# FURTHER INVESTIGATIONS ON THE NATURE OF ULTRA-MICROSCOPIC VIRUSES AND THEIR CULTIVATION

By FREDERICK WILLIAM TWORT, F.R.S.

*From the laboratories of the Brown Institution, University of London*

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## I. Introduction

Experiments on the cultivation of viruses are frequently conducted along orthodox bacteriological lines, and still it is sometimes assumed that these filter-passing agents are in reality extremely minute bacteria, which are likely to require the same fundamental conditions for their growth as are necessary for bacteria. The writer's first paper on ultra-microscopic viruses was published in 1915. The research was based upon theoretical considerations which incorporated several concrete conceptions that differed essentially from generally accepted ideas. It was not at the time possible to give a complete review of the theoretical position, but, in one section, experiments were carried out to test the possibility of growing the vaccinia virus on the living secondary micro-organisms associated with vaccinia. From these experiments followed the discovery of the bacteriolytic agents to be found in association with micrococci, with members of the coliform group, etc. At the same time, the fundamental characters of the agents were determined, and consideration was given to certain possible explanations of the phenomenon, to its bearing on cancer and to the preparation of vaccines. In the first and in several subsequent papers (Twort, 1915, 1922, 1923, 1930, 1931), the general nature of viruses was also discussed.

For a correct appreciation of the experiments to be described here, it will be well to give in advance the basic data and theories on which they rest, but to omit details until describing special experiments where the motives underlying their inclusion may appear to require some further explanation.

## II. AN ANALYSIS OF THE THEORIES DETERMINING THE POSITION OF VIRUSES IN NATURE

It is well known that all bacteria at times develop morphological changes, the nature and degree of which vary greatly in different varieties, and with the environment in which they are living. Even in the case of the dysentery bacilli, in which the variations are usually not marked, the writer succeeded in isolating three distinct forms, associated with granules, from a single "pure" colony (Twort, 1920). Instances have also been recorded in which a bacterium may develop exceptionally small forms, and pass a filter, a classic example being the motile "rovers" of certain nitrogen-fixing bacteria. The writer also once isolated a spirochaete from a mouse (Twort, 1921), a minute spirillum from grass and a minute bacterium from soil (Twort and Twort, 1921) and a tiny anaerobe from beef, each of which could be grown in a form that would pass a Berkefeld filter, although not with ease. In the writer's experience, one of the easiest methods of obtaining the passage of certain bacteria through a porcelain filter is to use rain water containing dead vegetation, which has been placed for a few days at about 25° C. These and other recorded examples represent instances in which the original micro-organism is always visible in the culture before filtration, although it does not necessarily follow that it is a visible stage which passes the filter. Whilst it must be recognised that still smaller varieties of bacteria may exist, it would nevertheless appear unlikely that this form of life is the causative agent of any true virus disease, such as anterior polyiomyelitis of man, distemper of dogs, or the mosaic diseases of plants. Although a small bacterium has been cultivated from a few conditions which are frequently included with the virus diseases, the writer does not agree that these bacteria should be placed in the true virus group, and they are not included when referring to that group in this paper. Some recent experiments on the filter passage of certain bacteria will be described later.

Before leaving the theme under review, it would appear profitable to consider as a possibility that a virus may represent some special reproductive stage in the life of a bacterium, comparable in nature to forms to be found in various cryptograms. The multiplication of most bacteria takes place by simple fission and the formation of two daughter cells. In other instances spore formation occurs, but as, usually, only one spore is produced, this is not a means by which bacteria increase in number. In the case of certain mould fungi, such as Mucors, many spores may be produced, and instances have been recorded of the occasional development of sexual cells. Moreover, the asexual spores of a fern pass through the prothallium stage before the production of

sexual forms, while many Algae produce swarm spores which are themselves capable of further division. Under these circumstances it would be unwise to overlook the possibility that a bacterium, if placed under the influence of a suitable stimulus, might produce many minute spores, biologically analogous to swarm spores, which might elude detection if they were sufficiently minute and were non-pathogenic. Such spores, if they existed, might be capable of multiplication by division while in this stage, and might require some further special stimulus to induce the reformation of the bacterial state. So far no direct evidence has been brought forward to support such a possibility, but it is one that has had to be considered, particularly when analysing the results of experiments and considering their significance.

The evidence available does not on the whole appear to support either the view that true viruses are extremely minute bacteria, or the view that they represent some normal sexual or other stage in the life of a bacterium, but rather indicates that they are representatives of a form of life which differs essentially from that of a bacterium. It has therefore been necessary to consider also other more probable explanations before embarking on cultivation experiments.

If life on this earth has been built up in accordance with the theory of evolution, then many forms, which are much more primitive than a bacterium, should have preceded the formation of what is known as a cell. If this be so, then those immediately preceding the evolution of an organised cell may be referred to appropriately as precellular units. It is suggested that a cell consists of an aggregate of *specialised* units. The cell, in the process of evolution, having been built up from a precellular unit which, instead of dividing so that its inherent characters passed on equally to daughter units to produce similar new independent forms, divided unevenly—or differentiated. As a result, the new units would have been impelled to remain in association to enable their respective specialised functions to act in unison for the purposes of carrying out the complete processes of life: the evolutionary laws being the same as those which brought about the development of the multicellular organism from the single cell form. In other words, it is suggested that there exists a precellular unit of life which bears the same relation to the collection of differentiated units forming a cell as a primitive cell does to the collection of differentiated cells forming an animal, and that there exist also a number of stages of development from the single unit to forms consisting of two or more differentiated units. It is further suggested that a virus represents a pathogenic variety of some such precellular form, some viruses being more highly developed than others. It would appear probable moreover that, at a very early stage in its development, primitive life produced some thickening or concentration of its substance to protect and to define the limits of the individual.

It would not follow that all viruses normally possess an independent existence apart from the cell they infect. It is quite possible that some arise from specialised precellular units—or biological molecules—of the more highly

organised cell, which have reverted to the less specialised form of an earlier evolutionary state, and incorporate in each unit sufficient of the essential functions of life to enable them to carry on an existence that is more or less independent of the cell from which they have arisen. Yet, by lacking some essential attribute for complete independence, these units might still require the association of a living cell to enable them to grow and multiply. It is suggested as probable that the transmissible bacteriolytic agents belong either to a normally independent precellular form or to those units of a cell that have reverted to that state; and that some viruses belong to one type and some to the other.

From a broad biological viewpoint, it is not unreasonable to conclude that a precellular unit, arising from a cell, might, under special circumstances, be capable of living and multiplying apart from the complete cell; for even specialised cells of a multicellular animal can be grown artificially apart from the remaining cells of the organism.

Indeed, it may be that living organisms not only progress in the direction of more highly specialised forms, but, in other instances, are capable of a process of evolution towards more primitive types, if nature offers here a gap with fuller opportunities for their multiplication and survival.

If some viruses arise from the cell host, it is possible that a type may exist which acts and increases in quantity by stimulating the cell host to produce more of the same "virus", although the units forming the virus may themselves possess no capacity for direct multiplication. It would obviously be impossible to cultivate a type of this kind on any artificial culture medium that was free from the living host cells, and it has previously been suggested (Twort, 1915) that the bacteriolytic agents may belong to this type.

The occasional appearance of a bacteriolytic agent in a bacterial culture that is presumed to be free from the agent may be explained on the assumption that our ordinary bacteriological technique of plating out is insufficient to render a culture free from such agents; but it may with equal force be argued that it has arisen afresh from the bacterial cell. Arguments can be brought forward to support each of a number of different views, but, although the problem is one of considerable importance, it is one also that is difficult to decide on the basis of definite proof. Attention may be drawn here to some experiments (to be described later) which show that, if the medium used and the associated conditions of cultivation are suitable, it is comparatively easy to induce the manifestations of a lytic agent in a "pure" bacterial culture. If viruses can originate from a cell, it would at once explain this "spontaneous" appearance of a bacteriolytic agent, as well as the associated phenomena of variations in the characters of different agents, and the existence of resistant strains of bacteria.

The conception of a cellular origin of viruses is not a new one, but is one of the possibilities put forward by the writer ever since his discovery of the bacteriolytic agents (bacteriophage) in 1915. It was explained in some detail

in two theoretical papers, published in 1922 and 1923, and it is interesting to note that it is a solution which appears to be receiving more serious consideration as additional facts are brought to light.

Recently Wollman (1920, 1925, 1934, 1935) has put forward an hereditary bacterial mutation theory to explain the phenomena associated with bacteriophages and viruses, the basis of the theory being that these agents originate from a bacterial or other cell. The author draws attention to the experiments of den Dooren de Jong (1931) with sporing bacteria in which he found that the lysogenic function was conserved in cultures heated at 85 and 100° C. Wollman at first concluded that in some ways these experiments constitute a condemnation of the parasitic theory. The experiments, however, in reality are not inconsistent with this theory, for, as Wollman points out in his later papers, if a bacterium in any form possesses the means of protecting its cellular contents from the effects of heat, then the same would also probably protect any intracellular bacteriolytic agent or virus.

