Arch. Mikrobiol. 84, 339–349 (1972)

Nutritional Control of Pigment and Isoprenoid Compound Formation in Extremely Halophilic Bacteria

M. B. Gochnauer, S. C. Kushwaha, M. Kates, and D. J. Kushner

Departments of Biology and Biochemistry,
University of Ottawa, Ottawa, Canada

Received March 14, 1972

Summary. 1. Cells of the extremely halophilic bacteria, *Halobacterium cutirubrum* and *H. halobium* grown in a chemically defined medium (BSMK) were red due to the presence of bacterioruberins (maxima, 370, 388, 494 and 527 nm). Adding 0.1% glycerol to BSMK stimulated growth, but cells rapidly lost bacterioruberins becoming greyish purple in the stationary phase. Acetone extracts of these cells were yellow with a broad absorption band at 360–390 nm, partly attributable to retinal. In BSMK medium with or without glucose, the bacterioruberin concentration increased until maximal growth was reached, then fell rapidly.

2. In complex medium (CM) cells formed less bacterioruberins than in BSMK. Adding 0.1% glycerol to CM stimulated growth but did not change pigmentation; adding glucose only slightly stimulated growth but greatly increased bacterioruberin formation. Exposure to visible light did not affect growth or pigmentation.

3. Addition of glycerol or glucose to BSMK increased the formation (relative to squalene) of dihydrosqualene, tetrahydrosqualene, and vitamin MK_8. Higher levels of these compounds were found in cells grown in CM than in BSMK. Though glycerol decreased the formation of bacterioruberins it increased the formation of β-carotenes. Glucose increased the formation both of bacterioruberins and β-carotene. A preliminary hypothesis to account for the effects of nutrients on pigmentation is presented.

Only a few reports have appeared on the influence of growth conditions on the pigments of extremely halophilic bacteria. Nandy and Sen (1963) studied the effects of different salt concentrations, moisture content, pH and temperature on growth and pigment formation (absorbance at 494 nm per unit weight of cells) by *Sarcina litoralis* growing on plates of complex media. These conditions could greatly affect growth, but pigmentation varied little. Brown and Stevenson (1971) found that the 494 nm pigment of *Halobacterium salinarium* growing in a liquid complex medium at 37°C rose to a maximum at about 3 days and then declined.

We recently studied the growth in defined amino acid containing media of extremely halophilic bacteria of the genus *Halobacterium* (Gochnauer and Kushner, 1969). Normally cultures were red or pink. If glyce-

rol, which stimulated growth, was added the color of the cultures quickly changed to greyish purple. These phenomena have now been studied in more detail by following changes in visible spectra of carotenoid pigments extracted from cells grown under different conditions. Pigments and related isoprenoid compounds known to be present in *Halobacterium* species are the red C_{50} bacterioruberins (Jensen, 1962; Kelly *et al.*, 1970), C_{40} carotenes (Kushwaha *et al.*, 1972), isoprenoid compounds such as squalene, dihydrosqualene, tetrahydrosqualene and vitamin MK_8 (Tornabene *et al.*, 1969; Kushwaha *et al.*, 1972), and retinal in the bacteriorhodopsin of *H. halobium* (Oesterhelt and Stoeckenius, 1971; Blaurock and Stoeckenius, 1971). Changes in relative amounts of most of these isoprenoid compounds were also followed by thin-layer chromatography and were correlated with the spectral changes.

