INVESTIGATIONS CONCERNING BACTERIAL LIFE
IN STRONG BRINES
by
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Introduction.

The remarkable occurrence of various organisms in media with relatively high salt concentrations has been known for a long time. Halophilic organisms occurring in salt lakes are described a.o. by ENTZ 1905 (25), TEODORESCO 1905 (94), NAMYSLOWSKY 1913 (62), and PEIRCE 1914 (66). In recent years many investigators, more especially BAAS BECKING and collaborators (3—7, 9, 15, 44) have studied the biocoenosis of salt lakes and the behaviour of halophilic organisms occurring therein. In their publications little attention has been paid to the bacterial flora, this being due to the fact that these organisms cannot be identified by direct microscopical examination. Nevertheless there can scarcely be any doubt that bacteria will form an essential link in the cycle of life occurring in these lakes.

A thorough study of the role of bacteria in this connection will ask for a continuous series of observations made at the spot, supported by isolations of the active organisms and an additional study of their cultural characters.

Owing to the lack of natural salt lakes in this country it was impossible for the author to carry out such a study. Still it seemed practicable to make some contributions to the problem stated. In the first place it was hoped that a bacteriological examination of various samples of crude salt, natural brine, salt mud etc. might throw some light on the composition of the normal bacterial flora

present in these materials. It may be stated at the outset that the results obtained in this part of the investigation have been very restricted. The principal cause which hampered the success of this part of the work has to be found in the relatively long time which had elapsed after the moment of sampling. There can be no doubt that a somewhat longer preservation of such samples will lead to a remarkable change into the proportion in which various bacteria occur. The special conditions under which these are kept will in these samples inevitably lead to an accumulation of special bacterial groups while others will be more or less completely suppressed. This means that no value can be attached to negative results of the examination. As regards the positive results not too much importance may be given to these either since with samples which are not taken with the special precautions necessary for bacteriological work the possibility of contaminations cannot be excluded. Nevertheless it seemed worth while to make some preliminary investigations.

Owing to these difficulties it was necessary to attack the problem in question from another point of view. It seemed quite possible to answer the question in how far bacteria normally occurring in a salt free environment are able to develop and thrive in suitable media containing high percentages of sodium chloride. If these experiments should be successful for special physiological groups it seems certain that representatives of these groups will also occur in salt lakes as soon as the nutritive conditions prevailing therein answer the special requirements of the group in question.

Moreover it might be expected that experiments in this direction would be of value to answer the question of the origin of halophilic bacteria. At the one hand one may surmise that in salt-lakes and natural brines an autochthonous bacterial flora exists. On the other hand it is acceptable that these bacteria are only adaptation forms of representatives of the normal soil flora. In the latter case it seemed possible to induce such adaptations in the laboratory. If the experiments would indeed show that many ordinary bacterial species possess such an adaptive power it seemed of importance to make some observations regarding the nature of this faculty. Is this adaptation process spontaneous or does the organism only develop in strong salt solutions after successive transfers in media with gradually increasing salt concentration? No less important is the question whether this adaptation is reversible or not.

These are the questions that are chiefly dealt with in this study. It should be stated here that in bacteriological literature already

many facts have accumulated which have a direct bearing on the problems under discussion.

In the first place one may find scattered observations regarding the occurrence of special bacteria in various salt lakes. Especially Russian investigators have already reported several results of their bacteriological examinations of „limans" [1]

As for the second and chief part of the investigation i.e. the behaviour of „normal" bacteria in brines already a good deal has been done by several investigators who endeavoured to obtain a practical knowledge of the process of the curing of food (ham, beef, pork, fish,cucumbers, sauerkraut, beans, endives etc.) and of other animal products (hides, intestines). However, most of these investigations are of an almost exclusively practical nature and the general principes involved have only found little consideration.

A full treatment of the literature will be given in the special chapters, as it proved very difficult to deal with our own results without immediate reference to preceding work.

In the two last chapters a report is given of investigations regarding the bacterial flora of a few salted vegetables.

CHAPTER I.

A preliminary investigation of the bacterial flora occurring in natural brines such as salt lakes and salt gardens.

§ 1. *Introductory remarks.*

As already mentioned in the introduction it seems desirable, and in many respects even necessary, to study the bacterial life in natural brines on the spot. Since it was impossible for the author to do this the investigation had to be restricted to a bacteriological examination of a number of brine samples which had been received in later years by the Botanical Institute at Leiden.

The following samples were examined:

1. sample of salt mud from the salt garden of Gersik-putih (Madura) collected in Sept. '31.
2. sample of salt mud from the salt garden of Grissee (Java) collected in Sept. '31.

[1] Liman is the name given in the neighbourhood of Odessa to shallow salt marshes which open into the Black Sea. The bed of these marshes is composed of black mud due to the presence of iron sulfide originating from the action of sulfate reducing bacteria. The salt concentration in the limans depends on the season and may differ from 4°-26°B°.

3. sample of salt mud from the salt garden of Pamekasan (Madura) collected in Sept. '31.
4. sample of crude salt from Searles Lake (California) collected in '31.

Direct inoculations on different salt containing solid media and enrichment cultures in special liquid media were made with these samples as inoculum.

Considering, however, that the samples were rather old it hardly could be expected that the results would be very favourable.

The time that organisms remain living in salt samples may be different. At first sight it seems improbable that living organisms will stand the exceptional conditions prevailing in dry salt during a long time. However, it has been found that *blue-green algae* often develop from samples kept for three years and the flagellate *Dunaliella* may even be raised from samples as old as seven years. (Hof and Frémy (38).

The length of time the sample is kept will certainly be of influence on the bacteria present in it. Not only will many bacteria die off during the long storage but also the nature of the bacterial flora may be quite different from that present at the moment of collecting. Waksman 1934 (96) states in his study upon the role of bacteria in the cycle of life in the sea, that samples brought in the laboratory may have within a very short period of time frequently within a few hours, a bacterial population which is quite different from the corresponding population in the sea.

So it cannot surprise that the bacteria obtained from our samples only belong to a few natural groups and that many common groups are not represented.

§ 2. Review of the literature.

At first a few remarks will be made regarding the use of the term halophilic. It appears from the literature that no unanimity exists regarding the meaning of this term.

Rubentschik 1929 (76) applies the term to bacteria which find their optimal development at a certain salt concentration, regardless of the ability of the form to grow in media without salt; if the form does not develop in fresh water media he calls it an obligate halophilic. When the bacterium develops better without salt he calls it „halotolerant".

Golikowa 1930 (34) reserves the term „halophilic" for bacteria that are unable to grow in media without salt, while all the other forms are regarded as halotolerant.

HOROWITZ-WLASSOWA 1931 (40) does not restrict the term to the organisms that are not capable of development without salt, but also includes the halotolerant forms. Obligate halophilic organisms are named "halobe".

In our publication on blue green algae occurring in strong brines (38) the term halophilic is used for organisms that are able to grow in solutions of more than three molar sodium chloride i.e. in sodium chloride solutions which are in equilibrium with an atmosphere of which the relative vapour tension is lower than 85 % [1]). This definition was more or less justified by the consideration given by WALTER 1931 (98). This author concludes a.o.: "das Hydratur Minimum von 85 % scheint das absolute Minimum für alle lebende Organismen zu sein". He makes, however, an exception to this rule for various organisms which are able to live in solutions of electrolytes which correspond with a smaller relative vapour tension. This would be due to a specific action of electrolytes on these organisms. This special quality seemed to us a justification for distinguishing these organisms as halophilic.

The manifold variation in behaviour of bacteria towards various salt concentrations in the medium makes it, however, very difficult to apply this terminology also in bacteriology. It does not seem rational to call a bacterium halophilic only because it develops in media containing more than three molar sodium chloride, whilst the optimal development may take place in salt free media. Nor seems it right to withhold from a bacterium the epithet "halophilic" although it has an optimal development at a certain salt concentration, just because it does not grow in a medium with more than three molar sodium chloride.

This holds the more since with bacteria it is possible to alter considerably the maximal and minimal salt concentrations tolerated by the organism (c.f. Chapter IV).

Under these conditions it seems to me that in bacteriology the terms "halophilic" and "halotolerant" may for the moment be best applied in the sense given to these words by Rubentschik.

After this discussion of the term "halophilic" mention will be made of the data found in the literature on the occurrence of several bacterial groups in natural brines.

The presence of several forms of bacterial life has been demonstrated from a direct examination of the natural brines.

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[1]) Miss RUINEN 1933 (79) in her work on green algae used the term halophilic in the same way.

Brine samples often show conspicuous development of bacteria e.g.
a. *Purple bacteria.*
   Rhodospirillum halophilum Baas Becking was observed by Prof.
   BAAS BECKING in samples from Sand Springs (Nevada) (4).
b. *"Red" bacteria.*
   A form similar to *Micrococcus morrhuae* Klebahn occurred in
   red brine samples from Sambhar-Lake (British India).
c. *Green Sulphur bacteria.*
   In a sample of mud received from the salt-garden of Gersik-
   putih (Madura) spontaneous development of green *sulfur bac-
   teria* took place, as proved by the appearance of a greenish
   colour and by the formation of sulfur in the medium.

As for the occurrence of other physiological groups of bacteria
in natural brines the following statements are found in the lite-
rature.

RUBENTSCHIK (1926) (73 and 74) demonstrated the presence of
urea bacteria in the Chadjibey liman near Odessa. He isolated a
number of *urea bacteria* and determined the amount of urea decom-
posed in media containing different percentages of several salts as
liman salt, NaCl, KCl, $CaCl_2$, and $MgCl_2$. It should be remarked
that all kinds of bacteria used in these experiments showed a
higher tolerance for liman salt than for one of the pure salts.
From the latter NaCl and KCl are tolerated in almost the same
concentration, $MgCl_2$ and $CaCl_2$ are more noxious. The maximal
concentrations tolerated by *Urobacillus hesmogenes* amounted for
liman salt 19%, NaCl 13%, KCl 14%, $MgCl_2$ 9% and for
$CaCl_2$ 6%.

The occurrence of *denitrifying bacteria* in natural brines has
been demonstrated by NADSON (61). This author mentions deni-
trification in the Weissowa-salt-lake. The concentration of the
brine is not stated.

Prof. BAAS BECKING (oral communication) states that when trying
to isolate green-salt-flagellates by means of agar shake cultures
(15% NaCl) isolated colonies cannot be obtained because of the
copious formation of nitrogen bubbles, resulting from the 0,5%
KNO_3 in the medium.

RUBENTSCHIK (1929) (77) investigated nitrification in the limans.
He was able to show that the first phase of nitrification: $NH_3 \twoheadrightarrow$
HNO_2 occurs up to 15% NaCl. He succeeded in isolating a saline
form of *Nitrosomonas.* The minimum concentration for growth
amounted to 0,2 mol. NaCl (= 1,2%). The optimal growth appea-
red in 1 molar NaCl. Rubentschik could not demonstrate the

occurrence of the second phase of nitrification, i.e. oxidation of $NO_2' \twoheadrightarrow NO_3'$ in the liman.

It is noteworthy that many investigators studying the nitrification in the sea failed to find the second phase. Notwithstanding this it is very probable that the second phase may also occur in the sea for WAKSMAN, HOTCHKISS and CAREY (97) have shown that crude cultures obtained by inoculation of a sample of sea-mud in a sea water medium to which the usual salts for the cultivation of *Nitrosomonas* were added, finally contained nitrate. They were not able, however, to demonstrate the oxidation of nitrite after direct inoculation of a nitrite-containing medium with the same sample of sea mud. Transfers of the old cultures mentioned above into a medium suitable for *Nitrobacter* gave, however, a positive result. Thus considering the investigations on *nitrifying bacteria* in the sea, it seems probable that the second phase too occurs in the liman, although Rubentschik in his laboratory experiments was unable to demonstrate the formation of nitrate.

The *sulfate reducing* bacteria which form an essential link in the cycle of sulfur metabolism are often present in brines and are able to reduce sulfates up to the highest salt concentration. Their general presence in salt gardens is immediately evident from the strong smell of hydrogen sulfide. Here the salters take much care to bring about a clean separation of the salt from the black mud. The latter consists of a hydrate of iron sulfide, formed by the sulfuretted hydrogen liberated by the action of the *sulfate reducing bacteria*. In order that the salt may be harvested without contamination with the black mud, the formation of an algal cover, consisting of the blue-green alga *Microcoleus chthonoplastes* Thur. (38) or sometimes of the green alga *Lochmiopsis sibirica* Woronichin (79) on the bottom of the salines is considered to be most desirable. VON BUSCHMAN (19) mentions this algal cover for several italian salines. FÜRER (31) remarks that the firmness of the bottom is of great importance for the purity of the salt.

Besides in the salt gardens the black mud is also present in the limans, where it forms the so-called "Heilschlamm". Many investigators have studied here the sulfate reduction. SAWJALOW 1913 (84) isolated a species of *Actinomyces* from a liman, which organism according to this author, was able to reduce sulfates. ISSATSCHENKO (according to BAARS (2), however, reports that the *Actinomyces* culture of Sawjalow was impure and also contained spirilla; in the experiments of Sawjalow it was to all probability the *Spirillum* and not the *Actinomyces* which reduced the sulfate.

ISSATSCHENKO 1930 (42) isolated from the Black Sea a sulfate reducing *Spirillum* closely related to *Spirillum (Vibrio) desulfuricans* Beijerinck and Van Delden.

RUBENTSCHIK 1928 (75) states that H_2S appeared in cultures of cellulose decomposers from the limans. Here the *sulfate reducing bacteria* use the fatty acids formed as a source of energy.

CHAIT 1924 (22) isolated from the Kujalnitzki liman a *Spirillum* that reduced sulfates in media without salt as well as in media containing salt up to 20% NaCl. Optimal development was found in media containing 8% salt.

SASLAWSKY 1928 (81) concludes that sulfate reduction is found in media containing up to 30% salt.

GAHL and ANDERSON 1923 (32) isolated *sulfate reducing bacteria* from oil-wells and studied the influence of salt solutions upon their metabolism. They distinguish three types:

type 1. develops in concentrations from o to 1% NaCl
type 2. develops in concentrations from 1 to 3% NaCl
type 3. develops in concentrations from o to 5% NaCl

Most of the isolated strains belonged to type 3.

The *colourless sulfur bacteria* which oxidise sulfur and its compounds are well represented in strong brines.

SASLAWSKY 1927 (80) isolated an obligate-halophilic species of *Thiobacillus*. The minimal concentration of NaCl in which this species was able to grow amounted to 2%, the maximal concentration to 22%. Optimal development was observed in 6% NaCl. As a criterion of development layer forming and titration of the culture liquid with iodine was used. First the thiosulfate is oxidised to sulfur and sulfate:

$$Na_2S_2O_3 + O \rightarrow Na_2SO_4 + S$$
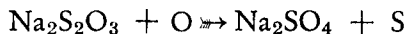
The sulfur produced may be oxidised further to sulfate, so that finally the thiosulfate is oxidised totally to sulfate.

SASLAWSKY and HARZSTEIN 1930 (83) investigated whether, in the case of obligate-halophilic bacteria, NaCl may be replaced by other salts. They used two obligate-halophilic species of *Thiobacillus*. One of the bacteria oxidised the thiosulfate only to sulfur and sulfate, so that the medium remained alkaline. The limiting concentrations of NaCl tolerated were 2—22%. The other bacterium oxidised thiosulfate completely, so that the medium became acid by the formation of H_2SO_4. The minimal concentration in which this bacterium was able to grow amounted to 1%, the maximal concentration was 22% NaCl. They found that NaCl may not be replaced by either KCl or NH_4Cl. In concentrations of 1 and

o,5 mol. of these salts no development appeared. In 1 mol. solutions of CaCl₂ and MgCl₂ the bacteria did not grow, but in o,5 mol. solutions of these salts development was observed. Both strains grew better in o,5 mol. MgCl₂ than in o,5 mol. CaCl₂. Combinations of two salts (wherein Na as well as Cl are represented: NaNO₃ + NH₄Cl; NaBr + KCl) were even not able to replace NaCl completely.

The investigators conclude that the action of NaCl on obligate-halophilic *Thionic-acid bacteria* is not only due to the osmotic pressure, but also is a specific action of NaCl.

The same result has been found in experiments with several other halophilic organisms. BAAS BECKING has stated this for the brine shrimp *Artemia salina* 1931 (15, 44) and for the flagellate *Dunaliella viridis* 1931 (6), PETTER 1932 (70) for *Bacterium halobium*, RUINEN 1933 (79) for *Lochmiopsis sibirica*.

GOLIKOWA 1930 (34) experimenting with an obligate-halophilic bacterium (producing no pigment) isolated from salted fish, however, came to the result that NaCl may be replaced by other salts.

ISSATSCHENKO 1927 described *Thiobacterium thioparum* f. *Tambi* from the Tambukan Lake (according to Benecke (11).

ISSATSCHENKO and SALIMOWSKAJA 1929 (43) isolated three species of *Thionic-acid bacteria* from salt lakes in the Krim. The maximal salt concentration that these bacteria could tolerate amounted to 24%.

VAN NIEL 1931 (63) mentions that Baas Becking has observed in brines a form related to *Thiobacterium thioparum* Beijerinck that forms sulfur globules within the cells.

According to BENECKE (11) Kolkwitz has found Beggiatoa in brine of 4,5%.

Purple sulfur bacteria were observed by BAAS BECKING 1928 (4) in saturated brine. He described a halophilic form: *Rhodospirillum halophilum*.

VAN NIEL 1931 (63) cultivated *purple sulfur bacteria* in brine of 30% NaCl. The strains he isolated at lower salt concentrations, or without salt, developed all up to 15% NaCl. The behaviour of the several strains in media with increasing salt concentrations did not show any differences.

BAAS BECKING 1930 (3) mentions the occurrence of *green sulfur bacteria* in salt lakes containing 7—8% salt.

BUTKEWITSCH 1928 (19) has found *Iron-bacteria (Gallionella* species) in seawater.

CHOLODNY 1931 (23) and PERFILJEW 1927 (67) observed *Gallio-*

nella in waters containing even more salt thans seawater.

The decomposition of cellulose in brines has been studied by ISSATSCHENKO and RUBENTSCHIK.

ISSATSCHENKO 1927 (according to BENECKE (11) found in the limans *Bacterium cellulosae album* that decomposes cellulose aerobically. This bacterium is active in a medium of 3,5° B°. At 15° B° (= ± 16%) the process was markedly retarded and at 24° B° no decomposition could be observed.

Issatschenko obtained anaerobic decomposition of cellulose only in media without salt.

On the other hand RUBENTSCHICK 1933 (78) has shown that anaerobic cellulose-decomposition occurs up to 15% NaCl. He also used mud from a liman as inoculum.

In harmony with the results of ISSATSCHENKO RUBENTSCHICK 1929 (76) obtained aerobic decomposition of cellulose up to 17% liman salt.

BENECKE 1905 (10) has demonstrated the occurrence of chitin decomposition in the sea. The decomposition is caused by *Bacterium chitonovorus* which is able to grow in a medium containing only chitin as source of carbon and nitrogen.

The occurrence of similar forms in brine is unknown. Indirect evidence seems to indicate that this decomposition — if it occurs at all — must be very slow. For in some natural brines chitin originating chiefly from the brine shrimp *(Artemia salina)* can often be found.

Few data are available on the decomposition of fat in brine. ISSATSCHENKO 1927 mentions the occurrence of bacteria that decompose fats in salt lakes in the northern part of the Kaukasus to a maximal concentration of 16% NaCl. (according to BENECKE (11).

HOROWITZ-WLASSOWA 1932 (40) described a fat-decomposing *Micrococcus* : *M. lipolyticus* from brine in which meat was salted. This form was able to grow in concentrations up to 18% NaCl.

The experiments of SASLAWSKY 1928 (81) show that in natural brines proteins are attacked. Saslawsky investigated the bacterial processes in the limans near Odessa. Egg-white, mussels and algae were used as organic material and the action of natural water (25,5° B°) was tested on these substrates. After soms months incubation he could demonstrate the presence of a large amount of putrefaction-products such as H_2S, NH_3, trimethylamin and indol. Finally a blood-red layer appeared on the surface of his cultures. The layer consisted of micro-organisms that could not be classified. Every sample consisted of many little globes (10—20 to many

hundreds) that resemble cocci. Probable he has observed a species of *Micrococcus* closely related to *M. morrhuae* Klebahn, M. *roseus* (see Petrowa 1933 (69) or *Tetracoccus carneus halophilus* Horo-witz Wlassowa 1931 (39). It is very probable that this organism had developed after the proteins had been attacked for it is known that organisms of this kind often occur in media containing decom-positions-products of proteins.

