MACROMOLECULAR VARIATION IN THE CHROMATOPHORES OF THE PHOTOSYNTHETIC BACTERIUM *RHODOSPIRILLUM RUBRUM*

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(Received November 16th, 1959)

SUMMARY

Antisera were prepared in rabbits by injection of purified chromatophore preparations from *Rhodospirillum rubrum* grown anaerobically in the light. The antisera contained antibody which precipitated the pigmented particles from cell extracts, and could therefore be defined as chromatophore antibody.

By absorption of antisera with extracts of dark-grown cells devoid of pigment, two types of chromatophore antibody could be distinguished; one fraction of antibody reacted only with extracts of light-grown cells, the other fraction of antibody capable of precipitating pigmented particles reacted also with colorless antigens present in dark-grown cells. It was concluded therefore that chromatophore antigens are of two types, specific components formed as a consequence of photosynthetic growth, and antigens structurally related to chromatophores which are carried along in dark-grown cells.

Specific cell surface antigens formed during photosynthetic growth were detected by agglutination titrations of light-grown cells.

Photosynthetically grown cells placed in the dark for a short time alter their cellular contents such that chromatophore antibody no longer precipitates the pigmented component present in cell extracts. The evidence indicates, therefore, that the photosynthetic unit is capable of dissociation into fragments in the dark. This dissociation may help to explain induction and chromatic adaptation phenomena in photosynthesis.

INTRODUCTION

Most strains of photoheterotrophic bacteria can be grown either anaerobically in light or aerobically in the dark. In his classical studies on this group of microorganisms, VAN NIEL[1] pointed out that similar metabolic pathways were used by the organisms under both conditions. The cells can use oxygen in the dark as terminal electron

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** This is publication number 48 of the Graduate Department of Biochemistry, Brandeis University.

acceptor, or can generate photooxidants as terminal electron acceptors for anaerobic growth in the light. Apart from the need for bacteriochlorophyll to provide photooxidants in cells growing anaerobically there are no known cellular alterations necessary for photosynthetic growth of these bacteria. Any cellular components formed exclusively during growth in the light could reasonably be considered essential parts of the photosynthetic apparatus.

This report describes an attempt to detect, by immunochemical means, cellular constituents formed solely as a consequence of photosynthetic growth. Such unique antigens have been found. Analysis of the formation and macromolecular alteration in these structures appears to permit new insight into the general problem of metabolic induction phenomena in photosyntheses.

MATERIALS AND METHODS

Rhodospirillum rubrum was grown in the medium of COHEN-BAZIRE *et al.*[2] either in completely full 1-l reagent bottles in the light, or in 500-ml volumes of media in 2-l Erlenmeyer flasks rapidly shaken on a rotary shaker in the dark at 30°. All antigen preparations were from extracts of cells prepared in a Raytheon sonic oscillator. A purified "small particle" fraction of chromatophores was used for injection into rabbits to obtain antisera[3]. Sera were obtained from three rabbits after one month of a series of intravenous injections. The sera were pooled, heated to inactivate complement and used for this study. The general immunochemical methods used have been described[4].

RESULTS

When *Rhodospirillum rubrum* is cultured and subcultured aerobically in the dark for many generations, the cells can be depleted of bacteriochlorophyll and colored carotenoids until there is no spectroscopically detectable pigment present in cultures. Typical absorption spectra of extracts of cells grown in light and dark are shown in Fig. 1. The loss of pigment is phenomic; all of the bleached cells are capable of photosynthetic growth when returned to anaerobic conditions. When cells which have been grown in the light are disrupted, the entire photosynthetic pigment complex is found in "particles" 50 mμ in diameter, called "chromatophores"[5], which can be isolated by differential centrifugation. Cells grown aerobically in the dark do not release particles of this size on disruption.

Antisera were obtained toward purified chromatophore preparations derived from *R. rubrum* cells grown in the light. Extracts of both light-grown and of dark-grown cells reacted with the antisera, as seen in precipitin titration data in Fig. 2. Although the equivalence point in the two titrations was obtained with approximately the same amount of cell extract, the extract from light-grown cells precipitated more nitrogen on reaction with antiserum. Since nothing is known about the nitrogen content of the antigens precipitating in the two reactions one must ask whether the extract of light-grown cells is qualitatively different, apart from the obvious difference that the precipitate from its reaction contains the bacteriochlorophyll and carotenoids of the cell.

