CL. A NOTE ON THE LIPOCHROMES PRESENT IN CERTAIN BACTERIA.

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THE presence of lipochromes in bacteria, *i.e.* in plant cells free from chlorophyll, should afford an excellent opportunity for studying both the conditions of formation and the functions of these substances, which, so far as chemical examination has shown, appear to be highly coloured, rather unstable hydrocarbons or simple derivatives.

Zopf has published a series of papers [1889, 1, 2; 1892] in which he has described the "chromolipoids" in eight species of bacteria, a summary of his work being given by Palmer [1922]. In three organisms, *viz.* *B. egregium*, *B. chrysogloia* and *M. (staph.) aureus*, Zopf recorded fairly definite evidence for carotene formation, for besides his colour tests with acids and solubilities he found that the alcoholic solutions showed two absorption bands 492–497 μ (covering the F line) and 456–442 μ (between the F and G lines). In *Sphaerotilus roseus* he found a yellow water-soluble pigment and a red alcohol-soluble one, the latter showing the bands of carotene. In the other four cases his evidence is not convincing.

Practically the only other recorded work on the occurrence of lipochromes in bacteria is that of Schrotter [1895], who briefly reported experiments to show that the pigments of *Sarcina aurantiaca* and of *M. (staph.) pyogenes aureus* give the solubility properties and colour reactions with conc. H₂SO₄ of "lipoxanthin," his general term for a lipochrome. However, he did not study the absorption spectra. Consequently it seemed desirable, in view of the improved technique due to Willstätter and his colleagues [1907–13], Tswett [1906, 1] and others, to examine in detail the pigments produced by some of the coloured organisms.

An additional stimulus to this enquiry lay in the more or less general association of vitamin A with lipochromes in the tissues of higher plants exposed to the light [Steenbock and Cross, 1919; Rosenheim and Drummond, 1920; Steenbock, Sell and Boutwell, 1921; Steenbock and Sell, 1922; Coward, 1923], and the absence of vitamin A but presence of lipochromes in etiolated plant tissues [Coward, 1924, 2]. This suggested the advisability of making an examination of the coloured organisms for the presence of vitamin A.

The two organisms investigated in this work are *Sarcina aurantiaca* and *Streptothrix corallinus*. Both are readily cultivated and are deeply pigmented, but differ in form and method of multiplication.

A. *SARCINA AURANTIACA*.

Large cultures of this organism were grown in Roux bottles on broth agar. After about ten days the growth was scraped off the surface, ground with anhydrous sodium sulphate, and boiled with alcohol for five minutes. Practically the whole of the pigment was thus extracted. Preliminary tests for the general lipochrome reactions were carried out on the material extracted by the alcohol and gave positive results.

Solubility. Insoluble in H_2O . Soluble in alcohol, ether, light petroleum and chloroform.

Colour tests. Greenish-blue colour with conc. H_2SO_4 , HNO_3 , and I in KI solution.

Kraus' phase test. The alcoholic extract was evaporated to dryness in an atmosphere of nitrogen, dissolved in 5 cc. light petroleum, and an equal volume of 90 % alcohol added. The pigment was almost entirely "epiphasic," so probably little or no xanthophyll was present.

At this stage it seemed evident that probably more than one lipochrome was present, so it was decided to adopt Tswett's chromatographic method of analysis. Saponification of the organisms with alcoholic potash and subsequent extraction with ether and light petroleum did not give satisfactory results, owing to the formation of yellow resinous material during saponification which is also extracted by light petroleum. The method finally adopted was that used by Coward [1924, 1] for higher plant tissues, the massed organisms being saponified with aqueous potash, and 20 cc. alcohol being added immediately before extraction with ether. The process was carried out as far as possible in an atmosphere of nitrogen. The ethereal solution was evaporated to dryness and the unsaponifiable matter redissolved in light petroleum (B.P. 40–60°). The chalk column was prepared according to Tswett [1906, 2] and Coward [1924, 1], and 10 cc. of the light petroleum solution, now presenting a strong pinkish yellow appearance, were used.

