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TRACE METAL COMPOSITION OF PHOTOSYNTHETIC BACTERIA

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SUMMARY

The manganese content of various photosynthetic bacteria (*Rhodospirillum rubrum, Rhodopseudomonas spheroides* and *Chromatium*) has been determined and compared with those of other metals (Cu, Zn, Fe) in cells grown photosynthetically and, in the case of the two photoheterotrophs, also aerobically in the dark. In addition, *Rps. spheroides* was grown photosynthetically in manganese-deficient media. The relevance of these results to the question of the role of manganese in bacterial photometabolism is discussed.

INTRODUCTION

Several reports[1–7] have presented evidence for participation of manganese in the oxygen-evolving step of green plant and algal photosynthesis. In addition, manganese is present in chloroplasts[8] in concentrations consistent with its proposed function. However, recent experiments in this laboratory have shown manganese to be present in comparable amounts in the chromatophores of photosynthetic bacteria-microorganisms which do not evolve oxygen. WIESSNER[9] noted that the manganese requirement of such bacteria was 1/100–1/1000 that of an algal culture although actual concentrations of manganese and other trace metals have not been reported. The trace metal composition of the photosynthetic bacteria, *Rhodospeudomonas spheroides, Rhodospirillum rubrum* and *Chromatium*, was investigated, therefore, to establish the various metal requirements in bacterial photosynthesis. *Rps. spheroides* and *R. rubrum* were also grown aerobically in the dark and their trace metal compositions compared to those of light-grown cells. In addition, the manganese component(s) of *Rps. spheroides* was further characterized by fractionation.

MATERIALS AND METHODS

Cell culture conditions

Rps. spheroides (2.4.1, VAN NIEL) and *R. rubrum* (1.1.1, VAN NIEL) were grown routinely on the modified Hutner medium of COHEN-BAZIRE, SISTROM AND STANIER[10] containing metals "44" and supplemented with ammonium succinate, yeast extract, peptone, sodium acetate and sodium glutamate. *Rps. spheroides* was grown, in addition, on the MS medium of LASCELLES[11] *plus* 10 μM iron citrate, and on a similar medium in which malic acid was replaced by succinic acid and supplemented with LARSEN's

trace elements solution[12] *minus* manganese. *Chromatium*, Strain D, was grown heterotrophically on a succinate medium containing LARSEN's trace elements solution, with Na_2S as the accessory electron donor. For photosynthetic growth, cells were cultured in liter bottles at 30–35° in an illuminated water bath. *R. rubrum* and *Rps. spheroides* were also grown aerobically in the dark on the same modified Hutner medium. The dark, aerobically grown cells were cultured on a shaker in milk-filter covered 2-l erlenmeyer flasks containing 500 ml of medium, as described by TANIGUCHI AND KAMEN[13]. Dark-grown cells were harvested in the exponential phase. Light-grown cells were harvested in the stationary phase.

The cells were collected by centrifugation for 10 min at 30000 × *g* in the cold in a Servall Model RC2 refrigerated centrifuge. Every batch of dark aerobically grown cells was examined under the microscope, after staining with an ethanolic solution of gentian violet, to assure cell purity. Following harvest, both dark- and light-grown *R. rubrum* and *Rps. spheroides* cells were suspended in cold 0.1 M phosphate buffer (pH 7.5) with a tissue cell homogenizer. *Chromatium* cells were suspended in 0.1 M phosphate buffer containing 1 % NaCl. The cell suspensions were centrifuged 5 min at 1000 × *g* to remove inorganic salts from the culture medium, then centrifuged at 30000 × *g* for 10 min to sediment the cells. The supernatant fluid was poured off and the cells were suspended in buffer for a second wash. Then, they were centrifuged at 30000 × *g* and the supernatant fluid discarded. Finally, the cells were suspended in buffer, or deionized water, and lyophilized.

Metal analyses

Approx. 1 g of lyophilized cell material was digested in a 30-ml Kjeldahl flask with 0.5 ml of concentrated H_2SO_4 and sufficient concentrated HNO_3 to yield a clear, colorless solution. The digests were diluted with water, filtered through Whatman No. 42 paper and washed to a final 25-ml volume of combined filtrate and washings. The digests, diluted as required, were analyzed by atomic absorption spectrometry, their metal content being determined by comparison with those of standard metal solutions in 2 % H_2SO_4. Standard metal solutions contained equal concentrations of Mg, Mn, Fe, Co, Cu and Zn in amounts from 0 to 3 ppm at intervals of 0.05–0.5 ppm. A Perkin–Elmer Model 303 atomic absorption spectrophotometer* equipped with a recorder readout accessory was used with the suggested instrument settings. Metal concentrations were determined with a 10-fold scale expansion by bracketing the sample concentration. Absorptions were then measured in the order: standards, sample, standards, sample and standards, for each determination. Standard digests were analyzed to determine recovery of digested material.