There is apparently one rather serious objection to the view that viruses *always* arise from cells. A virus, such as vaccinia, can be transmitted to man and to a number of entirely different types of animals, whereas it is recognised that the cells of one animal cannot be grafted on to the body of another type. It may accordingly be concluded that it is improbable that vaccinia has arisen from any precellular unit of a bovine cell. On the other hand, some viruses *are* very specific, and moreover, it is clear that any reversion such as that visualised would be in the direction of a more primitive common ancestor, in which case the objection would cease to carry much weight, providing the unit had undergone a sufficient degree of reversion towards the independent state to cause it to function as a common ancestor.

In the past, the writer has carried out many experiments based on the conception of the cellular origin of viruses, using unicellular animals in association with the bacteriolytic agents and viruses, but these gave no evidence that an animal such as an amoeba would satisfy the growth requirements of a bacteriolytic agent. It should be noted, however, that a bacterium is unlikely to represent a natural stage in the evolution of very primitive attributes of life towards higher living beings. More probably it represents a side branch that has developed in a special direction to a distorted end. The environment generally most suitable for its survival in nature affords evidence in support of this view. If this be the case, then a bacteriolytic agent—if arising from the bacterial cell—might find an amoeba unsuitable; in addition to which an amoeba may well possess sufficient inherited immunity to resist a bacteriolytic agent.

Experiments on the bacteriolytic agents have led to considerable controversy as to whether or no they should be classified as viruses. These differences of opinion would appear to be due largely to differences in the individuals' conception of the nature of the virus group. When the biological position of this group has been made clearer, it will probably be found necessary to make a

number of subdivisions. Then the subgroup occupied by a bacteriolytic agent will be more precisely defined, and the type name will be settled by the particular nomenclature selected.

In the writer's original description of the lytic agents, attention was drawn to there being a possible connection between these agents and the problems underlying the formation of cancers. In the case of the Rous "sarcoma", it is known that a filterable agent exists, although it cannot be said to be proved that a similar agent is associated with human cancers. Most workers are agreed that a cell may become cancerous under the stimulus of tars and certain mineral oils; moreover, several pure cancer-producing compounds have been synthesised, such as chrysene, 1, 2, 5, 6, dibenzanthracene, etc. It is not possible here to enter into a critical analysis of the many recent researches on cancer, but it would seem to be generally agreed that the results as a whole point to some special property of the cell as being one of the essential factors concerned. If, however, one considers both the virus experiments and the tar experiments on cancer, in relation to experiments with the lytic agents on the bacterial cell, then the pathological attribute of the cancer cell may be portrayed as a virus, produced, under the influence of some detrimental stimulus, from a precellular unit of the cell. Experiments indicate, however, that in most cases the pathological unit has not reverted sufficiently in the direction of its more primitive independent ancestor to be capable of living without the assistance of the remaining precellular units of the cell. Thus it remains intracellular, and so differs from the Rous "sarcoma" type which is filterable. On the other hand, it cannot be overlooked that it may be that the whole composite cell has undergone some reversion or other change, although possibly this is only another way of expressing the view that a number of associated units may be responsible for the loss of control in the cell.

## III. The probable conditions necessary for the growth of precellular forms of life

If the writer's theories correctly represent the fundamental position of viruses in the biological world, then in most cases the best conditions for their growth are likely to differ widely from those usually employed in researches on this group. From a brief consideration of the subject, it will be clear that the earliest manifestations of life must have obtained the necessary energy for their vital chemical reactions directly, or indirectly, from the physical forces available at the site of their existence; for these being the first stages in life, no organic compounds, arising from other types of life, would exist to supply the energy for their metabolism. It is true that, in the process of further development, some primitive forms, before reaching the evolutionary state of a complete cell, might in certain cases acquire the faculty of utilising the organic substances synthesised by other varieties. Moreover, those varieties that have

become pathogenic to animals and plants must have acquired the power of making some use of the tissues of their host. Even so, it would appear unlikely that all types would be able to dispense entirely, either with such physical forces as reach this earth from the sun and other celestial bodies, or possibly those arising from the earth itself. The utilisation of solar rays by micro-organisms is well illustrated by the classic example of the purple bacteria, which are able to absorb waves, chiefly in the near infra-red region. If then the virus group belongs to some primitive precellular form of life that, in most instances, cannot dispense with these natural physical forces, it follows that experiments on cultivation, particularly when seeking for natural wild varieties, should not be undertaken in the usual enclosed dark incubator, the interior of which is nearly surrounded by a double-jacketed copper tank.

The first manifestations of life would almost certainly include some form of synthesis and of oxidation. The food supply would consist of inorganic substances, comparable in simplicity with those which satisfy the needs of nitrifying bacteria. From our knowledge of photo-synthesis and allied photo reactions, it would appear probable that certain parts of the solar spectrum would be necessary for each function. In the case of green plants, the periods of day and night supply a suitable cycle for their metabolic processes, and these might also suffice for the most primitive forms of life. It would appear probable, however, that a more rapid change of conditions would be required for these minute primitive forms. Rapid microscopical changes certainly occur on the earth's surface; due primarily to its rotation, and to the movement of mineral particles brought about by tides, flowing water, rain, and winds. A rough rocky surface, containing various crystalline substances, on which the sun shines at a continually varying angle, would give rise to a changing type of wave energy that reaches the minute cracks and crevices. Such changes in the energy would be regulated by the processes of reflection, refraction, transmission, absorption and emission; while these processes would be further influenced by the presence of water cast into ripples by air currents. It is submitted as probable that it was in such situations that life started; there being not a blade of grass nor a fragment of moss to mask the controlling action of the mineral surfaces and nothing but moist rocky detritus in the crevices to act as foods. Moreover, the rocks being fixed, primitive life might lie and develop in a crevice under the stimulus of a *frequent repetition* of the essential changes, with little chance of their being washed away to an unsuitable position. In no way would the sea appear to offer such a satisfactory environment for the development of the type of life here portrayed, even if it presented a more suitable environment for the later evolution of early cellular forms.

There is another important aspect of the problem that must be taken into consideration when arranging experiments on the most primitive forms of life. If the position in nature, and if the requirements, of these forms have been correctly visualised, it would appear reasonable to conclude that, when

the physical conditions became favourable, life started and developed in many places about the same time. The first stages may well be depicted as some reversible chemical reaction, characterised by a photo-stationary state of equilibrium that varies under the influence of changing physical stimuli. This type of reaction is common in photo-chemical experiments, but from the nature of the elements or compounds taking part in many of these reactions, they clearly could not develop to a state or stage which would constitute the beginning of that type of life from which vegetables and animals have arisen. If one or more of these early forms, each starting in a number of similar situations, gradually evolved into the complex life we know, it is equally possible that, about the same time, other forms arose out of groups of entirely different chemical reactions. The differences, in fact, being so fundamental in nature that they would be impelled to develop along entirely different chemical lines, and these might be such that it would be chemically or physically impossible for development ever to reach a state comparable with that of the unicellular organism met with in nature. Such forms, indeed, would belong to another biological world. Yet it might not be impossible for some varieties to acquire the power of attacking certain compounds built up by the animal and vegetable kingdoms. They might indeed act as a kind of virus, and this possibility could not be overlooked in the arrangement of experiments in this general scheme of research.

## IV. DESCRIPTION OF SPECIAL EQUIPMENT USED IN THE RESEARCH

It was on the basis of the broad conceptions outlined above that the experimental methods employed in this research were evolved. It will not be necessary at this stage to enumerate all the contingent conclusions arrived at, as, where necessary, these will be considered when describing the relative experiments.

Owing to a lack of facilities it was not possible, until recently, to pursue the main investigation along the lines indicated by theory; but during the past few years the laboratories at the Institution have been re-equipped for that purpose, and this has enabled the research to be continued in accordance with an extended scheme mapped out.

One of the essential features of the research was that many of the experiments were to be conducted under the influence of different physical conditions, more especially as regards the effect of waves of different frequencies. Owing to the extent of the field to be explored, a considerable quantity of special apparatus was required, and circumstances made it necessary to construct the greater part of the more simple equipment in the laboratory workshop which was fitted out for that purpose. This took some time, as it was not always easy to plan or modify physical apparatus so that it could be used over a long and continuous period for biological experiments: especially so, as the experiments involved the testing of possible viruses on artificial media which throughout had to be maintained free from all contaminating bacteria.

The principle underlying the reorganisation of the laboratories was that they should be equipped on a permanent basis with fixtures that were likely to satisfy all requirements for the research. Further, that all unions for detachable units should be restricted as far as possible to a few suitable standards so as to facilitate changes and ensure the maximum speed consistent with efficiency in the setting up of any fresh series of experiments on a large scale.

The first problem that had to be considered was a supply of suitable electric currents. The mains being alternating at 220 volts, it was decided to install a high amperage, 14-volt accumulator, and a low amperage 360-volt type, in addition to Westinghouse metal rectifiers, to meet all probable D.C. requirements. Suitable transformers were also installed for obtaining low-voltage A.C. from the mains. All supplies were carried to a central switchboard table, on which were also placed the necessary sockets, switches and controlling units. High quality material was used throughout, double or treble fuses were inserted, the three-wire system was employed for the mains supply, and every care taken in fitting up to reduce the possibility of any breakdown to a minimum.

For many purposes throughout the research some form of accurate timing equipment was essential, and it will be most convenient to give here a brief survey of the different types employed. For comparatively heavy work, involving continuous motion, a fractional horse-power Klaxon geared motor was used, the simple induction type being selected when only an approximately accurate speed was required, and the synchronised type where accurate timing was necessary. These motors are particularly suitable for experimental work as they can be obtained with one of a number of different gears incorporated in the unit, and the working spindle can be set up vertically at right angles to the armature.