Chromatograph of pigment. As soon as the pigment entered the chalk, three separate bands appeared: (a) a wide yellow band; (b) a sharp yellow band, rather more pink than (a); (c) a diffuse yellow band at top; but on further washing with light petroleum all three bands travelled downwards and out of the tube, thus confirming the phase test, as the slightest trace of xanthophyll should have persisted as a sharp band in the chalk, even after repeated washing. Fractional filtration through the chalk was now adopted. 25 cc. of the light petroleum solution of the pigment were allowed to pass slowly through the column, and thus divided into 2 cc. fractions, each being examined spectroscopically.

Fraction	Band I	Band II	Band III
I	509-489	478-458	446-
II	508-490	476-458	446-432
III	510-491	475-457	447-435
IV	509-490	477-457	447-435
V	509-489	477-459	447-435
VI	490-475	466-451	448-435
VII	490-474	458-439	—
	—	466-451	—
	—	456-440	—
VIII	490-476	456-440	—
IX	492-475	453-440	—
X	492-475	453-440	—
XI	491-477	458-441	—

Width of slit 0.08 mm. throughout.

If fractions VI and VII are discarded as mixtures, these figures seem fairly clear evidence that the pigment is a mixture of lycopin and carotene. Compared with results of other observers, the separation seems satisfactory.

Lipochrome	Band I	Band II	Band III	Observer
Lycopin	509-490	477-458	447-435	Average fractions I to V above
"	509-492	479-461	447-435	Coward [1924, 1]
"	507-490	476-457	443-433	" "
"	510-499	480-468	440 to end	Willstätter and Escher [1910]
Carotene	491-476	456-440	—	Average VIII to XI above
"	489-471	456-441	—	Coward [1924, 1]
"	492-475	460-445	—	Tswett [1911]
"	488-470	456-438	—	Willstätter and Mieg [1907]
"	490-475	455-445	430 to end	Kohl [1902]

Large quantities of the organism were then grown, saponified and extracted as above, and an attempt made to get crystal formation by approximately separating the lycopin and carotene by means of the chalk column, evaporating each solution to dryness in an atmosphere of nitrogen and redissolving each residue in alcohol to which a little CS_2 had been added, or in light petroleum.

(a) Crystals closely resembling lycopin were obtained on two occasions, once from alcohol and CS_2 , as clusters of needles growing longer on keeping in the dark, and once from light petroleum, as long prisms with fish-tail ends [cf. Willstätter and Escher, 1910, Fig. 5].

(b) The solution of carotene was much less concentrated than that of lycopin, and so far no crystals have been obtained.

Since lipochromes were being formed continuously by these lower plant cells, it was decided to study the effect of conditions of growth on pigment formation. Numerous agar slopes were prepared from the same sample of broth, and then inoculated from the same parent culture.

I. *Light.*

(a) 24 slopes in ordinary test-tubes incubated at 22° .

(b) 24 slopes in black test-tubes with a close-fitting dark cap over the cotton-plug, but allowing free access to the air, incubated at 22° .

The growth and pigmentation per slope appeared constant throughout, diffuse daylight apparently being quite without effect; next an attempt was

made to compare more accurately the ratio of pigment formed per gram of organism in the dark with that in the light. Roux bottles were used and the amount of growth per bottle in a given time was approximately the same in the two cases. The temperature throughout was 20°. As more flasks were used for the dark experiment, the results will be expressed as three fractions as follows:

- (1) $\frac{\text{Total weight of growth in flasks in light}}{\text{Total weight of growth in flasks in dark}} = \frac{4.68}{11.3} = 0.40;$
- (2) $\frac{\text{Weight of unsaponified matter from material in light flasks}}{\text{Weight of unsaponified matter from material in dark flasks}} = \frac{0.041}{0.117} = 0.35;$
- (3) By Hellige colorimeter.

$$\frac{\text{Amount of pigment from material in light flasks}}{\text{Amount of pigment from material in dark flasks}} = \frac{26}{65} = 0.40.$$

For (2) and (3) the organisms were saponified with aqueous potash and extracted as for chromatograph work.

Considering the experimental difficulties and the number of processes involved, one may consider the above figures in good agreement.

