

XXII.—*Bacteriological Research from a Biologist's Point of View.*

By E. KLEIN, M.D., F.R.S., Lecturer on General Anatomy and Physiology in the Medical School of St. Bartholomew's Hospital, London.

THE memorable and classical researches of Pasteur on the relation of micro-organisms to various fermentative processes and to putrefaction have given an active impetus to investigations on the chemical activity of micro-organisms, and during the last 10 or 15 years a great number of facts have been brought to light, showing how enormous and important the rôle is that bacteria and yeasts play in the economy of nature (comp. Professor Frankland's paper, *Trans.*, 1885, 159). A great deal of this knowledge is real, and has been proved to be so by numerous accurate experiments. Thus it has been shown beyond any doubt that alcoholic fermentation is caused by the multiplication and activity of the yeast, known as *Saccharomyces*, that acetic acid fermentation is produced by *Mycoderma aceti*, that lactic acid fermentation is produced by *Bacterium lactis*, and it is also known that putrefaction is caused by a variety of species of bacilli. A good deal, however, of knowledge obtained with regard to chemical processes produced by micro-organisms, although put forward as perfect, on close examination is found to be in a very imperfect state.

I will illustrate this by a few examples, which I could multiply. There can be no doubt that the complex process spoken of as putrefaction of proteïds is due to bacteria, but when we come to inquire which part of the process is due to which bacteria, we receive no definite answer. Putrefaction is a process by which proteïds undergo changes, beginning with the formation of peptones, leading then to the formation of leucine and tyrosine, of indole, skatole, phenol, and a variety of substances belonging to the aromatic series, and further of certain alkaloïds known as ptomaines, ammonia and its salts, and nitrates, with the simultaneous development of sulphuretted hydrogen. Now no one has yet shown whether this whole series of changes is due to one kind of organism, or whether one kind of organism commences the process, and it is then carried on a step further by another kind of organism. We have certain well-established experiments by which it is shown that a certain kind of organism produces the change of sugar into alcohol, that is, the *Saccharomyces cerevisiæ*. After this has finished converting sugar into alcohol, another organism steps in and changes the alcohol into acetic acid. It is probable

that a similarly complex process occurs in putrefaction, that is to say, it is probable that one set of organisms only brings the proteïds down to a certain stage, preparing as it were the ground for another set which then begin their activity.

Another illustration which I can give of the imperfect state of our knowledge is this. The ammoniacal fermentation of urine, consisting as you all know in the conversion of urea into ammonium carbonate, is according to the account given by Pasteur and Cohn, due to a micrococcus called the *Micrococcus ureæ*. Professor Frankland told you in his address (*loc. cit.*, p. 178), that he found in ammoniacal urine a kind of vibrio which he considers to be the ferment of the ammoniacal change. Now I have myself examined a great many samples of urine that have undergone ammoniacal fermentation, but I have not found this vibrio. Had Professor Frankland isolated this vibrio, and, having isolated it by pure cultivations, had he then produced with it the ammoniacal fermentation in sterile urine, we should have been able to say that his so-called *Bacillus ureæ* is also an active agent in the conversion of urea into ammonium carbonate; but not having done so, and merely finding it present in ammoniacal urine, the assertion that it is the cause of the ammoniacal fermentation is open to question. To illustrate, at the same time, how an investigation of this nature ought to be carried out in order to command absolute value, I will mention to you experiments which were conducted last year by Leube and Graser (*Virchow's Archiv*, 100, 3). These gentlemen set themselves the task to determine which organisms produce the ammoniacal fermentation of urine. They proceeded in this way. They took ammoniacal urine, that is to say, urine after it had undergone ammoniacal fermentation, and isolated by careful experiments, in a way which I shall describe more minutely later on, the various bacteria present in such urine. Amongst the great variety of bacteria present, four kinds proved themselves capable of producing the ammoniacal changes of urea; first and foremost, a sort of straight bacillus, thick and rounded at its ends; secondly, a micrococcus identical with the known *Micrococcus ureæ*; the third, but of less pronounced activity, is a small short bacillus, oval in shape; whilst the fourth, still less active, is a very short bacillus, with truncated ends. These four kinds of organisms have been isolated, carried in pure cultivations through many successive generations, their morphological characters well ascertained, so that they might be recognised at once whether present or absent in a given cultivation; and with each species separately, sterile urine or sterile fluids containing urea were inoculated, and the chemical action of the several species studied.