For light continuous motion, a standard model of an eight-day English lever clock was selected, additional gear wheels with projecting spindles being added to give the required speeds.

Parts of apparatus, such as screens, switches, etc., requiring an accurately timed and precise movement, were controlled by an electro-magnetic master clock, through the intermediary of suitably constructed electro-magnetic secondary movements. The master clock selected for this purpose was a standard Post Office pattern, fitted with a seconds, a six seconds, and a half-minute impulse, as made by the Magneta Time Co. A separate switchboard, incorporating all the switches, plugs, fuses, resistances and meters, was constructed for this clock to facilitate accuracy and speed when bringing any piece of apparatus into use in any of the incubators or elsewhere.

The rest of the fixed laboratory equipment requires no special description, but before passing on to consider the different groups of experiments, it will be necessary to describe the essential features of the culture vessels, the sources of wave energy used, and the general arrangements in two of the special incubators.

As the research centred round the effect of waves of different frequencies,

this factor influenced the selection of vessels for holding media in the different classes of experiment. For most purposes some form of test-tube was used, a Pyrex glass tube measuring 6 in. by $\frac{3}{4}$ in. being the type selected for ordinary purposes. This size gave a sufficient surface of medium when used for plating out by means of pipetting a suitably diluted material over the surface. When it was desired to eliminate the effect of some of the absorption bands of glass, Vitrosil (fused silica) test-tubes were sometimes employed. In other cases a special type of open Pyrex glass tube was used. Although several models were made, it will be sufficient to describe one design. The tubes were made 6 in. long by $1\frac{1}{4}$ in. in diameter, and had a constriction below similar to a Roux potato tube. The mouth was contracted to an opening of only $\frac{1}{2}$ in., and to this was fused a short neck with a slightly expanded mouth, the contracted opening being made excentric so that its position extended from the centre of the main tube to one margin. When a medium such as agar was placed in these tubes, it was left to solidify with the tubes in a horizontal position with their necks uppermost. After setting, the tubes were stood upright for a short time to allow any condensation water to settle into the lower bulb which was made for that purpose. These tubes were sometimes used for ordinary cultivation experiments, but they were made for the special purpose of allowing some wave band to pass through the open neck on to the surface of the inoculated medium. When used for this purpose they were incubated in the horizontal position, with the neck in the lower half and the medium suspended in the upper, the cotton-wool plugs being removed at the latest moment of setting up the experiment, so as to minimise the possibility of contamination.

For certain experiments, in which a test-tube was of an unsuitable shape to use in association with the rest of the equipment, a special design of glass dish was selected as a standard. These dishes were of the type used by jewellers for small parts, being slightly constricted at the top, so that the lids fit with their sides flush with the circumference of the bottoms. The upper constriction was effective in preventing condensation water from flowing out of the dishes when they were set up with the bottoms vertical. The diameter of the dishes was $2\frac{3}{4}$ in., and the height $1\frac{3}{4}$ in. The bottom of each dish was etched on the outside with a line drawn across its diameter, and from the centre of this, and at right angles to it, a second line was drawn to the circumference. These lines were made to establish a definite position for the dishes, and in future descriptions they will be referred to as the T dishes. A number of duplicate aluminium lids, each with a central flanged hole, were made for these dishes, and into each was cemented one of a large number of different materials that could be tested as wave filters. After sterile hot agar had been placed into one of these dishes, the medium was allowed to set with the glass lid in position. When cool, the lid, covered on the inside with condensation water, was carefully removed, and the experimental lid substituted. Sometimes the dishes were used in the inverted position without any cover, the rest of the apparatus being suitably arranged for that purpose.

Small Pyrex glass and quartz flasks were also used in some experiments, but no special description of these is necessary.

Some consideration must now be given to the chief sources selected for obtaining a suitable supply of electro-magnetic waves. Whenever possible natural solar rays were employed, but in other cases recourse had to be made to some artificial source. As every artificial lamp is lacking in some of those frequencies which filter through the atmosphere from the sun, it was necessary to include a number of different types. The most important of these was the electric bulb containing a tungsten filament, many forms of which can be obtained and worked without difficulty. Three models were selected as best fulfilling most requirements. These were the Neron-Vitalux half-kilowatt sun lamp, running on 220 volts, the Metropolitan-Vickers 100-Watt projector lamp, running on 50 volts, the filament of which is enclosed in a bulb of boro-silicate glass, and the Metropolitan-Vickers 48-Watt, 12-volt car lamp, with a soda-glass bulb. Both the projector and the car lamps are made with very short filaments, so that they give practically a point light. It was desired to use also the tungsten filament, enclosed in a fused quartz bulb, but such a design could not be obtained. The use of mercury vapour lamps was confined to the small discharge burner, type T/M 5/354, made by the Thermal Syndicate. The more general standard type was not employed owing to its high cost, and the technical difficulties of running this form for long continuous periods. For some experiments in the infra-red region, open filament electric heaters were used. In other experiments various gas discharge lamps were employed. Gas-heated elements, such as the Welsbach mantle, were used only to a limited extent, owing to the formation of fumes and other disadvantages.

Two special wooden incubators, Nos. 7 and 8, were made to hold these bulbs, all the necessary transformers, fuses, switches, etc., being fixed on the top of the incubators, and permanently wired up so that any one or more could be brought into use without delay. The interior of each incubator was fitted out with various accessories such as screens, sliding shelves, etc., and, where necessary, parts were made interchangeable. Edison screw sockets were used throughout for all lamps, hanging ones being fixed to ball and socket joints for the purposes of adjustment. The temperature was maintained at any desired constant by means of a thermostat placed in the grid circuit of a thermionic relay which controlled the heaters. In most experiments the heat given off by the Neron Vitalux bulb or by a number of car bulbs exceeded that necessary to maintain the desired temperature, so in these cases they were placed in the heater circuit of the relay in place of the usual heater. This of course resulted at times in the lamps being automatically switched off by the thermostat, giving an irregular and intermittent influence from the lamps. When it was desired to screen the culture vessels from Hertzian or other longer electrical waves, they were placed in special aluminium or copper containers that were spun and shaped for the purpose. For complete shielding, lead, iron, copper or aluminium was used.

## V. THE CHOICE OF TECHNIQUE FOR EXPERIMENTS

From what has been said earlier in this paper it will be understood that the experimental tubes were but rarely subjected to the whole wave band emitted from the source. On the other hand, it was not considered desirable to carry out tests on narrow bands with precise physical apparatus until the field to be explored had been reduced. A large Hilger constant deviation spectrometer was obtained for such experiments, but clearly with this instrument only one narrow wave band, transmitted by one kind of prism, can be tested on one culture in each experiment. Further, as it was judged best to leave most of the virus experiments for periods varying between a week and six months, and as some thousands of test-tubes or other vessels were used, the employment of precise spectrometers was not a practical means of exploring the field on a large scale. Moreover, these instruments do not reproduce the more complicated conditions usually associated with the complex chemical structure and varied configuration of a natural terrestrial rock.

When drawing up details of any experiment, the basic factors that had to be considered were: the situation in which some member of the virus group might be found, the composition of the medium that would suit that particular type, the correct combination of physical conditions to use in association with the medium, and the manner in which growth or multiplication might be detected. Now, each of these factors—but particularly those relating to food supply and physical conditions—can be varied in many ways within the scope mapped out for the research, so the number of possible combinations is much too large to allow any haphazard selection of conditions any real chance of fulfilling the necessary requirements. The scheme adopted was first to analyse every known or observed fact, however small or vague, that might possess some significance in relation to the problem, no matter whether it concerned viruses, bacteria, plants, animals, or industrial processes, and to make such deductions as appeared reasonable. Then to carry out series of experiments in parallel with materials assumed to contain different forms of primitive life, in which one of the factors, and one only, was systematically varied along predetermined lines, until the experimental results gave definite indications of more favourable conditions. Then in other series of experiments to keep this factor constant to the improved conditions, while another factor was varied until this also was improved. By a repetition of these means it was hoped to reduce the field of possibilities, resting partly on theory and partly on isolated observations, to a much smaller one supported by broad experiments. The chief vital materials used in the parallel experiments were: a bacteriolytic agent in association with living bacteria, a possible active agent with dead bacteria, various living bacteria in nature, known viruses such as vaccinia and mosaic diseases of plants, and porcelain filtrates of muds, etc.

It must be emphasised here that the primary object of this research was not the creation of living matter, or indeed the reproduction of the first stages

of life, as has been concluded by some of my colleagues, but rather to discover suitable conditions for the growth and multiplication of viruses on, or in, artificial media *containing no other living matter*. It has been necessary to consider the evolution of the cell in order to define the fundamental conditions likely to be suitable for the growth of precellular forms. Moreover, the field has not been explored from the start of life towards bacteria; but from bacteria in the direction of more primitive forms. This method appeared to offer the greatest scope for progress, and technically it presents fewer difficulties. A highly developed precellular form would probably be easier to cultivate, and a positive result easier to determine than would be the case with still more primitive forms. Moreover, from the evolutionary standpoint, some viruses at least are likely to be nearer to a cell than to the first manifestations of life.

