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**Microbiology.** — *On some physiological artefacts.* By A. J. KLUYVER and J. K. BAARS.

(Communicated at the meeting of April 2, 1932.)

§ 1. *Introduction.*

The existence and the wide distribution of thermophilic microbes, i.e. of micro-organisms which only proliferate at temperatures of 30° C. and higher, offer several problems to the physiologist. Mention may be made in this respect of the problem of active life at temperatures of 60—75° C.,

temperatures at which all known proteins are coagulated or denatured. No less astonishing is the fact that often by lowering the temperature of cultivation already at 30° C. multiplication is altogether prevented, which means that at this relatively high temperature a process occurs which can be compared to the "rigor frigoris" ("Kältestarre") of the mesophilic organisms. Finally there is the problem of the very general occurrence of thermophilic microbes in nature.

It is regarding this latter question that we will make here some remarks based chiefly on experiences gathered in a study of the bacteriology and biochemistry of natural sulphate reduction [1]).

It has been known since long that the distribution of thermophilic microbes is not at all restricted — as might be expected — to those spots on earth where high temperatures prevail. On the contrary already for many years experimental proof was furnished that practically all samples of soil and water examined contain germs of thermophilic microbes.

Several authors have drawn attention to this fact and have attempted to offer an explanation for this remarkable phenomenon.

So LYDIA RABINOWITSCH [2]) suggested that the thermophilic bacteria would multiply in the colon of warm-blooded animals and would only reach the actual finding-places together with the excrements of the animals.

This view, however, was severely critisized by LIESKE [3]), who pointed out that practically everywhere in soil species are encountered which do not show any growth at temperatures below 40° C. Moreover for some thermophilic *Actinomyces*-strains LIESKE brought the experimental proof that they did not multiply at all on passing the colon of rabbits, notwithstanding the fact that the mentioned micro-organisms could always be isolated from the excrements of the said animals.

Another explanation was forwarded by MIEHE [4]) who made a special study of the factors underlying the spontaneous heating of various vegetable materials. MIEHE has brought experimental proof that thermophilic micro-organisms are indispensable in the establishing of high temperatures during vegetable decay. So one cannot wonder that MIEHE, and partly also NOACK [5]), is inclined to explain the general occurrence of thermophilic organisms in nature by accepting a secundary distribution from these primary centers of multiplication.

Already LIESKE points out that this explanation is not very satisfactory either, with a view to the wide distribution in regions for which conditions favorable for the production of high temperatures by vegetable decay are very unlikely to occur.

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[1]) For details regarding this study the reader is referred to: J. K. BAARS, Over sulfaatreductie door bacteriën. Diss. Delft 1930.

[2]) L. RABINOWITSCH. Zeitschr. f. Hyg. **20**, 154, 1895.

[3]) R. LIESKE, Morphologie und Biologie der Strahlenpilze, Leipzig, 1921.

[4]) H. MIEHE, Die Selbsterhitzung des Heues. Jena, 1907; 2te Aufl. Berlin, 1930.

[5]) K. NOACK, Jahrb. f. wissensch. Botanik **51**, 593, 1912.

NOACK (l.c.) attributes great significance to the heating of the soil by solar radiation, especially in summer, as a factor in the multiplication of thermophilic micro-organisms. LIESKE is prepared to accept this explanation partly, but emphasizes that it cannot give a complete solution of the problem, since thermophilic germs are found in equal frequency in soils which are exposed to solar radiation as in soils for which a heating to the minimum temperature for the growth of thermophiles is certainly excluded (soils in thick forests, in shadowed rock clefts etc.).

LIESKE gives finally as his opinion that the greater part of the isolated thermophilic micro-organisms are nothing but forms of ordinary mesophilic microbes, which have underlied sudden changes in properties in consequence of the conditions prevailing during isolation.

However, LIESKE was unable to support this view experimentally: all pure cultures of *Actinomyces* species tested were unable to adapt themselves to pronounced changes in the temperature of cultivation.

