JOURNAL OF BACTERIOLOGY, Nov. 1985, p. 749–756
0021-9193/85/110749-08$02.00/0

Vol. 164, No. 2

Mathematical Model for Determining the Effects of Intracytoplasmic Inclusions on Volume and Density of Microorganisms

JORDI MAS, CARLOS PEDRÓS-ALIÓ, AND RICARDO GUERRERO*

Department of Microbiology and Institute for Fundamental Biology, Autonomous University of Barcelona, Bellaterra (Barcelona), Spain

Received 5 April 1985/Accepted 29 July 1985

Procaryotic microorganisms accumulate several polymers in the form of intracellular inclusions as a strategy to increase survival in a changing environment. Such inclusions avoid osmotic pressure increases by tightly packaging certain macromolecules into the inclusion. In the present paper, a model describing changes in volume and density of the microbial cell as a function of the weight of the macromolecule forming the inclusion is derived from simple theoretical principles. The model is then tested by linear regression with experimental data from glycogen accumulation in *Escherichia coli*, poly-β-hydroxybutyrate accumulation in *Alcaligenes eutrophus*, and sulfur accumulation in *Chromatium* spp. The model predicts a certain degree of hydration of the polymer in the inclusion and explains both the linear relationship between volume of the cell and weight of the polymer and the hyperbolic relationship between density of the cell and weight of the polymer. Other implications of the model are also discussed.

Studies of the causes determining changes in volume and density of cells have only considered exponentially growing cells (14, 22, 26) without taking into account the conditions under which volume and density are most likely to change dramatically, namely, when inclusions are being accumulated (8; C. Pedrós-Alió, J. Mas, and R. Guerrero, Arch. Microbiol., in press). When population growth is not possible, inclusions are formed by the accumulation of some reserve substance (5). Since division is not occurring, this should cause increases in volume. Since the polymers are supposedly tightly packed in inclusions, they should also have a substantial effect on density.

The types and morphology of intracellular inclusions have been reviewed by Shively (27). The structure of some polymers, the enzyme systems involved in their synthesis and degradation, and the role they play as energy reserve substances have been summarized by Dawes and Senior (5). The early dates of both papers are both a tribute to the work of the authors and an indication of the little attention inclusions have received lately. The cases of poly-β-hydroxybutyrate (PHB), polyphosphate, and glycogen have more recently been reviewed by Merrick for phototrophic bacteria (17).

Reserve substances forming inclusions can account for substantial portions of the cell weight and volume. Glycogen has been reported to account for as much as 40% of the dry weight in certain mutants of *Escherichia coli* (3). PHB can constitute up to more than 90% of the dry weight in *Alcaligenes eutrophus* N9A (Pedrós-Alió et al., in press). Kinetics of accumulation of glycogen (12, 28), PHB (10, 23), polyphosphate (9), and sulfur (8, 32) into intracellular inclusions have been studied in many microorganisms. Heinzle and Lafferty (10) even developed a model of PHB synthesis under chemolithoautotrophic conditions, in which storage of PHB was inhibited by high specific contents of PHB. However, no studies have been done to examine the physical consequences for the cell of this or any other polymer accumulation in the form of inclusions. Also, sulfur globules (19, 24), polyphosphate (7), glycogen (13), and PHB (16, 34) have been analyzed to determine their chemical composition, but very few studies have been done to find out the in vivo state of such inclusions (17, 18, 21, 31), and the results are far from clear.

Thus, it was of interest to find out the effects of such substances on the volume and density of the cells, on the one hand, and to learn more about the physical composition of the inclusions themselves, on the other. First, we measured changes in volume and density of cells, together with changes in the inclusion being accumulated. Second, we developed a simple model, from theoretical considerations, to explain the relationships observed in several organisms and inclusions (8; Pedrós-Alió et al., in press). Then we fitted equations of the model to experimental data to test the model and, simultaneously, to quantify the parameters of the equations. In this way the empirical quantification could give information with biological meaning. Finally, we explored two sets of implications of such a model: for the organization of cellular inclusions and for the volume and density of the cells. Data from glycogen accumulation in *E. coli* and PHB in *A. eutrophus* were used to test the model. Then the model was applied to former results of sulfur accumulation in two *Chromatium* species.

MATERIALS AND METHODS

Experimental methods. The data used for testing the model were obtained by techniques and with strains already described (8; Pedrós-Alió et al., in press). Briefly, cultures of *A. eutrophus* N9A and *E. coli* K-12 were incubated with fructose and glucose, respectively, as carbon sources and no nitrogen source. *Chromatium warmingii* DSM-173 and *Chromatium vinosum* UA6002 were grown with 1 mM H_2S at an irradiance of 60 micro-einsteins m^{-2} s^{-1} of light at 28°C under anaerobic conditions. Cell volume, buoyant density, number of cells, and weight of the macromolecule were measured with time. Cell volume was measured with a Coulter Counter or in enlarged phase-contrast pictures. Buoyant density was determined in Percoll gradients (8). The number of cells was determined either with a Coulter Counter or by epifluorescence direct count of acridine orange-stained

* Corresponding author.

bacteria (35). PHB was measured by the method of Law and Slepecky (15). Glycogen was determined by the anthrone reagent method after extraction in boiling 30% KOH (11). Sulfur was measured in ethanol extracts by the method of van Gemerden (32).

