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THE INDEPENDENCE OF PHOTOSYNTHESIS AND AEROBIOSIS FROM STEROL BIOSYNTHESIS IN BACTERIA

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Key Word Index—*Rhodopseudomonas spheroides*; *Chromatium vinosum*; *Saccharomyces cerevisiae*; *Escherichia coli*; bacteria; photosynthesis; anaerobiosis and aerobiosis; sterol; phytol.

Abstract—Sterols were present in neither of two representative species of photosynthetic bacteria, *Rhodopseudomonas spheroides* and *Chromatium vinosum*. These organisms were grown under conditions commonly viewed as anaerobic. However, such conditions did not prevent *Saccharomyces cerevisiae* from biosynthesizing sterols, although they did induce accumulation of both 4,4-dimethyl and 4-desmethyl intermediates. Since the photosynthetic organisms did not biosynthesize sterols, bacterial photosynthesis must not be mated genetically or functionally to sterol biosynthesis. In contrast to what the literature records, *Escherichia coli*, grown under fully aerobic conditions, also failed to contain sterols which indicates that bacterial aerobiosis does not necessarily imply either the presence of sterol biosynthesis or a requirement for an exogenous source of sterols. Among the lipids of *E. coli* was a substance with the formula $C_{16}H_{32}O_2$ which moved in silica gel TLC at a rate similar to that of sterols and may have been a keto-alcohol of the same formula already isolated from coliforms. In the photosynthetic bacteria the major neutral lipid after saponification was phytol, in agreement with expectation based on the presence of bacteriochlorophyll-a.

INTRODUCTION

All photosynthetic plants so far examined from the prokaryotic blue-green algae to angiosperms biosynthesize sterols *de novo* [1]. On the other hand, sterol biosynthesis is far from a ubiquitous attribute of nonphotosynthetic systems. Among the organisms which lack this capacity are mycoplasmas, various bacteria, many protozoa, the pythiaceous fungi, the arthropods, the coelenterates, and the platyhelminthes [1]. Sterol biosynthesis seems to be invariably operational in nonphotosynthetic eukaryotes only in the vertebrates, the molluscs, the echinoderms, and those fungi which are not oomycetous [1]. This apparent dichotomy between photosynthetic and nonphotosynthetic organisms suggests the existence of an obligate functional link between photosynthesis and sterol biosynthesis. The purpose of this paper is to demonstrate that no such link exists, at least in bacteria. In particular, the photosynthetic bacteria, *Rhodopseudomonas spheroides* and *Chromatium vinosum*, were found to lack sterols. The older literature [2] suggests that *Rhodopseudomonas palustri* requires an exogenous source of sterol, but the bacteria we examined grew quite well without added sterol. Since these organisms were grown in the usual manner which is tantamount to semianaerobic conditions, the question arises as to whether the low level of oxygen would have prevented significant sterol biosynthesis even though the intrinsic genetics for biosynthesis were present. Unfortunately, fully aerobic conditions are not consonant with growth of these bacteria, and this precluded our doing the experiment which at first glance seems obvious. However, we have examined the ability of yeast (*Saccharomyces cerevisiae*) to make sterols under semianaerobic conditions and have found extremely low levels of oxygen are quite adequate to permit the biosynthesis of lanosterol and its conversion to 4-desmethysterols. The absence of sterols in the photosynthetic bacteria consequently indicates that photosynthesis and sterol biosynthesis are not intrinsically coupled in a functional sense. It also seems highly probable that they are not coupled in a genetic sense.

It has been argued that the primitive earth possessed a reduced atmosphere leading to the evolution of anaerobic organisms. If this were so, and if the photosynthetic bacteria arose under anaerobic conditions, we would have a satisfactory explanation for the absence of the genetics for sterol biosynthesis. An extension of the argument might then lead one to believe that only in the presence of oxygen would the genetics for sterol biosynthesis arise. It has not been possible to prove or disprove these views, but we have examined the problem of occurrence of sterol in *Escherichia coli*. This bacterium is believed by some [3, 4] to have had a primitive, anaerobic origin and consequently to have been on the earth for billions of years. During this time it has actually acquired the ability to live aerobically which might also imply the acquisition of sterol biosynthesis. Indeed, the literature records that it does contain sterols [5]. However, we have reinvestigated *E. coli* grown under fully aerobic conditions and are

unable to verify the earlier report. We believe the absence of sterols confirms at the bacterial level the much more fully documented evidence among eukaryotes that aerobiosis does not necessarily confer sterol biosynthesis on an organism.

