Analysis and Comparison of the Carotenoids of *Sarcina flava* and *S. lutea*

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SUMMARY

The carotenoid pigments of *Sarcina flava* (*Staphylococcus afermentans*) and *S. lutea* were extracted, purified and separated by thin-layer chromatography into seven fractions. These fractions were hydrocarbon or polar materials. The two bacteria appeared to synthesize identical pigments which could be complexed *in vivo* to different proteins or to different amounts of a protein, which would explain the apparent differences in the colour of colonies of the two bacteria when grown on nutrient agar.

INTRODUCTION

A number of non-photosynthetic bacteria synthesize carotenoids which are usually of a highly polar nature (Goodwin, 1954). Although the pigments of *Sarcina lutea* have been investigated by several workers, those of *S. flava* (*Staphylococcus afermentans*) have received little attention. Chargaff & Dieryck (1932) proposed that *S. lutea* had two carotenoids present, from the evidence of partition between 90% methanol and light petroleum (b.p. 60–80°). They assumed that the epiphase consisted of one compound which they considered to be a hydrocarbon (sarcinene). They inferred the presence of a more polar carotenoid (probably a xanthophyll) which was hypophasic in this system. Further work by Chargaff (1933) seemed to confirm this. Nakamura (1936) suggested that a single esterified pigment was present in *S. lutea* whereas Takeda & Ōta (1941), from the same bacterium, obtained a crystalline compound which they considered was a xanthophyll (sarcinaxanthin). Sobin & Stahly (1942) examined *S. lutea* and *S. flava* and reported that the former contained two carotenoids (carotenols), whereas the latter had only one carotenol, identical with one of those found in *S. lutea*. They stated that no esters or carotenoid acids were present in either of these bacteria. Since these results seem inconclusive, a new investigation into the pigments of *S. lutea* and *S. flava* was undertaken.

METHODS

Bacteria. *Sarcina lutea* (strain NCTC 196) and *S. flava* (strain NCTC 7503) were obtained from the National Collection of Type Cultures, London, and stock cultures were maintained by cultivation alternately on nutrient agar and in nutrient broth (Oxoid Ltd.) in the dark at 30°. Bulk cultures were grown on nutrient agar containing 5% glucose, in oblong aluminium dishes sealed and sterilized inside nylon tubing.

Chemicals. The solvents were methanol (Analar), hexane (reagent grade redistilled before use) and diethyl ether, made peroxide- and moisture-free.

Pigment extraction procedures. Two extraction methods were used; the results from each were identical although the extraction of pigment by method (b) was not so complete.

Method (a). The bacteria were harvested into 10 ml. volumes of 95% methanol in water in 50 ml. centrifuge tubes and subjected to 5 min. ultrasonic disintegration at 0°. The combined suspensions were transferred to a 500 ml. round-bottomed flask and the volume made to 100 ml. A porous chip was added, the flask made light-proof with black material and the suspension refluxed at 80° for 3 min. After cooling and centrifugation, the bacterial sediments were re-extracted with absolute methanol which left them white. The pigment-containing supernatant fluids were combined, concentrated and stored under nitrogen at 0° in the dark.

Method (b). This method was used as a check since Hughes & Cunningham (1963) stated that ultrasonic disintegration can produce very high temperatures and pressures around the cone. We wished to see whether ultrasonic treatment and heating as used in method (a) were not having any deleterious effects on the pigments. Unfired porcelain was ground to a fine powder in a ball mill, and the harvested bacteria, suspended in methanol, were mixed with an equal volume of this powder, homogenized in a Potter homogenizer, centrifuged, and the supernatant fluid decanted. After four such extractions, the bacterial remnants were virtually white; the combined supernatant fluids were concentrated and stored under nitrogen at 0° in the dark.

Purification of the total extract. The lipid precipitation technique of Blessin (1962) was followed and the ether of the resultant supernatant fluid was removed on a rotary evaporator under reduced pressure at 34°. A pigment solution was saponified in 10% (w/v) KOH in methanol overnight in the dark at room temperature and in an atmosphere of nitrogen. The unsaponifiable material was extracted into ether in the usual way.

Separation of the pigments. Thin-layer chromatography on silica gel G (Merck) washed with chloroform was used. To obtain reproducible R_F values, the conditions of Dallas (1965) were used. Layers 0.25 mm. thick on 200 × 200 mm. plates were used and the plates developed in an S-chamber (according to the design of Davies, 1963) in a constant temperature room at 15°. The plate and cover were clipped firmly together; this enabled development to be done in a normal thin-layer chromatography tank previously equilibrated with solvent. The solvent quoted by Rothblat, Ellis & Kritchevsky (1964) was used and was benzene + methanol + acetic acid (87 + 11 + 2 by vol.). In all, twelve plates were developed to ensure reproducibility. After development, the individual spots were identified by: (i) visibility; (ii) behaviour under ultraviolet radiation; (iii) spraying with a saturated solution of antimony trichloride in chloroform (Morton, 1942); (iv) treatment with iodine vapour (Truter, 1963); (v) spraying with 2.5 M-H₂SO₄. The last two detected not only carotenoids, but also their colourless precursors.

For preparative thin-layer chromatography, solutions of the carotenoids in ether were applied to a 0.5 mm. layer on 200 × 200 mm. plates by using the mechanical applicator produced by Desaga (Camlab (Glass) Ltd.). For optimum resolution only 200–300 µg. material could be applied to each plate. The plates were developed in the same solvent as before; the S-chamber was not used. After development of the plates, they were dried in a stream of nitrogen. The individual fractions were removed from the plates, eluted first into methanol and then into ether which was thoroughly washed

with water to remove any traces of acetic acid. The pigments were again taken to dryness and redissolved in methanol for storage as before.