The model. All through the paper we refer to an average cell in the culture. All contents are average specific contents obtained by dividing the amount of polymer per milliliter in the culture by the number of cells per milliliter. An error would be introduced if a significant portion of the cells were not accumulating the inclusion. However, upon observation of the cells by phase-contrast microscopy, very few cells were seen not accumulating inclusions: in all cases, less than 1%; therefore the possible error is not significant.

(i) Definition of terms. To clarify the terminology used, the main parameters are explained in Fig. 1. Essentially, we have three variables: density, volume, and weight. We discriminate among (I) the cell without any inclusion; (II) the whole cell with variable amounts of inclusions; (III) the whole inclusion (even though there is usually more than one inclusion body of the same substance per cell, all of them can be considered as a single one); (IV) the polymer in the inclusions, defined empirically as the sum of the polymer in all the inclusion bodies in the cell, as chemically measured; and (V) that part of the inclusions which is not polymer, but some other substance. This should be mostly water of hydration and, thus, proportional to the amount of polymer (hydration is assumed to be constant). If W_m is the weight of polymer, the weight of this other part of the inclusion will be proportional to W_m, let us say AW_m. Then, the weight of the inclusion will be $W_i = W_m + AW_m = W_m(A + 1)$. The constant A is a measure of the degree of hydration of the polymer in the inclusion.

(ii) Premises of the model. The following statements are assumed to hold true. (i) The weight and volume of that part of the cell which is not inclusion remains constant under conditions of accumulation of the inclusion (I in Fig. 1). That is, W_c and V_c are constant, and therefore D_c is also constant.

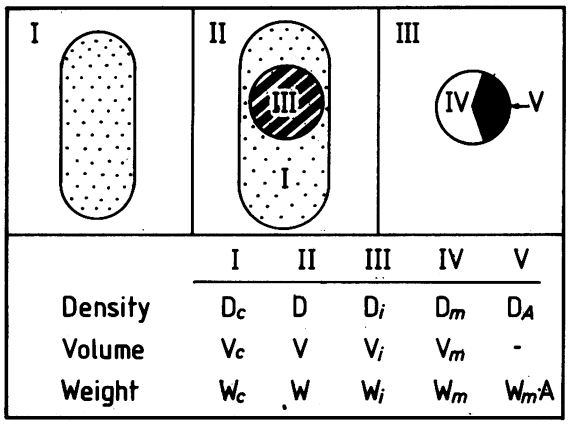

FIG. 1. Terminology used in the model. I corresponds to the cell without inclusions, II corresponds to the cell with variable amounts of inclusions, and III corresponds to the whole inclusion. Even if the polymer is distributed among several globules or inclusion bodies, they can be considered as only one, which would be the sum of all the inclusion bodies present. IV refers to that part of the inclusion which is polymer alone (i.e., dehydrated). V refers to that part of the inclusion which is not the polymer itself, but some other accompanying substance, probably water. The terminology used is presented in the lower half of the figure. See also the Appendix.

TABLE 1. Symbols used

Symbol	Definition
A (constant)	Constant relating the weight of water of hydration and the measured weight of the compound being accumulated
D (variable)	Density of the cell with inclusions
D_a (constant)	Density of water of hydration (1.000)
D_c (constant)	Density of cell without any inclusions
D_i (constant)	Density of inclusion bodies
D_m (constant)	Density of the compound being accumulated
n (variable)	Variable which equals the weight of inclusion bodies divided by the weight of the cell without inclusions (W_i/W_c)
V (variable)	Volume of the cell with inclusions
V_c (constant)	Volume of the cell without inclusions
V_i (variable)	Total volume of the inclusion bodies in a cell
V_m (variable)	Volume of the compound being accumulated
W (variable)	Weight of the cell with inclusions
W_c (constant)	Weight of the cell without inclusions
W_i (variable)	Total weight of the inclusion bodies
W_m (variable)	Biochemically measured weight of the compound being accumulated

(ii) The density of the inclusion is constant. That is, D_i is constant.

(iii) Measured variables. In our experiments with *E. coli* and *A. eutrophus*, conditions were such that everything was kept constant except for the accumulation of a given reserve polymer. Then we measured the following parameters along time courses: (i) dry weight of the cells per milliliter of culture (by weighing freeze-dryed cells); (ii) number of cells per milliliter of culture (with the Coulter Counter and by direct epifluorescence counting); (iii) buoyant density of the cells (by density gradient centrifugation in Percoll); (iv) weight of the storage material in milligrams per milliliter of culture (by standard biochemical methods); (v) volume of the cells in cubic micrometers (by the Coulter Counter or phase-contrast microscopy). With these measured parameters we could readily calculate the dry weight of each cell, the weight of storage material per cell, and the wet weight of the cell without inclusions.