Chlorophyll analysis

Bacteriochlorophyll was determined spectrophotometrically by extraction of lyophilized cells with methanol followed by conversion to bacteriopheophytin according to the method of VAN NIEL, with slight modifications**.

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** The time required for maximum extraction of bacteriochlorophyll and conversion to bacteriopheophytin was increased to 30 min and 1 h, respectively.

Preparation of cell extracts and particulate fractions

Freshly washed cells of *Rps. spheroides* were suspended in 0.1 M Tris buffer (pH 7.5) at a concentration of 200 mg/ml containing 1 μg/ml of calf-thymus deoxyribonuclease. The cells were broken by passing the suspension through a Servall Ribi cell fractionator Model RF-1 at 20000 lb/inch^2. The broken cell suspension was centrifuged at 30000 $\times$ *g* for 1 h. The sedimented material, designated 'large particles', was suspended in 0.1 M Tris buffer and lyophilized. The supernatant fraction was then centrifuged at 100000 $\times$ *g* for 2.5 h in a Spinco preparative ultracentrifuge, Model L, using the No. 30 rotor. The resulting supernatant fraction ('soluble fraction') was removed with a pipette and lyophilized. The precipitate ('small particles') was suspended in a small volume of deionized water using a Teflon tissue cell homogenizer and lyophilized.

Cell extracts were also prepared by sonication of a 10% suspension of freshly washed cells in the cold for a total of 20 min with the MSE 500-W sonic oscillator operating at 20 kcycles.

Electron paramagnetic resonance

Electron paramagnetic resonance spectra were recorded on a Varian E-3 X-band spectrometer operating at 100 kcycles/sec field modulation frequency. Spectra were observed on frozen aqueous suspensions in standard quartz tubes.

RESULTS

Table I presents the data from analyses on three successive growth experiments of each bacterium cultured anaerobically in the light. Duplicate analyses were made on some samples to determine their reproducibility. While Co was detected in each of the bacteria studied, concentrations could not be accurately measured by the above method. In Table II, the metal and bacteriochlorophyll concentrations are given relative to the Fe concentration along with average values. For comparison, the trace metal concentrations in spinach chloroplast lamellae are also included.

CLAYTON[15] has estimated that, in purple bacteria, the photosynthetic unit contains 30–40 molecules of light-harvesting bacteriochlorophyll for each of the reaction center molecules, such as the light-reacting cytochrome and specialized chlorophyll. ARNOLD AND NISHIMURA are reported[15] to make estimates of 30–60 bacteriochlorophyll molecules per photosynthetic unit based on flashing light experiments measuring substrate assimilation and ATP formation. Using the criterion of a photosynthetic unit of 30–60 bacteriochlorophyll molecules, the above results can be used to establish the maximum metal requirements for photosynthetic energy conversion in bacteria.

The results indicate that in the case of *R. rubrum* and *Chromatium* the Mn concentrations appear to be too low to be a functional part of the energy conversion unit, although these concentrations are equivalent to those found in spinach chloroplasts relative to the chlorophyll concentration. In the case of *Rps. spheroides*, Mn is concentrated to the extent of 2 atoms for every 40 bacteriochlorophyll molecules.

R. rubrum and *Rps. spheroides* were grown non-photosynthetically in the dark to determine if the high Mn concentration in *Rps. spheroides* was related to its oxidase system. The results are shown in Table III, as μmoles per gram dry weight. Com-

TABLE I

SUMMARY OF TRACE METAL AND BACTERIOCHLOROPHYLL ANALYSES ON WHOLE CELLS OF PHOTOSYNTHETICALLY-GROWN BACTERIA

a, b are duplicate analyses.

