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BACTERIAL ORIGIN OF SOME INSECT PIGMENTS AND THE ORIGIN OF SPECIES THROUGH SYMBIOSIS

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Prof. G.B. Poulton's classical work on *Colours of Animals* explains how most insects have a protective coloration. A typical instance can be provided by the homopterous insect, *Cicadella viridis*. It is coloured green as it feeds on weeds of the same colour. The insect is widely distributed in Europe particularly in Denmark and Italy. Practically any locality, where weeds are growing in a wet meadow, would offer some specimens of this insect. In places it has been found in such abundance as though nature had meant to cultivate it in a farm. Thus there is no scarcity of material and the findings described here can be easily confirmed.

Poulton explains that the gorgeous and iridescent colours of many butterflies and beetles are due to the physical phenomenon of interference. This has been further explained in a relatively modern work of Prochnow.[1] In such cases no pigment accounts for coloration. But it is quite conceivable that an underlying pigment can be superposed by interference, when the colour would appear somewhat different to the pigment itself. Probably this explains how the wings of the female *C. viridis* are bright green, as the name suggests, while those of the male have a bluish metallic sheen. Now the majority of insects do possess actual pigments. But their study started only when biochemistry developed into an independent branch of chemistry. Although dyes, like the cohineal, were studied when chemists were anxious to synthesize dyestuffs, pioneering work on insect pigments started with the isolation of a pterin from the wings of the cabbage-white-butterfly by Prof. Hopkins of Cambridge. By now pterins represent a group of important biological substances which includes folic acid. Work is also being continued at Cambridge, on colours of aphids, which was initiated by Lord Todd.

Considering the fact that the chemistry of insect pigments had hardly begun, their genesis has hitherto remained untouched. However, instead of its appearing a formidable problem, in some cases at least, there is a clue promising to facilitate research even on insect colours themselves. The symbiotic bacteria of two spittle-insects, *Aphrophora salicis* and *A. spumaria*, produce pigments similar in appearance to 'yellow ochre', and 'burnt-sienna,' which is reddish, The pigments of the symbiotic bacteria match with the colours of their respective insect hosts. Thus it would be easier to isolate the bacterial pigment after growing the germ as much as one desires, study the chemical constitution, and identify the same with the pigment of the insect body.

A problem becomes the more interesting as it proves to be many sided. Such happened to be the case with *Cicadella viridis*. The wings of the female, are yellowish green, while its body colour is lemon yellow; both are seen in Fig. 1 where the insect is shown in profile. Its head, in front, has a touch of orange, and traces of this colour are also present elsewhere on the body. The abdomen is seen best from underneath; Fig. 2 shows it as not being uniform in colour, there being traces of orange, as also of an olive-green colour. The abdomen of the male is clearly more orange, as also its head and legs. Of the two, the female is lighter, in all the shades of colours, thus representing 'the fair sex'. This reveals that the oxidation–reduction system differs in the two sexes. It may be stated in advance that the male is more orange due to the pigment being β-carotene, and the female paler because carotene has been transformed into vitamin A, which may be looked upon here as 'leuco-carotene'.

Within the abdomen, on either side of it, the insect carries tumours in its fatty tissue. The germs are bacteria so that the tumour should be designated bacteriotome, and not mycetome, which would imply that the contents represent fungal mycelia. A smear from the bacteriotome, however, would show not only the causal germ, but also cellular debris, resulting from the attack of the parasite up on host tissue. Figure 3 shows a smear of bacteriotome with large protoplasmic residues, P, staining blue with Giemsa, and possessing vacuoles, V. A cell is seen with a nucleus, N, being large enough to remind that giant-cell formation is a common feature in comparative pathology, representing tissue response to an invading germ. There are two bacteria, one long and isolated, R, which produces a red colony,

and another short, appearing clumped together, Y, which forms a yellow colony. When the germs were cultivated the red bacterium could grow independently, but the yellow always in association with the former. The mixed colony, of the red and yellow bacteria, appears to match with the main colour of the insect body. Figure 4 represents the bacterial colony, which may be compared with Fig. 1, showing the body of the insect. Thus the main colour of the insect body could be seen *in vitro* by the mixed colony of the two symbiotic bacteria.

