existed in the coelomic fluid this fact would have to be considered in any bacteriological examination of the alimentary canal.

Experimental methods and results.

A preliminary investigation of the coelomic fluid of the species of worms previously mentioned was made by the examination of films prepared from samples of the fluid taken from a number of positions along the coelomic cavity. The method employed in obtaining the samples was similar to that used in obtaining the samples of the gut contents. A large number of worms were examined and films stained by simple basic stains or/

or by Gram's method.

In all cases, numerous rod-like structures were observed. These structures stained well with the basic dyes employed and were consistently uniform rods with rounded or square-cut ends. They always occurred singly and gave a Gram-negative reaction. Samples of the coelomic fluid were examined directly, or diluted, by the hanging drop and wet mount methods for evidence of motility of these structures. Motility was never observed.

Smears were made from the external body wall, the internal body wall, external surface of the gut, the clitellum, nephridia, hearts, septa, ovaries, testes, seminal vesicles and calciferous glands. In all cases similar Gram-negative, rod-like structures were observed.

The species of the worm examined or the conditions under which it had been maintained did not appear to effect the incidence of these structures. Bacterial forms differing from these bacteroids were never observed unless the worm had suffered a recent injury. In this case, the coelomic fluid in the area of the injury showed a mixed microflora and large concentrations of amoebocytes.

These examinations had shown the prevalence of bacteroid forms in different species of earthworms under different conditions but had failed to demonstrate conclusively the bacterial nature of the structures. Samples taken from a large number of specimens of Lumbricus terrestris were examined by a variety of methods in order to obtain further information with regard to the nature of the bacteroidal forms.

Attempts to establish the presence of flagella or spores by a number of the standard techniques failed to indicate/

indicate the occurrence of either spores or flagella. When capsule stains were applied the bacteroids appeared to be surrounded by masses of amorphous material without any evidence of a distinct capsule associated with the structure. Films stained by the sudan black-safranin method did not indicate the presence of fatty material in the bacteroids. The structures were not acid fast. Negative staining with nigrosin failed to reveal any outstanding characteristics.

Films were stained with a variety of simple stains of acidic or basic properties. The bacteroids present in the films appeared to show a marked affinity for stains of the basic group. The acidic group of stains were taken up but much more weakly. This suggests a predominance of acidic over basic groups in the bacteroids which is a characteristic of bacterial protoplasm.

Wet mounts were prepared from dilutions of the coelomic fluid and dilute solutions of crystal violet, methylene blue, Congo red, neutral red or Gram's iodine were allowed to diffuse under the cover slip. At first the bacteroids became surrounded by the dye, but remained unstained. After a period the exterior surface of the bacteroid took up the dye and eventually the entire bacteroid stained. No changes were seen when iodine was applied.

A differentiation of a cell wall in the bacteroids was attempted by methods proposed by Khaysi (1941), and Robinow (1945). A considerable number of bacteroids showed what appeared to be a distinct cell wall and in some cases the longer bacteroids appeared to be divided by a thin septum.

The affinity of the bacteroids for basic stains,
their/

their reaction to vital staining and the evidence of a cell wall suggested that the bacteroids were, in fact, bacterial cells. On the basis of this evidence, it was decided to attempt the culture of the bacteroids of the coelomic fluid.

ORGANISMS OF THE COELOMIC FLUID.

ORGANISMS OF THE COELOMIC FLUID.

The available information concerning the nature of the bacteroids present in the coelomic fluid of the earthworm indicated that a satisfactory investigation of the gut microflora could not be conducted until it was established whether or not these bacteroids were viable and would interfere with the separation of the bacterial population found in the gut.

Method of sampling the coelomic fluid.

In all investigations of the coelomic fluid, the same technique was used. A worm was washed and killed as previously described. The exterior of the worm was sterilised by immersion in 80% ethyl alcohol, or a saturated solution of chlorinated lime, or by the application of a heated blade of a scalpel to the area to be incised. When chemical sterilisation was employed it was followed by several washings in sterile water to remove the excess disinfectant present. These methods seemed to be equally effective and did not appear to effect the bacterial content of the coelom. The worm, without recontamination, was then pinned to a sheet of sterile paper and a short incision made with a sterile scalpel through the ventral body wall, care being taken to avoid the alimentary canal and blood vessels. The body wall on one side of the incision was carefully pulled back with a sterile probe and a few septa cut. A sample was removed from the pocket thus formed with a sterile Pasteur pipette graduated to take 0.01 ml. The inoculum was then placed in 10 ml. of sterile tap water or Ringer's solution and the sample thoroughly mixed by agitation. Further dilutions of 1/10,000; /

1/10,000; 1/100,000, and 1/1,000,000 were prepared by the standard method and used as inocula. The platings were completed as rapidly as possible. A further 0.01 ml. sample of the coelomic fluid was then removed from the worm and spread over a 1 cm² area on a slide. The film thus formed was stained with basic fuschin and a direct count of the bacteroids present was obtained by the method commonly employed in direct counts of the bacteria present in soil or in milk. The figure obtained was subsequently used as a check against the number of colonies appearing on the plates.

Use of simple media for the isolation of the coelomic organisms.

Lim Boon Keng (1895) has reported the isolation in gelatine plates of several types of organisms, one of which was similar morphologically to the bacteroids. Pandazis (1931) has isolated an organism, which he considers to be a symbiont of the earthworm, by transferring nephridia to nutrient broth. Gianfurari and Cantoni (1936) report an organism of the Friedländer type as being constantly present in the coelomic fluid of the earthworm. They isolated the organism by streaking nutrient agar slopes with a loopful of the coelomic fluid. If these were, in fact, true representatives of the bacteroids, it appeared that a comparatively simple medium would suffice for their isolation.

In the initial attempt to isolate the coelomic organisms two types of media were employed, nutrient agar and Topping's agar (p. 51). A sample of the coelomic fluid was obtained from each of four worms and plates were poured with each/

each medium at dilutions of 1/1,000; 1/10,000; 1/100,000, and 1/1,000,000. Two plates of each dilution were incubated aerobically at 35° and two plates at 20°. The plates were examined at frequent intervals.

From one of the worms, the plates showed colonies on both media and at both temperatures of incubation within a few days. Examination of these plates after 14 days' incubation showed a wide variety of colony types and microscopical examination showed forms which varied widely morphologically. No growth was observed, even after 30 days' incubation, on any of the plates inoculated from the other three worms, save the occasional colony which was obviously a contaminant.

Effect of sterilisation of the external surface of the worm on the bacteria in the coelom.

These results suggested either that the colonies developing from the first worm were the result of contamination of the coelomic fluid by gut contents or that the sterilisation of the external surface of the remaining worms had been too drastic and had destroyed the bacteria present in the coelom.

In order to determine which was the case, four worms of a similar size were selected. Two were anaesthetized by placing them in the chloroform bottle for a period just sufficient to stop their movement. They were then removed and stabbed through a specific segment with a fine dissecting needle in such a manner as to rupture the gut wall. They were then placed in water and allowed to revive. After some time all four worms were killed and their external surfaces sterilised by identical treatment. Samples of the coelomic fluid from the stabbed worms were obtained from the area of the stabbing. It was observed that/

that the gut wall had been broken. Samples of the coelomic fluid were also obtained from the unstabbed worms. Nutrient agar plates were inoculated and incubated at 20°. It was found that a mixed flora developed on the plates inoculated from the stabbed worms, while those inoculated from the unstabbed worms showed no growth. It was subsequently determined that rupture of the gut wall of the earthworm occasionally occurred naturally through the presence of sharp bits of stone in the gut contents or through the violent muscular contractions which took place during the death agony. In all future samplings great care was taken to ensure that the gut wall in the sampling area was intact.

In order to test the efficiency of the sterilisation process nutrient agar plates were inoculated with samples of the final wash water and other plates were smeared with a sterilised worm. In no case did growth occur other than the occasional casual contaminant.

The results of these experiments indicated that the sterilisation process was satisfactory and did not effect the organisms present in the coelom. They also showed that the bacteroids were unable to develop on nutrient agar or Topping's agar when incubated aerobically.

Use of complex media for the isolation of coelomic organisms.

It was then considered that the organisms might require either a richer medium or anaerobic conditions of incubation. Platings from dilutions prepared from numerous worms were made with the following media and incubated aerobically and anaerobically at 20° and 35°.

1. Nutrient/

1. Nutrient agar enriched by the addition of 10% sterile ox blood.
2. Topping's agar enriched by the addition of 10% sterile ox blood.
3. Nutrient agar.
4. Topping's agar.
5. Tomato agar. (Stirling, 1951).
6. Soil extract agar. (p. 61).

Shake cultures were also prepared in tubes and incubated under similar conditions.

The anaerobic plates were incubated in McIntosh and Fildes jars or in jars containing a large tube of pyrogallie acid and NaCO_3 . Each jar also contained a tube of methylene blue oxygen indicator. The shake cultures were treated in a similar manner.

In no case was growth obtained.

The nature of the coelomic fluid suggested the possibility that an increased CO_2 tension might be required by coelomic organisms. Therefore, the experiment outlined above was repeated with the addition to the jars of tubes containing CaCO_3 and dilute HCl . Growth still failed to occur.

As these media failed to produce growth under a wide variety of conditions it was considered that still more complex media might be needed. It was also considered that 'anti-growth' factors might occur in complex organic media as had been reported by Foster, Hardwick and Guirard (1950). For this reason starch was added to some of the media as an adsorbent.

The /

The following media were employed:

1. Lemco beef extract, 1.0% w/v; Evan's peptone, 1.0% w/v; Yeastral, 0.25% w/v; glucose, 0.2% w/v; soluble starch, 1.0% w/v; agar, 1.5% w/v.
2. As medium 1 with starch omitted and 10.0% v/v sterile serum added.
3. As medium 1 with Lemco beef extract reduced to 0.5% w/v and 10% v/v sterile serum added.
4. As medium 3 with starch omitted.
5. Topping's agar.
6. Corn liver mash (tubes) (McClung and McCoy, 1934).

Plates were prepared from dilutions of the coelomic fluid of a number of worms and incubated at 22° and 35° under both aerobic and anaerobic conditions with and without excess CO₂. The tube cultures of corn liver mash were similarly treated.

After 14 days' incubation coelomic organisms had failed to develop in any of these media.

As all the media used had been adjusted to pH 6.6, it was thought that a pH effect might be suppressing growth. The six media mentioned above were prepared with final pH values of 6.0, 7.0, and 8.0; plates and shake cultures inoculated with dilutions of the coelomic fluid were incubated aerobically and anaerobically. After 14 days' incubation, growth of coelomic organisms had failed to occur.

It was apparent that if the bacteroid of the coelom was a living organism, as had been suggested by microscopical studies, the conditions so far provided would not permit its growth in artificial medium.

Isolation/

Isolation of coelomic organisms.

A study of the literature dealing with the isolation and cultivation of symbiotic bacteria associated with insects and of other types of fastidious bacteria was made, and the media employed were compared. It was decided to test, in the first instance, the medium devised by Lewis, Bedell and Rettger (1940), and modified by King and Rettger (1942) for the isolation of non-sporulating anaerobic bacteria.

The medium used was slightly modified from their formula and consisted of:

Bacto tryptone, 2.0% w/v; Lemco beef extract, 1.0% w/v; glucose 1.0% w/v; Na_2HPO_4 , 0.4% w/v; cysteine, 0.05% w/v; yeast autolysate, 10.0% v/v; agar, 1.5% w/v, and made to 1000.0 ml with distilled water. The glucose, cysteine and yeast autolysate were added after the agar and the other ingredients had been dissolved. The reaction was adjusted to pH 6.6. Two tablets of rezasurin were added as an oxidation-reduction indicator. The medium was then tubed immediately and sterilised by autoclaving to 22 lb. pressure. Before use the medium was heated to 100° and held at that temperature until the indicator had become completely reduced.

Plates inoculated with 1/100,000 and 1/1,000,000 dilutions of the coelomic fluid and incubated anaerobically permitted the growth of an organism which had a similar morphological appearance to those occurring in the coelom. Plates incubated aerobically failed to permit the growth of any colonies.

The experiment was repeated with dilutions of the /

the coelomic fluid obtained from each of six worms. With these six series of platings the effect of an increased CO₂ tension and the effect of an acidic, neutral and alkaline reaction of the medium was tested. In all cases colonies developed on those plates which were incubated anaerobically while no colonies developed on the plates incubated aerobically. The pH of the medium within the range of 6.0 to 8.0 did not appear to effect the growth of the organism. The organism grew equally well at 20° and 35°. An increased CO₂ tension appeared to stimulate growth but was not essential. The number of colonies which developed consistently represented about 10% to 20% of the number of rods in the coelomic fluid as determined by direct microscopical count.

Films were prepared from the colonies which developed and were stained by Gram's method. The films showed a regular Gram-negative rod similar in appearance to the rods appearing in the coelom. On two of the platings a few colonies of another organism occurred. It appeared to be a Gram-negative streptococcus. This organism, which was originally considered to be a contaminant, will be described subsequently.

Organisms from the nephridia of earthworms.

Pandazis (1931) reported the isolation of a specific aerobic organism from the nephridia of earthworms. Knop (1926) found by direct microscopical examination that large quantities of bacteria-like structures were concentrated in the ampulla of the nephridia.

A number of nephridia were dissected from various/

various worms and examined microscopically. There appeared to be large concentrations in the nephridia of the organism present in the coelomic fluid. Pandazis' method of isolation of this organism is of extremely doubtful reliability; the removal of nephridia from the earthworm is a slow and tedious process; the chances of contamination are very large; his method of inoculating nutrient broth directly with a nephridium would permit the rapid growth of any contaminants present. Furthermore, it is impossible to disassociate the nephridia from the coelomic fluid.

In order to check the occurrence of an aerobic organism in the nephridia, six nephridia were removed from different worms. The nephridia were crushed in a small quantity of sterile water and plates of King and Rettger's agar poured by the serial dilution method. It was found that sets of plates from two nephridia produced aerobic growth. The remaining sets only permitted the growth of the anaerobic organism. This evidence suggests that the organism isolated by Pandazis was, in fact, a contaminant and did not represent the organism present in the coelom.

Growth requirements of the coelomic organisms.

In order to determine the factor in King and Rettger's medium which permitted growth but which was not present in those media used previously, the following media were employed in a series of platings:

1. King and Rettger's agar (control).
2. King and Rettger's agar with yeast autolysate
replaced by Yeastrel.
3. King/

3. King and Rettger's agar with yeast autolysate replaced by soil extract.
4. King and Rettger's agar with yeast autolysate replaced by tomato juice.

Plates of each medium were inoculated with a 1/100,000 dilution of the coelomic fluid of a worm, and incubated at 20° both aerobically and anaerobically.

Growth of the organism occurred on the anaerobic plates of medium 1, but colonies failed to develop on media 2, 3, or 4.

The experiment was repeated with the same four media but with the replacement of cysteine by thioglycollate or ascorbic acid, and with the elimination of a reducing agent.

In these three series of platings growth once again occurred on the medium containing yeast autolysate and failed in its absence. The substitution of another reducing agent for cysteine, or its absence did not appear to effect growth although growth occurred more rapidly in the presence of a reducing agent. Growth failed in all cases on those plates which were incubated aerobically.

These results suggested that an essential growth factor was present in yeast autolysate but not in Yeastrel, tomato juice or soil extract. If cysteine was required as an essential growth factor, it appeared to be present in the other components of the medium in sufficient quantities to permit growth.

Pure/

Pure culture studies of the coelomic organisms.

A satisfactory method and medium had been secured for the isolation of an organism which apparently occurred regularly in the coelomic fluid of the earthworm. It was decided that a preliminary investigation of the physiological character and the function of this organism should be undertaken.

Fifteen colonies were selected from three different platings and replated in the same medium by the serial dilution method. After 7 days' incubation under anaerobic conditions at 20° well formed colonies had developed. A representative colony of each of the original isolations was selected from the first replating, and replated a second time in the same manner and also transferred to a vial containing the same medium as a semi-solid agar with 0.2% NaHCO_3 added as an additional CO_2 source. It was found that colonies consistently failed to develop on the second replating or on transfer from the first replating to the semi-solid medium.

Numerous attempts were made to isolate colonies which were capable of growth on the second replating. It was found in a few cases that colonies developed on the second replating or growth occurred in semi-solid agar on the second transfer. These colonies were, however, unable to survive any further transfers.

Glaser (1930 a,b) reported the isolation of a corynebacterium-like symbiont from the American roach, Periplaneta americana and from the German roach Blattella germanica. Gier (1936) indicated that only negative results had been obtained after hundreds of attempts to cultivate these/

these symbionts on many kinds of media. He considered Glaser's diphtheroides to be a slow growing contaminant of an unusual nature (Gier, 1937). Steinhaus (1947) reported that Glaser (personal communication) maintained that repeated transfers from a medium which had been inoculated with these microorganisms, but which was apparently sterile, were necessary before the organisms finally appeared as tiny pin-point colonies. According to Glaser, a period of slow adaptation to artificial conditions was necessary.

The possibility was considered that the organisms isolated from the coelom of the earthworm might initially carry with them a specific growth factor which would permit their original growth in artificial medium and that a quantity of this substance sufficient to permit growth might be carried to the first replating but then become exhausted. If such was the case it might be possible, by Glaser's method, to obtain a slow adaptation of the organism to the artificial medium.