Our search for sterols required a study of the neutral lipids of these various organisms. As a result it became possible for us to show that both species of the photosynthetic bacteria contain phytol. The phytol was presumably esterified *in situ* to bacteriochlorophyll-a which was demonstrated to be present earlier [6]. The presence of phytol was also documented previously in *R. spheroides* [7], although not in the green strain we used. Our own work constitutes, however, the first proof for the existence of phytol in *C. vinosum*. The data extend the existing observations [8–10] that the esterifying moiety of bacteriochlorophyll (fatty alcohol, farnesol or phytol) in photosynthetic bacteria is dependent on the structure of the porphyrin moiety. As we demonstrated previously [11], phytol is also present in blue-green and brown algae which are known to possess cholorophyll-a [12].

RESULTS AND DISCUSSION

Semianaerobic growth of yeast

Yeast was grown in a commercial fermentor in 6–7 l. batches in the presence of highly purified nitrogen and the absence of added sterol. The medium was also purged of oxygen with nitrogen prior to inoculation. Despite such precautions, the organism always grew to *ca* 10 million cells per ml during 3 days. When cells were grown under more stringent anaerobic conditions in sealed 1 l. flasks in the absence of sterol, no growth occurred [13]. Growth in the fermentor suggested that leaks in this large apparatus permitted enough oxygen to enter the system to permit sterol biosynthesis. This was confirmed by an examination of the neutral lipid of the cells. As shown in Table 1, there was 60 mg of 4-desmethylsterol and about the same amount of 4,4-dimethylsterol present per kilogram of wet cells. When ergosterol was added, much greater growth was obtained. The ratio of 4-desmethylsterol to cell weight remained about the same, but the 4,4-dimethylsterol disappeared. The 4,4-dimethylsterol from the unsupplemented incubations had the GLC R_t and MS (*m/e* 426 (M^+), 411 ($M^+ - Me$), 393 ($M^+ - Me - H_2O$), and 341 ($M^+ - Me - C_5H_{10}$)) expected of lanosterol. The 4-desmethysterol was composed of several sterols. The MS of the mixture showed M^+ values at *m/e* 384, 396 and 398, and a single dominant peak for M^+ less side chain at 271. A strong peak at 369 for 384 less 15 (Me) and none at 357 or 366 for 384 less 33 (Me and H_2O) and 18 (H_2O), respectively, indicated the 384-compound was zymosterol. The 398-component was accompanied by a fragment (*m/e* 365) derived from loss of Me and H_2O, indicating it cannot have been a Δ^7- or Δ^8-sterol and therefore must have been a $\Delta^{5,7}$-sterol with the 24β-methyl group and a saturated side chain (22-dihydroergosterol). The 396-component was also accompanied by a strong fragment for $M^+ - 33$ at *m/e* 363 showing it to be ergosterol. Other features of the MS agreed with these assignments. The UV spectrum of the mixture exhibited absorption peaks at 272, 282, and 294 nm for the $\Delta^{5,7}$-moieties. In GLC three major components were observed with the retention times expected of zymosterol, ergosterol and 22-dihydroergosterol in increasing amounts, respectively.

The sterol composition of the oxygen-deprived cells was markedly different from those grown either aerobically in the absence of sterol or semianaerobically in the presence of ergosterol (20 mg/l.). MS and ^1H NMR spectra as well as GLC showed ergosterol was the only component in the cells grown semianaerobically with added ergosterol. In the aerobic case, ergosterol was present but was accompanied by very small amounts of what appeared to be zymosterol and 22-dihydroergosterol.

