AN INVESTIGATION ON INFLUENZA.

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I. Introduction.

During the past year we have continued our investigation on influenza. The work may be divided into two sections: that which was carried out on cases of influenza, and with micro-organisms isolated therefrom, and that which was made with the object of throwing light on the subject by more indirect experiments.

Throughout the investigation attempts have been made to open up some entirely new lines of research, as were suggested by fundamental biological laws and conceptions, or those which might exist in theory, rather than to extend in more minute detail along lines that have now been followed without much change for many years, and are usually considered more orthodox. This method of investigation has been carried out because while orthodox methods are easiest to follow and may perhaps lead to small improvements in our technique, and may be followed by some increase in our knowledge, nevertheless it appears to the writers that, speaking in general terms, the usual lines

of investigation have now carried our knowledge nearly as far as such methods are likely to lead. On the other hand, if any fundamental advance is to be made, some entirely new lines of investigation must be followed; and although research of this nature usually gives negative results, it is, however, more likely to lead to important advances in our knowledge.

II. The Bacteriology of Influenza.

The preliminary work consisted for the most part in isolating every possible micro-organism from a few selected and typical cases of influenza. It will not, however, be necessary to go into these in detail, it is sufficient to note that the various micro-organisms isolated were similar to those usually met with by other workers. The influenza bacillus often associated with pneumococci, streptococci, and micrococci were most frequently encountered, and no evidence was obtained that the rarer types isolated, such as yeasts, coliform and diphtheroid bacilli, etc., had any direct relation to the disease.

All experiments carried out to test for the presence of an ultra-microscopic virus gave negative results. Mice, rabbits, and guinea-pigs failed to develop any condition resembling influenza when they were inoculated with filtrates obtained from influenza material, various methods of inoculation being employed. The pneumococci and streptococci isolated showed the usual pathogenic action on mice and rabbits. Inoculations carried out with different strains of the influenza bacillus on mice, rabbits and guinea-pigs showed no marked pathogenic action for these animals. With small doses there was merely some rise of temperature accompanied by a certain amount of malaise which, however, quickly passed off. With large doses mice and, to a less extent, rabbits showed more marked illness, and in the case of rabbits the temperature sometimes became subnormal. The animals, however, were usually well again by the second or third day. No monkeys were tested.

Our standard medium throughout this work for the isolation and cultivation of the influenza bacillus was prepared as follows:

> Ordinary peptone-beef-broth was sterilised in a flask, and to this when cool was added 5 per cent. of rabbit's blood, withdrawn from the vein of the ear with a sterile syringe. The whole was then incubated at 37° C. for one hour, repeatedly shaking to prevent the formation of much clot. About 20 drops of this fluid were added to ordinary agar tubes which had been melted and cooled to about 50° C. The tubes were then sloped ready for use. This proved a very satisfactory medium, and was free from the possibility of containing contaminating micro-organisms.

III. Conditions influencing the Toxicity and Pathogenicity of the Influenza Bacillus.

A considerable number of experiments were carried out to investigate the conditions under which cultures of the influenza bacillus might produce more toxic substances or become more pathogenic. In the first place fresh tissues and organs of normal mice and rabbits were removed immediately after the

animals had been killed, and were placed into test tubes containing a little sterile normal saline. Cultures of the influenza bacillus on such media were inoculated into mice and rabbits after one or more days' incubation of the cultures at 37° C. The results were not very definite, but cultures of the bacillus, grown in fresh kidney and liver media, appeared to be somewhat more toxic for the animals than cultures made in other media. Further experiments were then carried out to test the effect on toxin production, etc. when the media, instead of containing normal liver or kidney, contained one or other of these organs which had been taken from a mouse or rabbit which had previously been inoculated with a large dose of the influenza bacillus. Cultures obtained on such media appeared to show a distinctly more pathogenic action for the animals than did the previous cultures. This was what was expected on theoretical grounds, but experiments of this nature are complicated and possess so many obvious pitfalls that one would not be justified in drawing any definite conclusions until all possible errors and flaws have been investigated. Again, the possibility of combinations in such experiments is obviously very great, and if further investigation should support the general results suggesting increased toxicity and pathogenicity, it would still be necessary to work out the best conditions favouring these ends. If really toxic substances could be produced by some such means it might be of value in the preparation of vaccines and possibly also of an immunising serum. In the experiments described the best results were obtained by using the organs of the same species of animal as that to be afterwards inoculated. Two of the possibilities that were being tested were, firstly, does a preliminary dose prepare the organs so that they may break down and set free the bacillary toxins? or secondly, does the toxic effect come from the tissues that have been changed by the bacilli?