Six cultures which had developed on the first replating and which had survived transfer to the semi-solid medium in vials⁴ were selected. After 3 day's incubation two of these transfers were taken and, although no growth was visible, an inoculum of 1 ml. was transferred to fresh medium. The process was repeated every three days for a period of 30 days. The same procedure was followed with two cultures which had incubated 7 days with successive transfers occurring at seven-day intervals until six transfers had been completed. The remaining two cultures were transferred at fourteen-day intervals until four transfers had been/

been completed. In no case did visible growth occur.

As this method had failed to produce a strain adapted to artificial medium it was considered that the use of an enrichment culture might produce better adapted strains. Dilutions of 1/10,000, 1/100,000 and 1/1,000,000 of coelomic fluid were inoculated into vials of the semi-solid medium and into tubes of the same medium as a broth. Control plates were also inoculated at the same time. The tube cultures were incubated anaerobically and the plates were incubated aerobically. If growth occurred on the aerobic plates the inoculum was considered to have been contaminated and the series was discarded. After 7 days' incubation, vigorous growth had occurred in the semi-solid medium up to the 1/100,000 dilution and occasionally in the 1/1,000,000 dilution. Growth rarely occurred in the liquid medium and was never vigorous. Examination of films prepared from these cultures indicated that only Gram-negative rods of a type similar to those occurring in the coelom had developed. Platings were made from these cultures and although colonies developed in the first plating, these colonies were unable to develop on replating or on transfer to the semi-solid medium in vials.

At this point it became apparent that, before pure culture studies could be conducted, a method would have to be evolved by which the cultures could be maintained and remain viable after successive transfers.

The effect/

The effect of various carbon sources on the growth of the coelomic organisms.

The possibility that the organism might have specific energy requirements which had not been met by the media so far employed was considered. A selection of carbon compounds was made. The selection was purely empirical and based on the availability of the compounds in the laboratory at that time. A basal medium consisting of: Evan's peptone, 0.5% w/v; Bacto tryptone, 0.5% w/v; agar (Davis), 0.075% w/v; yeast autolysate, 10.0% v/v, was used.

The following reducing agents were added to the basal medium :

1. Thioglycollate 0.03% v/v.
2. Cysteine 0.05% w/v.
3. Ascorbic Acid 0.02% w/v
4. Glucose evolue 10.0% v/v.
5. No addition.

The media were adjusted to pH 6.8, and were tubed in 9 ml. quantities and sterilised at 15 lb. for 15 min. In the case of No. 3, the ascorbic acid was added aseptically at this stage as a Seitz-sterilised solution. When the sterilised medium had cooled to 45° 1 ml. of a suitable dilution of a Seitz-sterilised solution of the following compounds was added aseptically to the required number of tubes of each medium.

Compound/

Compound Percentage w/v.

Glucose	0.5
Fructose	0.5
Lactose	0.5
Mannose	0.5
Maltose	0.5
Sucrose	0.5
Arabinose	0.5
Salacin	0.5
Ethyl Alcohol	0.2
Glycerol	0.4
Na benzoate	0.2
Phenol	0.2
Na acetate	0.2
Malic acid	0.1
Malonic acid	0.1
Ca lactate	0.2
Na succinate	0.4
Citric acid	0.2
Pyruvic acid	0.4
Lactate plus acetate	0.1 each
Urea	0.5
Uric acid	0.16
Lemco	1.0
Tomato juice	1.0 v/v
No addition	0.0

Three cultures were selected that had been picked from an original plating of the coelomic fluid and transferred to vials of the semi-solid medium. Good growth had occurred in the semi-solid agar cultures. A series of each set of media was inoculated with a large loopful from each culture. The tubes were incubated for a period of 30 days and examined periodically. After seven days' growth had appeared in the media containing uric acid and a reducing agent. Growth failed to occur in any of the other media.

Platings/

Platings from cultures in the uric acid medium in King and Rettger's agar and in the uric acid medium solidified with agar failed to permit the growth of any colonies.

Utilisation of uric acid by the coelomic organisms.

Despite the fact that the organism could not be recovered by replating, the apparent growth of the organism in the uric acid medium suggested that further investigations should be conducted to determine if the organism was capable of utilising uric acid as a carbon and nitrogen source.

A large number of isolates from the coelomic fluid were selected and the uric acid medium previously described inoculated from them as a solid agar in plates, as a semi-solid agar in vials and as broth in vials. At the same time, the uric acid medium described by Barker (1942) was inoculated as solid agar in plates, semi-solid agar in vials and as broth in vials. Barker's uric acid medium consisted of: Uric acid, 0.3% w/v; K_2HPO_4 0.03% w/v; $H_2SO_4 \cdot 7H_2O$, 0.01% w/v; yeast autolysate, 0.5% v/v; phenol red solution 0.1% v/v; distilled H_2O to 1000.0 ml.

For solid agar 1.5% w/v agar was added; for semi-solid agar 0.075% w/v. The mixture containing approximately 900.0 ml. of water was boiled until the agar had dissolved and the boiling mixture was brought to a pH of 7.2-7.6 by the addition of 10% NaOH. Either 0.05% w/v cysteine or 0.01% v/v thioglycollic acid was added. If necessary, NaOH was added to maintain an alkaline reaction. The medium was then bottled or placed in vials and sterilised at 15 lb. for 15 min.

The/

The plate cultures were incubated anaerobically for periods of 7 and 14 days. The cultures in vials were incubated for periods up to 3 months and examined periodically. Colonies failed to develop on the plates. No apparent growth occurred in the broth cultures. Results from the semi-solid medium were variable. In some cases growth appeared to occur within three days. In others, growth, if it had occurred, was masked by the precipitation of uric acid salts and crystals of uric acid. In no case was it possible to recover the organism from the uric acid medium by transferring to King and Rettger's medium.

An effort was made to prevent the precipitation of the uric acid in the medium. Lithium urate is more soluble than sodium urate. Fresh medium was prepared which was neutralized by the addition of LiOH instead of NaOH. The experiment was repeated with essentially the same results as those reported above.

In order to determine definitely whether or not the coelomic organisms were utilizing uric acid Barker's medium was used in deep layers of agar in tubes and plates and inoculated directly with dilutions of coelomic fluid. After seven days' anaerobic incubation it was found that colony-like concretions of crystals had occurred both in the shake cultures and in the plates. The uninoculated tubes and plates showed aggregations of crystals but these were neither as hard and rock-like nor as distinctly formed. Those developing in the inoculated plates, when crushed and examined microscopically as a stained film, showed numbers of Gram-negative rods interspersed through, and associated with the crystals.

Efforts to recover the organisms by replating
in/

in King and Rettger's medium and Barker's uric acid medium again failed.

Effect of glycine on the growth of the coelomic organisms.

Barker and Beck (1941) have reported that Clostridium acid-urici utilised glycine in the presence of uric acid. It was thought that the glycine might act as a hydrogen donor and uric acid as the hydrogen acceptor. In order to examine the possibility that the presence of glycine might stimulate growth the following media were inoculated with two fresh isolates which had been transferred to King and Rettger's semi-solid medium.

1. Mineral salts base (p.); 0.2% w/v uric acid.
2. as 1 + 0.1% w/v glycine.
3. as 2 + 0.05% w/v cysteine.
4. as 1 + 10.0% v/v yeast autolysate.
5. as 2 + 10.0% v/v yeast autolysate.
6. as 3 + 10.0% v/v yeast autolysate.
7. King and Rettger's medium + 0.1% w/v glycine.
8. Barker's uric acid medium + 0.1% w/v glycine.
9. Thioglycollate medium (Difco formula).

The media were prepared both as broth and semi-solid agar and used in vials. After several weeks' incubation it was apparent that the presence of glycine had not stimulated growth.

Utilization/

Utilisation of nucleoprotein and nucleic acid by the coelomic organisms.

As growth of the organism had apparently occurred a number of times in a uric acid medium, it seemed possible that the organism might be involved in the nucleoprotein-nucleic acid metabolism of the earthworm. If such was the case, it seemed likely that crude preparations of nucleoprotein and nucleic acid would provide a source of available compounds. Crude preparations of nucleoprotein and nucleic acid (Hawk, Oser and Summerson, 1947), were made from fresh baker's yeast and incorporated in the following medium:

Yeast autolysate, 1.0% v/v; K_2HPO_4 , 0.03% w/v; $MgSO_4 \cdot 7H_2O$, 0.03% w/v; phenol red solution, 0.1% v/v; nucleoprotein or nucleic acid, 1.0% w/v; agar 0.075% w/v; cysteine, 0.05% w/v added after agar dissolved. Final pH adjusted to 7.4 or 6.6.

Vials of this medium were inoculated from a number of cultures of the organism in King and Rettger's semi-solid medium and from Barker's semi-solid uric acid medium. Visible growth occurred in most cases while in the remaining vials growth was slight or absent. There did not appear to be a preference for either nucleoprotein or nucleic acid. Replating in King and Rettger's medium failed to recover the organism and transfers to the nucleoprotein or nucleic acid medium did not produce growth.

Gram-negative rods appearing in agar preparations.

During the course of these investigations it was found invariably that inoculated agar media which failed to show visible growth contained an appreciable number of Gram-negative/

Gram-negative rods, similar morphologically to those found in the worm coelom, if films were prepared and examined microscopically. At first, it was thought that these rods represented a weak growth of the organism. On examination of uninoculated agar medium, however, it was found that similar rods were found. The possibility of thermophilic bacteria surviving the sterilisation process was considered and the period of autoclaving increased. The structures continued to appear in films prepared from uninoculated media. A wide variety of different agar media prepared by other workers in the department was then examined. In every case, from both solid and semi-solid media, Gram-negative rods were found in large enough quantities to suggest slight growth on microscopic examination. As many samples as possible of the different types and batches of agar that were in the laboratory were collected. The agars were dissolved in distilled water and autoclaved, at 20 lb. for 20 min., at a dilution of 0.05% w/v agar. Films were prepared and stained from these very weak agar gels. Gram-negative rods appeared in many fields. The source or time of preparation of the agar did not appear to effect the occurrence of the bacteria-like structure. They were equally common in Japanese agar of both pre-war and post-war batches, and in New Zealand agar. Further investigation revealed that they were capable of staining after 30 min. autoclaving at 30 lb. pressure, were only slightly disrupted when autoclaved at 22 lb. pressure in 0.1 N HCl and destroyed when autoclaved in 0.1 N NaOH.

Although the nature of these structures has not/

not been established it seems possible that they are dead bacterial cells. While they would present no difficulty in the examination of normally growing cultures, they make it impossible to accept the mere presence of Gram-negative rods in an agar medium as evidence of growth of a Gram-negative bacterium.

The possibility that the colonies which developed on the agar plates inoculated from the coelom might have represented a heat resistant contaminant of the agar was examined. Various samples of agar were ground and suspensions made in sterile water. Platings were made from these suspensions in King and Rettger's medium. Similar suspensions were heated until the agar had just dissolved. Platings from these suspensions were made in the same medium. The plates were incubated both aerobically and anaerobically. Numerous colonies developed from the unheated agar suspensions but no colonies developed from the heated agar suspensions. It may, therefore, be assumed that the colonies isolated from the worm coelom were not related to the structures appearing in the agar.

As the coelomic organism was unable to develop satisfactorily in a liquid medium and appeared to be capable of growth only in an agar medium, it has been necessary to accept a visible growth as the only reliable evidence of multiplication. In the following discussion only visible growth is considered.

Description/

Description of organism occurring in the coelomic fluid of earthworms.

Morphology.

Gram-negative, non-motile, regular rod with rounded to square-cut ends. Occurs singly or occasionally in pairs. Spores were not observed and the organism is not acid fast.

King and Bettger's medium.

Aerobic plates: No growth.

Anaerobic plates: Colonies small, opaque, entire, convex, smooth; brownish pigment formed.

Semi-solid agar in vials: Growth diffuse, spreading through the medium.

Solid agar stabs: Weak growth at bottom of stab.

Broth: Under anaerobic conditions very weak growth occasionally occurred.

Uric acid media.

Anaerobic plates: Organism interspersed through colony-like concretions of crystals.

Semi-solid agar in vials: diffuse growth.

Nucleoprotein or Nucleic acid media

Semi-solid agar in vials: Diffuse growth.

Other media.

Anaerobic or aerobic conditions: No growth.

Growth requirements.

The organism shows a specific requirement for some unknown component of yeast autolysate which could not be provided by Yeastrel, soil extract or tomato juice. The organism has a further requirement of an unknown nature which was/

was not provided by the media employed so as to permit of serial transfer. The extremely fastidious nature of this organism has so far made it impossible to study its physiological characteristics and functions.

Oxygen requirements.

The organism is a strict anaerobe. Growth in the presence of oxygen never occurred. The addition of a reducing agent to the medium enhanced growth.

Carbon dioxide requirements

An increased CO₂ tension was not essential, but appeared to enhance growth.

Temperature requirements

The organism grew equally well at 16° or 35°.

Taxonomic position of bacteroid occurring in the coelomic fluid of earthworms.

Because of the extremely fastidious nature of this organism it was not possible to obtain growth after two transfers on artificial media. Until further information can be procured it will not be possible to relate it to other described species. It seems likely, however, that this organism represents a type of bacterial symbiont specifically related to members of the Oligochaeta.

Lewis and Rettger (1940) review the taxonomic position of the genus Bacteroides and Lewis, Bedell and Rettger (1940) investigated the growth requirements of a number of Bacteroides strains. The morphological characteristics of the organisms of the worm coelom, namely, Gram-negative, non-sporulating/

sporulating, non-motile, non-capsulated rods occurring singly and occasionally in pairs, the complex growth requirements shown by the organism, the fact that it is an obligate anaerobe, and the fact that Bacteroides have seldom been observed outside of the animal body would suggest that a possible relationship exists between the organisms of the coelom and the genus Bacteroides.

The difficulty in obtaining adequate methods for the isolation and cultivation of the microsymbionts associated with insects and ticks has been emphasized by Steinhaus (1947). The work of Glaser and Gier with respect to the cultivation of the bacterial symbiont of the cockroach has been discussed previously. Webb (1940) attempted to cultivate the intracellular symbiont of Triatoma rubrofasciata without success although he could infect small laboratory animals with the organism. These two cases may be considered typical of many unsuccessful attempts, to establish conclusively the exact nature of many bacterial symbionts.

Organism of streptococcal morphology isolated from the coelomic fluid of the earthworm.

During the course of these investigations it was found that a streptococcus-like organism occurred in a number of platings and semi-solid agar cultures of the coelomic fluid. It was originally considered to be a contaminant but its reappearance as the only organism developing from the coelom other than the Gram-negative rod suggested that it occurred in coelom in small numbers which periodically increased. It never developed from dilutions higher than 1/100,000. It was found/

found capable of developing in tomato agar while the Gram-negative rod failed to grow in tomato agar.

From tomato agar plates twelve colonies were transferred to King and Rettger's semi-solid medium. Each of these cultures was replated twice in order to ensure pure cultures. The following is a description of the organism.

Morphology

Chains of regular cocci. The chains vary in length. Occasionally irregular forms appear in which the cells tend to elongate and become larger. The organism is non-motile and endospores were not observed.

Gram reaction.

Chains of regular cocci Gram-negative.

Irregular forms Gram-positive.

King and Rettger's medium.

Aerobic plates: No growth occurred until the organism had been grown for many generations on artificial media.

Colonies were then pin-point with sparse growth.

Anaerobic plates: Colonies small, opaque, yellowish to white, entire, convex, granular.

Semi-solid stab inoculation: Profuse growth of small punctiform colonies along the stab which later became joined by veil-like processes which spread along the bottom of the tube and up the sides between the glass and agar.

Solid agar stabs: Meagre growth along stab to within a few cm. of the surface of agar.

Broth (aerobic and anaerobic): No growth for several days; then/

then suddenly profuse growth occurred from the bottom which spread along the walls of the tube with the formation of comet-like processes. Under aerobic conditions the resazurin indicator became oxidized and then became reduced with the occurrence of growth.

Tomato broth and agar.

Growth similar to that observed in King and Rettger's medium.

Nutrient broth and agar.

No growth.

Gelatin.

After 10 days' incubation a faint trace of growth appeared along the stab. Growth did not increase and no liquefaction occurred.

Litmus milk

Acid formed, litmus reduced, no coagulation, no gas.

Litmus milk with iron nail in medium.

Acid formed, litmus reduced, solid soft curd, very slight gas formation which was probably due to an interaction between the acid and iron.

Arginine Medium (Niven, Smiley and Sherman, 1942).

Bead like colonies developed in the bottom of the tube at both 20° and 35° after 24 hr. incubation. A very slight trace of ammonia occurred after 40 hr. at both temperatures. The ammonia content did not seem to increase with further incubation.

Growth requirements.

Nutrient agar, Topping's medium and soil extract medium were unable to support growth. With the addition of either tomato juice or yeast autolysate growth occurred in these media. The addition of yeast autolysate or tomato juice to gelatin enhanced growth but without liquefaction. As the organism failed to grow in the absence of tomato juice or yeast autolysate it was not possible to determine the carbon sources which the organism could utilize as a source of energy.

Temperature requirements.

