Microbiology. — "*Pigments as products of oxidation by bacterial action.*" By Prof. M. W. BEIJERINCK.

(Communicated in the meeting of February 25, 1910).

The following experiments make it possible easily to find some notable bacteria more or less common in our surroundings and partly not observed hitherto.

1. *Formation of Protocatechetic acid from Chinic acid.*

As Löw [1]) had shown that 1 proc. solutions of calcium chinate become brown when exposed to the air by the formation of protocatechetic acid, EMMERLING and ABDERHALDEN [2]) have investigated this biochemism bacteriologically. They neutralised 10 proc. solutions of chinic acid with calcium carbonate, added 0.5 proc. peptone, 0.1 proc. kalium phosphate, and 0.1 proc. magnesium phosphate, inoculated this mixture with a few drops of an infusion of putrefying meat, and cultivated for some weeks at 35° C. They obtained protocatechetic acid together with a slimy bacterial mass in the liquid, from which it was possible to isolate a likewise slimy *Micrococcus*, which proved to be the cause of the production of the latter acid and was named *M. chinicus*.

The reaction goes after the formula:

$$C_7H_{12}O_6 + O = C_7H_6O_4 + 3H_2O$$

Chinic acid Protocatechetic acid

whereby at most 12 proc. chinic acid is converted; into what the remaining 88 proc. change is not noted by these experimenters. It is remarkable that only one atom of oxygen takes part in this reaction.

As the said authors had not applied in their experiments the so intense colouring of ferrisalts by protocatechetic acid, it seemed desirable to make use of it for the easier recognition of the inferred bacteria.

To this end the experiment was effected as follows:

For the rough or preliminary cultivation a liquid was used of the composition:

[1]) Berichte der Deutschen Chem. Gesellschaft, Bd. 14, pag. 450, 1902.

[2]) Ueber einen Chinasäure in Protocatechusäure überführenden Pilz. Centralblatt für Bakteriol. 2te Abt. Bd. 10, Pag. 337, 1903.

Tapwater	100.00
Dikaliumphosphate	0.05
Ammoniumchlorid	0.05
Calciumchinate $(C_7H_{11}O_6)_2\,Ca + 10H_2O$	0.1 to 10
Ferrichlorid	0.01

In a wide ERLENMEYER flask, so that a strong aëration occurs in the thin layer, inoculated with soil and cultivated at 20° to 25° or 30° to 35° C., the liquid colours deep black after a few days, in consequence of the formation of ferriprotocatechate.

To purify the bacterial culture a trace is transferred to a similar medium and cultivated at 20° or 30° C.

If this culture is sown on a medium of the same composition but solidified with agar and containing some ferricitrate[1]), colonies are obtained, from the cultures kept at 20° to 25° C., of different varieties of *B. fluorescens*, and from those kept at 30° to 35° C. chiefly of a *Micrococcus*, all lying amid diffusion fields of ferriprotocatechate of an intense violet or red colour. This *Micrococcus* belongs perhaps to the same species as that described by EMMERLING and ABDERHALDEN, but then certainly to another variety, for it does not produce slime, neither in presence of peptone nor of ammoniumsalt. This form, very common in our environment and which can be obtained with various other organic salts in a similar way as with chinate, I shall name *Micrococcus calco-aceticus*, as calcium acetate is very fit for its accumulation. Here it may be observed that acetates are also very useful for the accumulation of certain varieties of *B. fluorescens non-liquefaciens* which still grow at 30°C.

Streaks of these various bacteria on broth agar with one proc. calcium chinate, and a little ferricitrate, or on the above medium, give again deep black or red-coloured diffusion fields of ferriprotocatechate.

Part of the chinate oxidises directly to water and calciumcarbonate which precipitates as crystals dyed deeply violet, by having sucked up the ferrisalt of the protocatechetic acid during their crystallisation.

Other species, able to convert the chinic acid into water and calciumcarbonate or protocatechetic acid, but not found in the foregoing experiments, are mentioned in the following table where it is indicated by + and — whether the substances placed at the head are either or not formed. These experiments were made with broth agarplates with 1 proc. calciumchinate at 30° C., or use was made

[1]) Ferricitrate does not give a precipitate of ferriphosphate in the somewhat alkaline broth.

of the above named nutrient liquid containing ammonium chlorid, after its solidification with agar.