Materials and Methods

Organisms

The strains used were: *Halobacterium cutirubrum* NRC 34001 and *H. halobium* Verhoeven NRC 34020, the same as used in previous work (Gochnauer and Kushner, 1969). The media used were: a) Basal synthetic medium + potassium ion (BSMK), containing 15 amino acids, nucleotides, and the following salts (in g per 100 ml): NaCl, 25; KCl, 0.2; NH_4Cl, 0.5; $MgSO_4 \cdot H_2O$, 2; KNO_3, 0.01; K_2HPO_4, 0.005; KH_2PO_4, 0.005; sodium citrate, 0.05; $MnSO_4 \cdot 7H_2O$, 3×10^{-5}; $CaCl_2 \cdot 7H_2O$, 7×10^{-4}; $ZnSO_4 \cdot 7H_2O$, 4×10^{-6}; $FeCl_2$, 2.3×10^{-4}; $CuSO_4$, 5×10^{-6}; pH 6.5. b) Complex medium (CM), containing casamino acids, yeast extract, 25% NaCl, 0.2% KCl, 2% $MgSO_4 \cdot 7H_2O$, and other salts (Gochnauer and Kushner, 1969; Sehgal and Gibbons, 1960).

A preculture of cells in BSMK was grown for 2 days with shaking at 37° C. Inocula of 1/100 volume were made into the designated media (usually 100 ml in 500 ml Erlenmeyer flasks) equipped with sidearms for turbidity readings, as previously described (Gochnauer and Kushner, 1969). Cultures were incubated with shaking at 37° C, and growth was followed turbidimetrically. Cells were harvested by centrifugation, washed in a solution containing the salts of the BSMK medium and made up in a thick suspension containing 10—40 mg dry wt. of cells per ml. Protein content of these suspensions was determined by the method of Lowry *et al.* (1951).

Extractions of Pigments—One ml of cell suspension was diluted with 3.0 ml of acetone/methanol (1:1, v/v) and the mixture was vigorously shaken. After 1 h at room temperature the mixture was centrifuged, the supernatant extract decanted and the residue extracted three times with 2.0 ml portions of acetone/methanol (1:1). The combined extracts were made to a known volume (usually 10 ml) with acetone-methanol. Spectra were taken in a Hitachi Perkin-Elmer Visible Dual-Beam recording spectrophotometer in a 1.0 cm quartz cuvette. To standardize comparisons spectra were plotted as absorbance per mg cellular protein versus wavelength.

Material for Chromatography

Authentic samples of β-carotene and retinal were obtained from Sigma Chemical Co., and vitamin K_1 from General Biochemical Corporation. Squalene, dihydro-

squalene, tetrahydrosqualene and vitamin MK_8 were isolated from *H. cutirubrum* as described elsewhere (Tornabene *et al.*, 1969). Silica gel H for thin-layer chromatography was supplied by Brinkmann Instruments (Canada) Ltd. All solvents used were either spectral grade or purified according to Vogel (1956).

Chromatographic Separation

Portions of the total pigment extracts, derived from equivalent amounts of cells (based on protein estimations) were concentrated under a stream of oxygen-free nitrogen almost to dryness, and the residues were dissolved in a suitable volume (10—30 µl) of chloroform. The solutions were centrifuged and analyzed by thin-layer chromatography on 0.5 mm layers of silica gel H, previously washed with chloroform/methanol (1:1) and activated at 100°. Squalene, dihydrosqualene, tetrahydrosqualene and β-carotene were separated in the solvent system hexane/ethyl ether (99.6:0.4, v/v); vitamins K, in hexane/ethyl ether/acetic acid (90:10:1, v/v); red pigments (bacterioruberins; Jensen, 1962; Kelly *et al.*, 1970) in chloroform/methanol (93:7, v/v); and retinal in 0.5% ethyl ether in chloroform. The separated components were detected by their visual color or by spraying the plates with concentrated sulfuric acid and charring.