In cells obtained in the three different ways (Table 1), the 4-desmethylsterol was present at about the same cellular concentration (5 ± 1 fg/cell). In the oxygen-deprived cells grown without added sterol, it is clear that, while sterol biosynthesis occurred to the same overall level as in the aerobic case, all carbon passing along the pathway did not reach the state of ergosterol. This, together with an absolute reduction in the amount of biosynthesis possible due to the low concentration of oxygen, reasonably accounts for the drastic reduction (10^7 vs 10^8 cells/ml with ergosterol supplement) in the number of cells obtained semianaerobically. On the assumption that there are *ca* 10 mol of oxygen per mol of sterol biosynthetically required, we calculate that only 1 μm or 32 μl of oxygen per l. would account for the biosynthesis of the sterol found under semianaerobic conditions.

Table 1. Data derived from *Saccharomyces cerevisiae*

	Aerobic	Semianaerobic	
Sterol supplement	None	None	Ergosterol*
Total cells extracted	26.8×10^{11} cells	7.57×10^{11} cells	15.9×10^{11} cells
Culture volume	8 l.	60 l.	12 l.
Total mass of cells (wet)	116.0 g	63.5 g	135 g
Average mass/cell (wet)	4.33×10^{-11} g/cell	8.39×10^{-11} g/cell	8.48×10^{-11} g/cell
Desmethylsterol recovered	16.3 mg	4.0 mg	7.2 mg
Dimethylsterol recovered	none	4.4 mg	none
Desmethylsterol/cell (wet)	6.1×10^{-15} g/cell	5.3×10^{-15} g/cell	4.5×10^{-15} g/cell
Desmethylsterol/100 g cells (wet)	14 mg	6 mg	5 mg

*Supplemented with Tween-80 (15 ml/l.) and ergosterol (20 mg/l.).

Semianaerobic growth of photosynthetic bacteria

The bacteria were grown in the usual microbiological way without purging the medium of oxygen and without adding purified nitrogen to the space above the medium. This must have left more oxygen in the medium than in the *S. cerevisiae* case. The flasks were simply filled nearly to the stopper with medium. The neutral lipid after saponification and chromatography showed only one consequential material in the region of the chromatogram coincident with 4,4-dimethyl- or 4-desmethylsterols. This material appeared close to where lanosterol would have been expected. The substance was identified as phytol in the following manner for both bacteria.

Upon GLC, essentially single peaks were obtained with retention times relative to 1-octadecanol of 1.13 which is the same as obtained with authentic phytol. Geraniol and farnesol have relative retention times of 0.06 and 0.30, respectively. The ^{1}H NMR spectra (δ 4.14, *d*, $J = 8$ Hz, C-1 protons; 5.40, *t*, $J = 6$ Hz, C-2; 1.67, *s*, C-3′; 1.98, *t*, $J = 7$ Hz, C-4; 0.84, *d*, $J = 7$ Hz, C-7′ or C-11′; 0.85, *d*, $J = 7$ Hz, C-7′ or C-11′; 0.87, *d*, $J = 8$ Hz, C−15′ and C-16′; and 7.25, *s*, H on O) and MS (*m/e* 296, 278, 263, 250, 249, 221, 211, 210, 196, 179, 141, 137, 126, 71 and 43) were identical to those of authentic phytol. The bacterial material was acetylated and chromatographed on $AgNO_3$-Si gel TLC. The rate of movement of the principal band in each case was the same as that of authentic phytyl acetate but a minor component moved slower and was found in the region where farnesyl acetate was expected based on the R_f of a standard sample. After elution of the slower-moving material, GLC demonstrated it to be a mixture of at least three components. While insufficient material was available to achieve an adequate MS on the two slower-moving components, the fastest one yielded a spectrum which was similar to, but definitely different from, that expected of farnesyl or geranyl acetates. However, various characteristics of the spectrum, e.g. shifts of two mass units compared to the reference spectra, leads us to believe tentatively that the material represents partially reduced derivative(s) of the acetate of farnesol (and perhaps of other isopentenoids). Such compounds are reasonable biosynthetic intermediates to phytol, but they were not available to us for further study.