Growth occurred in tomato broth and litmus milk at 16°. One series in the same media developed in an incubator at 45°. Two series failed to grow in a 45° water bath suggesting that the maximum temperature for growth is about 45°. The organism grew equally well at 20° and 35°.

Oxygen requirements

The organism originally failed to grow unless anaerobic conditions were provided. After repeated transfers, however, the organism became adapted to aerobic conditions and weak growth occurred on slopes of King and Rettger's agar and tomato agar when incubated aerobically. In all cases much more vigorous growth occurred under anaerobic conditions.

Carbon dioxide requirements.

Carbon dioxide requirements.

An increased CO₂ tension did not appear to influence the growth of the organism.

Taxonomic position of the streptococcal form occurring in the coelomic fluid of the earthworm.

As in the case of the rod form there is not sufficient information to classify this organism. The Gram-negative reaction of the organism would, under the classification of Prévot (1933) which was adopted by Bergey (1948), suggest that it should be placed in the genus Veillonella of the family Neisseriaceae. The growth of the organism in chains is, however, more typical of the genus Streptococcus than Veillonella. The change from an obligate anaerobe to a facultative anaerobe on subculturing which occurred with this organism has been reported by Prévot as occurring with Streptococcus evolutus.

DISCUSSION.

Microscopical examination of the coelomic fluid has confirmed the universal occurrence of bacteria-like structures in a number of members of the Lumbricidae. The evidence obtained from different staining methods strongly suggested that some at least of these structures were bacterial cells. After many unsuccessful attempts a method was found by which an organism, which appeared morphologically similar to the structures in the coelomic fluid, could be consistently isolated. This/

This organism showed no relationship to organisms which had previously been reported as having been isolated from the coelomic fluid of earthworms. Lim Boon Keng (1895) isolated several types of bacteria using gelatin plates. Pandazis (1931) and Gianfurari and Cantoni (1936) isolated different organism by inoculating nutrient broth or streaking nutrient agar slopes. The fact that these organisms grew rapidly on simple media under aerobic conditions proves them to be entirely dissimilar to the obligate anaerobe with extremely complex growth requirements which was isolated in the present investigation. The fact that organisms failed to develop on a wide variety of media when the plates were incubated aerobically may be considered to offer fairly conclusive evidence that any aerobic organism developing from the coelomic fluid represents a contaminant. The methods employed by Pandazis and Gianfurari and Cantoni to isolate their organisms are obviously extremely unreliable.

As well as the Gram-negative anaerobic rod a Gram-negative streptococcal-like organism was isolated from the coelomic fluid on a number of occasions. Its repeated occurrence and distinctive characters suggest that it, too, represents a symbiont of the earthworm which occurs in much lower numbers than the rod form.

It was considered that a symbiotic relationship possibly existed between the rod and the streptococcus. This was tested by sterilising broth cultures of the streptococcus by Seitz filtration and incorporating them in sterile King and Rettger's medium. This medium was then inoculated from cultures of the rod. Stimulation of growth failed to occur. The inoculation of cultures of the rod with the streptococcus failed/

failed to produce a stimulation in the growth of the rod, although the streptococcus grew well. There did not appear to be an interaction between the two organisms.

The constant occurrence of these two organisms in the coelomic fluid and the failure of the amoebocytes to destroy them, while other types of bacteria, which may enter the coelomic cavity through the nephridia and dorsal pores, fail to persist (Keng, 1895; Cameron, 1932), strongly suggests that some symbiotic relationship exists between these two organisms and the earthworm. The nature of this relationship has not been established, but the apparent ability of the rod to utilise uric acid indicates an interesting approach for future investigation. Before such an investigation can be made, however, it will be necessary to establish more fully the growth requirements of the organisms.

For many years the essential nature of the coelomic bacteroids has been debated. Two schools of thought have developed, one that they are bacteria and the other that they are crystals of various substances. While this investigation has shown that some, at least, of the structures appearing in the coelomic fluid are bacteria, it is felt that others may well be inanimate inclusions.

The conclusions drawn by previous workers have been conditioned by their methods of studying these structures and the failure to cultivate the bacteria present in the coelom.

QUANTITATIVE STUDIES ON THE EFFECT OF WORMS ON
THE BACTERIAL FLORA OF A SOIL AND CHANGES
OCCURRING IN THE WORM GUT.

QUANTITATIVE STUDIES ON THE EFFECT OF WORMS ON THE BACTERIAL
FLORA OF A SOIL AND CHANGES OCCURRING IN THE WORM GUT.

INTRODUCTION.

The effect of earthworms on soil fertility and on physical and chemical transformations in the soil has been studied by numerous workers. It has frequently been suggested that earthworms influence the nature of the soil microflora but there is very little information available as to the quantitative and qualitative changes which actually occur in the worm gut or in the soil in which the worm is living.

Jensen (1898) reported that denitrifying bacteria were destroyed during passage through the worm gut.

Bassalik (1913) concluded that the microflora of the worm gut, worm casts and the soil were qualitatively similar. He found that in two soils the numbers of bacteria in worm casts were greater than in the soil and that with one soil a marked increase in numbers occurred in the gut. Unfortunately, his methods of sampling and the single comparisons of bacterial numbers do not provide reliable information.

Stöckli (1928) found that similar types of bacteria occurred in worm casts and in the soil and the physiological groups studied occurred in greater numbers in the cast than in the soil. He found that the bacterial population of the worm casts increased during the first five days following excretion.

Day (1950) found that after a worm had been 1 to 3 days in a soil the casts produced by the worm did not indicate any striking differences in the bacterial numbers present in the soil and in the cast. It may be considered, however, that the period/

period for which the worm was allowed to remain in the soil was not sufficient to permit the development of a specific flora in the gut, and that his sampling method was not adequate. He found that Bacillus cereus mycoides showed some reduction in numbers during passage through the worm gut and considered this to be due to the destruction of vegetative cells while the spores survived. Serratia marcescens was destroyed, apparently by the action of the pharyngeal secretion. Nitrifying bacteria did not appear to be effected by passage through the worm gut.

Dawson (1947) found that a decrease in the bacterial content of aggregates removed from the intestinal tract of the earthworm compared to aggregates formed by excreted casts and worm-free soil took place in the presence and absence of added organic matter. He considers that the ingestion of soil by the earthworm results in a decrease of the bacterial content of the eaten soil. He further states that after excretion, the number of bacteria in the excreted aggregates increases, in some instances surpassing that of the aggregates from worm-free soil. No significant differences were reported for the number of fungi found in aggregates from the worm intestines, excreted casts and worm free soil, although the gut contents gave lower counts.

The paucity of information available as to the quantitative effect of earthworms on the bacterial flora indicated that preliminary quantitative studies should be conducted.

GENERAL/

GENERAL METHODS.Soil sampling.

The soil samples used in all quantitative studies were obtained by the following method:

The surface layer of the soil was removed. A large sample was then taken to a depth of about 6 to 8 inches from the cleared area. This sample was passed through a 3 mm. sieve and the 3 mm. fraction air dried. After the sample was dried it was mixed thoroughly by quartering. The mixed, air dried sample was stored in large covered jars which permitted the ingress and egress of air. When a sample was required for a specific purpose a quantity of soil, greatly in excess of the requirements, was removed from the jars. This sample was again thoroughly mixed and the required weight of soil obtained by random sampling of the entire sample with a scoop spatula.

Method for plating soil.

Dilutions were made from a 10 g. sample which was shaken for 5 min. in 100.0 ml. of sterile water in a sterile stoppered bottle. The succeeding dilutions were made by adding 1 ml. of the previous dilution to 9 ml. of sterile water in a sterile tube or by adding 10 ml. to 90 ml. in a stoppered bottle. Each dilution was thoroughly mixed by agitation before the next dilution was prepared. A fresh, sterile pipette was used for each dilution. One ml. quantities of the required dilution were added to sterile Petri plates and about 10 ml. of liquid agar at 45° was immediately added and thoroughly mixed with the inoculum./

inoculum. Dilutions of 1/100,000; 1/200,000; 1/1,000,000; 1/2,000,000, and 1/10,000,000 were usually used in platings.

For quantitative work not less than four, and frequently five plates were poured from each dilution.

Platings were made as soon as possible after the preparation of the dilutions.

Media.

Soil Extract: Soil extract was prepared by autoclaving 1 Kg. of garden soil in 1 l. of tap water for 30 min. at 15 lb. pressure. A small quantity of CaSO_4 was added to the hot soil suspension and the soil extract obtained by filtering through paper by suction. The filtrate was made up to 1 l., 0.1% w/v K_2HPO_4 added and adjusted to pH 6.8.

Soil extract agar: 1.5% w/v agar was added to soil extract.

Topping's agar: 0.25% w/v Bacto peptone; 0.25% w/v Bacto yeast extract; 1.5% w/v agar.

PLATES



PLATE COUNTS OF BACTERIAL SPORES AND TOTAL BACTERIAL NUMBERS
IN A SOIL AND FRESH WORM CASTS OF THE SOIL ON DIFFERENT MEDIA.

The use of normal methods for a quantitative comparison of the microflora of the different sections of the earthworm gut with that of the soil would introduce an experimental error of such a magnitude that the value of the final comparison would be dubious. It was apparent that a method must be evolved which would not require weighed samples from the earthworm's gut. It was considered that if it could be established that bacterial spores passed through the earthworm gut unchanged, then a comparison between the number of spore-forming bacteria in different sections of the gut in relation to the number of bacteria in the same section developing on total count plates would relate the microflora of the gut to that of the soil originally ingested. Preliminary investigations were conducted to establish a heat treatment of the soils used which would successfully destroy all vegetative cells without destroying any of the bacterial spores present. When a satisfactory time-temperature relationship had been established a comparison was made between the spore counts of fresh earthworm casts and the soil in which the worms were living.

Methods.

Soil suspensions of suitable dilutions were treated by placing the dilution tubes in a hot water bath held at a controlled temperature. The bath was equipped with a mechanical stirring device. It was ascertained that the contents of the dilution tubes reached the temperature of the bath within a period of 3 min. It was decided to leave the tubes in the bath for a total period of 10 min. The tubes were cooled immediately after the heat treatment/

treatment by placing them in cold water. The required number of plates was inoculated from each dilution. Topping's agar medium was used throughout the preliminary experiments. Fresh worm casts were obtained by the method previously described. The inoculated plates were incubated for 14 days at 25° and counted. All figures are given as millions of colonies per g. of oven-dried soil.

Effect of various heat treatments on numbers of spore-forming bacteria.

The results obtained from four series of platings are given in Table 1. It is indicated that there is no decrease in the spore count until a temperature of 75° has been exceeded.

Table 1.

Bacterial numbers in millions per g. of oven-dried soil after 10. min. treatment at various temperatures.

Plating.	Soil sample	Temperature of treatment.				
		60°	65°	70°	75°	80°
1.	Garden.	1.9	1.4	2.0	2.3	
2.	"	2.6	2.2	2.2	2.6	
3.	"	1.9		1.8		1.2
4.	Worm cast of garden soil.	2.2		2.2		1.8

In order to ensure that the colonies developing at the lower temperatures were actually spore-forming bacteria, fifty colonies were picked from each of the 60° treatments of platings 2, 3 and 4 and transferred to slopes of the same medium. After incubation films were prepared from the growth on the slopes/

slopes and examined microscopically. With the exception of two cultures which proved to be micrococci all the remaining cultures were spore-forming bacteria. The micrococci were considered to be airborne contaminants and treatment at 60° for a period of 10 min. sufficient to destroy vegetative cells from the suspensions examined. It was decided, therefore, that future examination of samples for spore-formers would be made by subjecting the inocula to a temperature of 65° for 10. min., the increase of 5° was to ensure that no resistant vegetative cells would survive.

Comparison of numbers of bacterial spores in a soil and fresh worm casts of the soil.

A comparison was made between the number of bacterial spores in a soil and fresh worm casts of the same soil after 10 min. treatment at different temperatures. The results are tabulated in Table 2.

Table 2.

Numbers of bacterial spores in a soil and fresh worm casts of the soil after various heat treatments in millions per g. of oven-dried soil.

Treated at temperature of	Spore-forming bacteria.	
	Soil	Worm cast of soil.
60°	1.9.	2.2
70°	1.8	2.2
80°	1.2	1.8

The data given in Table 3, shows the relationship between the numbers of bacterial spores in a soil and fresh worm casts of that soil after 10 min. treatment at 65°.

Table 3. /

Table 3.

Bacterial numbers in soil and fresh worm casts of the soil after 10 min. treatment at 65° in millions per g. of oven-dried soil.

Type of soil.	Bacterial numbers.	
	Soil.	Worm cast.
Garden	2.3	1.7
	2.2	2.4
	2.7	2.6
	2.1	2.8
	2.4	2.1
Field	3.5	4.1
	4.7	3.9
	4.2	2.8
	4.3	4.6

These results would indicate that the numbers of bacterial spores occurring in fresh worm casts are not significantly different from those occurring in the soil in which the worm is living.

Comparison of total numbers of bacteria and of bacterial spores in a soil and fresh worm casts of the soil.

A preliminary comparison was made between total bacterial numbers in a soil and fresh worm casts of the soil to determine if any major alteration in the bacterial flora occurred within the gut of the earthworm.

The results of these platings, compared with the numbers of bacterial spores in the same soil samples after treatment at 65° for 10 min. is given in Table 4.

Table 4./

Table 4.

Total numbers of bacteria and bacterial spores in a soil and fresh worm casts of the soil in millions per g. of oven-dried soil.

6

Soil	Days incubated	Total numbers		Spores	
		Soil	Worm cast	Soil	Worm cast
Garden A	5	8.9	4.6	2.1	1.8
B	5	9.5	4.3	2.4	2.1
Field A	5	13.5	7.2	4.7	3.9
B	5	20.0	9.1	4.2	2.8
A	10	18.3	10.4	4.3	4.6
B	10	26.2	22.1	3.5	4.1

These results indicate an apparent decrease in the total bacterial numbers in the worm gut but suggest that bacterial spores present in the soil pass through the gut unchanged.

The medium used (Topping's agar), however, appeared to favour the growth of the zymogenous flora at the expense of the autochthonous. This view is suggested by the absence in the plates of the typical small, pinpoint colonies which commonly occur in soil platings and also by the fact that relatively low counts were obtained from comparatively rich soils. It was noted that an increase in bacterial numbers appeared to occur with the longer period of incubation. It was considered that the worms present in the soil might add some substance to the soil which is utilised by the bacteria present.

A comparison of the total numbers of bacterial colonies developing on media of different composition.

The/

A comparison of the total numbers of bacterial colonies developing on media of different composition.

The fact that the medium used was not considered to yield representative samples of the soil population made it apparent that changes in the composition of the medium should be investigated. Topping (1936) found yeast extract-peptone agar the most satisfactory of those investigated for the growth of soil bacteria. It seemed likely that one of the constituents now used was unsatisfactory. A comparison between different yeast extracts and peptones, and soil extract with and without added yeast extract was made. The media used had the following compositions:

1. Bacto peptone, 0.25% w/v; Bacto yeast extract, 0.25% w/v; agar, 1.5% w/v.
2. Evans' peptone, 0.25% w/v; Bacto yeast extract, 0.25% w/v; agar, 1.5% w/v.
3. Evans' peptone, 0.25% w/v; Yeastrel, 0.25% w/v; agar, 1.5% w/v $\frac{1}{2}$
4. Bacto peptone, 0.25% w/v; Yeastrel, 0.25% w/v; agar, 1.5% w/v.
5. Soil extract as described (p.) and 1.5% w/v agar.
6. As medium 5 + 0.2% Yeastrel.

Platings were made of suitable dilutions of a soil under three different treatments:

1. Field soil free from worms for 30 days.
2. Field soil with worms for 30 days.
- ~~3. Fresh soil with worms for 30 days.~~
3. Fresh worm castings from worms in soil 2.

The results of the platings are given in Table 5.

Table 5.

A comparison of bacterial numbers in millions per g. of dry soil from different soils in different media.

Medium.	Worm free soil Plating		Worm soil Plating		Worm cast Plating.	
	1.	2.	1.	2.	1.	2.
Bacto peptone + Bacto YE	24.5	11.8	26.2	18.3	22.1	- ϕ
Evans' " + " "	30.6	15.1	19.3	17.1	23.6	11.4
" " + Yeastrel	19.6	8.3	11.0	13.8	13.0	9.6
Bacto " + "	14.7	12.5	11.0	13.0	12.2	8.7
Soil extract + "	33.0	23.4	31.7	29.8	21.3	16.7
" " + Yeastrel.	20.8	19.9	30.4	23.8	21.3	12.2

ϕ plates overgrown with Bacillus mycoides.

Table 5.(SEE OPPOSITE PAGE).

It was suggested by these results that Yeastrel failed to provide some factor which occurred in Bacto yeast extract or the soil extract, or that it had an inhibitory effect. It was apparent that either a medium containing Bacto yeast extract or the soil extract medium would be preferable to a medium containing Yeastrel. The soil extract medium prevented the excessive growth of Bacillus mycoides and other spreaders which occurred on the Yeast extract-peptone media. It was decided to use the soil extract medium for future platings.

The effect of worms on the fungal population of a soil.