To illustrate, on the other hand, an inconclusive experiment, I will

mention the following examples. We are told by Professor Frankland that, according to Bell, *Mucor racemosus* is capable of producing alcohol out of sugar, that is to say, that it is also an alcoholic ferment. Now as far as I am able to say, this *Mucor racemosus* has not been isolated by exact methods; all that has been ascertained is that *Mucor racemosus* has been sometimes found to be present where alcoholic fermentation has been going on. But if you come to inquire more carefully into the matter, you will find that besides *Mucor racemosus*, *Saccharomyces* has also been present, and one would say with little hesitation that it was the latter organism which produced the alcoholic fermentation, whilst the mucer was merely present accidentally. A few years ago, Dr. Kern (*Biologisches Centralblatt*, II), described a peculiar bacillus, which owing to its containing a spore at each end he named *Dispora*, and because it was found in fermenting milk, used in the Caucasus as a drink under the name of "kephir" or "hippö," it was termed *Dispora Caucasica*. Now of this *Dispora Caucasica* Kern maintained that it is the active ferment in this alcoholic fermentation. Why did he maintain that? Because he found it always present in fluids undergoing this fermentation. Later on he was able to isolate it by pure cultivations, and then he became convinced that this bacillus had nothing to do with the alcoholic fermentation, but that the real agent in this process is a *Saccharomyces*; consequently this *Dispora Caucasica* is merely an accidental concomitant of this particular process. Similar criticisms might be applied to the various organisms which have been mentioned as the causes of the butyric fermentation by Fitz and Bell. As far as at present known, Van Tieghem and Prazmowski's *Clostridium butyricum* is the real ferment in this process. Many of the assertions made as to organisms having caused certain chemical processes are therefore untrustworthy, because they are due to either of the following sources of error: first, working with non-sterilised material; and, secondly, not isolating the organisms and testing their activity. If of any organism it is to be said with something like certainty that it really is the active cause of a specific chemical process, it must be shown, first, that when obtained pure, that is, when isolated it possesses certain well-defined characters; secondly, when introduced in this pure state into a suitable material, it must set up the specific action. These elementary conditions of experimenting are thoroughly appreciated and employed by most pathologists at the present day in their investigations into the relation of disease germs, but I am afraid they have not yet been fully understood by chemical investigators.

I will describe to you the methods which pathologists employ in ascertaining whether a given disease is due to a certain organism.

In the first place, the pathologist does ascertain whether an organism and which organism is present in the diseased tissues. In a variety of ways he studies the characters of such an organism by fresh examination, by methods of staining with various dyes, and by cultures. By this latter method, he not only studies their behaviour in various culture media, but at the same time isolates the organism in order to obtain pure cultivations. Having then obtained unmistakably pure cultivations, he proceeds to inoculate with them suitable animals, and ascertains whether this purified organism is capable of again producing the same disease from which it had originally been derived. And, further, in this so induced disease, the same organism must again be detected; by morphological study, and by the method of culture, it must become clear that in this new infection we meet with exactly the same organism as in the original disease.

I shall describe to you then, first, the method of cultivation and isolation, and, secondly, the method of testing the activity of the purified organism.

Supposing we have to do with the disease known as malignant anthrax: in this disease, the blood is the soil or locality in which the *Bacillus anthracis* finds its most suitable conditions for active growth and multiplication. The blood of an animal dead or dying of malignant anthrax very often contains vast numbers of this *Bacillus anthracis*. Every droplet of such blood inoculated into a suitable animal produces with certainty malignant anthrax and death in this animal. Its blood again teems with the *Bacillus anthracis*. But notwithstanding this we do not yet conclude that this *Bacillus anthracis* is the active ferment of this disease, because the *Bacillus anthracis* may be only an accidental concomitant; there may be another organised or non-organised ferment present, which is the real virus. In order to be able to say that the *Bacillus anthracis* is really the active virus, pathologists have found it necessary to isolate this *Bacillus anthracis* by cultivation. Koch was the first who achieved this cultivation. A drop of blood of an animal dead of anthrax, or a bit of the spleen of such an animal, was introduced into some suitable nourishing medium. He found that the *Bacillus anthracis* soon undergoes rapid multiplication by division, and that after a certain time, only hours sometimes, it formed in its interior bright oval seeds or spores. These spores were then introduced into fresh nourishing media, and they were seen to grow out or to germinate into bacilli, which rapidly multiplied by division. These bacilli, either after they had been forming spores, or before this period had been reached, were transferred to a fresh nourishing medium, in order to obtain a new crop of bacilli, and in this way by a number of successive cultivations he obtained the bacilli in a pure state, that is

to say, he obtained bacilli which had been far removed from their original nidus, *i.e.*, the blood. Now he found that however far removed cultures of the bacilli were, they always have the same action, namely, when introduced into a suitable animal, they invariably produce malignant anthrax and death, the blood and spleen of such animals containing vast numbers of the bacilli. In the same way, Koch proved that the bacillus known as *Bacillus tuberculosis* is the active agent of the pathological process known as tuberculosis.