Results

Effects of Glycerol on Pigment Formation

a) Spectra of Extracts. Strains of both *Halobacterium cutirubrum* and *H. halobium* were used in this work, but since the lipid and pigment composition of both appeared to be the same, and was generally affected in the same way by different nutritional conditions only results with *H. cutirubrum* will be reported. Spectra of acetone-methanol extracts of cells grown for 7 days in BSMK medium showed absorption maxima at 370, 388, 494, and 527 nm and a shoulder at 466—476 nm (Fig. 1, curve 1). These maxima correspond to those of bacterioruberin (Jensen, 1962; Kelly *et al.*, 1970; see Fig. 2). Adding 0.1% glycerol greatly stimulated growth but changed the color of the cells from pink to greyish purple. The extract of these cells was yellow and the remaining cellular material colorless. The spectrum of the extract (Fig. 1, curve 2) showed a great reduction in absorbance above 240 nm, and the characteristic peaks and shoulders due to bacterioruberin were virtually absent. At wavelengths below 420 nm, the extract of glycerol-grown cells showed a broad band between 360 and 390 nm, with absorbance higher than that in extracts of cells grown in BSMK medium alone. If the glycerol concentration in the medium was raised above 0.1%, absorbance of the extract showed an increase above 420 nm and a decrease below 420 nm. Even with the highest glycerol concentration used (2%), however, the absorbance of the extract did not rise to that of cells grown without glycerol (Fig. 1, curve 4).

b) Chromatographic Studies of Extracts. The following substances were identified by thin-layer chromatography in extracts of BSMK-grown cells: squalene, dihydrosqualene, and tetrahydrosqualene, vitamin MK_8,

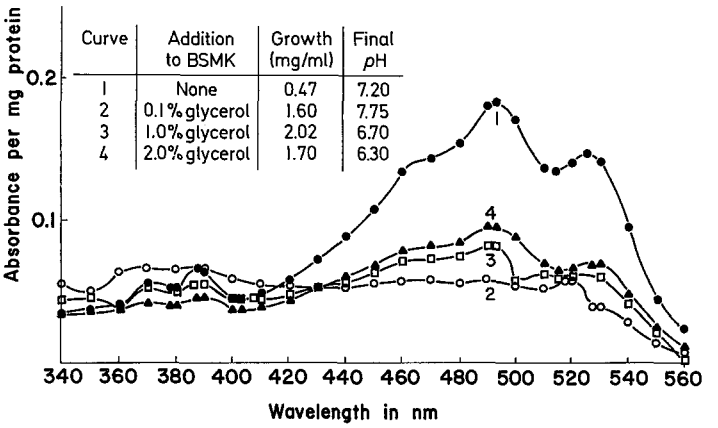


Fig. 1. Effect of glycerol addition to growth medium (BSMK) on spectra of extracts of *H. cutirubrum*. Cultures were grown 7 days before extraction. Amount of growth and final pH are shown in the figure

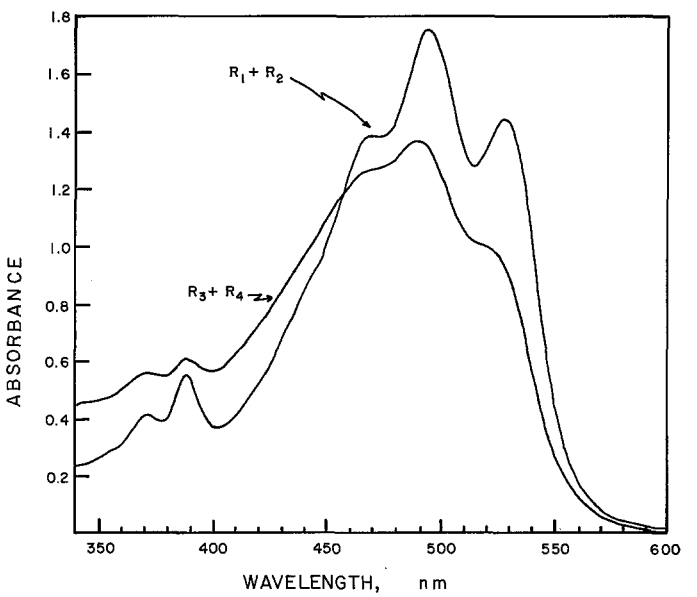


Fig. 2. Spectra in acetone of bacterioruberins isolated from *H. cutirubrum*. $R_1 + R_2$ bacterioruberin isomers; $R_3 + R_4$ anhydrobacterioruberin isomers

β-carotene, and several bacterioruberins (C-50 red pigments) (Fig. 3). The levels of tetrahydrosqualene and dihydrosqualene and vitamin MK_8 were low (relative to squalene) in extracts of cells grown in BSMK but addition