Nadson 1903 (61) demonstrated the decomposition of proteins in the Weissowa Lake. Several organisms seemed take part: *Bacillus mycoides, Proteus vulgaris, Bacterium albo-luteum, Bact. salinum* Nadson and three species of *Actinomyces*. The percentage of salt is not indicated; in the abstract of his publication it is mentioned that the percentage of salt was "average".

§ 3. *Experiments of the author.*

Only a short account will be given regarding the bacteria which developed from direct inoculations of material from salt lakes on solid media. This is a direct consequence of the scantiness of the results obtained from the few experiments made.

A sample of salt mud from the solar salt-garden of Grissée (Java) was inoculated on the following agar plates:
malt agar with 24% salt
nutrient broth agar with 18% salt
nutrient broth agar with 24% salt
peptone agar with 24% salt
yeast extract agar with 20% salt
After an incubation time of 16 days at 30° C. nothing had developed on the malt-agar-salt plate (pH = 5,6) but all the other plates showed very small colonies, that differed only little from each other.

On the peptone salt-agar three different kinds of colonies could be distinguished viz.
a. small, white colonies
b. larger, transparant colonies
c. red colonies.

Macro- and microscopical examination of the other agar-salt plates also showed the same three types of organisms.

From each of these three types I isolated one colony and obtained pure cultures of them in the usual manner. The three isolated strains which will be called A, B and C have been submitted to a more detailed examination.

Strain A was transferred from a culture-tube with peptone agar with 24% salt to peptone agar containing the usual 0,5% salt. It appeared that the strain developed well on this medium. The microscopical examination showed that the bacterium had formed terminal spores and that the cells were not swollen at sporulation. The vegetative cells were somewhat irregular in shape and were non-motile. On peptone agar with 6% NaCl motile rods were formed. However, on this medium sporulation did not occur.

On both media, (peptone agar with 0,5 and 6% NaCl) a pale orange pigment was produced. On the medium with 24% NaCl, on which the strain had been isolated, no pigment had been formed.

Inoculation in peptone gelatine showed liquefaction after two days. The type of liquefaction was crateriform to napiform.

Starch was not hydrolysed.

We were unable to identify this organism. BERGEY, in his "Manuel of Determinative Bacteriology" (12), does not describe a *Bacillus* species with terminal spores producing an orange pigment.

Strain B also developed better on peptone agar with 0,5% NaCl than on peptone agar with 24% NaCl. It appeared that also this strain formed terminal spores and likewise the cells were not swollen at sporulation. The vegetative cells formed winding chains which appeared somewhat swollen.

This strain, formed, like strain A, motile rods on peptone agar with 6% salt. Here sporulation was observed after two days.

On peptone agar with 0,5 and 6% NaCl a pale yellowish pigment was produced.

A culture in peptone gelatine showed liquefaction of the crateriform to napiform type. Starch was not hydrolysed.

We may therefore conclude that these two strains resemble each other closely. The only difference seemed to be that strain A forms an orange pigment and strain B a yellowish. It is possible that similar strains were observed by PETROWA 1933 (69) (isolated from salt of the saline of Kuüli Turkmenistan). Petrowa's strains formed a rose-coloured to yellow pigment. According to Petrowa the forms are closely related to *Bacillus mesentericus*. Petrowa does not mention, however, whether the spores were terminal or central. According to BERGEY (12) *Bacillus mesentericus* forms central spores and this character is not shown by our strains.

Strain C forms a red pigment. The cells are rod-shaped or sometimes almost spherical. Spore-formation was never observed.

It appeared that the form was obligate-halophilic for it did not develop on the usual peptone agar with 0,5% NaCl. On agar with 6% NaCl development was still observed. It appeared on microscopical examination of material from peptone agar with 24% NaCl that in water the more or less rod-like cells changed into irregular spheres of unequal size.

This form resembles *Bacterium halobium* Petter and also *Bacterium trapanicum* Petter. (70). *Bacterium halobium* is often the chief cause of the reddening of salted fish. KLEBAHN (according to BENECKE (11) thinks it acceptable that this form is a frequently occurring contamination of the salt used for the preservation. So it may be expected that this form will occur in samples from salt-gardens. A characteristic property of *B.halobium* is the formation of gas-vacuoles, macroscopically visible by the non transparent appearance of the culture. Gas-vacuoles, however, were never observed in the cells of strain C.

Bacterium trapanicum isolated by Miss PETTER from Trapani-salt is closely related to B. *halobium*, but does not form gas-vacuoles and the nutrient conditions are somewhat different. *B. trapanicum* for instance develops well in a medium with asparagin as sole organic substance and *B.halobium* does not. In order to investigate with which of these forms strain C should be identified the bacterium was transferred to a medium containing 1% asparagin, 0,02 K_2HPO_4, 0,02% $MgSO_4$ and 30% NaCl. For comparison *B.halobium* (strain No. 6 from Miss Petter) *B.trapanicum* and a strain of *B. halobium* isolated by the author from salted herring and containing gas vacuoles, were transferred into the same medium.

The result after 15 days incubation at 37° C. was:

	development
B. *halobium* (original strain of Petter No. 6)	—
B. *trapanicum* (original strain of Petter)	+
B. *halobium* (isolated by the author)	—
Strain C.	+

So it may be concluded that strain C is identical with *B. trapanicum*. It is remarkable that this form is so widely distributed and seems to occur everywhere in strong brines. Since Petter has shown that salt may be contaminated with this form, it is probable that it also may occur as the cause of the reddening of salted fish. It is possible that *B. halobium* is only a special form of *B. trapanicum*, which has adapted itself to life in media rich in decomposition

products of proteins, so that it has become unable to live in a medium containing only one amino acid such as asparagin.

B. *trapanicum* must be regarded as a typical halophilic form, in contrast with the two sporulating forms A and B. So it appeared that in the salt-mud sample typical halophilic forms as well as common, but halotolerant, forms are present.

Salt from Searles Lake (California) was inoculated on the same media indicated above, but no development was observed on any of these media. It seems probable that this is due to the fact that this sample of salt was too old. It was collected in 1931 and the experiments were carried out in 1934.

A sample of mud with blue-green algae from the salt-garden of Pamekasan (Madura), showed on direct microscopical examination numerous rods. From this sample which contained 24% NaCl streaks were made on peptone agar with 0,5, 3, 6, 12, 18 and 24% NaCl. After 6 days transparant colonies appeared on the plates containing 6, 12 and 18% salt. The plates with 24% NaCl showed no development. On the plates with 0,5 and 3% NaCl only few colonies appeared, which may probable be regarded as contaminations. The numerous transparent colonies on the plates with 6, 12 and 18% salt consisted of small motile rods.

On all three plates only one type of colonies could be found. After 12 days on the peptone agar plate with 24% NaCl the same type of colonies had appeared. Likewise these colonies consisted of small motile rods.

Pure cultures were isolated from the plates with 6, 12, 18 and 24% NaCl by cultivating them on agar with the same amount of salt.

The species appeared to be obligate halophilic. In contrast to strain C (B. *trapanicum*), however, the cells were not markedly changed by transferring them from a medium with 24% salt to water. No further characterisation of this form was attempted.

Since by direct inoculation few different bacteria could be isolated, enrichment cultures were made by inoculation of salt mud into special media. The results obtained were very scanty.

The presence of halophilic forms of *lactic acid bacteria, bacteria of the colon-group, bacteria causing methane fermentation of fatty acids, urea bacteria and denitrifying bacteria* could not be demonstrated.

Sulfate reducing bacteria were present. The development of this group could be demonstrated in salt concentrations up to 30%.

Therefore the following media were inoculated with mud from the salt-garden of Gersik-putih and incubated at 30° C.

tapwater	1000 cc
K_2HPO_4	0,5 gr.
NH_4Cl	1 ,,
$CaSO_4$	1 ,,
$MgSO_4$	2 ,,
Sodium-lactate	3,5 ,,
Mohr's salt	trace
NaCl	0, 3, 6, 12, 18, 24 or 30% (with % is meant number of gr. pro 100 cc of the liquid.)

In all concentrations reduction of the sulfate had taken place as shown by the appearance of a black colour and by a strong smell of hydrogen sulfide.

Development of *colourless-sulfur* bacteria was also obtained. Enrichtment cultures of *thionic-acid bacteria* were made in media with increasing amounts of salt. The medium of BEIIERINCK (13) for *Thiobacillus thioparus* was used:

aqua dest.	100 cc
$Na_2S_2O_3$	0,5 gr.
$NaHCO_3$	0,1 ,,
K_2HPO_4	0,02 ,,
NH_4Cl	0,01 ,,
$MgCl_2$	0,01 ,,

with 0, 3, 6, 18, 24 and 30% NaCl.

After sterilisation the media were inoculated with mud from the salt-garden of Grissée (Java). The incubation temperature amounted to 25° C. Development was confirmed by microscopical examination and titration with 0,025 n I. All flasks gave a positive result.

From the titration of the culture liquids with Iodine it may be concluded that the oxidation of thiosulfate occurred in all media. The following table gives the result of the titrations:

percentage of salt.	cc Iodine blank	cc Iodine after 8 days inc.	cc Iodine after 15 days inc.	cc Iodine after 23 days inc.
0	6.02	0.14	0.06	0.10
3	5.94	2.88	1.87	2.20
6	5.88	1.30	0.93	0.20
12	5.57	2.33	0.09	0.04
18	5.45	3.10	1.22	0.05
24	5.25	3.81	2.95	1.88
30	5.15	5.24	4.10	2.80

It is remarkable that most active oxidation occurred both in the medium containing no NaCl and in that containing 12% NaCl. The better suitability of the latter medium as compared with media containing 3 and 6% NaCl was also found in a second set of experiments. It makes the impression that the mud contained two types of *Thiobacilli* one being non-halophilic, the other adapted to a medium concentration of 12% NaCl.

Microscopic examination of all cultures showed the presence of small, non-motile rods.

Together with Prof. Baas Becking we observed a spontaneous development of *Green sulfur bacteria* in a bottle containing a sample of black mud from the salt-garden of Pamekasan (Madura) that had been placed in the light for some time. The brine in this bottle contained ± 12% salt. However, special enrichment cultures for these bacteria with various muds as inoculation material were unsuccessful.

Bacteria decomposing cellulose under aerobic conditions developed in media containing up to 9% NaCl.

The following medium was used as culture liquid:

K_2HPO_4	0,1 %
$CaCl_2$	0,01 „
$MgSO_4$	0,03 „
NaCl	0,01 „
$FeCl_3$	trace
$NaNO_3$	0,025 „

with 0, 3, 6, 9, 12 and 15% NaCl and with pieces of filter paper.

As inoculum mud from the salt-garden of Gersik-putih was used. After 9 weeks decomposition of the cellulose, marked by yellow spots, had taken place in the media with 0, 3, 6 and 9% NaCl. The media with 12 and 15% NaCl showed no decomposition of the cellulose.

This decomposition did not show a halophilic character for the strongest attack of the cellulose occurred in the medium with 0% NaCl.

Microscopical examination of the yellow spots showed the presence of mould-conidia and spherical and rod-shaped bacteria.

To demonstrate the occurrence of *bacteria which attack pectin* the following media were inoculated with mud from the salt garden of Grissée (Java) and incubated at 35° C.

citrus pectin	2	gr.
CaCO$_3$	2	,,
NH$_4$Cl	0,02	,,
MgSO$_4$	0,02	,,
K$_2$HPO$_3$	0,02	,,
tapwater	100	cc

with addition of respectively: 0, 3, 6, 12, 18, 24 and 30% NaCl. ·

The experiments were carried out under aerobic and anaerobic conditions. In both cases the pectin was evidently attacked in media containing up to 18% NaCl, as was shown by a strong development of bacteria in these media. However, although in the case of aerobic decomposition a few organisms could be isolated none of the strains obtained showed a marked ability to decompose pectin in pure culture.

Although in agreement with our expectation (c.f. the arguments given in § 1 of this chapter) the result of this part of the investigation is rather scanty, we may conclude from the foregoing observations that the various samples of salt-mud contain both typical halophilic forms (f.i. *Bact. trapanicum*) and non-halophilic forms which have succeeded . in maintaining themselves in these media.

It also appears — and this may be a fact of possible economic importance — that long storage (3—4 year) tends to destroy various bacterial forms.

CHAPTER II.

On the behaviour of bacteria, naturally occurring in a salt free environment, in media with high concentrations of salt.

§ 1. *Introductory remarks.*

In view of the unsatisfactory results reported in the preceding chapter it was decided to investigate the second point raised in the introduction viz. whether bacteria normally occurring in a salt free environment will be able to develop and thrive in suitable media containing high percentages of sodiumchloride. It seemed correct to suppose that representatives of different physiological groups which are able to develop in media containing large amounts of salt, will also be present in natural brines and may thrive therein as soon as the special nutritional conditions for these groups are realised.

The experiments were carried out in two ways. First the maximal salt-concentration was determined which still allowed development of several pure cultures present in the collection of the "Laboratorium voor Microbiologie" at Delft.

Considering the fact, however, that a bacterium in pure culture may lose a part of its ability to adapt itself to living in an environment differing from that in which it normally occurs, (KLUYVER and BAARS (47) it did not seem excluded that enrichment cultures made by inoculating salt-containing media with salt free material might give better results.

In fact it appeared that the maximal salt concentrations tolerated by several bacteria as occurring in ordinary soil are much larger than the concentrations tolerated by the corresponding pure cultures.

The latter method seems therefore better to answer the question which bacteria may be present in natural brines.

§ 2. Review of the literature.

Before communicating the results obtained it seems worth to discuss briefly the results of previous investigations.

Several publications mention the development of bacteria originating from a salt free environment in media with a relatively high amount of salt. For the greater part pure cultures were used for the experiments. Only few investigations were carried out with enrichment cultures.

Already for a long time attention has been paid to the subject in question. This is due to the fact that salt is used as a preserving agent. In earlier times the preservative action of salt was overestimated. In greek and roman times a salt solution was considered as to be completely inimical to life (BAAS BECKING (7). At the end of the last century a more critical attitude was taken.

KOCH 1881 (48) concluded from his experiments that relatively high salt concentrations possess only a very slight noxious influence on the development of bacteria.

MARTENS 1888 (57) mentions that NaCl showed only a very slight desinfecting action on Staphylococcus pyogenes.

DE FREYTAG 1890 (30) examined the time necessary to kill various pathogenic bacteria in saturated salt solutions.

PETRI 1890 (68) determined the time after which virulent germs, causing "Schweinerotlauf" were still present in salted pork. A decrease of virulence was stated after 70 days. After ½ year virulent germs still were present.

SILBERSCHMIDT 1896 (86) examined a case of meat poisoning and made also experiments with salted meat. He concluded that salting may not kill even micro-organisms without spores. He assumed that bacteria in infected meat are able to develop during the salting.

VAN ERMENGEM 1897 (26) states that *Bacillus botulinus* is able to develop in concentrations up to 6% NaCl.

STADLER 1899 (90) determined the maximal concentrations at which several bacteria still are able to grow. The maximal limiting concentrations amounted for *Bact. coli commune* to 8—10% NaCl, for *B. enteritidis* to 7—8% NaCl, for *B. morficans bovis* to 8—10% NaCl, and for *Bact. proteus vulgare* to 8—10% NaCl.

PETTERSON 1900 (71) found that a markedly noxious influence of salt appeared at a concentration of 20—25% NaCl. *Putrefying bacteria* are more sensitive to salt than others. *Obligate-anaerobic bacteria* tolerate as far as 5% NaCl.

ZWICK and WEICHEL 1910 (101) demonstrated that it takes a very long time to kill *B. enteritidis* in salt solutions. In a salt solution of 10—13% virulent germs still are present after 80 days. Development was observed in as high as 7% NaCl.

KARAFFA-KORBUTT 1912 (45) used various species of bacteria as *Bact. typh. abdom., Bac. mesentericus vulgatus* etc. and examined the maximal limiting concentration at which development could be observed. It seemed that *Bac. mesent. vulgatus* tolerates more salt (12%) than the other forms (4—7%).

TANNER and EVANS 1933 (92) determined the maximal limiting concentrations of several strains of *Bac. botulinus*. They found that the maximal concentration at which development could be observed amounted to 10% NaCl.

Apart from these experiments with a more or less restricted scope there are a few investigations that show a more general character.

LEWANDOWSKY 1904 (53) inoculated garden soil, cabbage and cow-dung in broth with increasing amounts of salt. At a concentration of 25% NaCl a coccus species and a bacterium could still be isolated.

SPERLICH 1912 (89) investigated which of the germs occurring in soil, water and air were able to grow in media containing 3% salt. He found that the number of colonies grown on aerobic plates with 3% NaCl was reduced to about one half. On testing the behaviour of anaerobic germs he found that only one quarter of the number of colonies remained. From the aerobic plates inoculated with tapwater he isolated a number of strains and investigated their behaviour in salt-containing solutions. He concludes that some

forms such as: *Micrococcus flavus, Sarcina lutea, Sarcina rosacea, Micrococcus luteus, Bact. constrictum* must be regarded as halophilic because they develop better in media with a slight amount of salt (½—6%) than in media without salt.

§ 3. *Experiments of the author.*

a. *Experiments with pure cultures.*

First several pure cultures present in the collection of the "Laboratorium voor Microbiologie" at Delft were tested on their ability to develop in media with increasing amounts of salt. The cultures were inoculated in media in which optimal development of the forms could be expected.

The following table shows the result:

Name of the organism	Percentages of salt						
	0	3	6	9	12	15	18
Pediococcus halophilus *)	+	+	+	+	+		
Streptobact. spec.	+++	+	+	—	—		
Bact. coli	+++	+	+	—	—	—	—
Clostridium acetobutylicum	+++	—	—	—			
Bacillus Pasteurii	++	++	++	+	—	—	—
Micrococcus denitrificans	+++	++++	+	—	—	—	—
Nitrobacter	++	++					
Azotobacter chroöcoccum	++	—					
Bacterium proteus	+++	+	+	—	—	—	—
Pseudomon pyocyanea	+++	++++	++	—	—	—	—
Pseudomonas putida	++	+	+	—	—	—	—
Pseudomonas spec.	++	+	. +	—	—	—	—
Staphylococcus pyogenes aureus	+h	+	+	—	—	—	—
Bacillus subtilis	+	+	+	+ 1)	+ 1)	—	—

* = Tetracoccus strain No. 1 of Orla Jensen.

1) compare the text.

It appears that most of the strains tested tolerate only a limited amount of salt, in so far as development in media containing more than 6% salt seldom occurs.

The relative high tolerance for salt of *Pediococcus halophilus* probable must be regarded in the light of the fact, that it was isolated out of pickled anchovy.

The strain of *Clostridium acetobutylicum* seemed to be very sensitive for salt for it did not even develop in a medium containing only 3% salt.

The strain of *Nitrobacter* (pure culture isolated by Mr. Dr. Ir. Г. Y. Kingma Boltjes) oxidised in a medium with 3% NaCl the

nitrite completely. It was not further examined if in the media containing more salt perhaps a part of the nitrite had been oxidised.

Azotobacter chroöcoccum did not develop in a medium with 3% NaCl. In the media with 1 and 2% NaCl development occurred.

Bac. subtilis transferred from the culture on peptone agar with the normal 0,5% NaCl in media with increasing percentages of salt gave development up to 6%. A transfer of the culture in 6% in a medium with 9% NaCl gave, however, development. Inoculation of this culture in a medium with 12% NaCl gave also development.

The same procedure applied to *Bact. coli, Bact. proteus, Ps. pyocyanea* and *Staph. pyogenes aureus*, however, was unsuccessful.

b. *Experiments with enrichment cultures.*

As it was expected that bacteria as occurring in a natural, although saltless environment would tolerate. high salt concentrations better than pure cultures, different media containing increasing amounts of salt were inoculated with materials from a saltless environment, such as common soil, well water etc. The media were first sterilised in order to exclude the possibility of development of bacteria eventually present in the added salt.

It was tested if representatives of the following groups originating from a saltless environment may possess the ability to develop in salt-containing media.

1. Lactic acid bacteria.
2. Bacteria belonging to the colon group.
3. Butyric acid bacteria.
4. Bacteria causing methane fermentation of fatty acids.
5. Urea bacteria.
6. Denitrifying bacteria.
7. Nitrifying bacteria.
8. Bacteria fixing atmospheric nitrogen.
9. Sulfate reducing bacteria.
10. Bacteria which oxidise sulfur or its compounds.
11. Bacteria which attack proteins.
12. Bacteria which attack cellulose under aerobic conditions.
13. Bacteria which attack pectins.