A large volume of antiserum was titrated to the equivalence point with an extract of dark-grown cells as indicated by the maximum in the curve in Fig. 2

(point a). The reaction mixture was allowed to stand 48 h in the cold and the precipitate was removed by centrifugation. The supernatant serum so obtained no longer reacted with extracts of dark-grown cells. It did, however, react with extracts derived from light-grown cells, as seen in the inset in Fig. 2. This supernatant serum, obtained by complete absorption with extracts of dark-grown cells, will be referred to as "specific antibody" since it contains antibodies which react with components present in light-grown, but not dark-grown cells.

As previously mentioned, the original antiserum precipitated bacteriochlorophyll from extracts of light-grown cells. Such a reaction can be defined as a chromatophore–antichromatophore reaction, because the antibody is capable of reacting with and specifically precipitating pigmented particles[4]. The chromatophore–antichromato-

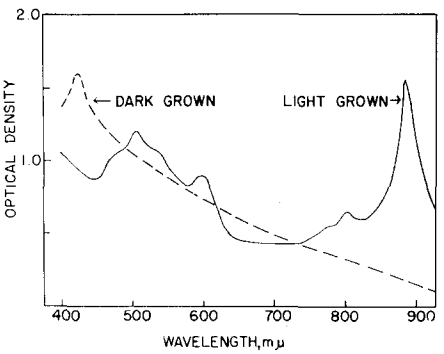

Fig. 1.

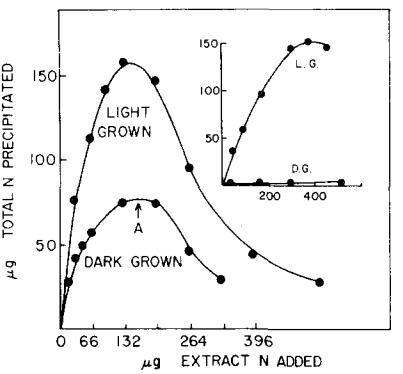

Fig. 2.

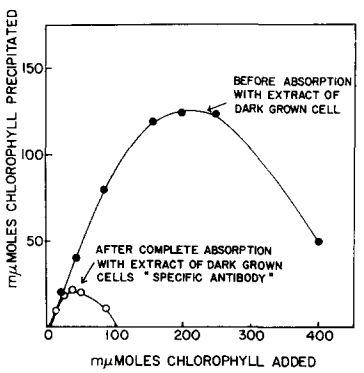

Fig. 3.

Fig. 1. Absorption spectra of sonic extracts of cells taken with Cary recording spectrophotometer. The extracts were adjusted to approximately equal protein concentration.

Fig. 2. Precipitin titrations of extracts and 0.1 ml antiparticle serum. Extract N plotted is acid-precipitable N only. The inset is a similar titration of serum equivalent to 0.5 ml after absorption at point "a" with extract of dark-grown cells.

Fig. 3. Reaction of pigmented particles from sonic extract with 0.5 ml antiserum and equivalent amount of antiserum after absorption at point "a" with extract of dark-grown cells.

phore reaction can be studied independently of other antigenically reacting species present in crude bacterial extracts because the precipitating antigen is a pigmented macromolecular complex which can be quantitatively measured by spectrophotometry of the immune precipitate. It is therefore possible to determine whether specific antibody and chromatophore antibody are the same, and if so, how much chromatophore antibody is specific only for light-grown cells.

Fig. 3 shows the results of a precipitin titration made with an extract of light-grown cells and the original antiserum, and a similar titration of the absorbed serum or "specific antibody". The data are adjusted to correct for dilution of the serum

which occurs in the first reaction to prepare specific antibody. Prior reaction of the serum with an extract of dark-grown cells reduces, but does not completely eliminate the ability of the serum to precipitate pigment bearing particles. Approximately 20 % of the antibody capable of precipitating pigmented particles is formed to unique cellular components present only in light-grown cells. The largest fraction of chromatophore antibody reacts also with extracts of dark-grown cells. This antibody fraction will be referred to as "related antibody" since it reacts with components which although present on pigmented particles are also present in some form in dark-grown cells.