IV. FILTER-PASSING SUBSTANCES WHICH BREAK DOWN AND DISSOLVE BACTERIA.

Since 1913 one of us (F.W.T.) has been investigating the biology of certain bacilli, notably members of the dysentery-typhoid-coli group, and the results are in course of publication[1]. This investigation may have some bearing on certain aspects of influenza, so the main results and theoretical considerations may be referred to here. Before dealing with this paper, however, it may be mentioned that one of us (F.W.T.) has shown[2] that "pure" cultures of bacteria may be associated with a filter-passing material which may entirely break down into granules the bacteria of the culture. This was most definitely demonstrated in cultures of micrococci which were isolated from vaccinia. Such cultures when plated out grew colonies, some of which could not be subcultured, but if kept they became glassy and transparent. The material of these colonies, when diluted about a million times and passed through a fine

[1] Researches on Dysentery, *Brit. Journ. of Exper. Pathol.* October, 1920.

[2] An investigation on the nature of Ultramicroscopic Viruses, *Lancet*, December 4th, 1915.

porcelain filter, was capable of infecting a fresh growth of the micrococcus, and this condition or disease can be transmitted to fresh "pure" cultures of the micro-organism for an indefinite number of generations, although the transparent material will not grow by itself on any known medium. The evidence obtained from the experiments supported the hypothesis that this material was not an ultra-microscopic virus growing on the living micro-organisms, but was a ferment secreted by the micro-organism for some purpose not altogether clear. A similar dissolving material was also found associated with the dysentery-typhoid-coli group of bacilli. Dr F. d'Herelle, at the Pasteur Institute, has confirmed these results in cultures of dysentery bacilli, although he believed that the dissolving material was an ultra-microscopic virus.

V. Special forms of Bacteria in Pure Cultures, and the possibility of the existence of Sexual Units.

To return to the investigation on dysentery, it is a well-known fact that various species of the dysentery-typhoid-coli group, when apparently in pure growth, produce forms that are considerably larger than the typical short bacilli, and variations in the size and shape of individual bacilli in a pure culture have often been described. Recently Major Hort and others have carried out a number of experiments with the object of throwing light on the significance of these different forms.

In certain experiments dealing with the dissolving material, one of us (F.W.T.) observed these long forms in greater number than usual and it was thought that they might in some way be associated with the dissolving material which had been found associated with this group. On the other hand, there was the obvious possibility that they might be distinct but symbiotic bacilli, or again they might represent a stage in the life history of the bacillus. It was also thought possible that they might be special forms with special work to do, like bees in a swarm, and might prove to be of importance in connection with the pathogenicity of the cultures and the production of immunity in the host, or they might represent nothing more than a mutation.

These large forms, after some difficulty, were isolated in apparently pure growth on a special litmus-maltose-agar medium. They were obtained from cultures of dysentery, typhoid and certain coliform bacilli. Further experiments with the Shiga type of dysentery bacillus showed that three fairly distinct types of the large or special forms could be isolated from the original "pure" cultures. Type A consists of long moderately thick threads which are sometimes twisted like a spirochaete. Type B shows long thick threads which are often markedly swollen either in the middle or at one end. The swollen portions may be split open setting free numerous granules, and a considerable number of these granules can be found free in most film preparations. Globular forms are also present, and these vary in size from that of a large coccus to that of a yeast cell. Type C consists of large bacilli which, however, are some-

what shorter than those of type B. The same swollen forms and free granules are present, and branching units are often observed, but in older cultures nearly all the bacilli are replaced by globular forms, often much larger than a coccus, and in these round forms the protoplasm is frequently collected as a semilunar mass round the circumference of the cell.

