Microbiology. — *"On the composition of tyrosinase from two enzymes"*. By Professor Dr. M. W. BEIJERINCK.

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The product of the action of tyrosinase on tyrosin is commonly called melanin, whose colour may be jet black, but takes all shades between light brown, pure red, brownish red, sepia and black in experimental conditions. These pigments are of uncommon stability and resist even heating with strong alkalies and sulfuric acid, whereby the black runs somewhat into brown but in chief remains unchanged. Even when boiled with nitric acid the melanin remains almost unchanged. It is accepted that the pigment of the hair and hide of higher animals is associated with these substances and is derived from tyrosin.

Melanin formation by symbiose of an Actinomyces with a bacterium.

On a culture plate of the composition: distilled water, 2 % agar, 0.1 % tyrosin (dissolved in a few drops natriumcarbonate) and 0.02 % K_2HPO_4, on which some centigrams garden soil are sown and which is kept at 30° C., hundreds or thousands of little sods of *Actinomyces* (*Streptothrix*) will develop after two or three days. The tyrosin serves at the same time as source of carbon and of nitrogen. But the agar itself also is attacked by these microbes, although with difficulty, and used as food. This is not surprising as many *Actinomyces*-species can even live on cellulose as source of carbon.

The common bacteria of the soil develop not or hardly on the tyrosin plate and cannot in the given circumstances compete with the slowly growing *Actinomyces* as they do on better media, e.g. on broth agar, where *Actinomyces* never occurs when bacteria are present.

As the delicate threads of this genus enter deep into the agar, the plates may be freed by washing from the bacterial colonies and the adhering soil; then the *Actinomyces* sods can be easily counted. In humus and humus containing soil their number is amazing. When they can freely multiply on plates which are poor in food their growth is unlimited and they produce sods of great extension, even of one or more decimeters in surface, commonly producing very fine mycelial-rings, which by turns bear spores or not. These rings are independent of light and suggest a periodicity in the nutrition not yet fully explained.

In somewhat extensive culture experiments, similar to the above, it may with certainty be expected that at some places brownish

red or jet-black spots will originate. The brown spots are caused by the oxidising action of some common soil bacteria, which produce a red or brown-red pigment from tyrosin; the black ones, caused by melanin, which will be more exactly considered here, have quite another origin.

In or near the centrum of these black spots always lies a colony of *Actinomyces*. Streaks on new culture plates of the said composition to obtain a pure culture, give the surprising result, that the organism can vigorously grow on the tyrosin but produces no pigment at all. A more minute examination shows further, that the black plants of *Actinomyces* lie under a thin, glassy, transparent layer of fine rod-bacteria. This layer covers like a crust the jet-black sods of *Actinomyces* and prevents them from producing spores, which does take place on that part of the mycelium, which develops outside the bacterial cover. If from this layer the bacterium is brought into pure culture, which is easily done on brothgelatin- or broth-agarplates, it proves to be an extremely delicate polar ciliate rodlet, which forms no spores and strongly liquefies culture gelatin. Streaks of the pure culture on a tyrosin plate produces no melanin at all, so that in this respect the bacterium resembles *Actinomyces*.

It is obvious that we here have a case of pigment formation reposing on the symbiose of the two organisms. Experience shows that this supposition is right: their combined streaks on a new tyrosin plate produce beautiful black spots of any extension. As they can both be very well grown on better media, such as broth-agar, the experiment is, the first isolation effected, easy and interesting. The experiment may be improved by providing the culture plates with a better source of carbon beside the tyrosin, for which glucose and peptone proved particularly useful. On the other hand, additions of an ammoniumsalt or of nitrates had no effect.

In order to ascertain which of the two organisms is the real cause of the melanin production, the following experiment was made.

On an agar-tyrosinplate of the said composition, parallel streaks of both organisms were drawn with some millimeters, distance between. The result was not dubious; after a few days the streaks of *Actinomyces* vigorously developed and covered with snow-white spores, but for the rest were quite colourless. The bacterial streaks, on the other hand, which had developed to a thin, hardly visible transparent layer, had become jet-black wherever they were near *Actinomyces*. The following must therefore take place: *Actinomyces* decomposes the tyrosin and produces from it a colourless chromogene which is converted into melanin by the bacterium and easily

diffuses through the agar, evidently without spontaneously oxidising at the air.

