The obligate autotroph — the demise of a concept[1]

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RITTENBERG, S. C. 1972. The obligate autotroph – the demise of a concept. Antonie van Leeuwenhoek **38**: 457–478.

Autotrophy is a life style in which inorganic compounds provide for all nutritional needs of an organism. Implicit in this definition is the capacity of an organism to derive all cell carbon from CO_2 and to obtain ATP either photosynthetically or chemolithotrophically. The existence of bacteria with such potentials has been known since the work of Winogradsky in the 1880's. The question explored in this paper is whether bacteria exist that must of necessity live autotrophically, i.e., the obligate autotroph *sensu* Winogradsky.

The evidence is briefly reviewed and leads to four conclusions. One: there is no obligatory coupling between phototrophy and autotrophy or between chemolithotrophy and autotrophy. Two: autotrophic bacteria are not uniquely inhibited by organic matter. Three: all putative obligate autotrophic bacteria so far tested assimilate and metabolize exogenously supplied organic compounds. Four: mixotrophy can exist with respect to autotrophic and heterotrophic biosynthetic mechanisms and/or to chemolithotrophic and chemoorganotrophic energy-generating processes.

Examples remain of bacteria that have not been cultured in the absence of an inorganic energy source or light. Such forms are appropriately described as obligate chemolithotrophs or obligate phototrophs. The available evidence, briefly categorized above, suggest that none of these bacteria is, at the same time, an obligate autotroph. From ecological and evolutionary considerations, an absolute dependence on carbon dioxide for all carbon makes little sense, and bacteria with such a requirement would be an anachronism on earth as it now exists.

I would like to dedicate this paper to the memory of Dr. Derek Hoare whose untimely death has saddened his friends and scientific colleagues. Those of you who have worked with photosynthetic and chemolithotrophic bacteria appreciate the contributions to our understanding of the physiology of these organisms that Dr. Hoare has made by his thorough and critical work.

[1] A lecture delivered before the third meeting of the Northwest European Microbiological Group, on August 18, 1971 at Utrecht, the Netherlands.

The autotrophic mode of life is a life style in which inorganic compounds provide for all nutritional needs of an organism. Implicit in this definition is the capacity of an organism to derive all cell carbon from carbon dioxide and to obtain ATP and reduced pyridine nucleotides either photosynthetically or chemolithotrophically. The existence of bacteria with such potentials has been known since the pioneering work of Winogradsky in the 1880's. The question explored in this paper is whether bacteria exist that must of necessity live autotrophically, i.e., the obligate autotroph *sensu* Winogradsky (1890).

I will make five points in this paper. They lead, I believe, to a negative answer to this question. One: there is no obligatory coupling between phototrophy and autotrophy or between chemolithotrophy and autotrophy. Two: autotrophic bacteria are not uniquely inhibited by organic matter. Three: all putative obligate autotrophic bacteria so far tested assimilate and metabolize exogenously supplied organic compounds. Four: mixotrophy can exist with respect to autotrophic and heterotrophic biosynthetic mechanisms and/or to chemolithotrophic and chemoorganotrophic energy-generating processes.

The term mixotrophy may be unfamiliar to you. It was introduced by Pfeffer (1897) to describe a physiology in which organic compounds were either stimulatory or necessary in the photosynthetic growth of certain lower plants and was later used by Pringsheim (1967) to describe the physiology of the *Beggiatoa* group, members of which grow autotrophically but whose autotrophic growth is greatly stimulated in the presence of specific organic compounds. The term, as used in this paper, implies the concomitant generation of energy by supposedly alternative mechanisms – that is, chemolithotrophically and chemoheterotrophically – or the concomitant use of autotrophic and heterotrophic mechanisms for biosynthesis (Rittenberg, 1969).

It will be noted that these four assertions directly challenge Winogradsky's concept of the inorgoxidant or obligate autotroph. I will present some data in support of each of these four statements. Unfortunately, I will not be able to present all the data now available on these points. If I appear to pay undue attention to work from my laboratory, it is only because slides were already available to illustrate these points. More comprehensive presentations of the available data can be found in recent reviews by Rittenberg (1969) and Kelly (1971).

The fifth point is that the existence of "obligate" autotrophic bacteria would not be predicted from ecological and evolutionary arguments. This statement is based on *ad hoc* and admittedly subjective arguments, and has little experimental support. Whether or not one agrees with these arguments will, I suspect, also be a matter of subjective judgment.

Let us consider now the first statement. It has long been known that photo-

trophy and autotrophy are not necessarily coupled in microbial metabolism. There was a long history of controversy about the physiology of photosynthetic bacteria in which Winogradsky and Molisch were the major protagonists. This controversy was clearly resolved when van Niel (1931) showed that some photosynthetic bacteria, the Thiorhodaceae and Chlorobacteriaceae utilize, either preferentially or exclusively, inorganic reductants such as hydrogen sulfide in their photosynthetic process. A second group, the Athiorhodaceae, utilize instead organic reductants (van Niel, 1944). It was initially believed that for the Athiorhodaceae, the organic compound served only as a reductant. This belief was strengthened by the work of Foster (1940) who demonstrated the quantitative conversion of isopropanol to acetone during photosynthesis by an unidentified Athiorhodaceae sp. However, it is now well known that the organic compound serves not only a reductive function but also as the major carbon source for growth of Athiorhodaceae during photosynthesis (Stanier et al., 1959).

