STUDIES ON THE GROWTH OF BACILLUS MEGATHERIUM DE BARY

by

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(with Tab. I).

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CHAPTER I.

STATEMENT OF THE PROBLEM.

It is a well-known fact that bacteria are able to develop under very extreme conditions. The influence of concentrated salt solutions on bacterial growth was investigated by Miss Hof, Estor and others. These authors were able to show that growth in concentrated salt-solutions depends upon the *density of infection.* No growth occured when a small amount of inoculation was used. Moreover the *incubation-time increased* with the salt-concentration of the medium.

It has been my aim to follow quantitatively the growth of an ordinary soil-bacterium in salt-solutions of various concentrations, in order to obtain a possible answer to the following questions;

1) In which way are growth-rate and maximal yield influenced by the salt-concentration?
2) What is the influence of the amount of inoculum?
3) What is the cause of the variability in incubation-time?

This inquiry might yield information to settle the question whether bacteria may *adapt* themselves to a new environment (with a concomittant change in their properties) or whether we are dealing with a *selection* of variants from a population.

CHAPTER II.

SURVEY OF THE LITERATURE.

§ 1. *The action of salt on bacterial growth.*

The literature on the influence of salt on bacterial growth, is both extensive and scattered. In this survey only a few more recent articles, which pertain to our own problem, shall be dealt with. It appears, in the first place, that *the growth-rate is decreased* by the presence of salt in the medium. On this point nearly all authors agree. (Eisenberg 1919, Estor 1927, Petter 1932, Hof 1935). Also *the incubationtime is much lengthened* by salt. Furthermore, *a heavy infection* of the medium *yielded better growth* of both

Bacillus anthracis and *Corynebacterium pseudodiphtheriae*, in high salt concentrations. (Eisenberg 1919). Slemmons (1926) had the same experience with *Bacterium coli* [1].

Young cultures (often called "young cells" by the authors) appeared to be *more sensitive than old ones.* This was established for *B. coli* and *Proteus vulgaris* by Sherman and co-workers (1923) and by Schultz and Ritz (1910) for *B. coli.* In these experiments a culture is considered as young when it is 3—6 hours old.

Kluyver and Baars (1932) met with the fact that *Vibrio (Sporovibrio) desulfuricans is more halotolerant when freshly isolated.* Old laboratory-cultures did not grow so well at lower temperatures.

Zobell and co-workers (1938), however, found quite the opposite when trying to grow marine bacteria on saltless media. When they cultivated their strains in solutions of decreasing percentage of seawater and nutrient-broth, they could not reach a lower percentage than 25 % seawater. But the strains that remained in the refrigerator for five months grew directly in saltless media. The authors offer the explanation, that by constant reinoculation they were dealing with "young" cultures and therefore with less resistent cells.

The results of Kluyver and Baars were corroborated, recently, by Starkey (1938) who found the anaerobic, sulfate-reducing organism to grow in a very different cell-shape at different temperature. At 55° it formed spores. Sewage and mud, heated over 98° before inoculation, yielded growth showing the sporulating form to pre-exist in nature. The sporulating form could be transformed into the non-sporulating form by gradual decrease of temperature. Also the common type, without spores, could be changed into the thermophilic race by gradually increasing the temperature. Cultures from the sporulating race, however, were able to grow directly at a temperature of 30°.

The *total yield* in various salt-solutions was measured by Estor (1927) for *Bacillus megatherium*, nephelometrically. The yield proved to decrease with increasing salt-concentration.

Hof (1935) could show for various types of bacteria that pure cultures *yielded forms of a lower halotolerance than enrichment-cultures.* She was able to isolate obligate-halophilic forms from garden-soil. Urea bacteria, for instance, isolated in a medium containing 24 % NaCl could not be grown in saltless media. She is inclined to consider the forms as something "new", something like a "Dauermodifikation" (Jollos 1921).

[1] I wish to express my sincere thanks to Dr. W. Slemmons, Oconowomoc, Wisc., U.S.A., who kindly send me a copy of his original data.

Kluyver and Baars (1932) interpret the halophilic and thermophilic organisms isolated from fresh and cold water by the assumption of a "heriditary change" in the potentialities of the bacteria caused by the new environment. They use the name "physiological artefacts".

Apart from these physiological changes we find a tendency to morphological changes of the bacterial cells in salt-solutions. Petter (1932) gives a survey of the literature on this subject. Eisenberg (1914) observed multicellular forms of *B. anthracis* and *Corynebacter*. Matzuschita (1910) and Hof (1935) mention the increase in size of the cells, Miss Smith (1933) a changed colour of the colony on agar-plates, working with *B. megatherium*. All authors agree that the changes disappear after re-inocculation in saltless media.

Therefore, with increasing salt-concentration, apparently

1) division-rhythm is retarded,

2) less cells survive.

From these two assumptions, the greater part of the above facts follow.

The interpretation of Kluyver and Baars, as well as that of Hof of the phenomena described by them, seems, however, to contain arbitrary elements. For, if in a statistical population of bacteria (a culture) all descendants of a single cell are considered, variability of the variants should be taken into account.

Suppose we have a unicellular alga which multiplies by division, of which some of the descendants lack chlorophyll in their plastids. These descendants are only able to multiply in organic media and in the usual, inorganic media they do not come to the fore. It may be assumed, with equal right, that the variability in the potentiality of a family of cells is such that an extreme milieu might select certain variants, which are unable to grow under "ordinary" conditions. As it is a well known fact that the milieu is an important factor for the characteristics, both morphological and physiological, of a culture, it must be born in mind how dangerous it may be to discuss the properties of a bacterium in its natural habitat, the soil, when only information is available concerning its properties in the pure-culture tube.

The reasoning stated above may also account for the failure of various authors to raise halotolerant forms from "young cells" (read "young cultures"). For the variability should increase with the number of variants and in an old culture the chance to meet an extreme variant is much greater. The success of a heavy inoculation

points to the same explanation. Slemmons (1926) expresses a point of view almost identical with ours.

As to the retardation of growth-rate, increase of incubation-time and decrease of crop-yield, they are factors which can be brought back, directly or indirectly, to a retarded division-rhythm. As this rhythm is strongly influenced by the velocity of the water-intake, salt may exert here an osmotic influence, while swelling-processes may also play a part (Petter, 1932). The occurrence of aberrant forms, especially in higher concentrations, show that these influences are by no means the only ones, however.

§ 2. *On the inadequacy of present nomenclature.*

In the above paragraphs we have met with *adaptation* and *selection*, both concepts used in phylogeny and genetics. However, these terms in themselves might apply to organisms which have nothing to do with genetics, as genetics is a science mainly dealing with the results of sexual reproduction.

We might adapt an organism to a new environment, without changing its genetical characteristics, we might select one individual from a population, by extreme milieu, and still not change its genetical make-up. And even when we do change its *genome*, the thesis might be defended that these changes fall outside the scope of present-day genetics, as genetical concepts are so closely interwoven with the combination and redistribution of characters by sexual reproduction that all of its nomenclature is pre-empted.

When a seed is X-rayed and a haploid dwarf-plant ensues, there is no term to express this phenomenon for if it is called "mutation", the word is used in quite an other sense than de Vries originally meant. Mutation, as a fixed heriditary change, only occurs in a strict sense in organisms subject to the laws of heredity or in other words, organisms in which sexual reproduction takes place.

As it seems best to reserve the word "mutation" for organisms which reproduce themselves by means of gamete-combination, the term "mutation" does not apply to changes that might occur in the offspring of vegetatively-developing cells. As bacteria, as far as we know, do not possess sexual reproduction, the words "mutation" and "mutilation" (van Loghem 1922, 1928) in bacteria should be replaced. A much better word is "Klonumbildung" as proposed by Lehmann (1916).

So, in studying the laws of constancy and variability, we have to seek analoga in vegetatively propagating cells of higher organisms. In sexually reproducing organisms we can find an analogon in the variability of the offspring of a "pure-line" (Johannsen) under

different milieu-condition, where the genotype remains always the same by self-fertilisation and therefore cannot interfere with variation.

When we consider a multicellular organism, which has developed from a single zygote it is, in this respect, comparable to a bacterial colony, which was formed by a single cell.

To be sure, the differentiation in the higher organism has gone so far that a great variety of cells, different both in form and function, has made its appearance. Moreover, these cells form a unity of a higher order, the organism.

In bacteria, the offspring of a single cell may also be differentiated in form as well as in response to environment. But this differentiation is much less advanced than that in the higher plant. Moreover the cells do not form a unity of a higher order, they do not form an organism. There seems to be, however, a fixed relation between the different kinds of cells originated as a result of the saiddifferentiation. This relation is reestablished in the progeny of every isolated cell. This indicates that the culture in this respect, acts as an individual, a "Ganzheit". (Avenarius, 1938).

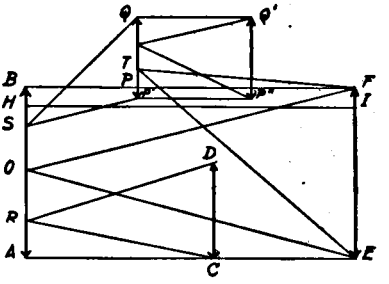


Fig. 1. Schematical representation of vegetative reproduction and its forms of differentiation.

The prospective potentiality of a single cell of a higher organism may be great or may be reduced to almost nil. Meristematic cells or embryonic cells may *retain* the power to reproduce almost an entire organism, or at least several of its tissues, while other cells may only reproduce their own kind. Still other cells have lost their power of vegetative reproduction when isolated. Vegetative reproduction may be represented graphically as follows (Fig. 1). On the line AB are represented the different types of cells of a higher organism. The abscissa represents time. The cell O, the zygote, is able to develop in the time AE to a state of differentiation EF, which is equal to AB. The cell R still has enough potentiality to develop, in the time AC, the potentiality CD. The cell H has lost its potentialities, in the time AE its offspring still shows the potentialities represented by I.

Now in certain instances the potentialities of the offspring of a certain cell may transgress those of its mother-cell. Bud-variations in higher plants such as laciniate or torquate leaves are instances of such a transgression. For the reasons mentioned above, it would

be incorrect to call these discontinuous changes "mutations". The neutral word "pedema" (το πηδημα = the jump) suggests itself as an equivalent of mutation.

In the schematical representation, cell S has, in the time BP, produced the pedema QP'. This pedema will, vegetatively, continue to reproduce itself. Sometimes, one of its meristematic cells T will suddenly revert to the original type, the laciniate beech shows branches with normal leaves. We might speak about an "*anapedema*" QP' and Q'P'', representing the sudden production of something different in a vegetative way and about a "*katapedema*" EF from T, in which the aberrant form reverts to the usual.

It seems reasonable to compare the variability of the offspring of a single bacterial cell with the ontogenetic differentiation in a higher organism, rather than with phenomena which imply sexual reproduction. In doing so we agree with Toenissen (1921) who compared the bacterial clone with the "Keimbahn" in the sense of Weismann.

The term "*mutation*" has to be abandoned, for reasons stated above. Moreover it is used in different senses in bacteriology, as well as in genetics. De Vries originally meant a spontaneous, genotypical change, occurring suddenly in a few individuals, among others, which remain unchanged. Modern experimental genetics, as it became possible to change genomes by means of X-rays, ultraviolet light etc., uses the word mutation in the broader sense of a change of the genome that is not the result of a sexual process, thus dropping its spontaneous character. Therefore many workers (e.g. Pringsheim 1910) abandon the term in bacteriology, while others, (Wolf 1909 and Beyerinck 1912) use the broader meaning. Most recent authors e.g. Seiffert (1936) and Lindegren (1935) go back to the de Vriesian conception.

The term "mutilation" (van Loghem 1921, 1928) only implies the *loss* of potentialities and therefore imposes too many restrictions, as many examples are known of "new" properties arising spontaneously (Beyerinck 1912, Gotschlich 1924). We shall, therefore, designate the transgression of normal bacterial potentialities as *pedemata*. The word of Lehmann's has the disadvantage that it cannot be applied in higher organisms.

From the above it follows that the introduction of the usual genetical terms into bacteriology seems not without danger. Many authors speak about "heridity", "genotype" and "phaenotype", some of them going so far as to mention non-disjunction of chromosomes as a cause of "mutation", where chromosomes and even nuclei never have been made visible in bacteria.

As without heridity (in a sexual organism) there is no genotype, there is also no phaenotype. Nobody will test the constancy of a genotype by vegetative propagation (vide the well-known experiments of Gaston Bonnier), while this is the only way a bacteriologist can work, as his objects refuse to act as gametes. There is no word expressing the inner potentialities of a vegetatively propagating cell, corresponding to *genotype*, which term expresses the inner potentialities of a sexually-reproducing organism. We venture to propose the word *hypartype* (ὑπαρ = real, in contrast to the 'unreal' dream). As the word *Phaenotype* is the logical mate of the word *genotype*, the word *doxatype* should correspond to *hypartype* (ἡ δόξα = appearance). We therefore propose, corresponding to the terms used in genetics, the following:

with sexual reproduction	*without sexual reproduction*
mutation = new genotype	pedema = new hypartype
Genotype-Phaenotype	Hypartype-Doxatype

The hypartype of all cells of a certain culture is the same when no pedemata are formed, and is characteristic for the species. The doxatype is the complex of properties that a certain cell and culture show at any given moment. The difference between these two types is what Drion (1936) has called *constitution*, the internal conditions, as far as determined by age, previous history and action of the milieu. When a cell forms a spore, its doxatype is changed, not its hypartype. When a culture is dividing rapidly, a few hours after inoculation, its doxatype is different from that of the same culture some hours later, when division has almost stopped.

Van Loghem and co-workers (van Loghem 1922, 1928) also made objection against the use of genetical terms in bacteriology. They consider the whole culture as the individual, and therefore comparable to the higher organism and not to the single cell. Two cells, formed by division from one cell, are no different organisms, but parts of one and the same individual. Thus a change inflicted upon a certain cell, must be transferred to its division-products.

When a culture changes its properties, van Loghem compares this phenomenon with a pathological change of a higher organism. When a person has recovered from a disease, he may remain immune for all of his life. In a bacterial culture, changing its resistance against a poison and retaining this increased resistance even on a medium without the poison we meet, according to van Loghem, with the same phenomenon. The lactose-negative, pathogenic, highly specialised *B. typhosum* is, according to this

author, phylogenetically originated from the lactose-positive *B. Coli.* When aberrant *typhosum* strains have become lactose-positive, this is looked upon as an *atavism.* So when barring genetics from bacteriology, this investigator introduces phylogeny. Instead of comparing a clone of bacteria to the "Keimbahn" of a Metazöon, van Loghem, more or less, compares it to the soma. Between the bacterial individual (after this reasoning) and the individual of the higher organisms, there is, however, this enormous difference, that the latter may die after reproducing its kind by the development of its gametes, while the former is unable to do so. In the example, stated above, the person is immunised, *but not his progeny.* Moreover, it is not true that vegetative reproduction implies that a changed cell also produces two changed cells. Before or after division, a changed cell has to make an amount of protoplasm equal to its own volume, and this protoplasm need not *per sé* be of the changed type. Jollos (1921) and Jennings (1929) could show that Protozoa, with abnormal cell-form, divided into two parts, one being normal, the other showing the deviation.

§ 3. *Adaptation versus Selection.*

A great number of authors has experimented or speculated upon the nature of bacterial variability. The analogy between bacteria and higher organisms is apparent. (Barber 1908, Pringsheim 1910, Eisenberg 1914, Baerthlein 1918, Jollos 1921, Jennings 1929, Rahn 1932, Lindegren 1935, Seiffert 1936).

By means of single-cell cultures Barber was able to show that descendants of the longest resp. shortest cell of a culture of *B. Coli* showed the same average length. Kelly and Rahn (Rahn 1932) could show that descendants with the fastest, resp. the slowest division-rhythm of *B. aerogenes* showed the same division-rhythm. Goodman (1908) succeeded in separating *B. diphtheriae* in two races, by selecting repeatedly the fastest and the slowest acid-formers. In all probability he met with dissociation. These experiments show Weismann's postulate to be false. Fluctuating variability is not transferred to the offspring, neither in bacteria nor in higher organisms. Numerous authors met with variability in the form of a changed outward appearance of a culture on a new milieu, which change disappeared when the culture was transferred to its old milieu. Changes in pigment-production, resistance, spore-formation, metabolism, virulence etc. were temporarily affected by agency of the culture, salts and other chemicals in the medium, culture in body-fluid, temperature etc. (Wolf 1909,

Pringsheim 1910, Eisenberg 1914, Baerthlein 1918, Gotschlich 1929).

The terms genotype, phaenotype, and mutation were used to cover analogous phenomena in bacteria, the latter term being applied to designate a change in one or more of the descendants of a single cell, occurring spontaneously, or as the result of an action exerted upon the culture, which change remains constant throughout a great number of generations, in the latter case even when the action from the milieu has ceased.

Prior to Pasteur's time, C. von Nägeli counted all bacteria under one species, in which species, therefore, an enormous variability should occur. The other extreme interpretation was caused by the methods of pure-culture designed by R. Koch. Often bacteriologists clung to the constancy of their isolated strains in a very dogmatic fashion, calling any aberrant cell; an "involution-form". The truth lay, as usual, in between. Bacteriological practice soon became aware of variability in bacteria.

Among the very first properties of micro-organisms Pasteur himself met with variability in toxicity and his observation became the basis of modern medical practice. His famous work on the attenuation of the chicken-cholera organism by ageing in broth, as early as 1880, the control of virulence of the *anthrax bacillus* by cultivation at abnormal temperatures, and the changes of the virus of hydrophobia after animal passage, are to be regarded as the classical examples of microbial variability.

Massini (1907) described a "bud-variation" of *B. Coli mutabile* on Endo-agar, which variation consisted in the appearance of small red buds on the margin of the white colonies. These variations were called "mutations" by Neisser in 1906, and this was the first time this term was used in bacteriology.

The doctrine of cyclomorphosis, according to which a bacterium should show a series of different developmental stages, temporarily attracted so much attention that much that might have been interpreted as variation or mutation, was incorporated in the "bacterial life-cyle" (extensive literature and criticism vide Henrici 1928, Hadler 1927, Petragnani 1932). Later investigations, especially by means of single-cell cultures, were able to show that many so-called important links in the bacterial cycle consisted of dead cells (Stapp 1931, Schlemper 1934, Pronk 1935, Buchsteeg 1935, Hobby 1935). In a few cases, however, the existence of different stages was made extremely probable (*B. radicicola*, Bewley and Hutchinson 1920, Bürgers c.s. 1937 unknown *Diplococcus*).

Another phenomenon, first described by de Kruif (1921) and Arkwright (1921), and often confirmed (a.o. Hadley 1927, Petragnani 1932, Lewis 1932, den Dooren de Jong 1933, Rettger 1935) is the so-called dissociation, in which a pure culture might become differentiated into two different types of cells and colonies. Schoute (1934), Kahn (1933) and others have demonstrated dissociation in the offspring of a single, isolated bacterium. In general two types of colonies appear which are called R(ough) and S(mooth). It is proved experimentally that influences from the milieu, e.g. Lithium-salts, may stimulate dissociation.

The same phenomenon seems to play a role in corals of the Fungia-type, animals where vegetative reproduction is of paramount importance.

The ideas of Kuhn (1931) and Hadley (1927), according to which dissociation might embrace several types, under which there should occur filter-passers, have been contested by other (Dienst 1933, Knaysi 1933, Schlemper 1934,s Pronk 1935, Rettger 1935, Swingle 1935, the latter giving extensive literature).

The experiments of Neisser and Massini, mentioned above were repeated by Benecke (1909) and Kowalenko (1910), who proved that even a single-cell culture of a lactose-negative *Coli* strain, growing with white colonies on Endo-agar, will produce small secondary colonies on the edge of the old ones showing by their red colour, that lactose is digested. Burri (1910) thought that every cell should have the potentiality to digest lactose, only the density of the population giving a few of them the opportunity to do so. Reiner Müller (1912) found that a great amount of bacteria, not able to attack various sugars, formed secundary colonies on agar-plates containing this sugar.