Comparison of the pigments. (i). Spectra were determined in spectroquality hexane (British Drug Houses Ltd.) and in methanol (Analar) with a Unicam SP.800 spectrophotometer. The wavelength calibration was checked with a holmium filter and was correct to within $\pm 1.4 \text{ m}\mu$.

(ii). Each fraction was partitioned between 95 % (v/v) methanol in water and hexane according to Petracek & Zechmeister (1956).

(iii). The *cis* or *trans* configuration of each pigment was determined by the iodine isomerization test (Zechmeister & Polgár, 1943).

(iv). The following method was used to test for 5-6 epoxides (Curl & Bailey, 1954; Jungalwala & Cama, 1962). The pigment was dissolved in methanol; to 3 ml. in a 1 cm. Unicam glass cuvette, one drop of a solution of 0.05 N-HCl in methanol was added. The λ_{max} were read before and after addition of the acid methanol and again after 10 min. A spectral shift of 20-25 $\text{m}\mu$ is characteristic of a 5-6 epoxide group.

(v). An HCl test outlined by Karrer & Jucker (1950) was done; a blue colour is given by certain structural features, e.g. epoxides, aldehydes and possibly by carotenoids with several hydroxyl groups.

RESULTS

Chromatographic comparison of the total pigment before and after saponification suggest that before saponification, some carotenoids tended to associate, probably by occlusion, with polar lipids which were saponifiable. There was no evidence for any simple carotenoid esters. Chromatography of the purified pigments from the two bacteria showed that both synthesized identical pigments. The results of the tests carried out on the individual fractions are shown in Table 1.

Table 1. *Characteristics of the pigments of Sarcina flava and S. lutea*

Pigment	R_F (S-chamber)	λ_{max} , methanol ($\text{m}\mu$)	λ_{max} , hexane ($\text{m}\mu$)	Partition coeff.	<i>Cis/trans</i>
1	0.96	—, 415, 439, 469	—, 415, 437, 466	0/100	All <i>trans</i>
2	0.60	—, 415, 439, 469	—, 415, 437, 466	27/73	All <i>trans</i>
3	0.42	—, 415, 439, 469	—, 415, 437, 466	71/29	All <i>trans</i>
4	0.41	331, 413, 436, 466	331, 412, 435, 464	71/29	<i>cis</i> isomer
5	0.26	331, 413, 436, 466	Insoluble	100/0	<i>cis</i> isomer
6	0.19	—, 415, 439, 469	Insoluble	100/0	All <i>trans</i>
7	0.06	331, 413, 436, 466	Insoluble	100/0	<i>cis</i> isomer

Pigment	HCl spectral shift	Colour with HCl	Colour with SbCl_3	Type
1	—	Nil	Brown	Carotene
2	—	Nil	Blue	Monohydroxy
3	—	Nil	Blue	Dihydroxy
4	—	Nil	Blue	Dihydroxy
5	—	Nil	Blue	Poly -OH
6	—	Nil	Blue	Poly -OH
7	—	Nil	Blue	Poly -OH

DISCUSSION

The extreme polarity of many of the compounds meant that their extraction was only possible with polar solvents such as methanol. The effectiveness of methanol may be aided by its denaturing effect on the bacterial proteins with which at least some of the pigments are complexed. Such pigment-protein complexes in *Sarcina lutea* were suggested by Matthews & Siström (1959), and it has been suggested that in *Micrococcus lysodeikticus* the pigments are bound to the cell membrane (Gilby, Few & McQuillen, 1958). That no deleterious effect on the carotenoid as a result of using ultrasonic treatment on organic solutions was found, agrees with the findings of Zolotoba (1965).

Thin-layer chromatography provided an excellent method with the solvent system used for the preparative isolation of seven pigment fractions in a chromatographically pure state. The advantage of the S-chamber in the early stages of this work meant that the spots were very compact and that R_F values were reproducible. The use of the S-chamber in an ordinary thin-layer chromatography tank overcame the problem of solvent evaporation from the solvent trough which can occur when using commercially available S-chambers.

Table I gives an indication of the type of carotenoids which these two bacteria synthesized. It would appear that the pigments of these two bacteria are similar. They have the same chromophoric group and, from the positions of the $\lambda_{\max}$, a system of nine conjugated double bonds is suggested. Colonies of the two bacteria do not look alike when grown on nutrient agar and this difference may be due to differences in the concentration of the individual pigments or to the way in which they exist *in vivo*. If the pigments are bound to protein, different proteins or different protein: pigment ratios may be involved, which might cause a shift in the $\lambda_{\max}$ in the visible region.

Rothblat *et al.* (1964) published details of the carotenoids of *Micrococcus lysodeikticus*; its pigments have a similar $\lambda_{\max}$ value to those of the sarcinas examined here, but the R_F values and partition values quoted mostly differ from those found here. *Micrococcus lysodeikticus* and *Sarcina flava* are possibly two of the many different strains of *Staphylococcus afermentans* (Dr S. P. Lapage, private communication). The characteristic of strains of *S. afermentans* is that they do not produce acid in peptone water + glucose or in the medium of Hugh & Leifson (1953). Thus it is not surprising that similarities were found; as Sobin & Stahly (1942) remarked, different strains of some species of bacterium may well be found to produce the same pigments.

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