Examination of the platings on different media, the bacterial counts of which have been given in Table 5, revealed the interesting fact that the fungal population of the soil in which the worms had been living for 30 days greatly exceeded the fungal population in either the worm cast or the worm free soil. Comparative counts of fungi are given in Table 6. It may be noted that the fungal colonies all appeared to be a similar species of Penicillium.

Table 6./

TABLE 6.

Fungal colonies in millions per g. of dry soil from different soils in different media.

Medium.	Worm free soil						Worm soil						Worm cast					
	Dilution			Dilution			Dilution			Dilution			Dilution			Dilution		
	1/10 ⁵			1/10 ⁶			1/10 ⁵			1/10 ⁶			1/10 ⁵			1/10 ⁶		
	Plating			Plating			Plating			Plating			Plating			Plating		
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1.	-	-	-	-	2.2	1.5	39.0	32.0	0.4	0.2	0.2	2.0	2.0	2.0	2.0	2.0	2.0	2.0
2.	-	-	-	-	1.9	1.2	42.0	36.0	0.2	0.1	0.1	2.0	3.0	3.0	3.0	3.0	3.0	3.0
3.	-	-	-	-	1.4	0.3	50.0	33.0	0.3	0.2	0.2	2.0	2.0	2.0	2.0	2.0	2.0	2.0
4.	-	-	-	-	1.0	1.3	40.0	29.0	0.3	0.1	0.1	2.0	2.0	2.0	2.0	2.0	2.0	2.0
5.	-	-	-	-	1.0	1.1	46.0	40.0	0.2	0.3	0.3	3.0	4.0	4.0	4.0	4.0	4.0	4.0
6.	-	-	-	-	3.4	1.2	55.0	47.0	0.5	0.4	0.4	3.0	4.0	4.0	4.0	4.0	4.0	4.0

Media: 1. Bacto peptone + Bacto YM
 2. Evans' " + " "
 3. " " + Yeastrel.
 4. Bacto peptone + Yeastrel
 5. Soil extract " "
 6. " " + Yeastrel.

Table 6.
(SEE OPPOSITE PAGE).

Examination of the plates suggested that the extraordinary increase in the fungal count in the 1/1,000,000 dilution as compared to the 1/100,000 dilution was related to the frequency of occurrence of spreading colonies of a type distinguishable from those of Bacillus mycoides. In plates of the 1/1,000,000 dilution there were, on the average, less than one spreading colony per plate while in plates of the 1/100,000 dilution spreading colonies exceeded an average of twenty per plate. From the results recorded and the appearance of the plates certain spreading colonies appeared to be antagonistic to the fungus.

It was also observed that the type of bacterial colony developing on the plates from the worm soil and the worm casts showed a marked difference in appearance to those developing from the worm free soil. This suggested that the presence of worms in a soil exerted some influence on the general nature of the microflora of the soil.

It appeared that in the soil in which the worms were living there was a substance which permitted the development of the fungal population and that this substance did not occur in the worm free soil. The marked decrease in the number of fungi in the worm casts indicated that an action destructive to fungal spores and mycelium occurred within the worm gut. This agrees with the results given by Day (1950) who gives counts of the fungal population in a soil and worm casts of the soil which indicate a reduction of the fungal population in the worm casts./

casts. An anti-fungal effect by earthworms is also reported by Smith, Raja Varma and Bhattacharya (1952) who found that the presence of earthworms in mite rearing tubes prevented fungal infestation.

Subsequent investigations failed to show a marked increase in the fungal population of soils containing earthworms. It seems likely that, in this case, the increase in fungi occurred through the introduction of some extraneous factor, possibly through the death of a worm.

In these platings (Table 5) it appears that a reduction occurs in the bacterial population of the worm cast at 30 days. Subsequent bacterial counts (Table 13) indicate a marked increase in the bacterial count of the worm cast at 30 days. It is possible that the reduction in bacterial numbers is related to the high fungal count in the worm soil through an antibiotic effect produced by the Penicillium species present. This suggests that the bacterial flora of the worm gut is more sensitive to the effect of the antibiotic than the bacterial population of the worm soil as the bacterial counts for both the worm-free soil and the worm soil as determined with soil extract agar closely correspond in three separate samplings.

The bacterial and fungal counts obtained in these samplings are compared in Table 7.

Table 7. /

Table 7.

Bacterial and fungal counts obtained on soil extract agar after incubation of a field soil with and without worms for 30 days in millions per g. of oven dried soil.

Sample.	Worm free soil.		Worm soil.	
	Bacteria	Fungi	Bacteria	Fungi.
1.	33.0	-	31.7	46.0
2.	23.4	-	29.8	40.0
3.	24.0	-	33.0	-

Comparative spore counts on media of different composition.

The effect of soil extract medium in the suppression of spreading colonies suggested that although this medium gave good total counts it might not be suitable for counts of spore-forming bacteria. Counts of bacterial colonies developing from inocula which had been treated at 70° for 10 min., on different media were compared. The results given in Table 8 are from the same soils and with the same media as those given in Table 5. The worms had, however, been in the soil for a period of 45 days.

Table 8. /

Table 8.

A comparative spore count in millions per g. of oven-dried soil on different media.

Medium.	Worm free soil.	Worm soil.	Worm cast.
Bacto peptone - Bacto Y.E.	4.3	-	-
Evans' " - " "	3.9	-	-
" " - Yeastrel.	4.2	-	-
Bacto " - "	2.9	-	-
Soil extract.	1.7	3.2	2.9
Soil extract - Yeastrel.	1.7	3.4	3.2

NOTE: At the dilutions used plates obtained from the worm soil and the worm casts were so overgrown with spreading colonies that satisfactory counts could not be obtained with the first four media.

These results indicated that soil extract medium was not satisfactory and also that a method of preventing the excessive growth of spreading colonies was required. It was considered that a decrease in the peptone and yeast extract content with an increase in the agar content might limit the size of the colonies developing in the medium.

The following media were compared:

1. Bacto peptone, 0.1% w/v; Bacto yeast extract, 0.1% w/v; agar, 2.0% w/v.
2. Bacto peptone, 0.25% w/v; Bacto yeast extract, 0.25% w/v; agar, 1.5% w/v.
3. Soil extract as described and 1.5% w/v agar.

Platings were made from suitable dilutions of a soil under two different treatments:

1. Field soil free from worms for 60 days.
2. Field/

2. Field soil containing worms for 60 days.

Three separate samples were taken from each soil and the dilutions used to provide inocula were heated to 70° for 10 min. and four plates poured from each.

The results from two series of platings are given in Table 9.

Table 9.

A comparative spore count in millions per g. of oven-dried soil on different media.

Plating.	Medium.	Sample.	Worm free soil.	Worm soil.
1.	1.	1.	1.8	5.9
		2.	1.8	3.9
		3.	1.4	3.2
	2.	1.	3.2	6.4
		2.	2.9	5.7
		3.	2.1	4.8
	3.	1.	0.6	1.1
		2.	0.8	1.1
		3.	0.8	1.2
2.	1.	1.	1.4	5.1
		2.	1.4	3.1
		3.	1.6	3.1
	2.	1.	2.4	6.7
		2.	2.1	5.3
		3.	2.1	4.1
	3.	1.	1.0	1.4
		2.	0.6	1.3
		3.	0.6	1.3

Media: 1. Bacto peptone 0.1% + Bacto YE 0.1% + agar 2.0%
 2. " " 0.25% + " "0.25% + " 1.5%
 3. soil extract + agar 1.3%

These results indicated that medium 2 consistently gave a higher count than the other two media but it also continued to permit an excessive growth of spreading colonies/

colonies which tended to negate the advantage of the higher count. Medium 1 prevented excessive spreading but gave a lower count while medium 3, which was most satisfactory in preventing the growth of spreading colonies obviously permitted only a portion of the spore forming population to grow.

It was decided to attempt to improve the number of colonies developing on the soil extract plates by enriching the medium with yeast extract and to determine if a lower peptone content with a higher yeast extract content would give as good results as medium 2.

Dilutions were prepared from a worm-free field soil and a similar soil which had contained worms for 15 days. Both soils had been brought to 80% of their water holding capacity and incubated at room temperature.

The dilutions were heated for 10 min. at 70°. Plates were inoculated with suitable dilutions and poured from the following media:

- A. Soil extract as described; Bacto yeast extract, 0.2% w/v; agar 1.5% w/v.
- B. As medium A, but Bacto yeast extract replaced by Yeastrel.
- C. Bacto peptone, 0.25% w/v; Bacto yeast extract, 0.25% w/v; agar, 1.5% w/v.
- D. Bacto peptone, 0.1% w/v; Bacto yeast extract, 0.25% w/v; agar, 1.5% w/v.

Counts were made from the 1/200,000 dilution after 14 days' incubation at 25°. The results from two series of platings are given in Table 10.

Table 10.

A comparative spore count in millions per g. of oven-dried soil on different media.

Plating	Medium	Worm free soil	Worm soil.
1.	A	6.0	7.3
	B	1.6	0.7
	C	5.7	5.0
	D	4.7	3.6
2.	A	5.3	5.6
	B	Growth suppressed	1.0
	C	5.0	5.7
	D	4.3	4.0

Media: A. Soil extract + 0.2% Bacto yeast extract
 B. " " + 0.2% Yeastrel
 C. 0.25% Bacto peptone + 0.25% Bacto yeast extract.
 D. 0.1% " " + 0.25% " " "

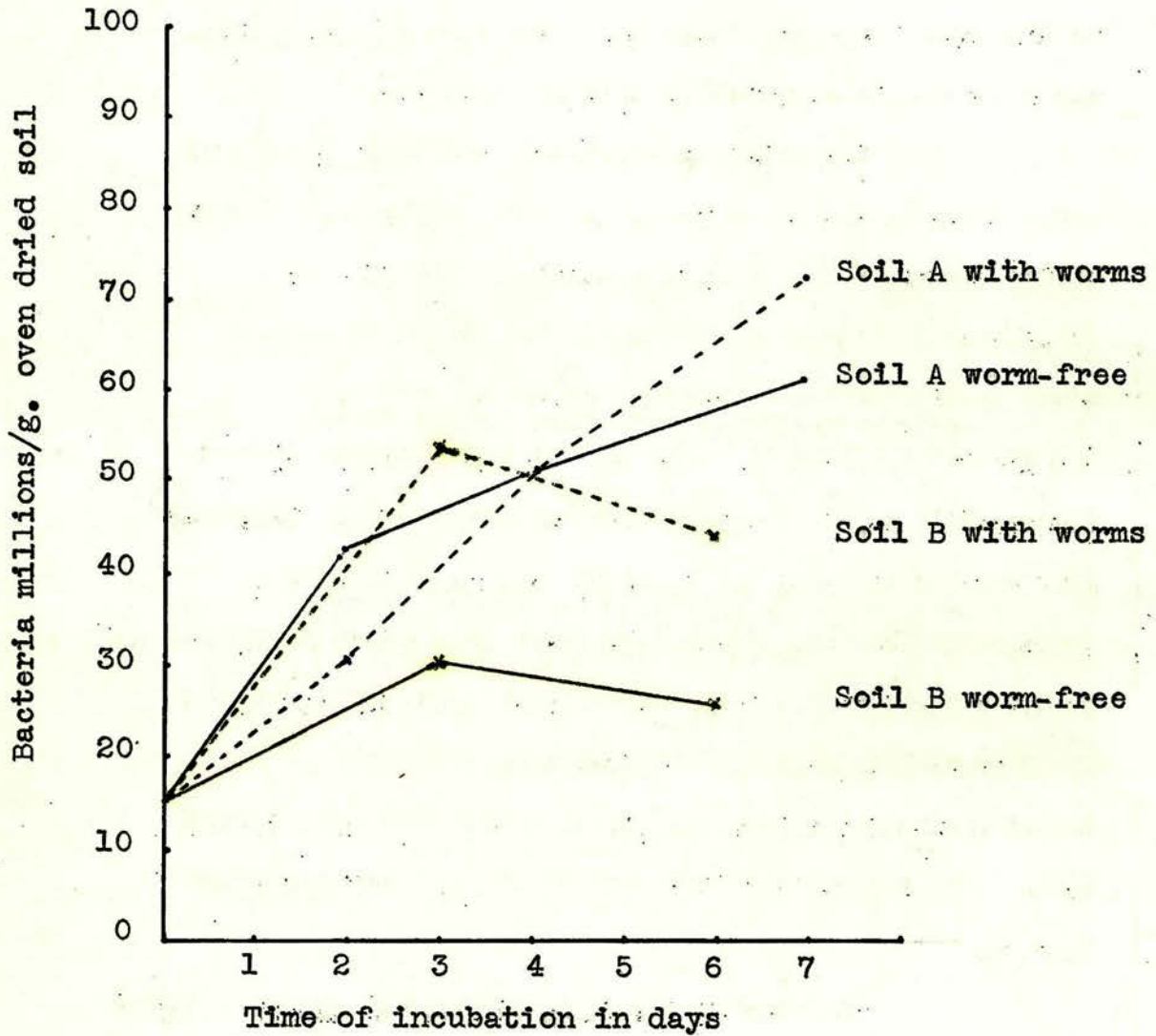
The 1/200,000 dilution which was used for counting was comparatively free from spreading colonies and gave counts approximately equal in numbers of colonies to the 1/100,000 dilution which had frequent spreaders.

Although all four types of media permitted the growth of spreading colonies medium A appeared to control excessive growth better than Media C and D and also appeared to give the highest counts.

As has previously been reported, Yeastrel appeared to have a definitely inhibitory effect on the development of bacterial colonies. The results also suggest that although some organisms require factors occurring in yeast extract, soil extract is capable of replacing peptone.

Graph 1.

Effect of worms in a soil on bacterial numbers.



A COMPARISON OF THE EFFECT OF WORMS IN A SOIL AND THE TREATMENT
OF A SOIL WITH WORM SLIME.

The effect of worms in a soil on the bacterial numbers of the
soil.

It had been indicated by the earlier experiments that when worms were present in a soil, the bacterial numbers in that soil increased (Table 4). The following experiment was conducted to determine if such was the case.

Four 200.0 g. samples of air dried field soil were placed in similar glass jars. The samples were brought to 80% of their water holding capacity. Two jars were designated as control A and control B. Three worms were added to each of the remaining two jars and the soils designated as worm soil A and worm soil B. The soils were incubated at 20°. Duplicate samples were taken at intervals from each of the worm soils and two series of dilutions prepared at the time of each plating. The sample designated as Series 1 represents a dilution of 1/1,000,000 and Series 2 a dilution of 1/2,000,000. Dilutions were prepared by the method previously given, and plates poured with soil extract agar. The plates were incubated at 20° and examined after 14 days.

The results are given in Table 11 and illustrated in Graph 1.

Table 11./

Table 11.

Bacterial numbers in millions per g. of oven-dried soil from a worm free soil and a soil containing worms.

Soil.	Time-days.					
	0	2	3	4	6	7
Control A.	15.0	43.3		51.7		61.7
Control B.	15.0		30.0		25.8	
Worm A Series 1		29.7		51.5		68.5
Series 2		30.3		48.3		75.0
Worm B Series 1			58.6		41.5	
Series 2			46.7		43.3	

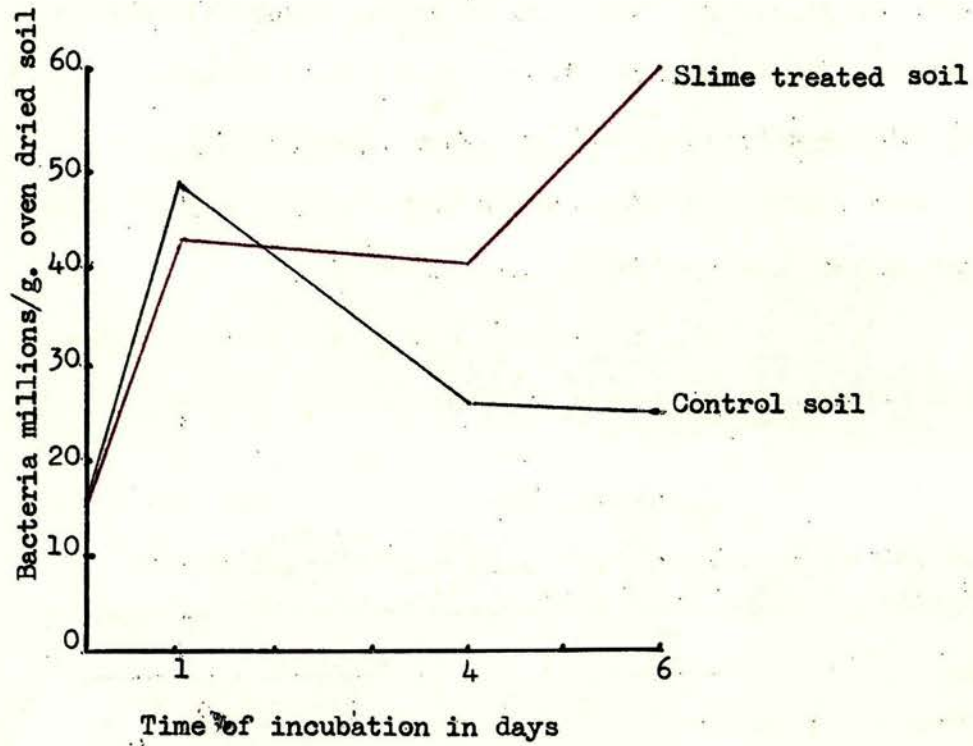
The effect of worm slime on the bacterial numbers of a soil.

