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7. Data used in this study represent ongoing efforts over several years of many members of the staff of the seismographic stations of the University of California with financial support from both the National Science Foundation and the Advanced Research Projects Agency, as well as the State of California in establishing and maintaining the long-term monitoring facilities. We particularly thank the Kaiser Natividad Quarry staff for their cooperation in timing explosions and D. Bussey, the present plant manager, for providing data on times and locations of specific explosions over the years. We appreciate also the receipt of an advance copy of the article by Scholz *et al.* (*5*).

30 July 1973

Functional Organelles in Prokaryotes: Polyhedral Inclusions (Carboxysomes) of Thiobacillus neapolitanus

Abstract. *The polyhedral inclusions of* Thiobacillus neapolitanus *have been isolated; they contain ribulose diphosphate carboxylase.*

Prokaryotic cells have not been reported to have metabolically functional organelles. Many blue-green algae and chemoautotrophic bacteria contain polyhedral inclusions that have identical appearances in thin section (*1*, *2*). We have isolated these inclusions from *Thiobacillus neapolitanus* (Fig. 1) and have discovered that they contain the enzyme ribulose diphosphate carboxylase (E.C. 4.1.1.39). We propose that these organelles, responsible for carbon dioxide fixation, be called carboxysomes.

The bacteria were grown in a 200-liter fermentor with constant aeration and *p*H control (*p*H 7.0) in the medium described by Vishniac and Santer (*3*). After the cells reached the maximum stationary phase of growth they were collected by centrifugation, packed in plastic petri dishes, frozen, and stored (−10°C) until used.

The purification procedures were monitored by electron microscopy and enzymatic assay. Fractions were collected, fixed with glutaraldehyde (1 percent final concentration), dialyzed, negatively stained (potassium phosphotungstate, *p*H 7.0) on carbon-coated, Formvar covered grids, and observed in a Siemens Elmiskop 1A equipped with a short focal length objective. Ribulose diphosphate carboxylase was assayed by modification of the method of McFadden and Denend (*4*). The final reaction mixture at *p*H 8.0 contained 0.01*M* tris(hydroxymethyl)aminomethane-HCl, 0.01*M* $MgCl_2$, 0.001*M* dithiothreitol, 0.001*M* ribulose diphosphate (tetrasodium salt, Sigma, St. Louis), and 0.02*M* $NaH^{14}CO_3$ (0.167 μc/μmole; New England Nuclear). The reaction was initiated by adding ribulose diphosphate after a 10-minute incubation period at 25°C. At desired intervals (0.5 to 2.0 minutes), the reaction was terminated by transferring aliquots into scintillation vials that contained an equal volume of glacial acetic acid. We used the scintillation fluid of Patterson and Greene (*5*) and a Packard Tricarb liquid scintillation spectrometer (model 3320) to measure radioactivity and a [^{14}C]toluene standard (New England Nuclear) to determine counting efficiency. One unit of ribulose diphosphate carboxylase catalyzes the fixation of 1 μmole of CO_2 per minute at 25°C under the conditions specified. All enzyme activities have been corrected for the minimal background fixation that takes place in the absence of ribulose diphosphate. Specific activities are expressed as the number of enzyme units per milligram of protein. Protein was estimated by the method of Lowry *et al.* (*6*) with bovine serum albumin as the standard.

The purification methodology, including the results of protein and enzyme assays, is presented in Table 1. Frozen packed cells (8 to 10 g) were resuspended in distilled water to a final volume of 20 ml. This suspension, containing 1620 mg of protein, had low enzyme activity; ribulose diphosphate does not effectively penetrate the cells. Deoxyribonuclease I (Sigma) was added (final concentration, 10 μg/ml), and the suspension was passed through a French pressure cell at 20,000 pounds per square inch external pressure. Nearly all the protein was recovered in the material that passed through the press (pressate), and the enzyme activity increased 15-fold (specific activity, 0.12).

After passing the material through the pressure cell, we performed all manipulations (resuspending, gradients) in the following buffer: tris(hydroxymethyl)aminomethane-HCl, 0.005*M*; ethylenediaminetetraacetic acid, 0.001*M*; $MgCl_2$, 0.001*M*; and $NaHCO_3$, 0.02*M*; *p*H 8.0.