The amount of the whole fraction moving slower than phytyl acetate in argentation chromatography was too small to quantitate, but appeared to be less than 1%. It was too little to be detected by GLC of the crude phytol, and phytol thus seems to have been the only functional side of the bacteriochlorophyll-a. The amount of phytol (105 mg/100 g wet wt of *C. vinosum* and 91 mg/100 of wet wt of *R. spheroides*, Table 2) was remarkably similar to our previous finding with blue-green (60 mg/100 g) and brown (60 mg/100 g) algae [11]. These results contrast sharply with those derived from tracheophytes. In the chromatographic fractions which would contain phytol from higher plants, we have found instead fatty alcohols, pentacyclic triterpenoids and 4,4-dimethysterols. Although phytol was presumably present, it was not among the major components in the many tracheophytes examined in this laboratory [14]. Had it been present at levels of 50–100 mg/100 g it would certainly would have been obvious. This difference in the lower and higher photosynthetic systems suggests the former may devote more of their mass to photosynthesis than do the latter.

Table 2. Data derived from photosynthetic bacteria

Material	*R. spheroides*	*C. vinosum*
Cells, wet wt	1250 g	1040 g
Cells, dry wt	219 g	240 g
Culture volume	325 l.	390 l.
Solvent extract	30 g	76 g
Phytol	1.15 g	1.10 g
Total sterol*	<0.2 mg	<5 mg

*No sterol was detected. The numbers given are the limit of our capacity to detect sterol in the two cases. See text for more details.

The absence of sterols was ascertained in the following way. The neutral lipids were chromatographed on alumina in a gradient of hexane–ether–methanol. Every fraction beginning with the appearance of phytol was monitored by GLC, and no sterol was found. The fractions containing phytol together with all subsequent fractions were combined and re-chromatographed in the same way with 50 mg of added cholesterol as an internal standard. The cholesterol appeared as expected in fractions immediately following phytol. This proved the fractions examined for sterol in the first chromatogram would have contained 4-desmethylsterol had it been present. The second chromatogram also permitted re-examination for sterols other than cholesterol and none was found. Fractions which should have contained sterol in the first chromatogram were combined and assayed by GLC. We should have been able to see a peak corresponding to *ca* 0.2 mg in the 1250 g of *R. spheroides* extracted and to *ca* 5 mg in the 1040 g of *C. vinosum*. A higher base line and other problems raised the limit of detection in the latter case, but after the second chromatogram with cholesterol the limit of detection for sterol with retention times different from that of cholesterol was *ca* 1.0 mg for the total amount of each organism examined. These various numbers indicate that the sterols, if any are present in these bacteria, are at levels one to two orders of magnitude less than in blue-green algae and cabbage (*ca* 30 mg/1000 g wet wt) [15, 16] which are distinguished by having the lowest sterol content of any organism or tissue we have examined. A more common value is at least 100 mg/1000 g.

4,4-Dimethylsterols, e.g. lanosterol, and monohydroxy pentacyclic triterpenoids were also excluded. They should have been in the fractions containing phytol, since the large amount of phytol completely blanketed the region of the chromatogram where these materials were expected. A 100 mg aliquot of the combined phytol-containing fractions was chromatographed on lipophilic Sephadex on which phytol, having only two-thirds the number of C-atoms as lanosterol, etc., moves much more rapidly than the polycycles of interest. Analysis of the appropiate fractions for 4,4-dimethyl compounds by GLC revealed nothing was present. We should have been able to

detect less than an amount corresponding to 0.2 mg in the entire sample of each bacterium.

Aerobic growth of E. coli

GLC of the neutral lipid after saponification showed that most of the material had a rention time less than that of cholesterol. An extremely weak peak (A) did appear close to the retention time of cholesterol and a large one (B) at 3.20 (relative to cholesterol). No peaks corresponding to 24-methyl- and 24-ethylcholesterol were observed. These sterols were reported to be present earlier [5]. The neutral lipid was chromatographed on Si gel TLC and peak A was located in the 4-desmethylsterol region (R_f 0.32). Most of the material in this region had a much shorter R_f than cholesterol in GLC, and nothing was present in this region with a R_f longer than that of cholesterol. Several substances with retention times relative to cholesterol of less than 0.70 (major mass), 0.70, 1.15 and 2.59 (probably a series of fatty alcohols) were found in the 4,4-dimethylsterol region (R_f 0.41). Peak B appeared in a still less polar region (R_f 0.81) along with an unidentified fluorescing substance.