From chinates result by	Protocatechetic acid	Calcium-carbonate as crystals	Remarks
Bacillus prodigiosus	+	—	
„ *punctatus*	—	—	
Aërobacter coli	—	—	
„ *aërogenes*	+	—	Some varieties
„ *liquefaciens*	—	—	
Pseudomonas aromatica	—	—	
„ *fluorescens non liquefaciens*	+	+	Some varieties
„ *fluorescens liquefaciens*	+	+	Some varieties
„ *pyocyaneus*	+	+	
Proteus vulgaris	—	—	
Microspira tyrosinatica	—	—	
Micrococcus calco-aceticus	+	+	All varieties
Acetic acid bacteria	—		
Yeast species	—		

This table shows that the common species which oxidise chinate to protocatechetic acid, namely the fluorescents, also embrace varieties devoid of this faculty..

The second column is but of relative value, for a number of bacteria oxidise the chinate and grow from it with great intensity without crystallisation of the thereby formed calciumcarbonate. The chinates, belong (with the malates) to the most easily assimilable organic salts for non-sporulating bacteria in general.

It is remarkable that there do not seem to exist spore-forming bacteria which produce protocatechetic acid, for I did not succeed in obtaining microbes from pasteurised materials, such as garden soil or canal mud, which, in solutions or on plates of the before given composition gave rise to an obvious change of colour. But by various spore-formers calciumchinate was changed into carbonate, though slowly.

With exclusion of air solutions of chinate are apt to come into fermentation, as was already observed by Löw, whereby carbonic acid, acetic acid and propionic acid are formed. Hydrogen was not found; the inferred microbes belong to *Aërobacter aëroyenes* and allied forms.

2. *Oxidation of Quercite to Pyrogallic acid by Pseudomonas aromatica.*

The knowledge that chinic acid derived from the hexamethylene ring (hexahydrotetraoxybenzoic acid) can so readily be converted by many microbes into an aromatic substance, easily demonstrated by the ferri-reaction, suggested the question if substances exist, related to chinic acid, that behave similarly.

This consideration induced to subject quercite to an investigation analogous to the foregoing, the structure of this substance being the hexamethylene ring, in which 5 atoms of hydrogen have been replaced by hydroxyl. It was proved that also here, under the influence of life, an aromatic substance is easily produced, but at the same time that addition of a ferrisalt to indicate that substance is superfluous; further, that but one single species of microbes seems to exist, of which only some varieties possess the faculty to form that substance.

A more precise investigation showed that here the chemical reaction proceeds quite correspondingly with the oxidation of chinic acid, but that the product is, after all probability, pyrogallic acid, evidently resulting thus:

$$\underset{\text{Quercite}}{C_6H_{12}O_5} + 0 = \underset{\text{Pyrogallol}}{C_6H_6O_3} + 3\,H_2O$$

Here, too, only one atom of oxygen per molecule of quercite is used. It should be noticed that in these experiments a large portion of the quercite vanishes in another way, probably as carbonic acid and water.

The microbes causing this conversion are very generally distributed in our surroundings, but although there occur among them a number of clearly distinct varieties, they all belong to one and the same species, namely that of the "aroma bacteria", well known in milk and milk products and for the first time distinctly described by MIGULA [1]) as *Pseudomonas aromatica*. It is a polarmonociliate short rodlet, little motile in plate cultures, more so in broth.

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[1]) System der Bakterien, Bd. 2, p. 880, 1900; with fig. Bd. 1, Tab. 1, fig. 8. This description is based on *Bacillus crassus aromaticus* TATAROFF. — Probable synonyms: *B. aromaticus lactis* GRIMMER, Centralbl. f. Bacteriol. 2te Abt., Bd. 8,

The very dark colour of the pigment in an aerated alkaline medium makes it easy to detect the quercite bacterium. If for example, on a broth agar plate with 0.5 proc. quercite, some drops of sewage water are spread, there is much chance that after one or two days at 30° C. some colonies appear that are jet-black, or lie amid a black diffusion field, distributed among the numerous non-pigment producing colonies, which latter are little troublesome, excepting *B. fluorescens liquefaciens*, whose secretion is injurious to the quercite bacteria.