In 1927 Stewart concluded that the sugar should exert influence when the bacteria were changing from their "vegetative form" into an other stage of their life-cycle, where autogamy and segregation of characters occur. The result is a sort of Mendelian inheritance. The *Bac. coli mutabile* is regarded as the heteozygous form and the race that ferments sugar is the homozygous recessive. This experimental evidence is not very convincing, however. Sexual reproduction is not yet proved (W. Seiffert 1936). Recently Lewis (1934) repeated these experiments, using lactose-negatives *Coli* strains cultivated on synthetic media. This author is of the opinion that only an extremely small part of the cells of his strains is able to use the

lactose and starts growing when this sugar is the only energy-source. His point of view is opposite to that of Burri. The lactose-positive cells should be formed in the normal course of development, even in the absence of lactose and always in the same ratio. Starting with a culture which had never come into contact with lactose, he could show that only concentrated suspensions of this culture grew on agar-plates, where lactose was the only energy-source. Having established the ratio between 'lactose-minus' and 'lactose-plus' individuals, he prepared a suspension so dilute that no cells from the former group came into play. This suspension was cultivated on lactose-free agar and when it had developed fully, the cells were suspended and inoculated on lactose agar. Suspensions from a certain concentration onward, were able to use lactose as only carbon-source. So 'lactose-plus' individuals were originated from 'lactose-minus' individuals on a lactose-free medium. According to Lewis the sugar had not changed the properties of the culture, but only selected those cells that could grow on it, the other part of the population remaining undeveloped.

In all other investigations on this subject, the strains of bacteria were cultivated on media containing other energy-sources, as peptone etc. besides the sugar. After Lewis every cell will develop in this case, using the other energy supply later on. When 'sugar-plus' forms are present, those will start to digest the sugar. So it is possible that Burri found a hundred percent yield of lactose-positive colonies, inoculating a lactose-negative *Coli* strain on peptone agar with lactose.

The foregoing example was extensively dealt with in order to show how the same problem was looked upon by different authors. Summing up Lewis' explanation; Secondary colonies, having the power to digest sugar which quality the original culture lacked and originated on a plate containing this sugar, are to be considered as "Mutations" (pedemata) developed spontaneously not by any action from the milieu, but selected out by that new milieu.

In Lewis' theory we meet selection as the principal phenomenon operating when bacteria show striking variability. Numerous other authors, however, support "adaptation" in this and similar cases. Often the two principles are combined; a part of the cells is thought to acquire the new property. Many workers, however, conclude to one of the said mechanisms, without considering the other (G. Seiffert 1912, Schmitz 1916, Schoetensack 1933).

Much work has been done on variability in metabolism and nearly all investigators in that line vote for adaptation. A recent summary of their work was given by Knight (1936).

The principal aim of all this work is to find means by which bacteria can be forced to grow on media they formerly refused, in the first place on synthetic media. We shall divide these groups of experiments in two parts, those where the "acquired" property did not last any longer than the influence from the milieu, and those, where this property persists.

To the first group belongs the work of Karström (1930) who investigated the connection between the presence of given carbohydrates in the nutrient medium and the development of the corresponding enzymes, in a number of bacteria such as *B. coli*, *B. aerogenes*, *Betacoccus*-strains and others. In accordance with his findings Karström divides the enzymes into "Constitutive enzymes" and "Adaptive enzymes." The former group is always present, and is built up by the cells independently from the milieu. The latter, however, consists of enzymes which are only produced by the influence of a particular milieu. A *Betabacterium*-strain, for instance, always showed the ability to digest arabinose and xylose, but glucose could only be attacked when this sugar was present in the medium. The formation of those enzymes sometimes requires a certain amount of time. *B. coli* was found to ferment glucose, fructose and mannose at once when cultivated in peptone-meat-extract, but it split mannitol only after 75 min. and sucrose after 105 min., while lactose required 165 min.

Similar to Karström's are the findings of Stephenson and co-workers (Yudkin 1932, Stephenson and Stickland 1933). A culture of *B. coli* in tryptic broth does not produce an enzyme capable of breaking down formate with evolution of hydrogen gas. If formate were present the enzyme showed its activity. In order to know whether selection might play a role in this process, the authors made the following experiments. First they could show that addition of formate did not alter the rate of growth. Secondly it was found that in a young culture the enzyme showed its presence in less than one hour, while the total amount of cells did not increase more than 18%. Stephenson c.s. drew the conclusion from their work that the formation of the formate-digesting enzyme was to be looked upon as a simple chemical reaction between the substrate and the cells. They even measured the velocity of the formation-reaction. The continued stimulus of the substrate is absolutely necessary to maintain the presence of the enzyme. On withdrawal of formate, the hydrogen-forming enzyme was completely lost. The explanation of these phenomena offer no difficulty whatever from our point of view. We only should like to drop the qualification "new" for the

"adaptive" enzymes, for to the potentialities of the species belongs the digestion of those substances that can be attacked by the said enzymes. This is a characteristic of the species as well as any other, and we cannot find any variation in it.

The second group is of more interest to our problem. Here we meet with the "training" of bacteria to synthetic media. Braun and Cahn-Bronner (1922) found one aberrant strain of *B. paratyphosum* that showed growth in a leucin-lactate medium after 14 days incubation time, and was able, after that period, to grow on subcultures in this medium. Also ammonium-assimilating strains were obtained from strains that ordinarily did not grow on ammonium-lactate. In most of their numerous experiments it was found that the ability to grow on a certain synthetic medium, once acquired, was not lost by cultivation in rich nutrient media. Most work was done, using the following technique. An extremely large inoculum was transferred to the synthetic medium, often the whole surface growth of a pure-culture tube was washed into it. This culture was incubated for a long time, in some experiments with *B. typhosum* for 7—12 days. From time to time test-samples were removed and inoculated into fresh synthetic medium. Transfers that yielded growth were constantly cultivated in the new milieu, and so the authors obtained a *paratyphosum-A* strain that had acquired the ability to assimilate ammonium. This strain was transferred to nutrient agar, and cultivated on it for four weeks, when transferred to the synthetic ammonia-medium it yielded directly a full growth. We shall refer to these experiments in a later chapter, but we wish to draw attention here to the work of Ingraham (1933) who found a much prolonged incubation-time for growth of various bacteria in nutrient solutions containing increasing percentages of Gentian-violet. She found that, before growth could begin, the initial unfavourable oxido-reduction potential of the medium had to be changed. This process was the cause of the prolonged "lag".

Fildes, Gladstone and Knight (see Knight 1936) used another technique for the same purpose similar to that applied by Kluyver and Starkey. They changed *B. typhosum* so that it grew on media where ammonium was the only nitrogen-source, by a method of serial sub-cultures in decreasing concentrations of tryptophane. At the end the bacterium was able to synthetisize tryptophane. The authors mentioned, as well as others, obtained similar results with various other bacteria. A detailed description of their work can be found in Knight (1936). It is of interest to note that in exceptional cases other changes are involved

together with the changes in metabolism e.g. loss of flagella. The authors just mentioned make no difference in their explanation between the two groups and consider the last cases also as an action of this milieu altering the mosaic of the enzymes. With this theory we cannot agree, because the influence is seen to last long after the stimulus has disappeared, and we do not like to introduce such drastic Lamarckian principles as long as other possibilities are open. In a following paragraph we will develop an other theory, which is meant to avoid such reasoning.

Many authors, however, vote for selection. Lewis' findings were stated above. Still clearer are those of Leitner (1930). He succeeded in isolating strains of dysentery bacilli of the *Kruse-Sonne* type, that were resistant against the bacteriophage, by its different colony-form. In every culture the resistant type proved to be present with a frequency of 1 on every 100 000 cells. On an ordinary agar plate the resistant colonies always escape attention but by the selective action of the milieu, they come to the fore.

Eisenberg (1914) found a pure-culture of *anthrax*-bacilli to consist of a sporgenic and a asporogenic type and was able to isolate them by selection.

Meller (1925) saw the development of two types of cells in stab-cultures of *B. vulgaris* in gelatin. As growth proceeded one part digested the gelatin anaerobically in the lower part of the tube, while the other type in the upper half of the tube digested its food aerobically. She succeeded in separating the two types that had become so totally different that one did not grow on the medium of the other.

Near the end of the growth-period aberrant forms begin to appear in every culture (Henrici 1928, Rettger and Gillespie 1935). Some of those cells are due to autolyze soon, others, however, are especially apt to carry on in the exhausted fluid, where a good deal of inhibiting substances and harmful wast-substances of metabolism will be present. These cells are responsible for the occurrence of secundary colonies on solid media, and the phenomenon of "second bloom" in broth. By constant inoculation on sterile filtrate of exhausted cultures Meller obtained a strain that did not grow on fresh broth. It seems reasonable to assume that by repeatedly transferring on fresh broth the non-aberrant cells overgrow the abnormal ones that are selected out however, when transferred to sterile filtrate. Miss Meller's work is in accordance with the classical experiments of E. C. Hansen (1906, 1907) on the differentiation of brewery-yeast into "bottom-yeast" and "top-yeast", whereby the principle of selection

was introduced into microbiology.

A most illustrative case of selection gives the recent work of Avenarius (1938).

When a culture has been dissolved by the action of a bacteriophage, often a clarified broth culture is seen to turn turbid again. Phage-resistant cultures may be isolated from these bacteria. d'Hérelle who studied this phenomenon himself, found that the cultures retained their resistance on solid media for a long time, and considered the "new property" as acquired. Gratia, however, regarded these so-called "secondary cultures" as descendants of cells already present in the initial culture, cells that by their stronger resistance escaped lysis. So Gratia supports selection. The initial culture is to be considered as always dissociated into a lysable, and a non-lysable race.

Avenarius worked on this subject with single-cell cultures. He succeeded by cultivation in Lithium-broth in bringing about a dissociation, and selecting a race of *B. Typhosum* and various other pathogenic forms that showed resistance against the bacteriophage. Of paramount importance is the fact that the selection was carried out without any influence of the bacteriophage itself. From single-cell cultures of lysable cultures resistant strains could be obtained and vice-versa. Avenarius' experiments thus strongly support Gratia's selection-theory. Resistant forms could be obtained after cultivating repeatedly in $LiCl_3$ broth. It is a well-known fact that $LiCl_3$ furthers dissociation. While, furthermore, all resistant strains showed a "rough" growth while the original cultures grew "smooth", Avenarius looks upon the variation as a dissociation.

Avenarius' results, together with the work of Lewis (1934), offer a severe criticism of the conclusions of Schlemper (1934). This investigator compared a large number of single-cell cultures isolated from one and the same pure culture, with one another, and found their behaviour against various media to be exactly the same. From this result he drew the conclusion that the isolated cells did not show any variability. This conclusion holds good for the full-grown cultures, but not for the cells themselves. For if we assume the original culture to contain e.g. "lactose-positive" as well as "lactose-negative" individuals, we may assume after Avenarius that the latter will develop in a medium where the lactose is not the one and only carbon-source, into a culture containing "lactose-positive" cells that will attack the sugar after a while.

In accordance with the foregoing experiments, a number of bacteriologists developed similar theories (Gildemeister 1916,

Gotschlich 1924, 1929, Klieneberger 1929, Sulmann 1933, Lindegren 1935, Ravich-Birger 1935, W. Seiffert 1936). A brief outline of these theories may be stated as follows.

A pure-culture, even a single-cell culture, consists of a large number of *different* individuals. The width of variation increases with the number. The variations are to be looked upon as spontaneous mutations, cropping up with as fixed a frequency as in higher organisms, but more often met with, because an enormous amount of cells is formed in a relatively short time.

"The ease with which bacteria are trained to new environments, probably depends solely on the occurrence of numerous genetically different forms, one or more of which is able to carry on under the new condition", as Lindegren puts it. And further on in the same paper: "it may be considered a general principle, that mutations occurring when the environment is changed, are not induced by these changes. The changed environment merely makes it possible to increase the number of a variant which is constantly present, but ordinary undetectable". The aspect of a culture on a certain medium will depend upon what kind of type is predominant in that milieu, or with the words of Ravich-Birger: "By transplanting such a mixture, the majority of colonies cropping up show the same character as the mutation predominant in the initial culture".

The ideas expressed in this chapter were partially suggested to me by Professor Baas Becking.

CHAPTER III.

THE CONCEPTS OF VARIABILITY AND MILIEU.

A theory, covering all known phenomena of bacterial variability, has to consider the above facts, besides those of Hof (1935), Kluyver and Baars (1932) and Starkey (1938), stated in a previous paragraph. A brief summary of their results might be useful.

It is possible to obtain, from ordinary garden-soil inocula, highly specialised organisms, such as obligate thermophilic- and halophilic bacteria. From various forms less specialised strains, growing at lower temperature resp. salt-percentage, are known. In most cases one is not able to grow these strains under the extreme conditions of the other races. So enrichment-cultures show a greater tolerance than pure-cultures. From sulphate-reducing bacteria two strains

can be isolated at 30° and at 55°, different in shape and seize. The latter race can grow directly at 33°, but the former fails to grow at 55°. By gradually cultivating at increased temperatures, the mesophilic race is completely changed into the thermophilic one. The same holds good for a halotolerant race of the same organism, that may be brought back to the normal race, and vice-versa by gradually decreasing, resp. increasing the percentage of salt. These experiments yielded better results when freshly-isolated cultures were used. Cultures that were more often transferred did not grow so well at milieus different from their own.

Let us try to develop a theory of bacterial variability, where the data, stated above, are looked upon from two different angles; variability and milieu.

Let us, to limit our thoughts, consider, mainly one property of the cells; their ability to divide in a certain percentage of salt which ability we shall call their halotolerance.

We shall divide variability into 1) fluctuating variability and 2) discontinuous variability, a division first made by Darwin and de Vries.

Let us consider first *fluctuating variability* in halotolerance.

The offspring of a single cell shall show fluctuating variability in regard to its halotolerance, the limits of this fluctuation shall be determined by the milieu. Suppose the halotolerance to vary in a saltless milieu (in future to be called 0 % milieu), in such a fashion that a few cells are able to grow in a milieu with saltpercentages from 0 %—6 %, in the same way a few from 0 %—12 %, while the majority is able to grow between 0 %—9 % NaCl. We have assumed a division into three distinct tolerances to simplify our argument, in reality we have a fluctuating distribution of the halotolerance of the cells.

When we transfer the culture to 6 % NaCl, all of the cells will develop. When transferring to 9 %, however, we shall get a full growth, but a part of the cells die. In 12 % salt we can only grow our culture when we transfer so many cells that we are sure to include into the inoculate the very small part with milieu-limits of 0 %—12 %. So a dilute suspension of our culture might refuse to grow in such a salt solution.

In another milieu, however, the fluctuating halotolerance might be shifted. We met in the literature such an enormous amount of facts, showing the modifying influence of the milieu, that we are allowed to make this assumption. On re-inoculation into the original milieu the original limits of fluctuation are restored, the change effected by the milieu does not persist in another milieu, and it is,

therefore, by no means a change of hypartype. Supposing a shift of fluctuating halotolerance in the new milieu of 6 %, we now obtain a majority of cells with tolerance 3 %—12 % and minorities becoming able to grow between 0 %—9 %, resp. 6 %—15 %. Starting from this culture it has become possible to obtain growth in 15 % NaCl. When we go back from 6 % to 0 %, however, a few cells shall not develop, those with tolerance 3 %—15 %. In this way we venture to explain the experiments of Kluyver, Baars, and Starkey, who by gradually changing the milieu could alter the properties of their cultures.

In the 6 %-milieu a few cells shall have halotolerance not including 0 %. We may assume that in very extreme milieus the amount of cells that can grow at 0 % is very small, and may even approach zero. In that case a direct transfer to 0 % will not be successful, though a gradual transfer might succeed.

Hof's experiments, however, do not fit into this concept. The direct isolation of organisms, growing in 24 % NaCl, and refusing to grow at zero percent, can not be explained in this way. It is necessary to add the following hypothesis. In every milieu organisms are formed that cannot develop in that milieu, but are due to autolyse. Their fluctuation lies between limits not corresponding to those of the milieu. For instance, in a milieu of 0 % NaCl cells are formed inevitably, according to the laws of chance, and therefore with a fixed frequency, with a halotolerance-range of 6 %—12 %. On transfer to 12 % NaCl they develop, when present in the inoculum. Also cells with halotolerance ranging from 12 %—24% might occur in the medium with 0 %, and especially in the natural habitat. In a medium of 24 % salt, it might be possible that the fluctuating-variability was so shifted, that none of the cells had a tolerance including zero percent. In this case re-inoculation to saltless media will not be possible, although, indirectly, via lower percentages, it might succeed. Hof, however, described no such experiments.

As the foregoing assumption may not be limited to a milieu of 0 %, we must assume that in a milieu of 12 %, for instance, cells will be formed with potentialities ranging from 0 %—6 %. This assumption might offer a useful explanation of the curious fact observed by many authors (Wilson 1922, Henrici 1928, Lewis 1932), that in every culture, in every stage of its development, and even, when division is most rapid, a certain amount of cells is seen to autolyse. Fig. 2 is an attempt to illustrate our reasoning.

A second complication is brought by the experiments of Meller (1925). She showed how a pure-culture was divided into two different cell-types, growing aerobically and anaerobically, and each refusing to

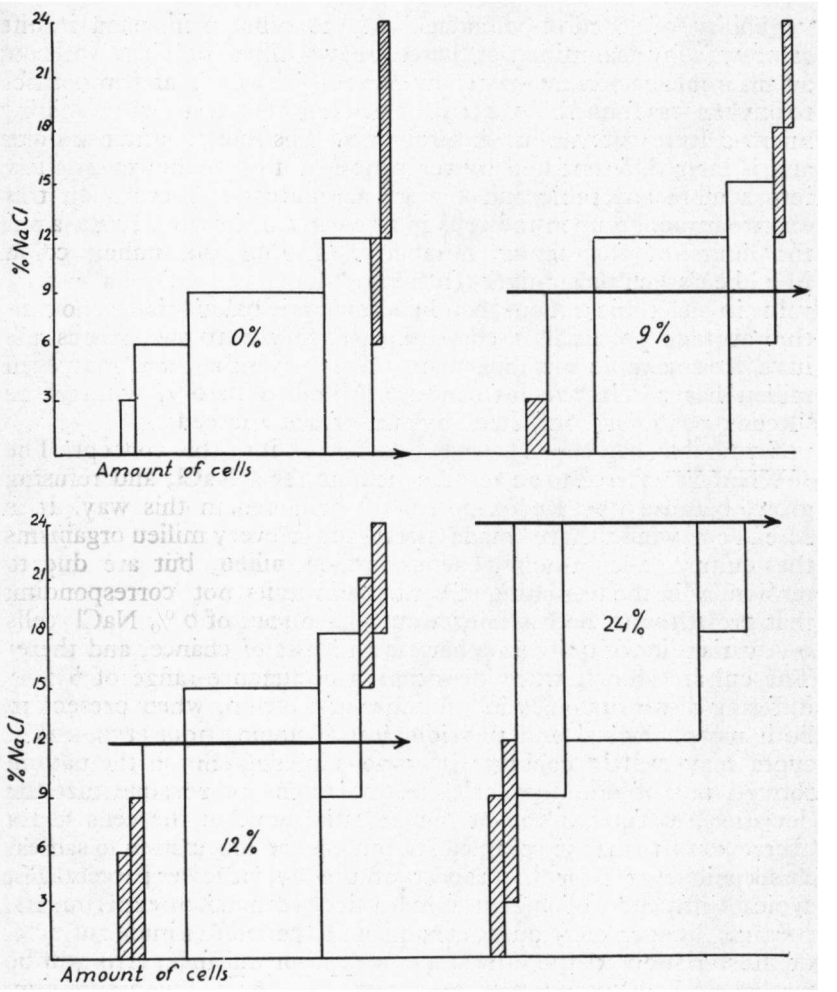


Fig. 2. Schematical representation of fluctating variability under the influence of milieu. Striated: those cells that are formed but are unable to develop.

grow in the milieu of the other type. We might assume also in this case an aberrant form of fluctuating variability. Whether a cell shall grow anaerobically or aerobically might depend upon inner physiological properties of that cell, for instance on its "Redox-potential". The only assumption we have to make now is a fluctuating

variability of "Redox-potentials" of the cells, influenced in the same way by the milieu as stated above. That individual cells of an anaerobic bacterium may show great differences in their resistance to oxygen was found by van Riemsdijk (1922) when examining micro-cultures of various anaerobionts. The milieu is first uniform and is later differentiated by the action of the bacteria themselves, into a more anaerobic and a more aerobic part. The milieu now exerts an action upon the cells in two ways. Primarily by adjusting the limits of fluctuating variability. The "Redox-potential" of Meller's culture might fluctuate around a different average value in anaerobic milieu, than in aerobic milieu. If this be the case, the average potential is changed in such a way that more cells have an anaerobic metabolism in the latter milieu. Secondly, the milieu has a selective influence in killing all cells in which the "Redox-potential" inflicted by the fluctuating variability, was unfavourable.