From the foregoing it is clear that *Actinomyces*, as well as the bacterium, can only be found in garden soil when germs of both species occur in each other's immediate vicinity. To promote this occurrence I have tried first on fit agarplates to grow *Actinomyces* and later floated them with a tyrosin solution, in which the melanin bacterium was present in so great quantity, that it could develop anywhere on the plate, after the tyrosin had diffused.

As the various species of *Actinomyces* are very vigorous, polyphagous microbes, which develop especially in dilute media at the side of the common bacteria, the most different food may be used for the first part of the experiment.

So, an agarplate, only containing some potassiumfosfate and ammoniumsulfate, was sprinkled with a little dry inulin mixed with garden soil. The soon developing flora was washed off under the tap by which the loosely adhering bacterial colonies together with the nondecomposed inulin, were removed. The agarplate was now clear again but in the surface were hundreds of *Actinomyces* colonies which had not been removed by the washing, as they had penetrated too deep into the agar. After treating with the tyrosin solution in which the melanin bacterium was suspended and a renewed cultivation for some days at 30° C., black melanin spots appeared around some six colonies of *Actinomyces*; this species must thus be rather common in the soil.

The tyrosin *Actinomyces* can also very easily be isolated from the roots of the elmtree (*Ulmus campestris*), in whose dead periderm cells an almost pure *Actinomyces* flora occurs, as I demonstrated before [1]). For the development of this flora some of the hairroots are carefully washed, to remove the adhering soil and are then ground in a mortar. The thus obtained brown paste is diluted with water, mixed with the tyrosin bacterium (which however is also rather common on the elm roots themselves), then sown out on a tyrosinplate of the above composition. After a few days numerous colonies of *Actinomyces* develop at 30° C., among which some jet-black ones.

Here it should be called to mind that the two organisms produce no pigment on peptone or broth-containing media, neither each for itself nor in combination. But herefrom cannot be concluded that at their cultivation from peptone no tyrosin originates. Nevertheless the conclusion must be drawn, that if at the splitting of the peptone

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[1]) Centralbl. f. Bakter. 2 Abt. Bd. 6, S. 2, 1900. Arch. Néerl. 1900, p. 327.

tyrosin is indeed formed, it is oxidised in another way but not to melanin.

That this *Actinomyces* must belong to another species than *Actinomyces chromogenes*, so common in our environment, is obvious. The latter namely is characterised by the production of a dark brown pigment from pepton, (but not from tyrosin) in which, as I have formerly [1]) shown, under certain circumstances chinon may be found.

Several other species of *Actinomyces* produce blue, red, or yellow pigments, whereby, as to the blue and red, the simultaneous presence of certain varieties of hay bacteria is favourable. In this case it is not tyrosin, but glucose, malates and nitrates that form the chromogeneous food, so that the symbiose is then evidently associated with other factors than those active in the production of melanin from tyrosin.

Hitherto I have not yet been able in liquid cultures with the help of *Actinomyces* and its symbiont to produce a somewhat considerable quantity of melanin. This could not be foreseen as this genus is as common in the mud of moats and canals as in garden soil. But some experiments as the above to find our *Actinomyces* in mud gave no result, so it seems that this species at least is a real inhabitant of the soil.

That pigment production in this case is difficult in liquid media, whereas *Microspira tyrosinatica*, which I described earlier [2]), produces it as readily in liquid as in solid media, is perhaps owing to the general propriety of *Actinomyces* to grow but slowly in solutions, probably in consequence of the little tension of the dissolved oxygen. *Microspira*, on the other hand, is as a true water microbe, evidently better adapted to that tension.

Theory of the melanin formation [3]).

In physiological chemistry it is generally accepted that at the tyrosin reaction from the tyrosin first originates homogentisinic acid, ammonia and carbonic acid after the formula

$$\underset{\text{Tyrosin}}{C_9H_{11}NO_3} + O_3 = \underset{\text{Homogentisinicacid}}{C_8H_8O_4} + NH_3 + CO_2$$

and that only afterwards by a new oxidation the homogentisinic acid is converted into melanin.

[1]) Centralbl. f. Bakter. 2 Abt. Bd 6 S 2, 1900. Arch Néerl. 1900, p. 327. Commonly the chinon is absent, which I did not know in 1900.

[2]) These Proceedings, XIII, 1066.

[3]) For the literature see Czapek, Biochemie der Pflanzen. Bd. 2, p. 462 and 478, 1905. Abderhalden, Physiologische Chemie, p. 362 and 365, 1909.

This might give a good explanation of the symbiose experiment, supposing that *Actinomyces* produces homogentisinic acid from tyrosin and that the symbiotic bacterium oxidises this acid to melanin. Taken for granted that these two processes are due to two separate enzymes, this conception may be called "the two enzymes theory" of the melanin production.