That chemolithotrophy and autotrophy need not be coupled was demonstrated only relatively recently. The original finding was made with *Desulfovibrio desulfuricans*, an organism which, in the 1950's, was considered to be a facultative autotroph. Supposedly it had the potential to grow with hydrogen as its energy source and CO_2 as its exclusive source of carbon. This ability was tested in two laboratories simultaneously using experiments of the same design.

Growth of *Desulfovibrio desulfuricans* was studied in media containing bicarbonate of known specific activity (Table 1; Mechalas and Rittenberg, 1960). A comparison of the specific activity of the final cell carbon with that of the starting bicarbonate gave a direct measure of the contribution of the latter to cell carbon. In mineral salts medium with bicarbonate, but lacking hydrogen, no growth occurred. In the same medium under hydrogen atmosphere there was a trace of growth but the specific activity of the cell carbon formed was only 29% that of the starting bicarbonate. In mineral salts medium supplemented with

Table 1. Growth yields and utilization of bicarbonate as a carbon source by *Desulfovibrio desulfuricans* grown in mineral salts solution plus supplements (recalculated from Mechalas and Rittenberg, 1960)

Supplements	Growth yield (mg C/liter)	Cell carbon from HCO_3^- %
HCO_3^- (0.1%)	–	–
HCO_3^- (0.1%) + H_2	0.6	29
Yeast extract (YE) (0.1%)	3.5	–
HCO_3^- (0.1%) + YE (0.1%)	3.9	12
HCO_3^- (0.1%) + YE (0.1%) + H_2	48.0	12

yeast extract, growth was relatively abundant. Again, though, cell carbon had only 12% of the specific activity of the starting bicarbonate. These data show clearly that in the bicarbonate–hydrogen–yeast extract medium, yeast extract supplies the major source of carbon. It will be noted that in the absence of hydrogen, growth on yeast extract or yeast extract plus bicarbonate is marginal. It was concluded from these data that energy generated chemolithotrophically from the oxidation of hydrogen is used to assimilate organic molecules in the yeast extract. It was further concluded that the marginal growth under putative autotrophic conditions was dependent on the presence of organic impurities in the medium. Thus, *D. desulfuricans* can grow as a chemolithotrophic heterotroph but not as a chemolithotrophic autotroph.

Postgate (1960) obtained very similar data (Table 2). Cell carbon in a yeast extract-bicarbonate–hydrogen environment is derived almost completely from the yeast extract. In the absence of yeast extract, the percentage of cell carbon from CO_2 is no greater than in its presence. Likewise, the contribution of carbon dioxide to cell carbon is no greater when *D. desulfuricans* is grown with an inorganic energy source than it is with an organic energy source.

Table 2. Fixation of labelled CO_2 in cell material during growth of *D. desulphuricans* (Postgate, 1960; *Courtesy of Z. allg. Mikrobiologie*)

H-donor	Yeast extract (%)	Incubation (days)	(% $^{14}C/C$)
H_2	0.4	4	3.24
H_2	0.02	4	7.9
H_2	0.01	4	6.85
H_2	0.005	5	8.05
H_2	–	8	7.3
Ethanol	0.1	3	3.38
Ethanol	0.01	5	8.4
Ethanol	–	10	5.3

A second chemolithotrophic heterotroph, *Thiobacillus perometabolis*, was described by London and Rittenberg (1967).

This organism is incapable of growth in a thiosulfate–mineral salts medium (Table 3). In the same medium supplemented with yeast extract reasonably abundant growth of the organism occurs. In the thiosulfate–yeast extract medium, growth increases in proportion to the yeast extract present, concomitant with a drop in pH. The data clearly indicate that the thiosulfate is being oxidized to sulfate. The organism grows in yeast extract broth in the absence of thiosulfate. However, growth is much less abundant and the generation time is much longer. The stimulation of growth by thiosulfate and the demonstration

Table 3. Growth of *Thiobacillus perometabolis* in mineral salts–bicarbonate medium supplemented with thiosulfate and/or yeast extract (YE) (London and Rittenberg, 1967; *courtesy of Arch. Mikrobiol.*)

Supplements %	Cell yield (mg dry wt/liter)	Generation time (hr)	Terminal pH
$S_2O_3^=$ —0.5	No growth	–	–
+ YE — 0.05	140	3.5	2.8
+ YE — 0.1	210	3.5	2.9
+ YE — 0.2	245	3.5	3.0
YE — 0.05	31	7	6.5
YE — 0.1	79	5	6.9
YE — 0.2	130	4	7.1

of its oxidation to sulfate, makes it clear that thiosulfate is an energy source. For this organism then, as for *Desulfovibrio desulfuricans*, an inorganic compound can provide energy and an organic compound carbon for growth, i.e., a coupling of chemolithotrophy and heterotrophy.

Both *T. perometabolis* and *D. desulfuricans* lack the potential to grow autotrophically. We have attempted to detect ribulose diphosphate carboxylase in both of these organisms after growth under a variety of conditions, but without success. Their inability to grow autotrophically, then, can be ascribed to a lack of a cyclic mechanism for CO_2 fixation.