When transferred to an aerobic medium the anaerobic race cannot grow, because its "Redox-potential" will be too high, but if Meller would have made a stab-culture in gelatin, so offering the culture a non-homogeneous milieu with different oxygen tension, as in the beginning of her experiment, we venture to assume that growth with both strains would have succeeded.

A variability of quite an other aspect is the *dissociative variability*. The culture shows, when dissociation occurs, two kinds of cells, differing discontinuously in a number of most important characters, both morphological and physiological (R type and S type). The types may remain constant in various media, but both may be formed out of one another. The milieu is apt to accelerate the deviation, to further one of the deviation-products, to injure the other, even to bar it completely, but the milieu cannot cause the dissociation, or alter the aspect of the deviation products. The typical influence of milieu (antiseptics, lack of oxygen, animal passage, hunger etc.) on one hand and the predestined character of the variation on the other, are so typically different from what we have called "pedemata", the analogon of de Vriesian mutation, that we cannot explain dissociation this way. We would rather point to the analogon with the ontogenetic differentiation process of multicellular organisms.

A very striking property of dissociation is the fact that the two types R and S of all the species investigated, show the same differences. The S type is always the more frequent, less resistent, motile, more virulent, stronger type. We venture to compare this process with the process of differentiation in ontogeny, where we see the

formation of the ectoderm and entoderm. We should not forget however, that the difference between the R form and the S form is very small compared with the conformity between them, they belong absolutely to the same species, the hypartype is not changed. We do not advocate the theory of Reed (1933), who thought that a typical gene-distribution might be the cause of the difference between R and S. Gotschlich (Kolle-v. Wassermann 1929) thinks pre-existing structural differences to be the cause, something like isomery in chemistry.

After our opinion we must explain the data of Braun and Fildes by means of dissociation-like phenomena. It was found that strains of *B. typhosus* and *B. paratyphosus A and B*, isolated from natural sources (e.g. cases of disease), when tested, already showed different nutrient requirements. 80 strains were isolated from 64 typhoid patients in the same way, and 62 gave no growth on ammonium-lactate, while the other 18 strains grew in this synthetic medium (Braun c.s. 1922 and others). Knight (1936) states that " .... it is possible that the organisms growing naturally under different conditions may be already partially adapted when first isolated". We doubt, however, whether it is logical to assume that out of 80 strains of typhoid-bacilli, isolated from 64 patients, 18 had been growing without any other nitrogen supply than ammonium-lactate. Therefore we should rather not regard freshly isolated strains that can assimilate NH_4 as "adapted", only while other strains seem to be unable to perform this feat.

Van Loghem tested all colonies of an ammonium-assimilating *B. typhosum*-strain and found that 25 % of these colonies were unable to use ammonium as only nitrogen supply (cit. Knight 1936). The cells are assumed to live on metabolic waste-products of the others and on dead cells.

The phenomenon of dissociation showed us that a bacterial culture may contain two different types of cells, that may be converted into one another spontaneously. Experimental evidence we have found in the work of Eisenberg (1914), Meller (1925) and Avenarius (1938). Although the milieu will play its twofold role and exert its usual modelling and selecting action, the natural potentalities of the species are not exceeded.

The Braun-technique as well as that of Fildes and associates will, after our opinion, only effect a strong shift in the relation between the two types. The observed difference in appearance of the two kinds of cells and the loss of flagella points in the same direction.

We might assume one type that can only use more complicated

carbon and nitrogen-compounds and a second race that can use ammonium as well, occurring beside one an other in the body of the typhoid-patient, in such a way that it is a matter of chance which type will predominateintheisolated culture. Cultivation in theusual nutrient media, however, chiefly yields the type with the narrower potentialities. Cells of the other type will be completely missing after a while. In dissociation-experiments long incubation is regarded as most efficient to provoke this phenomenon, so it seems reasonable to assume the same thing in the experiments of Braun. Braun and Cahn-Bronner's suggestions point in the same direction. They compare their interpretations with the well-known investigations of Weil and Felix whose work is a precursor of the dissociation hypothesis in its present form.

They do not consider the potentialities of the bacterial species to be changed by the milieu, but regard the long incubation time of a large number of cells as necessary" .... um den darin vielleicht enthaltenen wenigen Keimen welche die Fähigkeit der Ammoniak-assimilation innewohnt, Zeit zur Vermehrung zu geben". (Braun and Cahn-Bronner. 1922).

They drew attention to the fact that the experimentally changed cultures were exactly similar to ammonium assimilating cultures isolated directly from natural sources. The work of Ingraham (1933) might account for the extremely long incubation-time inherent to the technique employed.

The conversion will not be complete, as Van Loghem showed that a small number of cells will maintain themselves at the cost of metabolic waste-products and dead cells for quite a long time. This fact enables us to assume that the quintessence of the "change" consists in a shift of ratio between two kinds of cells. Braun c.s. themselves advocate this theory as follows from various passages of their work.

The formation of "pedemata", however, transgresses the hypartype of the cells. In the same way as multicellular organisms have their so-called "mutation-rate", bacteria have their statistically fixed amount of "pedemata", "jumping" to various directions. The important difference is that in bacteria they are so much oftener met with, because such an enormous amount of cells may be formed in a short time. The milieu can exert its selecting influence only here. "Pedamata" should be considered as spontaneous. In some cases the lack of any influence of the milieu was experimentally proved (Barber 1908, for *coli*-strains with extremely long cells and Leitner 1930 for phage-resistant *dysentery*-bacilli) in other cases made very probable (Lewis 1934, lactose-digesting *Coli mutabile*).

Wolf (1909) found a colourless strain of *B. prodigiosus* and *Staphylococcus aureus*, the former occurring after cultivation in various salt solutions, the latter spontaneous, both remaining constant. G. Seiffert (1912) cultivated a *coli*-strain that retained its resistance against Malachite-green on media without this poison. Although we cannot say what this kind of variants are exactly like, because the action of the milieu was not adequately investigated, we will regard them for the present as "pedemata". Fig. 3 gives a

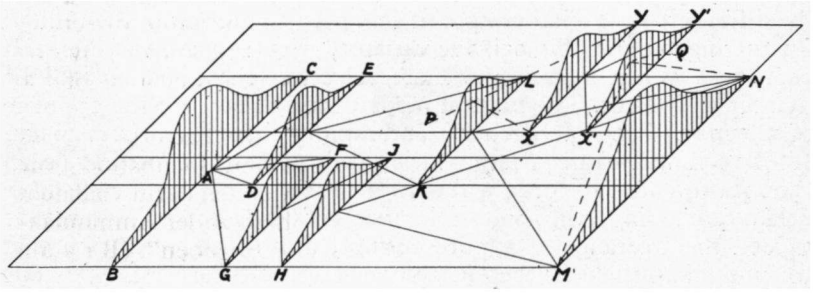


Fig. 3. Schematical representation of dissociation and formation of "pede imata" in bacteria.

schematical representation of our theory. In this figure BAC represents the initial culture, showing fluctuating variability in regard e.g. to halotolerance. Every cell A gives rise to both dissociation-products, DE and GF. Both forms may reproduce themselves, resp. KL and HI. Both may revert to the original culture-type MN. One cell of KL, however, P may give rise to a pedema XY that may form the anapedema X'Y', one cell of which; Q may revert to the original form MN. Katapedema.

It might be assumed that when some of the properties of the pure cultures described in the literature do not correspond to the aspects of the same organism in nature (Smit 1930, Meyer 1935), one of the reasons for this fact may be the selection of only a fraction of the potentialities as realised by an extreme milieu (*Zymosarcina ventriculi!*).

The last form of variability is the so-called cytomorphosis. It is a well-known fact that the offspring of a single cell in one and the same milieu will show different characteristics, both morphological and physiological, when studied at different times.

Henrici (1928) found that always cells of one culture differ in relative age, at every moment there are senile cells that do not divide. In a following chapter, where the growth-curve shall be

discussed, we shall meet his argument again. Here we should like to emphasize that it is absolutely necessary in physiological work on bacteria to compare two strains only when they are in the same relative phase of their growth.

After the reasoning given above, the milieu is of paramount importance for the aspect of the culture by its twofold action of determining the doxatypes and selecting out some of them at the cost of the other. Every culture is in a certain equilibrium in a given medium, this equilibrium consisting of a specific relation between the different types of all catagories that may be present in the milieu; fluctuating variants, dissociative variants, growth-phase variants, and pedemata. When a milieu is more selective, the selection will be sharper and faster. The natural media, the soil etc. are the opposite of selective. The heterogeneity and rapidly changing circumstances of the soil will cause a large variety of types to be present, each type represented by only a few individuals.

Hof, finding the salt tolerance of enrichment-cultures to exceed the tolerance of a pure-culture, draws the conclusion that the potential-milieu of the enrichment-culture is larger [1]. After our account a bacterial species as well as any other species has one strictly constant potential-milieu, although the potentialities of its cells may be different. We rather look for the solution of this problem in the action of the milieu upon the fluctuating variability as explained above. When a species is certain to give rise to a number of pedemata (shown by Leitner (1930) and Lewis (1934)), their potential milieu's should be added to those of the species.

Only one observation seems not to be in agreement with our theory. Kluyver and Baars found that a culture of *Sporovibrio desulfuricans*, growing at a temperature of 55°, was more difficult to cultivate at lower temperatures, when it was older. Freshly isolated cultures at 3 % NaCl showed the same phenomenon in regard to decreasing salt percentages. Old and new cultures were in the same phase of growth, differing only in number of transfers. In this case we cannot conclude to fluctuating variability alone. The effect is only a quantitative one, and the observations of ZoBell (1938) do not confirm it, although the different explanation ZoBell himself gives sounds reasonable, and is not in contradiction with Kluyver's results. When the data of Kluyver and Baars are confirmed, we have to assume that prolonged cultivation on the

[1] Baas-Becking defines potential milieu as the sum-total of all chemical and physical properties that enables an organism to grow in various milieus. (Ruinen 1933).

same milieu narrows the limits of fluctuating variability and also narrows, in this way, the potential milieu of the culture. We would meet thus in bacteriology a process similar to degeneration in higher organisms, the loss of extreme potentialities by the action of a milieu that makes no use of these potentialities.

CHAPTER IV.

MATERIAL AND METHODS.

§ 1. *The bacterium. Its isolation and culture.*

All experiments were carried out with *Bacillus megatherium* De Bary. This aerobic, Gram positive, spore forming rod has always attracted much attention owing to its considerable length and has been the object of many investigations. Its morphological and cultural properties were so adequatedly dealt with in the work of Den Dooren de Jong (1931), Henrici (1928), Knaysi (1933), and others, that we need not dwell at length upon this topic.

The bacterium was isolated after Den Dooren de Jong from garden-soil suspensions, heated to 80°, on malt agar. Pure cultures were prepared by means of numerous agar plates. Determination and identification was carried out with the aid of Bergey (1934) and Lehmann-Neumann (1926). Our strain consisted of long, motile rods, measuring 1.0—2.0 by 3.5—4.0 μ, occurring singly, in pairs and short chains (see plate no. I). On malt agar no spore-formation occurred, but on peptone "Witte", and various other media formation of central spores occurred.

Agar colonies are circular, thick, white to cream-coloured, old cultures become yellowish to light-brown on peptone. Gelatin is liquified, the optimum temperature is 35° C. From the pure-culture tube a single cell was isolated with the aid of a micromanipulator. This cell was incubated in a hanging drop of peptone-water adjusted to neutral reaction, and afterwards transferred to peptone-agar with 1 % glucose. The micromanipulator was of the Janse-Peterfi-type, manufactured by Zeiss. Isolations were made with micropipettes drawn from sterile glass tubing directly before use. The isolation-chamber was constructed after Schoute (1934), slightly modified. The cover-glasses were cleaned as recommended by Gee and Hunt (1928), and afterwards covered with a thin layer of 1½ % gelatin. This procedure, advocated by Peterfi (1924),

gave by far the best results. The isolation was carried out in the following stages:

1) A drop of a dilute suspension is sucked up in a sterile micro-pipette.
2) Under microscopical control micro-drops are deposited on one of the prepared cover-glasses. When one drop seems to contain only *one* cell this drop is controlled with stronger lenses.
3) With a second sterile pipette a drop of the sterile medium is placed on the second cover-glass.
4) With this pipette, the micro-drop, containing the isolated cell, is sucked up (see plate, no. II).
5) The cell is transferred, with this pipette into a large drop on the second cover-glass under microscopic control.
6) The cover-glass with the isolated cell is sealed on to a glassring, by means of sterile vaseline. The micro-culture is placed in a Petri-dish and incubated for 24 hours at 30° C.
7) Next day growth is controlled and transfers are made to agar-plates and to pure-culture tubes.
8) Next day growth on the plates is controlled.

§ 2. *Morphological changes in salt.*

Cultivation in salt solutions showed a curious phenomenon as far as we know never stated in literature. From 3 % up to 13 %, in fact in all percentages of salt investigated, the bacteria grew in long, winding chains. In broth cultures as well as on solid media although more prominent on the former. Sometimes many chains were twisted together forming clusters of a hundred and more cells. In non-aerated cultures in peptone-water, when growth seems completed, globules of about half a millimeter diameter are seen in an almost clear fluid. These clusters contain numerous twisted chains (see plate, no. III, IV, V, VI). Here hairpin-like structures, such as Kluyver and Van Niel described for *Bac. funicularius* (1926), project at the margin of the cluster. Studying the photographs one gets the impression that here the reason of this phenomenon must be a continuous winding of the whole thread round its longitudinal axis (see plate, no. VII). Recently Roberts (1938) found this structure to be the cause of the typical colony-form of *Bac. mycoides.*

We followed the growth of two chains, resp. in 6 % and 9 % NaCl in the constant-temperature room of 25° C. Figures 4 and 5 are camera-lucida drawings of this threads. In Fig. 4 we see that growth occurred over the whole length of the thread and is not restricted to the terminal cells. In Fig. 5 is depicted how the cells stick together.

At several places of the chains the cells are not attached to their neighbours endwise but lie more or less besides one another.

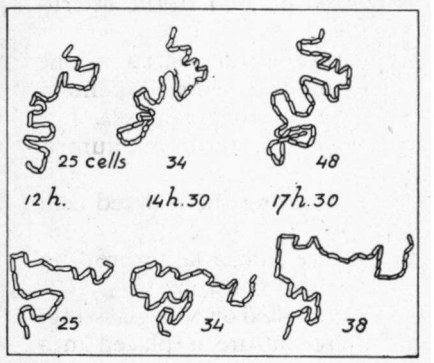


Fig. 4. Growth of a young chain in 9 % Peptone-water at 25° C. after 5 ½ hours.

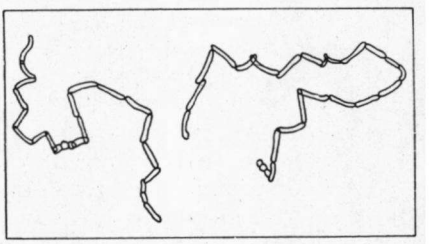


Fig. 5. Two short chains of a 36 hours-old culture in Peptone-water at 25° C. showing the attachment of the cells.

It seems that after the division the two cells tend to show "Post-fission movements" (H e n r i c i 1934).

We could not stop the cluster-formation, though we tried to do so by changing the media. Peptone, malt, and meat-extract, with and without glucose and chalk, showed the phenomenon in the same way. By blowing air through the culture-fluid under considerable pressure, the average number of cells per chain could be decreased. This average does not remain constant as growth proceeds. It has its highest value when growth is just starting and from that time on the chains become shorter. In exhausted cultures of low salt percentage we hardly see any clusters, the difference with a culture in saltless milieu becomes negligible because in ordinary media the cells often occur by twos and even threes. Fig. 6 and 7 give the average amount of cells per chain plotted against time during the whole period of growth at a given salt percentage. We shall call this number in the future "the cluster-factor". On solid media the colonies often showed a yellow colour as described by S m i t h (1933).

§ 3. *Statistical methods.*

The cluster-factor had to be found by taking the average of the number of cells in a number of chains. In almost every experiment the number of cells of the inoculum had to be known. In order to be more certain of the data statistical methods were applied. 10 mm^3

of the suspension were spread under a cover-glass of known dimensions, and at least 30 fields of vision of the microscope, the dimensions of which were known, were counted. The results proved to be

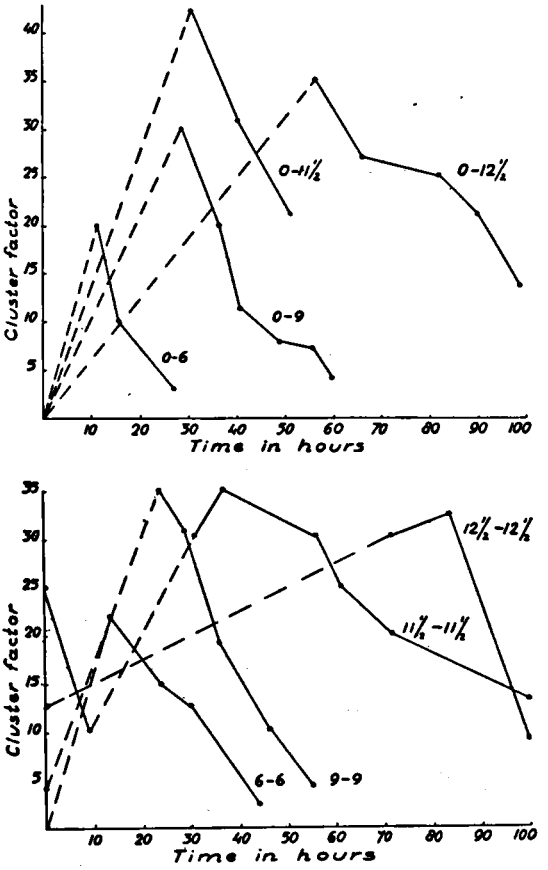


Fig. 6 and 7. Relation between "cluster-factor" and time. No. 6 inoculated with saltless pure cultures, no. 7 with salt cultures. Note the initial decrease of the 11½—11½ line.

sufficiently reliable when 2 or 3 cells per field were visible. In that case the frequencies of the different deviations from the average followed the law of P o i s s o n: The probability of the measured

amount n will be $e^{-m}\frac{m^n}{n!}$, where m is the average value.[1] Fig. 8 shows the agreement between the frequencies found and calculated.