So we may conclude that the problem of the wide distribution of thermophilic microbes in nature has remained unsolved until now. It seems to us that the observations reported below may throw some light on this question.

§ 2. *Observations on thermophilic sulphate reducing bacteria.*

It was BEIJERINCK [1]) who was the first to make a thorough investigation of the microbiological process of sulphate reduction and who gave a good description of the causal organism. This proved to be a strictly anaerobic bacterium the shape of which varied between that of a curved rod and that of a typical spirillum. In accordance with these findings the name of *Spirillum desulfuricans* — which was changed later into *Vibrio desulfuricans* — was proposed. His collaborator VAN DELDEN [2]) soon extended our knowledge of microbial sulphate reduction and succeeded in bringing *V. desulfuricans* into pure culture. Moreover VAN DELDEN isolated a closely related species *V. aestuarii* from mud of the North Sea coast and showed that this organism differed from *V. desulfuricans* by its ability to reduce sulphate in BEIJERINCK's medium only after the addition of 3 % sodium chloride.

Many years later ELION [3]) isolated a third species which was characterized by its ability to effect sulphate reduction at a temperature of 50—60° C. To this species, which was fully inactive at the optimal temperature of *V. desulfuricans* (30° C.) ELION gave the name of *V. thermodesulfuricans*.

It was a closer study of this latter species which led to some unexpected observations.

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[1]) M. W. BEIJERINCK, Centralbl. f. Bakt. 2te Abt. **1**, 49 und 104, 1895.

[2]) A. VAN DELDEN, Centralb. f. Bakt. 2te Abt. **11**, 81 und 113, 1904.

[3]) L. ELION, Centralbl. f. Bakt. 2te Abt. **63**, 58, 1924.

First of all it must be emphasized that *V. thermodesulfuricans* occupies a rather special place amongst the thermophilic bacteria described until now, owing to the fact that the great majority of these belong to the aerobic sporeforming bacilli. On the contrary *V. thermodesulfuricans* is a strictly anaerobic sporeless microbe of which no resting stages whatever are known. In accordance with this statement cultures of this bacterium are killed by the smallest traces of free oxygen as soon as the temperature is lowered to such a degree that active metabolism is prevented.

It is obvious that a bacterium with such properties is utterly unfit for a secundary distribution in nature and so one would expect that it could only be found in very restricted areas, where at some time temperatures higher than 30° C. would have reigned.

As a matter of fact experiences were quite incompatible with this deduction. ELION isolated the first strain of the new species from mud out of a ditch alongside the laboratory. Apart from the fact that the isolation was performed during the winter at a moment when the water in the ditch was covered with ice, it can be guaranteed that the temperature in the ditch and in the mud will reach under no conditions 30° C. Our first impression was therefore that we had to ascribe the said occurrence of *V. thermodesulfuricans* to an incidental cooperation of unknown factors.

However, it proved to be possible to find *V. thermodesulfuricans* quite regularly in all samples of mud tested and in many samples of soil as well.

The only way out of this impasse is to conclude that in nature no organism with the properties of *V. thermodesulfuricans*, i.o.w. an obligate thermophilic bacterium with sulphate reducing metabolism, exists. In this line of thought *V. thermodesulfuricans* should be nothing but an adaptate of the corresponding mesophilic organism to a higher temperature region. This means that we must consider *V. thermodesulfuricans* to be a physiological artefact produced under the conditions of the primary enrichment culture.

At first sight there seems to be a serious objection to accept this point of view. For if the pure culture of *V. desulfuricans* is tested on its ability to grow at a temperature of 55° C., no adaptation occurs and the result is quite negative.

However we were able to show that also in the pure culture remnants of the supposed adaptive power were still present on the understanding that adaptations to temperatures which are only slightly higher were still possible. Moreover it proved to be quite feasible to come to additional effects in this respect, i.e. to convert gradually a pure culture of *V. desulfuricans* into a pure culture of *V. thermodesulfuricans*.