A general description of the experiments will now be given, but as many gave negative results, it will be sufficient to consider them in groups, and to give details in selected instances only where the results seem to be of some significance. It will be essential also to describe certain of the special pieces of apparatus employed, and the technique of their use in conjunction with bacteriological equipment. It will be appreciated that, as the conditions in nature vary in many ways, so it was necessary to vary the conditions set up in the different experiments devised to imitate nature. Although experiments were frequently carried out in several sections at the same time, and were constantly changed between those on media and those on physical conditions, the description in each section will follow, as far as possible, the order in which results of interest were obtained, as it was these results which determined the precise course that was followed in subsequent experiments. At the same time, the gradual evolution of the best media will be given under one heading to ensure clearness.

## VI. Experiments on growth indicators

At an early stage in the research, consideration was given to devising an easy and reliable means of determining with certainty any indication of growth. With a bacterium, a successful cultivation can usually be detected by observing either the production of turbidity in clear fluid media, or the formation of closely packed masses on solid media. In the case of a true virus we do not know that either of these phenomena will occur, and as viruses are invisible, or at least indistinguishable with certainty from organic particles under the microscope, the problem of indisputable detection becomes a more difficult one.

In the case of a known pathogenic virus, a test can be made by inoculating washings from the implanted media into a susceptible animal or vegetable; but when varieties are being sought for in material of a mineral nature, this method of testing would be most unreliable. Moreover, it is necessary to exercise considerable care in the interpretation of small visible changes, as these frequently occur in a tube of medium along the line of inoculation, which

are produced by simple physico-chemical reactions, and, as far as is known, are in no way associated with the growth of any form of living matter. One notable example observed by the writer being the formation of definite raised particles on a clear agar, which could be "sub-cultured", but which were eventually proved to consist of some sulphur particle or compound, started in the first instance by the rubbing of the platinum loop on media containing sulphides. The multiplication of a bacteriolytic agent, we know, can be demonstrated by the effect it produces on the growth of a susceptible micro-organism, but a bacteriolytic agent, if it should be proved to be a virus, would have to be classified as a pathogenic variety. It is clear then that the orthodox methods used in bacteriology for detecting growth of bacteria cannot be relied upon when seeking non-pathogenic wild varieties of the virus group. It may be assumed, however, that if growth takes place in any medium, there will be some chemical change in the composition of the medium or surrounding atmosphere; but, except in special cases, it is not feasible to make an exact chemical analysis daily of the many small tubes of media used in such experiments. This being so, efforts have been made to discover substances which, when incorporated in the media, would act as indicators without having a detrimental effect on any primitive type of life that might be present. In all cases the initial tests were carried out on bacteria, so that the effect on growth could easily be observed.

The research on special growth indicators has given so far only one series of positive results, and a description of the experiments will best be confined to this series. It is known that most if not all bacteria contain some sulphur, and, in view of the close chemical relationship between sulphur and selenium, it was anticipated that if selenium were added to a medium in a suitable form it might be taken up by some bacteria and deposited in a state that would impart a red colour to the growth. Experiments showed this to be the case when the oxychloride compound was used and only a minute quantity added. Ordinary agar, containing beef extract, peptone and salt, may be used as a basis, and to each 100 c.c. should be added one or two drops of the selenium oxychloride. Other solid media, such as Dorset's egg medium, may be used in place of agar, but fluid media do not as a rule give satisfactory results. Sloped tubes of the agar medium are practically colourless, but when inoculated the growth of most bacteria acquires a bright red colour, and in some cases is so intense as to be almost black. The degree of intensity varies with different bacteria, with the age of the growth and with the quantity of selenium oxychloride added. A white micrococcus culture takes on a deep red colour and shows a vivid red deposit of growth in the condensation water. The colour of the coliform group is usually less intense, while that of the acid-fast group on an egg medium is marked. A brief consideration of the chemistry of selenium will be sufficient to explain the coloration produced.

It is generally stated that selenium in the form of a selenate possesses a detrimental effect on bacteria, and during the present research it has been

found that sodium selenate is quite unsuitable to use as a substitute for the oxychloride. Experiments on the oxychloride media have shown that most bacteria—including varieties isolated from waters and soils—usually give as good a streak growth on these media as on similar control media containing no selenium. The bacteriolytic agents also retain their activity on the selenium media. In some experiments to be described later, however, it will be shown that when a bacterium is subcultured on to a series of different media, the comparative extent of the growths forms a most unreliable basis on which to select the most suitable medium for isolating that particular bacterium. The only safe method of testing would appear to be to make the bacterium into a sufficiently thin suspension so that, when a drop is pipetted over each of the tubes of the sloped media, not more than about 50 colonies grow on the control tubes. When the oxychloride media were tested by this method, the results were disappointing. Most bacteria tested grew good colonies, but the number was usually greatly reduced, indicating that only a few of the strongest individuals from the community of a "pure" culture had grown into colonies. In some cases, however, the number of colonies showed no reduction, a notable example being certain varieties, to be described later, which were isolated from the ascophore fungus *Otidea aurantia*.

## VII. Experiments on media

The investigation of the nutritive requirements of viruses was conducted in conformity with the theories already given, the guiding principles being that many organic compounds contained in ordinary bacteriological media should be unnecessary, and might be detrimental, and that inorganic salts should satisfy the needs of many varieties, if the cultures were placed under the influence of a suitable combination of light or other wave frequencies.

For most solid media, agar was selected as a suitable and harmless substance to use for the purpose of solidification, and experiments were then made to determine the relative merits of different "waters".

An extensive series of comparative tests carried out in various directions soon gave a decided indication that rain water is far more suitable than the London water supply for incorporating into media, a fact that is not surprising in view of the processes of "purification" to which a town supply is subjected. Distilled water is also inferior to rain water, while stream waters show some variation and are therefore not to be recommended. In the collection of rain water, every care must be taken to ensure that its purity is not impaired, either by collecting off sooty, tarry, or painted surfaces, or from clouds that have passed through the impure air of a town or over recently burnt forest land. It is best collected off a clean glass or enamelled surface, and should be filtered and sterilised as soon after collection as possible. This is important, as turbidity due to the growth of bacteria soon occurs in mild weather, and this appears to deprive the water of some of its virtue. Sterilisation may be carried out in a steamer, although passing the water through a porcelain filter would be a

better method if it were reliable. But a filtrate so obtained might not be entirely free from bacteria, and it would certainly contain any virus form of life that was present in the water. If, however, the filtrate is either heated to 60° C. for 1 hour, or placed into fused quartz vessels and subjected to the action of a mercury vapour lamp, it may then be added unheated to sterile media.

Experiments were now made to ascertain if it is desirable to add beef extract, peptone and sodium chloride to media. One or another of these substances was incorporated in the rain-water agar, and the media tested with pure cultures of delicate bacteria obtained from animals, plants, rain water and muds.

It has already been pointed out, in an earlier section dealing with selenium, that streak cultures give very misleading results, and that if the relative merits of a series of media are to be correctly estimated, then all inoculations must be made from diluted suspensions of bacteria. Furthermore, in the interpretation of the results, it must be recognised that the number of colonies which develop on a medium is of more importance than the size to which they grow. A large number of colonies indicates that detrimental substances are not present in excess: a quick appearance of colonies shows that the general conditions under which they are being cultivated are good; whereas a large colony may simply signify that the medium is loaded with nutritive substances, a condition which is not always conducive to a healthy physiological state of individual bacteria, and may even prove poisonous to some of the more delicate varieties, as it undoubtedly may to the weaker individuals of any pure culture.

Experiments conducted in accordance with the technique described soon revealed the fact that beef broths or extracts act adversely on many bacteria, also that the activity of a bacteriolytic agent is diminished when these extracts are added to media. As regards peptone and sodium chloride, these, in reasonable quantities, had no harmful effect on the bacteria used in the earlier experiments. Moreover, parallel tests with a bacteriolytic agent in association with micrococci showed that both substances have a favourable influence on lysis.

The next series of experiments dealt with natural earths. The results may be summarised by recording that watery extracts of some clays undoubtedly improve the experimental media for many bacteria. They also give good results when demonstrating the action of a bacteriolytic agent. Among the best may be mentioned samples obtained from the Larcome and Rowland's Castle pits near Petersfield, and from the red marl pit at Radcliffe.

It was considered possible that some members of the virus group might require the addition of substances contained in the more primitive types of vegetation; accordingly experiments were carried out in this direction. The results, again, must be summarised. Watery extracts of certain leaf moulds and peat improved the simple agar media. Of the leaf moulds tested that from

the oak proved to be the best; but a bracken peat also gave fairly satisfactory results, so attention was now given to ferns. Watery extracts were made from the dried fronds, either green or dead, and incorporated in the experimental agars. About fifty species, including most of the British ferns, were tested. The results differed with different bacteria, particularly when green fronds were employed. Those giving the best growths with one or another bacterium were the green fronds of *Asplenium trichomanes*, *A. bulbiferum*, *Polystichum angulare*, and *Blechnum spicant*, while the dead fronds of *Allosorus crispus* and *Polypodium dryopteris* were good in some cases.