It may be noted that the large or special forms can always be isolated from normal cultures, and they appear to retain their characters after isolation and do not give rise to normal bacilli. Fermentation tests and agglutination reactions show but little variation from those of the original normal culture. It is clear from the experimental results that the special forms are not, as was at one time thought possible, distinct symbiotic bacilli, but are produced by the normal bacilli of the culture from which they were isolated. On the other hand the special forms do not appear to represent a degeneration, since they are best obtained by using good media and by keeping the cultures under good conditions.

It is of course possible that bacteria, like *Spirogyra* and other low forms of life, may multiply sexually as well as asexually, and the special forms already described may represent the sexual units. It is possible that a sexual form of multiplication is essential to the maintenance of a vigorous life after a certain number of asexual divisions. However, no experimental support to this theory could be obtained. Mixtures of the special forms, after repeated sub-culture in broth, failed to give colonies of normal bacilli, and it appears probable that if sexual forms exist they must be present in the normal culture, since it is from this that the special forms are produced. On the other hand the fact that the special forms cannot be induced to give rise to colonies of normal bacilli, is a strong argument against their being stages in a true life cycle. They may, of course, represent nothing more than mutations, but, if so, it is rather strange that although present in young normal cultures, yet they never appear to survive under ordinary conditions when a normal culture is grown on the usual laboratory media. It may be that a "dissolving material" plays a part in the disappearance of the special forms soon after they are produced.

VI. TOXICITY OF THE SPECIAL FORMS.

Another possible explanation is that the large forms are specialised units which are produced by the normal bacilli for some special purpose. They may assist in producing assimilable food or protective substances for the normal bacilli whose chief function is to reproduce. If such is the case, it might explain why the special forms do not survive in cultures made on laboratory media unless they are isolated from the standard small type. It might also explain certain points regarding successful infection, incubation period, the termination of infection, and the production of toxins, antitoxins, etc. In this connection it is interesting to note that in specimens from the human body of such material as faeces and urine, long forms of bacilli are often present in considerable number, and it does not seem at all impossible that these forms

may play an important rôle in producing the toxic effects in such conditions as infantile diarrhoea and vomiting and cystitis, etc.

Experiments were carried out to test the toxicity of the special forms for animals. The results appeared to show a greater toxicity of these forms than the normal cultures, but they were not sufficiently marked to draw any definite conclusions. It is, however, worthy of note that the special forms appear to lose their toxicity rapidly when they are isolated from the small forms, and it may be that their power of producing toxic substances is fully developed only in those units which are produced directly from the small bacilli. The fact that in a normal culture the special forms are always produced in this manner, and then die out, gives support to this suggestion. If effective toxin-producing units must be produced directly from the small bacilli, it is possible that the use of the dissolving material is to destroy them after they are no longer of any use to the bacterial community, or the dissolving up may be a means of setting free the toxic substances. In the case of the micrococcus which was isolated from vaccinia, the breaking down of the organisms may extend to the whole culture, and in a few hours there may be no units left, but the conditions of cultivation outside the body are very artificial, and it seems improbable that in the infected body this process would extend further than was beneficial to the life of the bacterial community.

If then, as seems possible, the function of some of the special forms is to produce toxins or other substances for the protection of the bacterium, then any conditions which might favour the growth of these forms would assist the normal bacilli to survive and multiply when they gained entrance to the human body. In this connection we carried out a series of experiments to test for the production of special forms in media containing a high percentage of sodium chloride, the idea being that the climatic conditions that favour an outbreak of dysentery would also produce a higher concentration of salt in their environment. In these experiments special forms were produced as in the previous ones, but in addition a few extremely large branching threads were observed. They looked more like the mycelium of a mould than anything else and contained a granular faintly staining protoplasm. The experiments were repeated several times with the same results, but attempts to isolate these forms failed, and their significance could not therefore be determined. It may be noted, however, that great care was taken to eliminate the possibility of contaminations.