The pressate was centrifuged in a Sorvall RC2-B refrigerated centrifuge (SS-34 rotor) for 1 hour at 48,000*g*. There were three readily separable fractions: the supernatant, the fluffy layer (easily resuspended by light shaking), and the hard pellet (resuspended only by using a stirring rod and hard shaking). About 80 percent of the total enzymatic activity was sedimented (fluffy layer and hard pellet). The results of MacElroy *et al.* (*7*) who reported that the ribulose diphosphate carboxylase of either *Thiobacillus thio-*

Table 1. Results of the purification of carboxysomes and ribulose diphosphate carboxylase of *Thiobacillus neapolitanus*. The numbers in parentheses indicate the amount of material carried forward to the next step in the purification. The specific activity of a fraction is the number of enzyme units divided by the protein in that fraction.

Fraction	Enzyme units	Protein (mg)	Specific activity
Cells	13.6 (12.9)	1620 (1539)	0.008
Pressate	180.5 (142.5)	1520 (1200)	0.12
Differential centrifugation			
Supernatant	28.6	561	0.05
Fluffy layer	58.7 (55.2)	283 (266)	0.21
Hard pellet	53.0	258	0.21
Sucrose step gradient			
Sample layer and 45 percent sucrose layer	16.0	235	0.07
60 percent sucrose layer	28.9 (27.2)	20.7 (19.5)	1.4
65 percent sucrose layer	4.5	6.7	0.67
$(NH_4)_2SO_4$ precipitate			
	11.6 (9.5)	9.4 (7.7)	1.2
Linear sucrose gradient			
Band 1 (25 percent sucrose)	4.4	2.0	2.2
Band 2 (55 percent sucrose)	1.4	3.4	0.41

parus or *T. neapolitanus* was not sedimented by centrifugation for 30 minutes at 105,000*g* may have been due to the rupture of most of the carboxysomes by their experimental procedures.

Even though 20 percent of the enzymatic activity was present in our supernatant, carboxysomes were not observed; it is reasonable to expect some breakage and subsequent enzyme release during the pressure cell treatment. However, it is possible that some of the enzyme exists free in the cytoplasm. Ribulose diphosphate carboxylase from *Thiobacillus denitrificans*, which does not have polyhedral inclusions, is not sedimented by centrifugation for 1 hour at 100,000*g* (*2, 4*).

The fluffy layer consisted primarily of cell envelope fragments and carboxysomes. The hard pellet contained a few whole cells and many partially broken cells and trapped carboxysomes. The hard pellet had a specific activity (0.21 unit/mg) as high as the fluffy layer; however, we could not further purify the carboxysomes from the combined sedimentable fractions (fluffy layer and hard pellet). The resuspended fluffy material was layered on the surface of sucrose step gradients consisting of 1.0 ml of 45 percent sucrose, 5.0 ml of 60 percent sucrose, and 9.0 ml of 65 percent sucrose (by weight). After centrifugation (2°C) for 16 hours at 81,000*g* in a Beckman preparatory ultracentrifuge (model L2-65B; swinging bucket rotor, model SW27, tube size ⅝ by 4 inches) approximately 50 percent of the added enzymatic activity was recovered in the 60 percent sucrose fraction (specific activity, 1.4 unit/mg). Carboxysomes (Fig. 2), consisting of a particulate component surrounded by a membrane, and some contaminating cell envelope fragments were observed. (The term membrane is used here only to indicate a surrounding barrier and in no way implies trilaminar structure). The isolated carboxysomes have swollen to an average diameter of 160 nm. The vast majority of the contaminating materials, as well as many ruptured, partially emptied, carboxysomes were retained in and above the 45 percent sucrose. The carboxysomes exhibited considerable instability when removed from the cytoplasm. We did not obtain a discrete carboxysome band and additional centrifugation did not result in increased carboxysome yield or band formation, but did increase contamination. The 60 percent sucrose with a density of 1.29 g/cm^3 at 2°C appears

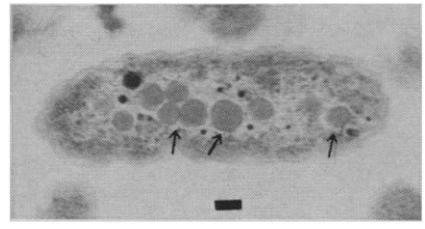

Fig. 1. Thin section of *Thiobacillus neapolitanus* showing the general structure of the cells. Carboxysomes are indicated by arrows. Marker bar represents 100 nm.

to be near the isopycnic point of the carboxysomes. The bodies ruptured, releasing their contents when the 60 percent sucrose fraction was diluted with ten volumes of buffer. At present, glutaraldehyde fixation is the only known method of stabilizing the carboxysomes.