(iv) The equations. We analyzed three relationships: between volume and weight of the polymer (equation 1), between density and cell volume (equation 2), and between density and weight of the polymer (equation 3). (See the Appendix for derivation of the linear forms of the three equations, and see Table 1 for definitions of the symbols used.)

$$V = \frac{W_c}{D_c} + \frac{(A + 1)}{D_i} W_m \qquad (1)$$

This equation is a straight line relating volume to weight of the inclusion material. If we experimentally get different values of both variables, $V_c = W_c/D_c$ (the intercept), and A (once the value of D_i is known from equation 2) can be estimated by linear regression (Table 2).

$$D = D_i + V_c (D_c - D_i) \frac{1}{V} \qquad (2)$$

This is also the equation of a straight line relating buoyant density to the inverse of cell volume, both of which can be measured directly in experiments. By linear regression we can then estimate D_i (intercept) and use this value in

TABLE 2. Dependent and independent variables for each of the three equations of the model, and parameters which can be estimated from intercepts and slopes of each equation

Equation	y	x	Intercept	Slope
1	V	W_m	$\mathbf{V_c}$[a]	$(A + 1)/D_i$
2	D	$1/V$	$\mathbf{D_i}$	$V_c(D_c - D_i)$
3	$(1 + n)/D$	n	$\mathbf{1/D_c}$	$\mathbf{1/D_i}$

[a] Parameters that can be calculated from each regression are shown in boldface.

equation 1 (Table 2). Also, knowing the slope and using D_i and V_c, D_c can be calculated.

$$\frac{(1 + n)}{D} = \frac{1}{D_c} + \frac{1}{D_i} n \quad (3)$$

Equation 3 is the linearized form of a hyperbola. It has several interesting features. If we know values for n and D and perform a linear regression, we can estimate D_c and D_i (given by the inverse of the intercept and of the slope, respectively) with known precision (given by the statistics of the linear regression). The value of D can be measured directly by density gradient centrifugation. However, the independent variable, n, equals $W_m(A + 1)/W_c$ (see Appendix, equation 7) and thus requires measuring W_m and W_c, besides knowing the value of A, which cannot be measured directly. We need equations 1 and 2 to estimate this last parameter. In the process, we can also estimate W_c as a bonus (Table 2).

The parameters of each one of these three equations could be found by linear regression with our experimental data. Finding the parameters was of interest because it allowed estimates of density, weight, and volume of the cell (D_c, W_c, and V_c, respectively) without inclusions, the density of the inclusion bodies (D_i), the degree of hydration of the compound forming the inclusion (A), and the density of such a compound in its anhydrous form (D_m). Also, it allowed prediction of the density of a cell from the weight of storage material.

All the statistical calculations were done with the *Statistical Package for the Social Sciences* (29) in the Digital VAX-11/780 at the Computing Center of the Autonomous University of Barcelona.

RESULTS AND DISCUSSION

Accumulation of storage materials as intracellular inclusions. Accumulation of reserve substances in the form of intracytoplasmic inclusions is one of the most striking features in procaryotes. It constitutes one of their adaptive mechanisms to changing enviroments. When conditions become unfavorable, bacteria change their composition to prepare for survival without growth. This may involve a highly complex series of events such as sporulation or just reorganization of the set of active enzymes (20). Within this range of possibilities, storage of polymeric compounds occupies an intermediate place: they confer survival value to the cells without the complexities of something like sporulation (2, 4, 5).

Whether it be glycogen, polyphosphate, PHB, or sulfur, inclusions contain large amounts of a potentially useful compound in a form that does not cause osmotic pressure changes and which is protected from degradative enzymes by nonunit membranes or by other means (5).

Microorganisms are able to regulate this storage capacity in accordance with environmental conditions. The case of glycogen accumulation in *E. coli* can serve as an example (Fig. 2). When carbon and nitrogen sources are present in the medium, glycogen content is low (but measurable), and the population grows (Fig. 2A). When the nitrogen source is removed from the medium while plenty of carbon is left, the population cannot grow because proteins and nucleic acids cannot be synthesized, but glycogen accumulates as a reserve of carbon (Fig. 2B). Finally, when neither carbon nor nitrogen are present, the cells first use up for maintenance energy whatever glycogen reserves they might have, and once the reserves are exhausted, they start to die (Fig. 2C).

Pertinent for our present purpose are the consequent increases in physical properties of the cells that this accumulation of substances in inclusions entails. There is an increase in weight and volume, since the cell incorporates new material. Moreover, since, as a matter of fact, the density of the reserve polymers is greater than the average density of the cell, there is an increase in cell buoyant density. Figure 3 shows this increase in density with increasing amounts of storage material in four different microorganisms, as predicted by equation 3 of the model. As can be seen, the relationship between specific content of storage polymer and cell density is hyperbolic regardless of the specific substance involved.

Testing of the model. The regression model explained in Materials and Methods was tested with data from two types of experiments, accumulation of glycogen by *E. coli* (Fig. 4) and accumulation of PHB by *A. eutrophus* (Fig. 5). The estimated parameters and 95% confidence intervals can be seen in Table 3. Also, for comparison, densities of storage polymers from the literature are summarized in Table 4. We will discuss each inclusion in turn.

Glycogen. Data from several experiments in which cultures accumulated only glycogen (Fig. 2B) were pooled together, and the three linear regressions expressed in the Materials and Methods section were performed to test the model. This analysis is summarized in Fig. 4. Equation 1