In view of the results obtained with dysentery and allied micro-organisms it was considered advisable to carry out somewhat similar experiments with the influenza bacillus, where units of considerable size are occasionally to be seen in cultures. Most media, as we know, are unsuitable for the growth of this micro-organism, so that possible changes in its constitution are strictly limited. On several occasions, however, we were able to isolate colonies that showed a considerable number of large forms, some of which were distinctly swollen in places. These bacilli, after sub-culturing several times on to our

standard influenza medium reverted back to the small forms. This, of course, may have been due to their never having been properly isolated from the small forms, but, be that as it may, it prevented further experiments with the cultures.

We then pursued our investigation in another direction. It would seem that infections of the kidney and possibly also of the liver play an important part in the toxic manifestations seen in an infected animal, and, if it be the function of some special form to produce toxic substances, we thought that these forms might be found in the kidney or liver. Cultures were accordingly made from these organs in a few post-mortem cases of influenza, and in one instance, a colony of an extremely large influenza bacillus was obtained. This strain, however, after being sub-cultured a number of times, reverted back to the small form as in the previous experiments, and this took place before we had an opportunity of testing its toxic action on animals. So far then we have been unable to obtain special large forms of the influenza bacillus showing the same degree of stability as in the case of the dysentery bacilli, but the experiments are being continued.

VII. EXPERIMENTS ON THE CULTIVATION OF NON-PATHOGENIC ULTRA-MICROSCOPIC VIRUSES UNDER THE INFLUENCE OF VARIOUS GASES.

The section of this investigation carried out by more indirect methods was chiefly confined to work on the general character and behaviour of ultra-microscopic viruses. It is obvious that if any light can be thrown on this group in general it will be of assistance in deciding more definitely the ultra-microscopic theory of influenza outbreaks, and in dealing with this group there are many reasons for believing that it can be best attacked by investigating certain other conditions. In the first place there is no definite evidence that such a virus exists in the case of influenza, and even if it does exist it would probably be more or less specific for man, and would therefore be difficult to demonstrate by animal inoculations. Certain diseases of animals, such possibly as vaccinia, should therefore give a greater prospect of success.

At the same time it is well known in the case of ordinary bacteria that every species that has a pathogenic variety has one or more non-pathogenic or wild varieties, and it may reasonably be supposed that the same general rule holds good with the ultra-microscopic group, various species of which are known to infect both animals and plants. Varieties of bacteria which are accustomed to grow outside the body and lead a non-pathogenic existence are usually much more easily cultivated than the strictly pathogenic varieties, and again it is not unreasonable to assume that this would also be the case with the ultra-microscopic group. The non-pathogenic series, which probably exists in such situations as water, soil, faeces, etc., would therefore appear to offer the greatest scope for successful cultivation, although it must be admitted that most if not all of such varieties would probably be very difficult to demonstrate by animal inoculations, unless any means could be adopted so to increase

their virulence by passage or otherwise that pathological lesions could be produced in animals.

Although it has often been claimed that certain pathogenic ultra-microscopic viruses undergo some multiplication on various media, more particularly on that of Noguchi, yet no definite growths, visible to the naked eye, have ever with certainty been obtained with any member of this group. This fact, in view of the extensive distribution in animals and plants of these viruses, suggests most strongly that the failure to obtain growths is not due to any delicacy in their requirements of food supply, but on the other hand is probably a result of some fundamental difference in their physiology. On the other hand, it must be remembered that if the living organic world has been slowly built up in accordance with the theories of evolution, then a bacterium and an amoeba must be highly developed organisms in comparison with much more primitive forms which once existed and probably still exist in nature. It may be that an ultra-microscopic virus belongs somewhere in this vast field of life which is even less organised than the bacterium or amoeba.