1. *Lactic acid bacteria.*

To obtain lactic acid bacteria at high salt concentrations, enrichment cultures were made in malt-extract containing 0, 3, 6, 12, 18, 24 and 30% NaCl. As inoculum garden soil was used. The glass-

stoppered bottles were incubated at 25° C. Only in the bottles containing 0, 3 and 6% NaCl development took place.

Enrichment cultures of *Betacoccus dextranicus* in yeast-water with 10% sucrose and increasing amounts of salt likewise showed only a positive result in the solutions with 0, 3 and 6% NaCl.

The same result was obtained by inoculation of sauerkraut in yeast-water with 2% glucose, 2% chalk and increasing percentages of salt.

From the experiments it may be concluded that *lactic acid bacteria* are inhibited in their development at a concentration between 6 and 12% NaCl.

This is in good agreement with the data of ORLA JENSEN 1919 (64) who found that most *lactic acid bacteria* are already checked in their development at a concentration of 5,5% NaCl. A few species of *Streptococcus* are able to develop in media with 10% salt. Only the various species of the genus *Tetracoccus* withstand higher concentrations of salt (15%).

HENNEBERG (37) has shown that the formation of acid in a 10% brine goes on very slowly.

From the experiments of FABIAN, BRYAN and ETCHELL 1932 (27) it may be inferred that in the fermentation of cucumbers maximal lactic acid is formed when the brine has reached 45° salinometer (= ± 12% NaCl). The authors mention the fact that the number of *weak acid* producers is increased by the addition of sugar after the salt concentration had reached 66° salinometer (= ± 18% NaCl). It was however not proved that amongst those forms *lactic acid bacteria* were present.

2. *Bacteria belonging to the colon group.*

To investigate the upper concentration limits of these bacteria enrichment cultures were made in lactose broth with an increasing percentage of salt. Well-water (with a colititer of 0,1 cc) was used as an inoculum. The glass stoppered bottles were inoculated with 5 cc water and incubated at 37° C.

Development of gas appeared only in the bottles with 0, 3 and 6% salt. The bottles with 12 and 18% NaCl still showed growth but no gas formation. The media with 24 and 30% NaCl remained without growth.

The maximal concentration at which *bacteria belonging to the colon group* still are able to grow seems therefore to be situated between 6 and 12% NaCl.

In harmony with these data several authors mention that *Bacterium coli* is inhibited at a concentration of 8—10% NaCl.

(PETTERSON (71), STADLER (90), KARAFFA KORBUTT (45), TATTEVIN (93).

3. *Butyric acid bacteria.*

Enrichment cultures were made in bottles containing yeast-extract with 2% glucose, 2% chalk and 0, 3, 6, 12, 18, 24 and 30% NaCl. The bottles were inoculated with pasteurised garden-soil and incubated at 35° C. The following table gives the results:

% salt	0	3	6	12	18	24	30
Days after which development appeared	3	3	3	—	—	7	—
Motile rods	+	+	+			+	
Sporulation	+	+	—			—	
Gas formation	+	+	+			+	

After three days fermentation had taken place in the bottles with 0, 3 and 6% NaCl. In all three bottles motile rods appeared but spores were only formed in the bottles with 0 and 3% NaCl. In the liquid with 6% NaCl no spores were formed, not even after some weeks. After a week in the bottle with 24% NaCl a vigorous fermentation ocurred. In the liquid long motile rods appeared; spores were not formed. The characteristic smell of butyric acid was present. The absence of spores is in agreement with MATZUSCHITA's observation (58) that spore formation of *Clostridium butyricum* is already inhibited at the low concentration of 2—4% NaCl.

According to MATZUSCHITA who worked with pure cultures the maximal concentration at which *Clostridium butyricum* is able to grow amounts to 5—6% NaCl.

The positive result of the enrichment culture in 24% salt as compared with the negative results of the enrichment experiments in the 12% and in the 18% media is very remarkable. Undoubtedly the development in the 24% medium will be due to the successful adaptation of the ordinary germ as occurring in soil to this special environment. Apparently this adaptation process is depending on many still unknown conditions and its occurrence is more or less a matter of chance. For otherwise it cannot be understood why also in the media with 12% and 18% salt the same process did not take place. This view is supported by the fact that I was unable to reproduce the positive result in the 24% medium in later experiments.

3. *Bacteria causing methane fermentation of fatty acids.*

SÖHNGEN (88) has shown that inoculation of salt free material in a butyrate medium with sea-water may cause a strong fermen-

tation of the butyrate. In order to investigate the ability of these forms to develop at higher salt concentrations the following media were prepared:

Ca acetate	2 %
NH₄Cl	0,05 „
K₂HPO₄	0,05 „
NaCl	0, 3, 6, 12, 18, 24 and 30%
in tapwater.	

These media were inoculated with ditch mud. The bottles were placed at 35° C.

In agreement with the experiments of Söhngen fermentation appeared in the flasks with 0 and 3% NaCl. In all other flasks, however, no fermentation took place.

No final conclusion may be drawn from this single experiment; it can only be said that it seems unlikely that the fermentation of acetates will occur at concentrations much higher than 3% NaCl. This result seems of some importance with a view to the improbability of the occurrence in salt mud of a methane fermentation of the anaerobic decomposition products of cellulose.

5. *Urea bacteria.*

Enrichment cultures were made in the following media:

Ca citrate '	2%
urea	3%
in tapwater with 0, 3, 6, 12, 18, 24 and 30% NaCl.	

After sterilization these media were inoculated with garden soil and incubated at 30° C.

Up to 18% NaCl formation of NH₃ was demonstrated but only from the cultures with 0, 3 and 6% salt urea bacteria could be isolated. In spite of the distinctly perceptible formation of NH₃ no urea bacteria could be isolated from the media containing 12 resp. 18% NaCl. On repeating the experiment the same result was obtained. Like in the first experiment two forms were isolated but neither these nor the first isolated forms were able to decompose urea.

The *Urobacterium* strain isolated from the enrichment culture with 6% NaCl was subsequently transferred to media consisting of 1% peptone and 5% urea and with increasing amounts of salt. Under these conditions a perceptible decomposition of urea took place even at 24% NaCl.

Since in the peptone medium the urea decomposition was so much better than in the Ca citrate medium new enrichment cul-

tures were made, this time in the peptone medium. As inoculum garden soil was used. This time urea was decomposed up to 24% salt. Pure cultures were isolated from the cultures with 0, 3, 6, 12 and 24% NaCl. The isolation from the culture with 24% salt was very difficult for the colonies on peptone agar with 24% NaCl were only very small. No isolation succeeded from the culture with 18% NaCl although in this case too a fair amount of NH_3 had been formed.

Quantitative data on the decomposition of urea by the isolated strains are reported in chapter IV.

It is surprising that *Urobacteria* isolated from garden soil withstand higher salt concentrations than *Urobacteria* isolated from a liman by RUBENTSCHIK (73). This author found decomposition of urea up to 18% liman salt. In solutions with pure NaCl he only found urea decomposition up to 13% NaCl. With a view to the experiments reported above regarding the behaviour of the strain isolated from the enrichment culture with 6% salt, it is difficult to ascribe this result to the fact that RUBENTSCHIK isolated his forms in a medium containing 5% NaCl.

6. Denitrifying bacteria.

Enrichment cultures were made in peptone water with 2% KNO_3 and 0, 3, 6, 12, 18, 24 and 30% NaCl. (Enrichment cultures in media with 2% Ca-tartrate in stead of peptone showed almost no gas formation). As inoculum garden soil was used. The glass stoppered flasks were incubated at 30° C.

In all flasks development of gas appeared. Only the incubation-time varied. In the flasks with 0 and 3% NaCl after three days a large amount of gas had been formed and in the flasks with 30% the same occurred only after 10 days.

7. Nitrifying bacteria.

Cultures for the enrichment of *Nitrosomonas* were made in media containing:

$(NH_4)_2SO_4$	0,1	%
K_2HPO_4	0,1	,,
$NaCl$	0,2	,,
$MgSO_4$	0,05	,,
basic Mg carbonate	1	,,
$FeSO_4$	0,001	,,

and moreover 0, 3, 6, 12 and 15% NaCl.

Garden soil was used as inoculum. The presence of nitrite was established with the reagent of Griess-Romijn after two weeks in

the media with o and 3% NaCl. In the liquids with 6, 12 and 15% NaCl no nitrite was present.

Enrichment cultures of *Nitrobacter* were made in the following media:

NaNO$_3$	0,1	%
K$_2$HPO$_4$	0,05	,,
MgSO$_4$	0,03	,,
Na$_2$CO$_3$	0,1	,,
NaCl	0,05	,,
FeSO$_4$	0,04	,,

and with o, 3, 6, 12 and 15% NaCl.

In the media with o and 3% NaCl the nitrite disappeared completely. In the other liquids the nitrite reaction stayed. It was not further examined if here the nitrite partly had disappeared.

8. *Bacteria which fix atmospheric nitrogen.*

KEUTNER (46) investigated fixation of atmospheric nitrogen in different salt concentration. On the basis of his experiments he considers *Azotobacter* as an euryhaline organism that seems to fix nitrogen even at 8% NaCl. The results of Keutner, however, do not seem very convincing and in the light of later investigations it appears that they are probably erroneous. The experiments of Keutner were made with crude cultures, so, that no certainty exists as to the nature of the organism responsible for the observed nitrogen fixation. LIEBERT (54) moreover supposes that during the experiments of Keutner the culture medium finally must have been acid as the amount of chalk added seems to have been insufficient. As the experiments took a very long time (some months) it is quite possible that NH$_3$ had been taken up from the air.

Liebert himself has experimented on the fixation of nitrogen in seawater, especially by *Azotobacter chroöcoccum*. For comparison he made enrichment cultures in a seawater-medium with garden soil as inoculum. In this manner he still obtained development of *Azotobacter* but less vigorous than in a medium without salt.

In the medium with salt a pellicle on the surface was never formed. On the bottom of the culture, however, he found many bacteria amongst which *Azotobacter*. In the enrichment cultures with salt more contaminations appeared than in the enrichment cultures without salt. Liebert did not succeed in isolating *Azotobacter* on agar media with 3% salt.

KORINEK (51) found that *Azotobacter* isolated from common soil developed very scantily on a medium with 3% salt.

Trials were made to obtain enrichment cultures of *Azotobacter* in media with different salt concentrations. The following medium according to Beijerinck was used:

glucose	2 gr.
chalk	2 „
K_2HPO_4	0,1 „
$MgSO_4$	0,02 „
aqua dest.	100 cc.

Moreover 0,0002 % Na_2MoO_4 was added (BORTELS (16) has shown that the addition of this salt gives a much better development of *Azotobacter*) and 0, 3, 6, 12, 18, 24 and 30 % NaCl.

After sterilization the media were inoculated with garden soil and incubated at 30° C. Development of *Azotobacter* was observed only in the media with 0 and 3 % salt. The culture without salt showed better development than the culture with salt, but still in the latter a pellicle appeared on the surface. After some time this pellicle had taken a brown colour. This enrichment culture seemed therefore to thrive a little better than that of Liebert which might be due to the addition of the molybdate. Transfers in a fresh medium always gave rise to good cultures, although the contaminations did not decrease. Plectridia were also present in the culture which might have belonged to *Clostridium Pasteurianum*.

Like Liebert we did not succeed in isolating *Azotobacter* from the enrichment culture with 3 % NaCl on agar media with the same amount of salt. Addition of molybdate to the medium, a decreasing of the amount of agar from 2 to 1 %, the use of washed agar, replacing the glucose by mannitol, nothing was successful. Development of *Azotobacter* could be observed on these media but the contaminations always seemed to develop better. Isolated colonies of *Azotobacter* did not appear, for if the media were inoculated with diluted suspensions *Azotobacter* remained absent and with heavier suspensions *Azotobacter* only developped amidst the contaminations.

Isolating of *Azotobacter* from the enrichment culture with 3 % salt on media without salt was neither successful. This is perhaps due to the fact that the contaminations had accumulated at continued culture.

The pure culture of *Azotobacter* from the enrichment culture without salt showed no development on media with 3 % NaCl.

9. *Sulfate reducing bacteria.*

In chapter I we have seen that *sulfate reducing bacteria* may be

active up to the highest salt concentration. BAARS (2) found that ditch mud inoculated in a lactate sulfate medium containing 3% NaCl gave a reduction of the sulfate. In order to investigate if the sulfate reducing bacterium, occurring in saltless ditch mud is able to develop in media with higher salt concentrations ditch mud was inoculated in the following media:

tapwater	1000 cc	
K_2HPO_4	0,5 gr.	
NH_4Cl	1	,,
CaSO_4	1	,,
$MgSO_4$	2	,,
Sodiumlactate	3,5	,,
Mohr's salt	trace	

with 0, 3, 6, 12, 18, 24 and 30% NaCl.

The media were sterilised before inoculation and the flasks were incubated at 30° C.

The bottles with 0, 3 and 6% turned black due to the formation of iron sulfide by the sulfuretted hydrogen produced.

It seems therefore that the *sulfate reducing bacterium* in natural brines is a specific halophilic form. With a view to the results of BAARS (2) it may, however, be possible to obtain a sulfate reducing organism from ditch mud, which is active in higher salt concentrations by transferring this form in media, increasing gradually in salt concentration.

10. *Bacteria oxidising sulfur and its compounds.*

Enrichment cultures of *thionic acid bacteria* were made in media with increasing amounts of salt.

The medium of BEIJERINCK (13) for *Thiobacillus thioparus* was used:

|		
aqua dest.	100 cc	
$Na_2S_2O_3$	0,5	%
$NaHCO_3$	0,1	,,
K_2HPO_4	0,02	,,
NH_4Cl	0,01	,,
$MgCl_2$	0,01	,,

with 0, 3, 6, 12, 18, 24 and 30% NaCl.

After sterilisation the media were inoculated with ditch mud. The incubation temperature amounted to 25° C. Development was confirmed by microscopical examination and titration with 0,025 n I. In the media inoculated with ditch-mud development appeared up to 6% NaCl. In all three flasks small motile rods were present.

The following table gives the amounts of 0,025 n Iodine in cc used for 5 cc of the culture liquid:

Percentage of salt	cc Iodine blank	cc Iodine after 8 d. incubation	cc Iodine after 15 d. incubation	cc Iodine after 23 d. incubation
0	6.05	2.23	0.04	0.04
3	6.20	3.07	0.10	0.18
6	5.85	2.40	0.20	0.21
12	5.72	3.54	2.05	2.10
18	5.36	5.23	4.80	4.80

So it appears that unmistakable oxidation of the thiosulfate had taken place up to 12% NaCl. From the positive solutions with 0, 3 and 6% NaCl pure cultures were isolated. It appeared, however, that the isolated strains were facultative autotrophic and developed well on peptone agar. Probable they are closely related to *Thiobacillus Trautweinii* Bergey at Al. (see TRAUTWEIN 95). This bacterium is the first *Thiobacillus* which had been proved to be facultative autotrophic. It develops well on ordinary media as peptone agar etc. The oxidation of the thiosulfate does not take place with formation of free sulfur as is the case with *Thiobacillus thioparum* Beijerinck (13). The latter species oxidises thiosulfate according to the reaction:

$$Na_2S_2O_3 + O \rightarrow Na_2SO_4 + S$$
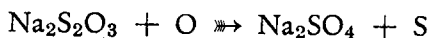
whilst *Th. Trautweinii* forms sulfate and polythionates. Trautwein concludes from quantitative data that the following reactions take place:

$$I \quad 4Na_2S_2O_3 + 12 O \rightarrow 4Na_2S_2O_6$$
$$II \quad 3Na_2S_2O_3 + 5 O \rightarrow Na_2S_4O_6 + 2Na_2SO_4$$
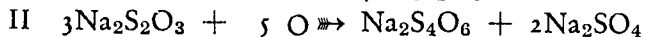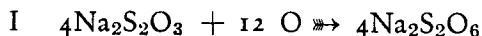
If nitrate is added to the nutrient solution this form is able to develop under anaerobic conditions, both autotrophic and heterotrophis. Trautwein claims that it is closely related to denitrifying bacteria as *Bact. denitrificans* (Stutzer and Burri) L. et N.

11. *Bacteria which attack proteins.*

The occurrence of these bacteria at high salt concentrations is of the utmost importance for the problem of the preservation of meat etc. in salt. Most investigators who have occupied themselves with this subject used pure cultures for their experiments and determined the maximal concentration at which development still appeared.

VAN ERMENGEM 1897 (26) concludes that the development of *Bacillus botulinus* is checked in a medium containing 6% NaCl.

The data of TANNER and EVANS 1933 (92) show that different strains of *Bac. botulinus* may differ from each other in regard to the maximal salt concentration tolerated. One of the strains used for his experiments still grew at 10 % NaCl and even persisted in forming toxins.

STADLER 1899 (90) found that *B. proteus* did not grow at a concentration of 8—10 % NaCl.

PETTERSON 1900 (71) concluded that in media with 5 % NaCl the development of all anaerobic bacteria seems to be inhibited.

In my own experiments I used mass cultures in a fibrin medium with increasing amounts of salt, inoculated with garden soil.

The percentages of salt amounted to o, 3, 6, 12, 18 and 24 %. The bottles were incubated at 35° C. The following table shows the result.

% NaCl	0	3	6	12	18	24
incubation time	6	6	6	6	34	60
Black colour due to H_2S formation	+	+	+	+	+	—
Spore formation	+	+	+	+	—	—
Motility	+	+	+	—	—	—

After 6 days in the bottles containing o, 3, 6 and 12 % salt fibrin was obviously decomposed. The bottles with o and 3 % NaCl had taken a black colour due to the formation of hydrogen sulfide which led to the precipitation of ferrous sulfide.

The bottles with 6 and 12 % NaCL also showed this black colour after 9 days of incubation.

In all four bottles spore formation could be observed although it seemed less pronounced in the bottles with salt than in the bottle without salt. Particularly in the bottle with 12 % NaCl very few spores were formed; the spore forming rods appeared to be very long. In the bottles with o, 3 and 6 % NaCl motile rods occurred but in the medium with 12 % salt no motile forms could be observed.

After 34 days the bottle with 18 % NaCl showed a distinct black colour and in the liquid non-motile, non sporulating rods were observed.

After 2 months the bottle with 24 % NaCl showed some development. The bottle had not taken a black colour but because of the disagreeable odor it was inferred that the fibrin had been attacked. Moreover numerous rods could be observed in the liquid. The rods were similar to those in 18 % NaCl non motile and without spores.

From the above it may be concluded that proteins may be de-

composed in high salt concentrations. The incubation time increases with the concentration of salt used. From the fact that the bottle with 24% salt showed no black colour it may be concluded that decomposition had taken place in a somewhat different manner.

Strong salt concentrations therefore only seem to cause a retardation in the process of decomposition of proteins, but total inhibition does not seem to occur.

That nevertheless it appears possible to preserve food by means of salt seems a strange fact. The strength of the brine used for salting amounts usually 12 to 25% so that certainly in the lower concentrations putrefaction can be expected. We must not forget, however, that the conditions under which the experiments mentioned above have been carried out are quite different from these at the preserving of food.

That no putrefaction takes place will chiefly be due to the fact that the temperature at which salted meat is kept, is usually very low. GRÜTTNER 1932 (35) recommends in his „Taschenbuch für Fleischwaren-Herstellung" a temperature of 6—9° C. This author remarks furthermore that the brine (which is used several times) is no more suitable for salting when it becomes very turbid and smells bad. From this we see that also in practice putrefying bacteria occur in the brine. It takes, however, a long time before they develop and in most cases this development is due to the fact that the brine had become too much diluted.

The time that meat stays in the brine amounts to about three weeks. This is rather short as we consider that at a temperature of 35° C. it still takes 34 days before putrefaction occurs. At the ordinary temperature of salt preservation this time will therefore be very much longer.

Another difference between salting in practice and the experiments is the number of germs present in the beginning. In the experiment a large number of germs was added with the inoculation material, whilst in the case of salting this number normally will be low.

This is also indicated by the fact that it is not recommendable to salt meat when already in state of incipient putrefaction. Apart of the fact that the number of germs will be much larger, in this case the brine will contain decomposition products of proteins which are more easily attacked. In that case e.g. the so-called „red" bacteria may develop. The incubation time of these forms is rather short (10 days in 30% brine at 37° C. (PETTER (70)).

In this connection it may be remarked that HOROWITZ-WLASSOWA

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(39) states that red spots only appear on salted intestines when they were not directly salted after the killing of the animals.