Bacterium	*Metals and bacteriochlorophyll (μmoles/g dry wt.)*				
	Mn	*Fe*	*Cu*	*Zn*	*Bacteriochlorophyll*
R. rubrum					
1a	0.0786	3.53	0.425	0.404	15.8
1b	0.0770	3.46	0.433	0.383	
2	0.0725	3.44	0.372	0.294	12.16
3a	0.173	3.68	0.306	0.328	12.12
3b	0.170	3.99	0.304	0.321	
Rps. spheroides					
1a	0.613	3.25	0.290	0.690	17.03
1b	0.634	3.23	0.288	0.694	
2	0.831	2.46	0.360	0.248	11.8
3	0.702	2.76	0.109	0.254	13.76
Chromatium					
1	0.0767	8.94	0.118	0.663	8.99
2	0.0494	8.96	0.102	0.631	9.08
3	0.0401	6.58	0.0945	0.497	7.49

TABLE II

MOLAR RATIOS OF TRACE METALS AND BACTERIOCHLOROPHYLL RELATIVE TO IRON

Bacterium	*Metals and bacteriochlorophyll (μmoles/g dry wt.)*				
	Mn	*Fe**	*Cu*	*Zn*	*Bacteriochlorophyll*
R. rubrum					
1	0.222	10	1.23	1.13	45.2
2	0.210	10	1.08	0.855	35.4
3	0.447	10	0.795	0.846	31.6
Mean	0.293	10	1.03	0.943	37.4
Rps. spheroides					
1	1.92	10	0.892	2.14	52.6
2	3.38	10	1.46	1.01	48.0
3	2.54	10	0.395	0.920	49.8
Mean	2.61	10	0.916	1.36	50.1
Chromatium					
1	0.0859	10	0.132	0.741	10.1
2	0.0550	10	0.114	0.704	10.1
3	0.0610	10	0.144	0.756	11.4
Mean	0.0673	10	0.130	0.734	10.5
Spinach quantasomes[8]	1	6	3		115

* Fe concentrations were arbitrarily taken as 10.

parison of Tables I and III shows that the Mn concentration decreases by about 45 % in dark aerobically-grown *Rps. spheroides* and by about 70 % in dark, aerobically-grown *R. rubrum*. The results suggest that the Mn content is not related to the oxidase system of either bacteria.

The possible participation of Cu in the photosynthetic unit appears to be marginal, as shown in Table II, particularly in the case of *Chromatium*. The Cu in *R. rubrum* and *Rps. spheroides* may be related in part to their oxidase systems, as evidenced by increased Cu content in dark-grown *R. rubrum* and *Rps. spheroides*. Although the copper protein, plastocyanin, has been isolated from green plants[16], no such protein has been found in photosynthetic bacteria.

TABLE III

TRACE METAL ANALYSES ON WHOLE CELLS OF DARK AEROBICALLY-GROWN BACTERIA

Bacterium	*Metals (μmoles/g dry wt.)*		
	Mn	*Fe*	*Cu*
R. rubrum			
1	0.0263	3.71	0.233
2	0.0484	3.36	0.811
3	0.0262	3.55	1.86
Mean	0.0336	3.54	0.968
Rps. spheroides			
1	0.401	4.42	0.652
2	0.405	3.80	0.408
Mean	0.403	4.11	0.530

It is of interest that all of the bacteria grown photosynthetically concentrate Zn to the extent of one atom (*R. rubrum* and *Rps. spheroides*) to almost 3 atoms (*Chromatium*) per 40 bacteriochlorophylls. However, no function has been ascribed to Zn in either green plant or bacterial photosynthesis.

The above results are perhaps particularly relevant to the role of Fe in electron transport and the energy conversion mechanisms in photosynthesis, since the role of Fe can be related to known constituents—the heme and non-heme iron proteins. On the basis of a photosynthetic unit of 40 bacteriochlorophyll molecules, the maximum number of Fe atoms per unit would be 11 for *R. rubrum* and 8 for *Rps. spheroides*, with as many as 38 for *Chromatium*. These numbers represent maximum values inasmuch as a significant fraction of the total iron is associated with the non-photosynthetic (soluble) portion of the cells. It would, therefore, appear that an investigation of the minimum apparatus for photosynthetic energy conversion would be more fruitful for *Rps. spheroides*.

The nature of the Mn in *Rps. spheroides* was investigated further. 150 ml of a sonicated cell extract was dialyzed against 2 changes (2 l each) of 0.1 M Tris buffer (pH 7.0) for a total of 72 h. An equal volume of cell extract was dialyzed against 0.1 M Tris buffer containing 0.001 M EDTA (pH 7.0). The dialyzed whole cell extracts were then lyophilized and digested, as previously described, for atomic absorption analysis of the Mn. In Table IV, the Mn concentrations are shown relative to bacteriochlorophyll along with an analysis of a portion of the initial cell suspension before

sonication. Table IV shows that about 29 % of the Mn is removed by dialysis against Tris buffer, while an additional 36 % is removed by dialysis against EDTA. The results indicate that while a portion of the Mn is easily removed by dialysis against buffer still another portion of the Mn is rather tightly bound to cell components.

Suspensions of freshly-washed whole cells and small particles of photosynthetically-grown *Rps. spheroides* were prepared in water, together with a soluble fraction.

