in my opinion that the O_2 consumption of the small animals is considerably larger than that of the bigger ones of the same kind. This difference is, I believe, to be attributed to the influence of the more rapid motions of the smaller animals and the more intense metabolism which a growing body possesses. TIGERSTEDT [1]), therefore, rightly says that in general, where assimilatory processes take place, they are accompanied by active dissimilatory ones. Here also belongs without any doubt the observation of WARBURG [2]) that the O_2 consumption after fertilisation of the eggs of the sea-hedgehog increased by 6 and 7 times the amount. The action of light, osmotic pressure and liquid-pressure, which might cause a certain influence of the area on the respiration, has not been settled. The influence of light I have not been able to separate from variation of motility.

Naples, Febr. 8, 1909.

Microbiology. — "*The decomposition of uric acid by bacteria.*" By Mr. F. LIEBERT. (Communicated by Prof. M. W. BEIJERINCK).

(Communicated in the meeting of April 23, 1909).

1. *Historical.*

The first investigators who studied the decomposition of uric acid by the action of bacteria were FAUSTO and LEONE SESTINI [3]).

They observed that uric acid in water when exposed to the air did not change, but that, as soon as the mixture was inoculated with a drop of rotting urine, the uric acid totally disappeared in some days.

Only when the experiment was interrupted before all the uric acid had been converted, they could detect urea.

Their conclusion was that the course of the process might be represented thus:

$$C_5 H_4 N_4 O_3 + 3\,O + 8\,H_2 O = 4\,NH_4 HCO_3 + CO_2$$

whereas as intermediate products alloxan and urea should occur, which latter substance they could really detect, but the former not. They have not worked at all with pure cultures.

The result obtained by E. GÉRARD [4]) was that two processes took place simultaneously.

[1]) Handbuch der Phys. Nagel. Bd. I 2e Halfte 2e Heft.

[2]) WARBURG, Hoppe Seyler Zeitschr. f. Phys. Chem. Bd. 57 blz. 1.

[3]) Landwirtsch Versuchsst. 1890, Vol. XXXVIII, p. 157.

[4]) Comptes rendus T. 122, p. 187 et T 123, p 185.

First, the bacteria should decompose the uric acid into urea and tartronic acid; secondly, the urea should be hydrolised to ammonium carbonate by the urea bacteria. According to my own experiments I cannot accept the first part of this supposition, as the formation of tartronic acid is but an hypothesis, GÉRARD not having been able to find this substance. But the presence of urea amongst the products he could point out.

The first who studied the action of pure cultures on uric acid was C. ULPIANI [3]). He obtained a micrococcus which attacked uric acid easily, and found that the maximum and minimum temperatures for this organism were respectively 40° and 29°, and he presumed that its optimum temperature should be 39°. This latter statement is evidently wrong and his supposition, too, that the isolated bacterium cannot assimilate glucose, clearly reposes on an erroneous experiment.

At his request CINGOLANI ascertained that the equation of the process is:

$$C_5 H_4 N_4 O_3 + 2 H_2O + 3 O = 2 NH_2 CO NH_2 + 3 CO_2.$$

The decomposition of uric acid by microbes has been studied by me after four methods. In the first place as an aërobic process; secondly as an anaërobic fermentation; thirdly as a denitrification; and finally as a sulphate reduction. As to the last I can be short: under none of the conditions chosen I could obtain with uric acid a sulphate reduction by bacteria.

As to the other cases, seven species of bacteria called for particular attention; the six first are aërobic bacteria, the seventh is a real anaërobic.

They are: 1. Bacillus fluorescens liquefaciens; 2. Bacillus fluorescens non liquefaciens; 3. Bacterium calco-aceticum (which is probably a new species but has already been obtained in different other ways in this Laboratory, where it is kept in culture by that name); 4. Bacillus pyocyaneus; 5. an aroma-producing species, which I wish to call *Bacterium odoratum*; 6. a urea-splitting bacterium *Urobacillus Musculi* and 7. a bacillus which causes an anaërobic fermentation in uric acid, *Bacillus acidi urici*.

