ASSOCIATION OF CAROTENOID PIGMENTS WITH PROTEIN COMPONENTS IN NON-PHOTOSYNTHETIC BACTERIA

by

SIDNEY SAPERSTEIN* AND MORTIMER P. STARR

Department of Bacteriology, University of California, Davis, Calif. (U.S.A.)

The association of plant pigments with protein has been demonstrated by several investigators, such as HERLITZKA[1] and LUBIMENKO[2]. That a similar situation held for the photosynthetic bacteria was suggested by LUBIMENKO[3]; this was substantiated when such preparations were made by other workers (LEVY, TEISSER AND WURMSER[4]).

The study on the photosynthetic bacteria was continued by FRENCH[5,6] who demonstrated that both the chlorophyll and the carotenoid complexes precipitated from extracts upon the addition of ammonium sulfate to half saturation. More recent work by PARDEE, SCHACHMAN AND STANIER[7]; SCHACHMAN, PARDEE AND STANIER[8] using the ultracentrifuge, showed that these pigments seemed attached to a specific protein in the photosynthetic bacterium *Rhodospirillum rubrum*. They believe that these complexes are comparable with the plastids of higher plants and should be termed chromatophores.

The present contribution stems from an interest in the carotenoid pigments of non-photosynthetic micro-organisms, particularly, the phytopathogenic coryneform bacteria. There are no prior reports on the association of carotenoids with other cellular constituents in these organisms.

The aim of this investigation, therefore, was to examine this association in the hope that this knowledge eventually would help elucidate the role of polyene pigments in micro-organisms.

The carotenoids in the different organisms studied here include compounds shown by STARR AND SAPERSTEIN[9]; SAPERSTEIN, STARR AND FILFUS[10]; SAPERSTEIN AND STARR[11] to be methoxyl, hydroxyl, hydrocarbon and ketonic polyenes. Demonstration with so diverse an assemblage of pigments, of an association of microbial carotenoids with protein components, would provide good support for the hypothesis that this association is general. The use of colourless mutants would also permit one to verify the lack of these particles in non-pigmented cells as observed previously with almost colourless *R. rubrum* cells grown aerobically in the dark (SCHACHMAN *et al.*[8]).

MATERIALS AND METHODS

Cultures. The organisms in this study were authentic strains of *Corynebacterium poinsettiae* CP2 (STARR[12]) and *Corynebacterium michiganense 4702, 4938, 4944, 4939:* yellow, orange, pink, and colourless, respectively (ARK[13]). In addition to these phytopathogens, cultures of *Mycobacterium phlei* and *Micrococcus agilis*, from the collection at this laboratory, were studied.

* Present address: Biochemical Research Laboratory, Special Products Division, The Borden Co., Yonkers 3, New York.

References p. 488.

Medium. The organisms were cultured in a medium having the following composition/100 ml final volume: peptone, 1 g; yeast extract (Difco), 0.5 g; glucose, 1 g; pH 6.8.

The cells were harvested by centrifugation in a Sharples Super Centrifuge after incubation for 4 days at 28° on a shaking machine as described by STARR AND SAPERSTEIN[9]. The cells were then washed once with distilled water.

Cell-free extracts. The cells were broken by grinding with alumina according to the method of MCILWAIN[14] and extracted with 0.05 *M* NaCl. The entire mixture was centrifuged at low speed (Servall SPX, 5,000 r.p.m.) to remove the alumina and coarse cellular debris. The supernate was clear and coloured (except for the colourless strain *49,39*); the colour corresponded to the colour of the cell mass before grinding.

Concentrated solutions of the protein-carotenoid complex were prepared by centrifuging the extracts (Spinco Model L preparative centrifuge) for 2 h at an average of 105,000 × *g*. The sedimented material was resuspended in 0.05 *M* NaCl or in distilled water. These concentrates were then examined in the analytical ultracentrifuge to observe colour boundaries, and to determine the sedimentation constants of the protein components. The Spinco Model E analytical ultracentrifuge, equipped with a Philpot-Svensen optical system, was used for this purpose. The average centrifugal field was 160,000 × *g*.

RESULTS

General properties of the protein-carotenoid complex. The coloured extracts obtained from the various organisms were examined with a Zeiss hand spectroscope; absorption bands were visible and corresponded to those observed when the major carotenoids from the various strains were dissolved in benzene. The extracts were also examined for absorption bands in the Beckman spectrophotometer Model DU. Results were similar to those described above. Addition of small amounts of iodine (*ca.* 0.02 ml of a 5% ethanolic solution), insolation, or boiling the extracts for several minutes, did not shift the position of the absorption maxima; nor did this treatment coagulate the carotenoid-bearing protein as tested by centrifuging the solutions for 10 min at 5,000 r.p.m.