Table 4 presents growth data for still another thiobacillus, *Thiobacillus intermedius*, isolated by London (1963). This organism differs from *T. perometabolis* or *D. desulfuricans* in that it does grow autotrophically in a thiosulfate–mineral salts medium. Growth is not abundant, but it does indeed occur. No growth occurs, however, in glucose–mineral salts medium. The organism possesses the reductive pentose cycle and cell carbon after autotrophic growth has the same specific activity as the starting bicarbonate (London and Rittenberg, 1966). In

Table 4. Growth yields of *Thiobacillus intermedius* (recalculated from London, 1963 and London and Ritterberg, 1966)

Medium	Growth yield (mg dry wt/100 ml)
Thiosulfate — 0.5%	5.2
+ Glucose — 0.3%	14.0
+ Glucose — 0.5%	28.0
Thiosulfate — 1.0%	10.6
+ yeast extract — 0.05%	20.6
+ yeast extract — 0.1%	40.0

a medium containing thiosulfate and glucose, or thiosulfate and yeast extract, growth is much more abundant than in the completely mineral medium (Table 4). In the presence of the organic supplement, 90% or more of cell carbon may be derived from the organic supplement, and 10% or less from the bicarbonate. This was shown by comparing the specific activity of cells grown autotrophically in the presence of $^{14}C\text{-}HCO_3^-$ with that of cells grown in the presence of non-radioactive organic supplements (Fig. 1). Even in the presence of very low concentrations of organic compounds, as for example 0.05% glucose, the specific activity drops over 90%. We can conclude, then, that even for an organism capable of growing as a chemolithotrophic autotroph, energy derived chemolithotrophically may preferentially be coupled to biosynthesis based on organic substrates rather than on carbon dioxide as the bulk carbon source.

Finally, with respect to the first point, an organism may grow without assimilating organic carbon and not be either chemolithotrophic or photosynthetic. This is true for *Pseudomonas oxalaticus* which utilizes the reductive pentose cycle and assimilates all cell carbon from CO_2 when growing on formate (Quayle and Keech, 1959*a*, *b*).

Turning to the second point, the original definition of the obligate autotroph included the concept that these organisms are uniquely sensitive to organic

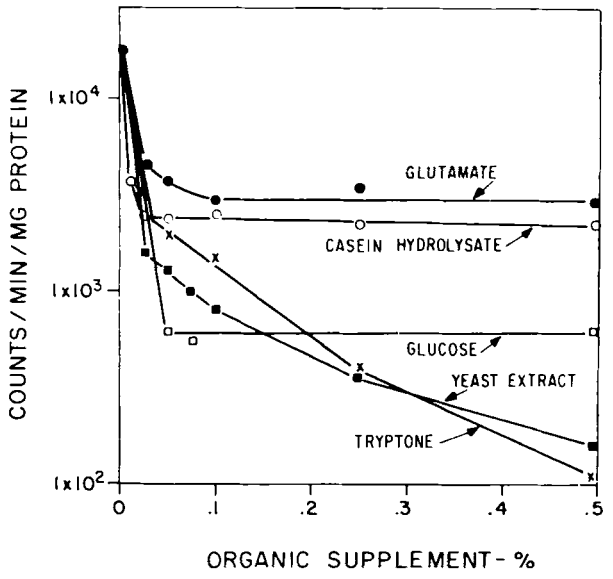

Fig. 1. Uptake of ^{14}C from $H^{14}CO_3^-$ by *Thiobacillus intermedius* grown in thiosulfate broth supplemented with organic compounds (London and Rittenberg, 1966; *courtesy of J. Bacteriol.*).

compounds. This claim no longer seems tenable. Inhibition of autotrophic growth by organic compounds is, of course, well documented. In all cases, however, where such inhibitions have been thoroughly investigated, they have proved quite specific, not general, and have been traced to mechanisms which also operate in heterotrophic growth. As is documented below, specific organic compounds may repress synthesis of enzymes of biosynthesis and of energy generation and also inhibit the functioning of such enzymes.

For example (Table 5), growth of two Thiorhodaceae on organic substrates results in reduced levels of ribulose diphosphate carboxylase (RuDP) activity, a key enzyme of the reductive pentose cycle (Hurlbert and Lascelles, 1963). It can also be seen that the two organisms, although qualitatively similar, do not respond identically. For example, growth of *Chromatium* on malate reduces RuDP carboxylase activity some 60%; growth of *Thiopedia* on the same substrate has no effect on the activity of this enzyme.

Table 5. RuDP carboxylase activity in *Chromatium* and *Thiopedia* organisms grown on organic substrates (Hurlbert and Lascelles, 1963; *courtesy of J. Gen. Microbiol.*)

Substrate	RuDP carboxylase activity[1]	
	Chromatium	Thiopedia
Acetate	41	45
Fumarate	46	77
Succinate	86	63
Pyruvate	25	41
Propionate	65	60
Malate	37	100

[1] Enzyme activities expressed as % of that in extracts of thiosulfate-grown organisms.

Inhibition of synthesis was shown in another way (Fig. 2; Hurlbert and Lascelles, 1963). *Chromatium* grown on an organic substrate was inoculated into a thiosulfate–mineral medium and the ribulose diphosphate carboxylase activity of the culture was followed with time. Enzyme synthesis continued until maximum specific activity was reached. If, during the course of growth on thiosulfate, pyruvate was added to the culture, there was a complete inhibition of further carboxylase synthesis.