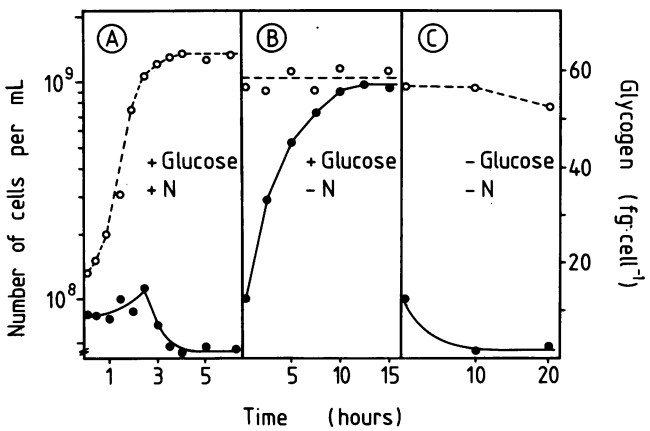

FIG. 2. Regulation of glycogen accumulation in *E. coli*. (A) In a culture with glucose and nitrogen, the cells are able to grow and reproduce; glycogen temporarily accumulates at the beginning of the stationary phase. (B) In a culture with glucose but no nitrogen, the cells are not able to grow and divide, but they accumulate large quantities of glycogen. (C) When neither glucose nor nitrogen are present, the cells first degrade whatever glycogen they have and afterwards start to die. Symbols: ○, number of cells per milliliter; ●, glycogen specific content.

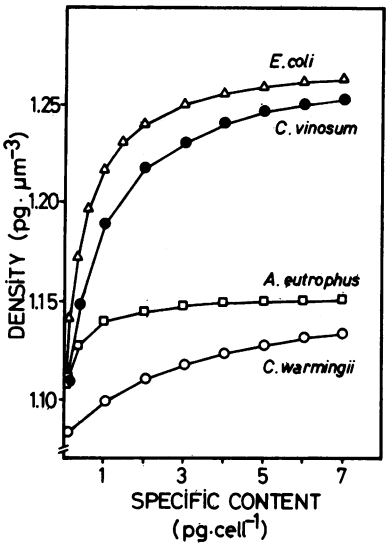

FIG. 3. Relationship between buoyant density and specific content (in picograms cell^{-1}) of three substances forming inclusions in different procaryotes: glycogen in *E. coli* (△), PHB in *A. eutrophus* (□), and sulfur in *C. vinosum* (●) and *C. warmingii* (○). Points and curves are those predicted by equation 3 of the model.

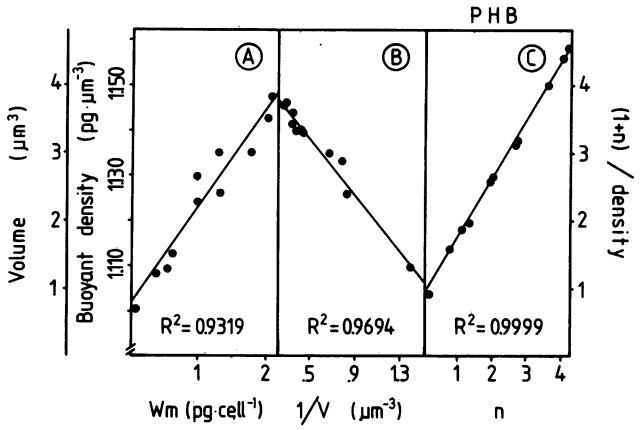

FIG. 5. Linear regressions for the three equations of the model as applied to PHB accumulation in *A. eutrophus*. (A) Relationship between volume and weight of PHB (equation 1). (B) Relationship between buoyant density and inverse of the volume (equation 2). (C) Relationship between (1 + *n*)/density and *n* (equation 3). The points are experimental data, while the lines were found by linear regression. The determination coefficients (R^2) are also presented.

gives the lowest determination coefficient (R^2 = 0.4807), reflecting the fact that the influence of glycogen on volume is small, as has been previously shown (R. Guerrero, J. Mas, and C. Pedrós-Alió, submitted for publication). This is also the cause of the wide 95% confidence interval for the estimate of parameter *A* (Table 3), although that of the volume of the cell without glycogen is reasonably narrow (Table 3). The other two equations have high determination coefficients: 0.8163 and 0.9814 for equations 2 and 3, respectively (Fig. 4B and C). Accordingly, 95% confidence intervals for parameters estimated from these two equations are narrow (Table 3). The results can be divided in two parts for convenience of exposition: some of them refer to the cell without glycogen and the rest refer to the inclusion of glycogen.

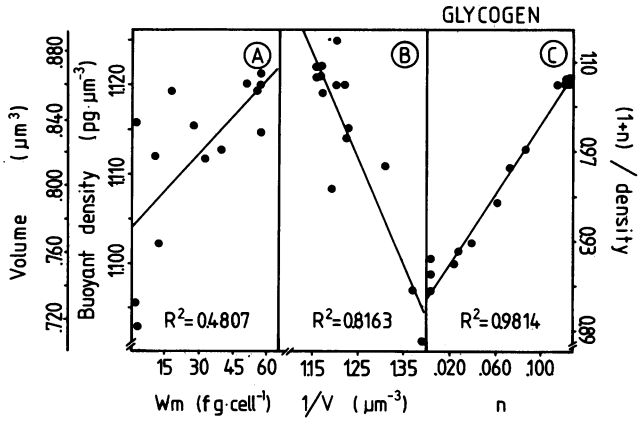

FIG. 4. Linear regressions for the three equations of the model as applied to glycogen accumulation in *E. coli*. (A) Relationship between volume and weight of glycogen (equation 1). (B) Relationship between buoyant density and inverse of the volume (equation 2). (C) Relationship between (1 + *n*)/density and *n* (equation 3). The points are experimental data, while the lines were found by linear regression. The determination coefficients (R^2) are also presented.

