Arch. Mikrobiol. 64, 103–110 (1968)

Growth Measurements of *Chromatium* Cultures

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Received July 18, 1968

Summary. The optical density of *Chromatium* cultures grown anaerobically in the light with sulfide as electron donor is mainly determined by the sulfur content of the cells. Since the sulfur content varies, growth cannot be followed in this way. However, optical density measurements are very useful to characterize the moment of sulfide depletion and maximal sulfur storage. In addition to protein or cell nitrogen, increase in structural cell material in growing cultures of *Chromatium* can easily be followed by bacteriochlorophyll determinations, provided that the illumination intensity is higher than the saturation intensity.

Growth of bacterial cultures can generally be followed by optical density measurements. In the case of Thiorhodaceae cultivated with sulfide or thiosulfate, growth cannot be followed in this way due to the accumulation of sulfur globules in the cells. For the same reason, dry weight cannot be applied as parameter for growth. Besides protein, bacteriochlorophyll can be used as parameter, but unfortunately the bacteriochlorophyll/protein ratio is markedly influenced by the light conditions at which the cells are cultivated (FULLER *et al.*, 1963).

The present study was performed to investigate whether or not optical density measurements do give some useful information, and whether increase in structural cell material can be followed by bacteriochlorophyll determinations. Structural cell material is assumed to include all cell material with the exception of reserve substances.

Materials and Methods

Chromatium strain *6412* isolated by PFENNIG, was grown either in 10 liter culture flasks or in 100 ml screw-cap bottles in media containing per liter: NH_4Cl 60 mg; KH_2PO_4 8 mg; $MgCl_2 \cdot 6\,H_2O$ 100 mg; $CaCl_2 \cdot 6\,H_2O$ 100 mg; $NaHCO_3$ 1000 mg; $Na_2S \cdot 9\,H_2O$ up to 780 mg; and 1 ml of each, a trace elements solution (HOAGLAND and SNIJDER, 1933; cf. HEWITT, 1952) and a Fe-EDTA solution (1000 mg $NH_4 \cdot Fe[SO_4]_2 \cdot 12\,H_2O$ and 800 mg $Na_2 \cdot$ EDTA per liter). The chemicals employed were of analytical reagent grade and distilled water was used throughout. $Na_2S \cdot 9\,H_2O$ and $NaHCO_3$ were sterilized separately as 7% and 5% solutions, respectively. To avoid the introduction of other sulfur compounds (POSTGATE, 1963) sulfide stock solutions were prepared under exclusion of oxygen by leading H_2S in solutions of NaOH. After sterilization, the solutions were cooled to room temperature under nitrogen. The pH was adjusted to 7.5 with HCl. Incubation occurred at 25° C and

ca. 2000 lux incandescent light, unless otherwise stated. In all experiments, a 1% inoculum taken from an exponentially growing culture with the same initial sulfide concentration was employed.

Optical density was estimated in air-sealed cuvets by measuring transmittancy of white incandescent light using a laboratory-made densitometer. O.D. measurements are expressed in relative units.

Sulfide was determined after the dimethyl-para-phenylene method as modified by PACHMAYR (1960). This modification has also been described by TRÜPER and SCHLEGEL (1964). Sulfide concentrations are expressed in mmoles/l.

Elemental sulfur was estimated after a newly developed spectrophotometric method based on the microscopical observation that the sulfur droplets in *Chromatium* were readily dissolved in ethanol. Ethanol extracts of cells with stored sulfur show a strong absorbance in UV (maximum at 260 mμ) as do solutions of sulfur in ethanol. Ethanol extracts of cells without stored sulfur show only a slight absorbance in the same range, which was found to be proportional to the absorbance at 772 mμ (bacteriochlorophyll). Therefore, in extracts of cells with stored sulfur a correction for the absorbance at 260 mμ not caused by sulfur was made on the basis of the absorbance at 772 mμ. Bacteriochlorophyll and sulfur were estimated in the same extract, which was prepared as described under bacteriochlorophyll. The calibration factor for sulfur was calculated from the absorbance of solutions of re-sublimated sulfur in 96% ethanol. Sulfur concentrations are expressed in mg at/l.

Sulfate was determined after a modification of the turbidimetric method given in "Standard Methods" (1955). Filtrates of membrane filtered samples were directly collected into beakers containing 1 ml acetic acid; CO_2 and H_2S were removed by evacuation. During standardized mixing, 2 ml of a 30% $Ba(Cl)_2 \cdot 2\,H_2O$ solution in water was then added to 25 ml filtrate and mixing was continued for one minute. After 30 to 32 min at room temperature, extinctions were measured at 365 mμ against a blank, which was prepared with un-inoculated medium. $(NH_4)_2SO_4$ served as a standard. To avoid sedimentation of $BaSO_4$, samples were diluted so that the sulfate concentration was less than 0.15 mmoles/l.