London and Rittenberg (1966) showed a similar effect of organic compounds on RuDP carboxylase formation in *T. intermedius*. Growth in the presence of glucose or yeast extract inhibits enzyme synthesis and, as previously noted, under these conditions cell carbon is derived from the organic compound and not from bicarbonate (Table 6).

Growth in the presence of organic substrates may also affect the chemoli-

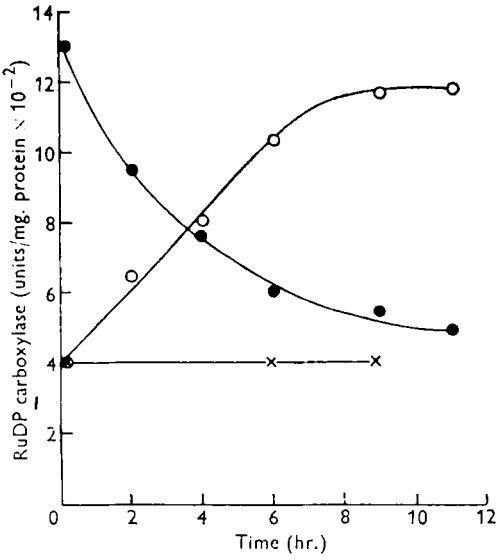

Fig. 2. RuDP carboxylase activity in extracts of *Chromatium* organisms transferred from pyruvate to thiosulfate (—○—), from thiosulphate to pyruvate (—●—), and from pyruvate to thiosulfate in the presence of 0.1 mM chloramphenicol (×). Substrate concentration was 0.01 M (Hurlbert and Lascelles, 1963; *courtesy of J. Gen. Microbiol.*).

thotrophic energy generating mechanism. The potential to oxidize thiosulfate decreases significantly when *T. intermedius* is grown either under mixotrophic or under heterotrophic conditions (Table 8; London and Rittenberg, 1966). The same phenomenon occurs in the hydrogen bacteria. Growth of *Hydrogenomonas* H 16 on fructose results in a marked decrease in the activity of both hydrogenase and of ribulose diphosphate carboxylase (Table 7; Eberhardt, 1966).

The molecular mechanisms of the inhibitions illustrated have as yet not been

Table 6. Carboxydismutase activity of *Thiobacillus intermedius* grown in thiosulfate broth with organic supplements (London and Rittenberg, 1966; *courtesy of J. Bacteriol.*)

Medium	Carboxydismutase		Cell carbon from HCO_3^-—%
	Activity[1]	Percent	
Thiosulfate — 0.5%	56	100	100
+ Glucose – 0.5%	24	44	3
+ Yeast extract — 0.05%	14	26	7
+ Yeast extract — 0.5%	5	1	1

[1] Activity — millimicromoles NADH oxidized per min per mg protein.
[2] Relative to activity of thiosulfate-grown organism.

Table 7. *Hydrogenomonas* H 16. Rate der Wasserstoffoxydation intakter Zellen, Hydrogenase- und RDPC-Aktivität nach Wachstum mit Fructose. Anzucht in Mineral-nährlösung mit 0,5% Fructose; Atmosphäre: Luft (Eberhardt, 1966; *courtesy of Arch. Mikrobiol.*)

Substrat	Vermehrung in heterotropher Kultur	Rate der Wasserstoffoxydation µl/Std/mg. Protein		Hydrogenase-Aktivität mE/mg Protein		RDPC-Aktivität mE/mg Protein
		$+CO_2$	$-CO_2$	lösliche Hydrogenase	partikelgeb. Hydrogenase	
H_2, CO_2	–	2535	509	1132	842	166
Fructose	$0{,}9 \cdot 10^2$	1313	592	860	563	25
Fructose	$1{,}0 \cdot 10^4$	976	499	255	528	15
Fructose	"unendliche Subkultur"	900	480	246	–	–
Fructose	"unendliche Subkultur"	855	501	365	270	22

Table 8. Effect of yeast extract in the growth medium on the oxidation of thiosulfate by cell suspensions of *Thiobacillus intermedius* (London and Rittenberg, 1966; *courtesy of J. Bacteriol.*)

Culture medium	Oxidation rate	
	A[1]	B[1]
Autotrophic — 0.5% $Na_2S_2O_3 \cdot 5H_2O$	15.0	100
+ 0.05% yeast extract	5.3	36
+ 0.10% yeast extract	2.9	19
Yeast extract, 0.5%, 1st generation	4.0	26
Yeast extract, 0.5%, 2nd generation	3.3	22
Yeast extract, 0.5%, 6th generation	4.6	30

[1] A-μmoles O_2/hr/mg protein; B — % autotrophic rate.

clarified. They appear similar, however, to the well known catabolite repressions that have been extensively studied in heterotrophic bacteria. It would be of considerable interest to determine the effect of cyclic AMP on these effects.