The three last mentioned bacteria were formerly unknown, or at least not yet produced in pure culture.

2. *The chemical transformation of uric acid by the bacteria of the aërobic flora.*

It was observed that in all the examined species under aërobic action the uric acid is oxidised by the bacteria. As it was probable

[3]) Rendiconti d. Acad. dei Lincei 1903, T. 12, p. 236.

that the species here active would produce compounds which are also obtained chemically by alcaline oxidation, I have inquired whether by their action allantoin originates from uric acid, and my result was that B. pyocyaneus, B. fluorescens liquefaciens, B. fluorescens non liquefaciens and B. calco-aceticum do indeed oxidise uric acid after the equation

$$C_5 H_4 N_4 O_3 + O + H_2O = CO_2 + \underset{\text{Allantoin}}{C_4 H_6 N_4 O_3}$$

whereas by the same bacteria the allantoin is further oxidised and hydrolysed thus:

$$C_4 H_6 N_4 O_3 + H_2O + O_2 = 2\,NH_2\,CO\,NH_2 + 2\,CO_2,$$

whereby consequently urea is formed.

In nature also allantoin is met with. It was found in the urine of sucking calves, in the allantois liquid of cattle, and in the juice of Beta vulgaris. Man also secretes it after the use of food containing tannic substances.

The urea as well as the allantoin I could point out chemically as follows: Into a large 1 L. ERLENMEYER flask, 1 G. of uric acid was filled, 150 mG. of K_2HPO_4 and 300 cM³. tap-water. After sterilisation this liquid was inoculated with the bacterium to be tested; then cultivated at 30°. After the course of a week the greater part of the uric acid had disappeared.

In order to isolate the allantoin, bariumhydroxid was added to precipitate the phosphates and the remaining uric acid, after which the superfluous baryta was removed by sulphuric acid. Now the liquid was evaporated to crystallisation and alcohol was added. This produced a white deposit which after re-crystallisation from water showed the characteristic crystals of allantoin. The thus obtained substance was further identified by the following reactions:

1. $AgNO_3$ and a trace of ammonia give a white deposit which is soluble in nitric acid and in an abundance of ammonia
2. Mercurinitrate produces a white precipitate.
3. FEHLING's cupric test-liquid is reduced after long boiling.
4. The murexid reaction has a negative result.
5. At the determination of silver in the silver-salt was found 40,61 % (calculated 40,75 %).

The urea was identified as follows: The alcoholic filtrate was vapoured to dryness and re-crystallised. Nitric acid gives the characteristic crystals of ureanitrate[1]); the substance also displays the beautiful

[1]) H. BEHRENS' Anleitung zur mikrochemischen Analyse. Heft 4. p. 18.

reaction of cupri-ammonium cyanurate[1]); with nitrous acid nitrogen is produced.

It was also biologically demonstrated that the thus formed substance was urea. To this end the crystals were dissolved in broth mixed with a quantity of bacteria, then kept for some hours at 50°. At this temperature there is no growth but the urea is transformed into ammonium carbonate, which in this experiment resulted in great abundance.

Use can also be made of the iris phenomenon [2])[3]) by adding some of the substance to yeastwater gelatin and making a plate upon which a strong urea bacterium is put. The NEWTON-rings, then occurring, prove that the added material contains urea.

As the media with uric acid in decomposition, are rendered strongly alkaline by the action of the urobacteria on the urea, the successive production of two distinct floras of bacteria is observed, one characteristic of the feebly acid or neutral medium, and one proper to the alkaline condition of the medium.

The allantoin produced at the oxidation of uric acid is itself an intermediate product, it being subject to further decomposition, which is in accordance with the fact that it is much more inconstant than uric acid.