The material from TLC with the R_f of 4-desmethylsterols was an oil containing crystals. MS analysis showed the sample to be overwhelmingly composed of a substance displaying a principal peak at *m/e* 256 corresponding to $C_{16}H_{32}O_2$. At a level of intensity of only 4% relative to the latter peak was one with *m/e* 386 corresponding to M^+ for cholesterol, but this peak was merely twice as high as the background peaks. While peaks did exist for expected fragments, they were also within the background. For instance, a peak at *m/e* 371 for $M^+ - Me$ occurred with an intensity of 64% of M^+ (expected: 32%), but a peak with the same intensity also occurred at, e.g. *m/e* 372, which has no significance in an authentic cholesterol spectrum. Still weaker peaks occurred at *m/e* 400 and 414 corresponding to 24-methyl- and 24-ethylcholesterol which were about half the intensity of the *m/e* 386 peak, but they were also too close to the background to be significant.

The major component (*m/e* 256) exhibited weaker (relative to *m/e* 256) but important peaks at *m/e* 213, 185, 171, 157, 149, 143, 139, 138, 137 and 131. Peaks as strong or stronger than the one at *m/e* 256 appeared at *m/e* 129, 115, 111, 97, 83, 73, 71, 70, 69, 60, 57, 55, 45, 43 and 41. Definite peaks also occurred at *m/e* 15, 18, 31, 165, 199, 227, 236, 250 and 264. The latter two may be impurities. Many of the peaks correspond to the homologous ions, $C_nH_{2n+1}O$, e.g. at 31 for MeO continuing on to *m/e* 213 for $C_{14}H_{29}O$ and the weaker *m/e* 227 for $C_{15}H_{31}O$. Although the interpretation of the spectrum is clouded by the fact that the compound was not entirely pure, the compound may be the optically active C_{16}-keto-tertiary-alcohol ($C_{16}H_{32}O_2$) of mp 104–105° isolated [17] from another coliform, *Aerobacter cloacae*, for which a completely detailed structure is lacking. The prominence of the fragment at *m/e* 213 which may be $M^+ - MeCO$ suggests the keto group may be next to one of the alkyl termini. Very strong peaks at the low mass end of the spectrum corresponding to C_nH_{2n+1} and C_nH_{2n-1}, e.g. at *m/e* 29 and 27 at the C_2-level on to 85 and 83 at the C_6-level, indicate the molecule probably has six or more unoxygenated C-atoms at another terminus.

The results of our study are summarized in Table 3 where they are also compared with the earlier report [5]. Based on the intensity of the GLC peaks both before and after TLC, there cannot have been more than 30 μg of cholesterol in the entire neutral lipid. Even the small amount (3 mg/100 g wet cells) of sterol in the blue–green alga, *Phormidium luridum* [15], is one hundred times as much. If there really were any cholesterol in the neutral lipid, which is not certain, the exceedingly small amount is best assigned to the status of a contaminant.

It has been suggested [18] that, in the absence of sterols or the presence of a very small amount of sterol, tetrahymanol or other pentacyclic triterpenoids may take the sterol's place in bacteria. It is interesting, therefore, that no consequential amount of such material was observed in *E. coli*. Material (RR_f 2.59) which appeared in the expected TLC region for triterpenoid did have the retention time expected of tetrahymanol, but the amount was no greater than that of 'cholesterol'. Peak B which was very much larger had a GLC retention time similar to that of the pentacycle friedelin, but GLC comparison with an authentic sample [14] proved otherwise. When friedelin with an RR_f of 2.98 was examined together with the material from the fluorescing region of the TLC, two distinct peaks were identifiable.