The work we have carried out on this group has been done under the influence of the above theoretical considerations. The first experiments, dealing with the possibility of an "essential substance" being the missing requirement for successful cultivation as was shown by one of us to be the case with Johne's bacillus (*Proc. Roy. Soc.* 1910), were carried out for the Local Government Board and were published in the *Lancet*, Dec. 4th, 1915. As the experiments were all negative they need not be referred to again here, and for the time being it was not considered profitable in connection with the present research to pursue this line much further, but rather to probe in several new directions. In the few experiments we carried out, no results of any importance were obtained.

Bacteria may be divided into aerobes and anaerobes, but many species are known to grow either with or without oxygen, while others may be best cultivated under a partial anaerobic condition. Pure oxygen and carbon dioxide are usually very detrimental to growth. Most bacteria, however, obtain their energy through the oxidation of some carbon compound.

In the case of some of the nitrifying bacteria, carbon dioxide is utilised as a source of carbon, and the energy required to split up the molecule of this gas is obtained by the oxidation of nitrogen.

We know also that other gases are essential to the cultivation of some species. This is notably so in the case of the sulphur bacteria which inhabit certain strata of water from which they can obtain oxygen from above and sulphuretted hydrogen from below. In this instance a certain tension of oxygen is required, and according to the vigour with which the sulphuretted hydrogen is given off from the organic material or sulphides in the mud, so the plane of bacterial growth rises or falls in the water. The iron bacteria are somewhat similar in their physiology, only bacteria of this group obtain their energy by the oxidation of soluble compounds of iron.

It was in view of these and other instances where special gases, etc. are required by bacteria that we started an investigation along similar lines with the ultra-microscopic viruses, bearing in mind that the living tissues of animals and plants at times give suitable conditions for their growth. A considerable number of experiments were carried out in atmospheres consisting of various mixtures of air, carbon dioxide, oxygen, nitrogen, ammonia, etc., but the results up to the present time have proved negative.

Some additional experiments were made with sulphuretted hydrogen and with media containing sulphur compounds, and as we obtained with these what at first appeared to be growths of more than one ultra-microscopic virus, it may be advisable to describe these experiments in some detail.

In the first experiments our standard blood agar medium was used as a basis, the only modification being that 0·05 per cent. of sodium sulphide was added before the addition of the fluid containing blood. On tubes of this medium inoculations were made from the filtrates of emulsions of garden soil obtained by passing the emulsion through an English Berkefeld filter. The tubes were incubated at 37° C. in an atmosphere of air expired from the human lungs. On the first cultures a few minute "colonies" appeared in two days, and these when sub-cultured on to fresh tubes of the same medium grew as a number of very minute colonies along the needle track. Uninoculated tubes which were streaked down with a sterile platinum loop showed no evidence of colonies. Further experiments showed that an emulsion of these supposed colonies when passed through a porcelain filter grew similar colonies when inoculated on to the same medium, although very few were present in the primary cultures. The power of producing colonies, however, appeared to be destroyed when the emulsion was heated to 80° C., but not so when heated to 70° C. The "colonies" grew best at 40° C., and the growth at room temperature was very slow, while at 60° C. no evidence of growth was obtained. Films were made from the colonies by every means considered likely to give stained preparations, carbol fuchsin, giemsa, and Zetnoff's flagella stain being amongst those tried. In every case, however, we were able to detect nothing but extremely minute granules. Hanging drop preparations also gave negative results. In the meantime somewhat similar "colonies" were grown from filtrates obtained from other materials, such as vaccinia, dung, and rotten apples. In view of the importance of deciding definitely whether any formed bodies could be demonstrated by the ultra-microscope, dark ground illumination, or by some photographic process, a number of "cultures" were handed to Mr Barnard who was working on this branch for the Medical Research Council.

So far the experiments had given satisfactory results, and it was hoped that we were dealing with a group of filter-passing viruses which were thermophilic, and non-pathogenic, and which grew in an atmosphere containing an excess of carbon dioxide. Further investigation, however, soon showed that an atmosphere of expired air was not necessary for the formation of "colonies," but that this took place equally well under anaerobic and aerobic conditions.