Experiments were next carried out to ascertain if agar media, prepared with rain water, clay and fern fronds, could be improved by the addition of any of the more common salts or elements which are believed to be essential to plants and animals. It was found that iron and calcium salts undoubtedly improve the media for some bacteria, and that they give the best results when added in the form of lactates, 0·01 per cent. being a sufficient quantity. It may be observed, however, that the effect of any salt is certain to vary according to variations in the composition of the clay selected. No benefit was observed with potassium or magnesium salts, or with sulphates, nitrates, or phosphates, while chlorides frequently gave a detrimental effect.

In consequence of the effects generally produced by chlorides, further experiments were made with the sodium salt, using not only the original test bacteria, but two other still more delicate wild varieties which had been isolated. These experiments confirmed the earlier ones in that they showed that most bacteria can tolerate 0·5 per cent. sodium chloride. On the other hand, the salt did not appear to be essential, and there was one organism on which it acted detrimentally. It has already been noted that sodium chloride proved to be advantageous to the activity of a bacteriolytic agent, but the question arises as to whether the visible result is to be ascribed to a beneficial effect on the agent or to some detrimental effect on the bacterium which makes it more susceptible to the action of the agent. The latter would appear to be the more probable, since the agent lives on the bacterium and not on the medium.

Experiments with chlorophyll indicated that both the water-soluble and oil-soluble chlorophyll may be added to some media with advantage.

During the research on the cultivation of Johne's bacillus from 1910 to 1914, extracts of many vegetable and animal materials were tested in attempts to find substitutes for the "essential substance" (or accessory food factor as it would now be called) which had been extracted from other acid-fast bacilli, and incorporated in a glycerine egg medium for growing Johne's bacillus. Most of these media were also tested with vaccinia, as well as various porcelain filtrates in which some virus may have been present. A list of most of the extracts tested is given in one of the later papers on Johne's bacillus (Twort and Ingram, 1914). As the experiments with virus materials were entirely negative, it will be unnecessary to give further details in this paper.

All recent experiments, in search of an "essential substance" or accessory food factor for viruses, were confined to extracts obtained from the sporophore and ascophore fungi, as it was the *Cantharallus* genus which gave the most definitely positive results with Johne's bacillus, and enabled this organism to be grown in primary cultures without the addition of extracts of other acid-fast bacilli. The fungi tested included *Otidea aurantia*, and although extracts failed to give satisfactory results, some cultivation experiments proved interesting and will be described later.

In the course of the present research, over a thousand batches of different media have been prepared and tested with various bacteria, vaccinia, porcelain filtrates and the bacteriolytic agents, under a variety of associated conditions. The agar media evolved as a result of these experiments will now be described in more detail.

The basis of all agar media being used at the present time consists of rain water and clay and fern extracts, and it is prepared in the following manner. 10 g. of Larcome clay are added to every 100 c.c. of clean filtered rain water, which has been sterilised within 24 hours of collection. At the same time a fern extract is prepared by adding 0·2 or 0·5 g. of the dried fronds of one or more suitable ferns to every 100 c.c. of rain water. Both of these are allowed to stand for an hour, then steamed for another half hour and passed through filter paper. Equal quantities of the clay and fern extracts are mixed, and to the mixture is added $1\frac{1}{2}$ per cent. powdered agar—Difco brand. This is autoclaved and then cooled to 50° C. The yolk and white of a hen's egg is mixed with an equal quantity of rain water, well shaken and added to the hot agar which is again autoclaved and filtered through a Chardin type of paper. Next is added 0·01–0·1 per cent. of water-soluble chlorophyll and, for some experiments, 0·01 per cent. of ferrous lactate, 0·01 per cent. calcium lactate and 0·01–0·1 per cent. peptone. If the oil-soluble chlorophyll is to be included 0·01 per cent. is added before the egg, so that any undissolved portion may be removed in the subsequent filtration. If any additional substances are to be tested these are added last, and occasionally a second filtration through Chardin paper is made. When finished the medium is placed into suitable test-tubes, autoclaved and set in slopes. When it is desired to incorporate some unheated material, it is best done by pipetting it into a tube of melted medium which has been cooled to about 48° C., the medium being immediately resloped and allowed to set.

## VIII. Early experiments on the influence of solar rays

In the early days of bacteriology it was recognised that direct sunlight—and to a less extent subdued light—has an injurious action on bacteria, and that the action is most marked in the blue-violet region of the spectrum, and less so in the near ultra-violet and green-red regions. It was established also that the nature of the implanted medium plays a part in determining the extent of the action. In the present paper it will be necessary to record results in which

similar injurious effects were produced, but it will be appreciated that the ultimate object of all experiments on bacteria was to obtain indications, directly or indirectly, of a combination of conditions which would produce a *beneficial* influence, and to apply the conditions to experiments on the cultivation of viruses.

One of the early problems to receive attention was that relating to the effect of different sections of the solar spectrum on the growth and activities of the various forms of vital material selected for the research. Experiments were carried out in the first instance with the different bands as they are reflected from the rough surfaces of the common rocks when the sun's rays fall upon them. The rocks included many of a composite nature, such as granites, serpentine, etc., and in all about 100 samples were tested. For these and similar experiments, a special incubator, which in future will be referred to as No. 4, was built against the inside of a window frame which faced south. The glass was replaced by wood, and this was bored to take various tubular fittings into which could be placed mounted lenses, filters and culture dishes. The incubator incorporated thirty-six of these fittings, all parts of which were made to a selected standard to take mounts for 2-in. discs or lenses, the lower twelve pointing downwards at a suitable angle to the sill for the reflection experiments, and the rest upwards for the wave-filter experiments. It will be understood that this incubator was restricted to experiments in which all parts, when once set up in position, remained stationary throughout the experiment. A thermostat in the grid circuit of a thermionic relay controlled the temperature. The lenses used were made of glass, quartz, fluorite or iceland spar, and in addition the pin-hole method of obtaining an image of the rock on the surface of the medium was employed.

Most of the solid media used in this section of the research consisted of an agar containing watery extracts of some clay and leaf mould, although the exact composition of the different batches was continually being varied throughout the experiments. In all cases the standard T dishes were employed, an inoculated drop of material being allowed to flow and spread from the centre towards the circumference. The dishes were set up so that the visible reflected rays from a rock were in focus on the surface of the medium, the cross of the T being set horizontally to ensure the rock image falling in practically the same position on the medium when the dishes were replaced after an inspection.

The experiments, which were carried out during the summer of 1933, were disappointing. In no case was any really positive result observed with any of the porcelain filtrates of muds or other materials. A certain degree of watery granulation was occasionally formed, but no evidence could be obtained that this indicated a phenomenon of any significance from the standpoint of the experiments. It should be noted, however, that these early tests were made before the better media, containing fern extracts, had been evolved, and it will be necessary to repeat them with suitably modified media as soon as climatic conditions become favourable.

## IX. EXPERIMENTS WITH NATURAL AND WITH ARTIFICIAL "LIGHT" FILTERS

In another section of the research, simple agar media, implanted with bacteria, viruses, bacteriolytic agents, filtrates of mud or other material, were subjected to the influence of solar or other rays; and the results compared with those obtained in duplicate tubes which were shielded in aluminium, copper, or wooden containers. In one series of experiments the wave bands impinging on the surface of the media were modified by interposing artificial or natural wave-band filters. The artificial ones comprised a large selection of Wratten gelatine light filters, a number of special gelatine and celluloid films made in the laboratory, and Nos. 2–14, signal green and flash ruby of the Chance-Parson's glass filters. The natural types included a few of animal origin, some of vegetable origin, and a large number of a mineral nature. The animal types consisted of such substances as split sheep parchment, horn, tortoiseshell, gelatine, etc. Those from the vegetable kingdom included rubber, cellulose, celluloid, dried leaves and thin sections of wood. The minerals comprised most of the common types to be found in nature, but special consideration was given to those varieties which contained what were judged to be the most essential elements associated with life in the animal and vegetable kingdoms. They included also quartz, fluorite, Iceland spar, rock salt, steatite, alabaster, marbles, etc., also many single natural crystals.

Many of the filters were used in several different ways in association with various kinds of media. In some experiments it was arranged for the filtered waves to act continuously on the surface of the cultures. In other experiments the cultures were completely shielded for varying intervals by means of revolving metal shields. In yet others two or more filters were brought into action consecutively. The technique will be best understood if one of the pieces of apparatus which was assembled for testing solar rays is described.

Twelve thin aluminium spinnings for holding the standard T culture dishes were taken and fixed in a ring on the flat bottom of a larger spinning, measuring 15 in. in diameter and $3\frac{1}{2}$ in. in height, which may be called the inner container. This container was then covered with its lid in which had been cut twelve holes, the centres of which coincided with the centres of the small dish receptacles. A small turned brass table, containing a boss with a central hole, was fixed centrally to the under-surface of this container, and the unit fixed, by means of a grub screw in the boss, to the vertical spindle of a suitably geared motor. This section of the apparatus was now placed inside an outer flat-bottomed spinning measuring 18 in. in diameter and 8 in. in height, and the motor bolted to the bottom so that the inner container when set in motion by the motor would revolve centrally in the outer container. The lid of the outer container was made with one, two or more circular holes, at the same radius from the centre of the lid as those in the inner container. Into the holes were placed gun-metal mounts containing the filters to be used. The outer container enclosed also two small electric bulbs to add to the heat given off by

the motor. These were placed in the heater circuit of a thermionic relay and controlled by a thermostat. The outer container was fixed to a heavy equatorial head which was set up in a position to ensure the sun's rays striking the filters vertically. The equatorial head was suitably geared to a fixed "secondary" revolving electro-magnetic movement which in turn was wired to the seconds impulse of the electro-magnetic master clock, the armature of the secondary movement revolving a quarter of a revolution at every impulse, and the equatorial head revolving at a speed of one revolution every 24 hours to follow the sun's path. When the inner container was set in motion, each culture consecutively was subjected to the influence of the sun's rays, after being filtered first through one filter and then another, these periods alternating with periods in which the cultures were shielded by the lid of the outer container. This arrangement was very suitable for testing the same combination of conditions on a number of different cultures, but for the testing of different conditions on similar cultures, the mounted filters were placed in the lid of the inner container instead of the outer one.