Of the two, the red germ alone could grow independently and its fresh growth, in a test tube, is illustrated in Fig. 5. It formed a thin film, with protruding nodules, like a typical mycobacterium. It was named *Mycobacterium carotinogen*. The bacteria grew long and close to one another, almost to see the identical form of growth in sections of the tumour, nor was it difficult to identify the longer germ in smear, Fig. 3, marked as R. The yellow germ would not grow independently. Smears of mixed culture, as in Fig. 4, showed they were clumped together like those of leprosy germ, and produced slime which explained why they adhered to each other, a feature which becomes obvious on critical examination of smear, Fig. 3. The yellow bacterium could ultimately be grown by itself when nicotinic amide was added to the culture medium. As the culture became older the colour became olive green, as in Fig. 6. But even here, a thin blister-like growth of the same colony shows it to be lemon yellow. As already pointed out the abdomen, examined from below, Fig. 2, shows a deeper shade of yellow than its sides, and even traces of olive green are visible. By now we are in a position to explain all the colours of the insect with their respective shades. The main colour is yellow, seen on the side of the abdomen, Fig. 1, and *in vitro*, on the mixed bacterial colony, Fig. 4. The deeper yellow colour of the underside of abdomen, Fig. 2, is the same as that of the yellow-blister, in the olive green colony, Fig. 6. Traces of orange, on the head of the insect, Fig. 1, is due to β-carotene of the red bacterium, Fig. 5. Traces of olive green, on the posterior end of the abdomen, in Fig. 2, is due to the olive green pigment of the yellow germ, Fig. 6. The brown or black stripes on the abdomen, in Fig. 1, is the melanin colour most common in the animal world, especially among insects. We are now left with the green colour of the wings, the colour which has given the insect its specific name, *viridis*. This colour is due to the olive green colour of culture, Fig. 6, with the phenomenon of interference added to it. The wings of the male insect have a bluish metallic sheen due to a greater concentration of the same pigment and with corresponding increase in interference.

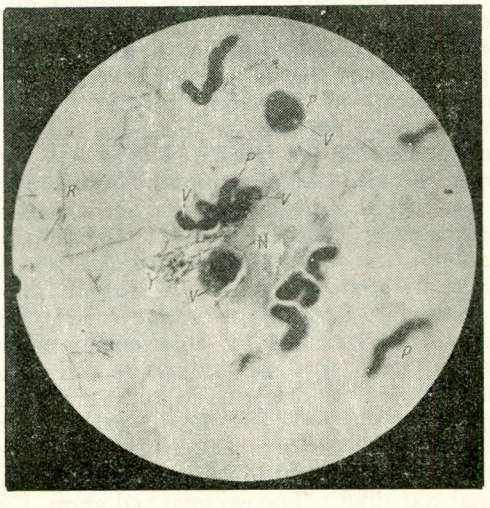

Fig. 3.—A smear of bacteriotome; P, protoplasmic pieces, stained blue with giemsa, and digestible with pepsin; V, vacuoles; N, nucleus; R, red bacterium; Y, yellow bacterium.

The abdomen of the male is more orange, so also its head and even legs, due to the carotene produced by the germ, Fig. 5, remaining as such. The female appears paler since carotene there is transformed into vitamin A which is colourless. Now both the cultures, red, Fig. 5, and the olive green, Fig. 6, were taken to Heidelberg, to the late Professor R. Kuhn. The red pigment was identified as β-carotene, the precursor of vitamin A. Its association with fat metabolism explained how the habitat of the bacterium happened to be fatty tissue of the insect. The olive green pigment, really of the old culture of the yellow bacterium, extracted with pyridine, gave a lemon yellow solution, with an absorption spectrum, identical with sarcinin, a bacterial colour, isolated by E. Chargaff. When the young bacterial colony was illuminated with ultraviolet light it fluoresced strongly, and so did the yellow abdomen of the insect. The identity of the bacterial pigment with the insect colour is thus supported . If a guess be ventured, this bacterial pigment will probably be a pterin. Thus the genesis of insect colours in the case of *C. viridis* could be traced to its pigment producing symbiotic bacteria.