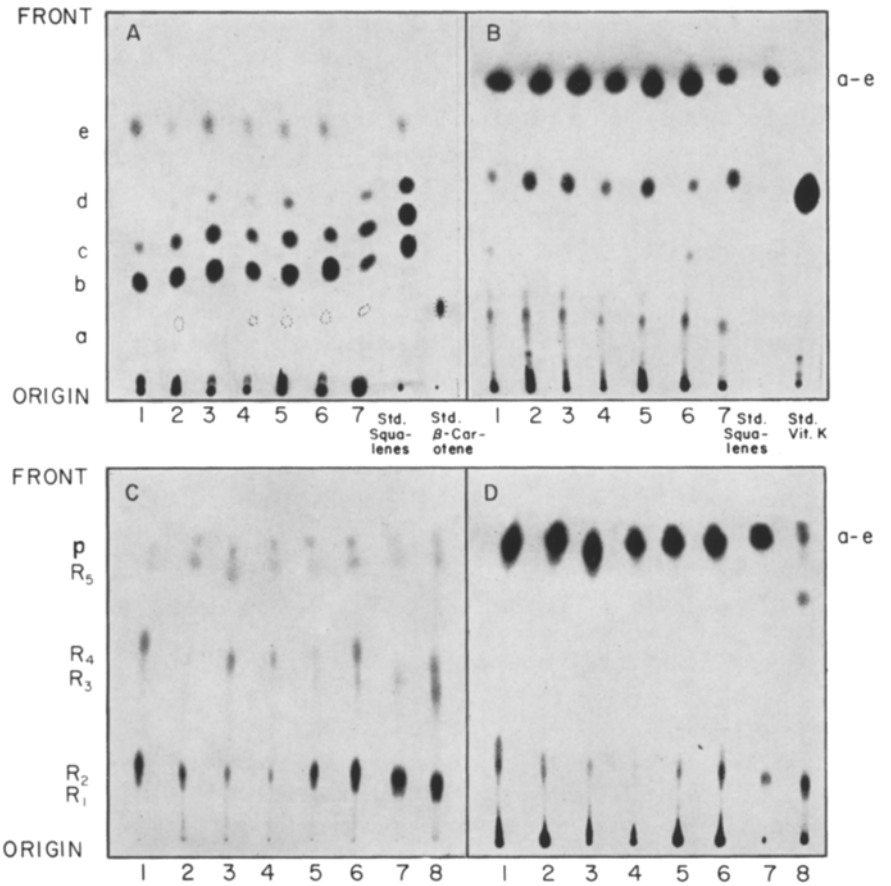


Fig. 3 A—D. Thin-layer chromatograms on silica gel H of acetone-methanol-soluble lipids of *H. cutirubrum* grown in media (BSMK) containing varying amounts of glycerol or glucose. Chromatograms were developed in A hexane/ethyl ether (99.6:0.4); B hexane/ethyl ether/acetic acid (90:10:1); C chloroform/methanol (93:7), uncharred; D same as C, but charred. Material chromatographed was obtained from cells grown in: *1* basal medium (BSMK); *2—5* basal medium plus 0.1, 0.5, 1.0 or 2.0% glycerol, respectively; *6* basal medium plus 2% glucose. Spot *7* total acetone-soluble lipids of *H. cutirubrum* grown in CM. Spot *8* total polar pigments of *H. cutirubrum* grown in CM (95% methanol-soluble fraction). Identity of spots: *a* carotenes; *b* squalene; *c* dihydrosqualene; *d* tetrahydrosqualene; *e* hydrocarbons; $R_1 + R_2$ bacterioruberin isomers; $R_3 + R_4$ anhydrobacterioruberin isomers; R_5 bisanhydrobacterioruberin; *P* unidentified yellow pigment

of 0.1% glycerol enhanced their formation (Fig. 3 A and B). One per cent glycerol appears to have lowered the relative amounts of dihydro- and tetrahydrosqualene and vitamin MK_8 but these were still greater than

those produced by cells grown in the BSMK medium alone. Maximum levels of dihydrosqualene and tetrahydrosqualene and vitamin MK_8 in the BSMK medium were obtained with the addition of 2% glycerol, but even higher levels, relative to squalene, were found in cells grown in the complex medium (CM).