The whole procedure is illustrated in Table I which will not want any further explanation, besides the statement that the figures given in the table represent mgr. $H_2S$ produced in the course of the experiment, calculated pro Litre of the medium.

But also the reverse process could be realised, as is demonstrated in

TABLE I.

Conversion of *V. desulfuricans* into *V. thermodesulfuricans*.

Initial culture a, cultivated at 30° C.

| Temperature of cultivation | 30° | 35° | 40° | 45° | 50° | 55° |
|---|---|---|---|---|---|---|
| Inoculated from *a* | $b_1$ : 510 | $b_2$ : 510 | $b_3$ : 420 | $b_4$ : 40 | $b_5$ : 20 | $b_6$ : 17 |
| „ „ $b_3$ | — | — | — | $c_1$ : 301 | $c_2$ : 36 | $c_3$ : 18 |
| „ „ $c_1$ | — | — | — | — | $d_1$ : 326 | $d_2$ : 112 |
| „ „ $d_2$ | — | — | — | — | — | $e_1$ : 415 |

Table II. In this Table the production of hydrogen sulphide (again calculated in mgr. pro Litre of culture medium) is given for successive

TABLE II.

Conversion of *V. thermodesulfuricans* into *V. desulfuricans*.

| Designation of culture | Temperature of cultivation | Quantity of $H_2S$ produced |
|---|---|---|
| Initial culture *a* | 55° C. | 480 |
| *b*, inoculated from *a* | 50° „ | 453 |
| *c*, „ „ *b* | 45° „ | 425 |
| *d*, „ „ *c* | 40° „ | 384 |
| *d'*, „ „ *d* | 40° „ | 481 |
| *e*, „ „ *d'* | 35° „ | 143 |
| *e'* „ „ *e* | 35° „ | 322 |
| *e''*, „ „ *e'* | 35° „ | 350 |
| *f*, „ „ *e''* | 30° „ | 180 |
| *f'*, „ „ *f* | 30° „ | 481 |
| *f''* „ „ *f'* | 30° „ | 510 |

cultures at gradually lowered temperatures. It appears from this table that at various temperatures it was necessary to have a short series of reinoculations before optimal metabolic activity was attained.

Taking into consideration the ubiquity of *V. thermodesulfuricans* together with the practical impossibility of a secundary distribution of this organism in nature, these results seem to warrant the view that this spécies is nothing but an artefact produced under the conditions of the accumul-

ation experiment out of a pluripotent sulphate reducing bacterium occurring in nature.

It seems probable that the same holds good for most thermophilic organisms with ubiquitous occurrence in nature.

§ 3. *Observations on the adaptation of V. desulfuricans to media containing various concentrations of sodium chloride.*

Whilst the general applicability of the forwarded theory of thermophily remains to be proven by further experiments, we can refer here to some experimental evidence which tends to support the correctness of this view.

It could be demonstrated namely that the pronounced adaptive power of the naturally occurring sulphate reducing bacterium as contrasted with the more limited power of the pure culture was not restricted to adaptations to abnormal temperature regions. As a matter of fact we met with an exactly corresponding situation in a comparative study of *V. desulfuricans* and *V. aestuarii* van Delden.

As already mentioned *V. aestuarii* was isolated by VAN DELDEN from mud soaked with seawater and moreover was shown to be inactive in the usual nutrient medium to which no sodium chloride was added. Since at the other hand *V. desulfuricans* was fully inactive in this medium in case 3 % of sodium chloride was added, these facts apparently fully justify the distinction in species as carried through by VAN DELDEN.

We could easily confirm the correctness of the above statements of VAN DELDEN.