Volume and density of the cell can be included in the first category. From these two, the weight of the cell without glycogen can be calculated (W_c in Table 3). It must be pointed out that this cell is an average stationary-phase cell, and thus, the volume (0.773 µm^3) and the density (1.1068 pg µm^{-3}) are lower than what could be expected from exponentially growing cultures of *E. coli* (22; Guerrero et al., submitted).

The second group of data gives us information about the nature of the glycogen inclusion. Density of the inclusion is between 1.257 and 1.292 pg µm^{-3} (Table 3), which coincides with the densities of glycogen measured in Nycodenz (Nyegaard & Co., Oslo) and metrizamide gradients (Table 4). These media, unlike CsCl or sucrose (with osmolarities above 2.4 osmol liter^{-1}), are designed to have osmolarities between 0.02 and 0.2 osmol liter^{-1}, closer to physiological levels, so that the degree of hydration of glycogen in such media is probably close to that in vivo. This would explain

TABLE 3. Estimated parameters for each of the two inclusions studied

Parameter	Glycogen		PHB	
	Estimate	95% CI[a]	Estimate	95% CI
Cell without inclusion				
Vol (V_c)[b]	0.773	0.037	0.741	0.471
Density (D_c)[b,c]	1.1070	0.095	1.1120	0.0378
Density (D_c)[d]	1.1066	0.005	1.1086	0.0039
Wt (W_c)[e]	0.856		0.823	
Inclusion				
Density (D_i)[c]	1.2572	0.043	1.1535	0.0027
Density (D_i)[d]	1.2920	0.088	1.1549	0.0017
A[b,c]	0.880	1.335	0.669	0.344
Density (D_m)	1.6658		1.2783	

[a] CI, Confidence interval.
[b] Obtained from equation 1.
[c] Obtained from equation 2.
[d] Obtained from equation 3.
[e] Obtained by simple calculation.

TABLE 4. Densities of different macromolecules forming inclusions[a]

Inclusion	Density (g cm^{-3})	Method[b]	Reference
Glycogen	1.29	Nycodenz	6
	1.25	Metrizamide	6
	1.480	Sodium metrizoate	25
Glucose + 40% H_2O	1.562	Sp gr	33
	1.282	Sp gr	33
PHB	1.23	Sp gr	5
	1.25	Sp gr	5
	1.19–1.23	NaBr	18
Sulfur	2.07	Sp gr	33
	1.957	Sp gr	33
	>1.143	Percoll + metrizamide[c]	8
	1.219	Indirect method[d]	8
Polyphosphate	1.23	Sucrose	7

[a] Data collected from the literature.
[b] Only the medium is specified when the method involves centrifugation in a density gradient. Specific gravity involves drying the purified material and measuring volume and weight.
[c] In *C. warmingii*.
[d] Measuring density of cells with and without sulfur in *C. vinosum*.

the excellent agreement with our results. Unfortunately, no data are available about the anhydrous specific gravity of glycogen, and thus our estimation of A cannot be compared with published data. In fact, ours is the first estimation of the in vivo degree of hydration of glycogen. It indicates that about 47% of the weight of the inclusion is water. However, densities of both anhydrous (D_m) and hydrated (D_i) glycogen in the inclusion can be compared with those of anhydrous glucose and glucose plus 40% water, respectively (Table 4). Although such a comparison can only be orientative, the good agreement gives some support to our results.

PHB. The treatment for the PHB results was identical to that for glycogen. The three regression lines can be compared with experimental data points in Fig. 5. Determination coefficients were remarkably high in the case of PHB (R^2 of 0.9319, 0.9694, and 0.9999 for equations 1, 2, and 3, respectively). Accordingly, 95% confidence intervals for estimates were narrow (Table 3). Data for the stationary-phase cell of *A. eutrophus* without PHB indicate a volume of 0.741 μm^3 and a density between 1.1086 and 1.1120 pg μm^{-3}. It can be seen that *A. eutrophus* is denser than *E. coli* and similar in volume.

The PHB inclusion had a density of approximately 1.1549 pg μm^{-3} (Table 3). This is considerably lower than that found for isolated PHB (Table 4), which goes from 1.19 to 1.25 pg μm^{-3}. However, the latter values were measured by weighing anhydrous material or by centrifuging in NaBr gradients. Both methods would give values close to that of anhydrous PHB. In fact, Nickerson (18) found two values for PHB inclusions in NaBr gradients: 1.19 and 1.23 pg μm^{-3}, the lower one corresponding to intact inclusions with their membrane and the higher one to inclusions without such a membrane. Taking into account an A value of 0.669 (Table 3), we can calculate that 40% of the PHB inclusion weight would be water. From this value, and using the measured densities of anhydrous PHB (1.23 and 1.25, Table 4), we can calculate (0.4 × 1.00 + 0.6 × 1.25 or 1.29) the theoretical hydrated values to be between 1.138 and 1.150 pg μm^{-3}, which are remarkably close to our results. Conversely, our estimate for anhydrous PHB (D_m = 1.2783) is close to the specific gravities found by other authors (Table 4).