Later we found that it was not necessary to add fresh blood to the medium, or in fact blood in any form. On the other hand, when the sodium sulphide was replaced by sodium sulphite in the medium no growth took place, and this was also the case when the percentage of sodium sulphide was increased to 0·5 per cent. In the latter case, however, it was thought possible that the alkalinity of the salt might be a detrimental factor in the experiment. This proved to be the case, for when the medium was neutralised after adding the sodium sulphide, it was found that 0·5 per cent. gave rather better results than 0·05 per cent.

A number of animals were inoculated with the supposed growth, but the results were entirely negative.

During these experiments we constantly put up controls which consisted either of uninoculated tubes or those which had been streaked down with a sterile platinum loop, and in all the first experiments the controls failed to give any evidence of growth, no colonies appearing on any of the tubes. In subsequent experiments, however, we found that when the controls that had been streaked down with the sterile platinum loop were left in the incubator for several days and then sub-cultured on to fresh tubes, a few minute colonies appeared along the needle track. These when again sub-cultured produced an abundance of minute colonies. Similar results with the control tubes were obtained on several occasions, and while the possibility of a contaminating ultra-microscopic virus could not be definitely excluded, nevertheless the evidence was against this being the explanation. The air of course may be loaded with non-pathogenic viruses of this nature, and if such were the case we do not know how far they could be excluded from a culture tube when the ordinary bacteriological technique is employed. On the other hand a more probable explanation of the results appeared to us to be that the colonies were nothing more than minute grains of sulphur which for some reason had started to be deposited on the surface of the medium. In view of the nature of the sulphur compound that was being used this possibility had been kept in mind throughout the experiments, but the fact that the medium was clear, that tubes streaked with a sterile platinum loop in the first experiments showed no evidence of colonies, and that in the tubes inoculated in sub-culture from the various filtrates the colonies remained confined to the needle track, in conjunction with the other experiments already described led us at first to think that even if sulphur formed part of the colonies, nevertheless this might have been deposited by the growth of some virus, in a manner similar to that which occurs with the sulphur bacteria.

We know of course in the case of solutions of various salts that some minute crystal or particle of dust is required to start the process of crystallisation, and a phenomenon of this nature is probably the correct explanation of the results described in these experiments. In order to test this point we inoculated some sterile tubes of our medium with some minute particles of sterile sulphur, and in sub-culture from these tubes we obtained minute colonies similar to

those obtained from filtrates of soil, etc., but in the primary cultures there were only a very few, and it was only in the sub-cultures that they were easily detected.

We found also that it was not necessary to add sodium sulphide to the medium, but that the "colonies" would appear on ordinary agar if sub-cultures were made on this medium and the tubes placed under a bell-jar which also covered a dish containing a solution of sodium sulphide to which had been added a small quantity of dilute hydrochloric acid.

VIII. THE POSSIBLE INFLUENCE OF LIGHT RAYS AND ELECTRICAL CHANGES.

Light is known to have an important influence on the growth of bacteria, and while most grow best in the dark, there are certain species such as *Bacterium photometricum* that are favourably influenced by certain light rays. This is sometimes also true with fructification, and may be demonstrated with the species *Philobolus microsporus*. Unless exposed to the light for a few hours the mycelium of this fungus is said to remain barren. The blue-violet and the yellow-red rays of the spectrum usually act quite differently on different species of bacteria. In view of the known action of light and other rays on the physiology of bacteria we have started some experiments along similar lines with the ultra-microscopic group, but so far without obtaining any positive results.

A few experiments have been carried out on the influence of certain electrical changes, but these also have given entirely negative results. Attempts were also made to cultivate an ultra-microscopic virus on certain media under the influence of the growth of bacteria or amoebae, but no evidence of successful cultivation was obtained.