In a previous paper I alluded to a simple experiment whereby aromatic milk results [1]).

To this end milk should be kept at a relatively low temperature, for example at 15° to 20° C., with full admission of air, so that it is left to spontaneous corruption by the aerobic germs it contains. The acidification is at first feeble on account of the low temperature, but it is then that the "aroma bacteria" increase very much and produce the characteristic ester which has not yet been nearer examined.

If of such aromatic milk streaks are made on a quercite plate of the above composition a large number of brown colonies of quercite bacteria appear after 2×24 hours at 30° C. An examination of their faculty of producing the aroma in milk proves that it does exist but only in a slight degree. The real "aroma bacteria", which develop by the side of the "quercite colonies" and correspond with these in all other respects, do not possess the power of producing pyrogallol from quercite, hence, though belonging to the same species, they represent other varieties. The quercite bacterium might thus be named *P. aromatica* var. *quercito-pyrogallica*. That *P. aromatica* is so easily distinguished as a species, makes it in this case possible to indicate a character by means of which forms found in nature and seemingly alike, may be recognised as belonging to different varieties. The oxidation function here, thus proves to be very variable, being present or

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S. 584, 1902. — *B. butyri aromafaciens* Keith, *Bacillus N°. 41* Conn; *Pseudomonas fragariae* Gruber, Centralbl. f. Bact. 2te Abt. Bd. 9, p. 705, 1902. — *Ps. fragariae* Gruber, Id. Bd. 14, p. 122, 1905, — and *Ps. fragaroidea* Harald Huss. Id. Bd. 19, p. 661, 1907. — Perhaps likewise the yellow-coloured *Ps. trifolii* of Harald Huss, Id. Bd. 19, p. 68 and 149, 1907, and several other different forms less easily recognisable in the literature are synonyms. — *Bacillus esterificans* Maassen, Arbeiten des Kais. Gesundheitamtes, Bd. 15, 1899, is quite another species, producing spores and belonging to the hay bacilli, and thus related with *Granulobacter polymyxa* Prazmowski.

1). Fermentation lactique dans le lait. Archives Neérlandaises, Sér. II. T. 13. p. 350, 1907.

lacking in closely allied forms which are themselves constant and differ only in this quality.

Another character by which the natural varieties of *P. aromatica* are mutually distinct, consists in their very unequal power of liquefying gelatin, this power being intense in some and quite absent in other varieties, with all intermediate degrees. The same is to be observed in the quercite bacteria; hence the variability of this property is in some degree a property of the whole group.

All varieties, apparently without exception, produce in glucose bouillon about 3 cm.^{3} N acid per 100 cm.^{3} liquid. For growth, oxidation and acid formation, peptones are wanted as source of nitrogen, ammonium salts and nitrates can hardly serve as such and only in pure cultures, but by no means in free competition with other microbes.

Although aromatic milk contains a great many quercite bacteria, its flora chiefly consists of other varieties of *P. aromatica*, but the following experiment, based on the principle: slow rising of the concentration of a good nutrient medium apt to produce a slight acidification, makes it possible almost exclusively to obtain the quercite bacteria.

Large glass beakers are filled with 1 L. of distilled water and therein are floated a few small dialysators of parchment paper, manufactured by SCHLEICHER and SCHÜLL, of the shape of experiment tubes, each filled with about 15 cm.^{3} of extract of greenmalt. This extract is prepared by rubbing two parts of greenmalt with three parts of water in a mortar and filtrating after some hours', digeration at room temperature. The clear solution contains relatively little maltose and is of course extremely fit for bacterial growth, where, likewise as in milk, lactic acid ferments are able to develop, but only little lactic acid can be formed on account of the low rate of sugar. Kept in a room where the temperature varied from 15° to 20° C. the spontaneously corrupted infusions in the beakers produced at repeated experiments, made in December 1910 and January 1911, so great an excess of quercite bacteria, that other species could hardly be found in the black mass, obtained by streak inoculations on broth-agar quercite plates.

If instead of submitting the malt extract to dialysis, different quantities of the extract itself were directly added to the water, then, with for the rest like conditions, much less quercite bacteria developed.