Sulfur. Sulfur accumulation was studied in *C. vinosum* and *C. warmingii* (8). Again, a hyperbolic relationship could be found between density of the cells and weight of the substance in the inclusion (Fig. 3). However, our data were not as extensive as with the other inclusions and, in *C. vinosum*, the asymptotic part of the curve could not be reached. For these reasons the estimates from linear regressions were not very precise. These drawbacks notwithstanding, some orientative results are presented in Table 5 for the sake of discussion. In addition to such problems, *Chromatium* spp. accumulate glycogen simultaneously with sulfur, so that changes in density could potentially be due to both inclusions, even though the effect of glycogen was probably low (8). Thus, we performed additional experiments in which the density of the sulfur globules was assessed indirectly (Table 5). In such experiments volume and density were measured before and after treatment of the cells with ethanol, which extracts sulfur and pigments but not glycogen. By several simple calculations, we could determine the density of the sulfur globule. The exact protocol and explicit calculations were presented for *C. vinosum* earlier (8). The good agreement between the densities of sulfur calculated by both methods gives further evidence of the fact that glycogen interference must have been very small.

The interesting conclusion from such experiments is that the density of the sulfur inclusion is different in the two *Chromatium* species. It could be argued that this would be the case for glycogen and PHB also, if different species had been studied. However, we do not think so, given that such polymers have well-defined macromolecular structures which are not likely to change from species to species. PHB seems to be a helical polymer which always has the same structure (5), and thus differences in densities of the inclusions among species would not be expected. Glycogen could show some differences when different degrees of branching are involved. This has been shown to occur in *Micrococcus lysodeikticus* (5). Thus, it would be interesting to know the density of glycogen in such a species. However, differences in density due to branching of glycogen can be expected to be very small, if one considers the data in Table 4. The density of glycogen in sodium metrizoate approximates the density of dry glucose, and the latter is a single molecule without any branching. Sodium metrizoate is the ionic derivative of metrizamide, and its osmolarity is considerably higher (see page 239 in reference 1). This agrees with the higher density of glycogen in metrizoate than in metrizamide (Table 4). Thus, differences in density between glycogen (a long, branched polymer) and glucose (a single unit) are not very significant, while both molecules show similar differences in density when measured by different techniques

TABLE 5. Estimated parameters for sulfur inclusions in two species of *Chromatium*

Organism	Vol of cell (μm^3)	Density of cell (pg μm^{-3})	Density of sulfur[a] (pg μm^{-3})	Density of sulfur[b] (pg μm^{-3})
C. vinosum	1.21	1.1150	1.2281	1.219
C. warmingii	7.44	1.0890	1.1321	ND[c]

[a] Estimated from equation 1 and experimental data.
[b] Indirectly measured by ethanol extraction experiments.
[c] ND, Not determined.

causing variable amounts of dehydration. This further supports the idea of degree of hydration as the main determinant of the density of the molecules in vivo.

In the case of sulfur it is not clear what kind of structure the inclusion has (19, 21, 24, 31; H. G. Trüper, personal communication). Nevertheless, it has been proposed that members of the family *Chromatiaceae* incorporate the external medium with sulfide into internal vesicles, which eventually develop into sulfur globules (21). If this was the case, it would be easy to imagine different species differing in the size and water content of their globules. In effect, our estimates of A are 0.44 for *C. vinosum* and 1.25 for *C. warmingii* (data not shown). The higher degree of hydration in *C. warmingii* is consistent with its larger size, given that large cells are less constricted by the presence and size of inclusions than small cells. The degree of hydration of sulfur in the small species is rather close to the values found for other inclusions in similarly sized bacteria. The assumption of constancy of density of the inclusion within one species, however, remains unproven.

Physical effects of inclusions. There are two types of conclusions about the physics of the accumulation of reserve polymers forming inclusions. In the first place, there are conclusions about the effects of the accumulation on volume and buoyant density of the cell, and in the second place, there are conclusions about the nature of the inclusion itself.

The relationship between volume of the cell and weight of the inclusion material was shown to be linear (Fig. 4A and 5A). Under the conditions of the experiments, the cells were unable to grow and probably were unable to synthesize new cell material. Therefore, the increase in volume without an increase in cell wall material would imply stretching of a somewhat flexible cell wall. In the case of *A. eutrophus* the effect is particularly dramatic, the surface area increasing from 7.5 μm^2 to 12.5 μm^2 (Pedrós-Alió et al., in press) owing to the high specific content of PHB. Whether this is a passive sort of expansion or whether there is degradation of some cell material to get building blocks for new cell wall is not known.

The relationship of specific content of the inclusion material with density is best described by a hyperbola (Fig. 3, 4C, and 5C). Some kind of asymptotic curve could be expected, since the maximum possible content of a substance is 100% of the dry weight, and at this concentration, the density of the cell would equal the density of the inclusion. Densities larger than this value are not possible; therefore, the density of the cell has to increase asymptotically with the increasing content of the inclusion.

As far as the inclusions themselves are concerned, parameter A assumes hydration. This water content should be proportional to the amount of the polymer. Because of this, we could assume $W_i = W_m(A + 1)$ (see Appendix). The degrees of hydration predicted by our model are quite reasonable when compared with those found by other methods (30).

These ideas can be easily extrapolated to macromolecules not forming inclusions. The only difference being that in the last case, the asymptote is never approached, because proteins and nucleic acids never reach the high specific contents necessary to have a strong influence on density (Guerrero et al., submitted). The relationship between density and macromolecules would obviously be hyperbolic if higher specific contents could be reached. Similarly, the set of equations has been expanded for more than one inclusion and for inclusions and macromolecules together (unpublished data). Moreover, gas vesicles and any other cell component affecting volume or density or both can be easily accomodated into our equations.