Addition of $[NH_4]_2SO_4$ to a final concentration of *ca.* 70% precipitated the carotenoid-bearing protein; the supernate became colourless. The precipitate was then redispersed by dialyzing against distilled water. Precipitation with $[NH_4]_2SO_4$ altered the protein, since the reconstituted protein-carotenoid complex showed a much higher sedimentation value following this treatment. Addition of ethanol to the extracts denatured the protein and the carotenoids were liberated into the aqueous ethanol. The carotenoids could be extracted from these alcoholic solutions with the usual fat solvents, *e.g.* petroleum ether, chloroform, and benzene.

The original protein extracts, and the sedimented protein-carotenoid complex, gave positive biuret reactions.

Ultracentrifugal and electronmicroscopic data. The ultracentrifugal patterns obtained with extracts from the organisms under study (before concentration by the methods described above) resembled those obtained from the pigmented and non-pigmented organisms studied by other workers (SCHACHMAN *et al.*[8] SIEGEL, SINGER AND WILDMAN[15]).

A typical pattern is shown in Fig. 1. When the carotenoid-bearing component was concentrated by differential centrifugation, before examination in the analytical centrifuge, a definite colour boundary formed. In extracts of *C. michiganense*, the pigment appeared to move with the protein component which had a sedimentation constant of *ca.* 35 *S**. In Figs. 3 and 4, two such patterns are presented: one represents the complex obtained from *C. michiganense 4938* (an orange colour mutant having as the major carotenoid the ketone canthaxanthin), the other represents *C. michiganense 4944* (pink

* Sedimentation constants are reported in Svedbergs: 1 *S* is 10^{-13} cm/sec/unit field.

References p. 488.

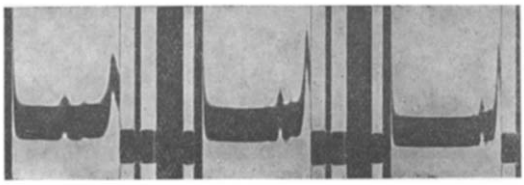

Fig. 1. Ultracentrifugal pattern of a dilute extract of *Corynebacterium poinsettiae* (high-thiamine cells). The right hand picture was taken 4 min after reaching a speed of 42,040 r.p.m. The succeeding pictures were taken at 4 min intervals.

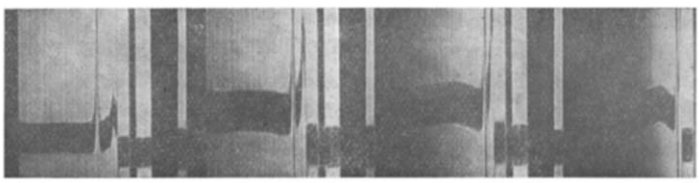

Fig. 2. Ultracentrifugal pattern of concentrated extract of *Corynebacterium poinsettiae* (high-thiamine cells). The pigment is moving with the first protein component: sedimentation constant of this fraction is 165 *S*. Sedimentation proceeds to left. Sedimentation constant of the second component is 30 *S*.

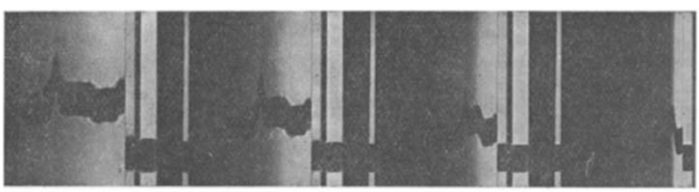

Fig. 3. Concentrated cell extract from *Corynebacterium michiganense 4938*. Pigment is moving with the 35 *S* component (first peak on the left side).

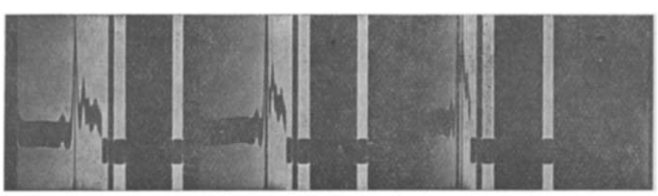

Fig. 4. Concentrated cell extract from *Corynebacterium michiganense 4944*. Pigment is moving with the 35 *S* component (second peak on the left side).