Crystalline ammonium sulfate was added to the dilute fraction (4°C, *p*H 8.0) to 70 percent of saturation and the resulting precipitate was collected by centrifugation, and redissolved in buffer. Only 43 percent of the enzymatic activity and 48 percent of the protein were retained and the specific activity decreased to 1.2 unit/mg. The redissolved precipitate was centrifuged in a linear sucrose gradient (5 to 65 percent by weight). There were two distinct bands at approximately 25 percent sucrose and 55 percent sucrose, but some enzymatic activity and protein were spread throughout the lower part of the gradient (below the band at 25 percent sucrose). The upper, enzyme band (Fig. 2, inset) had a specific activity of 2.2 unit/mg. The enzyme is approximately 10 nm in diameter and is similar to the ribulose diphosphate carboxylase of spinach chloroplasts and *Chromatium* (*8*). The lower band consisted of contaminating envelope fragments and carboxysome membranes with some adhering enzyme particles.

We have thus demonstrated that

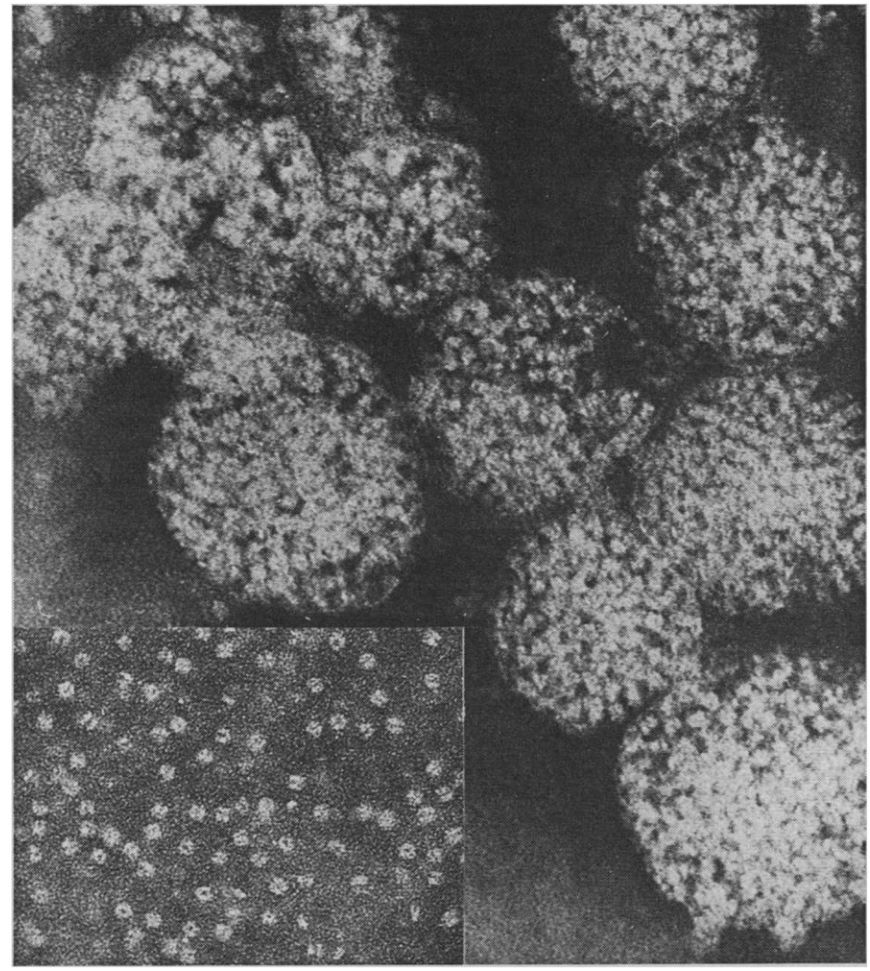

Fig. 2. Electron micrographs (negative stains) of the polyhedral inclusions (carboxysomes) and ribulose diphosphate carboxylase (inset) of *Thiobacillus neapolitanus* (× 187,000). The enzyme can be observed inside the inclusions.

much of the ribulose diphosphate carboxylase of *T. neapolitanus* is contained within membrane-bound, polyhedral shaped inclusions. We suggest that structurally similar inclusions in other autotrophic microorganisms may also be "packages" containing this enzyme.

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* Oak Ridge National Laboratory is operated for the U.S. Atomic Energy Commission by the Union Carbide Corporation.