The formation of C-50 red pigments decreased markedly with increasing concentration of glycerol up to 1%, but increased at 2.0% glycerol to a level still below that with the BSMK medium alone (Fig. 3C and D). As the C-50 red pigments decreased the C_{40} carotenes, which were present in very small amounts, appeared to increase. In addition one of the yellow pigments in acetone-methanol extracts of cells grown in BSMK + 0.1% glycerol had a mobility (R_f 0.51) on thin-layer chromatography identical to that of retinal. The same pigment was detected chromatographically in extracts of cells grown in complex medium. It was isolated by preparative TLC and further characterized by its ultraviolet absorption spectrum before and after treatment with sodium borohydride. These were identical with the corresponding spectra of authentic retinal (λ_{max} 381) and retinol (λ_{max} 325), respectively (Wald and Brown, 1956; Oesterhelt and Stoeckenius, 1971).

Effects of Glucose on Pigment Formation

a) Spectra of Extracts. Addition of glucose to the BSMK medium changed the intensity of pigmentation without changing the nature of the spectra. This is shown in Fig. 4, which presents spectra of extracts of cells grown 7 days in BSMK medium with 0, 0.5, 2, and 4% glucose added. All these glucose concentrations stimulated growth. One half per cent glucose stimulated pigmentation maximally; 2% glucose stimulated pigmentation at a smaller extent, and 4% glucose inhibited pigmentation. A glucose concentration of 0.1% affected neither growth nor pigmentation.

b) Chromatographic Studies of Extracts. Chromatograms of the extracts whose spectra are shown in Fig. 4 showed that addition of glucose to the BSMK medium increased the formation of dihydrosqualene, tetrahydrosqualene and vitamin MK_8, the greatest effect being given by 0.5% glucose. Addition of increasing amounts of glucose to the basal medium, in general, enhanced the formation of the red pigments, a maximum effect again being produced by 0.5% glucose. Essentially, the same effect was observed for β-carotene as for the red pigments.

Pigment Formation in Complex Medium (CM). Cells grew relatively rapidly in CM alone, even without the addition of glycerol. Adding 0.1% glycerol stimulated growth (Fig. 5), though not as much as in the BSMK medium (Fig. 1), and had no effect on the spectrum of the extract of cells.

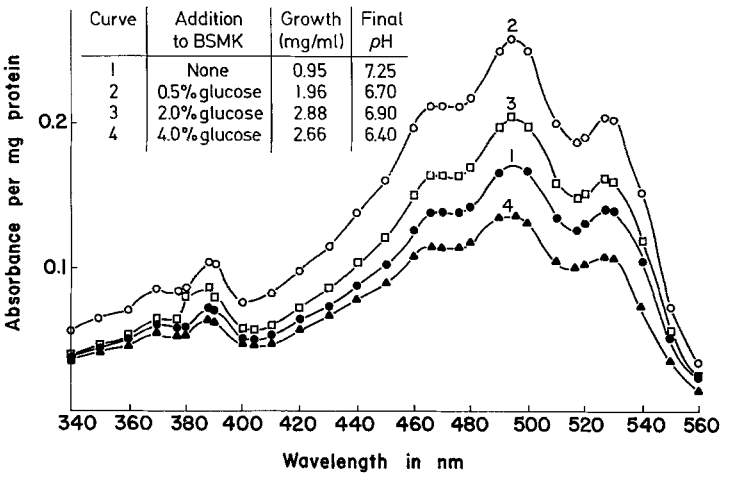


Fig. 4. Effect of glucose addition to growth medium (BSMK) on spectra of extracts of *H. cutirubrum*. Cultures were grown 7 days before extraction. Amount of growth and final pH are shown in the figure