In all these experiments diffuse daylight was used; but when cultures of *Sarcina aurantiaca* were exposed to direct sunlight, growth was poor and no colour developed [cf. Hefferan, 1904]. Even old cultures which appeared dense yellow in colour became bleached if placed near a window where the sun could shine on them. Two thicknesses of glass seemed to afford no protection; however, on sub-culturing and removing from the strong light the new growths developed pigment as usual.

II. Temperature.

- (1) 24 slopes in ordinary test-tubes incubated at 37°.
- (2) 24 " " " " 20°.
- (3) 24 " black " " 37°.
- (4) 24 " " " " 20°.

At the lower temperature (sets (2) and (4)) growth was much more vigorous than at 37°, but pigment formation per milligram growth was equal in the four cases.

III. Hydrogen-ion concentration.

Trypsin agar of varied p_H used throughout.

Temperature 20°.

6	test-tubes on trypsin agar	$p_H = 5$	no growth
6	" "	$p_H = 6$	good healthy growth, porcelain-like surface, orange colour
6	" "	$p_H = 7$	" "
6	" "	$p_H = 8$	good healthy growth, porcelain-like surface, colour not so deep.

Initial growth seemed slightly more vigorous on the neutral agar ($p_H = 7$), but the total growth and pigmentation after ten days was the same in the

tubes at $p_H = 6, 7$ and 8 . More accurately graded tubes were used to show the upper and lower limits for growth and pigmentation, the p_H being determined by Mrs Kerridge with the glass electrode [Kerridge, 1925].

Pigment appeared even with the slightest growth.

Lowest limit for active growth: $p_H = 5.30$.

Maximum growth: $p_H = 7.15$.

Highest limit for active growth: $p_H = 9.43$.

B. *STREPTOTHRIX CORALLINUS*.

This organism [Reader, 1925] presents a brilliant coral colour when grown at room temperature on agar plates. It was chosen as an example of the higher bacteria, as it was thought that conditions might be different in these organisms from those in a *Sarcina*. The technique used was exactly the same as described above for the *Sarcina*. The general lipochrome reactions all gave positive results, and the alcoholic solution appeared to resemble a lycopin solution closely. It was slightly more difficult to extract than the pigment of *Sarcina aurantiaca*, but previous grinding of the organisms in a mortar with a little kieselguhr overcame this difficulty. When preparing the solution for the chromatograph it was noticed that the light petroleum solution had not the pinkish appearance of the alcohol or ether solutions.

Chromatograph. (a) One thin sharp layer travelled slowly down the tube, became irregular and stationary. (b) One dense wide band 0.2 inch in depth stayed at the top. On washing with light petroleum it widened very slightly but neither divided nor moved downwards.

The material in fraction (a) was dissolved out in ether, but the faintly yellow solution was too weak for spectroscopic examination. Fraction (b) was dissolved out in ether, and evaporated down slightly. On spectroscopic examination this solution showed two well-marked absorption bands.

	Band I	Band II
Strong	509-485	465-455
	505-485	465-453
	508-486	465-454
	505-485	466-454
Weaker	507-485	467-455

Width of slit 0.08 mm. throughout.

Neither light petroleum of B.P. 40-60°, nor of B.P. 60-80°, caused this pigment to be washed through the chalk column as lycopin would be.

As it was possible that the position of the bands had been shifted somewhat as a result of the saponification, the following comparisons were made:

	Band I	Band II
Direct extraction with hot alcohol	508-485	466-453
	507-485	465-454
	508-485	456-453
After saponification	508-485	465-453

A few flasks seemed to have a yellow rather than coral appearance. However, since the extracted pigment from these gave the same absorption

bands, probably the effect was due to quantitative rather than qualitative differences.

Band I	Band II
507-482	465-451
508-483	466-454

All attempts to divide this pigment into two parts by fractional filtration, even into as many as ten fractions, were unsuccessful. Hence it was decided that it must be another lipochrome, the absorption spectrum of which had not been previously recorded. The suggested name is "coralin."