Some important conclusions can be derived from the above. There is no doubt that the main germ is the red bacterium, since the yellow one could not grow apart from it. Had there been, only the red germ, its pigment would have remained as "leuco-car otin," and the insect would have appeared white. A white insect, feeding on green weeds, would have been a misfit, giving rise

to an urge for protective coloration. The enquiry at this stage would then be, how did the insect acquire its green coloration. We can translate this problem by putting the legitimate question, which of the two germs has claims of priority. This has already been decided in favour of the red one; then the problem has been shifted further to the origin of the yellow germ. A rational hypothesis makes it a mutant of the red bacterium. A mutant, losing its power to synthesize nicotinic acid, became dependant on its parental red form, and the change also affected the pathway of pigment formation, from red to a yellow pigment. Many mutants must have occurred which can be visualized from actual mutations observable in bacterial cultures to be mentioned later on. Natural selection, in the Darwinian sense, must have fixed the symbiotes of *C. viridis*, the original as producing β-carotene, and a mutant producing yellow colour in fresh colonies, but an olive green pigment in old cultures. Thus it would appear that *C. viridis* descends from a white or pale parental form, with a single symbiote, and an increment in symbiosis explains its origin as another species. On going deeper, another question arises, what gave rise to bacterial mutations from which one was ultimately selected by nature, as the most suitable one, or as the offering one the best protective coloration to the insect. As explained before, the red germ lives in the fatty tissues of the insect. A white insect, conspicuously misfit in its environment, chased by its enemies, would have to fly from place to place and thereby face starvation. An animal system undergoing hunger is prone to acidosis. This is not directly proved to be the case in insect metabolism, but is to be inferred from general physiology. With acidosis is associated dehydration and these combined conditions in the fatty tissue of the insect would induce mutation among the bacteria inhabiting it. This can be confirmed by observations on bacterial colonies *in vitro*. When cultures become old the medium becomes dry and acidic, both at the same time. Mutations occur most under such conditions. To go into further details would make it an independent problem of general biological interest transcending the particular one that is being handled here. Then a white insect, carrying a red germ, cannot settle down on luxuriant vegetation and, on the contrary, fleeing from enemies suffers from disturbed metabolism of its fatty tissue, with cellular acidosis, which induces mutation of the original red bacterium. Of the bacterial mutants the host insect, which carried a germ which produces a yellow to an olive green pigment survived best, and became *C. viridis*.

It further means that the relationship between the red and the yellow symbiotic germs is that between the donor of something useful and that of recipient of something beautiful. Of primary physiological importance is vitamin A, of which the precursor, carotene, is supplied by the red germ. The mutant of this bacterium is the yellow green germ, which confers the beautiful green colour for its protective coloration. Here is a clear case of a symbiote of primary importance, giving rise to a mutant of secondary importance. This might remind one of Herbert Spencer's classical essay on *Use and Beauty*, which itself is based on the pregnant observation by Emerson, that a useful respiratory pigment of the snail later imparts its body colour, or what we might call its beauty. In our case out of the red germ, primarily representing *use*, emanates a yellow green one imparting *beauty*, or its external appearance.

We are left finally with the phenomenon of mutation particularly in the red germ. It is always safe in such a case to cite an independent authority as precedent. Lieske[2] discovered a species of mycobacterium which produced red pigment, and gave rise to a yellow mutant; he yublished a colour reproduction, showing yellow growth in the midst of a red one. I cannot imagine a more impressive example to support the wide occurrence of mutations in this group of bacteria. The mutants illustrated here cover a period of no less than 10 years. Attention remained focussed only on colour changes for to extend the study to morphological and serological changes at the same time was beyond the capacity of a single worker. Then taking the fresh culture of *Mycobacterium carotinogen*, this was illustrated in Fig. 5, where growth is like a thin film, but with nodules on the surface. Older cultures are depicted in Fig. 7, 8 and 13. The margin of such a colony, shown enlarged in Fig. 8, a mutant of the red germ, proved that of a ring-like colony, Fig. 9, which instead of spreading further *horizontally*, began to grow *vertically*, so that the colony was lifted up, leaving the surface of agar and getting dried in the air. Another mutant from the same, however, could spread sideways, yet its structure was sufficiently different to enable an easy separation; both the cultures, the original and the mutant, are seen in Fig. 10. A mutant from the red germ, Fig. 8, gave an orange colony, whose margin is seen. in Fig. 11, enlarged to the same extent as Fig. 8, to show the precise difference in colour between the original red and the secondary orange bacterium. The same orange mutant further mutated into a producer of a green colony, both are shown enlarged in Fig. 12, as found in the original test tube. The original red germ, Fig. 7, is shown again in Fig. 13, and an orange mutant in Fig. 14. This in turn gave a second orange mutant shown in Fig. 15, all three were sub-