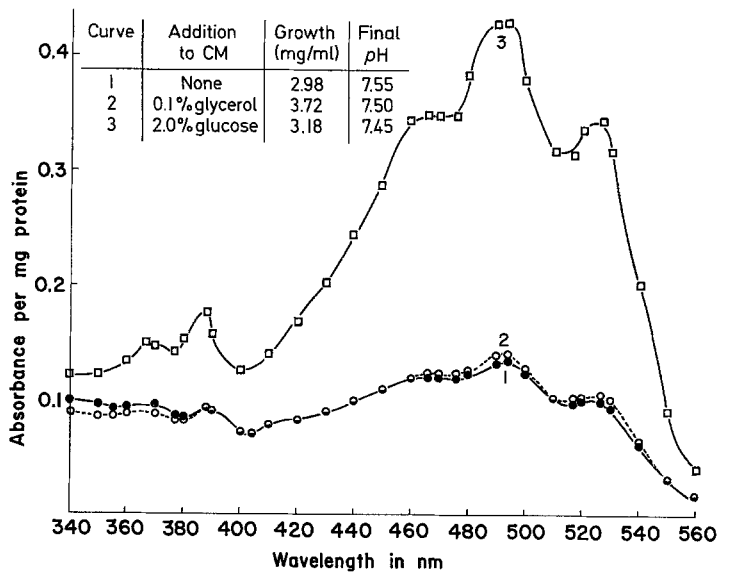


Fig. 5. Effect of glycerol and glucose addition to complex medium on spectra of extracts of *H. cutirubrum*. Cultures were grown 7 days before extraction. Amount of growth and final pH are shown in the figure

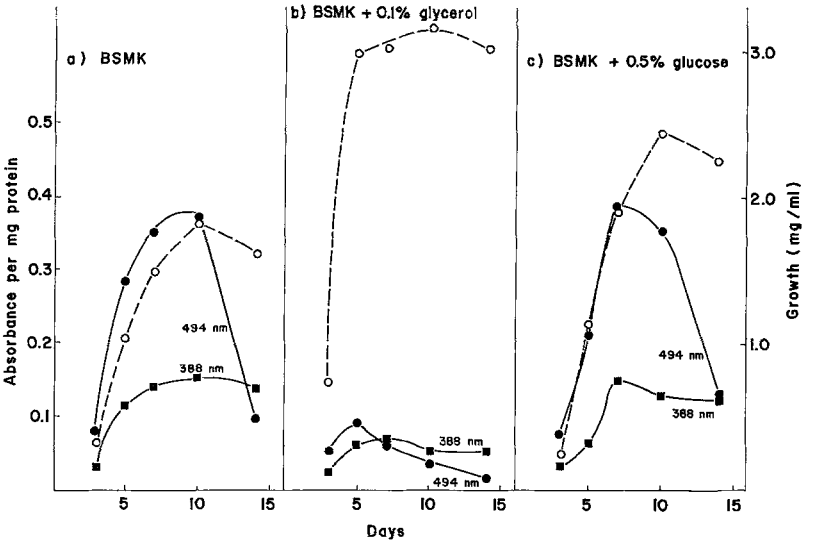


Fig. 6a—c. Time course of growth and pigment production by *H. cutirubrum* grown in: a BSMK; b BSMK+0.1% glycerol; c BSMK+0.5% glucose. Solid lines: pigment intensity at 388 and 494 nm; broken lines: growth curves

However, adding 2% glucose, which had little if any effect on growth, greatly increased the intensity of pigmentation (Fig. 5).