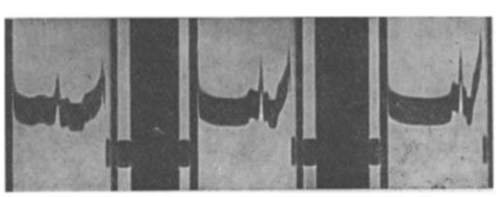

Fig. 5. Concentrated extract from *Corynebacterium michiganense 4939* (colourless mutant). Sedimentation constants of the faster moving components (left to right) are *ca.* 54 *S*, 42 *S*. Pictures were taken at 4 min intervals after reaching a speed of 59,780 r.p.m.

colour mutant having the methoxylated carotenoid spirilloxanthin as its major pigment). The colourless mutant of *C. michiganense, 4939*, did not show a pattern similar to the two colour mutants listed above, and the appearance of a 35 *S* fraction was very doubtful or, at least, markedly reduced (Fig. 5).

In the pattern obtained from *C. poinsettiae* grown under conditions corresponding to the high thiamine concentration used by STARR AND SAPERSTEIN[9], the pigment boundary moved with a non-homogeneous protein fraction having a sedimentation constant of *ca.* 165 *S* (Fig. 2). As shown by STARR AND SAPERSTEIN[9], *C. poinsettiae* synthesizes predominately the mono-hydroxy carotenoids cryptoxanthin and lycoxanthin, when grown on a high-thiamine medium; perhaps the type of carotenoid has some bearing on the sedimentation constant of the associated protein.

The concentrated protein-carotenoid complexes of *4944* and *4938*, collected by differential centrifugation, showed spherical particles in the electron microscope (Figs. 6 and 7); their estimated diameter was approximately 210 A. Estimation of particle size from sedimentation data gives a value of *ca.* 150 A (assuming a spherical particle).

The extracts of *C. michiganense 4702*, *M. agilis*, and *M. phlei* were not examined in the analytical ultracentrifuge. However, in all other respects, extracts from these cells resembled those which were studied in greater detail.

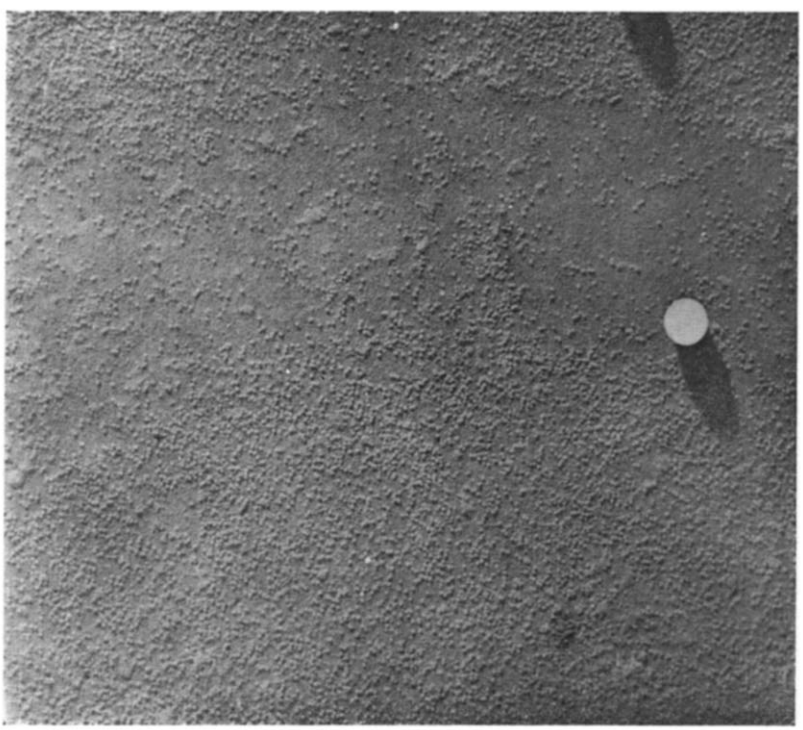

Fig. 6. Electron micrograph of the 35 *S* particles from *Corynebacterium michiganense* 4944 (pink mutant). The polystyrene particle in the photograph is 260 mμ in diameter.

References p. 488.