12. *Bacteria which decompose cellulose under aerobic conditions.*

To investigate the salt-tolerance of this group the same media as given on page 108 were inoculated with garden soil. After 8 days yellow spots appeared on the piece of paper in the culture without salt. After 12 days in the media with 3 and 6% NaCl development occurred also. A slight growth marked by the yellow colour of the paper appeared after 2 months in the liquid with 12% NaCl and after 4 months in the liquid with 15% salt. Microscopic examination of the latter cultures showed the presence of spherical cells.

From the results it may be concluded that addition of salt has a very retardative influence on the decomposition of cellulose.

13. *Bacteria which decompose pectin.*

From investigations on cucumber fermentation it is known that decomposition of pectin may occur in strong brines. The softening of cucumbers which seems to be chiefly due to decomposition of pectin may occur according to FABIAN, BRYAN and ETCHELL (27) in brine of 68° salinometer (= ± 18% NaCl). Le Fèvre (cited by Fabian, Bryan and Etchell) found that *Bacillus vulgatus* was able to decompose pectin ånd that this bacterium also was able to grow in a relatively high salt concentration and at a relatively low pH.

Some experiments were carried out on the decomposition of pectin in salt-containing solutions with garden soil as inoculum. The media given previously (on p. 109) were used. The experiments were carried out under anaerobic and aerobic conditions.

The glass stoppered flasks, incubated at 35° C. only showed development and gas formation up to 3% NaCl. In the flasks without salt motile sporulating rods appeared. In the medium with 3% NaCl motile rods appeared also but no spores were formed.

Under aerobic conditions development was apparent up to 18% NaCl. From these cultures some bacteria were isolated. It appeared that all isolated strains were sporulating rods. Reinoculation of these forms in a pectin medium showed distinct growth, but the added pectin did only disappear slowly, so that none of the isolated strains possessed a marked ability to decompose pectin.

It seems important to compare the salt concentrations tolerated by pure cultures with these with which corresponding enrichment cultures may be successful. For this purpose the results obtained were gathered in the following table:

Bacterial groups	% salt tolerated in enrichment cultures	Pure cultures of representatives of the group	% salt tolerated bij the pure cultures
Lactic acid bacteria	6%	Streptobact. spec. ..	6%
Bacteria belonging to the colon group	6%	Bact. coli .... Clostr. acetobuty-	6%
Butyric acid bacteria	24%	licum	< 3%
Urea bacteria	24%	Bac. Pasteurii	9%
Denitrifying bacteria	30%	Microc. denitrifocans	6%
Nitrifying bacteria 2nd phase	3%	Nitrobacter ...... Azotobacter chroö-	3%
Bacteria which fix atmospheric nitrogen	3%	coccum .... Vibrio desulfuricans	2% 2% *)
Sulfate reducing bacteria .	6%	B. proteus	6%
Bacteria which decompose		Ps. pyocyanea	6%
proteins	24%	Ps. spec.	3%
		Staph. pyogenes aureus	6%

*) (data of Baars (2)).

Comparing these results it may be concluded that generally the salt concentration tolerated in enrichment cultures is higher than that tolerated by the corresponding pure cultures. This fact indicates that the potential environment of a bacterium as occurring in nature is larger than that of the same bacterium in pure culture. (With potential environment is meant the sum total of the factors chemical as well as physical, under which an organism may occur BAAS BECKING (8) and (9). Continued growth under the same contions may cause that in pure culture the organism loses a part of its original potentialities.

The maximal salt concentrations tolerated by various pure cultures therefore will give an erroneous idea of that which may happen under natural conditions. For this purpose much more value must be attached to the experiments with enrichment cultures.

CHAPTER III.
On the possibility of an independent cycle of bacterial life in salt-lakes.

The data gathered in the preceding chapters enable us to discuss the cycle of bacterial life in a salt lake. A list of the various bacterial groups with their maximal tolerated concentrations will precede the more general considerations.

Several groups had to be omitted from this list because of lack

of own experience and since no data are found in the literature
as to their tolerance for salt (e.g. bacteria oxidising hydrogen or
methane, acetic acid bacteria etc.).

Bacterial groups	Maximal tolerated Concentration	
	mentioned in the literature	observed by the author
1. Lactic acid bacteria	15% (Orla Jensen)	9%
2. Bacteria belonging to the colon group	6—7% (Stadler)	6%
3. Butyric acid bacteria	6% (Matzuschita)	24%
4. Bacteria causing the methane fermentation of fatty acids	3% (Söhngen)	3%
5. Urea bacteria	18% (Rubentschik)	24%
6. Denitrifying bacteria	30% (Horowitz-Wlassowa)	30%
7. Nitrifying bacteria .... Nitrosomonas	15% (Rubentschik)	3%
Nitrobacter	0% (Rubentschik)	3%
8. Azotobacter chroöcoccum ..	3% (Liebert)	3%
9. Sulfate reducing bacteria ..	30% (Saslawsky)	30%
10. Bacteria oxidising sulfur and its compounds	30% (Saslawsky)	30%
11. Purple sulfur bacteria	30% (Baas Becking)	—
12. Green sulfur bacteria	8% (Baas Becking)	12%
13. Iron bacteria.	3% (Cholodny)	—
14. "Red" Bacteria	30% (Petter)	30%
15. Bacteria which decompose proteins	30% (Saslawsky)	24%
16. Bacteria which decompose cellulose	16% (Rubentschik)	15%
17. Bacteria which decompose pectin		18%
18. Bacteria which decompose chitin	3% (Benecke)	—
19. Bacteria which decompose fat	16% (Issatschenko)	—

From this list it may be concluded that most of the important
groups of bacteria are able to live in concentrations up to about
15% salt and that many groups are physiologically active even at
much higher concentrations.

Let us imagine a salt lake with a salt concentration of 15%.
In this lake the following cycles may occur.

Carbon cycle.

The carbon may be taken up from the air as CO_2 either photo-
synthetically or chemo-synthetically.

The following organisms which have the power of photosynthesis
may occur in the brine:

a) chlorophyll-containing flagellates as:
   Dunaliella (BAAS BECKING (6).
b) *Blue-green algae* as:
   Aphanocapsa (HOF and FRÉMY (38).
c) *Diatoms* (BUDDE (17).
d) *Purple sulfur bacteria* (VAN NIEL (63).
e) *Green sulfur bacteria* (BAAS BECKING (3).

Two autotrophic groups with chemosynthesis may be present in the brine:

a) *bacteria concerned with the oxidation of sulfur and its compounds.*
b) *Nitrifying bacteria.*

The assimilation products produced in the synthesis may enter in the cycle after the death of the organisms. From the three important groups of compounds: carbohydrates, fats and proteins, it is known that they may be decomposed at a NaCl concentration of 15%.

After several intermediate products, that cause in their turn the development of bacteria of various other groups (for instance the products of cellulose-decomposing bacteria may be used by sulfate-reducing bacteria) CO_2 may be liberated either by respiration or by fermentation processes. The higher organisms also contribute to the production of CO_2 by their respiration.

Nitrogen cycle.

It is very doubtful if at this concentration gaseous nitrogen may be fixed. It is improbable that *Azotobacter* or *Clostridium Pasteurianum* are able to live at 15% NaCl. The possibility remains, however, that the *blue-green algae* occurring in the brine may fix nitrogen. That *blue-green algae* are able to fix atmospheric nitrogen has been claimed already by Beijerinck and after many negative statements has been made probable again by DREWES (24) and by ALLISON and MORRIS (1).

Ammonium nitrogen may be used as source of nitrogen by various organisms or it may be oxidized to nitrite by *Nitrosomonas*. It has never been observed that the nitrite may be oxidized further to nitrate in natural brine. However, considering the fact that *Nitrobacter* originating from garden soil was able to withstand 3% salt, it still does not seem excluded that this form will also be active in the brine. The nitrate eventually formed may be reduced by *denitrifying bacteria* to lower nitrogen oxides or to gaseous nitrogen; finally it may also be used as a source of nitrogen by various organisms in the assimilation of proteins.

The proteins may be decomposed at the concentration of 15 % NaCl which means that the nitrogen will be liberated as NH₃.

Sulfur cycle.

The groups of organisms concerned with the metabolism of sulfur are well represented in brine.

The *sulfate reducing bacteria* are active even in saturated brine.

Apart from the sulfuretted hydrogen produced by the sulfate reducing organisms sulfuretted hydrogen is formed in the decomposition of proteins.

The H₂S liberated in this manner may be oxidized by the following groups:

a) *Purple sulfur bacteria* (anaerobic)
b) *Green sulfur bacteria* (anaerobic)
c) *Colourless sulfur bacteria* (aerobic)

All three groups may be represented in the brine. The *purple sulfur bacteria* and the *colourless sulfur bacteria* may be present even in saturated brine. The *purple sulfur bacteria* oxidise H₂S to sulfate, the *green sulfur bacteria* only to S. The *colourless sulfur bacteria* may produce sulfur, sulfate or polythionates.

The sulfate formed in this manner may once more be reduced by the *sulfate reducing bacteria* or may be used in the assimilation of proteins (cystein). '

It is to be remembered, however, that these considerations only are of a provisional nature since they are based partly on data relating to the behaviour of bacteria originating from salt-free media. It seems quite possible that an investigation *in situ* of the bacterial flora of natural salt lakes will prove the presence therein of several other groups, which could not yet be included in the foregoing scheme.

CHAPTER IV.
Some remarks on the origin of halophilic bacteria.

§ 1. *Introductory remarks.*

Little is known about the origin of halophilic bacteria. As already mentioned before (p. 94) it ·may be possible that the bacteria occurring in brines, whether natural or artifical, are specifically the same as those present in saltless media. If this supposition is true one may expect that ordinary bacteria from saltless media show a large tolerance for salt. This, however, seems not to be true at all if pure cultures are tested. We have seen in

Chapter II, that pure cultures of ordinary bacteria almost never show development in media containing 12% salt or higher. Nevertheless it seems possible to obtain several bacteria from saltless material that are able to develop in media containing 24—30% salt, by making enrichment cultures in salt containing media. It may therefore be that many bacteria occurring in salt lakes originate from a saltless environment. Most of the forms, however, isolated from salt lakes have a strong preference for media which contain high concentrations of salt and most of these forms even refuse to grow in salt-free media.

At first sight this behaviour is rather surprising when we accept the supposition made above that these forms originate from a salt free environment. It seemed therefore worth while to investigate whether freshly isolated adaptation forms from salt free materials also show some of the characteristics of the above mentioned truly halophilic organisms of the natural salt lakes.

Since it has appeared also from the experiments described in Chapter II that bacteria as occurring in nature possess a larger potentional environment than pure cultures, natural inoculation materials were used in these experiments.

For this reason pure cultures were isolated from the enrichment cultures made in media containing increasing amounts of salt, as described in Chapter II. As already has been stated salt-free inocula such as garden soil etc. were used in these experiments, while the media were always sterilised before inoculation to exclude contaminations from the salt added. From the "positive" cultures the pure cultures were isolated on media containing the same amount of salt.

In the description of these experiments the strains obtained are denoted by roman numerals, indicating the percentage of salt used in the enrichment culture and in the media for isolation and propagation.

These strains were then investigated as to their behaviour in media without salt and with increasing amounts of salt.

§ 2. *Review of the literature.*

Before proceeding to a report of these experiments a short survey of the scarce data found in the literature concerning the adaptation of forms, isolated from a saltless environment to salt containing media, will be given.

BAARS 1930 (2) has shown that such an adaptation form of *Vibrio desulfuricans* exists (see also KLUYVER and BAARS (47). The starting point of his investigation was the question whether *V.*

desulfuricans and *V. aestuari* were specifically identical. These
forms differ only from each other as to their behaviour in salt
containing media. *V. desulfuricans* develops better in a medium
without salt and its development is already checked in a medium
containing 3 % salt. On the contrary *V. aestuari* finds its optimal
development in a medium containing 3 % salt, while it does not
grow in a salt free medium.

Notwithstanding this Baars found that sulfate reduction may
appear in a medium with 3 % salt when saltless ditch mud was
used as inoculum. A pure culture isolated a long time (258 days) ago
from an enrichment culture obtained by inoculating the same mud
in a medium without salt, however, refused to develop in a medium
containing 3 % NaCl. After passage of media with 1 and 2 % NaCl,
however, this form was able to produce the same amount of hy-
drogen sulfide in a medium with 3 % NaCl as it first produced in
a medium without salt.

Likewise a strain of *Vibrio aestuari* isolated from sea mud which
first gave a very poor development in a medium without salt
developed very well in a saltless medium after passage of media
with 2 and 1 % salt respectively.

From these experiments it is clear that *Vibrio desulfuricans* is
able to adapt itself to a life in media containing 3 % salt. In
enrichment cultures of this form the adaptation is spontaneous but
when a pure culture is used for the experiments adaptation is only
attained by transferring the form in media gradually increasing
in salt concentration.

Kluyver and Baars give as their opinion that the facts reported
must be explained on the basis of the assumption that in nature a
pluripotent form of *Vibrio desulfuricans* exists which has the capa-
city of development under many abnormal conditions [1]). However
as soon as such a form is isolated in the laboratory and propagated
under the monotonous conditions to which pure cultures as a
rule are exposed, a loss in its properties occurs, which is responsible
for the disappearance or at least strong decrease of the adaptive
power originally present. It is for this reason that the said authors
give the pure cultures which have more or less irreversibly lost
their adaptive power, the name of "physiological artefacts".

Outside these data an indication for the existence of spontaneous
adaptation is found in a publication of Le Fèvre and Round (29).
These investigators isolated a number of bacteria from the scum

[1]) Besides the adaptation to media with high salt concentrations the adaptation
to higher temperatures was studied.

on the brine of fermenting cucumbers. Most of these forms did not develop in media without salt. The origin of the bacteria in question, however, is unknown. It is probable that they were brought in with the cucumbers, although it is not excluded that they had been present in the salt used. If we accept that they were brought in with the cucumbers they must be regarded as spontaneous adaptation forms of common bacteria.

A case of adaptation of *Protozoa* was found by Hoare (cited by Korinek (50). This investigator isolated *Protozoa* from fresh water, that were able to develop in a medium containing 3 % NaCl. After some time of cultivation in this medium they refused to grow in fresh water media.

Korinek (49) investigated the possibility of survival of fresh water-bacteria in the sea. He concludes that, although various common forms such as: *Bacillus subtilis, Bacterium pneumoniae, Bacterium pyocyaneum, Bacterium fluorescens*, etc. developed well on media with 3 % salt, they do not take part in the mineralisation process in the sea. This conclusion was based on the fact that pure cultures of these bacteria did not develop in a sterilised medium consisting of seawater with algae, while three species which he isolated from seawater grew well in this medium. It is difficult to understand that Korinek attached such a great value to this result for he had shown previously that the "land" bacteria he used for his experiments developed on a medium composed of seawater + Caulerpa decoction + 1 % peptone to which 2 % agar had been added. Moreover Korinek took a rather arbitrary choice of the "land" bacteria used in his experiments and it is very probable that when Korinek had inoculated the medium indicated previously (sterilised seawater with algae) with common soil, several bacteria would have developed.

In another publication Korinek (50) discusses the possibility of adaptation of the "land" bacteria to life in seawater. He cultivated a number of common bacteria for a long time in sea water media. It appeared, however, that after a year they still grew better in media without salt. Most of the marine bacteria in contrast did not develop on media without salt. It must be said, however, that Korinek only experimented with pure cultures originally isolated on media without salt. It is possible that the same experiments with cultures obtained from direct enrichment experiments would have given a different result.

Burke (18) studied the possibility of an interchange of bacteria between the fresh water and the sea. Therefore he inoculated sea-

water peptone gelatin and fresh-water peptone gelatin with marine
and fresh water samples. In general the number of colonies was
less on the heterologous medium than on the homologous medium.
Further experiments made it probable that the decrease of the
number of colonies on the heterologous media is due to the fact
that a part of the cells of every species present in the inoculum
is more sensitive and died on the heterologous medium and that the
failure to grow is not caused by the absence of certain species.
Burke concludes that the experiments suggest that fresh water
bacterial species are able to maintain themselves in the sea.

§ 3. *Experiments of the author.*

   a. The possibility of adaptation of *Bacterium coli* to a salt
      environment.

As mentioned in Chapter II enrichment cultures of *Bacterium
coli* were obtained by inoculating lactose broth containing o, 3
and 6 % NaCl with well water. From these cultures three forms
Bacterium coli O, III, and VI were isolated. To ascertain possible
differences between these strains in reference to their behaviour in
media containing increasing amounts of salt they were transferred
to peptone water with 2 % glucose and respectively o, 3 and
6 % NaCl. All three strains, however, developed as well in the
medium without salt as in the media with 3 and 6 % NaCl.

As a qualitative research did not give sufficient certainty, the
amount of glucose fermented after a certain time was determined.
Therefore the three strains were inoculated in peptone water with
2 % glucose and o, 3, 6 and 12 % NaCl. The flasks were incubated
at 37° C. and after two days of incubation the amount of glucose
was determined according to the method of SCHOORL (85). From
all cultures a sample (o,5 cc.) was taken at the same time and to
stop the fermentation to each sample 1 cc. 25 % H$_2$SO$_4$ was added.
Thereafter the glucose determinations were carried out in the usual
manner.

The following table gives the result:

incubation time	% NaCl	% glucose present				glucose fermented as % of the glucose added		
		blank	Coli O	Coli III	Coli VI	Coli O	Coli III	Coli VI
2 days	0	1.92	1.64	1.60	1.59	14.6	16.7	17.2
" "	3	1.94	1.58	1.55	1.53	18.5	20.1	21.1
" "	6	2.05	1.95	1.87	1.86	4.9	8.8	9.3
" "	12	2.29	2.26	2.33	2.32	0	0	0

From this table it appears that there is no difference in the behaviour of the three strains. That *B. coli VI* has fermented a little more glucose in the medium with 6 % NaCl than *B. coli O* in the same medium is of no significance, since the total amount of fermented glucose in this medium is very small. In order to promote a more complete fermentation of the glucose 2 % chalk was added to the media in the supposition that the combined action of the acid produced by the fermentation of the sugar and of the salt inhibited the development of the bacteria.

The percentage of glucose was determined in the same manner after 1, 2, 3, 8 and 10 days incubation at 37° C. Since in the former experiment in 12 % NaCl no development had appeared, the use of this medium was discontinued.

The following table gives the result:

incubation time	% NaCl	% glucose present			glucose fermented as % of the glucose added		
		blank	Coli O	Coli III	Coli VI	Coli O	Coli III	Coli VI
1 day	0	1.67	0.30	0.52	0.30	82.0	62.9	82.0
,, ,,	3	1.72	1.08	1.09	1.03	37.2	36.6	40.6
,, ,,	6	1.73	1.64	1.35	1.53	5.2	21.9	11.6
2 days	0	1.67	0	0	0	100	100	100
,, ,,	3	1.72	0.36	0.43	0.26	79.1	75.1	84.9
,, ,,	6	1.73	1.29	1.41	1.04	25.4	18.5	39.6
3 ,,	3	1.72	0	0	0	100	100	100
,, ,,	6	1.73	0.83	1.24	0.68	52.0	28.3	60.7
8 ,,	.6	1.73	0.32	0.45	0	81.5	74.0	100
10 ,,	6	1.73	0	0		100	100	

The addition of chalk indeed gives a marked acceleration of the fermentation. However, adaptation to life in salt containing media of *B. coli III* and *VI* apparently does not occur.

Although strain VI has fermented the glucose in the medium containing 6 % NaCl a little quicker than strain O and III, the difference is too small to interpret this as the result of an adaptation process. The conclusion seems to be justified that addition of salt has the same retardative influence on the development of all three strains.

To investigate whether the fermentation of glucose in a medium without chalk stopped at a certain pH, the three strains were inoculated in peptone water with 2 % glucose and 0, 3 and 6 % NaCl. After 3 days incubation the amount of fermented glucose

and the pH (by means of the glass electrode) were determined.
The results are shown in the following table.

% NaCl	% glucose present				glucose fermented as % of the glucose added		
	Blank	Coli O	Coli III	Coli VI	Coli O	Coli III	Coli VI
0	1.67	1.40	1.38	1.39	16.2	17.4	16.8
3	1.62	1.33	1.33	1.35	17.8	17.8	16.7
6	1.62	1.45	1.46	— *)	10.5	9.9	— *)

% NaCl	pH of the media		
	Coli O	Coli III	Coli VI
0	4.5	4.5	4.5
3	4.1	4.1	4.1
6	4.4	4.4	4.8 *)

A large difference in pH was not observed. It is remarkable
that the pH of the culture liquid without salt was not lower than
the pH of the liquids with 6 % NaCl since in the latter the amount
of fermented glucose is much smaller. It is, however, not certain
that the value of the pH measured of the liquids containing salt
is quite correct for there may be a salt error in the measurement.