Table 3. Data derived from *E. coli*

	Amount			
	This study		Schubert *et al.* [5]	
Material	Actual	Per 100 g dry cells*	Actual	Per 100 g dry cells
Cells	134 g (wet)	—	1800 g (dry)	—
Culture volume	60 l.	150 l.	600 l.	33 l.
Solvent extract	690 mg	1.7 g	69000 mg	3.8 g
Neutral lipid	90 mg	225 mg	3000 mg	167 mg
Sterol	<0.03 mg	<0.08	6 mg after 3 recrystallizations	0.3 mg

* Calculated on the assumption that the cells are 70% water. Our dry wt was then 40 g.

EXPERIMENTAL

Culture and extraction. *Rhodopseudomonas spheroides* strain Ga (a green mutant), kindly supplied by Dr. P. Leslie Dutton, and *Chromatium vinosum* (ATCC 17899) were grown anaerobically on a mineral medium containing succinate as sole carbon source [19]. Bacteria were grown at 27–29° with *C. vinosum* in continuous light and *R. spheroides* on a 12 hr light–dark cycle. Inocula were constituted by 2-day growths in 250 ml screw cap bottles filled to the top. The inocula were then transferred to 1 l. screw cap bottles for 3–4 days, and finally three 1 l. bottles were used to inoculate one 13 l. carboy filled to the top and closed with a rubber stopper. Cells in the carboys were harvested by centrifugation after 6 days. Following centrifugation and drying, the cells were extracted in a Soxhlet with Me_2CO for 24 hr, after which time no more colour remained in the cells.

A standard wild type K-12 *Escherichia coli* (ATCC 14948) was grown at 37° aerobically in 10 batches of 6 l. each in a phosphate-rich medium [20]. The bacterium was maintained at room temp. with serial transfers. Prior to each inoculation into the 6 l. flask, a culture was obtained from the one at room temp. and incubated at 37° for 24 hr. an aliquot of 5.0 ml was then taken from the latter culture to inoculate the 6 l. flask which was incubated in a commercial fermentor ('Magnaferm' from New Brunswick Scientific Co., Inc., New Brunswick, NJ) in the presence of a flow of air (0.5 l./min) scrubbed with $HgCl_2$ to sterilize it. After 76 hr the cells were collected by centrifugation. The combined pellets weighed 134 g. The collected cells were sonicated (30 min, 1.0 min bursts) in 300 ml Me_2CO in a 'Sonicator Cell Disruptor', Model W-185-F, Heat Systems-Ultrasonics, Inc., Plainview, NY Microscopic observation showed *ca* half the cells had been fragmented. The mixture was placed in a thimble and the Me_2CO soln from the sonication was used to extract the cells and cell fragments in a Soxhlet apparatus for 48 hr.

Saccharomyces cervisiae (ATCC 18790) was grown at 26° on a synthetic nitrogen base medium [21]. Inocula (40 ml) were from a semianaerobically grown continuous batch culture apparatus supplemented with 1 mg/l. ergosterol and Tween-80 (15 ml/l.) with transfers as previously described [13]. The fermentor was flushed for 18 hr with crude N_2 at 0.5 l./min and then for >6 hr with chromous chloride (0.4 M) scrubbed electronic grade N_2 at 0.5 l./min. The gas inlet was closed and no N_2 was allowed to purge the vessel during incubations. Cell populations were determined as previously described [13]. Cells were extracted with Me_2CO in a Soxhlet apparatus for 24 hr.

Chromatography and physical analysis. Me_2CO-extracted material was saponified in 10% methanolic KOH. After saponification the neutral lipid was chromatographed on 3% deactivated neutral Al_2O_3 using a solvent of Et_2O graded into hexane. TLC was performed on Si gel-G using 0.28 or 1.0 mm layers and developed with C_6H_6–Et_2O (9:1). Argentation TLC utilized 0.75 mm thick Si gel-G layers impregnated with 16% Ag^+ ion and developed with C_6H_6–hexane (4:5). Lipophilic Sephadex chromatography (Lipidex-5000) used a solvent flow of 20 ml/hr with 5% hexane in MeOH [16]. GLC was performed at 235° on XE-60 for sterols and 182° for phytol. GC–MS employed 3% OV 17 column. ^1H NMR spectra were obtained at 360 MHz.

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