There is another type of inhibition that has been studied over the last few years. It is well known that the autotrophic growth of microorganisms may be inhibited by the addition of individual amino acids to the growth medium. Different species, and, indeed, different strains of the same species, are in general sensitive to different amino acids. Recently we surveyed the L-amino acid sensitivities of three strains of thiobacilli (Table 9; Lu, Matin and Rittenberg, 1971). Our strain of *T. thioparus* is completely inhibited by phenylalanine and partially inhibited by cystine and glutamate. *T. neapolitanus* is completely inhibited by phenylalanine and histidine, and partially inhibited by four other amino acids, while *T. thiooxidans* is completely inhibited by cystine, methionine and valine. These inhibitions were demonstrated by the addition of individual amino acids at a concentration of 10^{-2} molar to an otherwise autotrophic medium. In all cases, these inhibitions were either partially or completely reversed if a balanced mixture of amino acids – that is, casein hydrolysate — was also present in the medium. Several of these inhibitions were looked at in more detail, but only the valine inhibition of *T. thiooxidans* will be referred to here. As already noted, the addition of valine to an otherwise autotrophic medium caused a complete inhibition of growth. If either leucine or isoleucine were added along with valine, the inhibition was almost completely overcome, and if both amino acids were present the growth rate was, in fact, stimulated (Table 10).

At the cell-free level, L-valine strongly inhibits the acetolactate forming enzyme which catalyzes the first reaction in the branched pathway leading to the biosynthesis of valine, isoleucine and leucine (Table 11). Isoleucine has a lesser inhibitory effect on this enzyme, and leucine none at all.

Table 9. Effect of casein hydrolysate (CH) on inhibition of growth of *Thiobacillus thioparus*, *Thiobacillus neapolitanus* and *Thiobacillus thiooxidans* caused by individual amino acids in otherwise autotrophic medium (Lu, Matin and Rittenberg, 1971; *courtesy of Arch. Mikrobiol*).

Medium[1]	Relative growth indices[2]					
	T. thioparus		*T. neapolitanus*		*T. thiooxidans*	
Autotrophic control	1.00		1.00		1.00	
+ CH	1.00		1.20		1.03	
	—CH	+CH	—CH	+CH	—CH	+CH
+ L-Phenyl-alanine	0.18(C)	0.26	0.16(C)	0.24		
+ L-Cysteine	0.27	0.36	0.34	0.76	0.05(C)	0.11
+ L-Glutamate	0.39	0.63	0.40	1.31		
+ L-Methionine			0.66	1.20	0.02(C)	0.24
+ L-Histidine			0.04(C)	0.90	0.22	1.10
+ L-Asparagine			0.24	1.00		
+ L-Valine					0.04(C)	0.46
+ L-Serine					0.34	0.86

[1] Mineral salts medium with 1.0% $Na_2S_2O_3.5H_2O$ plus amino acids as indicated at 10^{-2} M and casein hydrolysate (CH) at 0.05% (*T. thioparus*, *T. neapolitanus*) or 0.01% (*T. thiooxidans*).
[2] Growth index equals the ratio of thiosulfate used in experimental culture to that used in autotrophic control at the time of 50% utilization in the latter. Complete inhibition of growth indicated by (C) — all other cultures show 100% utilization of thiosulfate with continued incubation.

Table 10. Reversal of L-valine inhibition of growth of *Thiobacillus thiooxidans* by biosynthetically related amino acids (Lu, Matin and Rittenberg, 1971; *courtesy of Arch. Mikrobiol.*)

Medium[1]	Relative growth indices[2]
Autotrophic control	1.00
+ L-Valine	0.12(C)
+ L-Valine + L-leucine	0.88
+ L-Valine + L-isoleucine	0.87
+ L-Valine + L-isoleucine + L-leucine	1.40

[1, 2] See footnotes, Table 9.

Table 11. Effect of L-valine, L-leucine, and L-isoleucine on the acetolactate forming enzyme of *Thiobacillus thiooxidans* (Lu, Matin and Rittenberg, 1971; *courtesy of Arch. Mikrobiol.*)

Additions to reaction mixture[1]	Specific activity[2]	Per cent inhibition
None	90	–
L-Valine	32	64
L-Isoleucine	57	38
L-Leucine	90	0
L-Valine + L-isoleucine	33	64

[1] See Umbarger and Brown, 1958. Amino acids when present at 5×10^{-3} M.
[2] Enzyme units/mg protein.

These data, and very similar data obtained by Kelly (1971) and by Johnson and Vishniac (1970) support the contention that inhibition of autotrophic growth by individual L-amino acids is due to an amino acid imbalance in the medium. This phenomenon was first described by Gladstone (1939) for the anthrax organism. The mechanism of inhibition was clarified by Umbarger (1969) and others and shown to be due to a feedback inhibition by an end product of a biosynthetic pathway on the first enzyme in the pathway. The data presented here for the valine inhibition parallel similar data that could be presented for specific strains of *E. coli*.

Thus, available information suggests that the inhibition of chemolithotrophic bacteria by organic compounds rather than indicating a unique property of this group in fact indicates basic similarities between them and heterotrophic bacteria in mechanisms for regulating their metabolic activities.

Turning next to the assimilation of organic compounds by putative obligate autotrophs, we find that in every case tested, common metabolites are indeed assimilated and extensively metabolized. As an example, Hoare and Gibson (1964) showed that the presence of acetate in an otherwise autotrophic medium significantly enhanced the growth yields of three strains of *Chlorobium thiosulfatophilum*, a photosynthetic green bacterium (Table 12). They also showed that the acetate assimilated during growth is distributed in the major fractions of the cell. The label from either carbon one or carbon two of acetate is incorporated into the lipid fraction, the nucleic acid fraction, and cell protein.