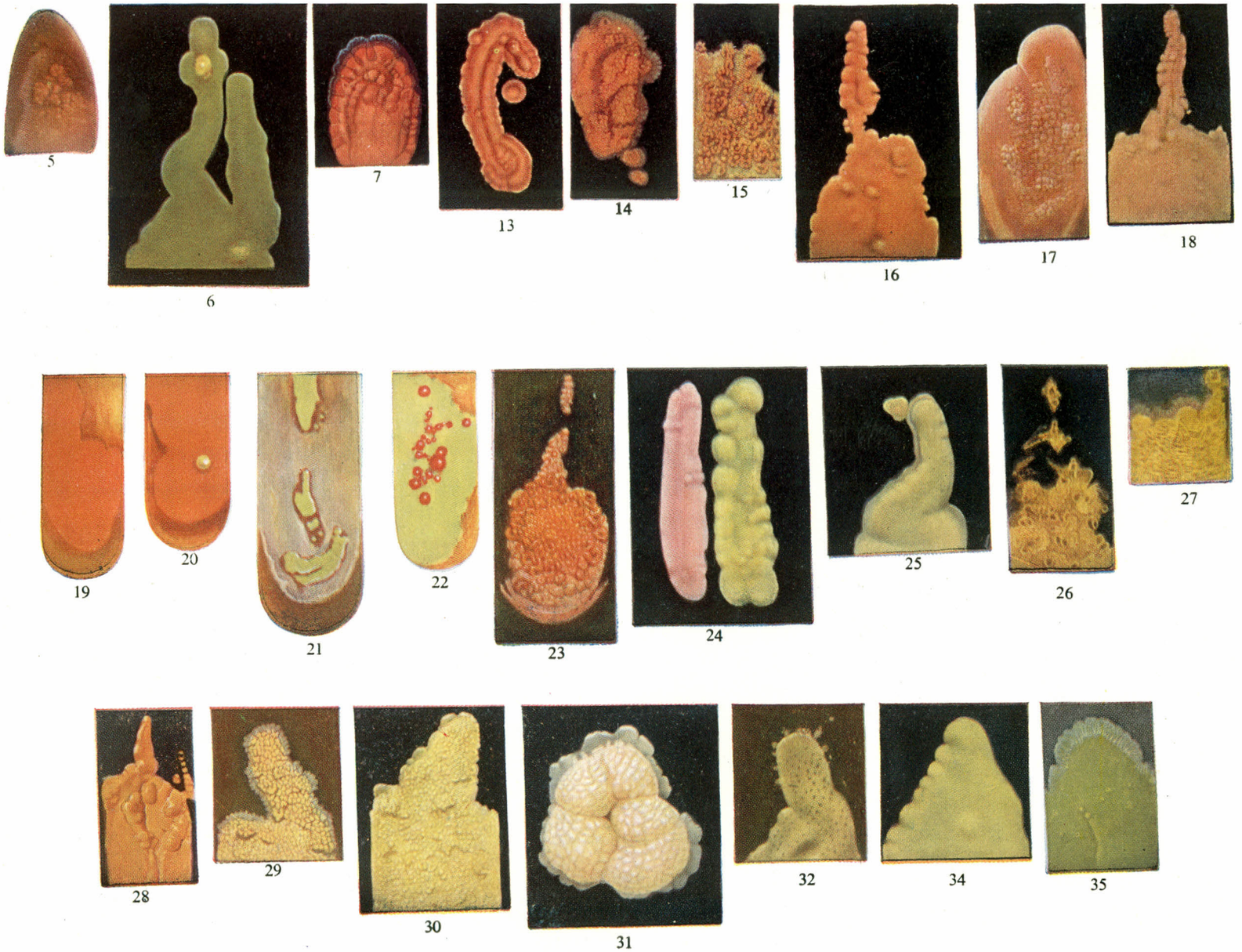

Fig. 5.—*Mycobacterium carotinogen*, the red germ, producing a thin film of fresh growth with nodule formations on the surface. Fig. 6.—An old culture of the yellow bacterium producing an olive green colour. Fig. 7.—Old culture of *M. carotinogen*, same as Fig. 5. Fig. 13.—*M. carotinogen*, grown at the same time as its mutants, Figs. 14 and 15. Fig. 14.—An orange mutant of *M. carotinogen*. Fig. 15.—Another mutant, more orange coloured than Fig. 14. Fig. 16.—A smooth salmon coloured mutant. Figs. 17 and 18.—A pink mutant, Fig. 17, in old culture resembling culture Fig. 16 but differing in shade; culture Fig. 16, grown again for comparison and shown as Fig. 18. Fig. 19.—A red mutant forming smooth colonies now named *Cornibacterium carotinogen*, and producing γ-carotene. Fig. 20.—An old colony of culture Fig. 19 producing a green mutant. Fig. 21.—A young growth of green mutant seen in Fig. 20. Fig. 22.—An old culture of the green bacterium, Fig. 21, producing a reverse red mutation. Fig. 23.—A rough colony of a red mutant producing a smooth pink mutant. Fig. 24.—The pink bacterium of Fig. 23, producing a white mutant, the two shown in streak cultures. Fig. 25.