Considering these experiments it must be said that nothing indi-
cates that an adaptation of *Bacterium coli* to life in salt-containing
media exists.

b. *Urea bacteria.*

From enrichment cultures in a medium containing 2 % Ca citrate,
3 % urea and increasing amounts of salt inoculated with garden soil
three strains of urea bacteria: *Urobacterium O, III* and *VI* were
isolated. All three strains developed on media containing o, 3 and
6 % NaCl. *Urobacterium O* grew much better on the medium
without salt (peptone agar with 2 % urea) than on the medium with
6 % NaCl. On the latter only one colony developed, while on the
medium without salt several colonies appeared although the same
suspension was used for the inoculation.

Urobacterium VI, however, developed on the medium with 6 %
NaCl as well as on the medium without salt.

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*) This culture became contaminated.

The three strains were transferred into peptone water with 5 %
urea containing 0, 3, 6, 12, 18 and 24 % NaCl. Development was
controlled by microscopical examination and production of NH_3.

The following table gives the results:

% NaCl	Urobact. O	Urobact. III	Urobact. VI
0	+	+	+
3	+	+	+
6	+	+	+
12	—	—	+
18	—	—	+
24	—	—	+

These results show clearly that *Urobact. VI* withstands much
higher salt concentrations than *Urobact. O* and *III*.

To investigate whether *Urobacterium VI* develops perhaps better
in a medium with a certain salt concentration than in a medium
without salt the amount of urea decomposed at different salt-
concentrations was determined.

Urobacterium VI was inoculated in peptone water with 5 %
urea and increasing amounts of salt. After a certain incubation
time the amount of NH_3 produced was determined. To prevent
the loss of NH_3 during the rather long incubation time the flasks
were closed by a rubber stopper. In this way, however, the culture
might lack oxygen and therefore air was passed through the culture
at regular intervals. The escaping air passed a solution of H_2SO_4,
so that the ammonia was absorbed and could be determined also.

The amount of NH_3 present in the culture liquid at the end
of the experiment was determined by titration with 0,1 n. H_2SO_4
with methyl red as indicator. As the peptone water showed buffer
action, the titration was not quite easy. By titration to a distinct
colour of methyl red between two colours differing only slightly
sufficient accuracy could be attained.

The percentage of salt in the culture liquids was determined by
titration with 0,1 n. $AgNO_3$ and K_2CrO_4 as indicator after Mohr.

The following table gives the results after an average of 25 days
of incubation at 30° C.:

Urobacterium VI in peptone water with 5% urea.

% NaCl	% urea decomposed	urea decomposed as % of the urea added
0	2.4	48
2.8	3.5	70
5.5	3.0	60
10.5	3.5	0
15.4	2.6	52
19.7	1.5	30

It appears from this table that the maximal amount of urea was decomposed at 2,8—15,5% NaCl. Since *Urobacterium VI* has been isolated from salt-free garden soil, this organism may therefore be regarded as another example of what KLUYVER and BAARS have called "physiological artefacts".

Comparison with the results of RUBENTSCHIK (73) on the amounts of urea decomposed by *Urobacterium psychrocartericus* isolated from the Kujalnitzky liman shows that *Urobacterium VI* decomposes much more urea in high salt concentration than *Urobacterium psychrocartericus*. This *Urobacterium* decomposes in a culture liquid with 18% liman salt ₁4,1% of the added 5% urea. In culture liquids containing pure sodium chloride at a concentration of 13% no urea was decomposed. In a 12% NaCl solution 9,8% of the 5% urea added had disappeared. Yet *Urobacterium psychrocartericus* was one of the best urea decomposers of the various forms of urea bacteria which Rubentschik isolated.

The fact that *Urobacterium VI* was able to decompose urea in a liquid with ± 20% salt, made it seem probable that it would be possible to isolate *Urobacteria* at higher salt concentrations than 6%. Therefore enrichment cultures were made in peptone water with 5% urea containing 0, 3, 6, 12, 18 and 24% salt.

Garden soil was used as inoculum. The flasks were incubated at 30° C. At all salt concentrations investigated development of NH_3 was observed. Pure cultures were isolated from the media with 0, 3, 6, 12 and 24% NaCl. As already mentioned in Chapter II isolation of a pure culture from the medium with 18% salt failed. This was caused by the fact that the colonies on peptone agar + 2% urea + 18% salt were only very small, so that on the second plate no colonies could be observed. Although the colonies on peptone agar + 2% urea + 24% salt were also very small isolation succeeded.

The isolated strains *III, VI, XII* and *XXIV* were tested on their ability to develop on media without salt. It appeared that the strains *III, VI* and *XII* developed well on peptone agar 2% urea 0% salt. In contrast to these strains *Urobacterium XXIV* did not develop on this medium. Also this bacterium is therefore obligate-halophilic, although it has been isolated from a saltless environment.

With *Urobacillus XII* (this strain is called Uro*bacillus*, because it forms spores; with the other strains sporulation was never observed) a series of experiments was made to investigate the amount of urea decomposed by it at different salt concentrations. It seemed desirable to check the determinations of the amount of urea decomposed by means of titration of the amount of ammonia produced by a method which allowed the direct determination of the amount of urea left in the culture liquid. First the method of Rubentschik was tested. This investigator determines the urea left by decomposing it with the aid of some c.c. of a broth culture of an Urobacterium (Rubentschik used *Urobacterium psychrocartericus*) at 50° C. The amount of ammonia produced was titrated after some time. Rubentschik claims very good results for his method.

To decompose the urea I made use of a culture of *Urobacillus Pasteurii*. The experiments were, however, not successful. Although *Urobacillus Pasteurii* is one of the best urea decomposers the amount of urea added was never decomposed completely.

As this method failed the method of VAN DER MEULEN (59) for determination of urea was used and with this method good results were obtained. In this method the urea is oxidised by bromine in a slightly alkaline environment. The amount of urea is not determined by measurement of the nitrogen produced as in former methods, but by establishing the amount of bromine necessary for the oxidation. A distinct quantity of bromine is added and the excess is determined by the addition of a solution of potassium iodide and titration of the Iodine produced with 0,1 n. sodium-thiosulfate.

The determination is carried out as follows: 0,5 c.c. of the urea containing liquid (Van der Meulen mentions that 40 mgr. may be present as a maximum) is put into a glass stoppered Erlenmeyer and diluted with 75 cc aqua dest. Then 10 cc 1 n. $KHCO_3$ is added and thereafter 10 cc of a bromine solution (40 gr. Br. + 100 gr. KBr dissolved to 1 liter). After addition of the bromine solution the stopper immediately is put on the flask. Now the mixture must stand for 45 minutes. Then 10 cc 1 n. KI is added

and after 15 minutes the Iodine produced is titrated by means of
0,1 n. thiosulfate. The difference in cc thiosulfate with a blank
indicates the number of mgr. urea present in the sample.

A blank experiment is performed in the following way: 10 cc
in. KI and 15 cc in. HCl are added to 75 cc aqua dest., then
10 cc of the bromine solution is added also. After a quarter of an
hour the Iodine produced is titrated by 0,1 n. thiosulfate.

To test the method some preliminary determinations were made.
First a blank was run and thereafter the amount of urea was deter-
mined in 0,3 and 0,5 cc of a solution which contained exactly 5 %
of urea. To investigate if peptone water also contained compounds
that react with bromine 0,5 cc peptone water was treated in the
same way.

The results were as follows:

	cc 0.1 n. thiosulfate	urea present in mgr.	% urea present in the liquid
blank	44.33	—	—
"	44.30	—	—
0.5 cc p.w. 5% urea	18.78	25.53	5.12
0.3 cc p.w. 5% urea	28.90	15.41	5.14
0.5 cc p.w.	41.97	(2.34)	(0.47)

It appears from these figures that this method gives fairly
reliable results. However, peptone water also contains a small
amount of compounds that react with bromine so that these must
be taken into account. Moreover, when determining the amount
of urea in a culture liquid of an *Urobacterium*, the ammonia pro-
duced must be removed first, since ammonia is also oxidised by
bromine under the conditions of the experiment. To remove the
ammonia 10 cc n KHCO$_3$ were added to 0,5 cc of the culture
liquid and through this mixture air was passed during such a
period that the escaping air no longer contained ammonia (using
Nessler's reagent as an indicator). Except that it took a rather
long time ($\pm$ 6 hours) before the ammonia was removed, this
method gave no further difficulties.

In this manner the amount of urea left in the culture liquid
was determined at the same time as the amount of ammonia pro-
duced.

The following table gives the result:
Urobacillus XII
in peptone water 5 % urea after a mean incubation time of 19
days at 30°C.

% NaCl	% urea blank	% urea decomposed as deduced from the determination of NH₃	% urea decomposed according to the bromine method	average % urea decomposed	urea decomposed as % of the urea added
0	4.81	4.21	4.15	4.18	84
2.3	4.83	4.46	4.25	4.36	88
4.1	4.79	4.11	4.27	4.19	84
8.8	4.70	— ¹)	3.64	—	72
11.7	4.48	4.17	4.01	4.09	82
14.3	4.52	4.02	4.06	4.06	80
14.7	5.—	— ¹)	4.16	—	84
18.3	4.45	3.72	3.99	3.86	78
21.9	4.78	2.10	1.86	1.98	40
25.0	4.70	0	0	0	0

¹) Incidentally NH₃ had escaped from these cultures so that this determination had to drop out.

From this table it appears that *Urobacterium XII* decomposes urea in high salt concentrations much more completely than *Urobacterium VI* isolated from the citrate-urea medium. The most remarkable point, however, is that *Urobacterium XII* decomposes urea in a medium without salt as well as in the media with salt. Up to 18% urea decomposition is in no way inhibited. At a concentration of 22% decomposition is reduced to half the normal value and at 25% no urea is decomposed at all.

Urobacterium XXIV was inoculated in peptone water with 0, 3, 6, 12, 18 and 24% NaCl. After 5 days of incubation 1 cc of the culture liquid was titrated with 0,1 n. H_2SO_4 with methyl red as indicator. Although in this way no exact data of the urea decomposed were obtained it was sufficient to compare the growth of the strain in the several salt concentrations. The result is given in the following table:

Urobacterium XXIV

in peptone water 5% urea and various salt concentrations.

% NaCl	cc 0.1 n. H_2SO necessary for 1 cc of the liquid	% urea decom	% urea decomposed of the urea added
0	0.66	0.20	4
3	0.42	0.12	2
6	3.62	1.1	22
12	6.72	2.0	40
18	5.75	1.7	34
24	2.21	0.7	14

As may be inferred from these results *Urobacterium XXIV*
proves to be strongly halophilic. Optimal development was ob-
tained in 12% NaCl. The amount of NH_3 produced in the media
with o and 3% NaCl is so small that certainly no growth had
occurred in these media. The strongly halophilic character of this
strain is most remarkable since it was isolated from garden soil.

The behaviour of the strains *Urobacterium VI* (from the urea-
citrate medium), *Urobacillus XII* and *Urobacterium XXIV* (from
the urea-peptone media) differ from each other. This is illustrated
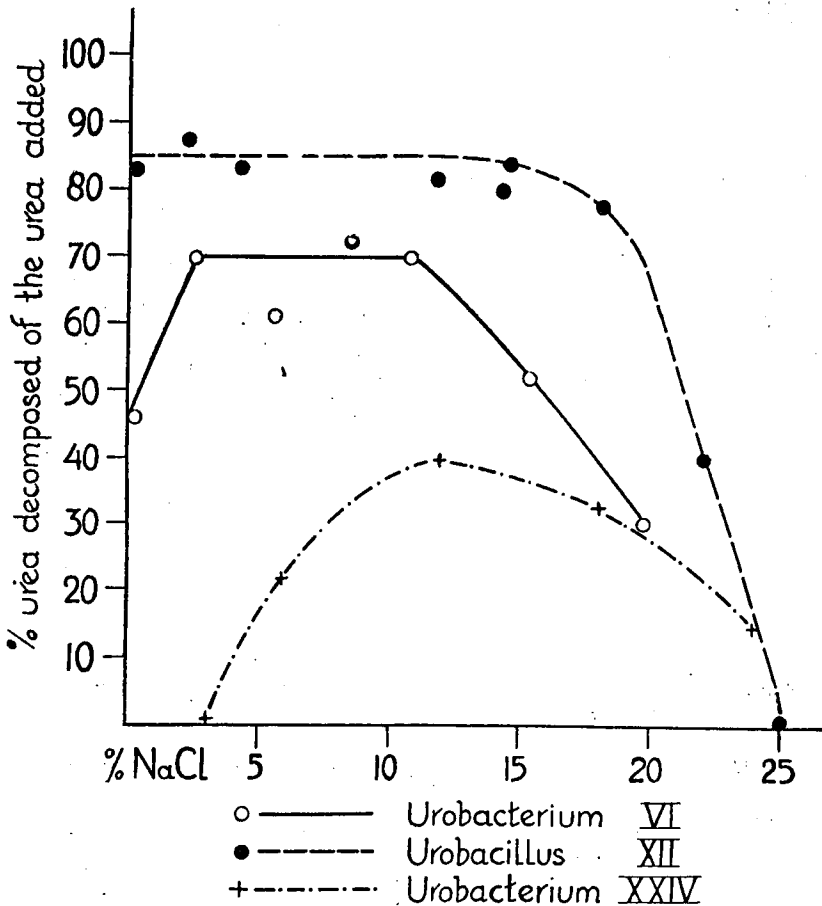in the following diagram.

Fig. 1. Development of *urea bacteria* in relation to the amount
of sodium-chloride present in the medium.

Each of the strains belongs to a special type. *Urobacterium VI* represents a *halophilic, Urobacillus XII* a *highly halotolerant* and *Urobacterium XXIV* an *obligate-halophilic* type. It is very striking that such different types may be isolated from garden soil. It cannot be accepted that such types with the potential environment which they possess after their isolation, are present in common soil. Particulary such an assumption seems to be excluded for *Urobacterium XXIV* as this strain does not develop in a medium without salt, even not when 3 % salt is added.

The three strains must therefore be considered as artefacts, that is they originate from common soil bacteria during the enrichment and isolation.

c. Denitrifying bacteria.

As already mentioned in chapter II denitrifying bacteria were obtained in enrichment cultures containing up to 30% salt. The enrichment cultures were made by inoculating peptone water + 2 % KNO₃ containing 0, 3, 6, 12, 18, 24 and 30 % salt with garden soil. From all enrichment cultures pure cultures were isolated. In the lower salt concentrations 0, 3, 6, 12 and 18 % even two different forms were obtained from each culture. All strains isolated appeared to be true *denitrifying bacteria.* The bacteria isolated from the salt containing media were tested on their ability to develop on media without salt. It appeared that the *denitrifying bacteria III, IV, XII* and one of the strains *XVIII* were able to grow on the common peptone agar (containing 0,5 % salt). In contrast to this the forms *XVIIIb, XXIV* and *XXX* did not develop on this medium. These forms thus were obligate-halophilic although they were isolated from garden soil. They developed well on peptone agar with 3 % NaCl. But even transfers from this medium to common peptone agar were not successful. So it must be said that these strains are adapted to life in a certain salt concentration. After some months some development was observed on the ordinary peptone agar, but this must be due to the drying up of the agar which is concomittant with the increase in the salt content, for on transferring material from this culture to a fresh plate of the same medium (peptone agar 0,5 % NaCl) no development could be observed.

All the strains isolated were transferred to peptone water with increasing amounts of salt. The following table indicates in how far growth occurred:

% NaCl		0	3	6	12	18	24	30
Denitrifying bact.	Oa	+	+	+	—	—	—	—
„ „	Ob	+	+	+	—	—	—	—
„ „	IIIa	+	+	+	+	+	—	—
„ „	IIIb	+	+	+	—	—	—	—
„ „	VIa	+	+	+	+	+	—	—
„ „	VIb	+	+	+	+	+	—	—
„ „	XIIa	+	+	+	+	+	—	—
„ „	XIIb	+	+	+	+	—	—	—
„ „	XVIIIa	+	+	+	+	+	—	—
„ „	XVIIIb	—	—	+	+	+	—	—
„ „	XXIV	—	—	+	+	+	+	+
„ „	XXX	—	—	+	+	+	+	+

From this table it appears that the percentage of salt at which the original enrichment experiment had been performed and at which the organism had been cultivated afterwards had markedly influenced the potential environment of the various strains.

To investigate whether re-adaptation may be possible the *denitrifying bacterium XXIV* was transferred successively in media with gradually decreasing amounts of salt. After passage in media containing 6, 3, 2, 1,75 and 1 % NaCl the form developed on media with 0,75 % NaCl. Thus it appeared that re-adaptation is possible. It must, however, be said that the re-adapted form persistently shows better growth on media with a higher percentage e.g. 6% salt than on the medium with 0,75 % NaCl. Continued cultivation of the form on the latter medium proved impossible. It does not seem excluded that longer propagation of the strain on the medium with 24% NaCl will result in a loss of adaptive power.

d. *Betacoccus dextranicus.*

Enrichment cultures of *Betacoccus dextranicus* were made in yeast extract with 10% sucrose containing 0, 3, 6, 12, 18, 24 and 30% NaCl. As inoculum ditch mud was used. Development of the bacterium was obtained in the media with 0, 3 and 6% NaCl. From all three media pure cultures were isolated. However, *Betacoccus III* and *VI* soon died on the media with the corresponding amounts of salt. Nevertheless it could be observed that they developed well on media without salt, *Betacoccus O* was inoculated in yeast extract with 10% sucrose and 0, 3 and 6% NaCl. In all three media development was observed. In the media with 3 and 6% salt

slime and capsules were formed, however, not so much as in the same medium without salt.

Therefore in the case of *Betacoccus dextranicus* no adaptation could be observed.

e. *Butyric acid bacteria.*

As mentioned in chapter II a butyric acid fermentation was obtained in yeast extract with 2% glucose, 2% chalk and 24% salt inoculated with garden soil. A butyric acid bacterium, however, could not be isolated from this culture, because no sporulation occurred. Shake cultures in yeast agar with ¼% glucose and 24% NaCl were made. Transfers of some colonies, developed in the lowest end of the tube, into yeast extract with 2% glucose, 2% chalk and 24% NaCl, however, did not give fermentation. Nevertheless an indication was obtained that the bacterium which had caused the fermentation was not able to develop in media without salt, for transfer of the crude culture into yeast extract with 2% glucose, 2% chalk and without salt did not give development and in the same medium but with 24% salt development could be observed.

So it is very probable that the bacterium which had caused fermentation in the latter medium has to be considered to be an adaptation form.

f. *Isolation of an obligate halophilic bacterium from an enrichment culture of garden soil inoculated into peptone water with 30% salt.*

A flask with peptone water containing 30% NaCl inoculated with garden soil and incubated at 37° C. gave development of bacteria. From this enrichment culture a bacterium was isolated in the usual way. It appeared that this bacterium was obligate halophilic, for it did not develop on peptone agar containing 0,5% salt. On peptone agar with 3% salt development was observed, but a transfer from this medium to peptone agar with 0,5% NaCl gave no development. Thus here again we meet with an example of an obligate halophilic organism originating from garden soil.

g. *Attempts to isolate obligate halophilic bacteria by direct inoculation of saltless materials on solid salt containing media.*

Malt agar plates containing 12, 18 and 24% NaCl; peptone agar plates with 12, 18 and 24% NaCl and nutrient broth agar plates with 9, 12 and 18% NaCl were inoculated with garden soil, canal water and sewer-mud and incubated at 30° C. From the plates

with 9, 12 and 18% NaCl several strains were isolated. The plates with 24% salt, however, remained without development. The strains isolated were tested on their ability to develop on common peptone agar. It appeared that nearly all strains grew very well, only one strain did not develop on this medium. This obligate halophilic strain consisted of small rods and was isolated from the nutrient broth agar plate with 18% NaCl which was inoculated with canal water.

h. *Bacteria received from Prof. Van Niel.*
Prof. C. B. van Niel was so kind to send to us 10 cultures of bacteria isolated from soil of Ames (Iowa) by means of enrichment cultures in yeast water with 20% NaCl. The bacteria were transferred to peptone agar with 0,5% NaCl. It appeared, however, that all strains developed very well on this medium, even much better than on peptone agar with 18% NaCl. They must therefore be regarded as halotolerant forms and not as adaptation forms.