IX. THE CULTIVATION OF THE INFLUENZA BACILLUS IN SYMBIOSIS WITH AMOEBAE ON BLOOD AGAR, ETC.

In other experiments attempts were made to cultivate the influenza bacillus with certain amoebae. It is well known that some non-pathogenic amoebae may be cultivated on an agar medium that is poor in nutriment, and the medium described by Musgrave and Clegg is often used for this purpose. The amoebae grow only with bacteria and frequently with those which belong to the coliform group. If such a culture of an amoeba is transplanted to ordinary peptone-salt-broth agar, the amoebae die out, and the explanation sometimes given for this is that an amoeba will not tolerate a highly nutritious medium. On the other hand the pathogenic amoebae, classified in the sub-group of *Entamoebae*, have never been grown for certain with bacteria, either on highly nutritious or on poorly nutritious media.

After some consideration we were led to favour the view that the failure to grow amoebae on highly nutritious media was probably due entirely to toxic

substances produced by excessive bacterial growth, and, further, that the reason that a coliform type of bacillus is frequently associated with the amoebae might be due partly to the fact that they outgrow the more delicate types, and partly because some of the pathogenic bacteria and other delicate types fail to grow in a medium that is poor in nutriment. It must also be remembered that bacteria grow in advance of amoebae in cultures, so that the cultivation of amoebae with delicate pathogenic bacteria on rich media could scarcely be expected unless special precautions were taken.

Experiments were carried out along the lines indicated above. We had in the laboratory an interesting amoeba that one of us (F.W.T.) had isolated from the faeces of a patient in Salonika. It was isolated on a special medium, but unfortunately we cannot give the exact composition as the Salonika notes have been mislaid. However, this is possibly of no importance as the amoeba has now been growing for several years on a plain water agar medium containing 1 per cent. of ordinary broth.

The elimination of the vigorous growing symbiotic bacteria at first presented some difficulty owing to the presence of a very motile bacillus. The following method, however, gave successful results. The associated bacteria were plated out on ordinary agar and two types isolated, one of which was very motile and the other less so. The less motile variety was then cultivated on Musgrave's medium, and a two days old culture was inoculated on the lower part of the streak with the amoeba-bacterial mixture, and the tube again incubated at 37° C. The upper part of the tube, having already a growth of bacterium upon it, proved less suitable for the more motile bacillus to grow up, and more suitable for the spreading growth of the amoeba, with the result that the amoebae rapidly grew away from the mixed bacterial growth below to the pure bacillary growth above, whence it was easily obtained in sub-culture with the less motile bacillus only. By using the same method it was found possible to eliminate the single type of bacillus from the culture, and substitute the influenza bacillus, providing a good blood agar medium was used, such as that described at the beginning of this paper. A two days old culture of the influenza bacillus on this medium was touched on the lower end of the streak with the amoeba growing with the single bacillus. The amoeba rapidly grew up the streak of influenza, and sub-cultures taken from the upper part showed amoebae and influenza bacilli only. From this culture it was found easy to eliminate the influenza bacillus and substitute such bacteria as typhoid, dysentery and spirilla if the same method was adopted, and it was particularly easy if ordinary agar or Dorset's egg medium was used, as on these media the influenza bacillus will not grow. The exact nature of the amoeba has not yet been settled, but it does not show the characters of *Entamoeba histolytica* or *E. coli*. It is interesting to note that the influenza bacillus appears to benefit by the presence of the amoeba, and it remains alive for a longer period than when grown without the amoebae. This then may be considered a case of symbiosis. Experiments on the production of toxin and various

immunity tests will now be carried out with such mixed cultures, and the research in other ways continued in the manner indicated in this paper.

X. THE CULTIVATION OF THE INFLUENZA BACILLUS IN SYMBIOSIS WITH CERTAIN BACTERIA FROM GRASS AND SOIL ON MEDIA CONTAINING NO BLOOD.