A pure culture of *V. desulfuricans* tested on its ability to grow in media with increasing concentrations of sodium chloride gave the following results:

| Concentration NaCl in medium : | 0 | ¾ | 1½ | 2¼ | 3 % |
|---|---|---|---|---|---|
| mgr. $H_2S$ produced pro Litre : | 397 | 325 | 200 | 87 | 40 |

Since the inoculation of the media involved the addition of a quantity of $H_2S$, corresponding to about 30 mgr. pro Litre, we learn from these figures that in the medium with 3 % NaCl practically no development of *V. desulfuricans* took place.

On the other hand a pure culture of a typical *V. aestuarii* gave the following results :

| Concentration NaCl in medium : | 0 | 1 | 2 | 3 % |
|---|---|---|---|---|
| mgr. $H_2S$ produced pro Litre : | 23 | 37 | 82 | 430 |

The pronounced divergency in behaviour of both species is sufficiently demonstrated by these figures.

Nevertheless we soon made the remarkable observation that it was quite feasible to isolate a strain with all the properties of *V. aestuarii* by starting from samples of mud which were practically devoid of sodium chloride.

It is obvious that this situation shows a great similarity to that which was reported in § 2 with regard to *V. thermodesulfuricans*. So we were led to the conclusion that the strains of *V. aestuarii* isolated from the salt free

mud again were artefacts produced under the conditions of the enrichment culture out of a pluripotent sulphate reducing bacterium.

But then it would be probable that also the pure cultures of both species would have preserved part of their adaptive power and therefore could be gradually converted into each other.

The experiments proved to be in agreement with this presumption. For the sake of brevity we will refrain from reproducing here the results, which can be found in the thesis of BAARS.

It seems of more importance to mention here another phenomenon which presented itself when various pure strains of *V. desulfuricans* were tested on their ability to grow in salt containing media. It was found that this ability was markedly dependent on the time during which the strain had been previously cultivated in the laboratory, notwithstanding all pure cultures were regularly reinoculated into fresh media about every four of five weeks.

This is demonstrated in Table III, in which the figures represent again mgr. of $H_2S$ produced pro Litre of culture medium.

TABLE III.

Relation between the age of culture of various strains of *V. aesulfuricans* and their ability to grow in media with increasing salt concentrations.

| Time passed since isolation | Percentage of NaCl in medium | | | | |
|---|---|---|---|---|---|
| | 0 | 3/4 | 1 1/2 | 2 1/4 | 3 |
| Strain *p* (258 days) | 397 | 325 | 200 | 87 | 40 |
| „ *q* ( 96 „ ) | 382 | 397 | 242 | 72 | 42 |
| „ *r* ( 45 „ ) | 351 | 390 | 290 | 212 | 90 |
| „ *s* ( 45 „ | 340 | 481 | 496 | 375 | 160 |

It is obvious from these observations that the longer the time the strain has been kept in pure culture the smaller is its adaptive power.

Quite the same phenomenon results from corresponding observations with *V. aestuarii*, as appears from Table IV.

Here too we observe that the longer the time which has elapsed since the isolation the smaller is the ability of the strain to adapt itself to unusual conditions. In other words the pure cultures of the bacteria under consideration are differentiated from the species as it occurs in nature by the loss of power to adapt themselves to sudden changes in environment. The longer the cultivation is continued the more this loss is accentuated so that by prolonged cultivation under constant conditions organisms result which might be called physiological artefacts.

TABLE IV.

Relation between the age of culture of various strains of *V. aestuarii* and their ability to grow in media with decreasing salt concentrations.

| Time passed since isolation | Percentage of NaCl in medium | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| strain p (570 days) | 23 | 37 | 82 | 430 |
| „ q (360 „ ) | 36 | 46 | 187 | 422 |
| „ r ( 20 „ ) | 52 | 130 | 340 | 441 |