Time Course of Pigment Formation. Experiments were carried out to measure changes in pigmentation and composition of extracts over an extended growth cycle of *H. cutirubrum* (Fig. 6a—c). In BSMK medium the absorbance at 494 nm (absorption maximum for bacterioruberin) rose rapidly during the early stages of growth to a peak at 10 days and fell rapidly after growth stopped. Absorbance of yellow pigments at 388 nm also rose in the early stages of growth, then remained almost stationary, so that by the 14th day (stationary phase) it was greater than the absorbance at 494 nm (Fig. 6a). In the presence of 0.1% glycerol (Fig. 6b), the cultures were slightly pink at first, then turned purple. Absorbance at 494 nm increased slightly up to day 5 and then decreased virtually to zero. Absorbance at 388 nm increased to a maximum at 7 days and then remained almost constant at a higher value than that at 494 nm (Fig. 6b). Addition of 0.5% glucose increased slightly the maximal intensity of absorbance at 494 nm in this experiment, and caused the maximum to be reached 2 days earlier (Fig. 6c). Chromatographic studies showed that the concentration of red pigments followed the changes observed in the

spectra of extracts during growth cycle. In contrast, in all media there was a gradual increase with time in the concentrations of squalene, dihydrosqualene, and tetrahydrosqualene.

Effect of Light on Pigment Formation. In the above experiments cultures were grown in an unlighted room at 37°C. In a further experiment, one set of flasks containing BSMK, BSMK + 0.1% glycerol and BSMK + 0.5% glucose was exposed to continuous illumination from a bank of three incandescent bulbs (65 watts total) placed around a circular (20 cm diameter) fluorescent lamp (Westinghouse FC 819-CW, 22 watts); total intensity, 100 foot candles. A second set of flasks was shielded from light by foil. No significant differences were seen in growth or pigmentation in comparable flasks from each set.

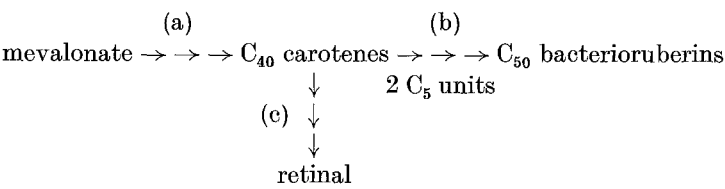
Discussion

Our results show that addition of small amounts of glycerol to a defined medium stimulates growth but almost entirely inhibits formation of bacterioruberins by *H. cutirubrum* and *H. halobium*. Such cells are purple and their acetone extracts contain retinal and other yellow pigments; the extracted cell residues are colorless. Binding to protein alters the yellow color of retinal to purple, in visual pigments (Hubbard *et al.*, 1965) and in the bacteriorhodopsin ("purple membrane") of *H. halobium* (Oesterhelt and Stoeckenius, 1971). It seems possible that bacteriorhodopsin may also be present in several other *Halobacterium* species, and further studies on this point are in progress.

Glucose stimulated both growth and bacterioruberin formation. Since the rise in bacterioruberin content paralleled growth in the basal synthetic medium (BSMK) it seems possible that the effect of glucose in this medium is connected with its effect on growth. The variation in the effect of glucose on pigmentation after 7 days incubation noted between different experiments (*cf.* Figs. 4 and 6) may have been due to the different levels of growth reached. Thus, cells from BSMK medium (Fig. 4) had not yet reached maximum growth, whereas growth was much higher in BSMK + 0.5% glucose in this experiment.

The most striking effect of glucose on pigment formation occurred in the complex medium, where growth was not stimulated (Fig. 5). Thus, not all effects of glucose on pigment formation are connected with effects on growth. Similarly the glycerol inhibition of bacterioruberin formation cannot be directly connected to its growth stimulation.

The pathway of synthesis of bacterioruberins is still unknown, but Kelly *et al.* (1970) have suggested that these C_{50} pigments arise from a C_{40} linear carotene by addition of two C_5 units, as follows:



In our experiments, glycerol decreased the formation of C_{50} pigments and slightly increased the formation of β-carotene. Growth in the presence of glucose increased both C_{50} pigments and β-carotene production. These results suggest as a working hypothesis, that in the above scheme glycerol may inhibit step (b), and glucose may stimulate step (a). Furthermore, 0.1% glycerol may also stimulate the formation of retinal [step (c)] which presumably is derived from C_{40} cyclic carotenes (Oesterhelt and Stoeckenius, 1971). Both glycerol and glucose increased the formation of dihydrosqualene, tetrahydrosqualene and vitamin MK_8. The mechanism for this increase is not known, but does not seem to be related to the effects of glycerol and glucose on pigment formation.