The aroma formed in the malt extract is of the same nature as that found in aromatic milk.

Other bacteria but the above named, producing a pigment from quercite, have not been found, neither by experiments with non-sporulating forms at higher temperatures nor among the microbes that remain alive in pasteurised materials.

Finally it may be remarked that quercite (which is not susceptible of alcohol fermentation) is attacked, when no air is admitted, by fermentation bacteria of the *Aërobacter*-group, such as *A. aërogenes*, under production of carbonic acid, hydrogen, and of organic acids which have not yet been more exactly examined.

3. *Oxidation of Tyrosine to Melanine by Microspira tyrosinatica.*

It is well known that the enzyme tyrosinase is able to oxidise tyrosine to a jet-black substance, which is formed at the air from dioxyphenyl acetic acid or homogentisinic acid. It is accepted that this substance originates after the formula [1]).

$$C_9 H_{11} N O_3 + O_3 = C_8 H_8 O_4 + N H_3 + C O_2.$$

Tyrosine Homogentisinic acid

In the experiments now to be treated I could not find ammonia which, according to the formula should come free, probably because all the nitrogen present in the tyrosine, is used for the growth of the bacteria.

Hitherto this conversion had only been studied as a consequence of the action of an enzyme occurring in higher plants and also in higher Fungi. Nobody, however, had as yet described tyrosinase-producing bacteria, whose existence will be referred to in the next lines. As they are rather easily cultivated and are able to produce great quantities of the black pigment formed from tyrosine, which is identic with or closely allied to the melanines of the human body, they are of importance for experimental physiology.

Tyrosine microbes are small vibrios, chiefly occurring in the sea and during the winter months present in the plankton. Fresh water is not however quite devoid of them and without much trouble they may be isolated from sewage water. The forms living in the sea produce, at least as regards the stronger varieties, besides tyrosinase, also tyrosine, and as this takes place from peptone they are to be recognised by the black stains which their colonies produce on broth-agar plates, which, as we have to deal here with inhabitants of the sea, should contain 3 proc. common salt. It is remarkable

[1]) ABDERHALDEN, Physiolog. Chemie. 2te Aufl. p. 367, 1909.

that these tyrosine-vibrios of the sea can be accumulated in seawater with addition of agar as sole source of carbon, ammonium chlorid as nitrogen food, and kalium phosphate. In this respect they show analogy to the gelase vibrios, which secrete the enzyme gelase by which agar is changed into sugar and which are also easily produced in this manner.

Accumulation of these microbes in seawater with tyrosine as source of carbon has not succeeded, as little as with their relatives from fresh water, by corresponding experiments. Endeavours to accumulate the latter from sewage water with tyrosine as source of carbon and nitrogen have produced fluorescents, which thus prove the stronger in the competition at such an "elective" cultivation.

The fresh-water form is fairly common in the sewage water of Delft; to obtain it in pure culture the undilute sewage water must be poured over a plate of the composition:

Tapwater	100
Tyrosine.	0.1
Natrium carbonate . . . ,	0.1
Dikalium phoshate	0.05
Agar	2

The superfluous water is allowed to flow off the plate, which is cultivated for some days at 30° C.

It is true that here the tyrosine is at the same time source of carbon and of nitrogen, but the method is now a "separative" one, as competition is excluded.

On the second or third day peculiar black spots are seen to appear around some colonies and slowly extend over a distance of some millimeters[1]). The black pigment proves able to diffuse only to a rather short distance, whilst the enzyme itself remains bound up with the bacterial bodies as belonging to the endo-enzymes. That here we have indeed to deal with a true enzyme, is more easily shown in the species of the sea than in the fresh-water microbes. To this end some material cultivated on broth agar is killed by the vapour of chloroform, then transported to a culture plate of the above composition, or to a nutrient liquid of the same preparation, but with omission of the agar. At a temperature of 40° C. the black-colouring is then rather quickly perceived but, of course, without development

[1]) As the so generally distributed fluorescent bacteria likewise attack tyrosine under production of a light red-brown pigment, there are always found spots of that colour on such culture plates, which can, however, by no means be mistaken for those of tyrosinase.

of the germs. As endo-enzymes may be considered as constituents of the protoplasm, it is not surprising that the reactions with such preparations, containing only dead material, are feeble, for the enzyme itself is for the greater part annihilated. Hence, in my opinion, endo-enzymes are best studied when still within the living cells themselves and by considering them as an essential part of the living protoplasm. Taken in this sense tyrosinase may be called a "respiration enzyme", and it is remarkable that as a product of respiration, beside the carbonic acid, ammonia is formed, instead of water as in the ordinary respiration.