§ 4. *Other presumable instances of physiological artefacts.*

Anyone who has given due attention to the remarkable results obtained by JAN SMIT in his study of the distribution of *Sarcina ventriculi* in nature [1]) will be struck by the great similarity between these dates and those presented in the foregoing paragraphs. Here again we meet with a bacterium which can be isolated according to the method given by BEIJERINCK from nearly every sample of soil. Still pure cultures of this bacterium mixed with sterilized samples of the same soils die quite rapidly, as may be inferred from the impossibility to reisolate after a very short time the organism out of the inoculated, previously sterilized, soil. Here again we are forced to conclude that an organism with the set of properties characteristic for *S. ventriculi* does not exist in nature. On the contrary it seems quite probable that this species is produced again out of a more or less common soil microbe under the special conditions of the enrichment culture: complete anaerobiosis and an extreme degree of acidity in the medium.

This view is not invalidated by the negative results of SMIT's experiments in which he tested several pure cultures of bacteria out of a "fertile" soil sample, on their ability to grow in the extremely acid, anaerobic enrichment medium. Again we must realize that the simple act of bringing the tested bacteria into pure culture will have damaged their adaptive power to a marked degree.

Finally we wish to draw the attention to some observations made by miss L. HOMANS in our laboratory. In studying the fermentability of "glutose" [2]) by bacteria miss HOMANS observed that from all soil samples tested a bacterium could be isolated which fermented glutose quite readily. This bacterium which proved to belong to the natural group of butyric acid bacteria, has received the provisional name of *Clostridium glutosicum* [3]).

[1]) JAN SMIT. Die Gärungssarzinen, Jena, 1930.

[2]) The rather vaguely characterized product resulting from the conversion of glucose and fructose in weakly alkaline media. Cf. W. COLTOF, Biochem. Zeitschr. **243**, 191, 1931.

[3]) An extensive report of the investigation of Miss HOMANS will appear later.

Now again it was most remarkable that from about 20 strains of the genus *Clostridium*, which had been isolated for the greater part from the same garden soil, none proved to be capable of fermenting glutose, even when cultivated in a suitable medium which contained a mixture of glucose and glutose. Since it seems excluded that ordinary garden soil will ever contain glutose, the occurrence of a special glutose fermenting bacterium in soil seems most improbable. Here again it appears much more likely that *Cl. glutosicum* is produced under the conditions of the enrichment culture from an ordinary butyric acid bacterium occurring in soil, which bacterium however looses its power of adaptation after being brought into pure culture in a glutose free medium.

In conclusion we wish to state explicitly that the views expounded above bear only a superficial resemblance to the theory forwarded by BEIJERINCK in his paper on the formation of physiological species. Whilst BEIJERINCK [1]) treated of irreversible changes of *pure cultures* of bacteria in answer to sudden changes in nutrient media, it seems to us that the adaptive power to sudden changes is restricted to the organisms as occurring in nature. The cultivation under ordinary laboratory conditions leads to an one-sidedness in properties which is most harmful to the adaptive power of the bacteria and which makes undoubtedly many of our pure cultures worthy of the name of physiological artefacts.

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*Delft*, March 31, 1932.

[1]) M. W. BEIJERINCK, These Proceedings, **16**, 1211, 1913.

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**Chemistry.** — *Der Einfluss der Korngrösse von Stoffen auf deren Schmelztemperatur.* Von N. SCHOORL. (Communicated by Prof. ERNST COHEN.)

(Communicated at the meeting of April 2, 1932.)

Vor längerer Zeit (1909—1910) wurde seitens PAWLOW [1]) die Aufmerksamkeit auf die Erscheinung gelenkt, dass von verschiedenen Stoffen kleine Teilchen bei niedrigerer Temperatur schmelzen als grössere Kristalle. Er beschreibt u.a. folgenden einfachen Versuch: wird die Versuchssubtanz auf die Innenwand eines Glasrohres zerstäubt, so zeigen die feinsten Teilchen an der Glaswand einen tieferen Schmelzpunkt als

[1]) PAWLOW, Z. physik Chem. **65**, 1 (1909); **65**, 545 (1909); **68**, 316 (1910); **74**, 562 (1910).