§ 4. *Discussion of results.*
The chief result of the investigations reported above is undoubtedly that the following obligate halophilic strains could be isolated from a salt free environment:

Denitrifying bacterium XXX
Denitrifying bacterium XXIV
Denitrifying bacterium XVIIIb
Urobacterium XXIV
Strain XXX from a culture in peptone water with 30% NaCl.
Strain XVIII from a nutrient broth agar plate with 18% NaCl.

Since these strains do not develop in media without salt, it seems excluded that they have been present in the soil as such. (All strains were isolated from soil, except *strain XVIII* from the nutrient broth agar plate which was isolated from canal water). They must therefore be considered as "artefacts", special forms that originate from common soil bacteria during the enrichment and isolation procedure. Consequently some common soil bacteria possess the ability to adapt themselves to the life in salt containing media and in doing so lose the property of development under the conditions of the original environment. It seems very probable that also under natural conditions in salt lakes some common bacteria, originating from a saltless environment, will yield adaptation forms. The above-mentioned results are therefore in favour of the assumption discussed in the introductory paragraph viz. that halophilic

bacteria from salt lakes etc. may be adaptation forms of common bacteria.

How should these "artefacts" be considered from a genetical point of view?

Since *denitrifying bacterium XXIV* could be partly readapted to media containing very small amounts of salt (0,75%) it is probable that they are not mutations, but must be classified under the so called "persistent modifications". It is, however, doubtful whether the genetical terminology as used for higher organisms may be applied to the description of bacterial forms. As is rightly emphasized by VAN LOGHEM (56) the clone rather than the bacterial individuum should be considered as an entity. In view of this bacterial variations should be considered as functional phenomena comparable to those occurring in the individual existence of higher organisms.

CHAPTER V.

Some observations regarding the bacterial flora of salted vegetables in the preservation of which a lactic acid fermentation occurs.

§ 1. *Some general remarks on salt preservation.*

In the preservation of vegetable products with salt two different procedures may be distinguished viz.:

a. *Preservation which aims at the occurrence of a lactic acid fermentation.* The lactic acid formed inhibits (sometimes together with the added salt) the development of putrefying bacteria. The amount of salt added is relatively low (0,5—10%). It may not be higher for then the lactic acid fermentation is also inhibited. In this way e.g. sauerkraut and cucumbers are preserved.

b. *Preservation in which the lactic acid fermentation does not take part.* The amount of salt added is much higher than in the first mentioned case (17—30%). Here the added salt is the chief factor that checks the development of bacteria. In this manner e.g. beans, tomatoes and endives are preserved.

Some observations were made regarding the inhibiting action of salt on the development of bacteria in sauerkraut and pickled cucumbers.

§ 2. *Sauerkraut.*

In the preparation of sauerkraut as a rule a certain amount of salt is added. According to HENNEBERG (36) the percentage of salt

added is 0,5—3 % of the weight of the cabbage. In the experiments of PEDERSON (65) 2,5 % salt is added.

For which purpose salt is added to the cabbage?

It may be supposed that it would serve:

1. to promote the development of the special "sauerkrautflora", which includes the inhibition of other bacteria.

2. to withdraw by osmosis water from the plant cells and through this cause the cabbage to be fully covered by the brine, which is essential to establish sufficiently anaerobic conditions. Moreover the continued action of the salt will cause the nutrient compounds present in the cell to enter more quickly into the brine.

In order to investigate in how far these two points hold good sauerkraut was prepared with 2,5 % salt and without salt.

On preparing the two krauts it was very striking that addition of salt had an enormous influence on the amount of juice formed. Whereas sufficient juice appeared on the cabbage with salt, tap-water had to be added to the cabbage without salt. This leaves no doubt that the second point is certainly of importance.

From time to time samples of the kraut juice were plated on yeast extract agar with 2 % glucose and 2 % chalk. At the time of each plating samples of juice were titrated with 0,1 n KOH and phenolphtaleïn as an indicator.

The agar plates of the two krauts showed no difference. On the plates inoculated with the kraut-juice with salt as well as on the plates inoculated with the kraut-juice without salt almost nothing but colonies of lactic acid bacteria appeared. Only sometimes a colony of a yeast or of a mould developed. A number of colonies of lactic acid bacteria was isolated from the various plates. It appeared that all forms consisted of short rods. To investigate whether the strains formed gas, shake cultures were made into yeast extract gelatin containing 2 % glucose. Moreover the production of acid in milk was examined. The following table gives the results.

Age of the krauts	Number of culture from Sauerkraut with 2,5% salt	Gasformation	Acid in milk	Number of culture from sauerkraut without salt	Gas formation	Acid in milk
3 days	1	+	—	7	+	—
	2	+	—	8	+	—
7 „	3	+	+	9	+	—
	4	—	+			
13 „	5	+	+	10	+	
21 „	6	—	—	11	—	—

According to the classification of ORLA-JENSEN (64) the strains No. 1, 2, 3, 5, 7, 8, 9 and 10 must be classified as belonging to the genus *Betabacterium* Orla Jensen, the strains No. 4, 6 and 11 as belonging to the genus *Streptobacterium* Orla Jensen.

On the basis of Henneberg's classification (37) all these strains should belong to *Lactobacterium brassicae fermentatum* Henneberg and its gas-forming variety.

Essential differences in the nature of the flora of the two krauts were not observed.

The results of the titration of the juices with 0,1 n KOH is given in the following table. The amount of acid is indicated as % lactic acid.

Age of the krauts	% acid as lactic acid Sauerkraut with 2,5% salt	% acid as lactic acid Sauerkraut without salt
3 days	0.32	0.40
4 „	0.34	0.46
5 „	0.34	0.48
6 „	0.63	0.65
13 „	1.11	0.75
21 „	1.61	1.13
60 „	1.67	1.37

Evidently there is no large difference between the acid formation in the two kraut juices.

That the amount of acid of the juice of sauerkraut with salt is somewhat higher than that of the juice of the kraut without salt will probably be due to the fact that the juice of the cabbage without salt had been diluted by the addition of tapwater.

The values for the acid formed correspond with the values found by other investigators experimenting on sauerkraut.

The results obtained warrant the conclusion that the most important reason for adding salt is to promote the formation of juice.

It was striking that the kraut without salt was much softer than the kraut with salt. It is not clear to which cause this is due. It may be that sauerkraut with salt after plasmolysis reabsorbs water from the juice. It is known that a gelatin gel placed in a salt solution first loses but then again takes up water (CALOW (21). It has been observed, however, that the latter phenomenon only occurs with gels consisting of proteins; a gel of agar-agar only loses water but does not reabsorb it.

HENNEBERG (36) is also of the opinion that salt is only added to withdraw juice from the cabbage.

That sauerkraut may be prepared without salt but by addition of

water is stated by GERSON (33). In agreement with our experience this author remarks that kraut prepared in this manner is very soft.

§ 3. *Pickling of cucumbers and gherkins.*

The amount of salt added in the preparation of pickled cucumbers is somewhat higher than that added in the preparation of sauerkraut. HENNEBERG (37) recommends the addition of about 6% salt. In the United States two procedures are used, to wit: Low-salt curing and High-salt curing (27). In the first method, the Low-salt curing, the cucumbers are placed in a 30° salinometer brine (= ± 8%). Water will be withdrawn from the cucumbers and the brine will be diluted. To compensate this for each hundred pounds of cucumbers nine pounds of salt are added. The brine is increased in strength at weekly intervals by adding so much salt that the brine raises three degrees salinometer at the time until it reaches 60° salinometer.

In the second procedure, the High-salt curing, the cucumbers are placed in a 40° salinometer brine (10%). As in the Low-salt curing nine pounds of salt are added for each 100 pounds of cucumbers. The brine is increased at the rate of two degrees on the salinometer per week until it reaches 50° salinometer after which it is increased at the rate of one degree per week until it reaches 60° salinometer. This method is especially used in hot weather and warm climates. The fermentation takes place much slower than in the first procedure.

In both procedures the initial concentrations are rather high if we consider that development of lactic acid bacteria is desired. In Chapter II it is mentioned that according to ORLA JENSEN (64) only the special lactic acid bacteria of the genus *Tetracoccus* are able to develop in media containing more than 10,5% salt. Of the other lactic acid bacteria only few species of *Streptococcus* (*Streptoc. liquefaciens* and *Streptoc. glycerinaceus*) are able to develop in a medium with 10,5% salt and even then the said species are very much inhibited in their development.

In fermenting cucumbers mostly a rod shaped lactic acid bacterium consisting of rather large long rods appears. It is called by HENNEBERG (37) *Lactobacillus cucumeris fermentatis*. According to this author the development of this bacterium is already checked in a medium containing 6,5% NaCl.

FABIAN, BRYAN and ETCHELL (27) studied the fermentation of cucumbers prepared after the two procedures mentioned above. They determined the number of bacteria together with the acidity and the salinity of the brine. It appeared that the highest acidity

is found at a salinity of about 40° salinometer in the Low-salt
curing and at about 45° salinometer in the High-salt curing.

The highest number of weak acid producing bacteria does not
coincide exactly with the highest amount of acid formed but
is found a few days earlier. With further increase of the salinity
the numbers of bacteria and the acidity decrease. According to
the authors the decrease in numbers of bacteria is not caused by
the increase in salt concentration but by the lack of available food
in the brine. When the salt concentration had reached 66° salino-
meter sugar was added to the brine. Immediately the number of
weak acid producing bacteria increased. It is however very doubt-
ful and even improbable that among those weak acid producers
lactic acid bacteria are represented.

It is not stated by the authors cited which lactic acid bacteria
cause the initial lactic acid fermentation of the cucumbers. Consi-
dering the rather high initial salt concentrations and the subsequent
gradual increase in strength of the brine it is at first sight hard to
believe that true lactic acid bacteria will show a sufficient deve-
lopment. It is possible, however, that with the gradual increase
in strength of the brine, halophilic adaptation forms of lactic acid
bacteria come into existance.
- To investigate if a spontaneous development of true lactic acid
bacteria may be obtained in a medium with 10% salt, gherkins
were placed in salt concentrations of 6% and 10%. Spontaneous
development of lactic acid bacteria took only place in the 6%
brine. In the brine containing 10% salt, however, motile bacteria
developed and the reaction became alcaline. No development of
lactic acid bacteria could be observed. Even after addition of
0,5% lactic acid and 0,5% glucose and inoculation with brine of
fermented gherkins no such bacteria could be found.

Repetition of the experiment gave almost no better result. This
time gherkins were placed in 6% and in 9% brine (the latter in
duplo). Although the 9% brine became acid, the percentage acid
formed was very low. The following table gives the acidities
observed expressed as percentages lactic acid:

Days past after the preparation	% acid as lactic acid		
	6% NaCl	9% NaCl I	9% NaCl II
4	0.30	0.24	0.19
5	0.42	0.23	0.20
11	0.59	0.30	0.23
15	0.68	0.29	0.27
23	0.49	0.28	0.29

Microscopical examination of the brine of 6% showed the presence of streptococci and rod-shaped non-motile bacteria. In the 9% brine these forms were also present, but not so numerous.

A rod-shaped *lactic acid bacterium* isolated from the gherkins with 6% NaCl proved to be a gas producing species (gas bubbles were produced in yeast extract gelatin with 2% glucose). It belongs therefore to the genus *Betabacterium* Orla Jensen. Inoculation in litmus milk gave acid production but no coagulation. The species gave still development in yeast extract with 2% glucose, 2% chalk and 9% NaCl. In the same medium with 12% NaCl no development was observed.

In this country cucumbers are fermented in a much weaker brine than the brine used in the procedures mentioned before.

A sample of brine of pickled cucumbers prepared at Amsterdam contained 3,6% NaCl. The percentage of acid produced in it amounted to 0,90% as lactic acid. Microscopical examination of the brine showed bacterial rods and oblong yeast cells. Inoculation of the brine on yeast extract agar with 2% glucose and 2% chalk gave development of a *lactic acid bacterium* and two *yeast* species viz. a species with folded and a species with smooth colonies. The *lactic acid bacterium* was isolated. It consisted of rather long rods. As in yeast extract gelatin with 2% glucose gas was produced the strain must be classified as belonging to the genus *Betabacterium* Orla Jensen. In milk no growth was obtained. In yeast extract with 2% glucose, 2% chalk and 6% NaCl growth was observed but when 9% was added the bacterium did not develop.

Gherkins are prepared in almost the same manner as cucumbers. Mr. P. de Jong, a salter at Roelofarendsveen, told me that the amount of salt added in the preparation of gherkins is varied according to their. size. To small gherkins less salt is added than to large gherkins. The gherkins are placed in old wine vessels together with the required amount of salt. The vessels are filled up with water.

A sample of brine from small gherkins was examined. The salt concentration amounted to 5,6%; the acid concentration to 0,84% as lactic acid. A *lactic acid bacterium* was isolated from the sample. This species showed no gas production in yeast extract gelatin with 2% glucose and must therefore be classified as belonging to the genus *Streptobacterium* Orla Jensen. Like the strain from the brine of the pickled cucumbers this strain developed in yeast extract with 2% glucose 2% chalk and 6% NaCl and did not grow in the same medium with 9% NaCl.

Similar *yeast* species as present in the cucumber brine occurred also in the sample of the gherkin brine.

The outcome of the preceding experiments may be summarised in such a way that they tend to confirm the results reported in Chapter II regarding the sensitivety of lactic acid bacteria towards higher salt concentrations present in the medium. While some species are still able to develop satisfactorily in media with as much as 6% salt, a concentration of 9% only allows a scanty multiplication of the bacteria in question.

This result is in agreement with those of preceding European observations; American investigators, however, report that growth of lactic acid bacteria in media with 9% salt still occurs.

Still the occurrence of lactic acid fermentation in media with a moderate salt concentration is of great practical importance since apparently the combined action of the acid formed and of the salt added in the preservation-practice inhibits the development of various types of bacteria, the growth of which would not be checked by the salt alone.

CHAPTER VI.

An investigation of the micro-organisms commonly present in salted beans.

§ 1. *On the general occurrence of a typical microflora in normal salted beans.*

In the preservation of beans in salt which has found such a wide application in this country it is only the salt that inhibits the development of putrefying bacteria. Lactic acid is not formed [1]).

This type of preservation is practised almost exclusively as an home-industry and then in a quite empirical way. This leads to large variation in the amount of salt added and on analysing the brines I found the salt content varying between 6 and 29%!

The addition of the salt causes withdrawal of water from the beans to such a degree that they sink into the brine. Addition of water is not necessary in most cases.

A great number of samples, partly of home-prepared beans, partly bought in the open trade, was subjected to a microscopical

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[1]) According to Henneberg (37) and Lafar (52) in some regions of Germany beans are preserved in such a way that a lactic acid fermentation corresponding to that in pickled cucumbers and sauerkraut occurs. In this case the amount of salt added is apparently kept low. (6—10%).

examination. I was greatly surprised to find that in all these samples, which apparently were all quite fit for consumption, numerous bacteria and yeasts were present. Although in the samples with the lower salt concentrations the flora was still more profuse yet the sample with the highest salt concentration also contained many bacteria and yeast cells.

From this result it may be concluded that the ordinary salt preservation of beans, which is of so common application, is far from being characterizised by a complete inhibition of bacterial life. Nor is there any indication that the micro-organisms present are of the acid producing type and therefore lead to the suppression of a putrefactive flora. By plating the various samples on a medium prepared from bean extract with 12 or in some cases 18% NaCl colonies were obtained, which as a rule were of two types. The first type proved to be a yeast species which will be described later in this chapter. The second type proved to be a short motile rod, which, as will be shown below, belongs to the genus *Pseudomonas*. In some cases also other bacterial species developed on the plates f.i. a few aerobic spore-forming rods and from time to time also *Micrococci*.

In regard to these findings it seems remarkable that salted beans only very rarely lead 'to food poisoning in this country.

§ 2. *On Pseudomonas Beijerinckii nov. spec. the agent of the purple discoloration of salted beans.*

Beans preserved with salt occasionally show the peculiarity that they become purple. Through the kind offices of the Director of the "Centraal Laboratorium voor de Volksgezondheid" at Utrecht a sample of salted beans in which a purple pigment had been formed was received from Mr. E. M. v. d. Zijl at Warffum (Groningen). The brine of the beans was reddish-brown. The cloth that had been placed at the top of the beans in the preserving jar was partly coloured purple in a batik-work like manner. The purple spots remained after washing. After some time the reddish-brown brine became purple and also the beans showed purple stains, particularly at those spots where the beans were pressed against the walls of the glass containing the sample.

At microscopic examination it was found that the brine contained many bacteria and yeast cells. It could, however, not be established in which cells the purple pigment was formed.

Transfers of the brine on peptone agar with different amounts of salt (o, 6, 10 and 18%) never gave a purple colony. Corres-

ponding shake cultures neither gave the result desired. The first idea, that the purple colour was due to the development of some species belonging to the Athiorhodaceae, seemed not to be correct, for it was soon found that the pigment was of a quite different nature. The purple bacteria contain, according to MOLISCH (60) two pigments: bacterioerythrin and bacteriochlorin. Bacterioerythrin dissolves easily in chloroform. It belongs to the carotinoids and gives, like all carotinoids, a blue colour with strong sulfuric acid. The pigment of the purple beans, however, decolorized on addition of sulfuric acid and did not dissolve in chloroform. It must therefore be concluded that the purple pigment does not belong to the group of the carotinoids. Hence it is also excluded that the purple colour of the beans is caused by a bacterium of the group causing the reddening of salted fish for PETTER (70) has shown that the pigments of these organisms belong to the carotinoids.

It was still possible that the yeast present in the brine formed the purple pigment. It is known that some yeast species produce a purple pigment of unknown composition (BEIJERINCK (14) when sufficient iron is present in the medium. At first sight this pigment resembles the pigment produced in the beans. To investigate if this idea was correct the two yeast-species isolated from the beans were cultivated on malt agar containing 0,001 % Fe NH_4 citrate but a purple pigment was not produced by the yeasts in question. On the contrary a culture of *Torulopsis pulcherrima* gave on the same medium the typical purple pigment as described by Beijerinck.

It appears from the above that the purple pigment could be produced by the bacterium so abundantly present in the brine. As on peptone agar (with or without glucose) there never appeared coloured colonies, the idea arose that the bacterium only produces the pigment if it grows in media made from beans. Therefore the purple brine was plated on bean extract agar with 18 % salt. In this way success was obtained. After an incubation time of 12 days at 25° C. there appeared purple colonies on the plate. The purple colonies consisted of small motile rods. A colony was transferred to another bean extract agar plate. On the second plate, however, none of the colonies took a purple colour. Notwithstanding this the pure culture of this bacterium produced, in sterilised salted beans, (made slightly alcaline by addition of a few drops of a $NaHCO_3$ solution) the purple pigment.

As already mentioned the bacterium was a motile rod. Flagellar staining showed that the bacterium possessed polar flagella.

Because of this polar flagella it seems desirable to classify the bacterium as belonging to the genus *Pseudomonas*. In agreement with the expectation in yeast-water with 2% glucose and 12% NaCl no gas is produced. Gelatin is not liquefied. The bacterium is aerobic and lacks the ability of denitrification, although nitrate is reduced to nitrite.

As to the behaviour of the bacterium in media with different salt concentrations it was observed that the development in salt containing media was much better than in those without salt. Transfers of the culture on common peptone agar (with 0,5% salt) gave development if a large mass of bacteria was used as inoculum, but the cells that were obtained on this medium were non-motile and irregular in shape.

If a normal suspension of the bacterium was streaked on peptone agar with 0,5% salt not a single colony developed. Inoculation of a thick suspension gave only a very scanty development. On bean extract agar without salt no development could be observed. On the same agar with 3, 6, 12 and 18% the development was very profuse. With 24% only a scanty development appeared and with 30% growth failed.

To investigate whether in normal salted beans, which had not taken a purple colour, the same *Pseudomonas* species may occur, a number of samples of salted beans were plated on bean extract agar with 12% salt. Microscopical examination of the brine of all the samples showed again the presence of yeast cells and rods, which often showed motility.

Some of the bean extract agar plates showed directly after incubation the occurrence of the bacterium producing the purple pigment. Another part of the plates showed no production of the purple pigment, but the bacterial colonies present on these plates closely resembled the pigment producing bacterium. In fact inoculation of the colourless colonies in sterilised salted slightly alcaline beans gave production of the purple pigment. Only from a few samples no pigment producing bacterium could be isolated.

The percentage of salt in the brine of each sample was determined by titration with 0,1 n Ag NO$_3$ in order to see whether the amount of salt would be of influence on the presence of the pigment-producing bacteria. The percentage of salt in the brine of the sample from Warffum was also determined and that of a sample received from Rotterdam that also possessed a purple colour.