The counted suspensions were most often heavily diluted before

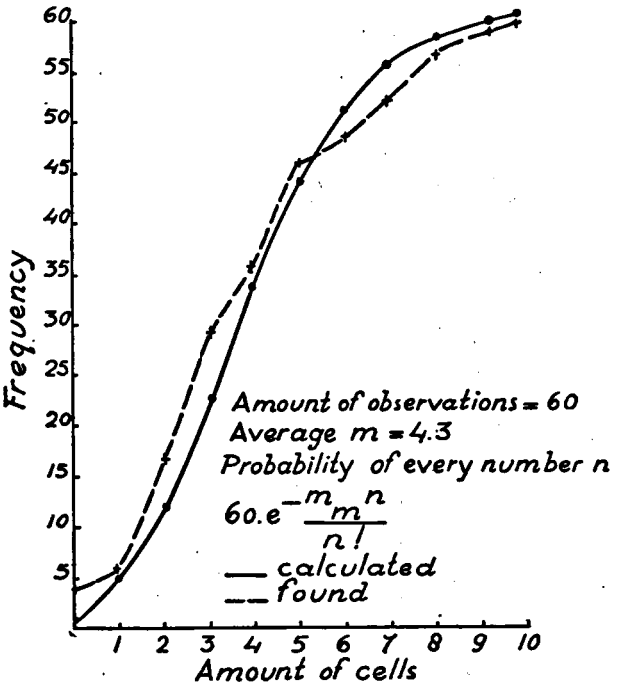


Fig. 8. The accuracy of the data obtained by visual counting of cells, as compared with the Poisson-formula.

inoculation, thus making the estimation of the number of cells, introduced in the medium, still more correct.

For the construction of the growth-curves it was necessary to collect a large amount of samples. To ensure sterility special culture-flasks were constructed of the following type (Fig. 9).

§ 4. *The culture vessel.*

The flask itself is cylinder-shaped and about 35 cm. high, shut by a pierced stopper. A glass-tube A, with a bulb B, filled with

[1] I wish to thank Dr. E. F. Drion. Batavia N.E.I. who kindly gave his advise in the mathematical method.

cotton-wool is inserted into the first hole. At the end of the tube is fitted the glass-filter C. Through the second hole of the stopper is placed the tube E ending in a capillary, which is closed by means of the rubber stopper F. Through the third hole passes the short tube G, with the bulb H filled with cotton-wool. The flask was placed in a water-bath of constant temperature of 30°. By means of a "Saja-pump" air was blown through tube A, after passing two wash-bottles with strong sulfuric acid. These bottles were suspended in the water-bath, in order to adjust the temperature of the air blown through the culture-fluid. When a sample was taken, the rubber tube connecting the washing-bottles with tube A, is removed, and the vessel is taken out of the waterbath. Air, being sucked in by a negative pressure, will be filtered through the cotton plugs B and H. Now the rubber stopper F is quickly removed and the end of tube E is flamed and plugged with cotton-wool. Tube A is closed with a small cork. Now tube G is connected with another pair of washing-bottles. When air is blown through these washing bottles, the solution in the vessel will rise through tube B. When it has risen to a few centimeters below the cotton-plug we can fix its position and sample through tube B. Afterwards, when we allow the fluid to fall again, air will be sucked in but it will be filtered through the cotton-plugs. In a large number of experiments we hardly met with any infection. In all experiments the vessel was filled with 25 cc. of the culture-medium. The percentage of NaCl of the medium was often controlled

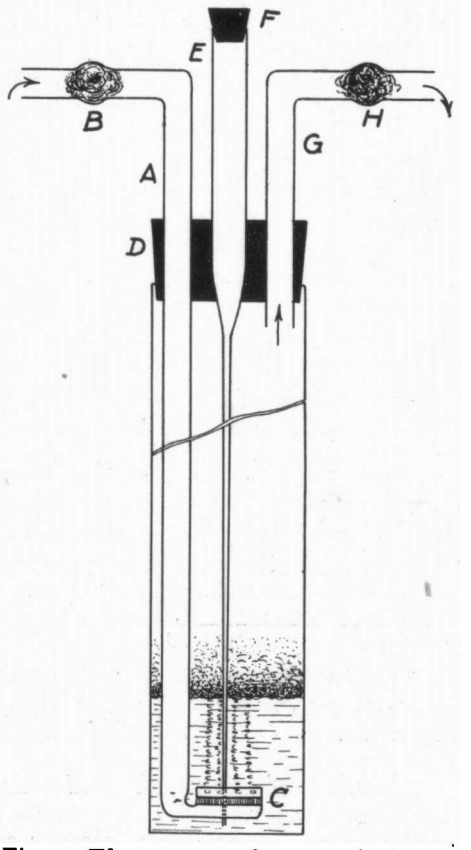


Fig. 9. The apparatus for aerated cultures.

after the end of an experiment, but was always found to remain constant. For plate-counts no more than 2 cc. was removed from the culture during the growth, about 0.5 cc. each time.

§ 5. *The plate count.*

Growth-curves were constructed by the plate method. We tried different ways of plate pouring and finally proceded as follows.

An amount of 0.1 cc. was sampled and poured out in a tube with melted peptone-agar-glucose, cooled till ± 45°. The tube was vigourously shaken, and poured in a sterile Petri-dish. After cooling and solidifying of the plate a sterile filter paper was placed in the lid of the dish and a drop of glycerol. The glycerol attracts water and so prevents the colonies cropping up to flow together. The amount of colonies was counted after an incubation of 24 or 48 hours at 30° or 35° C. Countings were made with the aid of a magnifying-glass. Plates that were overcrowded were discarded. For every point of the curve three plates were poured. In the graphs the curves are always drawn through the central point of a row of three. This point represents the average of the three data, the two extreme points representing those two of the three original data that deviate most of the average. So the reliability of the plate counts is directly visible from the graphs. Every curve is indicated by three numbers. The first refers to the salt percentage of the agar of the stock-culture, the second to the percentage of the peptone-water where growth was investigated, the third number to the agar used in the plate-count. This way of plate-pouring proved to be better than different other procedures we tried. When, for instance, we poured directly on solid plates the colonies flowed together on the surface, and when we poured the sample in the empty Petri-dish the colonies flowed together between the agar-layer and the glass.

We also prepared plate counts on agar-media containing the same amount of NaCl as the culture solution in which growth was studied. In the work with these plates we changed the technique on the following points. Because the salt-percentage had to remain constant during incubation at higher temperature, no glycerol paper was added. Secondly all plates were incubated in an atmosphere saturated with water the vapour-pressure of which corresponded to the salt percentage of the plates. For this purpose the plates were placed under a bell-jar with a basin containing some of the salt solution.

We had to wait longer for the colonies on the salt-plates. After counting, the plates were often reincubated and only discarded when prolonged incubation yielded no new colonies.

In the highest concentrations (12 % and 13 %), however, the plate-

method was replaced by the "Rolled-tube" method (W i l s o n 1922) that yielded better results, perhaps owing to better oxygen-supply. A very small amount of peptone-glucose agar (1 cc.) was melted in a tube, and cooled till 45°, we here used always 1 % agar instead of 2 % otherwise. The culture to be counted was poured into it, after which the tube was placed in a horizontal position, and rolled gently. The result is a very thin layer of solidified agar at the inner side of the tube. A piece of glycerol-paper was put into the tubes, which were incubated upside down. When comparing the "rolled-tube method" with agar of a lower percentage, with an ordinary plate-count as a control, an almost complete agreement was obtained.

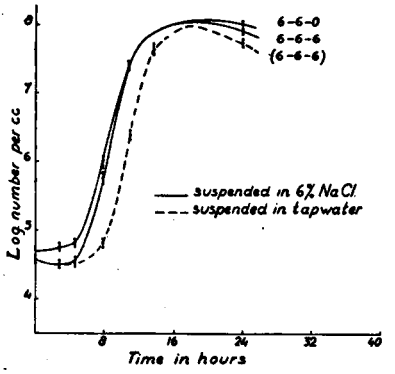


Fig. 10. Explanation see text.

When growth sets in, it is necessary to dilute the samples taken out of the growing cultures. This was done by means of tap-water adjusted before to 30° C. in the case of saltless plates and when salt-plates were applied, by means of salt solutions. Fig. 10 shows the difference between dilution in tap-water and in salt solution.

The salt cultures growing in clusters were studied microscopically before each observation, in order to find the cluster-factor. At least 30 chains were counted. In the very beginning of growth it was often impossible to find so many chains, and we had to satisfy ourselves with a lower amount. When microscopical inspection did show nothing at all, we used the cluster-factor of a previous observation. With this factor the number of colonies was multiplied.

§ 6. *The culture media.*

The plates gave a continuous control of the sterility of the cultures, because every infection would show as a different colony-form among the *megatherium* colonies. The percentages of NaCl were continuously controlled, as a rule after sterilisation in the autoclave, (15 minutes over 110°), also the salt percentages of the salt-plates after incubation. All glassware was heated in the oven during half an hour at 140°.

Culture media were

1) malt-extract. The raw extract, obtained from the brewery,

was diluted with twice its own volume of tap-water and adjusted to a neutral reaction,

2) peptone-water. 1 % peptone "Witte", 1 % glucose, ½ % NaCl. So when we mention in the following pages "saltless peptone-water", the actual percentage of salt is ½ %, as in most media, and "peptone-water with 6 % NaCl" means a *total* salt percentage of 6½ %,

3) peptone-agar. To the peptone-water 2 % agar was added. In the higher salt percentages, however, 1 % agar yields better results, because it did not coagulate so quickly. Duplicate plate series showed that the percentage did not interfere with the amount of colonies.

§ 6. *Nephelometric method.*

We also followed growth nephelometrically, using a photo-cell. The light of a small electric bulb connected with a large battery of accumulators, was thrown through a ± 20 cm. long horizontal glass-tube, shut by means of two plane glass-plates as used in polarimetry. The photo-cell is connected with a potentiometer and galvanometer in the usual way. The contents of the polarimeter-tubes was ± 12 cc. Therefore we could not fill the tubes with the culture solution itself, because every culture only contained 25 cc.. 2 cc., however, diluted with tap-water, proved to give satisfactory results.

CHAPTER V.

EXPERIMENTS ON GROWTH WITH CHANGING AMOUNT OF INOCULATED CELLS.

Preliminary experiments were performed in order to see whether growth of *B. megatherium* could corroborate the conclusions stated in Chapter I, and the assumptions in Chapter 3. For this series 50 cc. Schott-Erlenmeyer flasks were used filled with 10 cc. culture medium, with different percentages of NaCl. They were inoculated with an increasing amount of cells, from a 18—20 hours old pureculture, incubated at 30° C.

In the tables are given the respective times in hours, after which growth was visible with the naked eye. From every combination two duplicate-tubes were prepared.

Table 1. Exp. 20-V-'36. Malt extract, 25° C.

Amount of inoculated cells:	2.10^5	2.10^3	2.10^2
% NaCl.			
0	43-43	43-43	67-67
6	43-43	43-43	115-115

Exp. 11-IX-'36.

16 test-tubes with 1 cc. malt-extract each, and 16 test-tubes with 1 cc. malt-extract with 6 % NaCl.

Inoculated with ± 100 cells each, with the aid of the micromanipulator. Growth occurred at 30°C. after 24 hours in the saltless medium and after 96 hours in the other. Also single-cell cultures yielded growth in 6 and 7 % NaCl. In higher percentages, however no growth occurred. We repeated this experiment about 15 times with the same result. Refractometric control of the salt-percentage of the drops, showed it not to change during incubation.

Table 2. Exp. 8-IX-'36. Malt-extract at 30° C. × means that no growth occurred after an incubation of 14 days.

Amount:	2.10^6	2.10^3	2.10^2
% NaCl.			
3	24	48	48
6	72	72	72
7	72	72	×
8	72	240	×
9	288	288	×
10	366	×	×
12	×	×	×

For every salt-percentage transfers were made to agar-plates with the same percentage of NaCl, resulting in a luxurious growth on all of the plates.

Exp. 4-II-'36. Like in the previous experiment, the medium was peptone-water, the temperature 30° C. In this experiment transfers were made to saltless peptone-water, every day. Therefore, 25 mm^3. were inoculated into test-tubes with 1 cc. peptone-water and incubated at 30° C. When no growth occurred in these

tubes, it meant that the amount of viable cells in the original cultures was decreased to less than 1 cell to every 25 mm^3, this equalling 400 cells in the whole culture. To account for the absence of growth in the test-tubes, we might assume also that the cells in the original culture were in a sort of resting-stage and were not able to develop.

Table 3. Exp. 4-II-'36.

Amount:	10^7	10^5	10^3
% NaCl.			
3	24-24	24-24	24-24
6	48-48	48-48	48-48
7	48-48	48-92	×
8	96-96	×	×
9	120-120	×	×

The results of re-inoculation into pepton-water are graphically represented in Fig. 11. The logarithm of the amount of cells is plotted against the time in hours. When the subcultures yielded no growth it is assumed that the origin of that phenomenon must be sought in a decrease of the amount of cells rather than in a change of their viability. Starting from this assumption the graphs are drawn. In later chapters we shall try to offer experimental proof for this theory. When the original cultures were full-grown, a rough microscopical estimate of the total amount of cells was made. In every culture this amount proved to be of the order of magnitude of 10^8, in spite of the different salt-percentage. In the graphs the first points are given by the amount of inoculum, the endpoint is always 10^8. When subcultures yielded no growth, we plotted 2 for the logarithm of the amount, assuming it to be less then 400. We are aware of the fact that the lines constructed in this way are by no means to be regarded as growth-curves, they serve only to illustrate the results of re-inoculation, out of a culture growing in different salt-solutions.

Exp. 11-VII-'36. Like in the previous experiment. Peptone-water at 25° C. was used. In this set however, inoculations were made from pure-cultures, grown on agar media containing the same percentage of salt as the culture solution in which growth was studied. These pure-cultures were of various ages, but all of them had passed their optimum so as to be in the same period of their growth as the cultures used in previous experiments. Here also re-inoculations into saltless peptone-water tubes were made. The results of re-inoculation are represented in Fig. 12.

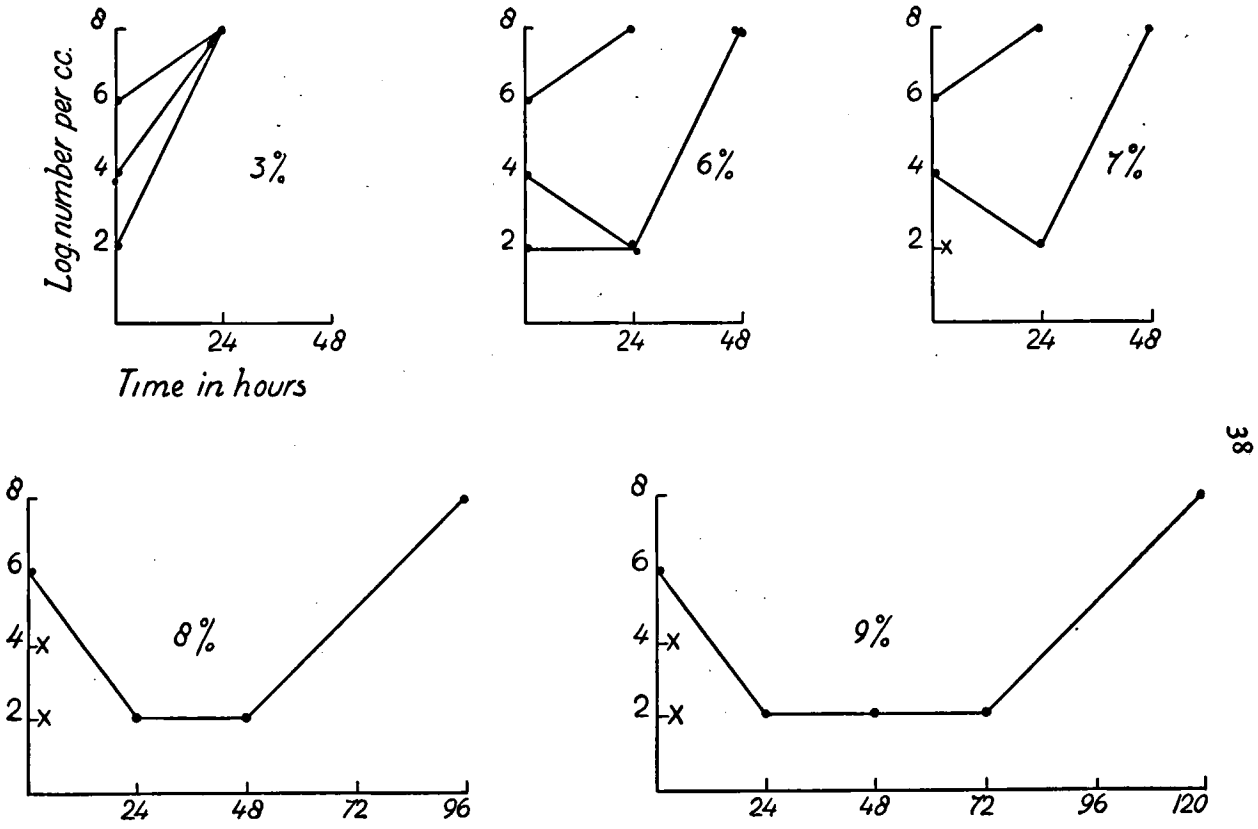


Fig. 11. Results of reinoculation in saltless peptone-water. x Stands for no growth in the initial cultures 2 for no growth in the subcultures. Inoculated from saltless pure cultures.

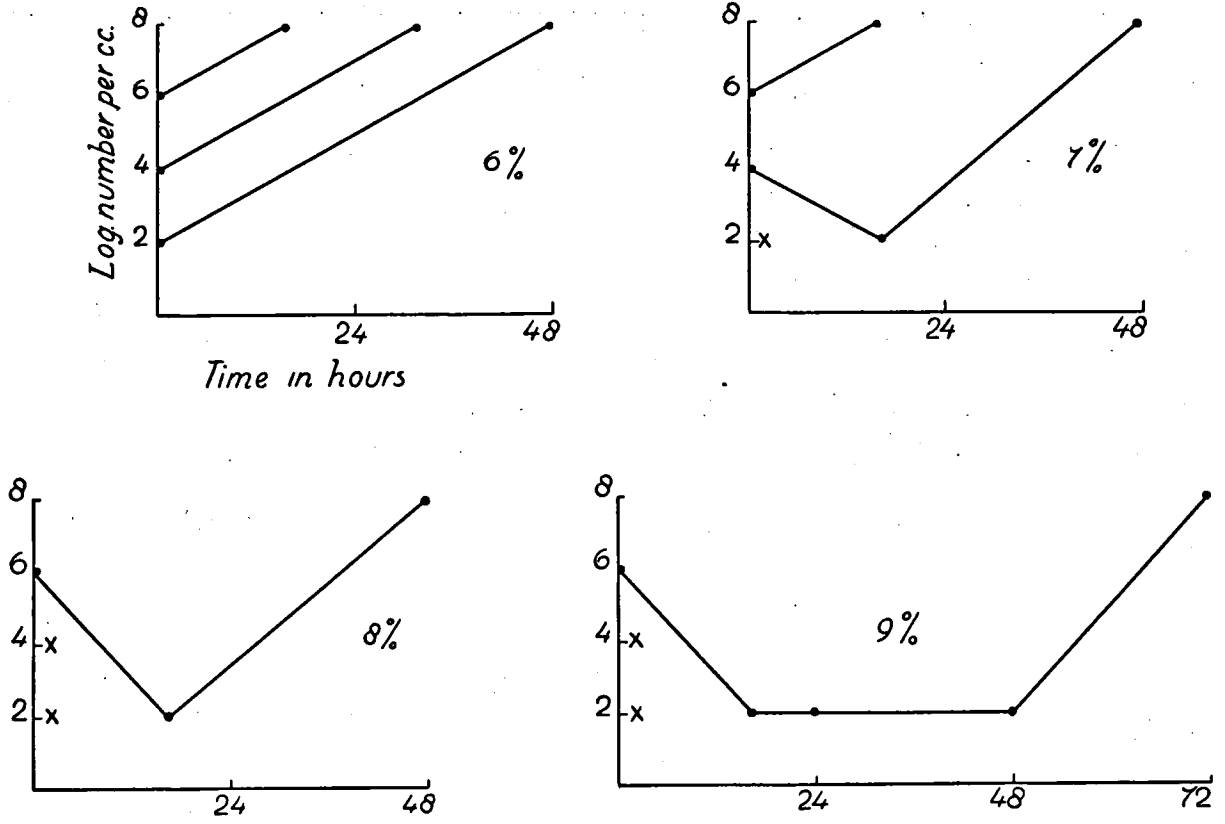


Fig. 12. Similar to fig. 11. Initial cultures inoculated with pure cultures with the same percentage of NaCl.