The field soil which was used to determine the effect of the presence of worms was used to determine the effect of worm slime in a soil.

The following method was used to obtain the worm slime. A number of worms were washed and placed in cold water for a period of 24 hr. Worms placed in water tend to defecate. A number were selected which showed clearly that their entire lower gut was free from casts. One of these worms was placed in a weighed 4.5 inch petri plate. The plate was then warmed by placing it over a 45° water bath. As the plate became warmer the worm exuded large quantities of slime which were spread over the bottom of the plate. The worm was then removed and the slime allowed to dry. The process was repeated with fresh worms until approximately 0.005 g. of slime had been collected. A sample of 50.0 g. of soil was weighed out and a small portion of the sample placed in the petri/

Graph 2.

Effect of worm slime on bacterial numbers in a soil.



petri plate. By grinding this soil against the bottom of the plate it was found that nearly all the dried slime could be collected. This mixture of slime and soil was then thoroughly mixed with the remainder of the sample and the entire sample transferred to a clean petri plate. The sample was then brought to 80% of its water holding capacity. A similar 50.0 g. sample of the soil was placed in another petri plate, brought to 80% of its water holding capacity and used as the control soil. Both soils were incubated at 20° and sampled periodically. Dilutions were prepared by the usual method and soil extract agar plates poured and incubated for 14 days at 20°.

The results of this experiment are given in Table 12, and illustrated in Graph 2.

Table 12.

Bacterial numbers in millions per g. of oven-dried soil from untreated and slime treated soils.

Soil.	Time-days.			
	0	1	4	6
Untreated.	15.0.	48.2	26.6	23.3
Slime treated.	15.0	43.3	40.0	60.0

Control soil B of the worm treatment, and the control soil of the slime treatment are in close agreement and give typical curves for freshly wetted soil. A marked increase in numbers occurs within 24 hr. which is followed by a decrease with a levelling of numbers. Control soil A of the worm soil gives an atypical curve and cannot be considered a representative sample. It is possible that some form of organic/

organic matter was accidentally incorporated in this soil.

Both the worm treated soils show a marked increase in bacterial numbers compared with the control soil after 48 hr. incubation. This increase is then maintained for the duration of the experiment. The increase is more pronounced in worm soil A than in worm soil B which may, possibly, be related to the relative activity of the worms in the two soils.

The slime treated soil shows the same initial increase as the control soil but the decrease in numbers which normally follows the wetting of a soil does not occur. This suggests that a portion of the bacterial population which is capable of utilising the slime develops while the balance of the population decreases in numbers. When the normal population reaches a stable level, the slime utilising organisms begin to show as an increase in the total numbers. The general level of bacterial numbers in this phase approximates that of the worm soil suggesting that the increased numbers occurring in a soil containing worms is related to the presence of worm slime.

The close correlation between the bacterial numbers in the two series of platings with the worm soil (Table 11), would suggest that the sampling and dilution techniques employed were adequate in providing representative samples of the soils.

Bacterial numbers in a worm-free soil, a soil with worms and fresh worm casts.

The preliminary investigations had indicated that marked changes in the total bacterial count of a soil occurred in the presence of earthworms and in the worm gut. For this reason an experiment was designed to establish, under carefully controlled conditions, whether or not this assumption was justified.

A field soil was selected. The 3 mm. fraction of this soil was air-dried and divided into a number of similar 2 Kg. portions which were placed in covered glass jars. The soil in each jar was brought to 60% of its water holding capacity as it was required. Earthworms were passed through five portions of the soil by the method previously described. When it had been ensured that the gut contents of the worm had been replaced two jars of the soil were moistened. One was taken as the control or worm-free soil and to the second the conditioned worms were added. The second was designated as the worm soil. The soils were covered and incubated at 20°. Samples of 10 g. were obtained periodically by coring to the bottom of the jars with a large, flamed cork borer. In order to obtain as representative a sample as possible a number of cores were obtained and thoroughly mixed before weighing. Samples of the worm casts were obtained by the method previously described.

A comparison of several types of media, as previously reported, had established that either a soil extract medium or a soil extract medium with 0.2% w/v Bacto yeast extract was most satisfactory for obtaining total counts of the bacterial population in these soils.

It was considered that over a long period of incubation types of bacteria might develop in the worm soil and in the worm cast which might have more complex nutritional requirements than could be provided for by a simple soil extract medium. On the other hand, it was considered that a medium as non-selective as possible should be employed in order to permit the maximum development of the autochthonous flora of the soils. A simple soil extract medium appeared most likely to fulfil this condition. For these reasons it was decided to conduct all platings for total counts on both the soil extract medium and the soil extract-yeast extract medium with a sufficient number of dilutions to examine the efficacy of the two media.

It was also considered advisable to use a selective medium which might permit the detection of types of organisms that would not appear at the dilution required for total counts. For this purpose a yeast-peptone medium as devised by Topping (1936) supplemented by the addition of 1/500,000 parts of crystal violet was employed. It was hoped that the crystal violet would inhibit the growth of many coryneform organisms and actinomyces thus permitting the development of Gram-negative types.

Dilutions of each sample were made by the method previously outlined and four plates of each dilution were prepared with each medium from each of the three materials. The plates were incubated at 20° and counted after incubation for 14 days.

The results are given in Table 13 in millions of bacteria per gram of oven dried soil.

Table 13.

Bacterial numbers in millions per g. of oven dried soil.

Soil.	Medium	Time of incubation of soils in days.								
		0	1	4	6	10	14	20	30	
Control	S.E.	33.6	67.9	54.6	49.0.	34.3	39.2	42.0	24.0	
	S.E.Y.	20.5	77.0	56.0	58.1	36.4	49.7	50.8	42.0	
	C.V.	1.5	9.8	21.7	14.0	7.4	8.4	20.3	16.8	
Worm soil	S.E.		58.8	38.5	79.8	79.8	80.5	61.6	33.0	
	S.E.Y.		79.1	60.2	93.8	79.8	91.0	77.0	56.0	
	C.V.		21.0	19.6	26.6	23.8	30.8	28.0	7.7	
Worm cast	S.E.		25.0	30.5	10.6	35.1	55.6	117.3	89.3	
	S.E.Y.		32.0	42.6	14.8	38.6	92.0	151.3	113.1	
	C.V.		7.8	3.6	1.7	5.4	42.0	122.8	62.9	

Media:

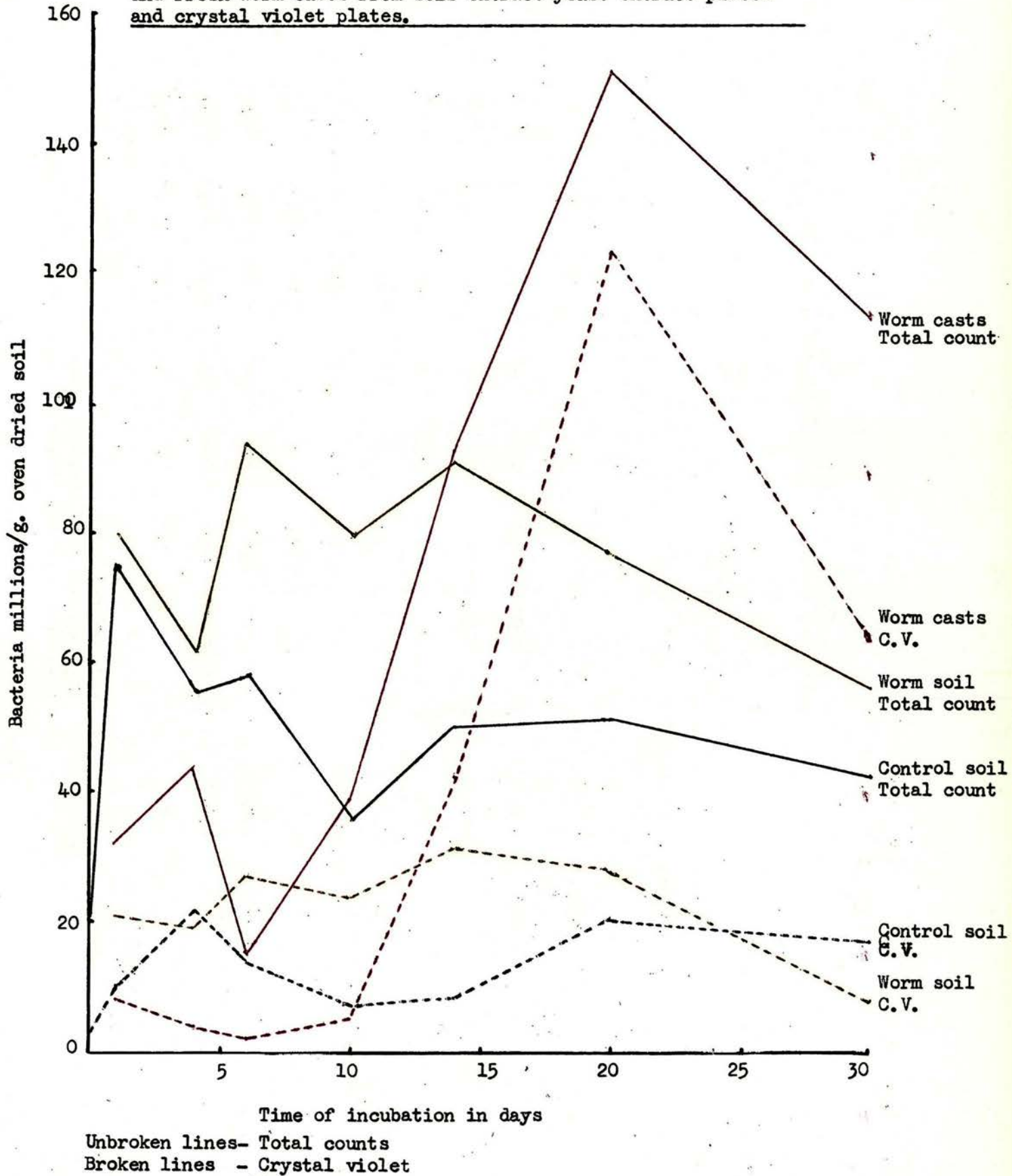
S.E. - Soil extract agar.

S.E.Y. - Soil extract-yeast extract agar.

C.V. - Topping's agar with 1/500,000 parts crystal violet.

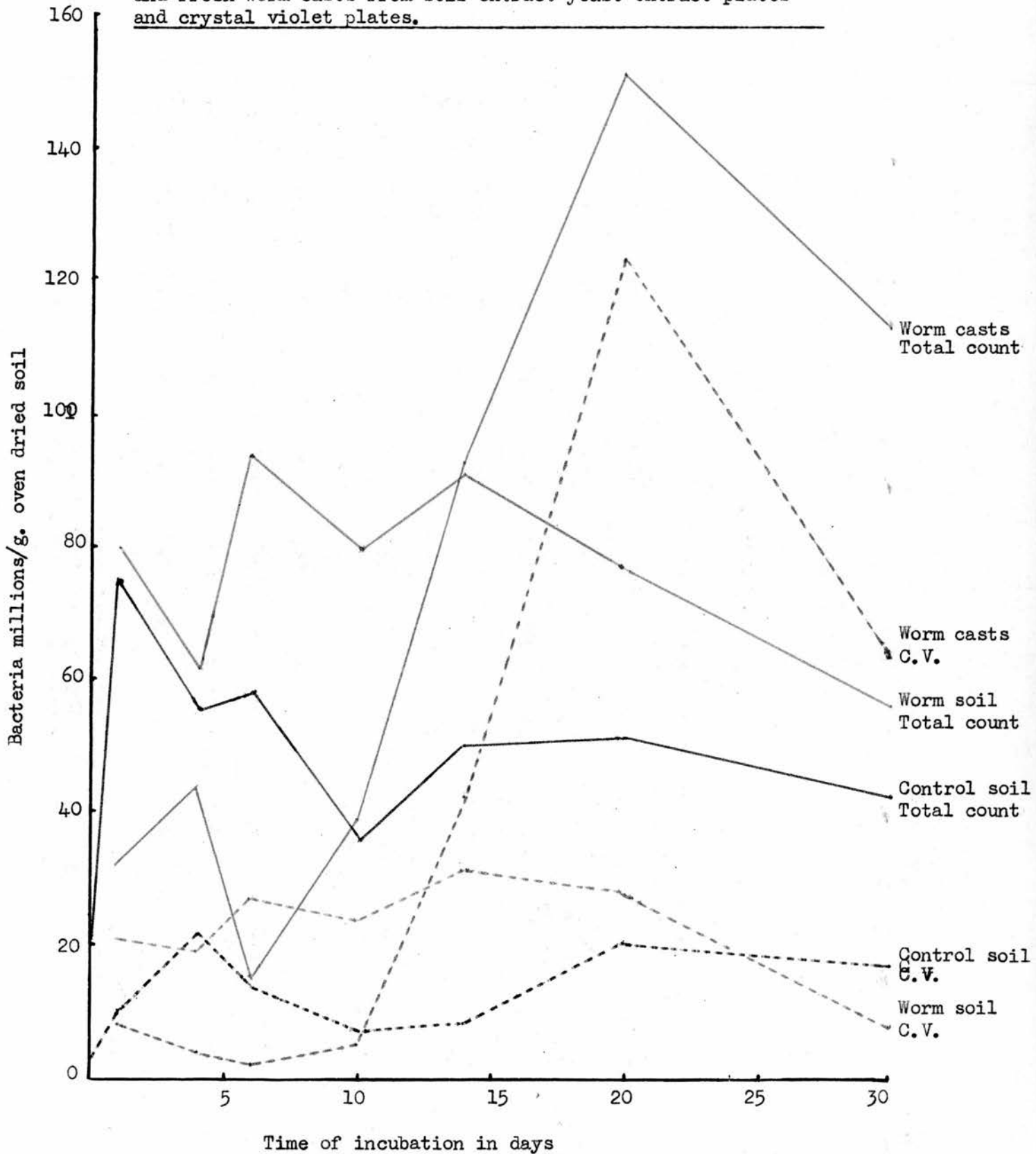
Graph 3

Bacterial numbers in a worm-free soil, a soil containing worms and fresh worm casts from soil extract-yeast extract plates and crystal violet plates.



Graph 3

Bacterial numbers in a worm-free soil, a soil containing worms and fresh worm casts from soil extract-yeast extract plates and crystal violet plates.



Unbroken lines- Total counts
Broken lines - Crystal violet

A graphical representation of the counts obtained from the soil extract-yeast extract medium and the crystal violet medium is given in Graph 3.

The counts obtained from the soil extract plates were consistently lower than those obtained from the soil extract-yeast extract plates. For this reason and in order to facilitate comparison of the curves, the curves for the soil extract platings have been omitted from the graph.

Graph 3

Table 13.

(SEE OPPOSITE PAGE).

The numbers of bacteria in the control soil appear to follow a typical curve for an untreated soil. Both the soil extract and soil extract-yeast extract media show a marked increase in bacterial numbers within the 24 hr. period following the wetting of the soil. In the case of the count obtained from the soil extract-yeast extract plates, the increase from 20.5 to 77.0 millions appears abnormally high. This increase is, however, repeated in the worm soil where a count of 79.1 millions occurs at 24 hr. It seems likely that the unusually high numbers are due to the development of types of organisms which fail to develop on the less complex medium. A similar curve is followed by the bacteria developing on the crystal violet plates although the stimulation produced by the wetting of the soil appears to be delayed.

The total count of the worm soil follows that of the control soil until 4 days after which a marked increase occurs in the worm soil. These results agree with those of a previous experiment reported in Table 11. It may be considered that the first effects of the earthworms in the soil are shown in/

in the period between 4 and 6 days. The counts for the worm soil on soil extract agar and on soil extract-yeast extract agar show essentially similar trends. The increased numbers obtained when yeast extract is provided are, however, more marked in the worm soil than in the control soil. A comparison of the numbers of colonies which appear on the total count plates and the crystal violet plates of the control soil and of the worm soil indicates that a greater difference occurs in the worm soil than in the control soil. This observation suggests that the organisms developing from the worm soil and the control soil in the crystal violet medium represent a more closely related population than do those developing in the total count plates of the same soils.

In the worm casts the initial increase in bacterial numbers produced by the wetting of the soil, which occurred in both the control and worm soil, did not occur. This would suggest that the portion of the soil microflora which is stimulated by the wetting of air-dried soil does not develop readily in the gut of the earthworm. A previous experiment reported in Table 11 had indicated that the bacterial numbers in fresh worm casts remained lower than those in the soil in which the worms were living until after 10 days. A similar result was obtained in this experiment. This occurs despite the fact that the worm casts represent a sample of the worm soil of not more than 48 hr. before the sampling of the worm soil. It would appear that certain groups of bacteria are stimulated by the presence of earthworms in a soil and that at least some of these bacteria fail to survive passage through the worm gut./

gut. The increase in bacterial numbers in the worm cast which follows the 10 day incubation period suggests that a selective action occurs within the gut so that certain bacterial types present in the worm soil proliferate as the others are destroyed. The nature of the curve of bacterial numbers in the worm casts, as determined from the crystal violet plates, differs greatly from those of the other two soils. It conforms to the curve given by the total count plates, and in the period from 10 to 30 days differs far less from the total count than in either of the other soils. This close relationship of the numbers obtained on a selective and on a non-selective medium would indicate that the worm gut contains a bacterial flora different from that of the control or worm soils.