The following table shows the result.

Number of sample	Pigment present in the sample when received	Percentage of salt	Presence of the purple pigment producing bact.
I (Warlfum)	+	16.9	+
II (Rotterdam)	+	10.6	+
III (Delft)	—	17.5	+
IV "	—	18.2	—
V (Den Haag)	—	12.2	—
VI "	—	16.4	+
VII "	—	10.1	+
VIII "	—	28.7	—
IX "	—	5.8	+
X "	—	27.4	+
XI "	—	17.8	+
XII "	—	12.4	+
XIII "	—	19.7	+
XIV "	—	18.7	+
XV "	—	15.4	+
XVI "	—	13.2	—

It is obvious that the amount of salt present in the brine does not determine whether the purple pigment-producing bacterium is present in the brine or not. Moreover it seems probable that the percentage of salt is also not of decisive influence for the production of the purple pigment in the brine.

The strains isolated producing a purple pigment in media made of beans were submitted to a further examination. They were tested on the ability to form nitrite out of nitrate, to produce indol, to decompose cellulose and to form acid from glucose.

No. of the strain	Nitrite formed	Indol produced	Cellulose decomposed	Acid from glucose (cc 0 1 n KOH necessary for 5 cc of the culure liquid)
I	+	—	—	0.70
II	+	—	—	0.77
III	+	—	—	0.47
IV	—	—	—	0.57
V	+	—	—	0.65
VI	—	—	—	0.51
VII	+	—	—	0.80
VIII	+	—	—	0.72
VI	+	—	—	0.82

Except that two of the strains do not reduce nitrate, all strains possess the same characteristics.

As far as could be ascertained in the literature no mention is made of the phenomenon of the purple pigment formation in salted

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beans. Yet it has been known to Prof. M. W. Beijerinck for in the "Lab. voor Microbiologie" at Delft a flask was found containing dried material of the purple bacterium of salted beans collected in 1905 [1]). With a view to this I have thought it appropriate to name the new bacterium *Pseudomonas Beijerinckii*.

§ 3. *On the factors which determine the production of pigment by Pseudomonas Beijerinckii.*

In the isolation experiments it had already appeared that *Ps. Beijerinckii* does not always produce pigment. In the first place the pigment-production depends on the nature of the medium. Besides, in media from beans, pigment production was observed by inoculating *Ps. Beijerinckii* in sterilised salted curly cabbage and sprouts. Although excellent growth of the bacterium was obtained in cauliflower, spinach and carrots pigment production did not appear in these media. On peptone agar, yeast extract agar, tomato extract agar and pea-leaf extract agar also never pigment production was observed.

It seems therefore that in beans, curly cabbage and sprouts a special compound is present from which the bacterium produces the pigment. Under the compounds occurring in beans stated by WEHMER (100) flavone is found. It was supposed that perhaps flavone may be a pre-stage of the purple pigment in question for it is known that flavones may be reduced to red pigments. Wehmer refers to a publication of SKALINSKA (87). This author investigated the pigments occurring in the seed-coat of beans. These pigments may be: yellow, brown or violet. Skalinska has shown that these pigments are related to each other. The yellowish and brown beans contain flavones, the yellow "pseudobases" and the violet beans anthocyanins. The presence of flavones is shown by extraction of the beans with alcohol. The extract gives with lead acetate a yellow precipitation and with $FeCl_3$ a greenish black one. These flavones may be reduced to anthocyanins.

The pseudobases are isomerisation products of the anthocyanins. By heating them with HCl the anthocyanins are formed back.

Although in the case of the salted beans no ripened seeds are present, it still is possible that already flavones or pre-stages of them are formed. It is probable that the production of flavones is not restricted to the seed-coat. A flavone containing extract was prepared from beans. The beans were extracted with boiling water (not with alcohol in order to avoid the dissolving of the chloro-

[1]) Trials to isolate from this material the purple bacterium were unsuccesful.

phyll). This extract was precipitated with lead acetate. The precipitate was washed and treated with H_2S to remove the lead. After sufficient treatment with H_2S the liquid was boiled and filtered. The filtered yellowish brown liquid was evaporated after controling that no more lead was present. After evaporation the obtained amorphous residue was dissolved in water. The solution still showed the reactions of the flavones (intensive yellow colour with KOH; with lead acetate a yellow precipitate and with $FeCl_3$ a greenish black one). Attempts to reduce these flavones were without result.

A few cc of the flavone solution was added to peptone water with 12% NaCl. This medium was inoculated with the purple pigment-producing bacterium from sample No. 1. The purple pigment, however, was not produced in this medium, notwithstanding the .fact that the bacterium had grown very well.

The supposition that a flavone is the compound from which *Pseudomonas Beijerinckii* produces the pigment seems therefore unwarranted.

The examination of the various samples of salted beans has shown that *Pseudomonas Beijerinckii* may be present in beans, although they have not taken a purple colour. It therefore must be concluded that even when the medium consist of beans the pigment is not always produced.

In order to inquire whether in ordinary practice pigment production in salted beans frequently occurs Mrs. Beijerinck (Wijster, Drente) was so kind to insert a note in the magazine: "In en om Woning en Boerderij". From the answers on this inquiry it turned out that formation of the pigment is not at all rare. As to the cause of the purple colour the opinion was given that it was formed only if beans from seed-beans were preserved; according to others it was dependent on the use of preserving pots of a special nature or on the piece of wood that generally is put on the top of the beans to support the heavy stones which are indispensable for keeping the beans in the brine.

In order to examine the first mentioned possibility beans from a brown seed variety and beans from a white seed variety were potted. Of each kind of beans one jar was inoculated with a pure culture of *Ps. Beijerinckii*, another jar was not inoculated. It appeared, however, that in all cases the purple pigment was produced at the top of the jars. From this we may conclude that the bacterium in question occurs regularly on various varieties of beans and that the special variety is of no influence on the production of the

pigment. This result could have been foreseen more or less since beans from brown seed varieties are only very exceptionally used for salt preservation.

It seems therefore that the pigment production in salted beans is not dependent on the variety of beans used for the preservation, but that only when *Pseudomonas Beijerinckii* develops under special conditions pigment production occurs.

In some preliminary experiments it was found that the purple pigment was only produced in sterilised salted beans when a few drops of a sodiumbicarbonate solution were added to the beans. Therefore it was thought that the alkalinity of the medium would have decisive influence on the production of the pigment. To investigate this point further the bacterium isolated from sample No. 1 was transferred into bean extract with 12 % NaCl and increasing amounts of a sodiumbicarbonate solution. After ten days incubation it appeared that in the tube without NaHCO₃ the pigment production had failed to come. The tube to which 2 drops of the NaHCO₃ solution were added had become faintly purple at the top. The other tubes with 4, 6, 8 and 10 drops NaHCO₃ solution showed at the top a distinct purple ring.

A new series was made and this time the pH of the media before inoculation and after incubation was determined with the glass-electrode. The following table gives the result.

Strain No. V in bean extract with increasing pH

Number of drops Na₂CO₃ solution added to 50cc extract	pH before inoculation	pH after incubation	pigment produced
0	5.4	8.3	+
2	5.6	8.3	+
4	6.2	8.6	+
6	6.8	8.5	+
8	7.0	8.6	+
10	7.6	8.6	+
12	8.1	8.6	++
14	8.1	8.6	++
16	8.4	8.7	++++
18	8.3	8.7	+++
20	8.3	8.7	+++
24	8.3	8.8	++++
28	8.4	8.8	++++
32	8.5	8.8	++++
36	8.7	8.9	+++
40	8.6	8.9	++++

Although in this series even in the medium with the lowest pH still a slight production of the pigment occurred, it yet is very obvious that an increase of the pH causes a stronger production of the pigment. That also in the media with the low pH some pigment is produced must be due to the fact that the development of the bacterium is concomittant with an increase of the pH.

To prevent large fluctuations of the pH during development of the bacterium, a buffer mixture was added to the bean extract. Although no sufficient buffer was added to maintain the same pH, the pH range was much less. The following result was obtained:

Media containing					
Buffer Mixture		The usual bean extract evapora-ted to the half of the volume	pH before inoculation	pH after incubation	pigment produced
2/15 mol Na₂HPO₄2aq	2/15 mol KH₂PO₄				
0.50cc	19.50cc	20cc	4.8	5.9	—
6 " "	14 " "	" "	4.8	6.0	—
" "	" "	" "	5.1	6.3	—
12 " "	8 " "	" "	5.1	6.3	— (very
" "	" "	" . "	5.6	6.7	+ faintly)
19 " "	1 " "	" "	5.6	7.3	—
" "	" "	" "	5.9	7.8	+
" "	" "	" "	5.9	7.9	+

The bacterium had developed in all cases, but pigment production appeared only in the media with the higher pH.

It may therefore be concluded that an essential condition for a marked formation of the purple pigment is the alkalinity of the medium. Probably a pH 8,0 is more or less optimal.

This is, however, not the only factor, for in many experiments the pigment did not appear, although sufficient NaHCO₃ was added.

Since it could be shown that extracts of salted beans were not always suitable to give pigment production, extract was made from dried beans (Vlinderco). This extract was prepared in the following way: from 1 parcel dried beans (about 85 gr.) 500 cc extract are made by boiling them with water for about one hour, then 1 gr. Na₂CO₃ and 60 gr. NaCl are added. The extract prepared in this manner gave, especially when 2% agar was added, a very excellent production of the pigment.

Not only the bacteria themselves were coloured on these plates but there was also pigment produced in the agar. It was remarkable that in the agar the pigment was very specially localized. If

a piece was cut out of an inoculated and incubated agar plate this could be observed still better. Under the colonies the purple pigment was produced directly under the surface, but on a distinct distance from the colonies a band of coloured agar was found on a small distance from the surface. The following figure may illustrate this.
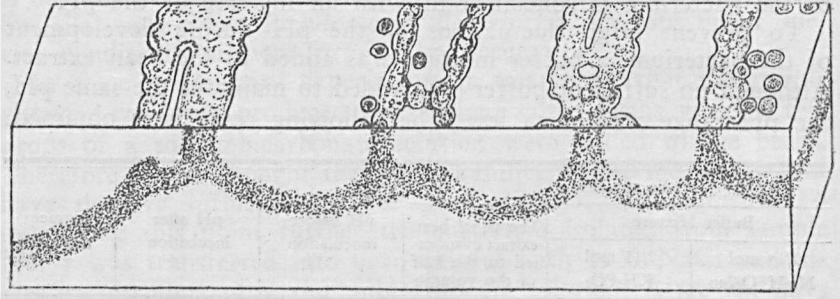

Fig. 2. Pigment production in a bean extract agar plate by *Pseudomonas Beijerinckii.* (The bacterium is only inoculated on the surface of the agar).

The localisation of the pigment suggested that a reduced oxygen-tension is an essential factor in the production of the pigment. This supposition was supported by the following observation. When a thick agar plate was inoculated and incubated pigment was only produced in the superficial regions of the plate. A piece of agar cut out of the lower part of such a plate was completely devoid of pigment. However, after a certain time (12 hours), the center of such a piece of agar became distinctly coloured. In contrast herewith the edges remained perfectly colourless (see figure).
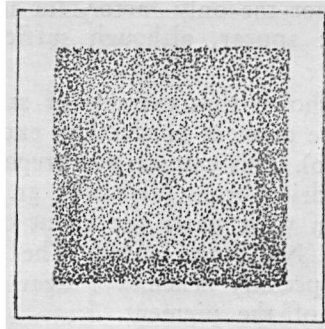

Fig. 3. Pigment production in a piece of agar cut out of a bean extract agar plate inoculated with *Pseudomonas Beijerinckii.*

When such a coloured piece of agar was cut into two parts the pigment did not show any change.

These facts lead to the following supposition concerning the formation of the pigment. The bacteria produce, by growing in a bean extract medium, a special chromogen. This chromogen is oxidized to the purple pigment only at a very specific oxygen tension. If the oxygen tension is too high the' pigment is not formed out of the chromogen; in all probability this substance is then oxidized in a different way. In the latter oxidation the purple pigment is obviously not formed as an intermediate product, for it was never observed that the colourless outer part of the agar piece first took a purple colour and moreover the pigment once formed is quite stable when it is exposed to the air.

If a piece of agar, as used in the last mentioned experiment, is not removed from the plate no pigment is formed in it. Since the chromogen is present in it, this must be. due to the low tension of the oxygen penetrating into the plate as influenced by the surface growth. Still the chromogen present in the deeper layers must be subject to chemical alteration for if the agar is removed after a longer time (f.i. 14 days) it remains colourless after exposition to the air.

§ 4. *Some observations regarding the properties of the purple pigment.*

As to the pigment itself experiments show that it is a very remarkable one. It cannot be included in one of the common natural groups of pigments.

If the purple brine is shaken with air the colour changes into brown. If then a few drops of a sodium hydrosulfite solution are added the purple colour comes back again. Addition of more hydrosulfite leads to decoloration of the pigment.

These experiments show that the pigment possesses a reduced purple form and an oxidized brown form. Probably the purple form may be reduced further to a colourless form. It must, however, be admitted that the brown form not always appears when purple brine is shaken with air; also it may occur that the brown form by addition of hydrosulfite does not give the purple form.

The pigment does not dissolve in organic solvents as methyl alcohol, ethyl alcohol, amyl alcohol, benzene, xylene, toluene, pyridine, acetone, dioxane or chloroform. It neither dissolves in water or in carbondisulfide. That it does not dissolve in water is shown by filtration of the brine through a Seitz filter. In this case a

purple coloured bacterial mass remains on the filter and the filtrate
is only of a pale yellow colour.

The purple brine decolorizes by addition of strong sulfuric acid
and does not become blue. Therefore the pigment does not belong
to the carotinoids which is also excluded because of its insolubility
in many organic solvents mentioned above.

Some varieties of beans possess purple to violet coloured pods.
As it seemed possible that this colour was caused by the same pig-
ment produced in the salted beans, some purple and violet varieties
were investigated. Seeds of the varieties "Schlachtschwert" and
"Heinrich Riesen" were kindly put at our disposal by Prof. J. A.
Honing of the "Landbouw Hoogeschool" at Wageningen. Plants
grew quickly out of these seeds and after 10 weeks both varieties
produced beautiful violet pods. It appeared, however, that this
colour was due to a typical anthocyanin and no relationship what-
ever could be detected between this pigment and the purple pigment
of the salted beans.

From the results of all these experiments it may be concluded
that the purple pigment of the salted beans neither belongs to the
carotinoids nor to the anthocyanins.

Since the pigment does not dissolve in any solvent tested it was
not possible to obtain it in a pure state which is necessary for a
further examination.

It was only more or less incidently that in the last months of
my investigation I made the following observation which had made
it possible to isolate the pigment from the bacterial cells.

On studying the influence of acids and bases on the pigment
I found that addition of diluted acetic acid to the purple brine
(20 cc of a 10% solution of acetic acid to 100 cc of the purple
brine) led to a decoloration of the purple bacteria while the super-
natant liquid took a yellow colour. After separation this liquid
from the bacterial residue by filtration through a Seitz filter a
clear yellow solution was obtained. On neutralizing this liquid the
purple pigment, however, did not reappear. Therefore I was much
surprised to find that on evaporating this solution sometimes a
purple residue was obtained.

A closer investigation showed that it was favorable to make the
evaporation proceed in vacuo, since exclusion of oxygen proved
to be more or less essential. Finally it was found that a slight
addition of sodium hydrosulfide to the solution — obviously in
order to prevent any oxidation of the pigment — was desirable.
On doing this the residue after evaporation had a yellow colour

which turned into a beautiful purple after the air had access.

Of cause the residue consists mainly of sodium chloride but since the pigment, also after the treatment described, is insoluble in water, the salt can easily be washed out, after which small quantities of an amorphous purple powder were obtained.

The complete insolubility of the isolated — although necessarily impure — pigment in all solvents tested has made it until now impracticable to make further inquiries into its chemical nature.

§ 5. *Other micro-organisms commonly present in salted beans.*

As already mentioned yeasts occurred also in the brine of the beans. It appeared that two species were represented: a form with highly wrinkled and a form with more or less smooth colonies. With the aid of STELLING DEKKER: Die sporogenen Hefen (91) and a publication of LODDER (55) it could be shown that the folded form is identical with *Debaryomyces membranaefaciens* Naganishi and the smooth form with *Debaryomyces Guilliermondi* Dekker.

Both species form almost round cells. Sporulation was not observed with certainty in the folded form. Only one cell was observed for which the presence of an ascospore was deemed probable.

This species forms a folded pellicle in malt extract. The colonies on malt agar are also folded. The buds remain often fixed to the mothercells, so that the so-called "Sproszverbände" are formed.

The fermentation of the various sugars is as follows:

glucose   +
galactose   + (very little)
saccharose   +
maltose   + .
lactose   —

Except the doubtful sporulation these properties correspond with those of the species *Debaryomyces membranaefaciens* Naganishi.

The smooth form showed after 4 days on peptone agar 0,1 glucose abundunt sporulation. One spore is formed pro ascus. A few copulation stages could be observed.

In malt-extract a folded pellicle is formed. The colonies on malt agar, however, are smooth. "Sproszverbände" were formed much less than by the former species.

The result of the sugar fermentation test was as follows:

glucose   +
galactose   + (very little)

saccharose +
maltose +
lactose —

This species corresponds rather well with *Debaryomyces Guilliermondi* Dekker.

From the other samples of salted beans evidently the same yeasts were isolated.

On old salted beans there often appears a brown coloured mould. This mould was isolated and could be identified with *Torula epizoa* Corda, which often appears on salted fish (Høye (41) and Farlow (28).

SUMMARY.

In recent years the biocoenonis of salt lakes has been particularly studied by Prof. Baas Becking and collaborators. In these investigations little attention has been paid to the bacterial flora. Subject of this study therefore was to investigate which bacterial groups may be present and physiologically active in natural brines.

As it was impossible for the author to carry out such an investigation on the spot, a number of samples of salt mud and crude salt were submitted to a bacteriological examination.

Bacterium trapanicum Petter and two *Bacillus* species were isolated from a sample of mud from the salt-garden of Grissee (Java) by plating on salt containing media. *Bacterium trapanicum* produces a red pigment and is obligate halophilic. The two *Bacillus* species which developed on common peptone agar (containing 0,5 % salt) could not further be classified.

A colourless, motile, obligate halophilic species, the characteristics of which were not further studied, was isolated from a sample of mud from Pamekasan (Madura).

From a sample of crude salt from Searles Lake (California), which was about three years old no bacteria could be isolated.

Enrichment cultures with the above mentioned samples as inoculum for halophilic representatives of *lactic acid bacteria, bacteria belonging to the colon group, bacteria causing methane fermentation of fatty acids, urea bacteria* and *denitrifying bacteria* were unsuccessful.

Sulfate reducing bacteria were present. The development of this group could be observed in salt concentrations up to 30%.

Colourless sulfur bacteria (thionic acid bacteria) developed also in media containing up to 30% salt.

Although a spontaneous development of *green sulfur bacteria*

was observed in a sample of mud from the salt garden of Pame-kasan (Madura) special enrichment cultures with various salt samples as inoculum gave no result.

Enrichment cultures of *bacteria which attack pectin* were positive up to the medium with 18 % NaCl under aerobic as well as under anaerobic conditions.

Although the result of this part of the investigation is rather scanty, chiefly caused by the relatively long time elapsed after collecting of the samples used, it may be concluded that the samples contain both typical halophilic forms and non halophilic forms. It also appears that long (3—5 year) storage of salt seems to destroy various bacterial forms originally present.

Since in the sample mentioned above the representatives of only few bacterial groups could be detected experiments were carried out to determine the maximal salt concentration which is tolerated by "normal" soil bacteria.

Bacteria which would develop in these experiments in culture media containing high percentages of salt may certainly be present in natural brines and thrive therein as soon as suitable nutritive conditions are fulfilled.

First several pure cultures (present in the collection of the "Labo-ratorium voor Microbiologie" at Delft) were tested on their ability to develop in media containing increasing amounts of salt. It appea-red, however, that the maximal concentrations tolerated were rather low. Most of the species tested tolerated 6 % NaCl at most. In a few cases f.i. with *Azotobacter chroöcoccum* growth was already inhibited at a concentration of 3 %.

Taking into account that bacteria as occurring in nature may adapt themselves better to a life under abnormal conditions than pure cultures (KLUYVER and BAARS (47)) enrichment cultures were made for various bacterial groups in media with increasing per-centages of salt and with garden soil as inoculum.