Fehleisen proved by the same kind of experiments that a micrococcus is the real cause of erysipelas, and I could mention a number of other instances, *e.g.*, septicæmia and swine fever, and many others, where a definite infectious disease has been proved beyond any doubt to be caused by a definite species of organism.

In all these experiments of pure cultivations, the material in which the organism is to grow must at the outset be made sterile, for if by any chance another organism, say a septic organism, happens to be present at the outset, this will suppress the disease organism that is introduced. The sterilisation of nourishing materials is therefore the first and I may say an essential condition for the success of the experiment; and this—namely, the sterilisation of the nutritive material in which the given organism is to grow, and in which it is to be obtained in pure culture—must be the first attempt in all experiments coming under this head. The sterilisation of nutritive materials is always effected by heat. The same of course applies to all vessels, instruments, &c., used in connection with such nourishing materials, the vessels that are to contain the nourishing material and the instruments that are used for the putting in or taking out the organisms. If you have to deal with a given nourishing material, it is necessary after sterilisation, or after supposed sterilisation, to keep it under such conditions as would facilitate the development of organisms. If the material remains unaltered, and barren of organisms, under these conditions you are justified in saying that this material is free from any germs. Having ascertained that this is the case, you introduce your organism which you wish to test in its chemical activity. Of course you have to be careful to introduce only that one organism which is to be tested, that is to say, you have to be careful of accidental contamination with organisms that are not wanted. There is at present a tendency to neglect accidental contamination by organisms contained in the air. You see in many pamphlets, in some books also, that it is recommended when introducing a definite organism into fresh nutritive material, that is to say, when inoculating nutritive material with a given organism, to expose the nutritive material to the free access of air during inoculation. Now there is no doubt that at certain seasons, and at some

places, nutritive materials can be exposed to free access of air, of course for short periods, say seconds, without any actual contamination with air organisms taking place. But anyone who has worked at this kind of research at all seasons of the year, and in a variety of localities, must have found that the danger from accidental contamination with air organisms is a real one. Professor Tyndall has drawn attention in a masterly way to these dangers, and has proved that, however short the time may be during which suitable nourishing materials are exposed to free air, accidental contamination with septic organisms does take place oftener and to a larger extent than can be desired by those who are engaged in such work. In London, free exposure of suitable nutritive materials to air is followed in a certain percentage of cases by contamination with the following organisms:—foremost are the spores of moulds; then *torula* or *saccharomyces*; then *micrococcus* and the spores of *bacilli*.

The isolation of bacteria which happen to be present in a given fluid or solid is achieved in a variety of ways. The method which is now employed to a great extent is that of plate cultivation, first introduced by Koch. It is based on the fact that when a variety of organisms are introduced into nutritive gelatin, previously liquefied and distributed, by shaking, through this gelatin, after the gelatin is allowed to congeal, the different organisms are kept permanently in different places. Then the conditions under which the gelatin is placed are such that organisms can multiply, it is found that each one of those different organisms will give rise to a colony made up of members of its own kind. In this way, it can soon be ascertained which kind of organisms has been introduced, and even how many different kinds have been introduced, provided that the original number introduced is not too large, so that the different colonies making their appearance in the gelatin remain sufficiently isolated. This is practically the method which Dr. Angus Smith first used for the study of bacteria in water. The better plan is that adopted by Koch, namely, after the introduction of the bacteria mixture into the liquefied gelatin, the latter is poured out on glass plates or similar vessels, previously sterilised, and then kept in a closed moist chamber. The gelatin of course is allowed to set, and is kept in the solid state, that is to say, the temperature at which these gelatin plates are kept must not be higher than the temperature at which the gelatin remains solid. After a few days, a number of spots are formed on these plates, each spot corresponding to a colony; the different colonies, when due to different species, show different characters (size, colour, liquefaction or not of the gelatin, &c., &c.) already to the unaided eye. From such plate cultivation, pure cultures can be made by inoculating from any of these different colonies fresh nutritive material contained in flasks

or test-tubes, or any other vessel, of course the nutritive material having been previously sterilised. This is the simplest method of isolation, and the one at present the most employed. It has its drawbacks, and it has its great advantages. The drawbacks are chiefly these: first, accidental contamination with air organisms is not excluded. You may therefore obtain a number of colonies in your plate cultivations which have not been represented in the fluid from which you derived your bacteria. This may not count for much when you have to deal with the isolation of an organism the special characters of growth of which you have previously already ascertained, for you may easily see whether in the plate cultivation your organism has made its appearance, and it makes very little difference whether there are any others present, or how many, but it is of considerable importance when you are first searching for and ascertaining the existence of an organism the characters of which you do not yet know.