—The white mutant of Fig. 24 in old culture showing only a trace of pink. Figs. 26–29.—The orange mutant, Fig. 11, producing in series, mutants coloured "chrome yellow", of different shades. They differed even when grown as parallel streak cultures. Figs. 30–31.—A pale coloured mutant, Fig. 30; its minor colony enlarged, Fig. 31. Fig. 32.—A similar pale mutant producing crystals probably of phosphates. Fig. 34.— Smooth mutant of Fig. 33. Fig. 35.—Another mutant, deeper green in colour, with a frilled margin of its colony.

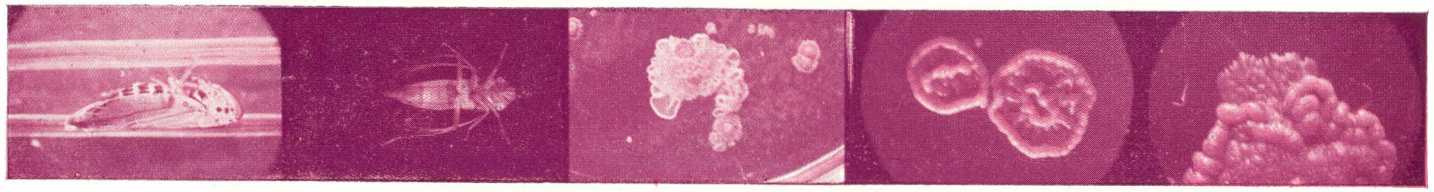

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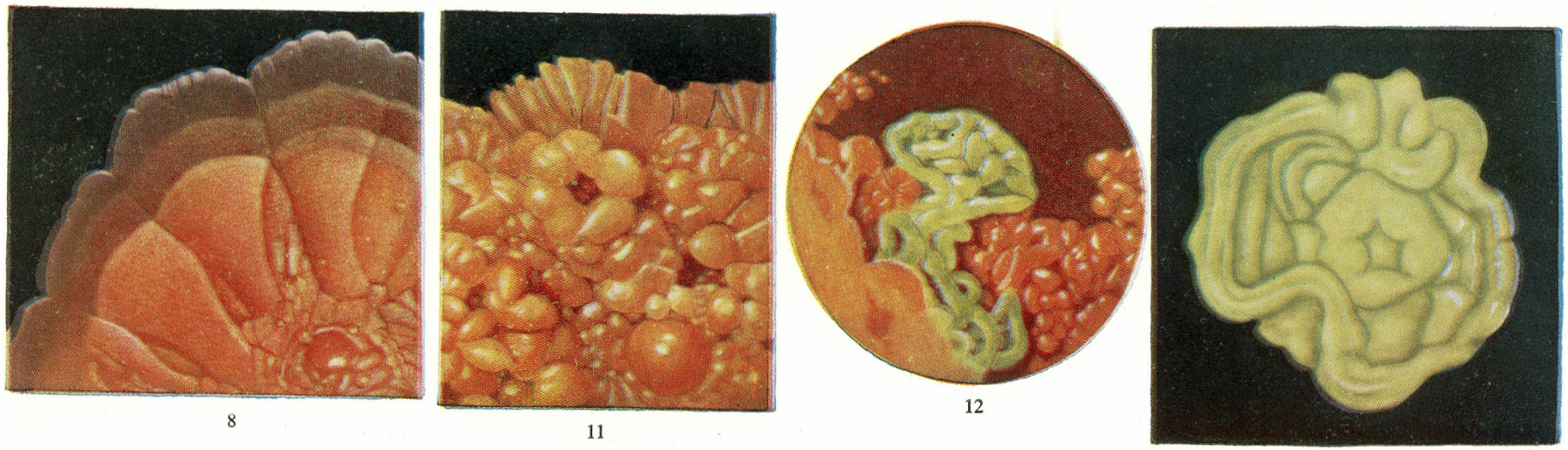
Fig. 1.—*Cicadella viridis*, female adult, with green wings and lemon yellow body showing traces of orange on the head and elsewhere. Fig. 2.—*C. viridis*, abdomen of female seen from below, coloured deeper lemon yellow with traces of orange and even of an olive green colour. Fig. 4.—Mixed colony of red and yellow bacteria; the main lemon yellow colour comparable with the body colour, Fig. 1. Fig. 9.—A colony of red mutant growing as a compact ring, not spreading horizontally but instead vertically; see text. Fig. 10.—A red mutant culture of Fig. 9 spreading horizontally.