In sewage water only a small number of tyrosine bacteria are found per cM^3. This number may be a little increased by leaving the sewage water for some time at room temperature, then making on plates streaks of the microbes accumulated in the layer at the surface. This microbe layer, very rich in infusoria and flagellates, produces, in particular as it seems in late summer, many more tyrosine bacteria than the sewage water itself. Nevertheless, as said above, it has not been possible to find a really good accumulation method of these tyrosine bacteria, although many trials have been made.

The black pigment can be prepared in great quantities by cultivating the pure microbes at 30° C. in large ERLENMEYER flasks of the said feebly alkaline solution of natriumtyrosinate with the required anorganic salts. The conversion is relatively slow, so that it is complete only after some weeks, but then a liquid is obtained which may be used as ink. Traces of ferrisalts favour somewhat the formation of melánine.

The tyrosine bacteria belong to the genus *Microspira* created by MIGULA. They are very small polar-monociliate, curved rodlets, somewhat varying in thickness, mostly thinner than the cholera vibrios, which for the rest they resemble very much. Like these they quickly liquefy broth gelatin and form on broth agar white, vigorously growing soft masses. Sometimes they are united in long chains; the longest individuals show distinct curves and remind of spirilli. If tyrosine is present in the nutrient medium, many individuals take partly a black colour, swelling up very much and sometimes becoming quite spherical, but the cilia do not become visible. They produce indol, but do not give the nitrosoindol reaction. They grow well in peptone solutions.

The fresh-water form colours broth agar without tyrosine not or only very late, but if tyrosine is added the brothagar grows rapidly black. The black-colouring begins still earlier on the before

mentioned culture medium, containing only tyrosine, although the growth on it is much slower than on brothagar.

As nobody had ever before observed tyrosinase formation by bacteria, there is reason to consider these microbes as new for science; the species occurring in sewage water may be called *Microspira tyrosinatica* [1]). It is an organism highly sensible to the nature of the nutrient substances, apt to lose the tyrosinase function by various not yet explained influences, but notwithstanding continuing for years in the laboratory as an hereditary constant species.

§ 4. *The brown pigment formed by the acetic bacterium Acetobacter melanogenum.*

When beer is left to corrupt at the air a film forms at the surface in which *Saccharomyces Mycoderma* and acetic bacteria develop, or only the latter, in accordance with the temperature and other culture conditions. If the corruption takes place at room temperature it will be perceived, when the beer is contained in beaker-glasses, that after the film has closed over the surface, some of the beakers slowly assume a dark brown colour and after two or three weeks get so dark, that the beer seems coloured by caramel.

For the isolation of the here active organisms streaks are to be made of the film on wort- or beer gelatin. Then these culture plates being kept *two or three weeks* at room temperature, they show deep brown spots evidently coloured by the same substance which originated in the beer itself, spots in whose centre the colony of a vinegar bacterium is lying. As a matter of course the plates are further covered with colonies of *Saccharomyces Mycoderma* and of ordinary vinegar bacteria.

Culture plates of 100 water, 10 gelatin, 2 peptone, 3 glucose, are also very good for growth and pigment production. The "brown" vinegar bacterium obtained in this way, I recently described under the name of *Acetobacter melanogenum* [2]). It is commonly but not always, a motionless organism, which can only develop on peptone as source of nitrogen and produces the pigment from this substance, if at the same time glucose or maltose is present. Other nitrogen sources but peptone have not been found. The sugar is during the

[1]) MIGULA's *Microspira nigricans*, System d. Bakteriën, Bd. II, p. 1013, does not liquefy gelatin, but colours it brownish black. Whether tyrosine and tyrosinase occur in this case has not been examined.

[2]) Pigmentbildung bei Essigbakterien. Centralblatt f. Bakteriol. 2te Abt. Bd. 29. S. 169, 1911.

growth partly converted into a strong acid, probably gluconic acid. In presence of alcohol much acetic acid is formed. Consequently beer acidifies with great intensity.

