growing species. The conception "species" would then comprise all the individuals possessed of all the variations in morphologic and biochemic properties, stated in the sporogeneous descendants of a single individual. Several moulds, hitherto considered as belonging to different species, will probably find their place between the limits of variation of other forms. It may furthermore be expected, that many cases of pleomorphy, and the doubt respecting the affinity of the Trichophyton group and the Favus moulds, will find their solution in that direction.

A particular attraction will be lent to such researches by the fact, that the chemical properties of the examined organisms can much more easily be observed than in the higher plants.

Besides the fungi there are, however, many other low organisms which multiply partly vegetatively (by division), partly sporogenously (by fructification). To these the bacteria belong, and here much has been discovered about the variability of properties, which has given rise to a sometimes very doubtful distinction of stocks, varieties, related forms, etc, and moreover to a great uncertainty as to the affinity of these forms and the possibility of their mutual transition.

If a nearer research might prove that, just as for the here examined lower fungus, also for bacteria and other organisms multiplation by spores produces stocks with another combination of properties than by direct division, an extensive field of investigation might possibly be opened.

A practical, simple method of arranging one-cell cultures, whereby the organisms remain vigorous and possessed of their normal properties is therefore required.

For that reason I will describe the method which enabled me to make the above observations.

Microbiology. — *"Method to cultivate micro-organisms from one cell."* By Prof. A. W. NIEUWENHUIS. (Communicated by Prof. M. W. BEIJERINCK).

(Communicated in the meeting of October 29, 1910).

Among the characteristics of the present period of investigation of micro-organisms and the application of the thereby obtained results in behalf of the life of modern society, this is certainly a salient one, that by the more profound study of the properties of those smallest of beings a great uncertainty regarding the constancy of their life functions becomes prevalent, in connection with doubts about

the independence and the more or less narrow affinity of certain, for instance of pathogenic and non-pathogenic species. To cite only a few examples, I mention, on account of their practical importance, the doubt about the constancy and affinity of many yeast species, of typhus and paratyphus bacilli and bacterium coli, and in mycology the contest as to the unity or plurality of the Favus- and Trichophyton moulds, especially in relation to their geographical distribution in the temperate zones.

Since the parasitic diseases in the tropics have been scientifically studied the existing uncertainty concerning organisms, such as typhus- and dysentery bacilli, still increases.

The important results obtained notwithstanding, which have become so valuable for industry, public health and physic, have somewhat broken the conviction that a solid, scientific insight into the morphological and biological properties of micro-organisms is necessary.

The great technical difficulties accompanying an investigation into the life conditions of some smaller microbes, have still more weakened that conviction.

For those, however, who seek the solution of the theoretical questions which are to form the firm base for important social measures, the study of the organisms themselves and of their properties continues to be a prominent demand.

Only recently Professor Dr. D.A. DE JONG, in his treatise "De Tuberkelbacillus", published as special number to the 4th year of "Tuberculose, het orgaan van de Centrale Vereeniging tot bestrijding der Tuberculose" pleaded eloquently for a conscientious investigation into the properties of the tubercle bacillus. How many important problems are still to be solved thereabout is shown even by some heads of this treatise as: 2. Properties of the tubercle bacillus in relation to the diagnosis; 3. Pseudo tubercle bacilli; 4. Culture of tubercle and pseudo tubercle bacilli; 5. Morphology of tubercle and pseudo tubercle bacilli; 6. Differences of tubercle bacilli mutually; 7 Tubercle bacilli of cold blooded animals. From the contents we further see, that also for these questions, touching the very heart of the matter, the differences of views are still very numerous.

For attaining our present standpoint, PASTEUR in the first place showed us the way by his evolutionary researches, and the great progress dates from the time when KOCH introduced his solid medium. According to many, however, this expedient does not afford the support, wanted for the following step to take on the long way before us of bacteriological and mycological research. Numerous are hence the other methods of investigation, whereby use is made of

biological properties of the organisms to state their independent existence or their affinity, for instance of serum reactions (agglutination, precipitation, etc.) together with color methods. Hitherto these methods could not, however, solve very grave questions, and, by their number, they prove in some way how little efficacious they are in certain cases.