8 11 12 33

Fig. 8.—Enlarged margin of a colony of Fig. 7. Fig. 11.—An orange mutant; margin of its colony enlarged. Fig. 12.—An orange bacterium, as in Fig. 11, producing a green mutant; the two bacterta seen in the original test tube and shown enlarged. Fig 33.—Green mutant Fig. 12 as a small rough colony, shown enlarged.

cultured at the same time and colour drawings made for comprison. I tried to show the difference in pigment formation by using a colorimeter, but the method did not prove easy enough to reveal the gradation in colour better than what I could easily detect and an artist could reveal in water colour drawings. A smooth colony, salmon coloured, also arose as a mutant, shown in Fig. 16. There further appeared a pink colony, which resembled Fig. 16, closely, but when grown side by side with it, they proved to be two different colour producing bacteria, Figs. 16 and 18. Figures 16 and 17 are identical whereas Fig. 18 is different from them.

Even though I tried to concentrate on colour changes of the mutants, morphological changes were also obvious. A red germ producing a smooth colony, Fig. 10, was handed over to Dr. Pradhan, who found that the pigment was no longer β-carotene but γ-carotene and morphologically the germ has to be named *Corynebacterium carotinogen*. In as much as the pigment of the germ, Fig. 5, was analysed in the Institute of Prof. R. Kuhn, and its morphological features studied under Prof. Oerskov, there can be no doubt of the form and function that characterised the original culture. That both these features changed has nevertheless to be admitted. The special advantage of studying mutation among colour-producing bacteria is the ease with which the human eye can detect the slightest change. The above study leaves the impression that probably other germs mutate likewise, and explains the numerous serologically different types we get, for example, in *B. coli*.

A mutant of the red germ, Fig. 5, which could be easily accepted as identical with the yellow/green, Fig. 6, was never met with. However others came close enough to maintain such a probability of having occurred in the fatty tissue of a starving insect body. An old culture of the smooth red colony, Fig. 19, produced a green mutant, Fig. 20; this germ Fig. 20, was not identical with Fig. 6, nor with the green mutant Fig. 12. Figure 21 shows a young growth of a green mutant, Fig. 20. An old colony of the same culture, Fig. 21, produced reverse mutation towards the red, appareatly identical with Fig. 19. These mutations were so frequent that I wondered if any two mutants, even though identical in colour, would prove to be the same even serologically. This is said to explain that an old colony of the green bacterium, Fig. 21, produced a reverse mutant towards the red, both shown in Fig. 22. If this red germ is identical with that of Fig. 19, or any other, can be questioned. And I should be inclined to state that no two mutants are identical, even though there appears no difference in colour. From a red mutant, producing a rough colony, arose a pink germ, forming a smooth colony both shown in Fig. 23. From the pink bacterium, Fig. 23, arose a faintly pink, almost white, mutant; for comparison both are shown in Fig. 24. The mutant was not quite free from pink shade, which is better seen in Fig. 25. The orange bacterium, Fig. 11, produced a series of mutants Figs. 26 to 29, of different shades of 'chrome yellow', the term being the one used in water colour painting. I can only assure the reader that the cultures were all pure and no time was spared to confirm this. Figures 30 and 31 show a pale colony, the latter has been enlarged. Figure 32 was the only culture characterized by a copious production of crystals, probably phosphates. Figure 33 is the green mutant illustrated in Fig. 12, here, as a small colony shown enlarged. Figure 34, shows a smooth mutant of Fig. 33. Figure 35 is another mutant, of culture, Fig. 34, whose green colour had a deeper shade, and its colony had a frilled margin.

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2. R. Lieskke, Morphologie u. Biologie der Strahlenpilize (1921). Leipzig. It contains a coloured plate.