In order to obtain more certainty regarding the correctness of this suppositon, I made some experiments with the soda salts of the homogentisinic acid ($C_8H_8O_4$) and compared the results with the conversion of the calcium and soda salts of the gentisinic acid ($C_7H_6O_4$). Both substances I owed to the Chemical Laboratory of the Technical University, the homogentisinic acid as lead salt, which I converted into the soda salt, the gentisinic acid in free state. Both behave towards microbes in a corresponding way, but the gentisinic acid oxidises more greater difficulty.

I also received from Professor PEKELHARING the lead salt of homogentisinic acid, prepared from urine, but this could not be distinguished from the other.

At the preparation with these substances of neutral or feebly alkaline agar plates, on which the oxidising microbes were to be grown, the difficulty arose that already during the heating at the air a brown colour appeared, which was not the case when cold. It could, however, with certainty be stated that, as was expected, *Actinomyces* produced no pigment from these acids; on the other hand, the symbiotic bacterium gave a dark brown colour, which may finally run into jet-black. As this bacterium produces some alkali, it might seem doubtful whether this alkali might be the cause of the more intense pigment production, or if any oxidising enzyme, produced by the bacterium, were active in this case. By cautiously neutralising the existence of an oxidase, which diffuses in the agar to a relatively great distance from the bacterial colony, could be ascertained. It is clear that the thus found enzyme might be called "homogentisinase". It will be seen by and by that it also occurs in higher plants and perhaps corresponds to the common laccase.

The formerly described *Microspira tyrosinatica* (l.c.) living in the sea and in sewagewater, oxidises tyrosin directly to melanin without intervention of any other organism. That this is done here also by a vigorously active tyrosinase is easily shown with the form living in the sea, the bacterium, when killed by chloroform, being still able to cause the melanin reaction. I think it is proved now, that also in this case the tyrosinase consists of two enzymes, as it is possible with *Microspira* to oxidise the homogentisinic acid to a dark pigment.

In order to ascertain how in this respect the tyrosinase of the higher plants behaves, I took strong tyrosinase preparations derived from the potato, the beetroot and latex of *Euphorbia Lathyris*[1]) which quickly colour tyrosin solutions deep black, and made them act on homogentisinic acid salts. The latex of *Euphorbia Lathyris* is extremely fit for these experiments as it can always be made to drip from the living plant, which supports our winters very well in the garden. A single drop on an agar-tyrosin plate at from 30° to 50° C. forms deep black melanin spots after a few hours already. But homogentisinic acid can also be oxidised with great velocity. For this experiment I used an agarplate of this composition: water, 2% agar, 0,5% natrium homogentisinate, 0,02% NH_4Cl and 0.02% K_2HPO_4.

On this plate drops of the latex were put and besides streaks were made of *Actinomyces* and the symbiotic bacterium. After some hours, at 30° C., dark brownish black fields appeared, evidently more readily formed than the black fields from the tyrosin.

After about 24 hours *Actinomyces* also began to grow but no pigment at all appeared, as was to be expected. The symbiotic bacterium did not develop under these conditions. But some broth being added to a like medium the bacterium could grow and oxidised the homogentisinic salt to melanin. So it is certain that also the tyrosinase of *Euphorbia Lathyris* must be a mixture of two oxidising enzymes; one of these, which may preserve the name of tyrosinase, produces homogentisinic acid from tyrosin, the other, "homogentisinase", forms melanin from the acid, and corresponds with the oxidase of the symbiotic bacterium. This enzyme requires no special name as "homogentinase" and "laccase" are probably identic.

Although the "two enzymes theory" of the tyrosinase may be considered as confirmed by what precedes, still it should be called to mind that, when a method of experimenting is used somewhat deviating from the described the above result with *Euphorbia Latyris* is not obtained. Such is, namely, the case when the milky juice of the plant is put on agarplates with homogentisinic acid salt, with addition of broth for the bacteria. Then the surprising fact occurs that the bacterium is active but the latex is not. Whereon this difference reposes is not clear.

Finally it may be mentioned that the existence of two enzymes in the tyrosinase of the beetroot was already made probable by P. C. VAN DER WOLK (Recherches au sujet de certains, processes enzymatiques chez *Beta vulgaris*, Nimègue 1912).

[1]) The latex of *Euphorbia palustris*, *E. Peplus*, *E. helioscopia*, *E Mysinitis*, contain no tyrosinase