Dry weight was estimated after drying of washed cells at 80° C till constant weight.

Cell number was determined microscopically. To save time during the experiments, parts of counting chambers were photographed which also eliminates the necessity for killing.

Bacteriochlorophyll was estimated spectrophotometrically in ethanol extracts. Samples were passed through membrane filters previously treated with H_2O, 96% ethanol and H_2O, in this order. The cell-sediment was washed with distilled water and extracted four times at room temperature in semi-darkness with 5 ml of 96% ethanol. Bacteriochlorophyll was found in the first two extracts only. The extracts were collected into 25 ml volumetric flasks, made up to volume and measured immediately against 96% ethanol at 772 mμ in quartz cuvettes (see estimation of sulfur). In this way, data are available within 10 min after sampling. Bacteriochlorophyll concentrations are expressed in *E* units (Extinction × 1,000) for 110 ml culture solution concentrated to 25 ml during extraction.

Cell nitrogen was estimated as NH_3 with Nessler's reagent after Kjehldahl digestion (GOLTERMAN, 1960). Cell-nitrogen concentrations are expressed in mg N/l.

Results and Discussion

1. O.D. Measurements

Optical density measurements appear to be useful to characterize the moment of sulfide depletion. Plots of cell number against optical density

for cells with and without stored sulfur demonstrate that the optical density is far more influenced by the storage of sulfur than by the number of cells (cf. Fig. 1).

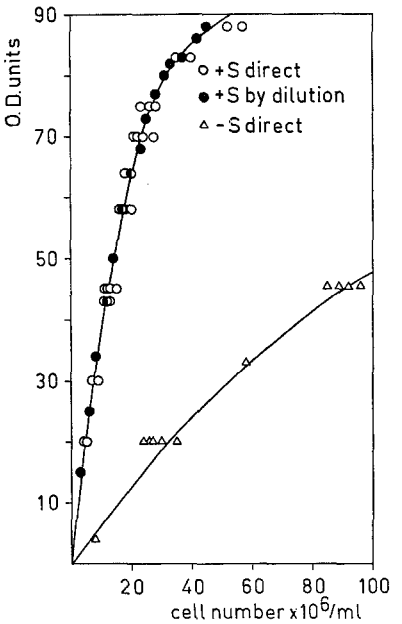

Fig. 1. Plots of cell number against optical density for cells containing stored elemental sulfur ($+S$) and cells not containing stored sulfur ($-S$). Curve for cells containing S was obtained either by using cultures in which sulfide was not depleted (direct) or by diluting such cultures with fresh medium (by dilution). Curve for cells not containg S was obtained by using cultures at the stationary phase

Sulfur is only accumulated in the presence of sulfide. In growing cultures, the optical density therefore increases as long as sulfide is still available, reaches a maximum at the moment of sulfide depletion and decreases as soon as the sulfur content of the cells decreases (cf. Fig. 2). The moment of maximal density is referred to as the Dm stage, and the stationary phase is referred to as the Df stage. Thus, during growth 2 phases can be observed; phase 1 in which sulfide is still present and phase 2 in which sulfide is absent while intracellular sulfur is still present. Fig. 2 shows that the optical density in phase 1 is not influenced by the external sulfide concentration.

Data on optical density, sulfide, sulfur and sulfate obtained in a growing culture are shown in Fig. 3, which demonstrates that maximal density (Dm) coincides with sulfide depletion and maximal sulfur storage.

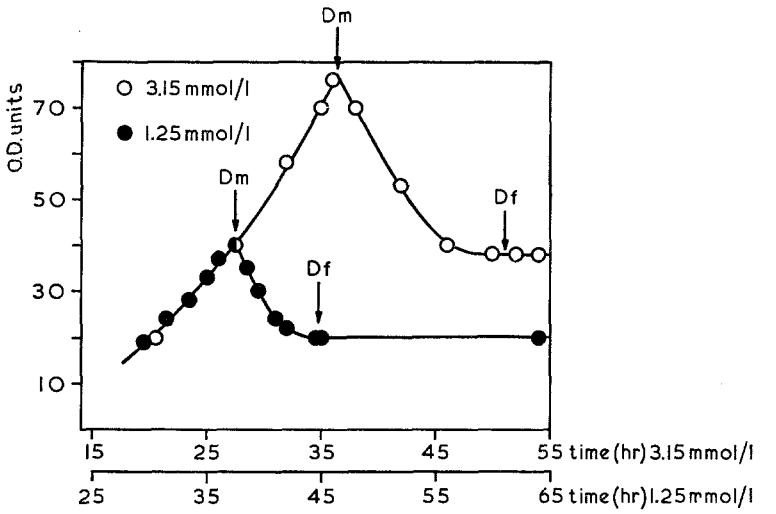

Fig. 2. Optical density measurements of *Chromatium* cultures with different initial sulfide concentrations. The different time scales are explained by the fact that the 1% inocula were taken from cultures with the same initial sulfide concentration as used in the experiments; the amount of inoculated cell material was thus less in cultures with lower initial sulfide concentrations