18 May 1973

Rabbit Blood Lymphocytes May Be T Cells with Surface Immunoglobulins

Abstract. *Rabbit peripheral blood lymphocytes and thymus cells do not respond to lipopolysaccharide mitogen in vitro, whereas spleen cells do. Soluble concanavalin A consistently stimulates 80 to 90 percent of rabbit peripheral blood lymphocytes, and the morphologic changes associated with such transformation may be observed within 18 hours after stimulation. Approximately 80 percent of rabbit peripheral blood lymphocytes have demonstrable immunoglobulin markers. These and other observations suggest that most rabbit peripheral blood lymphocytes are T cells with surface immunoglobulins.*

The observation that rabbit peripheral blood lymphocytes (PBL's) could be stimulated to undergo blast transformation with antiserum to immunoglobulin (anti-Ig) and antiserum to allotypic immunoglobulin (anti-As) provided the first convincing evidence that lymphocytes have surface immunoglobulins (*1*). The fact that up to 90 percent of rabbit PBL's could be transformed with anti-Ig indicates that most, if not all, rabbit PBL's have surface Ig (*2*). With the recognition of two cell types involved in the induction of immune responses, T (thymus-derived) cells and B (bone marrow–derived) cells (*3*), it became of interest to determine the classification of the rabbit PBL. Most observers concluded that the rabbit PBL is a B cell because of the association of surface immunoglobulin with mouse B cells and the inconsistent demonstration of surface Ig on mouse T cells [for example, see (*4*)]. Other methods of classifying T cells and B cells depend on their response to certain mitogens. Concanavalin A and phytohemagglutinin are believed to have mitogenic activity specific for T cells (*5*). In contrast, bacterial lipopolysaccharide or endotoxin (LPS) is believed to be a specific B cell mitogen (*5*). We now report that, on the basis of response to mitogens, the rabbit PBL behaves like a T cell.

Peripheral blood lymphocytes were obtained from rabbit blood that was defibrinated and sedimented with gelatin; the PBL's were cultured with different doses of mitogens (*1*, *6*). Four dilutions of each mitogen were tested on the cells of four or more individual rabbits; some of the results have already been presented (*6*). However, the most important mitogen, LPS, was not previously tested. Cultures of 3×10^6 PBL's in normal rabbit serum (18.5 percent) and Eagle's medium were set up, and doses of 5, 10, 50, and 100 μg of LPS (Bacto Lipopolysaccharide B, *Salmonella typhosa* 0901, control No. 553510, Difco) were separately added to individual cultures. Twenty-four hours later, 0.25 μc of thymidine was added, the incubation was continued for 24 hours, and the cultures were harvested. Part (1.5 ml) of each cell suspension was used for determining thymidine uptake and the remaining (0.5 ml) was smeared on a slide and stained with Jenner-Giemsa for the determination of the percentage of lymphocytes transformed. Similar experiments were performed with rabbit thymus (10×10^6 cells per culture) and spleen cells (3×10^6 to 5×10^6 cells per culture) (*6*) and graded doses of concanavalin A (*6*, *8*), pokeweed mitogen (Grand Island Biological, control A8218J), phytohemagglutinin, staphylococcal filtrate, anti-IgG (*2*), and anti-As (*1*, *6*).

Representative responses of spleen thymus and PBL's to the optimum doses of the various mitogens are given in Table 1. Cells from each source responded well to each of the above-mentioned agents. However, only spleen cells responded to LPS; PBL's and thymus cells give no responses even with doses up to five times greater and

Table 1. Optimum response of rabbit lymphoid cells to mitogens in vitro. Abbreviations: Con A, concanavalin A; PHA, phytohemagglutinin; LPS, lipopolysaccharide; PWM, pokeweed mitogen; Staph, staphylococcal filtrate; Anti-L, antiserum to rabbit light chain; Anti-γ, antiserum to rabbit IgG; and Anti-As, antiserum to rabbit allotypic determinants.

Agent	Spleen		Thymus		Blood	
	Blasts (%)	Radioactivity (count/10 min)	Blasts (%)	Radioactivity (count/10 min)	Blasts (%)	Radioactivity (count/10 min)
Control	20	4,123	5	5,000	<1	30
Con A	80	42,000	70	30,000	90	50,000
PHA	4+	22,000	2+	20,000	4+	2,000
LPS	50	17,000	6	4,500	1	50
PWM	60	15,000	35	20,000	30	1,200
Staph	60	14,000	30	10,000	60	4,600
Anti-L	50	35,000*	6	4,840*	90	6,000*
Anti-γ	45	34,000*	7	5,600*	80	5,000*
Anti-As	50	20,000	40	20,000	80	18,000

*Cultured in calf serum.