Many writers have pointed out that haemoglobin is essential for the cultivation of *B. influenzae*, but in some quite recent experiments I have found that it will grow on ordinary agar and on egg media without blood if it is grown with certain spirilla obtained from grass and certain extremely minute bacteria obtained from soil which pass a Berkefeld filter with some difficulty. The growth of these micro-organisms is very delicate, except when cultivated with the influenza bacillus, the combined growth being vigorous, dense, heaped up, and becoming brownish in colour. In such mixed cultures on Dorset's egg medium the influenza bacillus will live for five or six months, after which period it can easily be isolated on blood agar. The influenza bacillus has been isolated from mixtures with the assistance of these symbiotic bacteria. These results have been confirmed by repeated experiments with different strains of influenza bacillus. The results show how it may be possible for the influenza bacillus to survive outside the human body, and indicate other possible lines of research. Fuller details will be published later (F.W.T.).

XI. THE CULTIVATION OF THE INFLUENZA BACILLUS IN AN ATMOSPHERE OF PURE OXYGEN AND PURE CARBON DIOXIDE UNDER DIMINISHED PRESSURE.

Further recent experiments on the growth of the influenza bacillus under the influence of certain gases have shown that this micro-organism will grow on blood agar in an atmosphere of pure oxygen or pure carbon dioxide if the pressure is reduced so that there is an external atmospheric pressure over the internal of 450 mm. of mercury. Many other bacteria will also grow in such gases under reduced pressure.

SUMMARY.

(1) Our experiments support the view that influenza is caused by *B. influenzae*, and that pneumococci and certain streptococci are the most important agents of secondary infections. No new type of bacterium was discovered, and no evidence was obtained of the presence of an ultra-microscopic virus.

(2) *B. influenzae* appears more toxic for rabbits and mice when grown on fresh liver or kidney media than when grown on blood agar or in blood broth, and this is especially so when the kidney or liver is obtained from an animal of the same species that has previously been inoculated with a culture of the influenza bacillus.

(3) Three fairly distinct special forms have been isolated from cultures of *B. dysenteriae*. They are probably not sexual units or stages in a true life cycle. Special large forms have also been obtained from cultures of *B. influenzae*, but these reverted back to the normal small type after several sub-cultures.

(4) A filter-passing material has been found associated with certain micrococci from vaccinia and in pure cultures of members of the dysentery-typhoid-coli group of bacteria. This material breaks down and dissolves the bacteria of the cultures, and the "infection" can be carried to fresh normal cultures. The evidence is against its being a living ultra-microscopic virus infecting the bacteria, but it may have some connection with the special forms. No definite evidence of a similar dissolving material has been found associated with cultures of the influenza bacillus.

(5) There is some indication that one or more of the forms isolated from pure cultures of dysentery bacilli may be special toxin-producing units, also that this function is at its maximum when the bacilli are first produced by the normal small forms or by sexual units of a normal culture. This may possibly be the case also with special forms that have been found in cultures of the influenza bacillus.

(6) It is believed that non-pathogenic wild varieties of ultra-microscopic viruses must exist in nature, and that they should present less difficulty in cultivation than the pathogenic varieties. Cultivations were made from filtrates of soil, faeces and water on various special media, and the tubes incubated in various gases. The chief gases tested either alone or mixed were oxygen, nitrogen, carbon dioxide and sulphuretted hydrogen. All the results were negative, but some interesting deposit colonies were obtained on media containing a small quantity of sodium sulphide.

(7) The influenza bacillus grows in symbiosis with amoebae on blood agar, and in such cultures the bacillus lives considerably longer.

(8) The influenza bacillus grows in symbiosis with a small spirillum that was isolated from a grass emulsion, and in symbiosis with an extremely minute and delicate bacterium isolated from garden soil; after passing the emulsions through a Berkefeld filter. With these delicate bacteria the influenza bacillus will grow on media containing no blood, and if an egg medium is used the mixed growth is dense, heaped up and brownish in colour. In such cultures the influenza bacillus may live for months.

(9) The influenza and other bacilli will grow in an atmosphere of pure oxygen or pure carbon dioxide under diminished pressure.