Table 12. Effect of acetate on growth of *Chlorobium thiosulphatophilum* in thiosulfate–mineral medium (Hoare and Gibson, 1964; *courtesy of Biochem. J.*)

Acetate (mM)	Final cell yield (E at 600 mμ)		
	strain 8327 (OC)	strain 8346	strain 8227
0	0.750	0.660	0.760
2	1.020	0.825	0.920
5	1.190	0.925	1.280
10	1.240	0.880	1.230

Smith, London and Stanier (1967) examined the assimilation of a variety of metabolites by three blue-green algae and two thiobacilli — all of these organisms supposedly are obligate autotrophs. Table 13 shows the data for the three blue-green algae. It is evident that all compounds tested were assimilated at least to some extent. Under the conditions of the experiments, acetate and leucine provided up to 10% of the total cell carbon for all three algae, and aspartate was equally effectively utilized by one of them. Let me emphasize that

Table 13. Magnitude of incorporation of organic compounds by blue-green algae during photosynthetic growth (Smith, London and Stanier, 1967; *courtesy of J. Bacteriol.*)

Organic substrate (initial concn: 5 mM)	Amt (mg) of organic carbon incorporated per 100 mg of newly synthesized cell carbon		
	Anacystis nidulans	*Coccochloris peniocystis*	*Gloeocapsa alpicola*
Glucose	0.6		
Fructose	1.2	1.7	2.0
Pyruvate	3.0	5.6	0.6
Acetate	10.4	8.2	10.8
Succinate	0.4	1.3	1.0
Malate	0.2	1.4	1.0
Glutamate	1.0	0.3	1.8
Aspartate	0.5	0.2	9.9
Leucine	8.0	6.7	6.7

there is nothing in the experimental designs to indicate that the assimilation values are the maximum possible. Nor was there any attempt to combine organic substrates to determine how much cell carbon could be derived from appropriate combinations.

Table 14 shows similar data for *T. thiooxidans* and *T. thioparus*, two putative obligate autotrophs, as well as for *T. intermedius*, the mixotrophic organism mentioned previously. These organisms also incorporate carbohydrates, organic acids, and amino acids in significant quantities into cell material. It is of interest that little succinate is assimilated by *T. thiooxidans* while over 11% of

Table 14. Magnitude of incorporation of organic compounds by thiobacilli during chemoautotrophic growth (Smith, London and Stanier, 1967; *courtesy of J. Bacteriol.*)

Organic substrate	Amt (mg) of organic carbon incorporated per 100 mg of newly synthesized cell carbon		
	T. thiooxidans	*T. thioparus*	*T. intermedius*
Glucose			39.4(5)
Fructose	2.9(2)[1]		
Pyruvate	4.0(1)	2.6(2)	4.6(5)
Acetate	8.8(0.2)	9.2(2)	43.6(5)
Succinate	0.8(0.5)	11.6(2)	86.0(5)
Malate	3.7(2)		
Glutamate	3.0(2)	7.2(2)	87.8(5)
Aspartate	4.3(2)	7.3(2)	
Leucine	2.6(2)	2.9(2)	

[1] Figures in parentheses give the original concentration of the organic substrate in the growth medium (mM).

cell carbon can be derived from this compound by *T. thioparus*. Certain strains of *T. thiooxidans* are inhibited by low concentrations of succinate (Butler and Umbreit, 1966), but the mechanism of this inhibition has not yet been investigated.

Clark and Schmitt (1967) added very low concentrations, of the order of 10^{-7} molar, of individual ^{14}C-labeled amino acids to an otherwise autotrophic medium and measured the uptake of radioactivity and its distribution among major cell fractions of *Nitrosomonas europae*. The bar graphs (Fig. 3) show incorporation into, first, the cold-TCA-soluble fraction; second, the ether–ethyl alcohol fraction; third, the hot-TCA-soluble fraction; and last, the residue. The results with each amino acid differ somewhat. These differences are not significant to this discussion. It is pertinent that each amino acid not only was assimilated from the medium, but was also metabolized. For some amino acids, e.g., aspartate, glutamate, and histidine, most of the radioactivity was

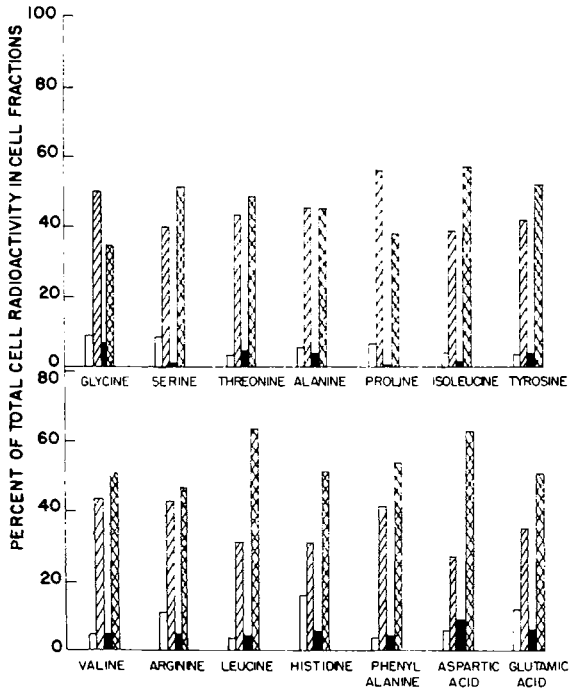

Fig. 3. Distribution of cellular radioactivity arising from ^{14}C-labeled L-amino acids supplied to growing *Nitrosomonas europaea* cells. Each set of bars represents, from left to right, cold trichloroacetic acid-soluble extract, ether–ethyl alcohol-soluble extract, hot trichloroacetic acid-soluble extract, and cell residue (Clark and Schmidt, 1967; *courtesy of J. Bacteriol.*).

found in the protein fraction. Others, e.g., glycine and proline, contributed more radioactivity to the lipid fraction than the protein fraction. It will be noted that some radioactivity also appeared in the nucleic acid fraction, as for example with aspartate.