A long time already there have been hopes for the great support of a practical method to carry out a one-cell culture under most varied circumstances, not only to observe the properties of the individuals among the micro-organisms, or their affinity or similarity to others, but also to cultivate organisms, which are difficult to study, because they cannot grow on solid media and should therefore be transferred from liquid to liquid. How much the want of such a means is felt, is proved by a few citations, referring to methods for one-cell culture already published before. When describing the method of S. L. SCHOUTEN, E. KÜSTER declares in his "Kultur der Microorganismen, Leipzig und Berlin 1907": "Eine ingeniöse Methode, welche die KOCH'sche zu ergänzen berufen sein könnte...." and when discussing a similar method of MARSHALL A. BARBER, H. PRINGSHEIM declares in the "Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten", Bd. 23, N. 6/9, Abteilung II: "Vielleicht ist die Methode BARBER's in noch höherem Grade, als der Autor annimmt, dazu bestimmt von den Einzelzellen ausgehend zu Reinkulturen solcher Microorganismen, wie z. B. mancher Flagellaten, Algen und Diatomeen zu kommen, die wegen ihrer Empfindlichkeit nur schwer in Anhäufungskulturen zu gewinnen sind und die sich deshalb von Bakterien und Schimmelpilzen nur schwer trennen lassen".

Such researches about the properties and affinities of certain organisms, cultivated strictly from one cell, were carried out by E. B. HANSEN in the Carlsberg-Laboratory near Copenhagen, relatively to some yeast species. They have given much insight into the properties of these yeasts and exerted a great influence in the brewing industry. With his method of working, which reposed on the detecting and noting of separate yeast-cells in a liquid medium, the relatively large size of a yeast-cell enabled him to apply a microscopical magnification of $\pm$ 60. Also for the modification devised by LINDNER such a large organism is required. Much smaller organisms, such as mould spores and bacteria cannot be found in this way and for these another method is necessary.

A practically good method to arrange one-cell cultures should answer the following requirements:

It must be fit to be applied with magnifications of 300 and higher; this needs no further explanation.

2. The organism to be isolated must be injured neither by chemical nor physical stimulants.

This is a demand which for other methods of research, too, is by no means generally recognised. It rests on the fact that as well higher plants as lower organisms react on even apparently insignificant stimuli, for example on a slight modification in nutrition, with a considerable deviation in their functions. Especially in biological isolation methods this principle is sometimes earnestly sinned against. As soon as temperatures to even 80° C., or chemical substances are used to kill other organisms present at the same time, it must be accepted that the remaining individuals are no more normal.

3. The greatest possible simplicity in the application is required, so that the method is within the reach of every experimenter, not exacting too much of personal dexterity, patience or time.

4. An easy maintaining of asepsis in the research. This, also, is sufficiently clear.

These demands are not satified by any of the hitherto published methods of research with one-cell culture. S. L. SCHOUTEN (1901), isolates the cells from suspensions in hanging drops at large magnification by means of two needles. MARSHALL A. BARBER (1907), does the same with fine glass capillaries and R. BURRI (1907) uses little drops of East-Indian ink.

The very ingenious method of S. L. SCHOUTEN is for a general application much too complex, as it requires too much from the dexterity and patience of the investigator; on account of the long time, too, wanted in the application it would be troublesome, even for a skilful experimenter, frequently to use it. Moreover, it has the drawback that the extremely fine, artfully made glass needles must be sterilised. How difficult this is with frequent use without stimulating and enfeebling the concerned micro-organisms by chemicals, the inventor himself proves on page 113 of his treatise.

MARSHALL A. BARBER substituted capillaries for SCHOUTEN's needles; with these he draws by suction some organism from a hanging drop. Especially when working with impure material the sterilisation of these capillaries must occasion still greater difficulties than SCHOUTEN's needles.

The "Tusche-method" of BURRI is again a culture method on solid substrata, in so far as it is used for the multiplication of an organism, whereas it does not properly effect the isolation of a single individual.