This conversion is analogous to that of the last named substance, and so here also is a flora of a neutral and one of an alkaline stage; the neutral flora produces an oxidation and an hydrolysis.

If a hydrolytic agent acts chemically on allantoin, then, after the degree of intensity of the hydrolysis, either allanturic acid and urea or glyoxilic acid results, whereas two molecules of the latter are converted into the more constant glycolic acid and oxalic acid.

Of these decomposition products, oxalic acid is also easily found in the biological hydrolysis. It could be proved that all the four bacteria which form part of the "neutral flora" of uric acid, namely B. fluorescens liquefaciens, B. fluorescens non-liquefaciens, B. calco-aceticum and B. pyocyaneus, produce this substance. This acid forms a deposit of calcium oxalate at the scratches of the vessel-walls. That the substance was indeed calcium oxalate was confirmed, besides by the characteristic forms of the crystals, by its solubility in hydrochloric acid, its insolubility in acetic acid and its power of decolorising acid permanganate. Moreover, after incineration a mixture of calciumoxid and calcium carbonate remained.

[1]) See footnote 1 on the preceeding page.

[2]) BEIJERINCK, Centralbl. f. Bakter. Bd. VII 1901.

[3]) SÖHNGEN, Kon. Akad. v. Wetensch. 12 Nov 1908.

The chemical oxidation of allantoin may give rise to several products, and finally calcium carbonate is formed.

In the biological oxidation of allantoin calcium oxalate also results. As it is probable that glycolic acid is at first produced it may be mentioned that in cultures, containing nothing but calcium glycolate as carbon food, which is not apt chemically to be converted into calcium oxalate, yet oxalate is produced by the bacteria. The process of oxidation does not however stop here, but the oxalate is further oxidised into carbonate; this takes place abundantly when to the liquid 0.05% calciumchlorid is added. The same occurs not only in accumulations with allantoin or calcium glycolate, but also with calcium oxalate as only carbon food, which is oxidised by the said bacteria, and chiefly by B. calco-aceticum; consequently this species is easily accumulated from garden soil with the said substance.

In order to control the rapidity with which the uric acid disappears in the pure cultures, the several isolated bacteria were put in tapwater to which a known quantity of uric acid and 0.1% bikalium phosphate had been added. Use was made of MERCK's acidum uricum pur. which was snow-white and contained no ponderable quantity of ashes. The acid was before weighing dried at 110° to constant weight. The rapidity of the decomposition may be determined in two ways; by controlling how much uric acid is converted in unit of time, and by determining the time in which the uric acid has disappeared. It seems that the former method is to be preferred here, as by the latter the retarding influence of the products of decomposition will be strongly felt and the results become less clear.

Nor will in the first mentioned case the determination of the decomposed uric acid offer any difficulty. After two days' growth at 30° the cultures were slightly acidified with strong hydrochloric acid and filtered through a GOOCH crucible. For washing was used a saturated solution of uric acid in water. In order to form a judgment of the error made in this way, 501 mG. of uric acid was added to a culture containing an abundance of B. fluorescens non-liquefaciens, analysing at the same time 499 mG. were found back, so the error is less than $^1/_2$ %.

If uric acid was used as sole carbon and nitrogen food, B. fluorescens liquefaciens assimilated 82 mG. in two days. For B. fluorescens non-liquefaciens, and B. calco-aceticum the quantities were respectively 85 and 96 mG.; the volume of the medium was 150 cM.^{3} and $^1/_{20}$ % K_2HPO_4 had been added.

Furthermore it was inquired whether the uric acid would be still

decomposed if at the same time other nutriment was present. To this end either $^1/_2$ % mannite or $^1/_2$ % peptone was added to the above culture liquid, which contained excess of uric acid. After two days mannite was still present.

	Uric acid assimilated in two days	
	in the solution with peptone	in the solution with mannite
B. pyocyaneus	151 mG.	91 mG.
B. fluorescens liquefaciens	82 "	48 "
B. fluorescens non-liquefaciens	41 "	35 "
B. calco-aceticum	79 "	60 "

These data prove that even if at the same time an easily assimilable substance is present, the uric acid is still attacked and is a good nutriment for the said species.