The apparatus was used also in other ways, for instance, when dealing with mineral crystals, the equatorial head was usually fixed in the line of the midday sun, in order to imitate the conditions produced by the changing angle of the sun's rays on a fixed rocky surface. For both the inner and the outer containers a number of duplicate lids were spun, so that variations could be made in the number and relative position of the holes, when it was required to incorporate other modifications for the systematic series of experiments carried out in this section of the research.

The first result of interest that emerged from these experiments was that solar rays are not nearly so detrimental to certain varieties of bacteria as is sometimes supposed, especially when the action is discontinuous. Sun rays filtered through the Wratten No. 18 A, which passes ultra-violet waves at a maximum of about 3600 Å., frequently produces no detrimental effect. It was also observed that the exact composition of the medium used plays a large part in determining whether certain parts of the spectrum will act detrimentally or otherwise. A beneficial effect indeed would scarcely be expected unless a combination of suitable wave-lengths with a fitting composition of the medium were used as a basis for the tests. In some cases the most detrimental waves appeared to be those between 4000 and 4700 Å. in combination with other waves. In other experiments there was a distinct indication that parts of the solar spectrum may be beneficial, but a full analysis of favourable effects must be deferred until a description is given of later experiments which were carried out with the 48-watt and 100-watt lamps.

Another result of interest with filtered solar rays was obtained in one of a series of experiments in which attempts were being made to cultivate some wild variety of one of the theoretical forms of life already discussed. It is probable that some of the conditions of the experiment were unnecessary; but, to avoid any possibility of omitting an essential factor, all the experimental

conditions must be mentioned, if only briefly. A simple agar medium, containing a watery extract of leaf mould, to which had been added 5 per cent. of ordinary peptone broth, was poured into a number of T dishes, and allowed to set. Each was then inoculated with one drop of a porcelain filtrate of mud emulsion obtained from a stream near Wisley. The dishes were covered with glass lids, and placed in the apparatus already described. A lid containing three holes was used for the outer container, and into these were placed Wratten gelatine filters Nos. 36, 61 and 88 respectively. The outer container was fixed at the position of the midday sun during September 1933, and the inner container arranged to revolve 50 times a minute. After 3 days one of the dishes was found to have grown a number of tiny whitish colonies. The T dish was now removed from the apparatus, and placed in an aluminium container on the bench at room temperature, and left there for 4 days. On re-examination it was found that a curious change had taken place: most of the colonies were still dense and whitish, but a compact group, and one group only, had become quite translucent. The change had not occurred as an outgrowth, nor was it limited to a part of any colony, as might have been expected if it represented a mutation or a form of degeneration. It was uniform in both extent and in appearance, and subcultures, when grown on a suitable medium, produced a pure translucent growth of a moderate-sized bacillus.

A close study of the cultures and the conditions under which the change may take place revealed a number of interesting points, and these will now be described. The bacillus is aerobic and spore-bearing. It grows best at about 30° C., but the most suitable temperature varies considerably with the medium used and with the conditions under which it is grown. When cultures are made on to ordinary agar containing beef broth, peptone and sodium chloride, and placed at 37° C., the dense colonies grow slowly, but the waxy translucent form shows no growth. If, however, a medium containing no beef extract is used, or if the temperature is lowered to about 28° C., then both forms grow. In all cases the distinctive characters of the waxy growth remain constant during cultivation. There is no erosion of colonies, and cultures retain their vitality for over a year. Both forms grow well in daylight and when placed 18 in. distant from a 100-watt projector bulb. When subjected to a temperature of 90° C. for 1 hour in a water bath, most of the spores are destroyed, although occasionally a few will survive this treatment. There are indications that in a normal culture the density of the growth is associated with the production of spores, and that the appearance of the changed waxy growth results from their destruction, as many shells and granules can be demonstrated in films made from the waxy growth. A fully grown culture of the dense form consists almost entirely of spores, whereas spores may be difficult to find in the waxy growth. This difference may also be demonstrated by comparing cultures made from suitably heated emulsions of each form.

As with bacteriolytic agents, the change may occur "spontaneously", and occasionally in old cultures the changed form gives rise to secondary dense

colonies, possibly arising from resisting spores, which are identical with the original dense growth. Experiments indicate that a spontaneous change occurs more frequently when the dense form is subjected to the sun's rays filtered through white marble, steatite, etc., than when it is shielded from those rays. It is essential, however, that suitable media should be used.

Although the distinctive characters of the change can be demonstrated without difficulty, an interpretation of the change is more difficult. In some ways it resembles that discovered by de Jong (1931) in a culture of *B. megatherium*, which has since been thoroughly investigated by de Jong and by Wollman. The change in *B. megatherium*, however, has been shown to be associated with the presence of a bacteriolytic agent, whereas with the bacillus here described, repeated experiments have failed to reveal the presence of a filterable agent. Moreover, if portions of dense and waxy growths are mixed, and the mixture subcultured on to fresh tubes, the resulting growth shows patches of both types. This may indicate, either that some extraneous influence is necessary to start the change, or that the spores can only become infected when they are forming inside a bacillus which has harboured the necessary agent in a resting or symbiotic state. The uniformity of the change indicates that a constant equilibrium is established, but this may be produced as a result of the selective activity. In any case it is clear that the main action is on forming or resting spores, and not on growing bacilli.

It might be reasoned that the waxy colony represents a symbiotic growth of a bacteriolytic agent with the bacillus, and that the absence of any visible action on the dense form by a filtrate indicates that the normal bacillus and its spore are resistant. If, however, to account for spontaneous changes, it is suggested that some agent has always been in symbiotic association with the normal bacillus, then, clearly, the waxy change must indicate a deviation from that symbiotic state, in so far that the agent has become pathogenic for a less resistant spore. But, in the primary plate culture, a group of about fifty colonies changed completely to the waxy form, while every other colony of the bacillus outside their area remained normal; a result which suggests the presence of some localised influence. A bacteriolytic agent undoubtedly shows considerable variation in the outward manifestations of its presence, and there may be some who would prefer to explain the phenomenon as being due to a type of this agent. The writer, however, believes that the characteristics of the change differ sufficiently to necessitate its being placed in a different category, and it is proposed in future to refer to the causative substance as a "sporolytic agent". The existence of such an agent is quite consistent with the theories already enunciated, and might denote that some precellular unit of the cell has undergone only a partial reversion to an independent state, thus necessitating a closer intracellular association than in the case of a filterable bacteriolytic agent.

It is necessary at this stage to give a short description of some interesting bacteria which were obtained from surface scrapings taken from the ascophore

fungus *Otidea aurantia*. Inoculations were made on to simple agar media containing extracts of one or another species of fern, and the tubes, as usual, placed under different conditions. Most of the cultures grew colonies of bacteria of no special importance, whereas a few grew in addition several interesting bacilli which were not easy to isolate. Three types, which were all chromogenic bacilli, may be mentioned. One started as tiny dull flat film-like colonies, which later lost their dullness in the centre and acquired a yellow colour. Another type grew as delicate green colonies, which subsequently acquired in the centre a red coloration, on which was superimposed a green iridescence. The third type, which grew later, had an intense deep violet hue. All types grew on a medium in which the fern extract used was obtained from *Blechnum spicant*, the conditions of cultivation being those given by a cool conservatory, glazed with Vita glass, facing south. After a few unsuccessful attempts, the yellow and red varieties were isolated, and later the violet type was obtained as a pure growth. The bacteria were found to be exacting in their nutritive requirements, and were occasionally used for testing experimental media. Experiments on filtrates obtained from *Otidea aurantia* and from cultures will be described later.

## X. ATTEMPTS TO GROW THE BACTERIOLYTIC AGENTS WITH DEAD BACTERIA

It has always been recognised that a bacteriolytic agent will not grow on dead bacteria, although Gratia and Rhodes (1924) showed that a suspension of dead bacteria undergoes dissolution when inoculated with a living culture and a portion of bacteriolytic agent. In view, however, of the results obtained under different conditions in the early experiments on agar media, it was decided to make a fresh series of tests with a bacteriolytic agent in association with dead bacteria only. Suspensions of micrococci were prepared with rain water containing the same substances as used in the agar media. They were heated to 60° C. or higher, and after being inoculated with a drop of filtered lytic agent were subjected to a variety of conditions as with the preceding experiments. One special combination of conditions should be mentioned. This consisted of an arrangement whereby the energy from each of two 48-watt car bulbs was passed respectively through two large glass prisms, set up to give one vertical and one horizontal spectrum, the two being superimposed, but at right angles to each other as they fell on the bacterial suspension. Now, bacteria in fluids when placed under the influence of light mostly remain suspended and in constant slow motion. The technique employed, therefore, not only ensured that different units of the bacteriolytic agent, at any moment, would come under the influence of every possible combination of two wave-lengths (within the limits of the prisms used), but at the same time resulted in a constant change of associated wave-lengths acting on each particle in the suspension. The suspensions occasionally showed some clearing, and this was accentuated when a fragment of some rock, such as limestone, Iceland spar, or felspar was added.