The above research on the "Individuality and Heredity of Tricho-

phyton albiscicans" I carried out by means of a one-cell culture method, which possesses all the above requirements. It can be applied with large magnifications (I used those of 300 und 450), and requires a glass needle of easy make and every time a new one, which renders disinfection unnecessary. The management does not require much dexterity and time, nor very complex instruments, whilst asepsis can easily be maintained.

Likewise as by the methods of S. L. SCHOUTEN and MARSHALL A. BARBER, I isolated the concerned organism from a drop of the suspended material hanging under the cover-glass, only with one needle, to subsequently transfer it to a drop of nutrient liquid in which the preliminary or the whole further development can take place. The two drops are hanging side by side under the cover-glass. The glass needle *a* is fixed on a stand *b* on which it can be moved mechanically in every direction by three micrometer screws (Plate 3).

As Plate 4 shows this stand can be placed beside every microscope *f*; the needle *a* should be so long that the point can be placed in the axe of the microscope. The end of the glass rod wherewith the isolation is effected, is a glass globule which may differ in size in accordance with that of the organism to be isolated.

At the previous research, whereby mould-spores of 2—$2^1/_2\,\mu$ and mycelium cells of 1—$1^1/_2\,\mu$ thickness, but of greater length, were transferred, I used globules of 20—30 μ diameter.

As to the execution of the isolation the following observations may be made.

a. The material from which the organism is to be isolated is distributed in a sterile liquid, in such a way, that it is suspended very finely divided, so that the spores in the drop under the microscope do not get too near one another, or too near strange ones. How this is to be contrived depends on the concerned material and may be arranged at will. I did it on a sterile object-slide in a flat glass box, likewise freed from germs. On the slide a drop of nutrient liquid was laid and with an iron needle a number of spores were distributed in it. To divide a bit of mycelium two iron needles were wanted.

b. The cover-glass on which a little of this material must be put, may be of the usual form, for example 22 × 26 mm.; it is, after a careful cleaning, very thinly smeared with pure vaseline on one side, and subsequently freed from germs by quickly passing that side a few times through a gas flame. The layer of vaseline should be extremely thin, as it only serves to prevent the drops from flowing over the glass surface.

It is advisable to prepare beforehand in a sterilised glass box a

sufficient number of these cover-glasses, for example for one day.

c. As nutrient liquid I used that which had proved most favorable for the organism. In order to exert no weakening influence I used the same liquid for mixing; in the above research 4 parts of glucose, 1 peptone, 100 water

d. A copper rim must serve as support for the cover-glass under the microscope, for as the experimenter works with the glass needle under the cover-glass with the hanging drops he wants a rather large free space. The most practical I found to be a copper rim 8 mm. high of the shape of three sides of a rectangle, having a side length of 18 mm. and from 1 5 to 2 mm. thick. The fourth open side serves for the introduction of the glass needle and for its movement.

To easily move the cover-glass I cement the copper rim with some vaseline on an ordinary object-slide lying on the stage. By greasing the flat top side of the copper square with a little vaseline, the coverglass adheres somewhat to it, which is desirable though not necessary.

e. The glass needle with which the isolation of the organism is effected is so simple of shape, and can so easily be made, that it implies the possibility of an extensive application of my method and much advantage over other needles. The part of the needle properly used is its terminal portion, which has the shape of a globule, in my investigation of 20—30 μ diameter, which dimension may, however, be varied according to the size of the organism. The foremost part was drawn out to $\pm$ 10 μ thickness and over a length of from 4—5 mm. bent upwards, with the globule at the top.

The making of such a needle is done as follows: two ordinary glass rods or tubes $\pm$ 4 mm. thick and $\pm$ 15 cm. long which touch each other with their ends, are melted together in a gas flame, then the still soft middle part is drawn out to 1 mm. thickness. After cooling the rod is divided into two by breaking it just in the middle.

To make the fine points a microburner is used and the flame lowered to a height of 2—3 mm. so that no yellow central part is seen. In this low flame the ends of the two glass rods are held in contact with each other. When half liquid they stick together and are drawn out to a thread of $\pm$ 10 μ thickness. After cooling and breaking in the middle, one has two of the desired needles but without terminal globule As the extremely delicate points must not lie in the axe of the rods but should be directed upwards, the drawing must be contrived so as to bring the needles in the same

plane at angles of ± 120°. The finest portions of the point then get also this direction.