As the large number of platings involved in this experiment precluded the practicability of conducting it in replicate, the possibility exists that some of the results may have been influenced by errors in sampling. It is, however, considered that the results are valid for the following reasons:

In the case of the control soil, the curves follow the typical pattern for an untreated airdried soil after it is wetted. The initially high bacterial numbers obtained at 24 hr. are duplicated by the worm soil which at this stage may be considered a replicate of the control soil.

A comparison of the curves for the worm soil with those obtained in an earlier study (Graph 1) shows a marked similarity. The initial peak in numbers was not obtained in the first experiment probably because no count was made at 24 hr.

In/

In the first experiment the second peak seems to occur earlier but this may be due to the greater concentration of worms per gram of soil. Following the second peak the same levelling off in bacterial numbers occurs in both experiments at an approximately similar level of numbers. An increase in numbers between 5 and 10 days is also shown by the experiment reported in Table 4.

A lower bacterial count in the worm cast than in the worm soil up to 10 days is reported in Tables 4 and 13. Dawson (1947) also reports counts in worm casts initially lower but later higher than in a worm-free soil. An extract from his results is given in Table 14.

Table 14.

Numbers of bacteria in millions in excreted casts and a worm free soil with and without added organic matter. (Dawson, 1947).

Soil.	Organic matter added.		No organic matter added.	
	Aug. 11	Aug. 29	Aug. 11	Aug. 29.
Casts.	55	131	29	81
Worm free	77	105	69	1

Bassalik (1912) also reports an examination of soil, intestines and excreted casts which gave populations of 20, 148, and 64 millions, respectively. These results obtained by Dawson and Bassalik provide some evidence supporting the view that the high bacterial counts obtained from the worm casts at 20 and 30 days are valid.

Numbers/

Table 15.

Percentage of bacteria capable of anaerobic growth as estimated by numbers of colonies developing in agar shake cultures.

Time of incubation of soil in days.	Control soil.		Worm soil.		Worm east.	
	Estimated numbers millions	% Total count	Estimated numbers millions	% Total count	Estimated numbers millions	% Total count.
0	10	50				
1	100	125	100	125	10	31
4	10	16	10	17	100	232
6	100	160	100	100	100	675
10	100	270	100	125	10	26
14	100	200	100	110	10	11
20	100	200	100	128	10	7
30	1	2.5	-	-	10	9

Numbers of bacteria capable of anaerobic growth.

A preliminary investigation was conducted to establish if a major alteration in the numbers of bacteria capable of anaerobic growth occurred in a soil in the presence of earthworms or in the passage of the soil through the worm gut.

The numbers of bacteria capable of growth in the absence of oxygen was estimated by colony counts in deep layers of agar in tubes. Two tubes of the soil extract-yeast extract medium enriched by the addition of 0.2% w/v glucose and 0.2% w/v peptone were inoculated from the dilutions used for the platings previously described (p. 71). The highest dilution which permitted the development of colonies under apparently anaerobic conditions was used as an indication of the approximate number of anaerobes or facultative anaerobes present in the soil. The estimated numbers and the percentages of the total plate counts that they represent are given in Table 15.

Allowing for the gross inaccuracies of this method, it would appear that there is little or no difference between the control and the worm soil. There does, however, appear to be a decrease in the incidence of bacteria capable of anaerobic growth in the worm gut after 6 days. This suggests that the bacterial population present in the worm soil which is capable of growth under anaerobic conditions is adversely effected when the soil is ingested by the earthworm

Table 16.

Percentage incidence of denitrifying bacteria as determined by gas production in Giltay's medium. Estimated numbers in 10,000's.

Time of incubation of soil in days.	Control soil		Worm soil		Worm cast	
	Estimated numbers.	% Total count.	Estimated numbers.	% Total Count.	Estimated numbers.	% Total count.
0	0.1	0.005				
1	0.01	0.0001	1.0	0.013	1.0	0.03
4	0.01	0.0002	10.0	0.17	10.0	0.23
6	0.1	0.002	1.0	0.01	10.0	0.68
10	0.1	0.003	10.0	0.13	10.0	0.26
14	1.0	0.02	100.0	1.1	100.0	1.1
20	10.0	0.20	100.0	1.3	100.0	0.7
30	10.0	0.24	100.0	1.8	1000.0 [*]	10.9 [†]

* Total count 113 million.

TABLE 16.
(SEE OPPOSITE PAGE).

Numbers of denitrifying Bacteria.

The numbers of denitrifying bacteria present in the three soils were estimated by inoculating three tubes of Giltay's medium (Fred and Wakeman, 1928) from the dilutions used to determine the bacterial count of these soils. The production of gas was considered indicative of the conversion of nitrate to gaseous nitrogen. The highest dilution in which gas appeared was used to estimate the number of denitrifying bacteria present in the sample. It is realised that an estimation by a dilution method of this type is highly inaccurate. It was, however, considered that any major differences in the soils would become apparent in which case more valid counts could be obtained. Table 16 shows the estimated numbers and the percentage incidence of these bacteria compared to the number of bacteria developing on the total count plates.

It is indicated by these results that no large increase occurred in any of the soils. There is, however, an indication that the worm soil and the worm cast contained more organisms capable of denitrification than did the control soil. This effect may be produced by more favourable conditions for these organisms existing either in the worm gut or in the worm soil.

Discussion/

Table 16./

TABLE 16.
(SEE OPPOSITE PAGE).

Discussion and general summary.

The quantitative investigations by Bassalik (1913) and Stöckli (1928) while providing some interesting information on changes which are likely to occur in a soil in the presence of earthworms, fail to provide sufficient information to permit a comparison of their work with subsequent quantitative studies.

Day (1950) provided more adequate counts of the bacterial population in a soil and in castings of the soil. His method of obtaining casts is, however, subject to some criticism. The worms were allowed to remain in a pot of the soil to be studied for a period of one to three days. They were then removed and placed in a fresh pot of the same soil. The castings which were voided were collected as quickly as possible and used as the cast sample. The counts obtained from the casts were compared with a sample taken from the second pot of soil. Casts obtained in this manner would not necessarily represent the changes produced in a soil by passage through the gut as the bacterial flora of the first pot might have been altered by the worm living in it. The exact age of the cast would not be known and even a few hours might effect the bacterial population of the cast. The removal of a cast from the surface of the soil on which it is resting introduces the possible error that some of the soil may be included with the cast. The results obtained however, are extremely interesting. Samples of the worm casts and the soil were collected at different times. In seven samples, the bacterial population of the worm cast exceeded that of the/

the soil by 14.65%, 56.80%, 310.77%, 90.38%, 340.00%, 254.29%, and 2.95% respectively. In six cases, the bacterial population of the worm cast was less than that of the soil by 46.90%, 71.66%, 25.47%, 92.32%, 86.68%, and 74.34% respectively. It was concluded from these results that the total numbers of bacteria were not consistently greater or less in fresh castings of the earthworm than in the soil. It may be noted, however, that a much more stable difference exists when the population of the cast is less than that of the soil than when it is greater. In the present investigation, the results given in Table 4 show a decrease in the bacterial population of the worm cast compared with the worm soil at 5 days of 48.3%, 54.7%, 46.6%, and 54.4%. In Table 13, the decrease at 24 hr. is 57.5%, at 4 days 26.2%, and at 6 days 86.7%. It thus appears likely that the counts obtained by Day which show greater numbers in the worm cast than in the soil represent the introduction of an extraneous factor such as the incubation of the worm cast or an increase in the population of the soil in which the worms were incubated being shown in the cast, but not in the worm-free soil. The conclusion by Day that the spores of Bacillus cereus mycoides pass through the earthworm gut unchanged while vegetative cells are destroyed is in agreement with the results obtained in this investigation.

The method employed by Dawson (1947) to obtain samples of the gut contents does not appear satisfactory. An average sample was obtained by opening the worm and removing alternate portions of the gut content along the entire length of the intestinal tract. While this method may give a figure which approximates the average number of bacteria present in the gut/

gut, it fails to demonstrate whether or not the bacterial population increases or decreases during passage through the gut. Direct microscopical examination has indicated that the population increases in the lower sections of the alimentary canal, and Day (1950) has demonstrated that certain types of bacteria are destroyed before they reach the intestine. Dawson sampled worm casts by removing them from the soil mass which is not satisfactory for the reasons previously stated.

Despite the inadequacies of the sampling methods the results reported by Bassalik, Stöckli, Dawson and Day are in general agreement with those obtained in this investigation, namely:

1. After an initial period the bacterial population of a soil in which worms are living exceeds that of a worm-free soil.
2. When worms are placed in a freshly moistened soil, the bacterial population of their gut is at first less than that of the soil in which they are living and then, after a period of some days, greatly exceeds it.

The destruction of denitrifying bacteria, reported by Jensen (1898), during passage of a soil through the worm gut was not observed in these studies.

In this work (see Table 6) and in the experiments of Day (1950), the fungal count of a soil was found to be reduced when the soil passed through the gut of an earthworm.

The addition of worm slime to a soil has been found to produce an increase in the bacterial population of the soil comparable with that obtained when worms are added to a soil. It thus appears likely that part, at least, of the effect of earth-
:worms/

worms in a soil may be attributed to the slime they secrete.

The quantitative studies have suggested that qualitative differences exist between the bacterial population of a soil in which worms are living and the bacterial population of freshly excreted worm casts. The increase in bacterial numbers which normally follows the moistening of an air-dried soil was not reflected in the counts obtained from the casts. A large proportion of the bacterial population of the control soil and the worm soil failed to appear on the crystal violet medium, but the microflora of the casts did not show this repression. There is thus evidence that some types of bacteria multiply in the worm gut while of the original soil population ingested some portions are destroyed while others are probably unaffected.

Evidence has been obtained that bacterial spores pass unchanged through the worm gut. This observation should make it possible to examine quantitatively the population of various sections of the alimentary canal without the necessity of weighing the samples. The method could be as follows: Total counts and spore counts would be made of the soil in which the worm is living. The worm would be killed and the samples obtained with a loop from various sections of the alimentary tract; a total count and a spore count would be made on each sample. The spore count would show the weight of soil examined and thus the total count per gram could be calculated.

QUALITATIVE STUDIES ON THE EFFECT OF WORMS ON
THE BACTERIAL FLORA OF A SOIL AND CHANGES
OCCURRING IN THE WORM GUT.

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THE BACTERIAL FLORA OF A SOIL AND CHANGES
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NUTRITIONAL CLASSIFICATION OF CULTURES ISOLATED.

One of the most difficult problems still facing soil bacteriologists is the evolution of a satisfactory method for the classification of the autochthonous microflora of the soil. While considerable progress has been made in the study of many of the more specialised forms of soil bacteria which are marked by their specific physiological activities, the failure of the more abundant types of soil bacteria to respond to the classical morphological and biochemical groupings has hindered the progress of qualitative bacteriological studies of the soil.

An approach to this problem has been made by Lochhead and his co-workers. West and Lochhead (1940) proposed a system of grouping based on the growth response of soil bacteria to three nutritional media of increasing complexity. This nutritional grouping was expanded by Lochhead and Chase (1943) by increasing the number of media of varying complexity to seven. The media employed, which have been used by the Ottawa group in subsequent nutritional studies, provided the following groupings of soil bacteria:

1. Those that are capable of growth in a simple, basal medium containing mineral salts, and glucose and nitrate as the only sources of carbon and nitrogen.
2. Those that are capable of growth on the addition of ten amino acids to the basal medium.
3. Those capable of growth when cysteine and seven growth factors are added to the basal medium.
4. Those/

4. Those that require the addition to the basal medium of both the amino acids and growth factors.
5. Those that require the addition of yeast extract to the basal medium.
6. Those that require the addition of soil extract to the basal medium.
7. Those that require the addition to the basal medium of both yeast and soil extracts.

These nutritional groupings have provided the means of investigating a number of soil problems amongst which may be mentioned studies of the rhizosphere effect by West and Lochhead (1940) and Lochhead and Thexton (1947); seasonal and manurial effects by Katznelson and Chase (1944); the influence of cropping on the bacterial flora of the soil by Wallace and Lochhead (1948), etc.

Some objections have been raised to this system because of the media used by Lochhead and his associates. Many bacteria are capable of utilising inorganic ammonium salts as their source of nitrogen, but are unable to obtain their nitrogen requirements from nitrate. Because of this, many bacteria which should be placed in the simplest group may be relegated to a group requiring more complex media (Taylor, 1951). The use of heat sterilised glucose may also be questioned since it is known to be inhibitive to several types of bacteria which are capable of utilising glucose sterilised by filtration. A nutritional classification based on a single test of the growth requirements of an organism may also be criticised. The original inoculum may carry sufficient growth factors to initiate a growth response which would not occur in subsequent transfers to the same medium.

Despite/

Despite these objections the usefulness of the nutritional system of grouping soil bacteria can not be denied. Lochhead (1952) states: "It should be emphasized that any procedure for the nutritional classification of soil bacteria is an arbitrary system, and the grouping of organisms studied depends upon the observance of a rigid set of conditions with respect to the number and composition of the differential media, the time and temperature of incubation and the manner of assessing the growth response."

Materials and methods.

From the soil extract-yeast extract plates and the crystal violet plates used for counts of bacterial numbers in the worm-free soil, the worm soil and the worm casts, reported in the previous section (pp.), cultures were isolated and grouped according to their nutritional requirements. The plates were incubated for approximately 21 days at 20° before colonies were transferred to slopes of soil extract-yeast extract agar enriched by the addition of 0.2% w/v Bacto peptone and 0.2% w/v glucose.

Colonies were systematically picked so that all the colonies on opposite sectors of a plate were included. A total of 36 colonies were picked from each plating. The platings at 24 hr. and at 10, 20 and 30 days were chosen as being likely to show the changes which occurred in the soils and the worm casts. In all 864 isolations were made.

The slope cultures were incubated at 20° and examined every 24 hr. until visible growth occurred. At this time each isolate was transferred by loop inoculation into the following/

following media:

1. Basal medium consisting of glucose, 0.1%; K_2HPO_4 , 0.1%; KNO_3 , 0.05%; $MgSO_4$, 0.02%; $CaCl_2$, 0.01%; NaCl, 0.01%. $FeCl_3$, 0.001%; distilled water, 1 l. The medium was heated to 100°, filtered and adjusted to pH 6.8.
2. Amino acid medium consisted of the basal medium plus 0.5% Bacto vitamin-free casamino acids.
3. Growth factor medium consisted of 750.0 ml. basal medium. 250.0 ml. soil extract; 0.1% Bacto yeast extract.

All percentages are w/v. In order to prevent any possible addition of growth factors to the basal and amino acid media all glassware involved in their preparation, and also the test tubes used, were soaked overnight in cleaning solution and thoroughly rinsed in tap and distilled water.

The nutritional classification was based on the use of modified forms of the three nutritional media originally employed by West and Lockhead (1940) rather than the more comprehensive media of Lockhead and Chase (1943), as it was considered that these three media would indicate any major changes in the bacterial populations to be studied. The necessity of combining the nutritional studies with the quantitative studies previously reported (pp.11-77) also made it impracticable for a single worker to do the work entailed in the employment of seven nutritional media.

The cultures were incubated at 20° and examined for growth after 24 hr., 3 days, 7 days and 14 days. Growth response in the nutritional media was determined by the method given by West and Lockhead (1940). Each isolate was considered separately and the tube showing the heaviest growth was assigned

a turbidity value of 4. Other cultures of the same organism were rated by comparison. Slight differences in growth were not considered significant.

Nutritional groups.

On the basis of their growth response in the three media all the organisms studied fell into one of the following five major nutritional divisions.

1. Bacteria with simple requirements.

These bacteria are capable of a maximal development in the basal medium. Their growth is not significantly improved by the addition of more complex nutrients.

2. Bacteria which require a mixture of amino acids.

These organisms are capable of obtaining all their growth requirements from the amino acids present in the vitamin free casein amino acids provided. They showed no significant stimulation by the provision of growth factors. They are incapable of growth in the basal medium or develop slowly at a submaximal rate.

3. Bacteria which require growth factors.

These bacteria produce maximum growth only in the growth factor medium. In the great majority of cases they show no growth in either the basal or the amino acid medium.

4. Bacteria with undefined requirements.

These bacteria fail to grow on the soil extract slopes, or if developing on the soil extract slopes fail to grow in any of the nutritional media.

5. Bacteria inhibited by amino acids.

Table 17.

Percentage incidence of major nutritional groups (36 isolations from each plating; actinomycetes eliminated).

Group.	Medium	Control soil.				Worm soil				Worm cast.			
		1	10	20	30	1	10	20	30	1	10	20	30
1.	SY	14	22	19	14	8	22	14	22	14	11	17	14
	CV	17	33	28	3	41	25	28	86	8	31	19	36
2.	SY	31	14	19	39	33	17	55	8	14	19	17	72
	CV	22	6	17	17	6	41	36	11	33	28	25	58
3.	SY	25	44	31	11	47	31	19	52	33	58	64	8
	CV	17	39	44	61	22	17	33	3	22	33	52	6
4.	SY	22	8	14	8	8	22	8	8	17	8	3	3
	CV	41	6	8	8	17	6	3	0	33	8	3	0
5.	SY	3	3	11	3	0	8	3	0	6	0	0	0
	CV	3	17	3	6	14	0	0	0	3	0	0	0

Media: SY = Soil extract-yeast extract agar.