Previous work on immune reactions of pigmented particles from the photosynthetic anaerobe *Chromatium* has established that chromatophore antibody reacts with intact cells[4]. Data in Table I show that specific antibody can agglutinate intact cells of *R. rubrum* growing in the light. Such cells placed under aerobic growth conditions in dark lose the ability to be agglutinated however, and the reaction reappears when the culture is returned to anaerobiosis in the light. These data further indicate the cell surface nature of some of the specific components of the photosynthetic apparatus.

TABLE I

AGGLUTINATION OF INTACT *R. rubrum* CELLS BY "SPECIFIC ANTIBODY"

Specific antibody, 1:5 in reaction mixture (ml)	*2 · 10⁹ cells each type*		
	Light-grown cells	*Aerated-dark 7 h*	*Returned to light 13 h*
1.0	4 +*	+ —	4 +
0.8	4 +	+ —	4 +
0.7	3 +	—	3 +
0.6	2 +	—	2 +
0.5	2 +	—	2 +
0.4	2 +	—	2 +
0.3	+	—	—
0.2	+	—	—
0.1	—	—	—

* A reaction of 4 + indicates that all of the cells added were strongly agglutinated by visual estimate. The relative extent of agglutination was graded downward from this estimate by visual comparison. Total reaction volume 1.2 ml containing $2 \cdot 10^9$ cells and indicated amount of serum diluted in saline.

We have studied the ability of the types of chromatophore antibody to detect the presence of their respective antigens in cells growing in light and dark. The antigen is particulate, and consequently its titer may depend on its state of fragmentation during preparation of extracts by sonic rupture of the cells. Therefore, a series of control experiments was performed to test the reproducibility of the assay with cell extracts. The precipitin titrations were found to be reproducible on extracts provided an equal amount of cell breakage had been obtained. Data from a typical control experiment are given in Table II. Inquiry into the formation of chromatophore antigens, therefore, seemed possible.

It has been shown by COHEN-BAZIRE *et al.*[2] that aeration of photosynthesizing cells of *R. rubrum* causes an immediate cessation of chlorophyll synthesis but does not inhibit exponential growth of the cells. The data in Fig. 4 confirm the findings

TABLE II

TEST OF REPRODUCIBILITY OF PRECIPITIN REACTIONS ON SONIC EXTRACT

Values are given for the maximum in the precipitin titration curve. The shapes of the curves were identical with all extracts. The original antiserum, diluted 1:10, was used. Titrations were done on 1-ml aliquots of diluted serum.

Sonic oscillation (min)	*Precipitin titration of sonic extract*	
	Bacteriochlorophyll precipitated (mμmoles)	*Total N precipitated (μg)*
1	23.7	148
4	24.2	151
10	23.2	146

of COHEN-BAZIRE *et al.* and include serological analysis of cell extracts prepared from *R. rubrum* during growth in light and dark. A culture of *R. rubrum* growing exponentially under anaerobic conditions in the light was transferred to Erlenmeyer flasks, shaken vigorously on shaker for 10 h in the dark, and then returned to anaerobiosis in light. Growth and chlorophyll synthesis were measured spectrophotometrically, and aliquots of the culture were taken to prepare cell extracts for antigen titration. The growth curve of the culture and the relative amount of chlorophyll per cell are plotted in Fig. 4. Aeration resulted in an immediate inhibition of chlorophyll synthesis and consequent exponential decrease in the amount of chlorophyll per cell as growth continued aerobically. Returning the culture to anaerobiosis in light caused a resumption of photosynthetic growth and chlorophyll biosynthesis. No lag phase in growth was detectable in the culture during the conversion of cells from photosynthetic to aerobic growth or their return to photosynthesis, and no chlorophyll destruction took place in the cultures under any conditions. Precipitin titrations on extracts of these cells, using specific antibody, are also given in Fig. 4.