The terminal globule is simply made by passing the broken end of the 10 μ thick glass thread so quickly through the microflame, that it just for a moment has a yellow blaze. If it is done too slowly the hair-thin glass thread melts into too large a globule.

When drawing out the glass to a thread of about 10 μ it often breaks or the ends get a somewhat irregular shape, particularly when the drawing goes too far. After the globule has been formed the point sometimes needs improvement on account of an abnormal bent. A very simple manner is then to keep the end of the needle for a moment over the flame, it grows soft and the rising current of hot air may place it in the right position.

After a few trials the making of the needles is quite easy for every experimenter.

Now that every one is able after a little trying to construct the desired needle within some ten minutes or less, without any other implements but a few glass rods and a microburner, it is in the first place possible to repair a needle that has become useless by refounding it, instead of by disinfection, further it may be replaced by others easily made in store.

f. There are diverse stands with which the thereon adjusted objects may very slowly and regularly be moved in three directions by means of micrometer screws; if they have only about the height of the stage of a microscope they may be rendered serviceable to this method by supplying them with a glassneedle. As Plate 4 shows, such a stand *b* is placed quite free beside a microscope *f*, only the needle *a* must reach to a certain height over the table that it may be placed with its point under the objective.

The stand used by me is shown on Plate 3 in all its parts, and consists of a foot *j*, on which a column *h* may be raised by screw *d*; on table *i*, adjusted on this column, is a sliding-piece destined for the fixation of the needle-holder and movable to the right and the left by screw *c*. The movement backwards and forwards is effected by screw *e*, which makes the whole upper part of the foot turn round on its base. All these movements are regulated by spiral-springs, which counteract the movements of the screws. The needle holders of which two, with their needles fixed by means of gypsum, lie beside the stand, are placed loose on the sliding-piece and are fastened by two pins in corresponding holes. The whole is made of copper. The up and downward movements, caused by screw *d*, were accompanied in this stand with a slight rotation; for

the rest the movements of the point of the needle *a*, as seen under the microscope, were quite-regular, so that the apparatus proved very useful.

g. Any microscope, either with or without a nose piece may be adapted to this isolation method if only the room between the objective and the stage is large enough to place in it the glass slide, with the copper square and the cover-glass with the two drops. If the stand is not constructed for the microscope in use, the desired height can be obtained by placing disks of the required thickness under the base.

As may be seen on Plate 4, a movable stage of ZEISS was adjusted on the microscope *f*, by which the slide *l* and thus the copper square *m*, too, and the cover-glass *n* may be quite regularly moved in the horizontal plane. The movements of the ZEISS instruments I applied later instead of those of the stand as the screws ran more gently. With the stand only, without the movable stage *k* the method can still be very well carried out.

h. Finally, for moist chamber I made use of the following simple arrangement which can easily be sterilised. An objective is sterilised in a gas flame and subsequently placed on a $\pm$ 7 m.m. high glass ring of 20 m.m. diameter inside and 1 m.m. thick. By holding such a ring with a forceps in the flame, it is soon freed from germs, and in that warm state greased with vaseline on one side it adheres well to the glass slide and prevents the air from entering. In a sterilised glass box a whole series of such moist chambers may be kept in store for some research. To cultivate in it the organism in the hanging drop, the top edge is also rubbed with some vaseline and the cover glass pressed so much that, also here all access of air is excluded.

At first, as is often advised, I put a drop of liquid on the bottom of the room to prevent evaporisation of the culture drop. This, however, gives some trouble by the condensation of vapour on the under surface of the cover-glass, which does not occur when the drop at the bottom is left out. When a hanging drop of for instance 4—5 m.m. diameter and $1^1/_2$—2 m.m. thick is used, the slight evaporisation wanted to saturate the small room I never found injurious. Vapour at the bottom of the chamber is sufficiently prevented by placing it on a solid object in the warmed room by which the glass-slide becomes warmer than the coverglass.

The isolation and subsequent cultivation of an organism are managed as follows:

First the objective, with the copper square fixed on it with vaseline, is placed on the stage of the microscope so that its axis passes

through the centre of the square and the open side is directed to one of the sides, as on Plate 4 to the left.