In summary, we have a set of equations which fit the experimental data extremely well. The parameters of such equations are not only empirically determined coefficients, but they also have biological meaning. Such parameters can be estimated by linear regression from four simple measurements: cell volume, cell number, cell buoyant density, and weight of the polymer being studied. As in any other linear regression, the determination coefficients give the percentage of the dependent variable explained by the independent variable, as well as an indication of the goodness of fit. The excellent way in which the model equations fit experimental data leads one to conclude that the assumptions used in deriving the model are consistent with the available evidence. Finally, the results throw light on the structure of inclusions and on the relationships of volume and density with such inclusions in procaryotes.

APPENDIX

Equation 1. In a cell with an inclusion,

$$V = V_c + V_i \tag{1}$$

or

$$V = \frac{W_c}{D_c} + \frac{W_i}{D_i} \tag{2}$$

Assuming that the inclusion is composed of the polymeric substance plus a certain amount of water of hydration, the weight of such water has to be proportional to the weight of the polymer, that is W_mA, where A is a measure of the degree of hydration of the polymer. Then

$$W_i = W_m + W_mA = W_m(A + 1) \tag{3}$$

substituting in equation 2

$$V = \frac{W_c}{D_c} + \frac{(A + 1)}{D_i} W_m \tag{4}$$

which is the equation of a straight line relating volume of the cell to weight of the inclusion polymer.

Equation 2. Let n be equal to the proportion between W_i and W_c

$$n = W_i/W_c \tag{5}$$

so that

$$W_i = nW_c \tag{6}$$

From equations 3 and 6 we have that

$$W_cn = W_m(A + 1) \tag{7}$$

Substituting in equation 4 we get

$$V = \frac{W_c}{D_c} + \frac{W_cn}{D_i} \tag{8}$$

and isolating n in the right-hand term

$$\frac{VD_i}{W_c} - \frac{D_i}{D_c} = n \tag{9}$$

Substituting this value of n in equation 21 we get

$$\frac{1 + \frac{VD_i}{W_c} - \frac{D_i}{D_c}}{D} = \frac{1}{D_c} + \frac{1}{D_i}\left(\frac{VD_i}{W_c} - \frac{D_i}{D_c}\right) \tag{10}$$

simplifying and passing D to the right-hand term

$$1 + \frac{VD_i}{W_c} - \frac{D_i}{D_c} = \frac{DV}{W_c} \tag{11}$$

and rearranging

$$\frac{DV}{W_c} = \frac{D_c - D_i}{D_c} + \frac{D_i}{W_c} V \tag{12}$$

passing V and W_c to the right-hand term

$$D = \left(\frac{D_c - D_i}{D_c}\right)\left(\frac{1}{V}\right)\left(\frac{W_c}{1}\right) + \left(\frac{D_i}{W_c}\right)(V)\left(\frac{W_c}{V}\right) \tag{13}$$

eliminating terms

$$D = \frac{W_c}{D_c}(D_c - D_i)\frac{1}{V} + D_i \tag{14}$$

which is equivalent to

$$D = D_i + V_c(D_c - D_i)\frac{1}{V} \tag{15}$$

This is the equation of a straight line relating density to the inverse of the volume, both of which can be measured experimentally.

Equation 3. By definition

$$D = W/V \tag{16}$$

In the case of a cell with an inclusion this can be decomposed in:

$$D = \frac{W_c + W_i}{V_c + V_i} = \frac{W_c + W_i}{\frac{W_c}{D_c} + \frac{W_i}{D_i}} \tag{17}$$

Substituting nW_c for W_i (from equation 6) in the previous expression

$$D = (W_c + nW_c)/(W_cD_i + nW_cD_c)/(D_cD_i) \tag{18}$$

and rearranging

$$D = \frac{(1 + n)D_cD_i}{D_i + nD_c} \tag{19}$$

passing $(1 + n)$ to the left-hand term and inverting both terms

$$\frac{(1 + n)}{D} = \frac{D_i}{D_cD_i} + \frac{D_c}{D_cD_i} n \tag{20}$$

from which follows

$$\frac{(1 + n)}{D} = \frac{1}{D_c} + \frac{1}{D_i} n \tag{21}$$

which is the linearized form of a hyperbola relating density to the parameter n, which, in turn, is a transformation of the variable W_m, given by equation 7.

ACKNOWLEDGMENTS

J.M. was supported by a predoctoral fellowship from the Spanish Ministry of Education and Science. We thank the Departament d'Ensenyament of the Generalitat de Catalunya for an Ajut per al Perfeccionament de Professors to C.P.-A.