These data show that upon conversion of cells to aerobic growth in the dark, the chlorophyll in the cell becomes non-precipitable by specific antibody. On return to photosynthetic growth conditions, the ability of pigmented particles to react with specific antibody reappears. No chlorophyll destruction takes place during aerobic

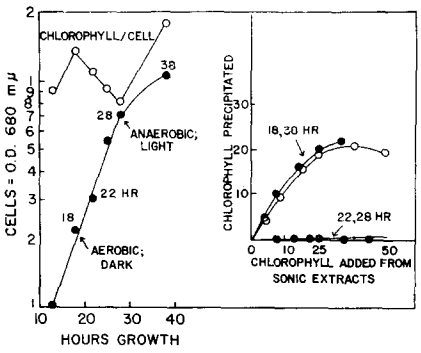

Fig. 4. Growth curve of *R. rubrum* culture initially growing exponentially in light. The bacteriochlorophyll content per cell was estimated from the ratio of O.D. at 880 and 680 mμ. In the precipitin titrations (inset) of cell extracts, standardized on chlorophyll content, specific antibody equivalent to 0.5 ml antiserum was used.

growth. The amount of chlorophyll added, in the form of crude bacterial extract, is the same for titration of all extracts. It is concluded, therefore, that photosynthetically grown cells of *R. rubrum* which have subsequently been allowed to grow for a short time aerobically in the dark alter their cellular contents in a manner which renders their chlorophyll non-precipitable by specific antibody.

The question arises whether related antibody, which can react with both pigmented particles and dark-grown cell extracts, reacts with the pigmented particles in the aerated cells. Data in Table III show that there is a large decrease in the

TABLE III

REACTION OF PIGMENTED PARTICLES WITH ORIGINAL ANTISERUM

The original antiserum was diluted 1:10 and 1-ml aliquots used for titration. Data are given for the maximum in the titration curves.

History of cells	*Precipitin titration of sonic extracts*	
	Bacteriochlorophyll precipitated (mμmoles)	*Total N precipitated (μg)*
Initial culture growing in light anaerobically	25.0	158
Placed in dark 4 h, aerobic	4.5	136
Returned to light for 10 h	26.4	156

ability of the original antiserum to precipitate pigmented particles from the extract from aerated cells. This decrease is greater than can be accounted for by loss of reaction by specific antibody alone. However, the presence of non-pigmented reactants in these cells is indicated by the amount of nitrogen in the precipitate from the aerated cell extract.

Extracts prepared from photosynthetically grown cells of *R. rubrum* which had been washed and aerated in phosphate buffer did not lose their ability to precipitate pigmented particles on reaction with specific antibody. These data are given in Table IV. Therefore, growth of the cells is required to alter the photosynthetic apparatus.

We next attempt to analyze the nature of the loss of reactivity by the pigmented particles of *R. rubrum* during aerobic growth. It may be asked whether the unreactive particles are uniquely different from those originally present in photosynthesizing

TABLE IV

STABILITY OF PIGMENTED PARTICLES IN AERATED RESTING CELLS

Specific antibody equivalent to 0.5 ml original serum was used.

Initial cells, light grown		*Cells aerated 3 h, 0.05 M phosphate, pH 6.8*	
Precipitin reaction, bacteriochlorophyll (mμmoles)		*Precipitin titration, bacteriochlorophyll (mμmoles)*	
Added	*Precipitated*	*Added*	*Precipitated*
18	12	16	12
36	22	32	22
54	14	64	14
72	12	96	12

cells. By aeration of growing cells for short periods of time, a partial loss of reactivity of particles in the cell extract could be obtained. The extract from aerated cells was then titrated with specific antibody to the equivalence point and the new absorbed serum obtained was retitrated with an extract of photosynthetically grown cells. The data are given in Table V. It can be seen that absorption of specific antibody with the extract of aerated cells which had a reduced titer completely removed all antibody capable of precipitating pigmented particles. It appears, therefore, that there is no preferential destruction of some specific antigens on the macromolecules in aerated, photosynthetically grown cells, accounting for the loss of reactivity.

TABLE V

ABSORPTION OF SPECIFIC ANTIBODY BY EXTRACT FROM AERATED CELLS

	History of cells	*Chlorophyll precipitated at equivalence (mμmoles)*
a	Initial light grown	27
b	Aerated	18
c	Returned to light	30
d	Reaction of c after absorption with b at equivalence point	0

TABLE VI

PRECIPITATION OF MIXTURES OF EXTRACTS FROM PHOTOSYNTHETICALLY-GROWN AND AERATED CELLS

Specific antibody equal to 0.5 ml original antiserum used per tube. When antigen mixtures were added, the aerated cell extract was added first.