3. *Bacteria active in the aërobic decomposition of uric acid.*

As said above these bacteria belong to two consecutive floras; the first is found as long as the medium reacts neutrally or is feebly acid, the second, characteristic of the alkaline phase, may be called "urea-flora", as the urea, produced by the first flora, is by it converted into ammonium carbonate.

a. Flora of the acid or neutral phase.

Like my predecessors I used uric acid at the same time as source of carbon and of nitrogen. Two different liquid culture media were prepared:

1. The liquid of GÉRARD (l.c.): 1 G. of uric acid, 12 G. K^2HPO^4, 1000 cM.^{3} tapwater.

2. 1 G. of uric acid, 0.1 G. K^2HPO^4, 1000 cM.^{3} tapwater.

Between these two culture liquids there exists, besides in the rate of phosphate, a remarkable difference: In GÉRARD's liquid all the uric acid is dissolved and the reaction is neutral. In the other it is acid to the end, although feebly, by the excess of uric acid, even if by the bacteria ammonia is produced. Both liquids were filled into small ERLENMEYER flasks so that the layer was $\pm$ 1 cM. high. Garden soil was used for the inoculation. At 30° in both tubes a rich culture was observed after 2×24 hours, and the transfers also went very well.

The reaction of GÉRARD's liquid was then still neutral, whereas the other under these conditions reacted still feebly acid.

The flora which first appeared proved in both cases to be the same and consisted of B. fluorescens liquefaciens, B. fluorescens non-liquefaciens and B. calco-aceticum. After repeated transports B. fluor. liq. vanished completely, the optimum temperature of this species being nearer to 25°, whereas that of the other is about 30°. In the neutral liquid of GÉRARD this microbe could however maintain itself longer than in the other.

The isolation of the bacteria was done on broth-gelatine.

On agar-plates, which besides a little phosphate contained some uric acid, by which the whole plate was quite opaque, the uric acid disappeared around the bacterial colonies under the influence of the formed ammonium carbonate. As this compound increased a ring of crystals occurred in which calcium urate could be pointed out. If the growth took place not at 30° but at 37°, it was found that (from garden soil as well as from canal water) the same bacteria as from the cultures at 30° were obtained, with the exception of those cases when B. pyocyaneus occurred in the material, as this species dispels the others more or less completely.

Uric acid thus proved to be a very good food for this latter microbe too, although the power of secreting pyocyanin was lost and could only be restored by repeated transplantations in peptone solutions.

By many experiments it was proved that during the winter of 1907—1908, as well from canal water as from garden soil at Delft, by this method a culture was constantly obtained in which B. pyocyaneus positively predominated. But in 1909 no more a growth was to be had containing this species, and when material was used from a garden at the Helder, the results were also negative.

With a view to get more certainty about this point, I added to a rough accumulation with uric acid at 37° a trace of a culture of B. pyocyaneus. How small soever the number of introduced germs might be, they were always able to maintain themselves, even in the transports. It seems thus proved that the occurrence of B. pyocyaneus in the soil is but accidental.

b. Flora of the alkaline phase in the decomposition of the uric acid.

As has been described in the preceding pages B. fluorescens produces urea from uric acid. If a crude accumulation is allowed to stand it may thus be expected that the urea bacteria which, like B. fluorescens, were present in the original inoculation material, will find in the liquid containing urea the conditions wanted for their growth. This is indeed the case as is shown by the intense production

of ammonium carbonate, for a culture obtained in this way had a titre of 0,18 N. The urea flora did not however directly follow that of B. fluorescens and allied species, for when a rough culture is only some days old it evolves a pleasant odour of a compound ester, and cultures show that the original species are replaced by an aroma producing microbe. Milk used as medium for this bacterium favours very much the production of the aroma which after some days reminds of the flavour of young cheese. This bacterium, B. odoratum, does not however produce urease, although it is capable of splitting urea by catabolism.