The formation of pigment under different conditions was studied and has been reported in detail in another paper [Reader, 1925]. The agar slopes were prepared as for *Sarcina aurantiaca* and arranged in sets as before. It was observed that the cultures at a low temperature (20°) were highly pigmented both in the light and in the dark, but that even after six weeks no pigment had been formed at 37°. A second series more accurately graded showed that maximum coloration appeared at 18°, and that above 27° only traces of colour could be found. Growth was vigorous from 22° to 37°.

Slides were made regularly from every set and stained by Gram's stain. It soon became apparent that the development of pigment was parallel with the segmentation of the bacilli or rods. This is only the case when a broth or other complete medium is used. In synthetic medium (inorganic salts + glucose) there is no pigmentation even at 18°. When trypsin broth or agar is inoculated with this organism, both the segmentation and the development of pigment depend on the temperature, as above 27° the growth is rapid, filamentous, and colourless.

Direct sunlight did not have the effect of decoloration on this organism.

PRESENCE OF VITAMIN A IN THESE PIGMENTED ORGANISMS.

Considerable quantities of these organisms were grown in Roux bottles, saponified with alcoholic potash for 3 hours, extracted with ether, washed with boiled water, dried over sodium sulphate and evaporated nearly to dryness, the whole process being as far as possible carried out in an atmosphere of nitrogen. The unsaponifiable matter was mixed with medicinal paraffin in order to feed it in varying doses to rats which had become steady in weight on a vitamin A deficiency diet. No growth of the rats was obtained; also the colour tests for vitamin A [Rosenheim and Drummond, 1925] were negative. In the case of *Streptothrix corallinus* all the usual tests (Salkowski's reaction, Liebermann's reaction, Tschugaieff's reaction, Neuberg's reaction, etc.) for cholesterol and related compounds were negative. In the light of the recent work of Steenbock [1925] and of Drummond and Coward [1925] on the formation of the antirachitic factor by exposing cholesterol to ultra-violet light, this is interesting. The extract from *Sarcina aurantiaca*, however, gave good sterol reactions.

In a recent publication Luce and MacLean [1925] claimed that the presence of traces of vitamin A in ordinary yeast could be proved if large enough doses

of unsaponifiable matter were fed to vitamin A-deficient rats. In this connection one interesting fact has been noted in my preliminary extractions of unsaponifiable matter of large cultures of yeast. A concentrated solution of this extract was yellow in colour and gave definite carotene absorption bands when examined spectroscopically in both chloroform and ether solutions. Observations in these solvents by Kohl and by Coward are recorded for comparison.

Solvent	Band I	Band II	Observer
Chloroform	505-480	465-450	Kohl [1902]
"	505-480	465-450	Reader
"	506-483	465-450	"
"	506-483	465-450	"
Ether	490-475	455-445	Kohl [1902]
"	491-476	456-440	Reader
"	489-471	456-441	Coward [1924]

This unsaponifiable matter from yeast does not give the vitamin A colour tests with dimethyl sulphate, arsenic trichloride or trichloroacetic acid (50% solution in chloroform), so possibly it is only the antirachitic factor which is formed, for Steenbock [1925] has shown that this factor is frequently found in the absence of the growth-promoting fat-soluble vitamin. Using two methods of testing for the presence of antirachitic activation, viz. (a) the induction of growth and calcification in the rat during phosphate inhibition and (b) the cure of rickets produced by deficient phosphate and excess of calcium in the diet, he found that a large number of naturally occurring materials can be activated antirachitically by exposure to ultraviolet light. Among these are yeast, commercial casein, ox bile, lanolin and grains, and their commercial products such as starch, meals, flours, breakfast foods and oils.

SUMMARY.

(1) *Sarcina aurantiaca* contains two lipochromes, carotene and lycopin, the latter predominating.

(2) *Streptothrix corallinus* contains one lipochrome, apparently distinct from the well-known ones, for which the name "coralin" is suggested.

(3) Neither of these organisms forms appreciable quantities of vitamin A, even under the most favourable conditions as regards growth and pigment formation.

(4) Yeast contains traces of carotene.

(5) Some of the higher bacteria, e.g. *Streptothrix corallinus*, appear to contain no cholesterol or phytosterols.

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