Table 4. Exp. 11-VII-'36.

Amount:	2.10^6	2.10^4	2.10^2
% NaCl.			
6	18-18	30-30	42-42
7	18-18	42-42	×
8	42-42	×	×
9	60-60	×	×

From the above experiments we may draw the following conclusions:

1) When more cells are used as inoculum growth may take place in higher salt-percentages.
2) At one and the same percentage growth begins earlier when a large inoculum was applied.
3) During growth in higher salt-solutions, there is a period in the beginning, where the amount of cells that may develop in saltless peptone-water has decreased in comparison to the initial number of inoculated cells.
4) When cultures were used which were cultured before on solid media with the same amount of NaCl as the solutions, growth began much earlier.
5) Although we inoculated with cells grown in media with the same percentage of salt, a slight inoculum yields no growth in the higher percentages.
6) Here too the amount of cells viable on re-inoculation to saltless peptone-water, may be less than the initial amount.

The results obtained are in agreement with the literature cited in chapter I, and especially with the work of S l e m m o n s (1926). That the range of variation is a function of the number, is in accordance with our views. L a n g e (1922) found that heavy suspensions of *B. coli* and *Micr. pyogenes* showed an increased resistance to desinfection as compared with dilute suspensions, and proved the existence of a mutual protection of the cells. A dilute suspension showed the same resistance as a heavy one, when a heavy suspension of killed cells was added to it.

L a n g e only found the said effect in suspensions much more concentrated than mine, while in dilute suspensions the effect was negligable. This is not in accordance with my data because in 7 % NaCl solution I found a marked difference between 10^4 and 10^2 cells. Therefore we do not believe the protecting action described by

Lange to play a role. A set of experiments was carried out to prove this assumption.

Exp. 16-VI-'38. Peptone-water, 25° C. In this experiment we worked with two series, the first similar to the other experiments, while to the other series was added a autoclaved suspension of a full-grown culture inoculated in peptone-water the day before. To each tube 2.10^6 cells of the killed suspension were added. Microscopically the killed suspension could not be distinguished from a living one, all the cells seemed quite intact.

Table 5. Exp. 16-VI-'38.

Amount:	2.10^2	2.10^2 + † S.	2.10^4	2.10^4 + † S.	2.10^6	2.10^6 + † S.
% NaCl						
3	24-24	24-24	24-24	24-24	12-12	12-12
6	36-36	36-36	24-24	24-24	24-24	24-24
7	64-64	64-64	64-64	64-64	36-36	48-48
8	× ×	× ×	90-90	90-90	60-60	60-60
9	× ×	× ×	× ×	× ×	90-90	90-90

According with our view, no acceleration of growth was found by adding killed suspension to a growing culture, so a large number of cells did not exert a protecting influence as in Lange's work. We, therefore, do not intend to use this phenomenon in our explanation of the fact that growth in stronger salt-solutions only occurred with a heavy inoculum.

After these preliminary experiments it seemed useful to follow the growth in salt more closely and to compare the track of the different growth-curves in different solutions. We shall, for this reason, discuss the growth-curve in bacteria, from a general point of view, in the next chapter.

CHAPTER VI.

THE GROWTH CURVE IN BACTERIA.

§ 1. *Methods of following growth.*

By growth curve we understand a graph, expressing the relation between the amount of bacteria and the time.

Ever since the oldest investigations of Nägeli and Schwen-

dener, much work has been done to establish this relation. Extensive historical surveys are given in the works of Chesney (1916), Jensen (1928), Henrici (1928) and Rahn (1932) and need not be repeated here.

A short discussion of the principal arguments may follow here, according to the papers of the above mentioned authors and those of Penfold (1914), Wilson (1922) and Meller (1925), while we also consulted Buchanan's textbook. (Buchanan and Fulmer 1930).

One may obtain a growth curve in three totally different ways;

1) The amount of cells may be counted at different times, either in the culture itself, or in its dilutions, microscopically or indirectly, e.g. with a nephelometer.
2) One of the chemical changes the bacteria are bringing about, e.g. gas-formation, may be measured.
3) A sample of the culture, diluted if necessary, may be put into a solid culture medium and the germs developing into colonies, may be counted after incubation; so-called "plate-count". A sample of the medium can also be prepared so as to form a range of dilutions. After bringing these in a suitable medium the initial amount of cells in the sample may be computed when we know the ultimate dilution in which growth occurs. This methods only gives an approximate value.

The first method gives the most exact expression of the amount of cells that is present at every moment.

The nephelometric method, however, needs a severe correction, when the cells do occur in clusters of different magnitude, as in our case, because the light-absorption of a certain amount of cells will be totally different, when the cells stick together and form chains and clusters.

The main draw-back of microscopical methods is that one can only begin to apply these methods, when a fairly large amount of cells has been formed, because, otherwise the chance to see any bacteria through the microscope is too small. So when working with a small inoculum one may only follow the end of the proces. Secondly, visual methods establish no difference between dead and viable cells. Knaysi (1935) succeeded in working out one method for *B. coli* with "neutral-red" but it is understood that in every special case a new method has to be carefully tested before use.

The second group of methods postulates that metabolism shall keep pace with growth. This is certainly not the case, and this group of methods, historically the oldest, are scarcely used now.

By means of the third method we only count cells that are viable

on the medium used. One is interested, however, in the amount of cells that are present at every moment in the growing culture. It may be asked, moreover, how many of these cells will be dividing. On the plate the cell is only counted when it has grown out to a full-grown colony. When it stops its divisions, after a number of cells have been formed not large enough to be noted on the plate, the cell is not counted together with the viable part of the culture. Moreover, the medium is constantly changing in the growing culture as a result of the action of the cells themselves. The medium of the plates, on the other hand, always remains the same, and from what happens on *that* medium conclusions are drawn about what the same cells would have done, when they had remained in their own medium.

From this survey we may see that we can only get a vague impression of what goes on in a growing culture. R a h n puts it in the following words: "None of these methods gives an absolutely correct conception of the increase of living matter. Nor can any method be suggested which would give a true measure of living matter because we do not know enough about it".

In daily bacteriological practice, however, the methods described above proved to yield fairly reliable and comparable values, at least when only the ascending part of the curve is concerned. (W i l s o n).

§ 2. *The different parts of the curve.*

When, against time on the abscissa, the logarithm of numbers is plotted the general track of the curve is as follows.

Starting from a 24-hour old culture of *B. megatherium*, for instance, cultivated on peptone-agar with 1 % glucose, and inoculated into peptone-water with 1 % glucose, aerated, and incubated, as stated in chapter III, one finds the first part of the curve to run parallel to the abscissa, for about two hours. This phase is called the "latent period". Than follows, during a few hours, a part of the line running continually steeper, indicating an increase of the average growth-velocity of the cells. This period is called the "lag-phase". Some authors mean by "lag time" the whole periode from the beginning till the maximum-velocity has been reached. After our opinion this habit is the cause of much confusion and should be abandonned.

In the following period the growth-velocity is constant and has reached its optimum value. The growth-curve is in its "logarithmical period", represented by a straight line. Than follows the "period of negative acceleration", during which the graph falls with increasing velocity until it runs more parallel to the abscissa, during

the "stationary phase". Until now the curve is S-shaped. After that, the amount of cells is seen to decrease, but by far not so regularly as it was increasing before. Two cultures, grown under the same conditions will show almost the same growth-curves until this stage is reached, but the second part of both lines may deviate considerably. In this period, however, growth no longer occurs.

Most often, the growth-curve will descend slowly, but sometimes a second rise occurs, forming a second maximum in the curve. This process escapes attention when a broth-culture is studied without plate counts but when on a solid medium, the process causes the formation of so-called "secondary colonies". On the edge of a full-grown colony that had stopped its growth long ago a new small colony is seen to crop up.

In every special case, however, the difference between the various phases will be not so-marked. The "latent period" and the "lag-phase" always make their appearance, but sometimes it seems as if from the straight part of the curve only the point of inflection remains. Also the horizontal part, round the optimum, may be reduced to almost nil.

The growth-process has a marked temperature-optimum, the curves being considerably shifted by changing temperature.

It has been shown experimentally that the total yield is independent of the mass of the inoculum, an extremely large inoculum excepted.

The "latent period", however, is lengthened by a small inoculum, and the maximum is reached slightly later. With an extremely large inoculum the growth-velocity can be seen to decrease before a "logarithmical phase" has been reached. The growth-velocity seemed unable to reach its maximum in that case. Numerous investigations have been made to obtain an insight in these phenomena.

The "lag-phase" is considered as originating by the variation of "latent periods" of various cells. Every single cell starts dividing with maximum velocity, directly after it has overcome its "latent period". During the "latent period" hardly any cell is dividing, during the "logarithmical period" the overwelming majority is dividing, the "lag-phase" is the period in between when every moment more cells begin to divide, thus causing a continuous rise in the average growth-velocity of the culture.

Henrici (1928) and Jensen (1928) offered experimental proof for this assumption by following the growth of isolated cells under the microscope. This reasoning may also give an explanation for the decrease of the "latent periode" when a large inoculum is used.

In that case, we have more chance to meet fast-growing cells,

which can convert the "latent period into the "lag-phase". The latter phase, however, lasts much longer when a larger inoculum was used. The reason is essentially similar. In a large inoculum the chance to meet extremely slow growing cells will be greater too. As long as there is a number of cells that refuse to divide, the average growth-velocity cannot reach its maximum value. In Fig. 13 we give

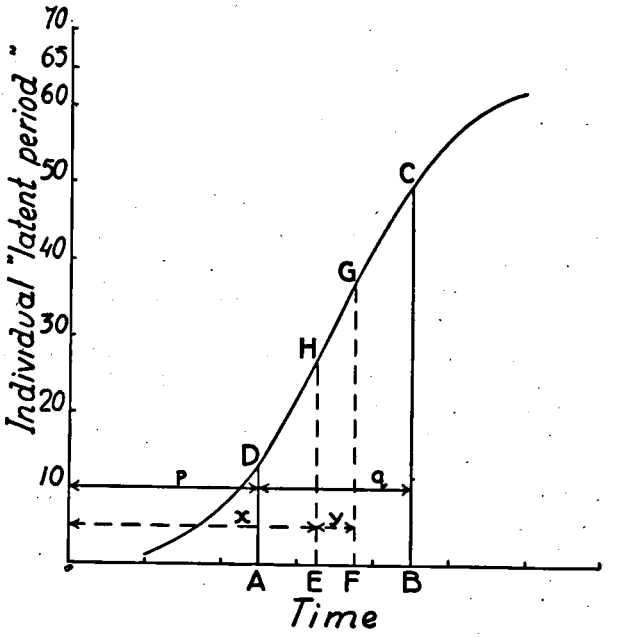


Fig. 13. Schematical representation of the relation between "latent-period" resp. "lag-period" and seize of the inoculum. Inoculum ABCD yields latency p, lag q. Inoculum EFGH yields latency x, lag y ($x > p$ and $y < q$ but $x + y < p + q$).

a schematic representation of this theory. When "lag" is overcome, the average growth-velocity will be independent of the amount of inoculated cells. It seems to be possible, however, when working with extremely large inoculata, that the maximal growth-velocity is reduced. A possible explanation of this phenomenon might be, that the growth was inhibited by the influences to be discussed later on, *before* a maximal growth velocity could be attained. The "lag-phase" is assumed to pass directly on to the "period of negative

acceleration". We venture to propose this theory to explain our results reproduced in figure 15.

§ 3. *The explanation of the "latent period".*

The reason that a "latent period" exists, must be sought in the cells itself and not, as older workers did, in the medium. Young cultures were seen to grow without any "lag" in fresh media, if transferred from a culture in its "logarithmic phase", a culture that had overcome "lag" itself. (C h e s n e y 1916) The same phenomenon could be shown with single-cell cultures (J e n s e n 1928).

Also traces of inhibiting substances, transferred with the bacteria from the old medium into the new one, cannot play a part, because in that case, the "latent period" had to increase with heavy inocula instead of doing the opposite.

P e n f o l d (1914) and C h e s n e y (1916) demonstrated clearly the correctness of M ü l l e r's assumption, that the reason for the latent period should be sought in the phenomenon that cells, when transferred into a new milieu, go on for a short time, behaving in the same way as in their old milieu. C h e s n e y transferred from a growing culture to the same medium and found that the curves, indicating what happened to the transfers, ran parallel to the growth curve of the initial culture. The reason why bacteria do not grow directly with maximal velocity, is the same as why they stopped growing in their old milieu.

During the horizontal part of the curve, and still more during the decline afterwards, it is apparent that several cells autolyse. Also a large part of them shall not be autolysed yet but do no longer belong any more to the viable part of the culture.

This was found by W i l s o n (1922), who compared the results of the plate-count with those obtained by visual methods. The latter always proved to yield higher values. The difference was enormous in the second part of the growth-curve, but it was also evident during the ascending part in the beginning. W i l s o n assumes that always a fraction of cells shall die, even in fast-growing young cultures. After the maximum this process prevails, before the maximum it only plays an unimportant role. This theory was attacked by K e l l y and R a h n (R a h n 1932) because they could obtain a 100 % growth in their single cell-cultures. H e n r i c i (1928) and L e w i s (1932) saw several times the atrophy of a cell, when working with micro-cultures, from *B. megatherium* and *B. coli.* When the percentage of cells that are autolysed is small, it will be difficult to prove the existence of this process by means of single cell-cultures.

§ 4. *The termination of growth.*

The reasons why growth comes to a stand-still are the following;

1) lack of food.
2) accumulation of dangerous waste-products of metabolism.
3) inhibiting substances formed by the cells themselves.

The first reason is of least importance as, among others, Meller (1925) and Rahn (1932) could demonstrate. They proved that exhausted culture media still may contain much food, for when these cultures are autoclaved and reinoculated with the same bacterium growth may once more set in. This process may even be repeated several times. Also waste-products are exceptionally limiting factors for growth. In the experiments of Rahn and Meller such products as *skatole* and *lactic acid*, were not removed by sterilisation and still growth went on. Moreover, when we compare the increase in generation time (the time the culture needs to double its amount of cells) and the increase in waste products, the former is enormous and the latter only slight.

The third reason is by far the most important one, though there are cases known where the first and second actions exert a strong inhibiting influence.

The first to assume auto-inhibition was Eykman in 1905. His work was later on improved and corroborated by Meller (1925), who demonstrated the existence of such inhibiting substances bejond doubt. She even distinguishes two kinds, thermostabile and thermolabile ones, and develops a theory, after which, the "latent period" and the fall after the optimum, are considered as resulting from the action of those substances. She made some most important experiments with old cultures. When she inoculated a large amount of duplicate-tubes with old cultures, they behaved very contradictory, some of them giving no growth at all. Old cultures proved to grow much better on autoclaved filtrate of exhausted cultures of about the same age. In these media they even yielded more growth than in fresh broth. She drew the following conclusions from her experiments.

1) In cultures, which had developed beyond their maximum, a greater variability among the cells has occurred. Whether or not this process is influenced by the medium remains undecided.
2) In the beginning of growth in extreme media, (old filtrates for young cultures and fresh broth for old cultures), a selection may take place of those cells that grow better in these media, the other cells being killed.

Our own theories, developed in the previous chapter, agree with

those stated here. We already took the opportunity to draw attention to the curious fact that even in actively dividing cultures some cells must be assumed to die, directely after they have been formed. We also pointed out the enormous difference between two stages of one growing culture.

Henrici, studying the morphological changes during growth with various bacteria, found them to be uniform in shape during the first part of the ascending branch of the growth curve. In the "negative acceleration phase", however, and later on, a great variety in form and size was seen. How are we to consider these aberrant forms?

A good deal of them is due to autolysis and to be considered as "involution forms", the "Absterbe-Erscheinungen" of german authors. As growth proceeds, the chance to meet "pedemata" will increase and a minority might be considered as such.

The bulk of aberrant forms, however, is to be considered as products of far-advanced fluctuating variability because as the milieu influences are stronger as growth proceeds, their modelling influence on the doxatype of cells will produce extreme forms continuously. The forms mentioned occur so regularly and so clearly as a response to the action of the milieu, and also in such an amount in every culture, that we do not think that pedemata will play an important role.

CHAPTER VII.

OWN EXPERIMENTS ON GROWTH IN DIFFERENT SALT CONCENTRATIONS.

§ 1. *Scope of the experiments.*

In continuation of the preliminary experiments the growth in various percentages of NaCl was followed quantitatively, and growth curves were constructed. Our aim was to find a possible answer to the following questions:

1) Do all of the cells develop, when transferred to liquid-saline media from saltless media, or does only a fraction of them do so (Selection or no selection)?
2) Will the cells, viable in the new milieu start to grow at once, or only after a while (during which time they might be thought to "adapt" themselves)?
3) What kind of growth curve will describe their growth in the different media?

4) What will be the relation between the different growth velocities?
5) Will there be a depression in the total yield?
6) When we transfer cultures, grown in saline milieus to saltless ones, what will be the answer to the first three questions?
7) What will be the answer to the foregoing questions for the growth of cultures, grown in saline media before, after transfer to liquid media containing the same percentage of salt?

We followed growth in two aerated cylinders as reproduced in Fig. 9, with the aid of a double series of plates, one series of peptone-agar-glucose plates, the other series containing a certain amount of NaCl besides. One of the cylinders contained peptone water with a certain percentage of salt, the other, serving as a control, peptone water alone. In every experiment the salt percentages of the cylinder and the plates were the same. Our intention was, by transferring from both cylinders to both kinds of plates, to fix the situation at any desired moment and to learn, in such way, which part of the cells was able to grow on either of the two milieus, the old and the new. It is necessary to assume, therefore, that when a cell can develop in a certain *liquid* milieu it will do the same when transferred to the conformable solid agar-milieu. This is tacidly accepted in the greater part of all quantitative work. W i l s o n (1922) found only a very slight difference between the results of the plate count and visual countings in the ascending part of the growth-curve. As we saw he explains this discrepancy by assuming that the cells that were present but did not grow out on the plates, were neither able to develop in the culture. All workers, however, who paid any attention to this problem, agree that the amount of viable cells that can form colonies on solid media is closely related to the amount that is dividing in the growing culture. The other methods of measuring growth cannot be used for our purpose, which follows directly from the consideration of the questions we stated in the beginning of this chapter.

§ 2. *Growth in salt solutions of pure cultures from saltless media.*

Fig. 14 shows a growth curve, where the increase of cells in normal peptone water-glucose is followed, by means of saltless plates. The apparatus used in all of the following experiments is that given in Fig. 9. Contrary to the other curves this one has been followed till after its optimum, to show the above stated phenomenon of "second-bloom". Usually we did not proceed further than the maximum. All the phases described in chapter 6 are visible here, also the phenomenon of "second bloom".

Fig. 15 gives a set of curves also concerning growth in saltless medium, but with different inocula. Here, as in all other cases, only the ascending part is drawn. This figure illustrates what was said about different inocula in the foregoing chapter. The curves I, II, III and IV show, in the order mentioned, the decrease of the "latent period". The logarithmic growth, however, is the same in all cases. The numbers V, VI and VII show a still smaller "latent period",

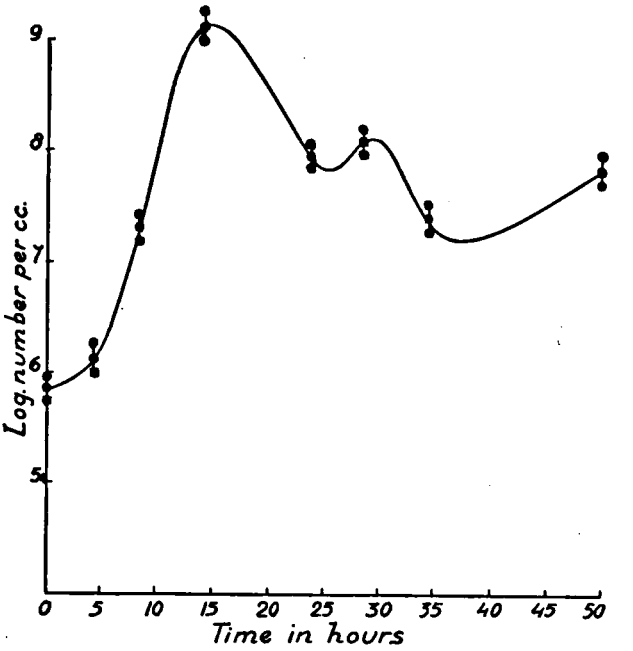


Fig. 14. Growth-curve followed beyond the maximum.

but they hardly have a horizontal part, no. VI begins from P on to rise less rapidly, and no. VII from Q on. In this case we assumed that the inhibiting action began before the maximal velocity was attained.