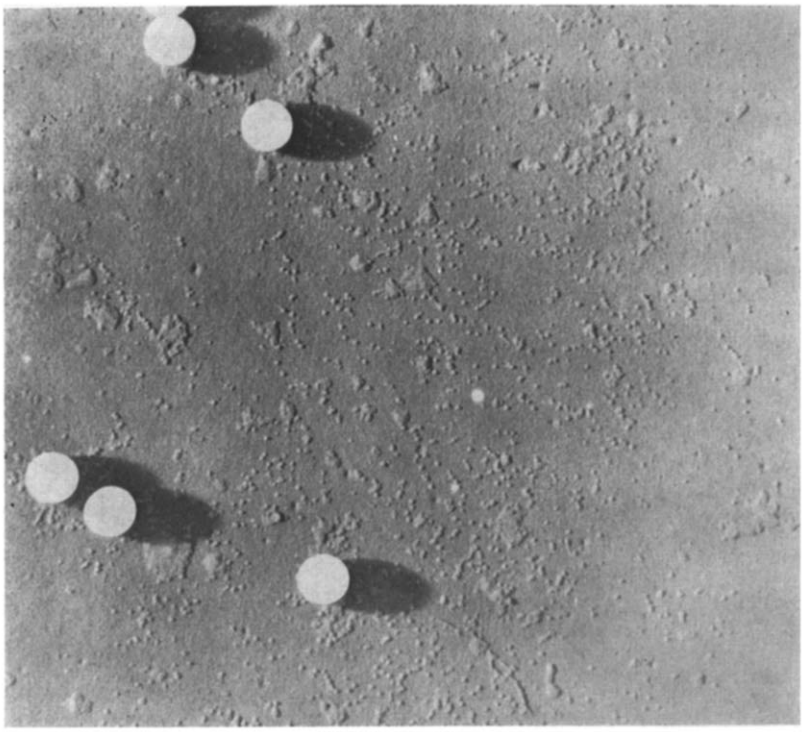

Fig. 7. Electron micrograph of the 35 *S* particles from *Corynebacterium michiganense 4938* (orange mutant). The polystyrene particles are 260 mμ in diameter.

DISCUSSION

The carotenoid pigments of the non-photosynthetic microorganisms which we have studied, in concordance with observations by others on the photosynthetic bacteria (LEVY *et al.*[4]; FRENCH[5]; and SCHACHMAN *et al.*[8]), are associated in these cells with specific protein particles. These studies include three different genera; association of carotenoid with protein, therefore, may be widespread in bacteria and not limited to photosynthetic micro-organisms. With *C. michiganense*, as with the photosynthetic bacterium, *R. rubrum*, when the formation of pigments is hindered, the formation of the specific protein component is likewise hindered. The only condition examined for *C. michiganense* was the inhibition of carotenogenesis in an induced colorless mutant. In this strain there was a marked decrease of the 35 *S* particle fraction when equivalent amounts of the colourless cells were extracted and examined in the analytical ultracentrifuge.

It is, of course, not possible to decide from these observations whether the formation of carotenoid pigments in these micro-organisms is dependent upon the existence of the protein(s) with which they are found associated. Indeed, the failure of the writers to find a 35 *S* particle fraction in the cell extracts of *C. michiganense 4939* (colourless mutant) does not preclude the existence of such a protein in these cells. It is possible that the sedimentation constant of the protein-carotenoid complex is in part governed by the attachment of the carotenoid; failure of the cell to produce the pigment could then alter the sedimentation constant of the associated protein.

References p. 488.

In the extracts, absorption maxima were observed at about the position corresponding to that of the isolated pigment dissolved in benzene. However, when the extracts were subjected to conditions (*e.g.* light, I_2) which generally result in *cis*⇄*trans* isomerization and concurrent shifts in absorption maxima of carotenoids in organic solvents, no such shifts in absorption maxima were observed. These results suggest that the conditions which cause isomerization of carotenoids when in solutions of organic solvents, do not affect carotenoids associated with protein, possibly because the carotenoid molecule is so attached to the protein that rotation around certain ethylenic bonds is prevented.

It seems a reasonable supposition that the carotenoids of bacteria are formed in the cell in an association with specific protein. Conversely, one might speculate on the possibility that the pigment may be required for protein synthesis.

Further exploration is of course needed to test these hypotheses.

ACKNOWLEDGEMENTS

We are indebted to Drs. R. C. WILLIAMS and H. K. SCHACHMAN, Virus Laboratory, University of California, Berkeley, California, in whose laboratories the electron micrographs and ultracentrifugal data were obtained. We thank Dr. R. Y. STANIER, Department of Bacteriology, University of California, Berkeley, for the discussions which led to this study.

SUMMARY

The relationship between carotenoids and other cellular constituents was studied in the phytopathogenic coryneform bacteria *Corynebacterium poinsettiae* and *Corynebacterium michiganense* (including colour mutants), and also in two non-pathogenic bacteria: *Mycobacterium phlei* and *Micrococcus agilis*.