Solutions of 10 proc. glucose and 2 proc. peptone in tapwater with 10 proc. calcium carbonate at 25° or 30° C. grow black after a few weeks, the carbonate changing at the same time into calcium-gluconate.

Although for the formation of the pigment the simultaneous presence of sugar and peptone is required, there is cause to admit that the pigment is an aromatic substance, taking rise from peptone alone, whereas this reaction only occurs during the growth of the microbe, for which growth also sugar is wanted. In an earlier paper I gave to such processes the name of auxobolisms.

By the formation of the pigment in the gelatin plates the gelatin not only becomes deep brown, but at the same time quite insoluble in boiling water, which is the more remarkable as the newly isolated stocks of *A. melanogenum* liquefy the gelatin in the beginning (probably by the intense acid production and not by a specific enzyme). Older stocks lose this liquefying power, probably as they become slower in producing acid; their pigment formation, however, remains the same.

Only very few substances render gelatin insoluble in boiling water as for example, formalin and chinon, whilst among the microbes, as far as known, only *Actinomyces chromogenes* (*Streptothrix chromogena*) has the same effect on gelatin by chinon production from peptone. As, moreover, the brown-coloured gelatin reduces silver in an ammoniacal solution of silver nitrate, and produces metallic mercury from an alkaline mercury solution, there is reason to admit that *A. melanogenum* does really produce chinon, this substance giving the same reactions. However the most characteristic reactions of chinon could not be obtained, namely, the blue-colouring of guajac emulsion and the production of iodium from hydroiodic acid. But the secretion product of the brown vinegar bacteria gives quite well the black-colouring with ferrisalts, also characteristic for chinon.

Summary.

The oxidation of chinic acid to protocatechetic acid is brought about by number of microbes belonging to very different groups and is easily demonstrated with ferrisalts. In particular *Micrococcus calco-aceticus* and some varieties of *B. fluorescens non liquefaciens* possess this faculty in a high degree and hence can be found and isolated from mixtures of bacteria.

The oxidation of quercite to pyrogallol is caused only by certain varieties of *Pseudomonas aromatica*, so that we have here a very specialised function. Green-malt extract allowed to grow "aromatic" by spontaneous corruption at low temperature abounds in that species and always contains numerous quercite bacteria which besides, are fairly common in sewage and even in canal water as also in "aromatic milk".

Melanine formation from tyrosine is proper to certain sea-vibrios and to *Microspira tyrosinatica* not uncommon in sewage water and easily found by this reaction. It is a microbe closely allied to the cholera and the photogenic vibrios. The tyrosinase function is sometimes suddenly lost by unknown influences, but may return in the same stock. Notwithstanding, the species can be considered as fairly constant and remains so for years in the laboratory.

Beer, poor in extract, colours dark brown when corrupting at the air. This is owing to the presence of a vinegar bacterium, *Acetobacter melanogenum*, which produces a pigment reminding of caramel from peptone. By the secretion products of *A. melanogenum* gelatin is as it were tanned and becomes insoluble in boiling water. Perhaps chinon is inferred in this process.

In natural varieties of the species of microbes, which in all other respects show no difference, the oxidation function in regard to certain substances may be either or not present, but if present it may be very constant in these varieties.

Zoology. "*The Eutherian and the Metatherian early blastocyst*". By Prof. A. A. W. HUBRECHT.

The careful description of the early development of the Marsupialia by Prof. J. P. HILL in vol. 56, pt., of the Q. J. of micr. Sc., has been anxiously awaited by numerous vertebrate embryologists, who, being acquainted with HILL's important contributions (together with WILSON) to the ontogeny of Monotremes, expected that a firm basis would henceforth be established on which the mutual relationships of the more primitive and the more specialised Mammalia might be built up. In this respect however the valuable publication, above referred to, is a deception. Far from being exhaustive it presents the limited number of observations available in the light of an interpretation in which the distinction of what is normal from what is abnormal, is largely dependent on numerical relations and in which the representatives of the so-called abnormal blastocysts are not fully introduced to the reader, nor sufficiently described at length, to enable the interested student to form an opinion for himself.