Figure 16 shows, on the same coordinates, the growth of an amount of cells, (indicated by the arrow, 10^4 in every cc.) in peptone water with 1 % glucose, counted on peptone agar plates, besides the growth of a culture in the same medium with 6 % NaCl, counted in two ways, viz. on peptone-agar plates, and also on peptone-agar plates with 6 % NaCl. The inoculated cells originated from a

18-hour old pure culture, cultivated on peptone agar at a temperature of 30°.

The lines are indicated by three consecutive numbers, the first number refers to the salt-percentage of the pure culture, from which the inoculated cells originated, the second number refers to the

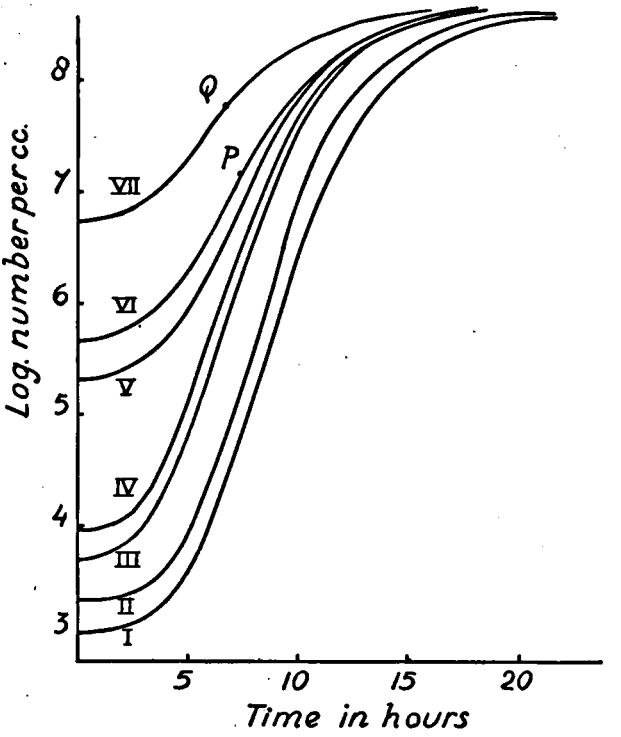
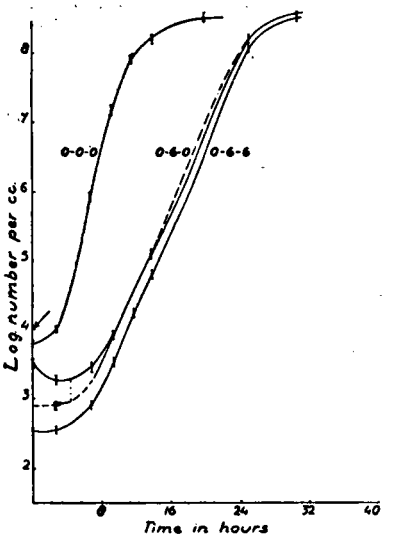
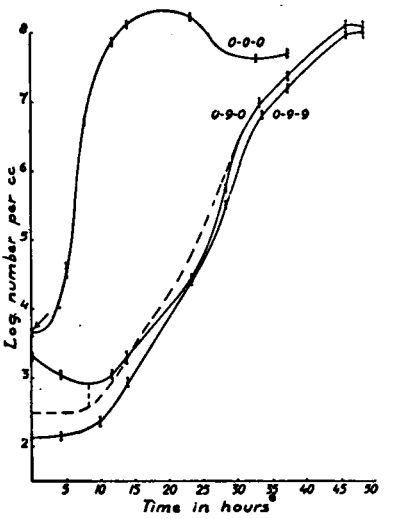


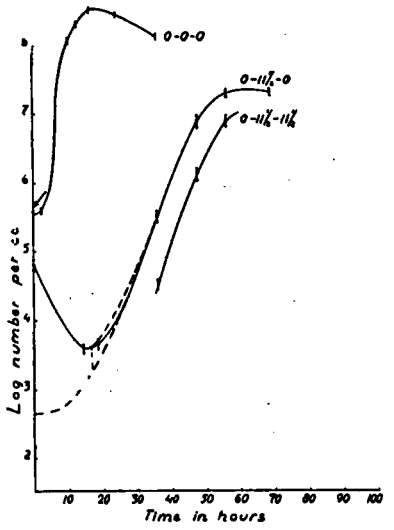
Fig. 15. Effect of increasing inoculum on the growth curve.

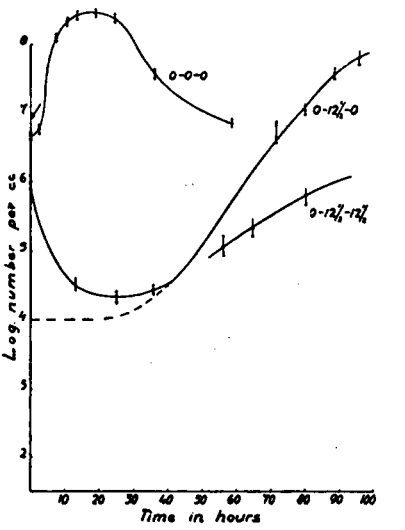
salt-percentage of the liquid medium where growth was studied, the last number refers to the salt-percentage of the agar-plates. The "cluster-factors" in Figs. 6 and 7 are average-values from many experiments. In the following graphs, no such figures shall be depicted. In all following experiments the same nomenclature is used. These graphs allow of the following conclusions:

1) In the 0 %-milieu not all of the inoculated cells could grow, the arrow being situated higher than the first point of the 0-0-0 curve.









Figs. 16, 17, 18, 19. Explanation see text.

2) The crop yield in the 6 %-milieu is the same as in saltless medium. This result is in contradiction to E s t o r's findings (1927) who found a considerable decrease, on reoculating the same organism to the same salt-percentage. E s t o r measured nephelometrically and did not mention any cluster-formation. Moreover, his cultures were not aerated, perhaps these differences may account for the different results.
3) Not all of the inoculated cells formed a colony on 6 %-peptone-agar.
4) The plate counts from the 6 %-culture on 6 %-agar plates, the 0-6-6 line, give results that remain always below those of the 0-6-0 line, indicating the amount of cells from the 6 %-culture that showed growth on saltless plates.

So we see that no cells are formed that can grow only in 6 % NaCl and not in 0 % NaCl.

5) The 0-6-0 line, however, shows a curious initial decrease, in a similar way as we found in the preliminary experiments described in chapter V. The same curve was found by S h e r m a n c.s. (1922) who studied growth in dialysed media, without a trace of salt, with the aid of ordinary plates with a normal supply of inorganic salts. The two curves both referring to the growth in 6 % salt, deviate largely in the beginning but later on they run parallel. The slight deviation might be explained in this way that a certain percentage of cells forms colonies that cannot be seen on the salt plates, or perhaps the percentage of cells that cannot develop on a solid medium increases with the salt percentage of the medium. Be this as it may, the second part of both curves yields a good impression of cell-increase, although the upper line is the most correct one. The shape of the first part of the curves cannot be explained so easily, however. The first point was found as follows; directly after the cells, grown on saltless medium were transferred to the 6 %-peptone water medium, a sample was taken and plated out on 0 %-peptone plates. The difference between the amount of colonies developed in this case and in the other case, where we sampled from the saltless culture, is apparent. So we must assume that a number of cells loses the power of reproduction, directly after contact with the new milieu. It seems reasonable to assume that this process of destruction is not ended exactly at the time we took our sample, but proceeds afterwards. We might suppose that the first decline of the 0-6-0 curve indicates the death-rate of the cells that refuse to develop in the 6 %-milieu. However,

we should also take into account the fact that, when these cells are autolysing, those that have the ability to grow shall increase in number and the cells formed by that fraction can form colonies on 0 % as well as on 6 % NaCl-agar. The increase of the latter disturbs the image of the decrease of the other fraction. The 0-6-0 line is thought to be composed of two curves, the growth-curve and the death-rate curve. The curve that expresses the true course of cell-increase in the salt milieu is not identical with either of the two curves. For we saw that in the later stage of growth, the 0-6-6 curves show the same course as the 0-6-0 curves yielding, however, slightly lower values. Therefore the first part of this line can give no correct data either; the true values might be expected to be higher also in this part of the curve. The true course might be approached best, when we take the ascending part of the 0-6-0 line and try to exterpolate it to time "zero". For this purpose we draw aline parallel to the 0-6-6 line. The death-rate curve should cross the growth curve at a certain instant. At this instant the amount of cells that grow on 0 % is twice the amount that grows on 6 %. So when we draw a line parallel to the 0-6-0 line and 0.3 logarithmic units (measured along the ordinate) removed from it, the point of contact of these two lines will also be a point of the death-rate curve. This curve, however, may be constructed directly. For if the assumption be true that a part of the inoculum dies, we should find the death-rate curve by following a culture raised from very few cells, in which no growth occurs (see next chapter). It might be also possible that in the first part of the growth the cells passed into a sort of resting-stage and did no longer grew on solid media. In our case this period would have lasted 5 hours, and be followed by another 5 hour-period during which the culture "revived" again and gradually yielded its original amount of colonies. A disadvantage of such a reasoning would be that after the resting period had passed, growth had to start without any "lag" or "latent period", because the curves are seen to rise directly after their minimum. If such a process would take place, however, we should not be able to find the initial decrease by means of visual counting. As will be stated in a following chapter, we also measured growth by means of a nephelometer and were able to confirm the phenomenon. We anticipate on purpose because the experiments on growth have to be stated in relation to some definite theory.

6) In every experiment on growth in salt solutions we determined the "cluster-factor" before any observation. In the beginning, however, growth did not permit microscopic examination, so we only could use this correction-factor in the latter part of the curve. It stands to reason that the observations just before the introduction of the "cluster-factor" will need a correction. This correction is indicated by a dotted line. Figs. 6 and 7 (see chapter IV) give the average course of the "cluster-factor" at various salt percentages.

Fig. 17 shows, in a similar way, the growth in a 9 % milieu. From this figure it follows that;

1) The curve for the growth in the saltless medium which was reconstructed in every series to serve as a control, agrees completely with the curve of the preceeding series.
2) The general relation between the three curves is the same as before.
3) Growth proceeds in 9 % NaCl medium much slower than in a 6 %-medium.
4) There seems to be a slight increase in the crop-yield. The logarithm of total amount of cells being 8.1 in stead of 8.5 in the 6 %-milieu.
5) The percentage of cells that yielded colonies on the 9 % NaCl-agar directly after inoculation is smaller than the corresponding percentage in the 6 %-series.
6) The "cluster-factor" is increased as compared with the 6 %-milieu.
7) A considerable correction in the shape of the 0-9-0 curve has to be made, because the "cluster-factor" apparently has increased from 0 to 30 in a relatively short time.

Fig. 18 shows growth in the same nutrient medium with 11½ % NaCl. From these data it follows, that:

1) The 0-0-0 line, serving as a control, runs parallel to the lines of the other series.
2) Growth is decreased still further.
3) The total yield did again decrease (antilog 7.5).
4) The general relation between the three curves was re-established.
5) The percentage of cells that did grow in this salt percentage must have fallen considerably, because the first plate-counts showed no colonies. This means that less than 1 cell out of 0.1 cc. was able to form colonies on 11½ % NaCl-plates.

6) For the first time a marked difference between the amount of colonies on the salt-plates and the amount growing on the saltless plates is apparent. The difference between the 0-11½-0 and the 0-11½-11½ lines amounts to 1.3 log. units on the ordinate. Here the saltless plates give a much better impression of the growth-process than the salt plates. A constant difference between the plates and the liquid cultures seems to be in the aeration, lack of oxygen might be a cause of this discrepancy.
7) If we construct the point of contact between the death-rate curve and the exterpolated growth curve and if we connect this point with the starting point of the 0-11½-0 line we have approximated the course of the death-rate curve. This course will be seen to coincide with the 0-11½-0 line itself. This seems reasonable, because at the very beginning the lysis of the majority of the inoculum will be the only process that takes place, those cells that will grow afterwards having not yet overcome "latency" and "lag".
8) The "cluster-factor" is again increased.
9) The point showing the number of cells capable of growth indicates that only few cells were able to develop. Therefore no colonies appeared on the first salt plates.

Fig. 19 shows growth in a 12½ % NaCl nutrient medium. From these data it appears that;

1) The control is again in agreement with the 0-0-0 lines obtained before.
2) The general relation of the three curves remained the same.
3) Growth proceeds still more slowly.
4) The total yield is decreased as compared with the 0 %-milieu, but seems similar to that in the 11½ %-milieu.
5) We see, as in the preceding series (11½ %) the peculiar course of the first part of the 0-12½-12½ line.
6) The difference between the amount of colonies in both series is much increased, at the end it even attains a value of about 2½ log. units.
7) The "cluster-factor" did probably not increase, compared with growth in 11½ %. This cannot be concluded with certainty from the data obtained, because we do not know what had happened before the first observation.

§ 3. *Summary of results.*

Table 6. Summarizes the data obtained, giving the fraction of inoculat cells that starts growth in the different percentages.

Table 6.

Percentage of NaCl in the medium	Fraction that starts growth
0	83 %
6	8 %
9	6.3 %
11½	0.16%
12½	0.3 %

Table 7. Maximal growth velocity during the "logarithmic phase" computed from the ascent of the curves during 8 hours (in the case of the 0 %-curve we took 4 hours and doubled this value).

Table 7.

Percentage of NaCl in the medium	Relative growth velocity.
0	4.10
6	2.45
9	1.90
11½	1.25
12½	0.65

These data are graphically represented in Fig. 20.

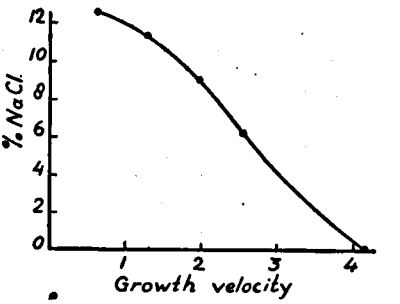


Fig. 20. Explanation in the text, table 7.

Table 8. Relative time in which the growth-maximum is reached.

Table 8.

Percentage of NaCl in the medium	Relative time to reach the maximum.
6	20
6	32½
9	48
11½	64
12½	104

This was graphically represented in Fig. 21.

If we now compare the graphs that give the best image of growth in the various salt-percentages, we see that, but for a slight increase in total yield, the only difference is the decreased division velocity. If we want to compare the curves more closely, they should be recalculated, changing the abscissa by making the time to reach the maximum the same. The correction factor for every graph was taken from table 8. The result of such an operation is seen in Fig. 22. where we collected some of the curves from Fig. 15, besides the graphs for the growth resp. in 6 %, 9 % and 11½ % NaCl, all recalculated to a time for maximal growth of 20 hours. In the cases where the total crop-yield showed a marked decrease it was necessary to make a correction for this phenomenon. The result shows that the three seemingly different curves run in almost the same way and fit very well in the bundle of curves copied from Fig. 15, representing growth in saltless media with various density of inoculation. So it is possible to derive the growth curve for various salt percentages from the "normal" curve by very simple means.

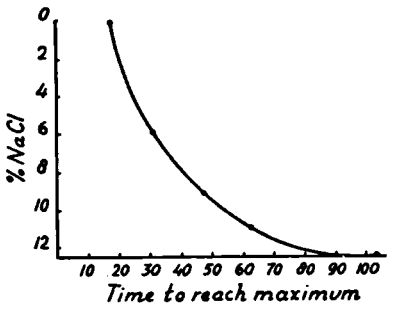


Fig. 21. Explanation see text, table 8.

For 12½ % NaCl solutions the above operations were not carried out because the extremely low velocity of growth in this medium, involves a large correction-factor with, consequently, the possibility

of large errors. Moreover, the reconstruction of the first part of the curve seemed, for the same reason, too uncertain to warrant conclusions.

Summarizing the results we may say;

1) The data given in this chapter are in accordance with the preliminary experiments.
2) From all cells, inoculated in a solution of higher salinity, only a fraction starts growing, this fraction is smaller in higher percentages.
3) Those cells that cannot develop in the given medium are due to die.
4) The growing cells show in essence the same growth-curve as is established in salt-free milieu, but for a deformation, more pronounced in increasing salt-percentages, along the ordinate. In other words, the division rhythm was retarded.
5) The total crop-yield remains constant in the lower percentages, and shows a slight decrease in the higher ones.

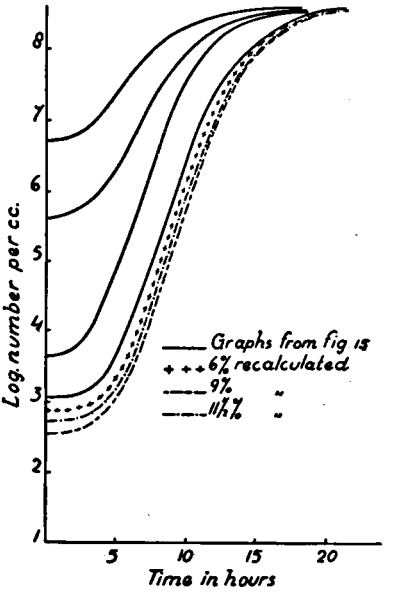


Fig. 22. Explanation see text.

CHAPTER VIII.

INOCULATION WITH PURE CULTURES FROM PEPTONE AGAR WITH DIFFERENT PERCENTAGES OF SALT.

§ 1. *Description of the curves.*

Growth was studied in strains cultivated on salt-agar and transferred to a nutrient solution containing the same percentage of salt. In order to obtain comparable results, the experiments were made with cultures of physiologically the same age. From the results of the previous series, the times were computed corresponding to 18 hours in saltless medium.

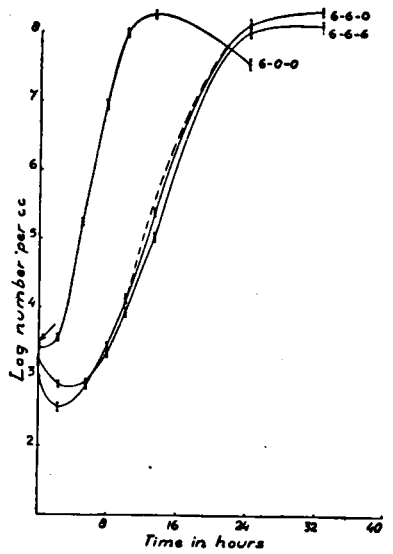


Fig. 23.

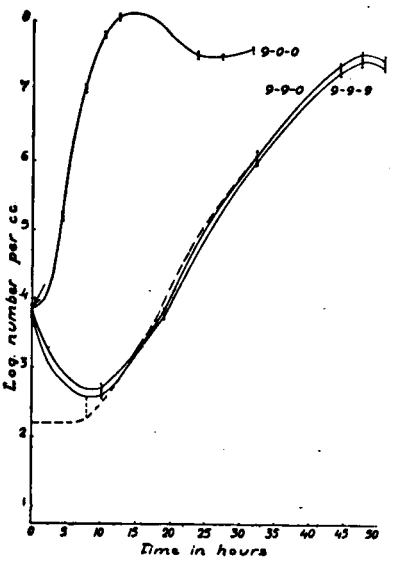


Fig. 24.