To obtain the urea flora which succeeds that of B. odoratum, the culture was streaked on broth gelatin with addition of 1% urea and 0,1 % ammonium carbonate. But no ureabacteria developed, which did take place on yeastwater gelatin with the above additions. A description of the thus isolated single species was not found; it shall be called *Urobacillus Musculi*. The temperature of 30° is the most favourable for its culture as, especially on broth-agar urea and ammonium corbonate, then an abundance of urease-producing colonies occur. Sporulation has not been observed and gelatin is not liquefied. The maximum quantity of urea converted into a 1% peptone solution amounts to 3,6 % [1]).

Addition of peptone is not quite necessary for the splitting of urea, but it highly favours it [2]). Calcium malate, calcium lactate and glucose, together with urea, gave only rise to a very slight decomposition.

3. *Denitrification with uric acid as carbon food.*

If a stoppered bottle, quite filled with water and uric acid, with addition of 1 % kaliumnitrate and a little phosphate, is inoculated with soil, then after some days' cultivation at 30° under these anaërobic conditions, gas evolves, which proves to consist of a mixture of carbonic acid and nitrogen.

A plate culture on broth gelatin shows that the denitrifying bacterium, B. Stutzeri [3]), is cause of the process, hence this species may be very well accumulated in the said way. It also denitrifies strongly with uric acid in pure culture. In three days, for instance, 250 mG. KNO_3 were completely decomposed in 250 cM^3. of the culture medium, so that sulphuric acid and diphenylamin did no more react.

At denitrification in general the escaping nitrogen originates from the

[1]) Beijerinck, Centr.bl. f. Bacteriol. II, VII Bd. 1901.

[2]) Söhngen, Kon. Akad. v. Wetensch 12 N 1908.

[3]) van Iterson, Centralbl. f. Bakteriologie II, XII Bd. 1902.

nitrate or the nitrite, and not from the concerned organic compound. With uric acid there was reason to suppose that this could be otherwise, as perhaps the amido groups might react in the feebly acid medium with the nitrous acid and thus per molecule of nitrate two molecules of nitrogen should escape. Quantitative tests have however shown that this was neither the case here.

The same denitrification experiment being repeated at 37°, B. pyocyaneus was obtained in the winter of 1907—1908. When this bacterium was not present in the soil, as in 1909, the denitrification remained feeble, 37° being too high for B. Stuzeri, which is then also the originator of the gas.

4. *Fermentation of uric acid by an anaërobic.*

In the same way as in the preceding case, stoppered, 100 cM.^{3} bottles were used for these experiments; they were filled with 1 G. of uric acid and further with tapwater to which 0,1 % kaliumphosphate had been added.

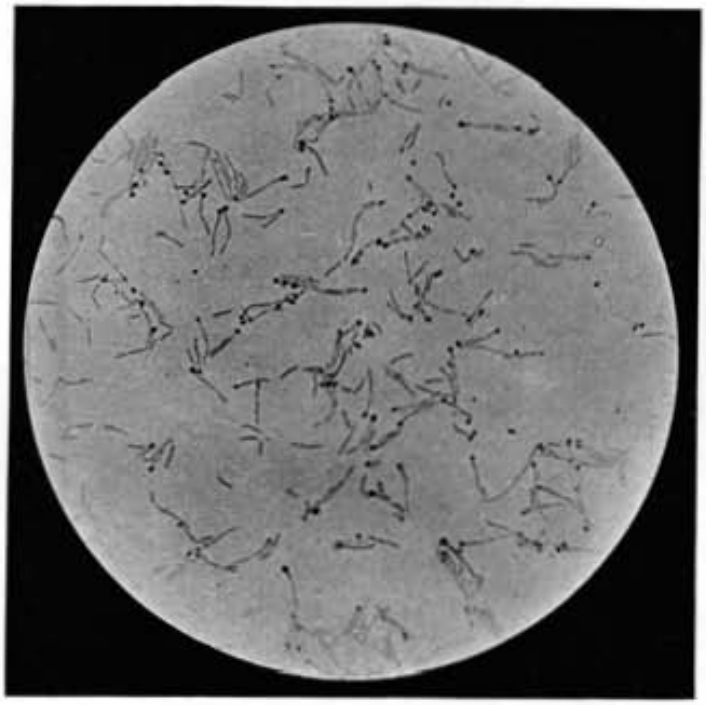
For the inoculation I took garden soil containing carbonates, from which by the free uric acid some carbonic acid evolves. This slight gas production however soon ceases. Canal mud, too, served well as inoculation material. With heath soil adhering to Erica roots, the process was slower and a greater quantity of inoculation material was wanted to bring about a good fermentation.