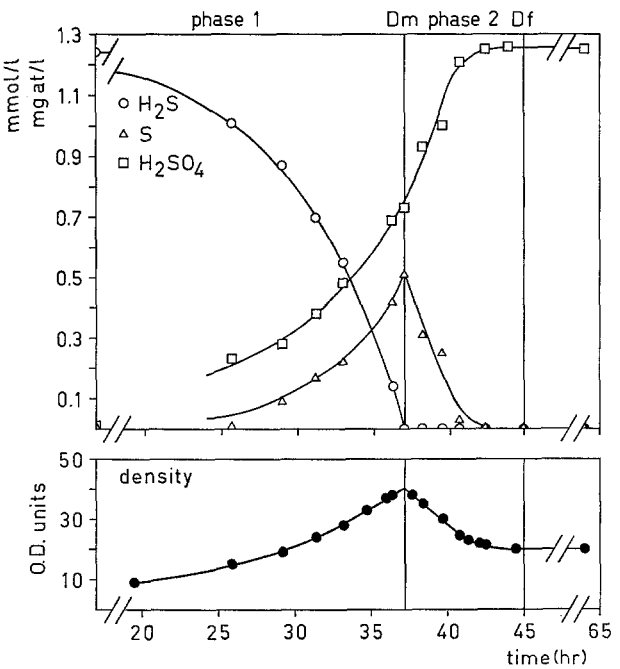

Fig. 3. Time courses of optical density, sulfide, sulfur and sulfate in a growing *Chromatium* culture. Medium prepared with 1.24 mmoles/l sulfide

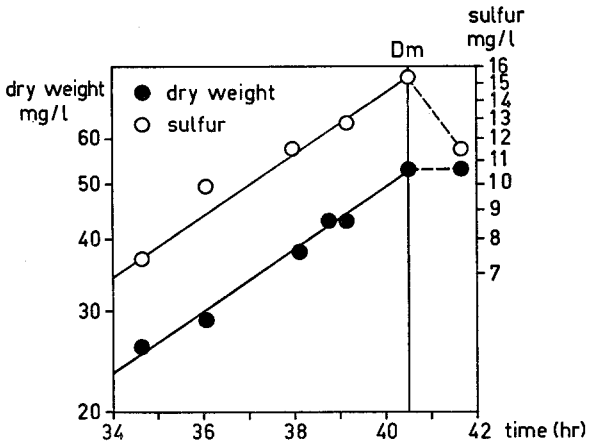

Fig. 4. Dry weight and intracellular elemental sulfur in phase 1 of an exponentially growing culture of *Chromatium*. Scales on ordinate are logarithmic, scale on abscissa is linear. Equal slopes for dry weight and intracellular sulfur indicate that the sulfur content of the dry weight is constant

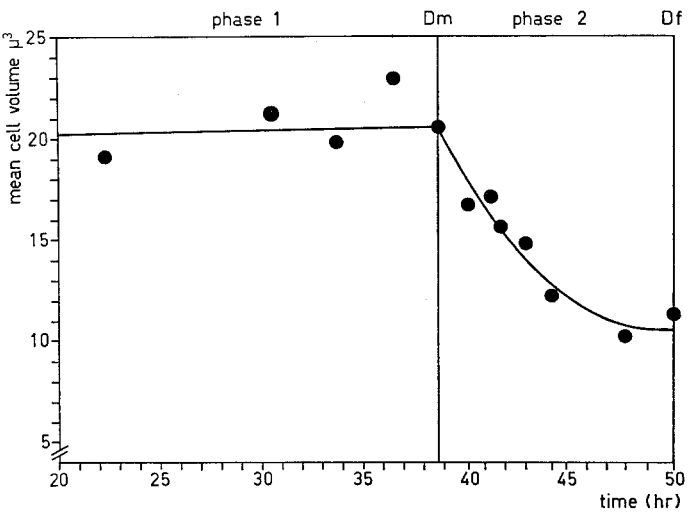

Fig. 5. Mean cell volume in an exponentially growing culture of *Chromatium*. Cell length an cell width were measured with the aid of photo-micrographs. Volumes were calculated with the assumption that the cells are cylinder shaped. Each point represents the average of 100 cells

Throughout phase 1, the content of intracellular sulfur per mg dry weight is constant (cf. Fig. 4). This means that when the culture is still in phase 1, growth can be followed by optical density measurements and dry weight measurements. However, such data would neither be com-

parable to experiments in which the organisms are cultivated with organic electron donors, nor with cultures in the stationary phase. As soon as the cells are in phase 2, neither optical density nor dry weight can be used as growth parameter.