Now the stand *b* with the needle *a* is placed on the left side of the stage, in such a way, that the needle reaches over that side, its point being placed under the objective within the copper square (fig. 4). It is further desirable already now to bring the head of the needle in the axis of the microscope, which after some practice under control of magnifications of for instance 50, may be done with the hand or by means of the micrometer screws. When the head of the needle is in the desired position, the glass rod *a* is so far lowered by the corresponding screw, that its head is somewhat below the top edge of the copper square, whereby the head, of course, continues to move in the axis of the microscope. When now the cover-glass with the hanging drops is put in position the drops do not touch the head.

The cover-glass under which the organism is to be isolated, has been described before (see *b*). The hanging drops are transferred to it with the following precautions. With a sterilised glass rod a drop from a tube of germ-free nutrient liquid is laid on the fatted side, which drop is to serve for the cultivation of the organism and will therefore be called culture drop. Its size is best at 4 mm. diameter and $1^1/_2$—2 mm. thickness. With the same glass rod a small quantity of the suspended material can now be taken up and placed at little distance, for instance 1—$1^1/_2$ mm., as second or "material drop" beside the culture drop. It is best to give the material drop an oblong shape, so that a long side may be turned towards the culture drop. Micro-organisms situated near the edge are most easily isolated, and the small distance between the drops is desirable to make the way for the organism to be isolated as short as possible. This cover-glass supplied with two drops is now removed with a forceps from under the sterile cover glass under which they were formed, then quickly inverted, so that the drops do not coalesce which, however, does not easily occur on the fatted side; the cover-glass *n* with the two now hanging drops is placed on the copper square in such a way that the drops are in the middle and do not touch the edges.

By removing the glass slide *l*, eventually with the movable stage, it is possible to bring the space between the two drops in the axe of the microscope, which is easily controlled by fixing the edges of the drop.

After these preparatives the transference of an organism must be conducted as follows: The simplest way is to pick up the indivi-

dual to be transferred at the side of the material drop which is turned towards the culture drop. Then the needle is screwed up by *e* so much that the rounded point is vaguely visible and consequently lies beneath the field.

The transferring itself reposes upon this, that, when the point of the needle is placed in the material drop, just against the greased under surface of the cover-glass, and the needle is then horizontally moved by screw *e* out of the drop, it carries along with it over the cover-glass a small quantity of the liquid, and at the same time the corpuscles present in it. The size of the droplet depends on that of the glass globule, the nature of the liquid, the degree of greasing, etc., but with the above given proportions of a globule of 20—30 μ, the quantity of liquid, when moving slowly, is large enough to carry along a suspended spore of $2^1/_2$ μ. By the greasing the liquid will, soon after its exit from the edge of the material drop, contract at the cover-glass into a droplet, in consequence of which it does not flatten out on its way. If now by means of screw *e* the globule is continually moved in the direction of the near culture drop, the latter and the droplet containing the spore will soon coalesce.

If thus the point of the needle is placed in the material drop in contact with the organism to be transferred (for easily working it is best in the centre of the field), it can in the way described be conveyed to the "culture drop" and there develop uninjured.

That the particles to be conveyed should be as far from each other as possible in the "material drop" is evident and explains the necessity of strong dilution.

The easiest way is to isolate an individual from a pure culture; however, from a mixture it is also very well possible, and as all can, to a certain extent, be controlled, at any magnification, contamination of the culture drop seldom occurs after some practice. Continued suspension of the organism during the transference is most desirable, but when the way becomes too long the quantity of the liquid diminishes by the lagging droplets, and a spore, for instance, will adhere to the under surface of the cover-glass. By placing the point of the needle behind it, then moving again, such a particle is carried along, but the less frequently this occurs the better on account of possible injury. This explains why the two drops are placed as close as possible to each other. Especially long, slender organisms are strongly apt to adhere.

The greasing with vaseline has also much influence in this respect, the thicker the layer the more trouble it causes. Contamination of the needle by the vaseline cannot be wholly avoided and cleaning

is very troublesome. hence a simple refounding of the needle to obtain a new pure one is desirable.