The most favourable temperature lies at about 35°; at 45° the fermentation is less energetic. Temperatures below 30° are also less fit, as then the fermentation does not always go on regularly.

After a day's cultivation the first traces of gas are already observed, and then it rapidly increases in quantity. But when it has reached a certain height the fermentation strongly relaxes. This relaxing could not be ascribed to want of uric acid, as by the murexidreaction it could yet in abundance be indicated, both in the fluid and in the precipitate.

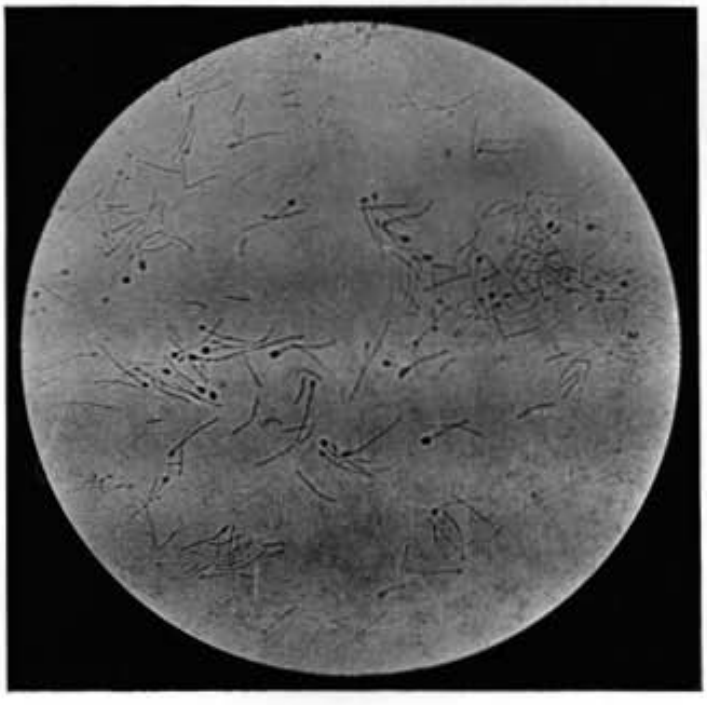
A new supply of uric acid did somewhat accelerate the fermentation, but as was to be expected, gave no positive amelioration. The great concentration of the ammonium compounds from the uric acid was found to be cause of the abatement. This noxious influence was however eliminated by the addition of neutral magnesium phosphate ($MgH\,PO_4 . 7\,H_2O$). This salt, with difficulty soluble in water (0.01 gr. per L.), combines with the ammonium carbonate to ammonium-magnesium phosphate, hence the reaction continues neutral. That the addition of magnesium phosphate really has this result is at once

Fig 1.



Bacillus acidi urici, coloured with carbol-fuchsine (720).

Fig 2.



Bacillus acidi urici, not coloured (720).

evident when comparing the image of a culture in its first phase of fermentation to that of one where the fermentation has ceased. In the former case the irregular uric acid crystals and the boat-shaped ones of the magnesium phosphate are only to be seen; in the latter the characteristic "coffins" of ammonium-magnesium phosphate.