In phase 1, growth can also be followed by cell number determinations. However, in phase 2 this is impossible because the mean cell volume is not constant (cf. Fig. 5). The decrease in cell volume in phase 2 cannot be accounted for by the disappearance of intracellular sulfur; Fig. 4 shows that in phase 1 from each 100 mg dry weight about 30 mg is sulfur. Assuming that the water content of cells without stored S is 80%, then 380 mg wet cells in phase 1 contain 30 mg sulfur. Assuming that the specific weight of cells not containing stored sulfur is 1, and that of sulfur is 2, then 365 µl cells contain 15 µl sulfur. That is, the S content of living cells in phase 1 will be approximately 4% v/v, whereas the decrease in cell volume in phase 2 is nearly 50%.

2. Bacteriochlorophyll as Parameter for Structural Cell Material

A prerequisite for applying bacteriochlorophyll as parameter for structural cell material is that its concentration in the stationary phase is directly related to the initial sulfide concentration.

Theoretically, this holds for any light intensity applied, provided that the amount of light available per cell is kept constant. However, in growing cultures illuminated by the same light source throughout, the amount of light available per cell decreases with increasing cell number. It is a well-known fact that the chlorophyll content of photosynthetic organisms is influenced by the light intensity at which the cells are cultivated; for *Chromatium* this was demonstrated by FULLER, CONTI and MELLIN (1963). For technical reasons, therefore, increase in structural cell material can only be followed by chlorophyll measurements in those experiments in which the illumination intensity is such that the decrease in the amount of light available per cell due to increased optical density does not affect the chlorophyll content. Above an illumination intensity of 1000 lux, neither the specific growth rate nor the ratio bacteriochlorophyll/H_2S was influenced by the changes in illumination intensity due to growth (cf. Table 1). Therefore, as a rule *Chromatium* was cultivated at illumination intensities ranging from 1500 to 2500 lux.

Table 2 shows that the bacteriochlorophyll concentration in the stationary phase of cultures grown at ca. 2000 lux was directly related to the initial sulfide concentration.

Table 3 shows that the ratio bacteriochlorophyll/cell nitrogen was constant in a growing culture.

Table 1. *Specific growth rate and bacteriochlorophyll/H_2S ratio in* Chromatium *cultures grown at different illuminiation intensities*

Illumination intensity	Specific growth rate	Bacteriochlorophyll at Df stage per mmole sulfide/l oxidized to sulfate
(lux)	(hr^{-1})	(*E* units)
200	0.043	410
300	0.058	—
400	0.067	—
500	0.079	—
600	0.079	—
700	0.081	—
800	0.098	—
900	0.098	—
1000	0.097	258
2000	0.098	264
3000	0.098	256

Light conditions were established by changing the distance between light source and culture. The illumination intensities listed were measured at the front side of the cultures. Specific growth rates were calculated from optical density readings in phase 1 of cultures grown in 100 ml screw-cap bottles. With increasing optical density, decreasing growth rates were observed in those cultures which were grown at illumination intensities below 800 lux. The values given above were the highest observed.

Table 2. *Ratio bacteriochlorophyll/initial H_2S concentration in the stationary growth phase of* Chromatium *cultures grown at ca. 2000 lux*

H_2S at start	Bchl at Df stage	Ratio Bchl/H_2S
1.20	302	252
1.17	304	260
1.13	302	267
1.05	258	246
0.94	242	258
0.60	151	251

Table 3. *Ratio bacteriochlorophyll/cell nitrogen in a growing culture of* Chromatium

Time (hr : min)	Bchl (*E* units)	Cell N (mg N/l)	Ratio Bchl/cell N
32 : 30	54— 54	1.1—1.6	34—49
35 : 00	76— 77	1.6—2.0	38—48
38 : 00	115—115	2.7—3.0	38—43
41 : 00	175—177	4.1—4.5	39—43
42 : 20 (Dm)	210—212	5.0—5.1	41—42
44 : 00	265—266	6.4—6.8	39—42
48 : 00 (Df)	350—353	8.6—8.8	40—41
49 : 00	351—356	8.5—8.7	40—42

Minimal ratio obtained by dividing lowest Bchl value by highest cell-N value, maximal ratio obtained by dividing highest Bchl value by lowest cell-N value.

Since the determination of chlorophyll can conveniently be coupled with that of elemental sulfur, increase in structural cell material is most easily measured by using chlorophyll as the parameter.

Acknowledgements. The author is indebted to Dr. H. L. GOLTERMAN, Prof. A. QUISPEL, and Prof. H. VELDKAMP for valuable discussions. Thanks are also due to Mrs. K. JOBSON-DE BOER for skilled technical assistance.

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