The final example in this sequence is taken from the elegant but little known doctoral thesis of Still (1965) who studied the uptake of acetate and alanine by *T. thioparus* and the distribution of carbon from these substrates into the various amino acids of the cell. As the data show (Table 15), the alanine label appears in all amino acids examined with a considerable excess in alanine as would be expected. In contrast, acetate carbon was found only in glutamate, proline, arginine and leucine, and was not incorporated into the other amino acids examined. These data suggest that *T. thioparus* does not have a complete TCA cycle and more specifically that it lacks alpha-ketoglutarate oxidase. Subsequent work (Kelly, 1967; Hoare, Hoare and Moore, 1967; and others) has shown the same hiatus in other so-called obligate autotrophs.

Table 15. Incorporation of the labelled atom of (2-^{14}C)-DL-alanine, (1-^{14}C)-acetic acid and (2-^{14}C)-acetic acid into the cellular amino acids of *Thiobacillus thioparus* (Still, 1965)

Cellular amino acid	Activity in cellular amino acid (μC/μmole amino acid)		
	(2-^{14}C)-DL-alanine	(1-^{14}C)-acetic acid	(2-^{14}C)-acetic acid
Glutamate	0.005	3.27	2.84
Proline	0.005	3.98	3.41
Arginine	0.005	3.55	3.12
Leucine	0.009	3.41	3.12
Valine	0.007	–	0
Aspartate	0.002	0	0
Threonine	0.002	0	0
Lysine	0.005	0	0
Isoleucine	0.005	0	0
Methionine	0.003	0	0
Alanine	0.108	0	0
Serine	0.001	0	0

The ability of these photosynthetic and chemolithotrophic organisms to utilize an individual organic compound for a few percent of the total cell carbon implies that heterotrophic and autotrophic biosynthetic processes function concomitantly within a cell. In terms of the definition previously given, these organisms are therefore mixotrophic with respect to carbon assimilation and biosynthesis.

Mixotrophy has also been demonstrated in the hydrogenomonads (Ritten-

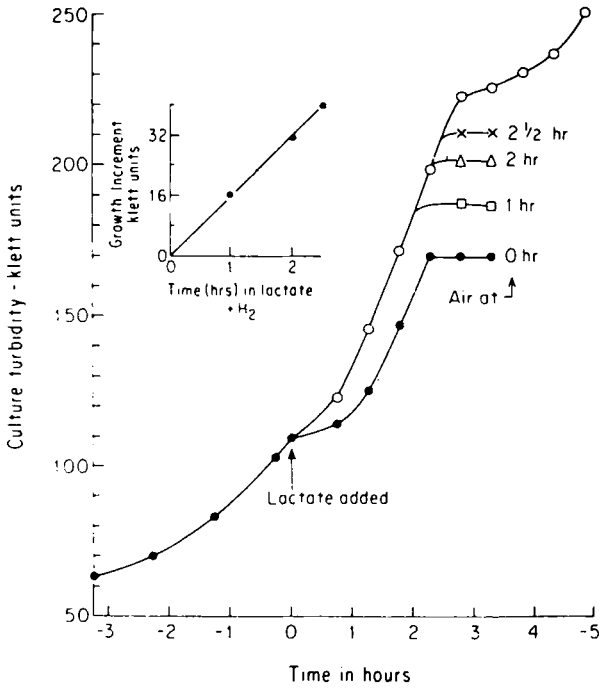

Fig. 4. Growth of *H. eutropha* in the presence of lactate (8.0 μmoles/ml) and hydrogen. Five replicate cultures started in autotrophic medium. Lactate was added to all cultures at zero-time; air was substituted for hydrogen atmosphere in individual cultures at indicated times (Rittenberg and Goodman, 1969; *courtesy of J. Bacteriol.*)

berg and Goodman, 1969), in *Nitrobacter agilis* (Smith and Hoare, 1968) and in *Beggiatoa* species (Pringsheim, 1967). The critical experiment with *Hydrogenomonas eutropha* is illustrated in Fig. 4 (Rittenberg and Goodman, 1969). A series of identical cultures was prepared in mineral salts solution and incubated in an atmosphere of hydrogen, oxygen and CO_2 — that is, under autotrophic conditions. When these cultures reached a convenient rate of development, a small amount of lactate, sufficient for one to two doublings, was injected into each culture. In one culture, air was substituted for the gas mixture at the time of lactate injection. In other cultures, the substitution of air for the gas mixture was delayed for 1, 2 and 2½ hours, respectively, after lactate addition. The longer the delay — or, put in another way — the longer the culture was in the presence of dual substrates, the higher the final growth yield. In fact, as the insert shows (Fig. 4) the growth increment over the heterotrophic control was directly proportional to the time in the presence of dual substrates.