In the deposit at the bottom we observe numerous highly motile rods, whose motility rapidly decreases at access of air; so, there is reason to believe that the excess of magnesium phosphate acts also beneficially on the bacteria, by enabling them easily to reach the places with the most favourable oxygen tension.

If the fermenting fluid is poured on the plate with admission of air, no microbes occur which ferment the uric acid; but B. calco-aceticum does develop.

The fermentation organism proper which was hitherto unknown, and shall be called *Bacillus acidi urici*, is a spore-forming, strongly motile, obligative anaërobic microbe, whose photographic image is given here.

The middle length of the rods is 5 μ; their width is 0.7 μ, and the diameter of the spores is about 2.5 μ. The rods are excessively motile but by cultivation on plates the motility gets quite lost. The spores are round and placed at the ends, but eventually they are oval. The colonies on broth-agar are transparent, round or of more irregular shape, and very variable in size.

As to the products of fermentation the following was established. The escaping gas consists solely of carbonic acid. Quantitative tests proved that per one molecule of uric acid three molecules of carbonic acid are liberated. It was further observed that the urea groups are converted into ammonium carbonare, and that acetic acid is formed.

The quantity of this acid greatly depends on the nature of the fermentation; a crude accumulation produces more acetic acid than a fermentation that has been repeatedly transferred, or has been obtained by pure culture.

As to the chemism of the fermentation the production of carbonic acid corresponds with the formula as given by STRECKER [1]) for the hydrolytic decomposition of uric acid:

$$C_5H_4N_4O_3 + 5\,H_2O = \underset{\text{Glycocoll}}{C_2H_5NO_2} + 3\,CO_2 + 3\,NH_3$$

But glycocoll I have not been able to point out. It is however very well possible that this substance is reduced to ammonium acetate by the further action of the bacteria.

[1]) LIEBIG's Annalen. Bd. 146, pag. 142, 1868. Zeitschr. f. Chemie 1868 pag. 215.

It is also possible that the decomposition takes place after the equation:

$$C_5H_4N_4O_3 + 6\,H_2O = 3\,CO_2 + \underset{\text{Glycolic acid}}{CH_2OHCOOH} + 4\,NH_3$$

However I have as little been able with certainty to detect this acid[1]), but then, here too, the possibility exists that it is reduced to acetic acid through the influence of the bacteria, which point will be a subject for later inquiries.

Summary.

In the preceding it has been proved:

1. That when uric acid is attacked by aërobic microbes this acid is transformed into carbonic acid and ammonia, whereas as intermediate products allantoin, urea and oxalic acid are formed. The bacteria causing this chemism may be divided into two groups: a flora of the feebly acid or neutral phase of the medium, to which belong B. fluorescens liquefaciens, B. fluorescens non liquefaciens and B. calco-aceticum; and a flora which develops in the culture liquid after it has become alkaline, and which consists of *B. odoratum* and *B. Musculi.*

2. That B. pyocyaneus and B. Stutzeri with uric acid as carbon food, in presence of saltpeter, cause denitrification.

3. That under absolutely anaërobic conditions uric acid may be fermented by the spore-forming *Bacillus acidi urici* n. sp., in which process ammonia, acetic acid, and 3 molecules of carbonic acid per molecule of decomposed uric acid are produced.

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EXPLANATION OF THE FIGURES.

Fig 1. *Bacillus acidi urici.* Magn 720. (Zeiss. 2,5 mM. Proj. oc. 2). Colony on broth-agar, coloured with carbol fuchsine.

Fig. 2. *Bacillus acidi urici.* Magn.-720. Colony on broth-agar, taken after life.

[1]) In order to find this acid I made the fermentation take place in a Chamberland-bougie, quite immersed in sterile water, so that the intermediate products might diffuse in it; but it was of no avail.