LITERATURE CITED

1. **Birnie, G. D., and D. Rickwood.** 1978. Centrifugal separation in molecular and cell biology. Butterworths, Ltd., London.
2. **Boylen, C. W., and J. C. Ensign.** 1970. Intracellular substances for endogenous metabolism during long-term starvation of rod and spherical cells of *Arthrobacter crystallopoietes*. J. Bacteriol. **82**:578–587.
3. **Damotte, M., J. Cattanéo, N. Sigal, and J. Puig.** 1968. Mutants of *Escherichia coli* K12 altered in their ability to store glycogen. Biochem. Biophys. Res. Commun. **32**:916–920.
4. **Dawes, E. A.** 1976. Endogenous metabolism and the survival of starved prokaryotes. Symp. Soc. Gen. Microbiol. **26**:19–53.
5. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microb. Physiol. **10**:135–266.
6. **Ford, T. C., D. Rickwood, and J. Graham.** 1983. Buoyant densities of macromolecules, macromolecular complexes, and cell organelles in Nycodenz gradients. Anal. Biochem. **128**:232–239.
7. **Friedberg, I., and G. Avigad.** 1968. Structures containing polyphosphate in *Micrococcus lysodeikticus*. J. Bacteriol. **96**:544–553.
8. **Guerrero, R., J. Mas, and C. Pedrós-Alió.** 1984. Buoyant density changes due to intracellular content of sulfur in *Chromatium warmingii* and *Chromatium vinosum*. Arch. Microbiol. **137**: 350–356.
9. **Harold, F. M.** 1966. Inorganic polyphosphates in biology: structure, metabolism, and function. Bacteriol. Rev. **30**:772–794.
10. **Heinzle, E., and R. M. Lafferty.** 1980. A kinetic model for growth and synthesis of poly-β-hydroxybutyric acid (PHB) in *Alcaligenes eutrophus* H16. Eur. J. Appl. Microbiol. Biotechnol. **11**:8–16.
11. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 209–344. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press, Inc., London, Ltd., London.
12. **Holme, T., and B. Cedergren.** 1961. Demonstration of intracellular polysaccharide in *Escherichia coli* by electron microscopy and by cytochemical methods. Acta Pathol. Microbiol. Scand. **51**:179–186.
13. **Holme, T., T. Laurent, and H. Palmstierna.** 1958. On the glycogen in *Escherichia coli* B: variations in molecular weight during growth. II. Acta Chem. Scand. **12**:1559–1563.
14. **Kubitscheck, H. E., W. W. Baldwin, and R. Graetzer.** 1983. Buoyant density constancy during the cell cycle of *Escherichia coli*. J. Bacteriol. **155**:1027–1032.
15. **Law, J. H., and R. A. Slepecky.** 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. **82**:33–36.
16. **Lundgren, D. G., R. M. Pfister, and J. M. Merrick.** 1964. Structure of poly-β-hydroxybutyric acid granules. J. Gen. Microbiol. **34**:441–446.
17. **Merrick, J. M.** 1978. Metabolism of reserve materials, p. 199–219. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
18. **Nickerson, K. W.** 1982. Purification of poly-β-hydroxybutyrate by density gradient centrifugation in sodium bromide. Appl. Environ. Microbiol. **43**:1208–1209.
19. **Nicolson, G. L., and G. L. Schmidt.** 1971. Structure of the *Chromatium* sulfur particle and its protein membrane. J. Bacteriol. **105**:1142–1148.
20. **Preiss, J.** 1969. The regulation of the biosynthesis of α-1,4-glucans in bacteria and plants. Curr. Top. Cell. Reg. **1**:125–160.
21. **Remsen, C. C.** 1978. Comparative subcellular architecture of photosynthetic bacteria, p. 31–60. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
22. **Schaechter, M., O. Maaløe, and N. O. Kjeldgaard.** 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19**:592–606.
23. **Schlegel, H. G., G. Gottschalk, and R. von Bartha.** 1961. Formation and utilization of poly-β-hydroxybutyric acid by knallgas bacteria (*Hydrogenomonas*). Nature (London) **191**: 463–465.
24. **Schmidt, G. L., G. L. Nicolson, and M. D. Kamen.** 1971. Composition of the sulfur particle of *Chromatium vinosum* strain D. J. Bacteriol. **105**:1137–1141.
25. **Scott, R. B., and W. J. S. Still.** 1970. Properties of liver glycogen

isolated in density gradients of sodium iothalamate. Arch. Biochem. Biophys. **139**:87–92.
26. **Shehata, T. A., and A. G. Marr.** 1975. Effect of temperature on the size of *Escherichia coli* cells. J. Bacteriol. **124**:857–862.
27. **Shively, J. M.** 1974. Inclusion bodies of prokaryotes. Annu. Rev. Microbiol. **28**:167–187.
28. **Sirevåg, R., and J. G. Ormerod.** 1977. Synthesis, storage and degradation of polyglucose in *Chlorobium thiosulfatophilum*. Arch. Microbiol. **111**:239–244.
29. **SPSS Inc.** 1983. SPSS users guide. McGraw-Hill Book Co., New York.
30. **Tanford, C.** 1961. Physical chemistry of macromolecules. John Wiley & Sons, Inc., New York.
31. **Trüper, H. G., and J. C. Hathaway.** 1967. Orthorhombic sulphur formed by photosynthetic sulfur bacteria. Nature (London) **215**:435–436.
32. **van Gemerden, H. G.** 1968. Utilization of reducing power in growing cultures of *Chromatium*. Arch. Mikrobiol. **64**:111–117.
33. **Weast, R. C. (ed.).** 1972. Handbook of chemistry and physics, 52nd ed. Chemical Rubber Co. Press, Cleveland, Ohio.
34. **Williamson, D. H., and J. F. Wilkinson.** 1958. The isolation and estimation of poly-β-hydroxybutyrate inclusions of *Bacillus* species. J. Gen. Microbiol. **19**:198–209.
35. **Zimmermann, R., and L.-A. Meyer-Reil.** 1974. A new method for fluorescence staining of bacterial populations on membrane filters. Kiel. Meeresforsch. **30**:24–27.