CV = Topping's agar + 1/500,000 parts crystal violet.

A small group of the bacteria isolated failed to grow or are severely inhibited in the amino acid medium but are capable of good growth in both the basal and growth factor media. Subsequent examination indicated that many of these organisms are incapable of development on nutrient agar slopes or in glucose peptone agar stabs. They also failed to develop in glucose peptone broth. Organisms of this type were reported by West and Dochhead (1940).

Experimental results.

The percentage incidence of each of these major nutritional groups is given in Table 17. The percentages are calculated on the basis of the thirty-six isolations made from each plating. The few actino-mycetes which were isolated have been eliminated as the type of growth which they showed in the solutions did not permit of satisfactory evaluation. The capability of bacteria to grow in the basal medium was judged from several successive readings. Some organisms showed an equal growth response at 24 hr. or 3 days in all media but by 7 days showed submaximal growth in the basal medium. It was considered that the initially good growth was sufficient to justify placing these organisms in group 1 since the subsequent increased growth in the amino acid or growth factor medium seemed to be conditioned merely by a larger supply of nutrients.

It appears that the percentage incidence of group 1 organisms isolated from the non-selective total count plates is essentially similar in the control soil, the worm soil and the worm casts. No major fluctuations occur and the group represents a fairly stable proportion of the bacterial populations/

populations.

The crystal violet plates show less stability, marked increases in the incidence of group 1 occurring in the worm soil at 24 hr. and 30 days.

The percentage incidence of group 1 on the crystal violet plates is, in most cases, greater than on the total count plates.

Group 2, the organisms requiring amino nitrogen, showed considerable shifts in percentage incidence in each of the three materials examined. The shifts did not always take a parallel course on the two media employed. The figures for the control soil, the worm soil and the worm casts do not show any clear relationship to each other. These results could have been obtained if group 2 is not homogenous but comprises several types of bacteria which showed independent numerical fluctuations.

Group 3, the organisms requiring a complex medium, likewise show irregular variations. In this group, the fluctuations were more marked on the crystal violet plates than on the total count medium.

Organisms of groups 4 and 5 appear to represent small but stable fractions of the soil population in the control and worm soils and in the worm casts. Group 4 shows the highest incidence at 24 hr. thus indicating that it responds to the original wetting of the soil. The group does not appear to be effected by passage through the worm gut.

In summary, these results suggest that groups 1, 4 and 5 represent a fairly stable percentage of the soil population while the major changes in the bacterial equilibrium of the soil are caused by shifts in the percentage incidence of/

of groups 2 and 3. Fluctuations in the bacterial equilibrium of the control soil, the worm soil and worm casts as determined by the non-selective total count medium appeared to be less pronounced than those shown by the crystal violet agar. This observation suggests that the dominant organisms in the soil are not as susceptible to changes in the environment as are certain minority groups. Numerical changes in the nutritional groups have been found to be associated with the presence of earthworms, but a reliable generalization on this question does not appear to be possible on the basis of the evidence so far secured.

BACTERIAL/

BACTERIAL BALANCE INDEX.

The use of the three nutritional media permitted a study of the changes in the microbiological equilibrium of the control soil, the worm soil and worm casts by the application of the "bacterial balance index" as calculated by West and Lochhead (1940).

West and Lochhead found that in every soil investigated a balance existed between two general nutritional groups of bacteria. One group was capable of rapid growth in a simple mineral salts-glucose-nitrate medium, while the other group was dependent for its development on adequate supplies of specific amino acids or growth factors. If one group increased through favourable conditions then the proportion of the other group fell. The bacterial balance index is a numerical expression of this microbiological equilibrium. The percentage of bacteria capable of maximum growth in the basal medium is assigned a negative value. The percentage of bacteria requiring more complex media for growth is assigned a positive value. The sum of these two values is designated as the bacterial balance index.

The value of the bacterial balance index as a means of demonstrating changes in the microbiological equilibrium of a soil has been shown by West and Lochhead (1940) and by studies in relation to the incidence of certain soil-borne plant diseases by Hildebrand and West (1941), Katznelson and Richardson (1948) and Rowatt and Atkinson (1950).

It was considered that an application of the bacterial balance index to the bacterial flora of a worm-free soil

Table 18.

BACTERIAL BALANCE INDEX.

Soil.	Medium	Days Incubation.					
		1		10		20	
		Meth.1	Meth.2	Meth.1	Meth.2	Meth.1	Meth.2
Control soil	ST	42	64	30	38	28	42
	CV	36	77	5	11	39	47
Worm soil	ST	80	88	36	58	75	83
	CV	14	31	25	31	61	64
Worm cast	ST	28	45	80	88	72	75
	CV	33	66	39	47	69	72

Media:

ST = Soil extract-yeast extract agar.

CV = Topping's agar + 1/500,000 parts crystal violet.

a soil containing worms and worm casts would aid greatly in showing if any major changes occurred in the microbiological equilibrium of these soils.

Method.

The bacterial balance indices were calculated from the growth response in the nutritional media after 7 days' incubation at 20°. These readings were selected arbitrarily for the reasons given by Lochhead (1952). They differ slightly from the values found for the nutritional groupings where the growth response was judged from successive readings as had been reported previously (p.92)

The index was calculated by two methods. Firstly, by the method employed by West and Lochhead (1940). This method assigns a negative value to the percentage of bacteria which show maximum growth in the basal medium. Those that are unable to grow in the basal medium but show maximum growth in the amino acid medium or the growth factor medium are given a positive value. The percentage of bacteria which show submaximal growth in the basal medium is not considered nor are those which failed to survive original isolation. The second method is similar to the first but modified to include the percentage of organisms which failed to survive original isolation by assigning it a positive value. It may be noted that the two methods gave similar results but the index as calculated by the second method was higher. This suggests that a fairly constant relationship is maintained between group 4 organisms and those which are unable to develop in the basal medium but are capable of growth in more complex media.

Results./

Table 19.

Percentage incidence of Group 2 organisms and the bacterial balance index in the worm soil.

Total count plates				Crystal violet plates			
Incubation period days	% exp. 2	B.B.I.		% exp. 2	B.B.I.		
		Meth. 1	Meth. 2		Meth. 1	Meth. 2	
1	33	80	88	6	14	31	
10	17	36	58	41	25	31	
20	55	75	83	36	61	64	
30	8	49	57	11	11	11	

Results

The calculated bacterial balance indices are given in Table 18 and represented graphically in Graphs 4, 5, 6 and 7. The curves for the control soil appear to be typical of a soil which has been dried and then remoistened. The tendency for an untreated soil to show an increased bacterial balance index on holding has been reported by Katznelson and Richardson (1943) and Katznelson and Chase (1944).

The worm soil showed an unstable equilibrium, thus indicating that changes occurred in the composition of the bacterial population during the course of the experiment. In the worm soil organisms of group 2 had a notable effect on the level of the balance index. This is brought out by the figures given in Table 19.

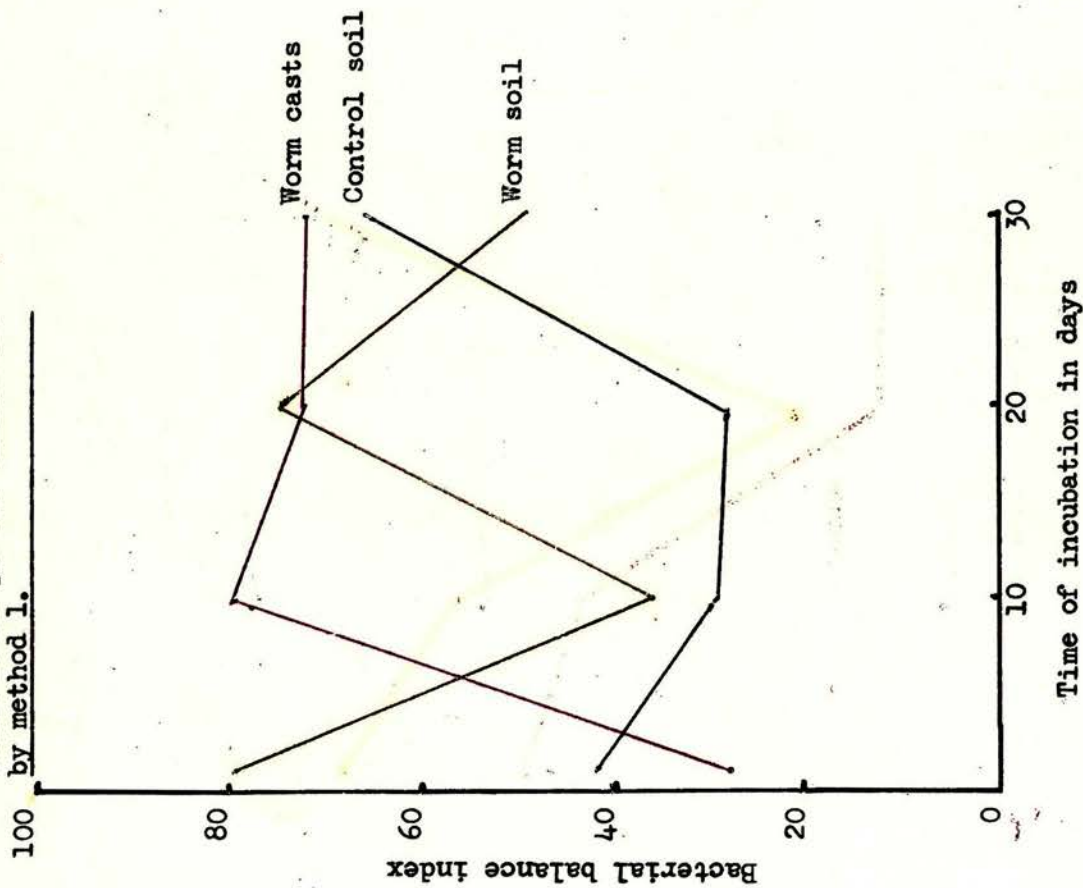
Table 19
(SEE OPPOSITE PAGE).

It has already been reported (Table 12) that the addition of worm slime to a soil increased the bacterial numbers in the soil. Katznelson and Chase (1944) have reported that the quantity of carbohydrate added to a soil effects the level of the bacterial index. It seems possible that fluctuations in the quantity of worm slime would lead to variations in the balance index.

The worm casts, unlike the soil in which the worms are living, shows a relatively stable bacterial equilibrium. This may be taken as evidence that the bacterial population of soil is appreciably altered in composition during the passage of the/

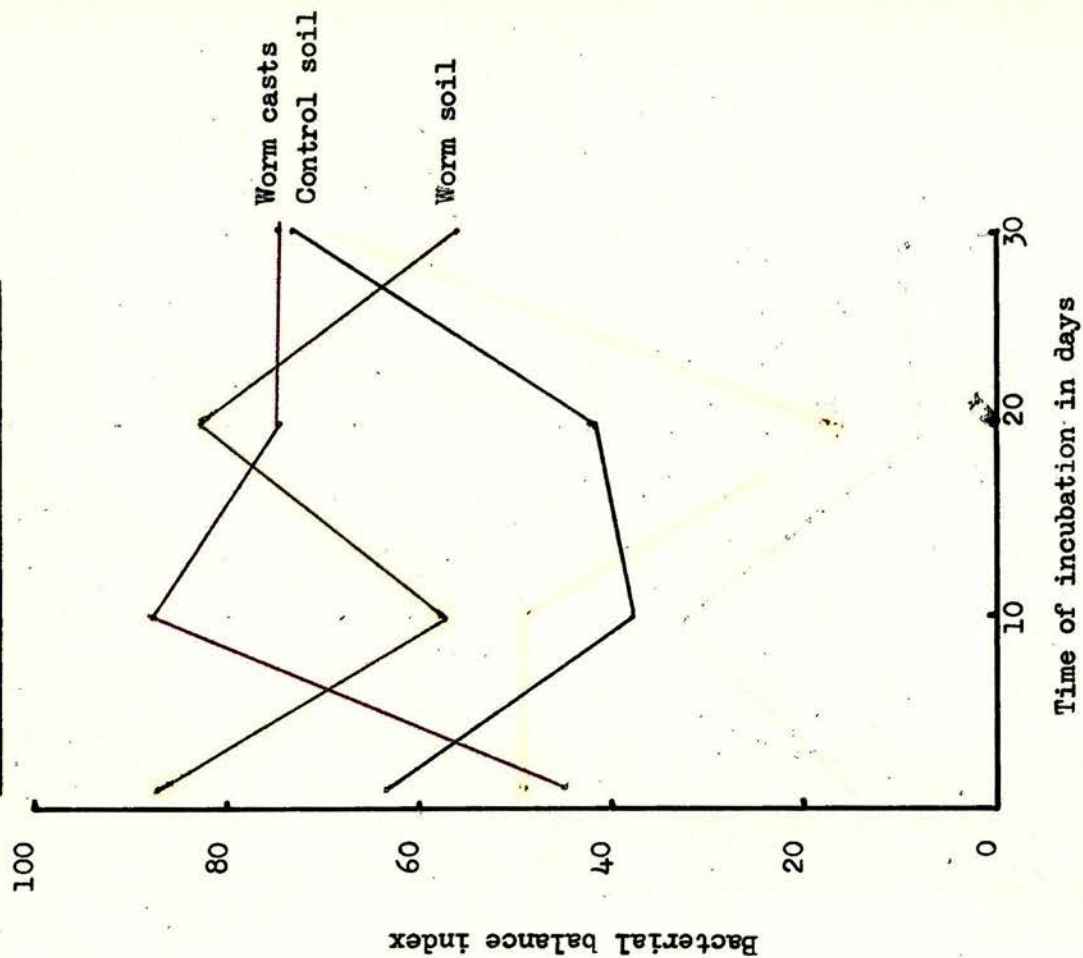
Graph 4.

Bacterial balance index from
total count plates calculated
by method 1.

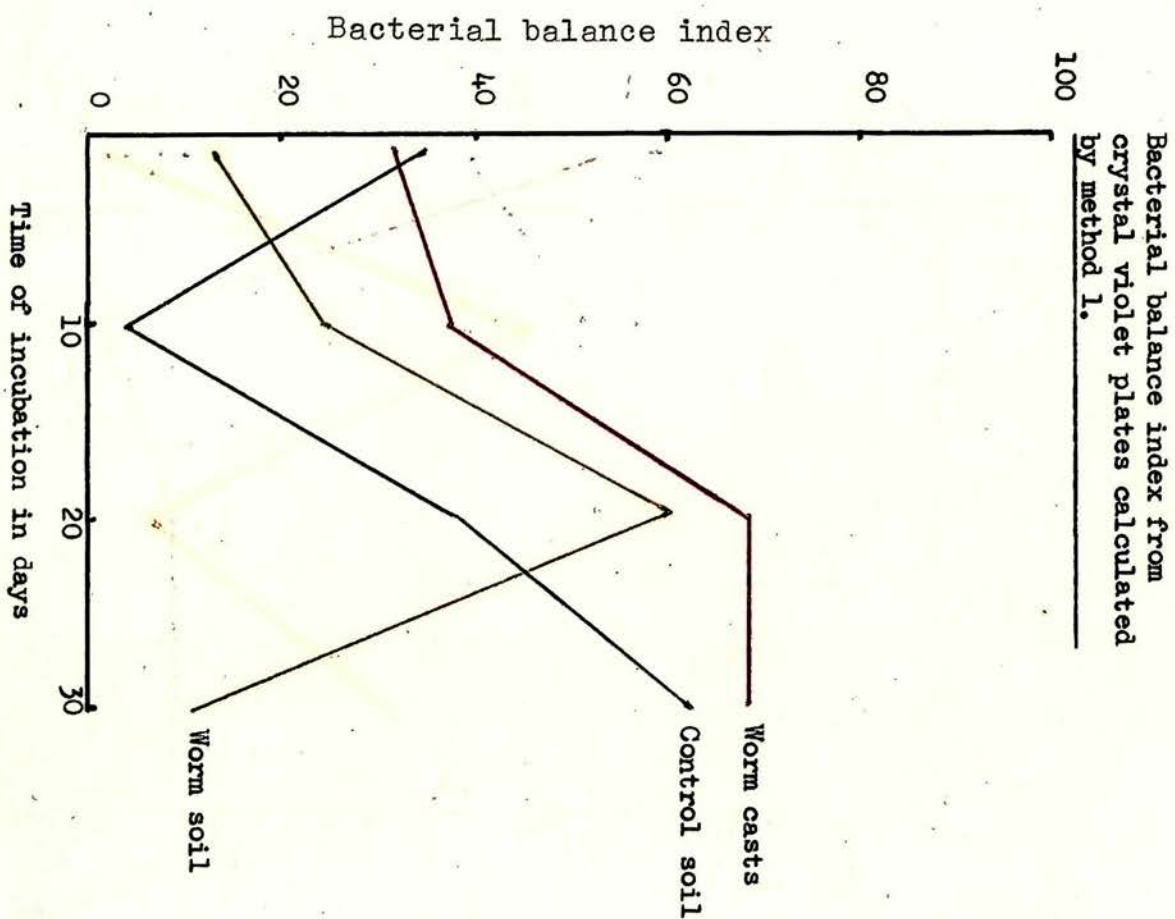


Graph 5.