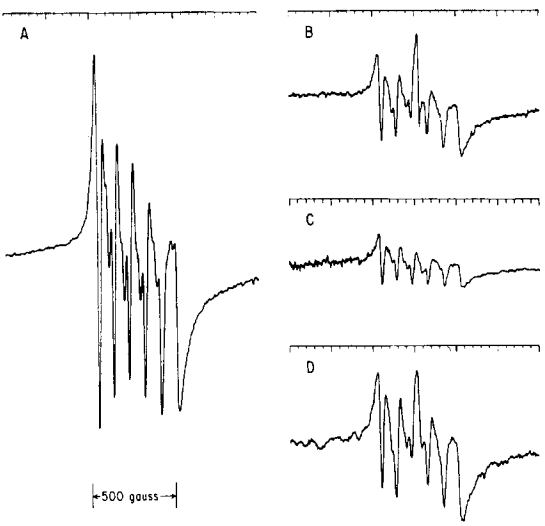

Fig. 1. EPR spectra of cell components of photosynthetically grown *Rps. spheroides*. Spectra observed at −90°, 50 mW microwave power, scan range ± 2500 Gauss, 0.3 sec time constant. A. $1.0 \cdot 10^{-5}$ M $MnSO_4$. Modulation amplitude 0.5 × 10 Gauss. Receiver gain 1.5 × 10^5. B. Whole cells $4.03 \cdot 10^{-5}$ M in Mn. Modulation amplitude 0.63 × 10 Gauss. Receiver gain 2.0 × 10^5. C. Small particles $5.00 \cdot 10^{-5}$ M in Mn. Modulation amplitude 0.63 × 10 Gauss. Receiver gain 2.0 × 10^5. D. Soluble fraction $1.24 \cdot 10^{-5}$ M in Mn. Modulation amplitude 2.5 × 10 Gauss. Receiver gain 2.0 × 10^5.

Aliquots of the solutions were lyophilized, digested and analyzed for Mn as described above. The EPR spectra (Fig. 1) of the solutions were observed along with a standard solution of $MnSO_4$. Comparison of the size of the signals at the appropriate instrument settings indicates that less than 3 % of the Mn in the whole cells and cell fractions can be observed in each of the solutions relative to the spin signal from the $MnSO_4$ solution. We conclude that the Mn in *Rps. spheroides* is not present primarily in an aqueous environment as Mn (II). This fact is reflected in the finding that only 29 % of the Mn is removed by long-term dialysis against Tris buffer.

Particulate and soluble fractions of light-grown *Rps. spheroides* were prepared (as described in MATERIALS AND METHODS) to determine the location of Mn in the cell. Particulate fractions were suspended in deionized water or Tris buffer and lyophilized. The lyophilized cell fractions were analyzed for bacteriochlorophyll, and for Mn after digestion. The results are presented in Table V as per cent of total Mn in each fraction and as the ratio of Mn to bacteriochlorophyll in the particles. Approx. 64 % of the manganese is associated with the particulate fractions of the bacteria, and the concentration relative to bacteriochlorophyll is highest in the small particles containing the highest concentration of bacteriochlorophyll.

TABLE IV

EFFECT OF DIALYSIS ON THE Mn CONTENT OF A SONICATED EXTRACT OF PHOTOSYNTHETICALLY-GROWN *Rps. spheroides*

a, whole cells before sonication and dialysis; b, after sonication and dialysis against Tris buffer (pH 7.0); c, after sonication and dialysis against Tris buffer (pH 7.0) *plus* 0.001 M EDTA.

Sample	*Mn*	*molar ratios*	
		Bacteriochlorophyll	*% of initial Mn*
S_a	1	19.6	
S_b	1	27.4	71.5
S_c	1	56.0	35.0

TABLE V

Mn CONTENT OF CELL COMPONENTS OF PHOTOSYNTHETICALLY GROWN *Rps. spheroides*

a, normal cells cultured on the modified Hutner medium; b, manganese-deficient cells cultured on the succinate–glutamate medium.

Fraction	*% total Mn**	*Ratio of Mn to bacteriochlorophyll*
Soluble		
a	35.7	
b	39.9	
Small particles		
a	21.4	2:41.6
b	25.8	1:982
Large particles		
a	42.9	1:38.4
b	34.2	1:437

* Per cent of total Mn found in the three fractions.