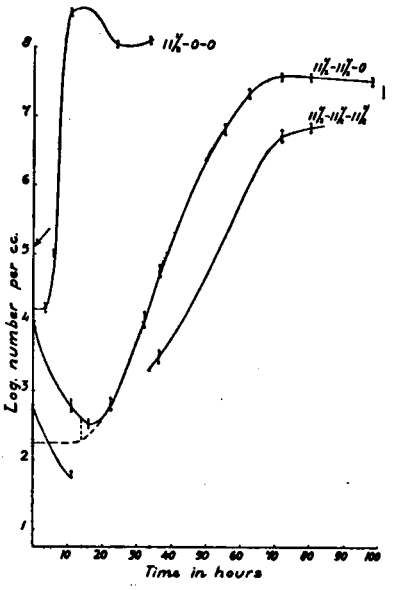


Fig. 25.

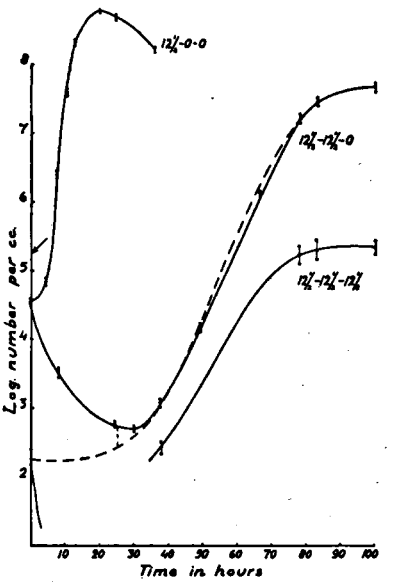


Fig. 26.

Fig. 23 shows the result on a 6 % NaCl solution. We may conclude;

1) Retransfer to saltless medium gives exactly the same curve as obtained by inoculation with a saltless pure culture.
2) At zero time we see that nearly all of the cells from the 6 % peptone-water culture yield colonies on salt plates as well as on saltless plates. At the end of the growth at 6 % in the previous series the same phenomenon was observed.
3) The latter part of the 6-6-0 and 6-6-6 lines runs completely parallel to the lines of the previous series, where we inoculated with saltless pure-cultures. The place of the maximum, the growth-velocity and the mutual relation between the two curves is the same as in the previous series.
4) Here it also appears that in the saltless plates more colonies develop than in the salt plate series.
5) A curious fact is the initial decrease when the cells are transferred from a solid medium of 6 % NaCl to a liquid medium with the same salt-percentage. In a following paragraph we shall deal at length with this unexpected fact.
6) The inoculated cells from the pure culture tube had a negligible "cluster-factor", so we could only use the correction-factor in the latter part of the curve, and had to introduce a correction in the part just before the first "cluster-factor".

In Fig. 24 we give the corresponding results for 9 % NaCl. We see that;

1) The growth in saltless milieu is the same as in the previous case.
2) As in the 6 %-series, all cells from the 9 %-culture develop in both plate-series.
3) The curves for the 9 %-solution run exactly in the same way as in the previous series, but for the first part.
4) The initial decrease appears in the same way as in the 6 %-lines of the previous experiment, but this decrease leads to lower values.
5) The initial "cluster-factor" was 4. Later on, microscopic examination revealed nothing at all, so, for the want of better, we used this value throughout.

Fig. 25 gives the growth in 11½ % NaCl solution. It appears that;

1) The curve for saltless milieu is again similar. (As in the foregoing case the difference in the ordinates must be taken into account).
2) As in the same percentage of the preceding series, considerably more cells from the salt culture could develop in the saltless

plate series than in the other series, where we cultivated on plates with 12½ % NaCl.

3) From the total amount of cells inoculated in the 12½ %-solution initially only a fraction could develop on the agar-plates, a still smaller part developing on the salt-plates.
4) The 11½-11½-0 line runs parallel to the 0-11½-0 line from the previous series, at least when only the latter parts of both curves are considered Also the maximum is almost the same.
5) The initial decrease becomes markedly stronger.
6) The 11½-11½-11½ line is broken off, in cultures corresponding to the deepest point we saw no colonies at all on the plates.
7) The "cluster-factor" showed a curious feature. It began with a value of 25, and ± 9 hours later we found only 10. No further observations could be made before growth had started.

Fig. 26 represents the growth in a milieu of 12½ % NaCl. The following conclusions may be drawn;

1) The growth-curve for 0 % is again the same.
2) The discrepancy between the data of the salt-plates and those of the saltless plates is increased compared with earlier results.
3) Again only a fraction of the inoculated cells was able to develop even in saltless medium.
4) The 12½-12½-0 line does reach the same value as the 0-12½0- line, but only runs parallel to it later on.
5) The initial decrease is again more marked.
6) Here again the deepest part of the graphs for the salt plates approaches zero.

But for the 6 %-cells, all cultures from salt-agar tubes grew in the characteristic winding threads in the pure culture tubes and also in all salt solutions. When transferred to 0 % peptone water, however, the normal aspect of the cultures was directly restored. The morphological change proved, therefore, to be a temporary one.

§ 2. *The initial decrease.*

A complication is the above-mentioned initial decrease in all of the curves of this series, those depicting the behaviour on salt-plates as well as those from saltless plates. The latter part of all curves runs parallel to those from the previous series.

From a pure culture, grown in agar with a certain percentage of NaCl for a long time and transferred each week, a certain amount of cells was transferred to a liquid nutrient medium with the same percentage of salt. When a certain sample is taken out of the solution at the very beginning, at zero time, a percentage of the said cells,

high in the case of low salt contents and low in the stronger solutions, was able to develop on agar plates without salt and the same percentage developed on salt-free agar. When taking a sample somewhat later, however, we find a marked decrease in the amount of such cells. In all events things cannot go on in exactly the same way in the solution as on the solid media because, when after a certain time e.g. 80 % of an amount of cells dies, we should expect not more than 20 % colonies on the plates poured during this period, when a cell transferred to agar plates is supposed to behave in the same way as in the liquid medium. We saw in the previous chapter that it is generally assumed that most of the cells that can divide in the fluid, shall do so on the solid plate. Our case, however, looks like the opposite. In the literature there are a few cases known where a normal growth-curve (growth in saltless broth, counted by means of saltless agar plates) showed a slight fall in the beginning. In some papers we found this phenomenon had even escaped the attention of the author. We met evidence of such an initial decrease in the work of Sherman and co-workers (1922), Chesney (1916), Schultz and Ritz (1910) and Hüne (cit. Rahn 1932). The latter's data concerning the growth of *B. coli* in meat-extract are reproduced in table 9.

Table 9.

Time.	Amount.
15 min.	9720
45 „	6340
120 „	17300
240 „	36400

Again at a certain time a certain amount of cells may grow out into colonies, and some time later, only a fraction of the same amount can do so. Those workers who met with this fact and only refer to it, as Schultz amongst others, seem to have missed the significance of this phenomenon, for this author states: „. . . . in seltenen Fällen, anscheinend nach einer geringen Verminderung . .".

It is not possible, however, to account for the curious phenomenon, that must follow from their reasoning namely that the same decrease does not take place when a cell is transferred to a plate. Because if this were the case, we could expect more than the lowest value for all of the plate counts during the decrease. In our opinion the decrease may be accounted for in three different ways;

1) In reality the initial amount of cells remains constant. The decrease is only apparent and is caused by a temporary decrease of germinating-power of the cells. Such an assumption has, however, the following disadvantages;

a) We saw that in the extremely short period between inoculating and sampling (neglected in the curves, and never exceeding 5 minutes), a large amount of cells has died, as appears from the discrepancy between the first point of the 12½-12½-0 line and 11½-11½-0 line and the arrow indicating the magnitude of the inoculum. It seems reasonable to assume that in the inoculum always an amount of non-viable cells is present. These cells might autolyze in fresh peptone-water even if its salt percentage is the same, just as Meller saw the autolysis in fresh broth when she inoculated with very old cultures. This process will not stop after 5 minutes, but go on. The above-stated hypothesis does not take this process into account.

b) If we make such an assumption than the true course of growth should be represented by a long horizontal line during the time of the decrease. The growth-curve constructed in this way may by no means be transformed into the curve for growth in saltfree media.

2) A second theoretical probability is the following. From the beginning the clusters might increase by fusion. Here, however, we have the following disadvantage;

a) The "clusters" should reach, in this case, an enormous size, in the case of the 12½-12½-12½ line from the order of magnitude of 800. We never found filaments of more than 60 cells, with an average of 40.

b) In one case working with a large inoculum, we were able to find a number of clusters during the decreasing period of the curve (11½-11½-0 and 11½-11½-11½) (see Fig. 7). The initial "cluster-factor" for the inoculum was 25, and 10 hours afterwards we found a value of 10. The decrease over this period amounted to 1.1 log. units. When following the growth of two filaments we found them to grow by normal fission of all of their cells and not by mutual fusion (see Fig. 4).

3) A third hypothesis may obviate the above-mentioned disadvantages. The decrease in the lines of the 0-6-0 type described in the previous chapter, where we transferred to the old salt free milieu, was interpreted as the result of a autolysis-curve and a growth-curve of the other cells. As we were forced to

accept a difference between the behaviour of cells in agar and cells in broth at higher saltpercentages (and even exceptionally according to the literature, in saltfree media) we might apply this interpretation to this case.

It is, therefore, assumed that if cells from old agar-cultures were transferred to broth-cultures of the same saltpercentage, only a fraction of them can grow, the rest being autolyzed. During this process, however, part of the autolyzing cells may be "revived" when retransferred to agar-media.

In order to find the true course of growth in salt solutions, we may separate the two contributory influences and thus obtain the growth-curve and the death-curve separately. When this analysis was carried out we obtained the same graphs as in the previous chapter where saltless pure cultures were used. In the following chapter we shall try to offer experimental proof for the assumption that also in this case, a number of inoculated cells has died.

The different behaviour of agar and fluid might be object of further physiological investigation, which falls outside the scope of this paper. We should like to make one suggestion, however, in calling attention to the work of Walker and associates, who found that a cell must have a certain CO_2-pressure in its vicinity before it may divide. A current of CO_2-free air was able to postpone growth for more than 24 hours. It is apparent that a cell can create a good CO_2-atmosphere better in a solid medium than in a liquid one. (Walker cit. Knight 1936).

Some consequences of our hypothesis are;

1) The phenomenon of initial decrease is more apparent in higher salt concentrations, but it is chiefly present (though perhaps not perceptible) in "saltfree" media (read: media with about ½ % NaCl). It seems reasonable to assume that in extreme media the cells are in a less favourable condition after the end-point of growth, than in a normal milieu. Meller (1925) clearly demonstrated that in old cultures a large number of cells are unable of development in fresh broth. The transmission to liquid should affect the cells more in high salt percentages than in lower ones.

2) It may be of interest to discuss the question whether this assumption does interfere with our former deductions. As we remarked before, our interpretations were founded on the assumption that a cell, capable of development in a certain liquid medium, can do the same thing on the corresponding agar-medium, and vice-versa. The latter assumption

seems no longer tenable. We supposed a fraction of cells to die in aerated peptone-water and to survive in agar-media. Our aim is to approach the growth in the solutions. On all occasions, however, those cells that developed in fluid, could also grow out in agar. The data computed from the agar-plates are, for this reason, perhaps too high, but certainly not too small.

§ 3. *Conclusions and summary.*

Summarizing the results stated in this chapter it might be stated that;

1) Cells cultivated on saline pure-culture media, grow in liquid media with the same percentage of salt in the same way as cells that were not in contact with salt before.
2) With increasing salt percentages a considerable percentage of non-viable cells proved to be present in the inoculum.
3) Growth in salt peptone-water solutions proceeds in the same way as with inocula from salt free agar, as long as only the second part of the curves is concerned.
4) In the beginning all cells prove to grow as well on salt-plates as on saltless agar plates. In the higher percentages, however, the saltless plates yielded much better results.
5) The growth in salt solutions shows a decline at the start in both series.
6) This decrease may be explained by the autolysis of these cells that cannot stand the transfer from salt agar to saline liquid, but may be restored when transferred back to agar in time.
7) This phenomenon was mentioned in the literature as an exception in normal, saltfree, media.

In table 10 a summary of significant data is given. Comparison of these data with those from tables 7 and 8 clearly shows the conformity between the two sets.

Table 10.

Salt percentage	Relative growth-velocity	Time to reach maximum
6	2.58	32½ hrs
9	1.85	52½ ,,
11½	1.00	72 ,,
12½	0.90	100 ,,

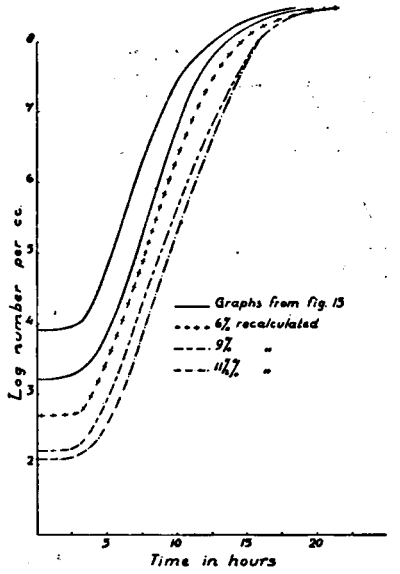


Fig. 27.

In Fig. 27 we represent the result of the recalculation of the curves for 6 %, 9 % and 11½ % NaCl from this chapter (Fig. 23, 24, 25, 26) together with some graphs taken from Fig. 15, where the growth in saltless broth was represented. We see that the recalculated graphs, corrected for retarded division-rhythm and diminished cropyield (the 6 %-line excluded), fit very well into the bundle of the other curves.

CHAPTER IX.

EXPERIMENTS CONCERNING AUTOLYSIS.

§ 1. *Nephelometric experiments.*

To prove the reality of the initial decrease, described in the previous chapters, the amount of cells present at given times was measured with the aid of a nephelometer. If the dead cells autolyze the nephelometer data might allow of direct interpretation.

The results are represented in the following tables. The first column indicates the relative time in hours, the second gives the resistance in Volts used in the circuit of the photocell. This resistance is proportional to the current and to the amount of transmitted light, but reciprocally proportional to the turbidity of the liquid. In Fig. 28 the data are represented graphically, the resistance plotted against time.

Table 11 gives the accuracy of the method. The tube was filled with tap-water. After every observation the voltage of the accu-

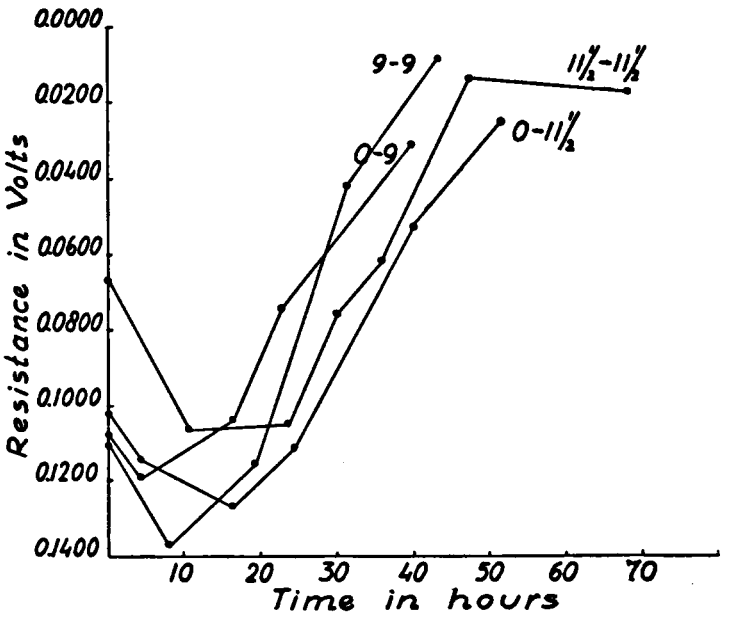


Fig. 28. Explanation see text.

mulator was adjusted against a normal element. The observations were made with three minute-intervals.

Table 11.

Photocel	Accumulator
0.1292 V.	1.0784 V.
0.1295 V.	1.0782 V.
0.1290 V.	1.0784 V.
0.1295 V.	1.0784 V.

Table 12. 25 cc. peptone-water 11½ % NaCl inoculated with a ± 60 hours-old agar culture in peptone-agar 11½ % NaCl, at 30°. Inoculum ± 4.4×10^6 cells per cc. Samples were taken at intervals, 2 cc. each time. Diluted with distilled water to fill the photometer tubes.

Table 12.

Relative time	Resistance
0 hrs.	0.0670 V.
10½ ,,	0.1069 V.
23½ ,,	0.1052 V.
30 ,,	0.0768 V.
35 ,,	0.0644 V.
47 ,,	0.0149 V.
71 ,,	0.0180 V.

From 30 hours after inoculation onward the beginning of the growth was visible with the naked eye. Because the "cluster-factor" is changing during growth, no efforts will be made to calculate the amount of cells at every moment. A heavy suspension in peptone-water 11½ % NaCl (from the order of antilog. 8—9 cells per cc.) showed a transmission corresponding to 0.0129 Volts and for the sterile fluid 0.1323 Volts, which values give an idea about the amount of cells. The data given in the two following tables are not mutually comparable, in a strict sense, as the use of different peptone-water caused a slight change in colour, perhaps owing to resterilization of one of the media.

Table 13. Conditions as in previous experiment. 9 % NaCl inoculated with ± 18 hours old culture on peptone-agar inoculum ± 2.10^5 cells per cc.

Table 13.

Relative time	Resistance
0 hrs.	0.1087 V.
4 ,,	0.1200 V.
16 ,,	0.1046 V.
22½ ,,	0.0754 V.
40 ,,	0.0314 V.

Table 14. Conditions as in the previous experiment 9 % NaCl inoculated with ± 40 hours-old culture on peptone-agar, 9 % salt. inoculum ± 2.10^5 cells per cc.

Table 14.

Relative time	Resistance
0 hrs.	0.1105 V.
8 ,,	0.1380 V.
19½ ,,	0.1246 V.
31 ,,	0.0424 V.
43 ,,	0.0087 V.

Table 15. Conditions as in the previous experiment. 11½ % NaCl inoculated with ± 18 hours-old culture on peptone-agar. Inoculum ± 2.10^5 cells per cc.

Table 15.

Relative time	Resistance
0 hrs.	0.1019 V.
4 ,,	0.1145 V.
16 ,,	0.1275 V.
22½ ,,	0.1100 V.
40 ,,	0.0525 V.
51 ,,	0.0256 V.

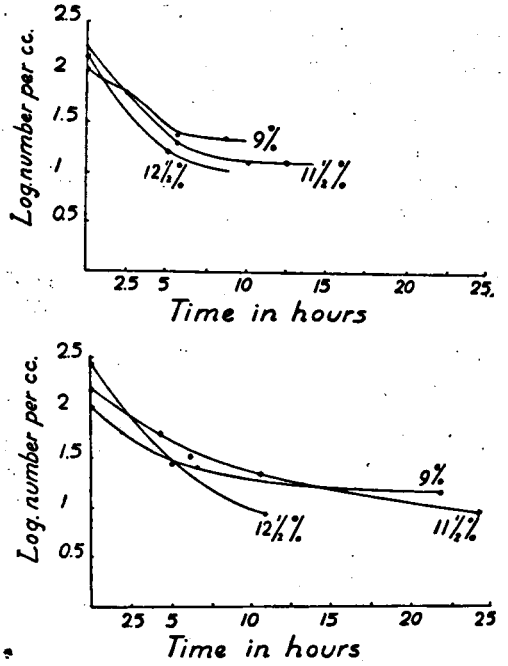
The results of these four experiments agree completely and show clearly that a decrease in the beginning is also demonstrable by means of a direct method.

§ 2. *The death-rate curves.*

If it be true that a fraction of the cells is autolyzing, decrease in the amount of the inoculum may cause complete cessation of growth, autolysis being the only remaining process.