Bacteriochlorophyll added as sonic extract of (mμmoles)		*Bacteriochlorophyll precipitated (mμmoles)*
Light-grown cells	*Light-grown cells + aerobic growth**	
15	0	14
30	0	26
0	17	2
15	7	14
15	15	12
15	29	6

* The light-grown culture was placed under exponential aerobic growth conditions in the dark for 10 h.

It seemed likely that photosynthetically grown cells formed non-pigmented reactants on aeration which could prevent precipitation of any intact chromatophores by specific antibody. The presence of such "inhibitors" in aerated cells was demonstrated by studying precipitation of extracts which reacted to precipitate chlorophyll, alone and in the presence of extracts from aerated cells. Typical data are given in Table VI, which shows that the precipitation of pigmented particles from reactive extracts by specific antibody could be inhibited by extracts of pigmented cells grown aerobically. This suggests the presence, in the aerobically grown cells of components which, although non-pigmented, react with specific antibody.

Finally, more precise characterization of the macromolecular change accompanying the inability of chlorophyll to be precipitated from aerated cell extracts was attempted by simultaneously studying antibody, chlorophyll and ^{32}P precipitation from extracts prepared from pigmented cells before and after aerobic growth. ^{32}P labeled cells were obtained by growth of the cells in $^{32}PO_4$ containing media to label the chromatophore phospholipids, which constitute the majority of the phosphorus of the particle, and consequently, provide a measure of precipitating antigen independent of chlorophyll content. The antibody used was a preparation of specific antibody conjugated with dimethylaminonaphtalene sulfonyl chloride by the method of REDETZKI[6]. The relative amount of antibody in the precipitates was determined directly by measurement of the fluorescence of the washed specific precipitates in a Farrand fluorometer. The data are summarized in Table VII, and show that extracts of the aerated cells react with and precipitate specific antibody from the serum, although such extracts precipitate less chlorophyll on reaction. Furthermore, the composition of the specific precipitate formed by extracts of aerated cells is different from that precipitate formed by the contents of photosynthesizing cells sampled initially. There is less chlorophyll per unit phospholipid on the precipitating antigen from the aerated cells. These data are all consistent with a release of chlorophyll from its bearer antigen during aerobic growth of pigmented cells of *R. rubrum*.

TABLE VII

CHANGE IN COMPOSITION OF PIGMENTED ANTIGEN ON AEROBIC GROWTH OF *R. rubrum*

The data refer to different points on the respective precipitin curves.

Analysis of specific antibody precipitates from extracts							
Initial cells (light-grown)				*90 min aerobic growth*			
*Antibody**	*^{32}P (counts/min)*	*Bacteriochlorophyll (mμmoles)*	*Ratio***	*Antibody*	*^{32}P (counts/min)*	*Bacteriochlorophyll (mμmoles)*	*Ratio*
24	27,000	1.1	2.45	18	30,000	1.1	2.72
21	49,000	2.8	1.75	14	39,000	1.4	2.78
14	41,000	2.6	1.57	10	25,000	1.1	2.27
10	25,000	1.5	1.66	10	20,000	0.8	2.50

* Galvanometer readings indicating relative fluorescence of precipitates.
** ^{32}P, counts/min/bacteriochlorophyll $\times 10^4$.

DISCUSSION

This investigation establishes the presence of unique antigenic components in *Rhodospirillum rubrum* which are formed as a specific consequence of photosynthetic growth. Although the chemical nature of these antigens is unknown at present, one of them appears to be a cell surface component, because specific antibody reacts with and agglutinates intact cells growing photosynthetically. A cell wall polysaccharide serologically related to the pigmented particles in the photosynthetic anaerobe, *Chromatium*, has been isolated[4]. It remains to be determined whether *R. rubrum* contains a similar surface antigen.