No proof, however, could be obtained of any increase of the bacteriolytic agent under any combination of conditions employed.

An investigation into the cause of the clearing showed that a suspension of dead bacteria, containing no lytic agent, may undergo considerable dissolution when placed under the influence of the sun's rays. It was observed that the change is slower and less marked when a suspension is subjected to the influence of a 100-watt projector lamp, and may be unobservable in suspensions placed in a dark Hearson's incubator. These observations are being further investigated.

## XI. Bacteria isolated from porcelain candle filtrates

When preparing a porcelain filtrate of some mud or other material, it was usual to make up the primary emulsion with 200 c.c. of sterile saline or rain water. This was passed through a Doulton white porcelain filter at a pressure of 200 mm. into a sterilised flask which in some cases contained 100 c.c. of sterile clay water. This clay water was prepared by filtering an emulsion twice through filter paper before sterilisation in the pressure flask. When about 60 c.c. has passed the Doulton filter, the flask was disconnected, and 10 c.c. of its contents pipetted into each of six Pyrex glass test-tubes. From one of these a small quantity of filtrate was removed and pipetted over the media to be tested in some direct cultivation experiment. The filtrate tubes of each experiment were then placed in a dark wooden cupboard at room temperature for periods varying from 1 to 12 months. A periodic inspection occasionally revealed a slight turbidity in one or more of the tubes, although in nearly every instance no growth had occurred on any of the direct cultures made from these in the primary experiment.

Cultivations were made periodically from the opalescent filtrates on to tubes of each batch of experimental medium as it was prepared. Some tubes were placed on shelves in the laboratory at room temperature, where they came under the influence of subdued daylight after its passage through a plate glass window facing due north. Other tubes were put into wooden incubators, at temperatures varying from 15 to 35° C., under a variety of conditions such as have already been described. For some experiments a special revolving lantern was made. This lantern contained a fixed table on which were fitted a small mercury vapour lamp, type T/M 5/354 as made by the Thermal Syndicate, and a 24-watt, 6-volt car bulb. A ball-bearing turn-table supported the inner table on its central spindle, while an outer revolving cylinder held the lantern. Like most apparatus of this type it was driven by an electro-magnetic secondary movement connected by interchangeable gears to the lantern, and timed by the master clock, a speed of the order of one revolution every minute being usually employed. The lantern was fitted with four interchangeable "light filters", and the culture tubes placed in a circle round the lantern, at distances varying from 12 to 24 in.

from the sources of energy. Parallel experiments were also carried out with a 100-watt projector lamp fixed in the centre of a wooden incubator, and the cultures placed round at a distance of 18 in. Many of the test-tubes were hung by their rims from holes in the top of the incubator, so that the mouth of each tube was outside to encourage a diffusion of fresh air through the cotton-wool plugs. In many cases control tubes were shielded in wooden or in copper containers.

The importance of pure air was made manifest during the experiments. In order to simplify inspection in the case of tubes shielded by wood, several boxes were constructed with sliding doors, and for each a sliding test-tube stand to hold 108 tubes was made, the whole being then varnished and dried in a wooden incubator for 14 days. Cultures of delicate bacteria placed in these cases frequently gave negative results, although duplicate tubes in a copper container grew well. From additional experiments it was concluded that detrimental fumes were being emitted from the varnished surfaces, and the use of these boxes had to be discontinued.

A bacterium was eventually grown from quite a number of the filtrates. Nearly all the bacteria formed small raised colonies showing a certain amount of red coloration which varied in intensity and shade with the different bacteria and with the medium employed. Some shade of salmon pink was the most common colour. Most of the colonies were found to consist of fair-sized bacilli with a marked tendency to pleomorphism, and a few showed motility. Although most of the salmon-coloured colonies appeared to belong to the same class, they showed variations in detail. Some were much more sensitive in their nutritive requirements than others. A noticeable feature of the group was a tendency to produce two types of colonies, a few being much more vigorous than the majority which grew as delicate colonies between the larger ones. The primary filtrates from which they were grown were obtained from various sources, but chiefly from muds, and diseased plants and trees. A selection of the most sensitive varieties was used for testing the merits of the experimental agars, and it was largely from tests with these varieties and with the Wisley mud bacillus that improvements in the media were worked out. As the media were modified, further cultivations were made from those filtrates which had so far failed to give any growth. Eventually, an old filtrate obtained from enlarged seed capsules of *Papaver rheas* grew some very delicate colourless colonies which differed considerably from all varieties so far isolated from other sources. The growth proved to be that of a moderate size pleomorphic bacillus, showing no special characters of note, but it represented a type that was very sensitive to small changes in the composition of media, and proved a valuable addition to the series of test bacteria used for experiments on media. It was from results obtained with the selected test bacteria that the later modifications in the composition of agar media were evolved.

## XII. Granule "colonies" associated with opalescence of the medium

The remaining old filtrates, mostly from diseased vegetation, muds and rain water, from which no bacterium had so far been grown, were inoculated on to media incorporating a watery extract of some fern frond, and placed as usual under a variety of conditions. The early results may be summarised by stating that many filtrates produced minute granules on the inoculated surface of a few of the experimental agar media. The best results were obtained with those which contained an extract of the dried fronds of *Asplenium bulbiferum*, *A. marinum*, *A. trichomanes* or *Allosorus crispus*, the media being prepared with clay water as already described. The most recent modifications have included an extract of bracken ash, prepared by making a 0·25 or 0·5 per cent. emulsion in rain water, autoclaving, and passing through filter paper. At first all attempts to obtain "subcultures" failed, but eventually a definite granulation, associated with a milky opalescence of the medium, developed in several tubes. Even so, for some time frequent failures were encountered, until a close analysis of the positive results revealed the fact that in nearly every instance the granulation and opalescence had developed on media which had been prepared and inoculated on the same day. When further subcultures were made on to freshly autoclaved and sloped tubes of a suitable medium, positive results were obtained without difficulty. So far, the best results have been with cultures subjected to subdued daylight at room temperature, that is, under conditions which were shown to be suitable for the growth of some of the delicate bacteria already isolated from other filtrates in the earlier experiments. Filtrates made from materials collected from various plants, muds, etc., have given the same general results, although, in detail, distinct differences were observed, particularly as regards the extent of the changes. On a selenium medium, the growth acquired a pale pink coloration. When a tube of medium is autoclaved, most of the contained gases are driven off, and some little time elapses before they are reabsorbed. Experiments carried out to test the effect of placing cultures under a reduced pressure gave negative results. The phenomenon did not occur on media set some days previously: it did not increase when it had already developed on freshly autoclaved media grown at normal pressure, and it was not more marked on media placed under a reduced atmospheric pressure immediately after being inoculated, than when the cultures were first left at normal pressure.

The problem now was to establish the significance of the phenomenon, and more particularly whether the granulation or the opalescence represents a physico-chemical change which can be started and continued by the action of some catalytic agent, or whether either indicates the growth of some precellular form of life, such as was envisaged in the earlier theoretical section of this paper. Additional experiments showed that an opalescence along the line of a streak inoculation can be produced without the presence of an unheated filtrate, thereby demonstratin g that a freshly autoclaved simple agar

medium is in an unstable physical condition, and that opalescence cannot be regarded as proof, or even as evidence, of any growth. At the same time, as some bacteria produce an opalescence, a similar condition might be expected to occur if there were any growth of a more primitive form of life. The second manifestation of the phenomenon—the small raised granules—have so far only been obtained in streak subcultures when the primary inoculations were made from some unheated filtrate. It is not proposed at this stage to give a description of the minute particles to be seen in film preparations, as it is particularly desired to avoid any suggestion that their presence should be accepted as evidence of the existence of any form of living matter, even though irregularities in shape might be explained away by attaching the word pleomorphic to the description. It is recognised that a bacteriolytic agent or a virus may adhere to any organic particle, as also it may to a crystal, but the presence of such particles or crystals constitutes no proof that they represent the virus, although in certain cases they might indicate the presence of (or result in consequence of) the activity of some virus.

No inoculations have been made into any of the rodents, as some of these animals when kept in cages are prone to develop respiratory affections which may become more acute and be accompanied by a rise in temperature if the animal is subjected to any adverse disturbance. Inoculation experiments under these circumstances may give misleading results, particularly when a large dose of material would be required to produce any effect.

So far no proof has been obtained that either the opalescence or the granules represent the presence of any form of living matter, but suitable experiments are being made to throw further light on the problem.