Rps. spheroides was grown photosynthetically on the malate–glutamate medium with and without the addition of Mn and also on the succinate*–glutamate medium with and without added Mn to determine if this bacterium has an absolute requirement for this metal. Cultures were incubated with *Rps. spheroides* cells grown on the modified Hutner medium. Several successive transfers were then made to dilute the Mn in the initial inoculum. Cells grown on the malate–glutamate medium without added Mn gave a yield 80 % of that of the cells cultured with added Mn. The yield on the succinate–glutamate medium without added Mn was 95 % that of cells grown with added Mn. The cells were analyzed for bacteriochlorophyll and Mn. These results are shown in Table VI. The Mn concentration of cells cultured on both the MS medium and the succinate–glutamate medium (containing added Mn) is considerably higher than that of cells cultured on the modified Hutner medium. The Mn to chlorophyll ratio in cells grown without added Mn is greatly decreased and much smaller than one atom per unit of 40 bacteriochlorophyll molecules.

Finally, the distribution of Mn in cells grown on the succinate–glutamate

* Malic acid was replaced by succinic acid because the former contains appreciable concentrations of manganese.

medium without the addition of Mn was examined to determine if the residual Mn would be associated with the small particle chromatophore fraction. The results are presented in Table V along with the distribution of Mn in cells cultured on the modified Hutner medium. There is no significant change in the distribution of Mn in the three fractions of deficient cells, while the Mn to bacteriochlorophyll ratio is, in fact, higher in the large particle fraction.

TABLE VI

EFFECT OF MANGANESE DEFICIENCY ON THE Mn TO BACTERIOCHLOROPHYLL RATIO IN PHOTOSYNTHETICALLY-GROWN *Rps. spheroides*

a, malate–glutamate medium without added Mn; b, succinate–glutamate medium without added Mn.

Culture medium	*Concn. (μmoles/g dry wt.)*		*Molar ratios*	
	Mn	*Bacteriochlorophyll*	*Mn*	*Bacteriochlorophyll*
Malate–glutamate	1.42	8.61	1	6.06
Low Mn_a	0.054	10.6	1	196
Succinate–glutamate	1.83	6.31	1	3.45
Low Mn_b	0.019	8.12	1	428

DISCUSSION

Based on a photosynthetic unit of 30–60 bacteriochlorophyll molecules, one can conclude that Mn is not functionally involved in the energy conversion apparatus of either *R. rubrum* or *Chromatium*. On the other hand, *Rps. spheroides* concentrates Mn to the extent of 2 atoms per 40 bacteriochlorophyll molecules under normal conditions of cell growth. The major portion of the Mn was located in particulate fractions of the cell and only partially removed by long-term dialysis. It was found, however, that *Rps. spheroides*, cultured on a manganese-deficient medium, gave a greatly reduced Mn to bacteriochlorophyll ratio, although the cell yield was not reduced greatly. This result would imply that this bacterium also does not have an absolute photosynthetic requirement for Mn. This view is consistent with previous reports relating the Mn in green plants to oxygen evolution, a function absent in photosynthetic bacteria. The possibility remains, however, that Mn is necessary for maximum photosynthetic activity (in *Rps. spheroides*) so that manganese deficiency results in some decreased cell yields. Some workers[17] have noted that reduced photosynthetic activity in manganese-deficient algae could be rapidly restored by the addition of manganese salts to whole cells. Furthermore, the conditions of deficiency did not produce a gross alteration of pigment composition[4,18]. As in the case of manganese deficiency in algae, there is an increase in the bacteriochlorophyll concentration of cells grown under manganese-deficient conditions. The reduced Mn to bacteriochlorophyll ratio in the manganese-deficient cells could reflect a change in the size of the photosynthetic unit or alternatively a decrease in the number of active photosynthetic units. However, light particles have been prepared from *Rps. spheroides* cells grown under normal and manganese-deficient conditions by fractionation of small particles on a sucrose gradient according to the procedure of WORDEN AND SISTROM[19]. Preliminary observations on the light-induced absorption changes of the particles indicated that there

was no significant difference in the extent of chlorophyll bleaching in the two particles.

The function(s) of the large concentration of Mn found in *Rps. spheroides* cells grown under normal conditions is unknown. Partial trace metal analysis of digests of *Rps. spheroides* cultured without the addition of Mn shows, however, that neither Fe, Zn nor Cu replaces Mn in the deficient cells. Cells grown on the succinate–glutamate medium contain the highest concentration of Mn although growth rates are similar to those of cells cultured on the same medium without the addition of Mn. The increased Mn content of cells grown on the succinate–glutamate medium compared to that of the Hutner medium, therefore, does not appear to reflect an increase in the requirement of Mn for a specific organic substrate.

Zn and Cu, for the most part, are present in these bacteria at sufficient concentrations to warrant further investigation into their potential functions in bacterial photosynthesis.

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