The great speed with which *R. rubrum* can alter its metabolism from photosynthetic to aerobic growth suggests that little change in intracellular architecture is required for conversion. We find from analysis of the immunochemical data that the changes occurring are principally characterized by the chlorophyll of the cell entering a non-precipitable form. The fact that extracts of aerated cells still react with and remove specific antibody from sera indicates that the unique chlorophyll-bearing antigens in these cells are neither masked nor destroyed; they are separated from the chlorophyll of the cell. Furthermore, the chlorophyll present in aerated cells is not precipitable by related antibody either, which indicates that an appreciable fraction of the macromolecules are disorganized, not just specific components formed in light.

Further data lending support for the view that aerobic growth leads to chlorophyll dissociation from the photosynthetic apparatus come from recent work on pigment mutants of *Rhodopseudomonas spheroides*. These cells lack unsaturated carotenoids and are killed by simultaneous exposure to light and oxygen[7]. Death of the cell results from a chlorophyll-mediated photooxidation of an unknown essential cellular constituent. DWORKIN has recently found that a short period of aerobic growth will protect the cell from photokilling[8]. This also suggests that during dark aerobic growth, the chlorophyll has entered a different form than its state in photosynthesizing cells. In the aerated mutant cell, chlorophyll may be no longer in contact with the essential cell component, and cannot cause death.

All of the data presented in this paper lead to the view that during aerobic growth, the chlorophyll of the cell leaves its bearer antigens. Whether this change involves alteration of the tertiary structure of the photosynthetic unit, or more extensive changes, is unknown. The dissociation does not appear to release free chlorophyll because there are no spectral changes of chlorophyll accompanying the reaction. However, we may ask how the separation might affect the organisms physiologically and whether this finding can be used to explain events following subsequent illumination of the cells. There is a long history of reports of "induction phenomena" in photosynthesizing cells placed aerobically in the dark for a period of time and subsequently returned to the light[9]. Theories attempting to explain the lag in photosynthesis and other physiological changes which occur in green cells returned to light have generally assumed that during the dark period the photosynthetic apparatus remained intact. The present results show that, in *R. rubrum* at least, such an assumption is untenable and immediately suggests an alternative explanation for metabolic induction periods; that it is simply the time required for chlorophyll to reorient itself in its site in the photosynthetic apparatus. Furthermore, some aspects of "chromatic adaptation" of green cells may be explained on this basis; cells grown in light which is not absorbed by chlorophyll may be expected by analogy with dark-grown cells to dissociate their photosynthetic unit and contain "inactive" chlorophyll of the type shown in this study to be separated from its bearer antigen(s) in the cell.

ACKNOWLEDGEMENTS

I am indebted to Professors M. D. KAMEN and H. GAFFRON for discussions of this work, and to the Society of American Bacteriologists for a President's Fellowship in 1959.

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Biochim. Biophys. Acta, 42 (1960) 34–43

HAEMOGLOBIN OF THE LAMPREY, *LAMPETRA FLUVIATILIS*

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(Received December 23rd, 1959)

SUMMARY

The haemoglobin of the brook lamprey (*Lampetra fluviatilis*) has been investigated. It has a molecular weight of 17,200 and an absorption spectrum in the visible and Soret regions very similar to human haemoglobin. The tryptophan fine-structure absorption band in the u.v. and amino-acid composition of lamprey and human haemoglobins are significantly different. Lamprey haemoglobin has one sulfhydryl group per mole of native and 1.9 group per mole of denatured protein. All 26 specimens examined showed one minor and two major components on electrophoresis. Lamprey haemoglobin is of special interest in providing a clue to the evolution of haemoglobin in the vertebrates.

INTRODUCTION

Cyclostomes are the most primitive living vertebrates. Together with the extinct ostracaderms, the most ancient fossil vertebrates, they form the super-class *Agnatha*, which has followed an independent evolution since earliest vertebrate origins. Cyclostomes are also the most primitive animals known to have haemoglobin enclosed in erythrocytes. Haemoglobin and myoglobin are two proteins which have been intensively studied both from the structural and the functional point of view. Investigations of haemoglobins in different species[1] show how the protein has been modified in the course of evolution to fulfil the physiological needs of a wide range of organisms. The haemoglobin of the lamprey is of special interest since it lies intermediate in structure and properties between myoglobins and haemoglobins of higher