Another disadvantage of this method is that although many organisms are capable of growth in nutritive gelatin, such as is commonly used, there are other organisms, and some very important organisms, at any rate important to pathologists, which refuse to grow in gelatin. Again, there are other organisms which, although not incapable of growth in gelatin, will not grow in it, or far too slowly, at the temperature at which gelatin remains solid; they require much higher temperatures in order to develop into colonies that can be recognised by the unaided eye. These disadvantages apply also to water analysis by plate cultivations, such as have been minutely described and extensively used by Dr. Percy Frankland.

Another very good method of isolation is that by "dilution." In this method a given number of different bacteria are distributed over or in a large quantity of previously sterilised indifferent fluid, so that a definite quantity of this fluid contains only one organism. With this definite quantity of fluid nutritive material is inoculated, and then such nutritive material becomes the seat of the growth of that one kind of organism. This is the method which was first used by v. Naegeli, and afterwards with great success by Lister, in his investigations of *Bacterium lactis*.

A third method of isolation is that known under the name of "fractional cultivation," first used by Klebs. In this method the fact is made use of that different organisms grow to a different degree in different nutritive media. It is within the knowledge of everybody who has worked with bacteria, that different bacteria require different materials to grow in—some grow better than others in a given nutritive medium. Now supposing we have a fluid or a solid which contains, as the microscope proves, a variety of bacteria,



on inoculating with this mixture of bacteria one and the same kind of simple nutritive medium contained in several test-tubes, and exposing these to suitable conditions of growth, it will be found that after one or two days there is present in this particular nutritive medium the offspring, not of all the organisms that have been introduced, but pre-eminently and predominantly, perhaps, only those of one species. If that be the case, there is no difficulty in obtaining pure cultures from this one species, provided we use only a trace of this growth for the new cultivation. The chances are that by inoculating a trace of that growth into a fresh nutritive material of the same kind as above, we introduce only one species, because one species is only present predominately. Growing different bacteria in different media in this way, it is then found that they multiply or grow with different degrees of rapidity. In a similar way success may be achieved by adding to or subtracting from the nutritive medium certain substances.

In starting the cultivation of organisms, various methods are used for inoculation. There is Koch's method, by which a platinum wire, heated and subsequently cooled, is used to introduce the trace of the organism into nutritive gelatin or other material, contained in test-tubes, plugged with sterile cotton-wool. In this process, the cotton-wool plug is altogether removed. The test-tube containing the nutritive material, which is solid, is inverted, and then the inoculation is performed with the platinum wire by pushing the infected end of this wire into the solid material; the plug is replaced, the test-tube again put upright and exposed to the suitable conditions favouring the growth of the organism. In this process of inoculation, the chances of accidental contamination are of course not avoided. In the summer months, and in London, this method of inoculation is followed in a considerable percentage of cases by undesired accidental contamination.

I myself prefer the plan in which accidental contamination is reduced almost to *nil*. I take a pointed capillary pipette, freshly made, pass one end through the cotton-wool plug (previously drawn up halfway) down into the culture tube from which inoculation is to be made, allow a drop to ascend into the capillary tube, withdraw it, and pass it through the cotton-wool plug into the tube in which growth is to be started. In this way the chances of accidental contamination, owing to the lifting out of the plug, are reduced to the smallest dimensions, provided, of course, that the cotton-wool plug is sterile. By the plate cultivation, and by cultivations in test-tubes, the characters, both morphological and of the mode of growth, can be studied and ascertained; and it will be found that many different species have in this respect different characters. Some grow in spots, others in flat patches, others as films, uniform or com-



posed of minute droplets; some grow best in the depth, others best on the surface; some liquefy gelatin, others do not liquefy it, and so on.

Having, then, ascertained the characters of an organism, and obtained it in pure cultivations, the organisms are then tested for their activity by inoculating with them the suitable nutritive material. If this organism, by multiplication in this material, is capable of starting a specific chemical action in this new suitable material, then we are bound to say that it is this organism which is the cause of the specific action. The whole chain of evidence must be complete in the sense which I have indicated, in the sense in which it is considered as complete by pathologists of the present day, and only then, and not till then, will the statements put forward by chemists command that value which they claim. Not till chemists come to look upon the matter in the same light in which we look upon it, namely, to obtain the organisms pure, to render nutritive material sterile, to be able to produce with this pure organism the specific chemical activity you wish to obtain—not till you have fulfilled all these conditions, can you claim to have established the fact that a definite organism is the cause of a definite chemical process.

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