Therefore, a very small inoculum was used. The limit was rapidly reached, because reliable results cannot be obtained when more than 0.1 cc. samples are taken. Growth was followed in the usual way by means of one series of plates (only saltless). At a percentage of 12½ % NaCl it proved sufficient to use an inoculum of 300 cells per cc. At 11½ % and 9 % resp. 150 and 100 cells per cc. were used. At 6 % growth was always observed with inocula of 100 cells per cc. which is the limit for the method of observation. An inoculum of ± 50 cells in the whole vessel did not show any growth, but single cell-cultures in small drops yielded always a luxurious growth. The

effect of aeration may account for this difference. The results are represented in Figures 29 and 30, the former gives the results of inoculationsfrom saltless agar-cultures, figure 30 gives the results from inoculation with strains cultivated before on agar with the same percentage of NaCl as the solution itself. We are aware of the fact that these curves are by no means to be regarded as correct death-rate curves. We therefore shall make no attempt to compare them with curves which were the result of carefully planned experiments to study the death-rate. We only wanted to use these curves to check our reasoning.



Figs. 29, 30. Death-rate curves. Inoculated with saltless pure cultures resp. cultures grown on salt.

CHAPTER X.

TRANSFER TO SALT-PLATES FROM A CULTURE GROWING IN SALTLESS BROTH AT SUCCESSIVE PERIODS.

In this chapter we want to settle the following questions, with the aid of the same technique as used before.

1) Inoculating with a ± 18 hours old culture, grown on peptone-agar without salt, in saltless peptone-water, shows that only a small fraction of the bacteria was able to form colonies on salt plates. The question remains whether or not this fraction is constant in all phases of growth.

2) In the later stages of a broth culture in 6 % or 9 % NaCl we found that nearly all cells are able to develop on both media. At the higher percentages we found the salt-plates to lag behind in development. When inoculating 0 % peptone-water with a pure culture, grown on salt agar, what will be the relation between the amount of colonies developed on both media and how will this relation change during growth?

We therefore prepared a series on peptone-agar with different amounts of salt, besides a series of plate-counts on 0 %-agar. These experiments were carried out along with those mentioned in the previous chapter. In the Figures concerning these experiments, the salt-free plate-count curve, the 0-0-0 curve from the previous series, is always given as a control.

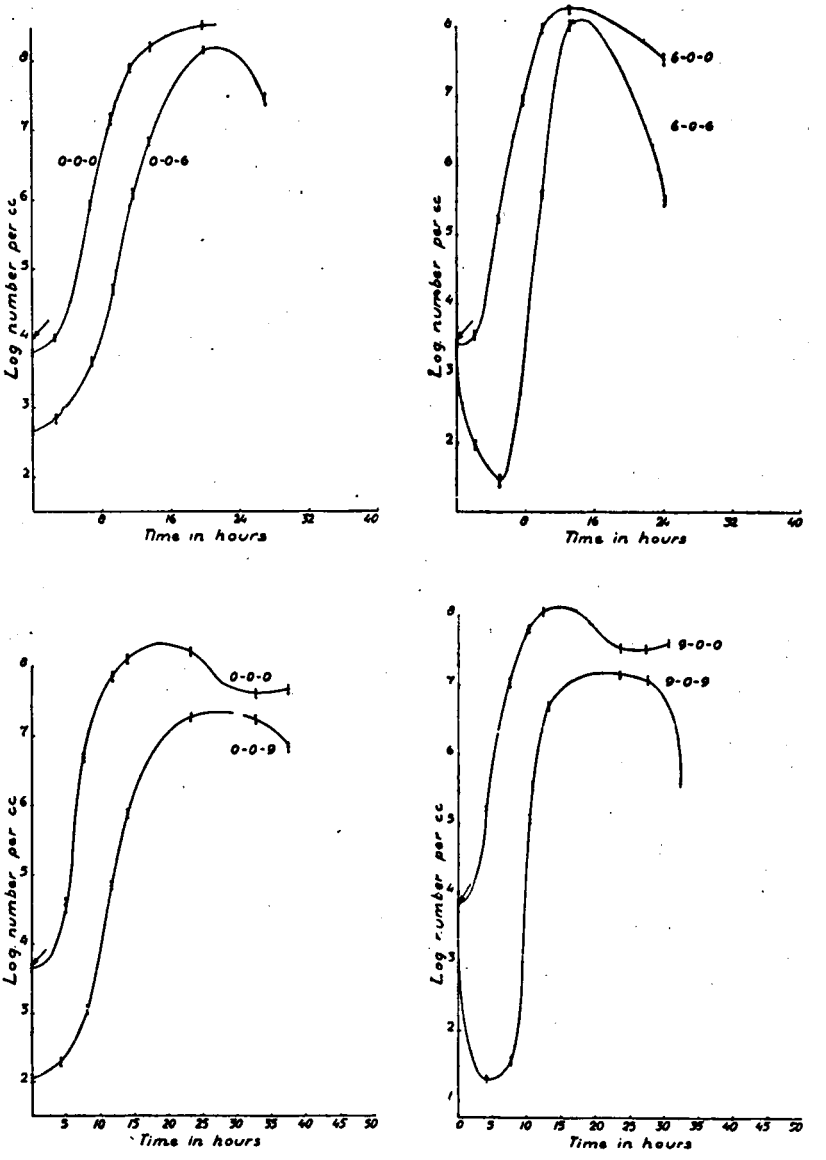
Fig. 31 gives the result of plating out samples from a culture, growing in 0 % NaCl and inoculated with a saltless pure-culture, on a 6 % agar-medium.

We see that the surviving fraction is not at all constant. It falls considerably during the "lag-phase", and has a minimum in the "logarithmic phase". In the beginning the distance between the 0-0-0 curve and the 0-0-6 one is only 1.1 in logarithmic units, after 7½ hours 2.5 and after 10 hours again 2.5. When the curves ascend towards their maximum, the fraction increases as well, the distance between the two lines being only 0.3. Afterwards the fraction decreases again and after ± 25 hours it has regained the same value as in the beginning. This is not strange because in the beginning we used a culture of similar age, although cultivated on agar. These observations show that the potentiality for growth on salt agar media is highly dependent upon the relative age of the culture. Cells in the "logarithmic period" proved to be most severey inhibited by the NaCl-action. The sensitivity shows its minimum at the maximum of the culture. This observation corroborates the work of Sherman c.s. (1929) and Schultz and co-workers (1910). Jensen (1928) found similar differences between cells of *B. coli* of various ages.

Fig. 32 represents the results of similar plate counts for growth in the same liquid medium, 0 % inoculated with a ± 48 hours old pure-culture on agar with 6 % NaCl. We see that;

1) Initially all cells are able to develop in both media, exactly as we found in the end of the liquid culture in Fig. 16.

2) The cells that are formed in saltfree peptone-water, however, do not retain the property to form as many colonies on 0 %-agar as on 6 %-agar, but we see the 6-0-6 curve to descend



Figs. 31, 32, 33, 34. Explanation see text.

rapidly until the distance between the two lines is the same as in Fig. 16.

3) Here again the fraction that can grow on salt plates reaches its maximum at the developmental maximum of the culture, to fall afterwards. The latter part of the curve reminds one strongly of the latter part of the o-o-6 line.

4) As already stated in a previous chapter, the normal outward appearance of the cells was restored directly as division began in the o %-milieu. The "clusters" typical for the salt media disappeared directly after retransfer to saltless media.

Figs. 33 and 34 represent the same phenomena in 9 % NaCl, and Figs. 35 and 36. those in 11½ % NaCl. We see in Fig. 33 that the distance between the two lines has increased, as was to be expected, considering the preliminary experiments which showed that in order to obtain growth in the higher percentages, we had to use a larger inoculum. Although a fairly large inoculum was used no colonies were found on the salt plates in the first observations. Only when the amount had reached a value of about 10^8 cells per cc. the first colonies started to grow.

Fig. 35 illustrates the same phenomenon for 11½ % of NaCl. Here we cannot find the close conformity between the data of the two series of plate counts in the same way as we saw in Fig. 18. It seems to be a heavy task for the cells to grow out on 11½ %-agar plates, although they grew in 11½ % NaCl nutrient solution.

Fig. 35 shows, however, the same relation as seen in the previous graphs. It is apparent that a distance between the two lines of $\pm$ 4.5 log. units indicates that no colonies may be expected in the beginning when the inoculum contained $\pm 10^5$ cells per cc. The usual samples of 0.1 cc. in this case contained $\pm 10^{4.5}$ cells.

Fig. 37 and 38 show similar experiments in a percentage of 12½ % NaCl and a similar reasoning may be applied in this case. The difference between the amount of colonies that grows out on o %-or 12½ %-media is striking.

There seem to about 900 times as many cells capable to develop on the former milieu.

Summarizing it may be said that;

1) The number of cells, able to grow on salt plates is not constant during the course of growth in saltless peptone-water.

2) This number shows a maximum at the developmental maximum of the culture, a minimum during the "logarithmic phase" and shows an average value at the start and at some hours after the maximum.

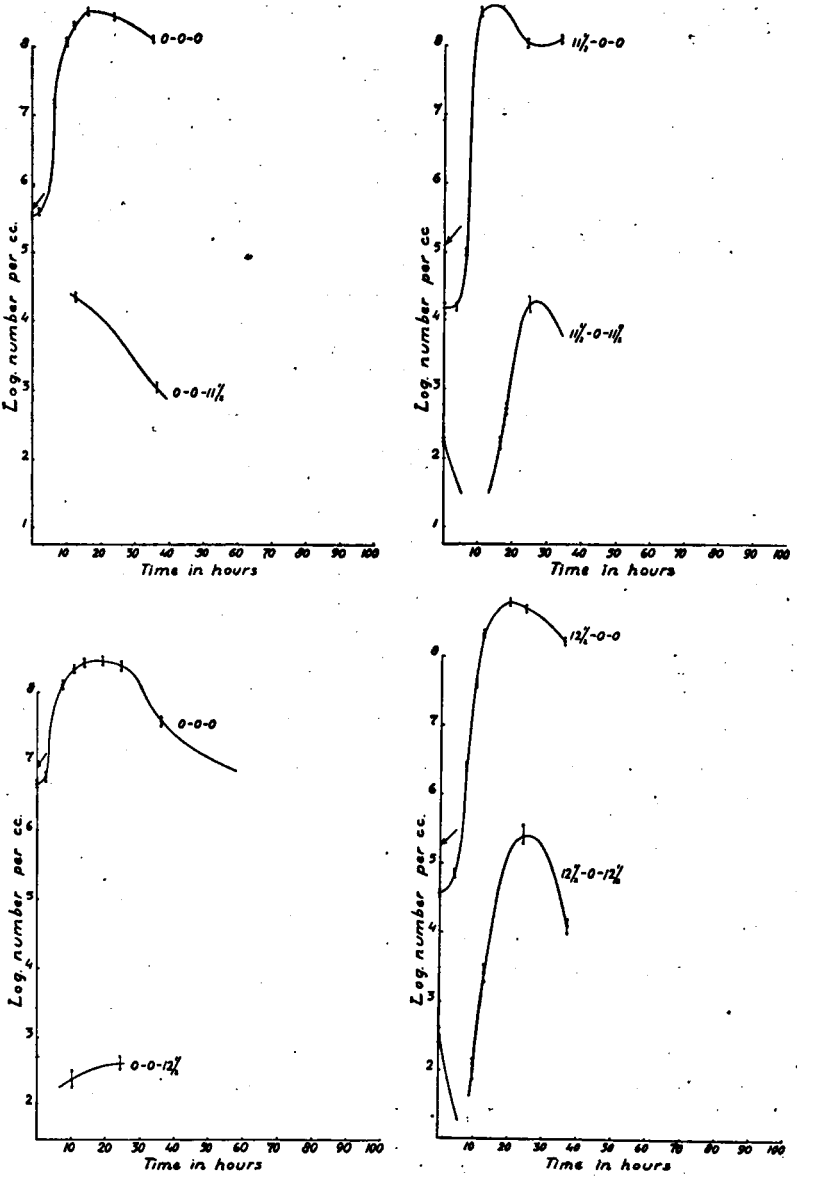


Fig. 35, 36, 37, 38. Explanation see text.

3) All cells that are formed in saline milieu have the property to form nearly as many colonies on salt plates as on salt free plates, as is withnessed by the parallel course of the 0-6-0 and the 0-6-6 lines, the 0-9-0 and the 0-9-9 lines etc. Futhermore the coincidence of the first point of the 6-0-0 and 6-0-6 line and 9-0-0 and 9-0-9 etc. is indicative of this fact.
However, as soon as the cells start developing in the 0 %-milieu this property disappears with the morphological difference; the formation of so-called "clusters".

CHAPTER XI.

DISCUSSION OF THE RESULTS. GENERAL SUMMARY.

When we consider our results in the light of the theory developed in Chapter 3, we come to the following conclusions.

Not all of the cells of a certain inoculum start growing when transferred to strong salt solutions. We always saw less colonies on our salt-plates than on the control plates without salt, although the same amount of cells was introduced. This is in accordance with the preliminary experiments which proved that a small inoculum gave no growth. The part that grew showed a normal development which, however, was much slower than in saltless milieu. We saw that the retardation of growth was a function of the salt-percentage. The property of salt-cultures to give colonies on salt-agar plates for nearly 100 %, compared with saltless media, disappears when the cultures are retransferred to saltfree media. In the same way the morphological change that proved to be so characteristic for all salt-percentages is directly lost. From these data we can draw the conclusion that those cells that differ from the greater part by their ability to grow out in stronger salt solutions, do not have a different "hypartype". Fluctuating variability gave them a halotolerance, including the resistance against the salt percentage in question so that they could survive while others had to die. After that the milieu exerted its modifying action on the constitution and shifted the limits of variability, and therefore shifted the "doxatype".

The changed appearance of the cells, therefore, did last no longer than the milieu-action itself. If only selection had been active the "new" qualities should have been constant.

Rahn (1932) draws attention to the fact that the offspring of resistant cells show an average resistance and not an increased one. In a detailed survey of the literature he shows the experimental proof for this assumption as offered by various authors. The same thing holds true in our experiments. Rahn, however, supposes that as to the so-called thermoresistance, the primary factor is not resistance, but survival-time; the latter being a matter of chance. We thought it better to assume a variability in resistance. Between Rahn's cells there is only a quantitative difference, all of the cells are destined to die, but in our experiments there is a qualitative difference as well, for some of the cells are to die but the rest will develop.

We saw also that not only the transfer to a medium that shows a marked difference with the old one, causes a selection, but also that this selection takes place on every occasion, be it not in such a marked way. Transfer from salt-agar cultures to liquid cultures with the same percentage of NaCl also gave rise to a selection. Such a phenomenon is not qualitatively different from what occurs in salt-free (read ½ % NaCl) media.

The experiments of Meller (1925) seem to us of general importance, we want to regard them as extreme cases of phenomena that do occur always, but often in such a small degree that we do not notice them. A brief summary of our ideas in this matter may be given as follows. As growth proceeds, the milieu is changing gradually, in some instances even giving rise to inhomogeneous conditions. The culture is growing and a large number of new cells are formed, the chance to meet with variants (in the broadest sense of the word) of various kinds, will also increase. By its selective action and by its ability to change the limits of variability, the changing milieu will generate a changing culture. In ordinary media these phenomena will not play such an important role, but in more extreme milieus, they will be the cause of the death of a large fraction of the inoculum.

In all other parts of biology investigators shrink from accepting a constant change of potentialities brought about by an action of the milieu. However, we saw a number of authors advocating such an action in microbiology, in spite of the striking analoga between bacteria and multicellular organisms. We, however, thought it well to try all other possibilities first before accepting such a radical hypothesis.

At the end of our work we want to formulate some, chiefly experimental, desiderata. When we started our experiments we hoped to meet with similar phenomena as Hof. Obligate halophils were not met with, however. It might yield good results to apply our

method of double plate-count to such cases where irreversible variations are due to occur. The experiments of Lewis seem very promising in this respect; the work with plate-counts on both normal agar and on Endo-agar.

From our work we obtained the impression that it will be of great importance to study bacteria in their *natural milieu* and not in the pure-culture tube on routine media. The work of various authors with silica-plates, direct microscopic examination of buried glass slides, etc. meant to rule out the action of the nutrient medium, seems very promising for the advance of soil- and water-bacteriology. Winogradsky showed some 35 years ago how fundamentally important questions of metabolism could be solved by direct microscopic observation.

We wish to end with a quotation of M. Soule: "When any progress is to be made in bacteriology, workers must draw their attention from the problem of what bacteria *can do*, to the problem of what bacteria *really are*".

SUMMARY.

1) A survey of the literature concerning the morphological and physiological properties of bacterial growth in strong salt solutions is given.
2) A review of the literature on variability of bacteria revealed the fact that, although various authors advocate a modifying action of the milieu on the potentialities of the bacterial species, others explain the same phenomena without such an assumption.
3) A theory was developed in which all variation-phenomena were classified into three groups: fluctuating variability, dissociative variability, and "jumping" variability. A new terminology was proposed, dropping all genetical concepts.
4) Quantitative experiments on growth of *B. megatherium* in NaCl-peptone-solutions confirmed the data given in the literature on this subject. The growth-velocity decreased with increasing salt-percentage, the incubation-time was prolonged, a larger inoculum was indispensable in higher percentages.
5) During growth in NaCl solutions there proved to be a period, directly after inoculation, where retransfer to salt-free peptone-water yielded no growth.
6) With the plate count method growth-curves were constructed for growth in various percentages of salt.

7) The curves show a decrease in the first hours, while the first point showed that only a fraction of the inoculum was able to develop.
8) The initial decrease was explained as the resultant of two relations; the growth curve of those cells that did develop, and the death-curve of those that autolyzed.
9) It was possible to separate the components. The curves obtained in this way, recalculated to comparable growth-velocity, fit very well into a bundle of curves for growth in salt-free milieu.
10) The autolysis of a fraction of the inoculum could be demonstrated by nephelometric methods.
11) With extremely small inocula no growth at all was observed in percentages over 6 %, and the curves obtained this way gave an expression of the autolysis.
12) All cultures grew in all percentages in tortuous filaments; the so called "clusters". On retransfer to saltless media, the original morphology was restored. The amount of cells per "cluster" was low in the beginning and in the end, but maximal in the time that growth had its maximum.
13) Cultures growing in salt-solutions had the property to form nearly as many colonies on salt-plates as on salt-free agar. Cultures growing in salt-free peptone-water did not show this property. These cultures showed a maximum susceptability against salt during the "lag-phase".
14) Because the property to form nearly 100 % colonies on salt-plates was directly and completely lost on retransfer to saltless media, and also because of their altered morphology, the colonies were looked upon as results of the modifying action of the milieu on the limits of fluctuating variability.

This investigations were carried out in the Botanical Laboratory of the University of Leyden. I am very much indebted to its Director, Prof. Dr. L. G. M. Baas Becking, for his suggestions and valuable help for which I wish to thank him particularly.
Furthermore I wish to thank the technical staff of the Laboratory for constant help, especially Mr. J. E. Bevelander who carefully prepared the figures.

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EXPLANATION OF THE PLATE I.

Figure I. *24 Hours old peptone-water culture at 30° C. Showing aspect of normal pure-culture in saltless media.* × *500.*

Figure II. *Isolation of a double cell with micropipette.* × *150.*

Figure III. *220 Hours old culture on peptone-agar. 12½ % NaCl at 30° C.* × *850.*

Figure IV. *"Cluster" from 48 hours old culture in 6 % NaCl. Aerated at 30° C.* × *850.*

Figure V. *"Cluster" from 48 hours old culture in 9 % NaCl. Non-aerated at 30° C.* × *850.*

Figure VI. *90 Hours old culture on peptone-agar. 9 % NaCl at 30° C.* × *375.*

Figure VII. *Non-aerated culture in peptone-water. 9 % NaCl at 30° C. 50 Hours old. Showing "hairpin-structure".* × *250.*

(Photographs no. I and VII were made by J. W. Varossieau).

TABLE I.