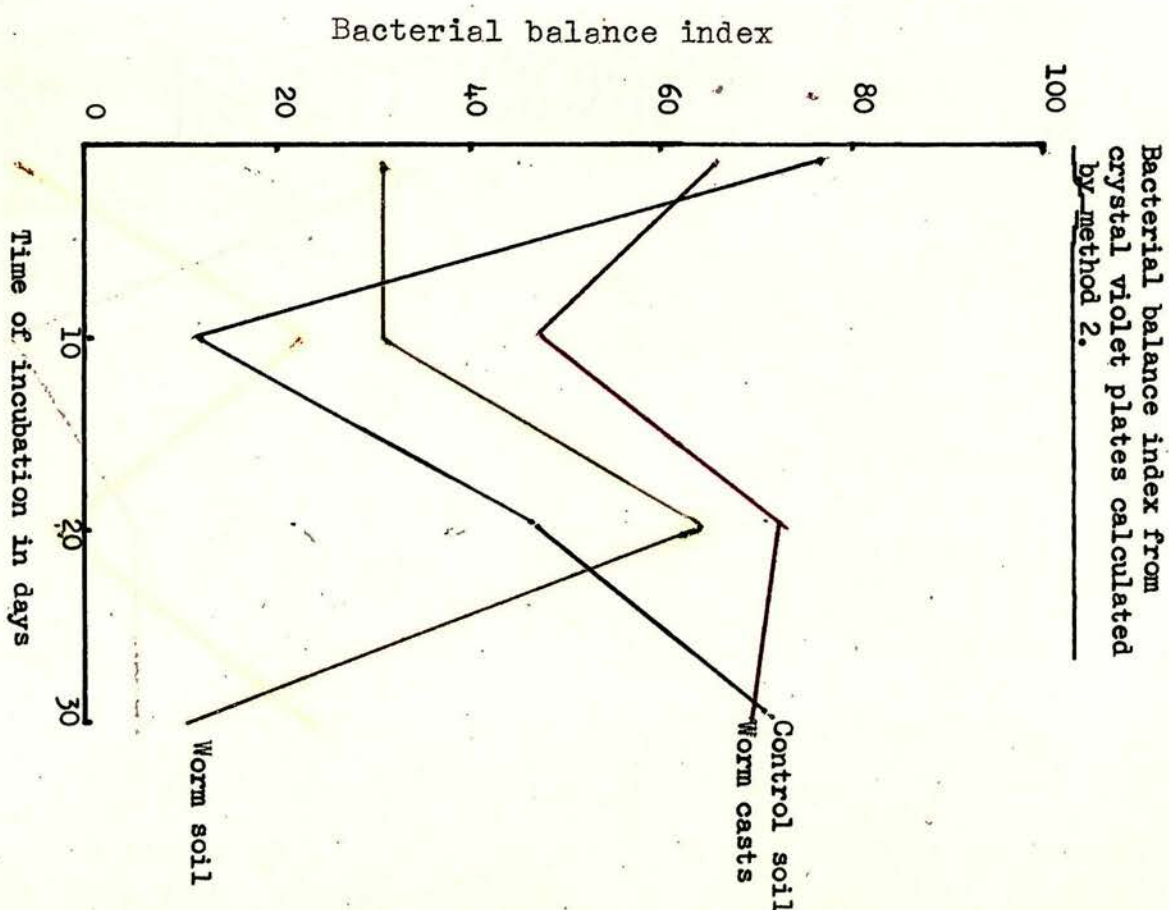
Bacterial balance index from
total count plates calculated by
method 2.



Graph 6.



Graph 7.



of the material through the alimentary tract of the animal.

The figures in Table 13 show that the counts on soil extract-yeast extract agar are higher than those on soil extract agar. The difference is least noticeable in the control soil and most noticeable in the worm casts. It may be noted that when the bacterial balance index is high, indicating a high incidence of organisms with complex growth requirements, the difference in counts obtained from the two media is relatively large. This evidence suggests the use of the yeast extract medium in future work.

The possibility that the changes in the bacterial balance index of these soils are attributable to limitations of the sampling method has been considered. While such a possibility cannot be entirely dismissed, the uniform trends indicated by the bacterial counts, the typical results obtained from the control soil and an earlier test of the sampling technique (Table 11) would suggest that representative samples had been obtained.

ADDITIONAL INVESTIGATIONS OF THE BACTERIA ISOLATED FROM WORM-FREE AND WORM SOIL AND FROM WORM CASTS.

Lochhead and Chase (1943) report that the ability of bacteria to reduce nitrates, liquefy gelatine, or produce acid in glucose does not appear to be associated with the nutritional requirements of the organisms. They also found that there is no clear correlation of morphology with nutritional needs. Nevertheless, it was considered desirable to discover some additional characters of the organisms that occurred with the greatest frequency in the worm gut and the worm soil.

Materials and methods.

The slope cultures obtained from the colonies picked from the platings of the control soil, worm soil and worm casts (p. 72) were examined every 24 hr. until visible growth appeared. At this time, the motility of the organism was determined by the hanging drop method and a film was prepared and stained by Gram's method.

If the stained film showed a Gram-positive organism, or a coryneform type, or if spore formation was suspected fresh films were prepared after a further 6 days' incubation. Litmus milk and glucose-peptone broth consisting of glucose, 0.5% w/v; Bacto peptone, 1.0% w/v; brom thymos blue, 0.001% v/v of a 1.6% solution and containing a fermentation tube, were inoculated from each culture.

The cultures were incubated at 20° and examined after 24 hr., 3 days, 7 days and 14 days. Further examinations of the litmus milk cultures were made at 21 days, 30 days and 60 days.

At/

At a later date a proportion of the strains were transferred to other media as will be subsequently reported.

General results.

It was found to be impossible, with the limited information obtained, to devise a grouping which could be applied satisfactorily to all the cultures. Nevertheless, among the Gram-negative rods several compact groups could be recognised owing to the similarity in each of the morphological, physiological and nutritional characters. Since these groups appeared to have made an important contribution in distinguishing the bacterial populations of the control soil, the worm soil and the worm casts, they will be described here in so far as their characters were determined.

Groups of Gram-negative rods.

1. Coliform group.

In this group were placed the motile, Gram-negative rods which proliferated rapidly on agar media, produced acid and gas from glucose and an acid reaction in litmus milk. All cultures grew well in the mineral salts-glucose-nitrate solution. Ten of the organisms were tested and found to give a negative Vokes-Proskauer reaction.

2. Pseudomonads and related types.

Here are included strains of motile, Gram-negative rods which multiplied rapidly on agar media and in the mineral salts-glucose-nitrate solution. A proportion of the cultures produced a soluble fluorescent pigment in one or more of the media/

media employed. All of the cultures showed at least slight acid production as determined by the brom-thymol-blue indicator in the glucose broth. The strains varied in their proteolytic action on litmus milk.

3. Non-motile rods with yellow growths.

Abundant yellow growth appeared on soil extract-yeast extract agar and on nutrient agar. In glucose broth the organisms produced an alkaline reaction of a weak acidity as determined by the brom-thymol-blue indicator. All strains were proteolytic and produced an alkaline reaction and ropiness in litmus milk. The organisms failed to grow in the mineral salts-glucose-nitrate solution but grew well in the amino acid and growth factor media.

4. Motile rods with yellow growths.

This group contains motile organisms which produce abundant yellow growths on agar media. All strains give an alkaline reaction in glucose broth and litmus milk. The milk is digested and becomes ropy. The organisms fail to develop in the basal nutritional medium but grow well in both the amino acid and growth factor media.

5. Organisms with undetermined growth requirements.

This series of organisms showed only an extremely thin growth on the soil extract-yeast extract slopes after 7 to 14 days' incubation. The only other media in which macroscopic evidence of growth was secured was the growth factor medium used in the nutritional grouping. When the soil extract-yeast extract slopes were inoculated the butts of the slopes were also/

also stabbed. A proportion of the organisms in this group produced gas in the stabs. Unfortunately, the substrate that yielded the gas was not identified. Attempts were made to study this group more fully at a later date. It was then found to be impossible to transfer the cultures despite the fact that at this time a distinct growth was apparent on the slopes and microscopical examination showed apparently normal cells. The attempts at recovery were conducted with a variety of media and under aerobic and anaerobic conditions and with increased CO₂ tension.

This type of organism appears to have growth requirements of an undetermined nature which are carried in sufficient quantities to initiate growth on isolation but which are not adequately supplied by the media that were used. The Ottawa group of workers have reported the occurrence in soil of organisms which develop on isolation in a soil extract semi-solid agar medium, but which fail to grow in any of the media employed for testing nutritional requirements. The extremely slow rate of growth shown by this type of organism in the present studies suggests that the 5 day incubation period used by the Ottawa workers would not permit the appearance of macroscopic growth. It is possible that a proportion of the cultures reported by them as failing to grow would have shown growth in their complex growth factor medium if a longer incubation period had been used.

6. Organisms sensitive to amino acids.

This group of strains represents a portion of the nutritional group 5, and a few organisms of nutritional group 1. Although capable of growth in the mineral salts-glucose-nitrate solution/

Table 20.

Percentage incidence of groups of Gram-negative bacteria.

Group	Media	Control Soil				Worm Soil				Worm Casts			
		1	10	20	30	1	10	20	30	1	10	20	30
1.	SX CV	-	-	-	-	11	8	3	1	-	-	-	-
2.	SX CV	-	-	-	-	-	-	-	6	-	-	-	3
3.	SX CV	-	-	-	-	-	-	-	11	14	-	-	17
4.	SX CV	6	3	-	-	-	-	-	-	3	-	-	25
5.	SX CV	3	-	11	3	17	3	11	8	-	-	19	3
6.	SX CV	-	11	-	-	-	19	6	6	-	-	-	-

Media:

SX - Soil extract-yeast extract agar.

CV - Topping's agar + 1/500,000 parts crystal violet.

solution they fail to proliferate in the amino acid solution and they show little or no growth on nutrient agar or in glucose broth. Litmus milk remains unchanged by the organisms.

The relation of Gram-negative rods to the earthworm.

The incidence of each of the groups is given in Table 20 and is expressed as a percentage of the total isolations so that a comparison can be made with the nutritional grouping and the bacterial balance index. The actual number of strains of each group isolated and the counts which the colonies represent are given in Tables 21 and 22 respectively. These tables are appended to permit an easier assessment of the relative importance of the groups.

The figures in Table 20 indicated that in the presence of earthworms, there is an immediate increase in the soil of the proportions of coliform organisms (group 1) and of bacteria possessing complex nutritional requirements (group 5). Later in the experiment the worm soil showed increased proportions of the organisms forming yellow growths (groups 3 and 4) and of the pseudomonad group (group 2).

The examinations of worm casts indicate that coliform organisms are to some extent destroyed during their passage through the animal. The organisms of groups 2, 3, 4 and 5 are at least unharmed by conditions in the worm gut; the plate counts shown in Table 13 provide evidence that the organisms producing yellow growths (groups 3 and 4) and the organisms with complex nutritional requirements (group 5) are capable of multiplication in the alimentary tract of the worm.

The significance, in relation to soil fertility, of changes that have been detected in the bacterial populations of the earthworm's gut, and of the soil to which the animal's secretions and casts have been added, still remains to be explored. It is generally acknowledged that a stable crumb structure is one of the most important factors determining soil fertility. Hopp & Hopkins (1946) and Slater & Hopp (1947) have confirmed many field observations relating an increased stability of soil aggregates to the occurrence of earthworms in soil. Dawson (1947) found that soil aggregates removed from the earthworm gut were more water-stable than earthworm casts which were in turn more stable than aggregates removed from a worm-free soil. Martin (1945) and later investigators have shown that microorganisms are capable of producing polysaccharides and other aggregating materials. It thus seems likely that a portion at least of the bacterial population of the worm gut is involved in the production of aggregating materials. The Gram-negative rods which produce yellow growths (groups 3 and 4) are among the organisms that seem to deserve investigation from this point of view. They produce aropy condition in milk and viscous growths on agar media, showing that they synthesise a bacterial gum; they also gave evidence of being capable of multiplying in the gut of the earthworm.

The findings reported here and the conclusions drawn from them must necessarily be regarded as tentative. It is believed, however, that they provide some basis from which the problems might be attacked in a more extensive and more detailed investigation.

Table 21.

Number of colonies of groups of Gram-negative bacteria.

Group	Medium	Control soil				Worm soil				Worm casts			
		1	10	20	30	1	10	20	30	1	10	20	30
1.	SY CV	-	-	2	-	4	1	1	-	-	-	-	-
2.	SY CV	-	-	3	-	-	2	3	2	-	3	1	10
3.	SY CV	-	-	-	-	-	2	4	-	-	-	1	6
4.	SY CV	2	-	-	-	-	-	2	2	-	1	-	9
5.	SY CV	1	4	1	6	6	1	4	-	-	7	9	1
6.	SY CV	-	-	1	-	7	2	-	-	2	2	-	-

Media: SY - Soil extract-yeast extract agar.

CV - Topping's agar + 1/500,000 parts crystal violet.

Table 22.

Numbers of bacteria in millions per g. oven-dried soil of groups of Gram-negative bacteria.

Group.	Medium	Control soil.			Worm soil.			Worm Casts.		
		1	10	20	30	1	10	20	30	30
1.	SY CV	-	-	1.1	-	847 4.6	2.2 0.7	2.1	-	-
2.	SY CV	-	0.8	1.6	-	-	1.3	2.3	3.2 3.8	0.5
3.	SY CV	0.3	-	-	-	-	1.3	8.6 3.9	-	-
4.	SY CV	4.3 0.3	-	-	-	-	-	0.8	0.4	0.2
5.	SY CV	2.1	4.0	1.4	-	13.2 0.6	2.2 0.7	8.6 2.3	-	7.5 0.2
6.	SY CV	-	1.0	0.6	-	4.0	4.4 0.3	-	-	0.4

Media: SY = Soil extract-yeast extract agar.
CV = Topping's agar - 1/500,000 parts crystal violet.

GENERAL SUMMARY AND CONCLUSIONS.

GENERAL SUMMARY AND CONCLUSIONS.

Preliminary investigations were conducted with the purpose of developing satisfactory methods of examining the microflora of the gut and coelomic cavity of the earthworm. For the purpose of showing the final effect on the soil microflora of passage through the gut a method was developed by which the final gut contents of an earthworm could be obtained in the form of a fresh cast which did not come in contact with the soil.

The nature of the so-called bacteroids of the coelomic fluid was investigated. Microscopical examinations indicated that some, at least, of these bodies are bacterial cells. Cultural studies demonstrated the occurrence in the coelomic fluid of a Gram-negative rod which grows only under strictly anaerobic conditions. This organism has complex nutritional requirements, the nature of which was not determined, and growth was never obtained after more than two transfers following isolation on any of the media tested. A streptococcus-like organism which requires anaerobic conditions for isolation occurred in smaller numbers in the coelomic fluid. After repeated subculturing this organism becomes adapted to the presence of oxygen and is then capable of producing weak growth on aerobic agar slopes. These two organisms are considered to represent the natural microflora of the coelomic fluid of the earthworm, and other types of bacteria only occur as the result of infection.

Direct microscopical examinations of the gut contents/

contents of a number of species of earthworms failed to show any obvious differences between the gut microflora and the microflora ~~of~~ the soil in which the worms were living.

Soil free from earthworms and soil containing worms were incubated in the laboratory at 20°. Quantitative comparisons of the bacterial flora of the soils were made with the object of showing the total effect of the castings and excretions of the animal. It was found that after about 4 days the soil containing worms had a larger bacterial population than the worm-free sample. These increased numbers were maintained for the duration of the experiments. Evidence was also obtained which relates some, at least, of this increase in bacterial numbers to the presence of worm slime in the soil. Quantitative examinations of the bacterial flora in the anal region of the gut indicated that for an initial period of about ten days the bacterial count in this part of the alimentary tract was lower than that of either the worm-free soil or the soil in which the worms are living. After this period a rapid increase occurred in the bacterial count of the gut and the counts obtained greatly exceeded those of the soils.

Qualitative studies were made of cultures isolated from the worm-free soil, the soil containing worms and the final gut contents of these worms. These studies utilised a nutritional classification and an application of the "bacterial balance index" as used in soil microbiology. Additional characters of some of the dominant organisms were also obtained. From these investigations, evidence was obtained which related the increased numbers of bacteria in the soil containing worms to the multiplication of certain bacterial groups. No evidence/

evidence was secured that the earthworm gut contains a specific microflora. Apparently, however, the high bacterial counts obtained from the fresh worm casts, after worms had been in a soil for a number of days, were related to the occurrence of certain groups of soil bacteria which are capable of proliferating in the worm gut. Other groups of soil bacteria appear to pass through the gut unaffected while others appear to be destroyed to some extent.

It was established that bacterial spores pass through the worm gut unaltered. By obtaining the ratio between the spore count and the total bacterial count for a weighed sample of the soil in which worms are living and by making spore counts and total counts on small unweighed samples from the alimentary tract, it would be possible to obtain quantitative data for gut contents without requiring to weigh these samples.

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