In this way cultures were obtained of *lactic acid bacteria* in media containing up to 6 % NaCl, *bacteria belonging to the colon group* up to 6 % NaCl, *butyric acid bacteria* up to 24 % NaCl, *urea bacteria* up to 24 % NaCl, *denitrifying bacteria* up to 30 % NaCl, *Azotobacter chroöcoccum* up to 3 % NaCl, *sulfate reducing bacteria* up to 6 % NaCl, *colourless sulfur bacteria* up to 12 % NaCl, *bacteria which attack proteins* up to 24 % NaCl and *bacteria which attack pectin* under *aerobic conditions* up to 18 % NaCl.

Comparison of the maximal concentrations tolerated by pure cultures with those tolerated in the corresponding enrichment cul-

tures shows that in general, the latter are much higher. Pure cultures seem therefore to, possess a smaller potentional environment than the corresponding forms which occur under natural conditions.

The possibility of an independent cycle of life in natural brines was discussed on the basis of the results obtained and of those found in the literature. In a salt lake containing brine of about 15 % the most important bacterial processes may take place.

Since it appeared that many bacteria occurring in garden soil tolerated rather high percentages of salt the question arose whether the bacteria occurring in natural brines might be adaptation forms of "normal" soil bacteria. Forms isolated from natural brines as a rule possess a high preference for salt. Many of them do not develop in media with a small amount of salt. If the supposition that they are adaptation forms of "normal" bacteria is true, some "normal" soil bacteria should be able to adapt themselves in such a way that they lose the property to develop in media without salt. To investigate if it is possible to isolate such adaptation forms from salt free material several bacterial strains were isolated from the enrichment cultures in salt containing media with garden soil as inoculum. The media for isolation and propagation contained the same percentage of salt as the enrichment cultures. The isolated forms were tested on their ability to maintain development on media without salt.

The possibility to give adaptation forms was investigated for *Bacterium coli*. Strains of this form were isolated from enrichment cultures with well water as inoculum and with o, 3 and 6 % NaCl. The three strains, however, did not show any difference as to their behaviour in salt containing media. So the existence of an adaptation form of *Bacterium coli* could not be demonstrated.

Urea bacteria were isolated from enrichment cultures in media containing 2 % Ca citrate, 2 % urea and o, 3 and 6 % NaCl and inoculated with garden soil. *Urobacterium* O and III (the Roman numerals indicate the percentage of salt present in the enrichment culture and in the media for isolation and propagation of the form) developed in media with o, 3 and 6 % NaCl, in a medium with 12 % no growth was observed. *Urobacterium VI* developed in media with o up to 24 % NaCl. So the latter form tolerated much higher salt concentrations than the forms O and III.

From enrichment cultures in peptone water with 5 % urea and o, 3, 6, 12 and 24 % NaCl and with garden soil as inoculum once more urea bacteria were isolated. The forms O, III, VI and XII developed on peptone agar with 2 % urea and 0,5 % NaCl, but

Urobacterium XXIV did not give development on this medium. Consequently the latter form must be considered as a typical adaptation form.

Quantitative data of urea decomposed in media with increasing percentages of salt were obtained for *Urobacterium VI* from the citrate medium and for *Urobacillus XII* and *Urobacterium XXIV* from the peptone medium. The diagram of these data (p. 140) shows that each of the strains used represents a special type. *Urobact. VI* is a halophilic type, *Urobacillus XII* is highly halotolerant and *Urobacterium XXIV* is obligate halophilic.

Denitrifying bacteria were isolated from enrichment cultures with o, 3, 6, 12, 18, 24 and 30% salt. It appeared that the forms III, VI, XII and XVIIIa developed on common peptone agar (with o,5 NaCl). The forms XVIIIb, XXIV and XXX, however, did not give development on this medium. So the latter are adaptation forms and may be considered to be "artefacts" originated from "normal" soil bacteria during the enrichment and isolation.

No adaptation forms could be isolated from *Betacoccus dextranicus*.

Enrichment cultures of *butyric acid bacteria* were obtained in media with o, 3, 6 and 24% salt. A pure culture could not be isolated form the medium with 24% salt because sporulation did not occur. Nevertheless it was made probable that in this culture an adaptation form had developed for transfer in a medium without salt gave no development and in a medium with 24% salt again fermentation appeared.

From a culture in peptone water with 30% salt inoculated with garden soil an obligate halophilic form was isolated.

A number of bacterial strains were isolated from different salt containing agar plates inoculated with salt free material as garden soil, canal water and sewer mud. Only one of the strains isolated appeared to be an adaptation form for it was the only one that did not develop on common peptone agar.

Prof. C. B. van Niel was so kind to send ten strains of bacteria isolated from a soil sample from Ames (Iowa) by enrichment cultures in yeast extract with 20% NaCl. It appeared that all strains were halotolerant for they developed better on peptone agar with o,5% salt thans on peptone agar with 18% salt.

From the results obtained it may be concluded that in fact it is possible to isolate from salt free material forms which have adapted themselves in such a way to a life in a salt environment that they do not develop subsequently in media without salt. There-

fore it is very probable that many if not all forms occurring in natural brines are adaptation forms from "normal" soil bacteria.

Finally some observations were made on the bacterial flora of a few vegetable salt preserves viz. sauerkraut, gherkins, cucumbers and beans.

In order to investigate for which purpose salt is added in the preparation of sauerkraut, krauts were prepared with 2,5 % and without salt. The result showed that salt chiefly is added to obtain sufficient juice. An important influence of the addition of salt on the bacterial flora and the acid formation was not observed in the experiment made.

In about the same manner as sauerkraut gherkins and cucumbers are preserved. In this case the percentage of salt added is as a rule much higher.

FABIAN, BRYAN and ETCHELL (27) mention in their publication on cucumber fermentation that in America cucumbers are fermented in relatively high salt concentrations (8 % salt in the Low salt curing and 10 % in the High salt curing). During the fermentation the strength of the brine is still increased.

As they do not mention by which kind of *lactic acid bacteria* the fermentation was caused an effort was made to obtain a spontaneous lactic acid fermentation in a 10 % brine. Therefore gherkins were placed in brine of 10 % and of 6 % NaCl. A lactic acid fermentation, however, appeared only in the brine of 6 % NaCl. From this brine a species of the genus *Betabacterium* Orla Jensen was isolated which was able to develop in a medium with 9 % NaCl. In a medium with 12 % NaCl this form gave no development.

In our country the amount of salt added in the preservation of cucumbers and gherkins is much lower. A sample of brine of fermented cucumbers from Amsterdam contained 3,6 % NaCl. From this sample a species of *Betabacterium* was isolated.

A sample of brine from Roelofarendsveen contained 5,6 % NaCl. From this sample a *Streptobacterium* species was isolated.

In both samples yeasts were present.

During the preservation of salted beans no lactic acid fermentation appears. The percentage of salt added is as a rule much higher than in the preservation of gherkins and cucumbers. Several samples of salted beans were investigated. The percentage of salt varied from 6—29 %. To my surprise in all samples numerous bacterial and yeast cells were present.

Occasionally salted beans become purple. A sample of such beans

received from Mr. E. M. v. d. Zijl at Warffum (Groningen) was submitted to a furher investigation. It was found that a *Pseudomonas* species to which the name *Pseudomonas Beijerinckii* was given is responsible for the formation of the purple colour. This bacterium produces the purple pigment only when it grows in bean extract or extracts of some other vegetables. In peptone water or yeast water no pigment is produced.

The same bacterium was found in a number of samples of salted beans, which did not possess a purple colour. It appeared that the pigment was only produced when the bacterium grows under certain conditions. The formation of the pigment is chiefly dependent on the pH of the medium and of the oxygen tension.

The purple pigment could not be identified. It is insoluble in water as well as in carbon disulfide as in all organic solvents tested. When shaking with air the purple pigment turns to brown. By addition of hydrosulfite the colour again turns to purple. By addition of acids f.i. diluted acetic acid a yellow solution is obtained. Evaporation of this solution in vacuo at 60° C. gives a purple amorphous residue. Even in this form the pigment does not dissolve in any solvent.

In the brine of salted beans yeasts always occurred. They were identified as *Debaryomyces membranaefaciens* and *Debaryomyces Guilliermondi.*

On old salted beans we often encountered *Torula epizoa* a brown mould.

CONCLUSIONS.

1. Salt samples contain typical halophilic as well as halotolerant forms.

2. Long storage of salt samples seems to destroy various bacterial forms which were originally present.

3. Pure cultures of "normal" bacteria originating from a saltless environment tolerate only a relatively low amount of salt in their culture liquid.

4. The salt concentration tolerated by pure cultures is as a rule lower than the concentration at which corresponding enrichment cultures are still successful.

5. In a salt lake, containing brine of about 15% most of the known important bacterial processes may take place.

6. Since it is possible to isolate obligate halophilic forms from

salt-free material it is very probable that bacteria, occurring in salt lakes etc. originate from "normal" soil bacteria.

7. Addition of salt to cabbage in the manufacture of sauerkraut has no important influence on the bacterial flora and on the acid formation in the kraut.

8. The acid formation in gherkins placed in 9% brine is very low. This is the more surprising as in the U.S.A. cucumbers are fermented in brine of 8 and 10%.

9. In the brine of salted beans numerous bacterial and yeast cells are always present.

10. The occasional occurrence of purple brine on salted beans is due to the action of a new species of bacteria: *Pseudomonas Beijerinckii*. Although this bacterium is nearly always present in salted beans the purple colour does only exceptionally appear. Its formation is dependent upon the pH of the medium and upon the oxygen tension.

11. The purple pigment of the bacterium is insoluble in water and in all organic solvents tested. A yellow solution could only be obtained in diluted acetic acid. The purple pigment may be reobtained from this solution by evaporation in vacuo at 60° C. The chemical constitution of the pigment is still unknown.

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LITERATURE.

1. A l l i s o n, F. E. and M o r r i s, H. J. (1930). Science. New. Series 71, p. 211.
2. B a a r s, J. K., (1930). Over sulfaatreductie door bacteriën. Diss. Delft.
3. B a a s B e c k i n g, L. G. M., (1925). Ann. of bot. 39, p. 613.
4. ———— (1928). Tijdschr. Ned. Dierk. Ver. 3 Ser. Afl. 1, p. 6.
5. ———— (1930). Contributions to marine biology, Stanford p. 102.
6. ———— (1931). Journ. Gen. Physiol. 14, p. 764.
7. ———— (1931). Scientific Monthly 32, p. 434.
8. ———— (1931). Gaia, of Leven en Aarde. Inaugural adress. The Hague. M. Nijhoff.

9.　———— (1934). Geobiologie. W. P. v. Stockum & Zn. N.V. The Hague.
10.　Benecke, W., (1905). Bot. Ztg. I 63, p. 227.
11.　———— (1933). Bakteriologie des Meeres. Handb. d. biol. Arbeitsmethoden von E. Abderhalden. Abt. IX Teil 5 Heft 6.
12.　Bergey, D. H., (1934). Bergey's Manual of Determinative Bacteriology. 4e Ed. Baltimore.
13.　Beijerinck, M. W., (1904). Bakt. Zentralbl. II 11, p. 593.
14.　———— (1928). Arch. Néerl. de Phys. de l' homme et des animaux. 2, p. 609.
15.　Boone, E. and Baas Becking, L. G. M., (1931). Journ. Gen. Physiol. 14, p. 753.
16.　Bortels, H., (1930). Archiv f. Mikrobiologie 1, p. 333.
17.　Budde, H. (1932). Ber. d.d. bot. Ges. 50, p. 343.
18.　Burke, V., (1934). Journ. of Bact. 27, p. 201.
19.　Buschman, J. O. von, (1909). Das Salz. (Leipsic) 2 Vols.
20.　Butkewitsch, W. S., (1928). Ber. d. wiss. Meeresinstitutes. Lief. 3. (Russian with german summary).
21.　Calow, E. H., (1932). Report of the Food investigation Board. London.
22.　Chait, S., (1924). Ber. d. wiss. Forschungs Inst. Odessa. 1 No. 10—11, (Russian) (cited by Rubentschik (75)).
23.　Cholodny, N., (1931). Beih. Bot. Zentralbl. I 48, p. 399.
24.　Drewes, K., (1928). Bakt. Zentralbl. II 76, p. 88.
25.　Entz, G. Sr., (1904). Math. Naturw. Ber. Ungarn 19, p. 89.
26.　Ermengem, E. von, (1897). Ztschr. f. Hygiene 26, p. 1.
27.　Fabian, F. W., Bryan, C. S. and Etchell, J. L., (1932). Agric. exp. Station. Michigan State College. Sect. of Bact. Techn. Bulletin No. 126.
28.　Farlow, W. G., (1886). Bulletin of U.S. Fish Commission.
29.　Fevre, E. Le and Round A. L., (1919). Journ. of Bact. 4, p. 177.
30.　Freytag, C. J. de, (1890). Archiv f. Hygiene 9, p. 60.
31.　Fürer, F. A., (1900). Salzbergbau und Salinenkunde. (Braunschweig).
32.　Gahl, R. and Anderson, B., (1928). Bakt. Zentralbl. II 73, p. 331.
33.　Gerson, M., (1930). Meine Diät, p. 96. Berlin-Ullstein.
34.　Golikowa, S. M., (1930). Bakt. Zentralbl. II 80, p. 35.
35.　Grüttner, F., (1932). Taschenbuch der Fleischwaren-Herstellung einschl. Konservierung (Braunschweig).
36.　Henneberg, W., (1916). Das Sauerkraut. Sonderabdruck aus „Deutsche Essigindustrie" XX No. 21—22.
37.　———— (1926). Handbuch der Gärungsbakteriologie. 2e Aufl. Parey. Berlin.
38.　Hof, T. and Frémy, P., (1932). Rec. trav. bot. neérl. 30, p. 140.
39.　Horowitz Wlassowa, L. M., (1931). Bakt. Zentralbl. II 85, p. 12.
40.　———— (1931). Ztschr. f. Unters. d. Lebensm. 62. H. 5, p. 596.
41.　Høye, K., (1906). Bergens Museum Aarbog. No. 12.
42.　Issatschenko, B. L., (1929). Proceed. Intern. Cong. plant. Sc. Vol. 1. p. 211.
43.　Issatschenko, B. L. and Salimowska, A., (1929). Proceed. Intern. Cong. plant. Sc. Vol. 1, p. 220.
44.　Jacobi, E. F. and Baas Becking, L. G. M., (1933). Tijdschr. Ned. Dierk. Ver. 3e serie 2 Afl. 2—3 p. 145.
45.　Karaffa Korbutt, K. von, (1912). Ztschr. f. Hygiene. 71, p. 161.
46.　Keutner, J., (1904). Wiss. Meeresunters. Abt. Kiel N.F. 8, p. 29.
47.　Kluyver, A. J. and Baars, J. K., (1931). Kon. Akad. Wetensc'. A'dam. Proc. 35, p. 370.

48. K o c h, R., (1881). Mitt. aus d. Kaiserl. Gesundh. Amte Bd. I, p. 273.
49. K o r i n e k, J., (1926). Bakt. Zentralbl. II 66, p. 500.
50. —— (1927). Bakt. Zentralbl. II 71, p. 73.
51. —— (1932). Bakt. Zentralbl. II 86, p. 201.
52. L a f a r, F. (1906). Handbuch der technischen Mykologie.
53. L e w a n d o w s k y, F., (1904). Arch. f. Hyg. 49, p. 47.
54. L i e b e r t, F., (1911). Verh. uit het Rijksinst. voor het onderzoek der
      zee. Derde deel.
55. L o d d e r, J., (1932). Bakt. Zentralbl. II 86. p. 227.
56. L o g h e m, J. J. v a n, (1931). Proc. Koninkl. Ak. v. Wetensch., A'dam.
      34.
57. M a r t e n s, (1888). Virchow's Archiv. 112. p. 369. (After Karaffa
      Korbutt (45)).
58. M a t z u s c h i t a, T., (1902). Zur Physiologie der Sporenbildung der
      Bacillen. Diss. Halle.
59. M e u l e n, J. H. v. d., (1930). Chem. Weekbl. 1930, p. 550.
60. M o l i s c h, H., (1907) Die Purpurbakterien nach neuen Untersuchungen.
      Jena.
61. N a d s o n, G., (1903). Micro-organismen als geologischen Factoren. I
      St. Petersb. (Russian). Ref. Bot. Zentralbl. 96. p. 591.
62. N a m y s l o w s k y, B., (1913—1914). Bull. intern. ac. sc. Cracovie.
      Ser. B 1913, p. 88. 1914, p. 526.
63. N i e l, C. B. v a n, (1931). Archiv f. Mikrobiologie. 3, p. 1.
64. O r l a—J e n s e n, S., (1919). The lactic acid bacteria. Kopenhagen.
65. P e d e r s o n, C. S., (1930). New York State Agric. Exp. Station.
      Technical Bulletin No. 168.
66. P e i r c e, G. J., (1914). Carnegie Inst. 1919 p. 193.
67. P e r f i l j e w, B. V., (1914). Journ. micr. St. Petersb. T. 1. p. 179
      and 204.
68. P e t r i, R. J., (1890). Arb. aus dem kaiserl. Gesundh. Amte. Bd. 6,
      p. 266.
69. P e t r o w a, E. K., (1933). Archiv f. Mikrobiologie. 4, p. 326.
70. P e t t e r, H. F. M., (1932). Over roode en andere bacteriën van
      gezouten visch. Diss. Utrecht.
71. P e t t e r s o n, A., (1900). Archiv. f. Hyg. Bd. 37, p. 171.
72. P e u c h, F., (1887). C. R. p. 105.
73. R u b e n t s c h i k, L., (1926). Bakt. Zentralbl. II 67, p. 167.
74. —— (1926). Bakt. Zentralbl. II 68, p. 161.
75. —— (1928). Bakt. Zentralbl. II 73, p. 483.
76. —— (1929). Bakt. Zentralbl. II 76, p. 305.
77. —— (1929). Bakt. Zentralbl. II 77, p. 1.
78. —— (1933). Bakt. Zentralbl. II 88, p. 182.
79. R u i n e n, J., (1933). Rec. des trav. bot. neérl. 30, p. 725.
80. S a s l a w s k y, A. S., (1927). Bakt. Zentralbl. II 72, p. 236.
81. —— (1928). Bakt. Zentralbl. II 73, p. 18.
82. S a s l a w s k y, A. S. und C h a i t, S., (1929). Bakt. Zentralbl. II 77,
      p. 18.
83. S a s l a w s k y, A. S., und H a r z s t e i n, N., (1930). Bakt. Zentralbl.
      II 80, p. 165.
84. S a w j a l o w, W., (1913). Bakt. Zentralbl. II 39, p. 440.
85. S c h o o r l, N. en R e g e n b o g e n, A., (1917). Chem. Weekbl., p. 221.
86. S i l b e r s c h m i d t, (1896). Korrespondenzblatt f. schweizer Aerzte.
      (cited after Karaffa Korbutt (45)).
87. S k a l i n s k a, M., (1925). Comptes Rendus Soc. de Biol. 93. p. 780.

88. Söhngen, N. L., (1906). Het ontstaan en verdwijnen van waterstof en methaan onder den invloed van het organische leven. Diss. Delft.
89. Sperlich, A., (1912). Bakt. Zentralbl. II 34, p. 406.
90. Stadler, E., (1899). Archiv f. Hyg. 35, p. 40.
91. Stelling—Dekker, N. M., (1931). Die Hefesammlung des „Centraalbureau voor Schimmelcultures", I. Teil. „Die sporogenen Hefen." Verh. Kon. Akad. van Wetensch. A'dam. Afd. Natuurk. 2 Sectie. 28. No. 1.
92. Tanner, F. W. and Evans, F. L., (1933). Bakt. Zentralbl. II 88, p. 44.
93. Tattevin, L., (1927). Le sel et les microbes. Diss. Nancy.
94. Teodoresco, E. C., (1905). Beihefte Bot. Centralbl. 18, p. 215.
95. Trautwein, K., (1921). Bakt. Zentralbl. II 53, p. 513.
96. Waksman, S. A., (1934). Scientific Monthly 38, p. 35.
97. Waksman, S. A., Hotchkiss, M. and Carey, C. L., (1933). The Biological Bulletin 65, p. 137.
98. Walter, H., (1931). Die Hydratur der Pflanze. Jena. Fischer.
99. Wehmer, C., (1897). Bakt. Zentralbl. II 3, p. 209.
100. —— (1929). Die Pflanzenstoffe. 2e Aufl. Jena. Fischer.
101. Zwick und Weichel, (1910). Arb. aus dem Kaiserl. Gesundh. Amte. 33, p. 250.