## XIII. ADDITIONAL EXPERIMENTS WITH THE BACTERIOLYTIC AGENTS

In view of the phenomenon obtained with porcelain filtrates inoculated on to freshly autoclaved simple media, somewhat similar parallel experiments were carried out with different combinations of a bacteriolytic agent, living micrococci and suspensions of dead micrococci. The conditions were varied as regards the composition of media and the time which had elapsed since they were autoclaved; the temperature at which the tests were made and the presence or otherwise of the influence of a 100-watt projector lamp. The results so far obtained must be summarised. A bacteriolytic agent on some media containing a fern extract, when placed under the influence of a projector lamp, retained its full activity for days. This was shown by subsequently inoculating the tubes with living micrococci. Under no combination of conditions was it possible to obtain any proof that a bacteriolytic agent had increased in quantity when the agent alone was inoculated into a suspension of dead micrococci, however the suspension might be prepared. Moreover, the addition of material obtained from filtrates, which had given granulations and opalescence of a medium, in no way affected the result. When a living micrococcus only was

inoculated into suspensions of dead micrococci, some clearing of the suspensions frequently followed, but the extent varied with the conditions. In the absence of light, it was slow and not marked, but when placed under the influence of a projector lamp, a fair degree of clearing frequently took place. This clearing, however, varied greatly according to the composition of the fluid used in making the suspensions, but still more so with the condition of the medium on which the micrococcus used for the suspensions had been grown. The clearing was most marked and most rapid with suspensions prepared from micrococci grown on recently autoclaved media. Such suspensions, killed in a water-bath, inoculated with a living micrococcus, subjected to the action of a 100-watt projector lamp at a distance of 18 in., and incubated at 37° C., usually showed marked clearing in 24 hours. From the extent of the clearing, one gains the impression that the growing micrococci also must have undergone some autolysis. When, however, the partially cleared suspensions were passed through a Doulton filter, the filtrate usually showed no evidence of the presence of a bacteriolytic agent, although, as has already been pointed out, the spontaneous appearance of a bacteriolytic agent is more likely to take place under the influence of a projector lamp than when cultures are placed in darkness.

It has often been suggested that vaccines used in vaccine therapy would be improved if the intracellular toxins were set free by breaking up the bacteria. The writer originally suggested that the use of a bacteriolytic agent might supply a means of obtaining a better vaccine, and the experiments described in this paper indicate methods of freeing intracellular toxins without the use of a bacteriolytic agent.

The experiments are also of interest in connection with Wollman's theory of "hereditary factors", which he has submitted to explain the phenomena associated with bacteriolytic agents. In so far as the essential characters of all animals and plants are inherited, so it will be recognised that the character of a bacterium which determines its susceptibility to a bacteriolytic agent has been inherited. Moreover, if a bacteriolytic agent can arise from a bacterium, then its spontaneous appearance in a normal culture must indicate that some member of the bacterial community has mutated to display that character, even if it has arisen from the same attributes of the cell which normally produce an autolytic enzyme. Unless the individual bacterium at the same time acquired a special immunity it would immediately be destroyed by its own agent, and could therefore not transmit its characters to daughter cells. If at the same time it *did* acquire a resistance and survived to produce daughter cells to which it transmitted the same characters, then one would expect to be able to isolate such a type from a culture undergoing spontaneous lysis, but the writer has never succeeded in isolating a colony of any bacterium which retains the character of a constant production of a bacteriolytic agent which will produce lysis only in another culture of the same species of bacterium. This aspect of the phenomenon, therefore, gives no indication of the existence of a

*special* hereditary factor. Moreover, the production of an autolytic enzyme by a bacterium depends largely upon the conditions under which it is grown. This is proved by the fact that a suspension of dead bacteria, prepared from cultures which have been grown on a freshly autoclaved medium, will undergo autolysis when placed under the influence of a 100-watt projector lamp. No evidence has been obtained during the experiments to suggest that the conditions, which are favourable to autolysis, in any way stimulate a bacterium to transmit any special autolytic characters to subsequent generations, nor has any evidence been obtained that a bacteriolytic agent can be generated by an autolytic enzyme. The question therefore arises in the writer's mind as to the exact nature of the hereditary factor associated with Wollman's theory of the phenomenon of transmissible bacteriolysis. At the present time, experiments are being carried out with suspensions of dead bacteria under modified conditions in attempts to stimulate an autolytic enzyme to become transmissible. At the same time other experiments are being made to acclimatise a bacteriolytic agent to multiply in a suspension of dead bacteria.

The general research on viruses is being continued along all the lines indicated in this paper, with the financial assistance of the London University and the Medical Research Council, who have jointly supported the research, and to whom reports were submitted in 1934 and 1935.

## XIV. SUMMARY

A survey has been made of the different theories put forth to explain the nature of ultra-microscopic viruses, and it has been concluded as probable that they are representatives of some precellular forms of life. The writer's views have been given, and the probabilities have been expounded regarding the constitution of these forms, their evolution towards a composite cell, the conditions under which they exist, the means by which they obtain the necessary energy for their vital processes, and their food requirements. It has been suggested that at times some unit of a cell may undergo a partial or complete reversion to the independent precellular form. The possibility of the existence of other primitive forms of life, belonging to another biological world, has also been considered. The theories discussed have been analysed in relation to the known facts concerning virus diseases, transmissible bacteriolysis and cancers.

The reorganisation of the laboratories and the fitting up of various electrical and timing equipment, which were necessary to test the theories experimentally, have been explained, and a description has been given of some of the special pieces of apparatus made in the workshop for the experiments.

Experiments were carried out on an extensive scale with animal and vegetable materials containing known viruses, with muds, etc., presumed to contain precellular forms of life, and with bacteriolytic agents. Certain delicate bacteria, isolated from porcelain filtrates, were used as controls.

Attention was first given to indicators for growth, and it was found that minute quantities of selenium oxychloride incorporated in media resulted in many bacteria growing with a bright red colour.

From an extensive series of experiments on nutritive requirements, it was determined that beef extracts usually act detrimentally on bacteria, and they should not be used. The detrimental effect was marked with the bacteriolytic agents. Good agar media were gradually evolved. Rain water proved to be better than water from a town supply. A watery extract from certain clays supplied sufficient nourishment for many bacteria, but the media were improved by the addition of watery extracts of bracken ash and of the fronds of certain ferns, notably those belonging to the *Asplenium* group.

The associated conditions used during cultivation were varied in many ways, particular attention being given to the influence of solar rays and the energy emitted from projector lamps. In different experiments the wave energy was modified by reflection or filtration, different bands being tested both consecutively and intermittently. The materials used consisted of lenses, prisms, natural rocks and crystals, as well as animal, vegetable and chemical filters.

Many bacteria were found to tolerate solar rays when cultivations were made on suitable media, particularly if the action was intermittent. They were least tolerant to the region of the spectrum around 4000 Å.

A spore-bearing bacillus has been obtained from cultures of a mud filtrate on a leaf mould extract medium, placed under the intermittent influence of filtered solar rays. A group of the colonies developed a homogeneous waxy change, which was proved to be associated with a dissolution of the spores. It is suggested that the change is due to the presence of a "sporolytic agent", although it has not been found possible to demonstrate the agent in a porcelain filtrate.

In a number of cases pleomorphic bacilli have been cultivated from old porcelain filtrates obtained from various diseased plants and muds. Many were found to be very sensitive as to their nutritive requirements. Most of them grew well in subdued daylight and under the influence of a 100-watt projector lamp.

Other porcelain filtrates produced granules on the surface of certain experimental media. When subcultures were made, granules associated with a marked milky opalescence of the medium occurred along the line of inoculation. No bacterium could be found in films made from the granules. The phenomenon appeared only on tubes of media which were autoclaved, set and inoculated on the same day. The milky opalescence can be produced without the presence of an unheated filtrate. The significance of the granules is being investigated.

Many comparative experiments have been carried out with suspensions of dead micrococci, either uninoculated or inoculated with living micrococci or a bacteriolytic agent or with both. It has been shown that suspensions of dead bacteria undergo more marked clearing when placed under the influence of

solar or other rays than when placed in darkness. The effect is increased when the suspensions are prepared from cultures grown on tubes of media which have been autoclaved, set and inoculated on the same day, and it is particularly marked when the suspensions have been inoculated with living micrococci. The significance of these and other results has been considered, and attention has been called to the utilisation of the factors concerned for the purpose of freeing intracellular toxins in the preparation of various vaccines.

In conclusion I should like to record my appreciation of the valuable services rendered by my only assistant, Mr H. Frankham, the laboratory attendant and caretaker of the Institution.

## REFERENCES

GRATIA and RHODES (1924). *C.R. Soc. Biol.* **90**, 640.
DEN DOOREN DE JONG (1931). *Centralbl. f. Bakt.* **120**, 1 and **122**, 277.
TWORT, F. W. (1915). *Lancet*, ii, 1241.
—— (1920). *Brit. J. Exp. Path.* **1**, 237.
—— (1921). *Lancet*, ii, 798.
—— (1922). *Vet. J.* **78**, 283 and 324.
—— (1923). *J. State Med.* **31**, 351.
—— (1930). *Lancet*, ii, 1064.
—— (1931). *Ann. Inst. Pasteur*, **47**, 459.
TWORT, F. W. and TWORT, D. N. (1921). *J. Hygiene*, **20**, 85.
TWORT, F. W. and INGRAM, G. L. Y. (1914). *Centralb. f. Bakt.* **73**, 277.
WOLLMAN, E. (1920). *C.R. Soc. Biol.* **83**, 1478.
—— (1925). *Ann. Inst. Pasteur*, **39**, 739.
—— (1934). *C.R. Acad. Sci.* **198**, 1642.
—— (1935). *Lancet*, ii, 1312.

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