The polyene pigments in these organisms are associated with protein components. The pigment-protein complex is extracted from the ruptured bacterial cells with the usual protein solvents. The complex is relatively stable and, while attached to the protein moiety, the carotenoids do not show reversible *cis*⇆*trans* isomerization.

Analytical ultracentrifugation shows that the carotenoids of *C. michiganense* are associated with particles having a sedimentation constant of about 35 *S*, and that such particles were not demonstrable in colourless mutants.

RÉSUMÉ

Les auteurs ont étudié la relation entre les caroténoïdes et les autres constituants cellulaires chez les bactéries coryniformes phytopathogènes, *Corynebacterium poinsettiae* et *Corynebacterium michiganense* (ainsi que des mutants colorés), et chez deux bactéries non pathogènes: *Mycobacterium phlei* et *Micrococcus agilis*.

Les pigments polyéniques de ces organismes sont associés à des constituants protéiques. Le complexe protéine-pigment peut être extrait des cellules bactériennes désintégrées, par les solvants usuels des protéines. Le complexe est relativement stable et, tant qu'ils sont fixés à la moitié protéique, les caroténoïdes ne subissent pas d'isomérisation *cis*⇆*trans* réversible.

L'ultracentrifugation analytique montre que les caroténoïdes de *C. michiganense* sont associés à des particules dont la constante de sédimentation est d'environ 35 *S*, et que de telles particules sont absentes chez les mutants incolores.

References p. 488.

ZUSAMMENFASSUNG

Die Beziehungen zwischen Karotenoiden und anderen Zellbestandteilen in den coryneartigen, phytopathogenen Bakterien *Corynebacterium poinsettiae* und *Corynebacterium michiganense* (einschliesslich Farbmutanten), sowie in den zwei nicht pathogenen Bakterien *Mycobacterium phlei* und *Micrococcus agilis*, wurden untersucht.

Die Polyenpigmente dieser Organismen sind an Eiweisskomponenten gebunden. Der Pigment-Proteinkomplex kann aus den geplatzten Bakterienzellen mit den üblichen Proteinlösungsmitteln extrahiert werden. Der Komplex ist verhältnismässig stabil und solange die Karotenoide an die Eiweisskomponenten gebunden bleiben, tretet an ihnen keine reversible *Cis*⇆*Trans* Isomerisierung ein.

Mit Hilfe der analytischen Ultrazentrifuge wurde festgestellt, dass die Karotenoide von *C. michiganense* an Teilchen, welche eine Sedimentationskonstante von 35 *S* aufweisen, gebunden sind, und dass solche Teilchen in farblosen Mutanten nicht nachweisbar waren.

REFERENCES

1 A. HERLITZKA, *Biochem. Z.*, 38 (1912) 321.
2 V. LUBIMENKO, *Rev. gen. botan.*, 39 (1927) 619.
3 V. LUBIMENKO, *Botan. Zhur. S.S.S.R.*, 6 (1921) 107.
4 R. LEVY, G. TEISSER AND R. WURMSER, *Ann. physiol. physiochim. biol.*, 1 (1925) 298.
5 C. S. FRENCH, *J. Gen. Physiol.*, 23 (1940*a*) 469.
6 C. S. FRENCH, *J. Gen. Physiol.*, 23 (1940*b*) 483.
7 A. B. PARDEE, H. K. SCHACHMAN AND R. Y. STANIER, *Nature*, 169 (1952) 282.
8 H. K. SCHACHMAN, A. B. PARDEE AND R. Y. STANIER, *Arch. Biochem. and Biophys.*, 38 (1952) 245.
9 M. P. STARR AND S. SAPERSTEIN, *Arch. Biochem. and Biophys.*, 43 (1953) 157.
10 S. SAPERSTEIN, M. P. STARR AND J. A. FILFUS, *J. Gen. Microbiol.*, 10 (1954) 85.
11 S. SAPERSTEIN AND M. P. STARR, *Biochem. J.*, 57 (1954) 273.
12 M. P. STARR, *J. Bact.*, 57 (1949) 253.
13 P. A. ARK, *J. Bact.*, 61 (1951) 293.
14 H. MCILWAIN, *J. Gen. Microbiol.*, 2 (1948) 186.
15 A. SIEGEL, S. J. SINGER AND S. G. WILDMAN, *Arch. Biochem. and Biophys.*, 41 (1953) 278.

Received October 25th, 1954