As soon as the organism is arrived in the culture drop the glass needle *a* is lowered by means of screw *e*, and the transported organism now lies free in the culture drop. Before the cover-glass can be placed on the moist room for further development of the germ, the material drop must be removed. This is done by means of a piece of filter paper, sterilised by being passed through the gas flame. The cover-glass is removed with a forceps from the copper square, and then a point of the filter paper is cautiously held in the bottom part of the material drop, which is quickly sucked up. Minimal rests of moist do no harm. By placing the cover-glass with the hanging drop in the moist chamber, as aforesaid, with addition of some vaseline, one has an opportunity to make the development proceed either with or without control of the microscope.

When working with strongly contaminated material, so that it is hardly possible to isolate the wished for organism from the material drop, it is convenient to place a "washing drop" closely beside the two others. With the greatest care, the spore is then first conveyed from the "material drop" to the sterile "washing drop" in the above manner. With the point of the needle the spore is conducted through the latter, whereby other accompanying organisms may be left behind and, with the same needle, or if this is supposed to be contaminated, with a new one, the spore is led into the culture drop. Material- and washingdrop are removed with the filter paper before the organism is placed in the moist chamber.

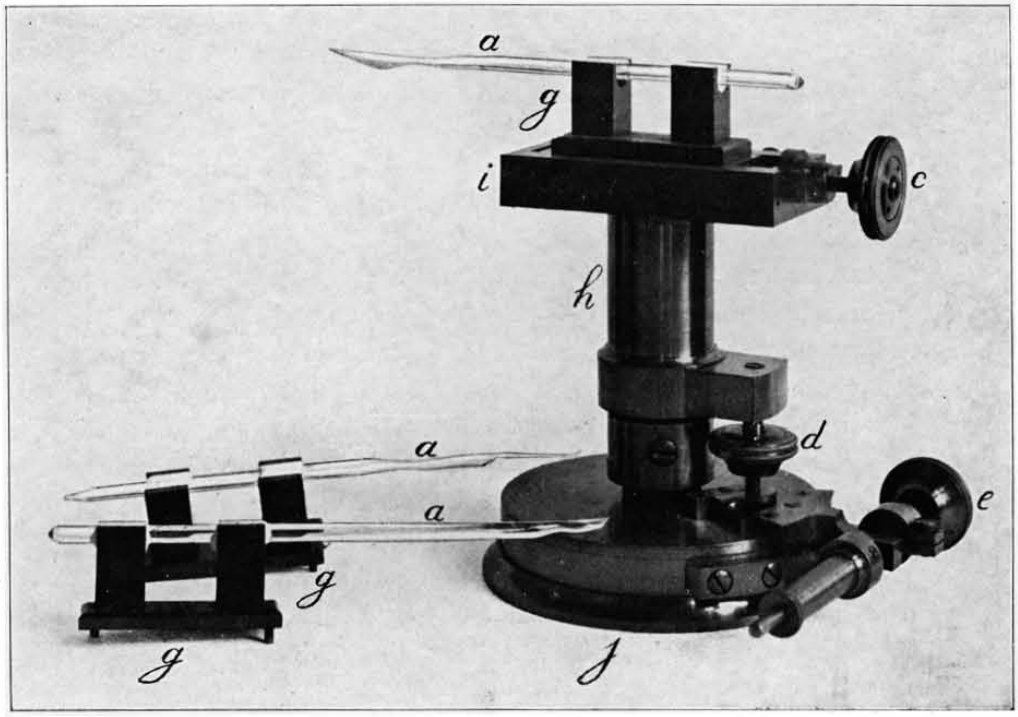
The relative simplicity of this method renders it possible to modify it according to circumstances, which will certainly further an extensive application.

Such a modification of the foregoing manipulation I have devised myself by using the movable stage of ZEISS *k*. With this it is easy to move the slide *e*, and hence the cover-glass *n* with the hanging drops, in the horizontal plane. For the isolation proper of the organism it is the same if either the needle or the cover-glass is moved. It depends chiefly on the fine workmanship of the used instruments which method is to be preferred. On Plate 4 we see the micoscope and stand with the movable stage of ZEISS.