In conclusion, small variations in growth media can have considerable effect on pigment production in two species of extremely halophilic bacteria. The physiological basis of such changes might be difficult to determine because of the nutritional complexity of these organisms. However, the ability to affect pigment production so drastically might prove a valuable tool in studying carotenogenesis, and the physiological role of pigments, in these organisms.

Acknowledgements. This work was supported by grants from the National Research Council and the Medical Research Council of Canada. We are indebted to Mrs. P. Fejer and Mrs. V. Garg for technical assistance.

References

Blaurock, A. E., Stoeckenius, W.: Structure of the purple membrane. Nature New Biol. **233**, 152—155 (1971).

Brown, R. H., Stevenson, J.: Variation in the composition of a purified membrane fraction from *Halobacterium salinarium* with the phase of growth. Antonie v. Leeuwenhoek **37**, 89—99 (1971).

Gochnauer, M. B., Kushner, D. J.: Growth and nutrition of extremely halophilic bacteria. Canad. J. Microbiol. **15**, 1157—1165 (1969).

Hubbard, R., Bownds, D., Yoshizawa, T.: The chemistry of visual photoreception. Cold Spring Harb. Symp. quant. Biol. **30**, 301—315 (1965).

Jensen, S. L : The constitution of some bacterial carotenoids and their bearings on biosynthetic problems, p 157. Trondheim: Bruns 1962.

Kelly, M., Norgard, S., Liaaen-Jensen, S.: Bacterial carotenoids. XXXI. C_{50}-carotenoids. 5. Carotenoids of *Halobacterium salinarium*, especially bacterioruberin. Acta chem. scand. **24**, 2169—2182 (1970).

Kushwaha, S. C., Pugh, E. L., Kramer, J. K. G., Kates, M.: Isolation and identification of dehydrosqualene and C_{40}-carotenoid pigments in *Halobacterium cutirubrum*. Biochim. biophys. Acta (Amst.) **260**, 492—506 (1972).

Lowry, O. H., Rosebrough, N. J., Farr, S. N., Randall, R. J.: Protein measurement with the folin phenol reagent. J. biol. Chem. **193**, 265—275 (1951).

Nandy, S. C., Sen, S. N.: Effect of environment of growth and carotenogenesis of *Sarcina litoralis*. Canad. J. Microbiol. **9**, 601—611 (1963).

Oesterhelt, D., Stoeckenius, W.: Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. Nature New Biol. **233**, 149—152 (1971).

Sehgal, S., Gibbons, N. E.: Effect of some metal ions on the growth of *Halobacterium cutirubrum*. Canad. J. Microbiol. **6**, 165—169 (1960).

Tornabene, T. G., Kates, M., Gelpi, E., Oro, J.: Occurrence of squalene, di- and tetrahydrosqualene and vitamin MK_8 in an extremely halophilic bacterium, *Halobacterium cutirubrum*. J. Lipid Res. **10**, 294—303 (1969).

Vogel, A. I.: Practical organic chemistry, pp. 161—175. London: Longmans, Green 1956.

Wald, G., Brown, P. K.: Synthesis and bleaching of rhodopsin. Nature (Lond.) **177**, 174—176 (1956).

Dr. M. B. Gochnauer
Department of Biology
University of Ottawa
Ottawa, Ontario, K1N 6N5, Canada

Prof. Dr. M. Kates
Department of Biochemistry
University of Ottawa
Ottawa, Ontario, K1N 6N5, Canada

Dr. S. C. Kushwaha
Department of Biochemistry
University of Ottawa
Ottawa, Ontario, K1N 6N5, Canada

Prof. Dr. D. J. Kushner
Department of Biology
University of Ottawa
Ottawa, Ontario, K1N 6N5, Canada