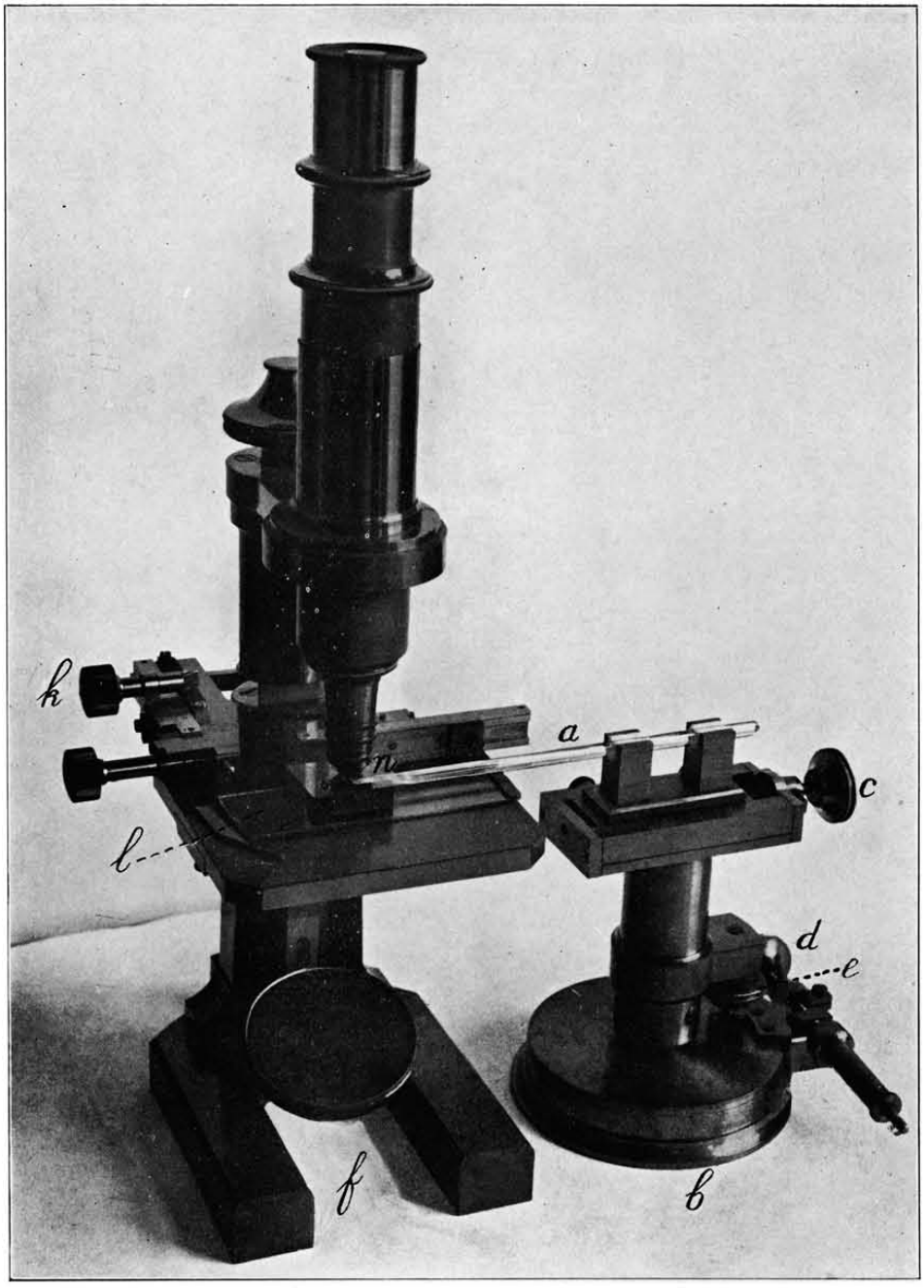
I now wish to bring my kindest thanks to my colleague VAN ITALLIE for his receiving me in his Laboratory, and for his help and sympathy.

A. W. NIEUWENHUIS. "Method to cultivate micro-organisms from one cell."

Plate 3.



Copper stand for the movement of the glass needle a in three directions.



Microscope and stand for the separation of a microorganism and the culture from one cell.