A plot of the growth increment per hour of mixotrophic growth against the culture development rate at zero time, that is, the rate at which the culture was growing autotrophically just before lactate addition, shows a direct relationship between these two parameters (Fig. 5). These and other results suggest that the autotrophic growth potential existing at zero time continues to function under heterotrophic conditions. In other words, in the environment provided, each cell was growing autotropically and heterotrophically as well as chemolithotrophically and chemoorganotrophically at the same time.

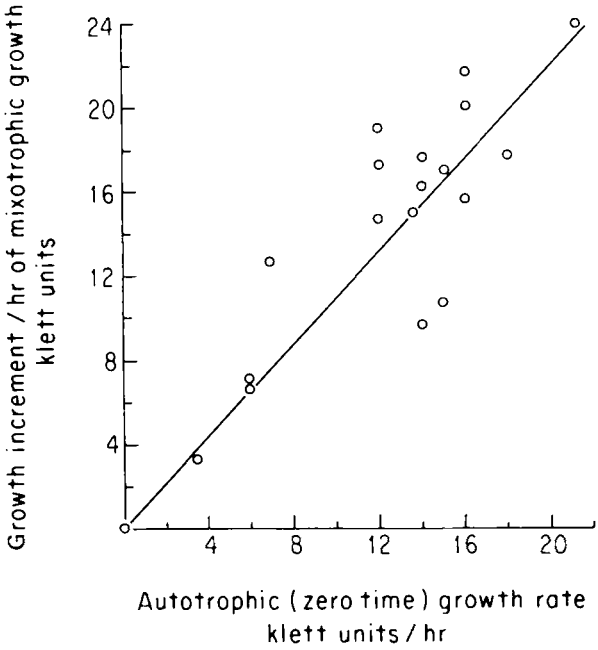

Fig. 5. Relation between increased growth yields (mixotrophic growth and heterotrophic growth) and the zero-time (autotrophic) absolute growth rates of the starting cultures (Rittenberg and Goodman, 1969; *courtesy of J. Bacteriol.*).

Examples remain of procaryotic organisms that have not been cultured in the absence of an inorganic energy source or of light. Such forms are appropriately described as obligate chemolithotrophs or obligate phototrophs. The available evidence, briefly categorized above, suggest that none of these bacteria is at the same time an obligate autotroph. Some of these bacteria may, indeed, have an absolute dependence on the reductive pentose cycle for the assimilation of a significant portion of their cell carbon. In this sense, then, it would perhaps be proper to still speak of obligate autotrophs but not in the sense of Winogradsky.

Even in this sense, the question is moot as to whether such an absolute dependence exists. I have shown you data indicating a significant assimilation of carbon from organic sources by such organisms. No one, to the best of my knowledge, has deliberately attempted to formulate a mixture of organic compounds that would permit growth of these organisms with either light or an inorganic substrate as an energy source in the absence of a functional reductive pentose cycle. It might be interesting, as a laboratory exercise, to attempt the concoction of such a medium. One would have to bear in mind that repression of the energy-generating mechanism would have to be avoided. Likewise, one would also have to avoid feedback inhibition and other inhibitory mechanisms which might prevent growth. This, as I said, might be an interesting type of experiment to do, but such an experiment begs the really important question. That is, how do these microorganisms function in Nature?

We have almost no knowledge regarding this question. In Nature, these organisms are rarely, if ever, growing in the type of artificial environment we use for their cultivation in the laboratory. In fact, I can think of no natural environment supporting microbial growth where there would not be at least traces of organic substrates available. Massive growths of the Thiorhodaceae are found in organically rich environments, and chemolithotrophic oxidations go on in similar environments. You may remember that it was Schloesing and Muntz, in 1877, who first demonstrated that nitrification was a biological process. What they in fact demonstrated was that organic nitrogen in sewage is converted to nitrate under appropriate conditions and that this conversion is prevented by disinfectants. The phenomenon they described is reproduced on a large scale in modern sewage disposal plants. Table 16 shows data for the activated sludge system in which the sewage from the City of Los Angeles is processed. The material entering the sludge tank is high in organic materials and free of nitrate-nitrogen. The material leaving the tank is low in soluble organics and much of the nitrogen has been converted to nitrate. This, then, is chemolithotrophy in the presence of an abundance of organic matter.

Recently Brock and coworkers (1971) showed that sulfide, added to water

Table 16. Nitrification during sewage treatment by activated sludge process (Data provided by W. F. Garber, Bureau of Sanitation, Department of Public Works, Los Angeles)

	Raw sewage (mg/liter)	Effluent-activated (mg/liter)
Soluble organics	170	<5
Kjeldahl nitrogen	60	15
Ammonia nitrogen	18–20	trace
Nitrate nitrogen	trace	15

Table 17. Sulfide stimulation of uptake of various labeled compounds (Brock et al., 1971; *courtesy of J. Bacteriol.*)

Labeled compound	Counts per min per cover slip		
	No sulfide	Sulfide	Sulfide + 4% formaldehyde
^{14}C-glucose (0.1 μCi/ml)	823	1,366	776
^{14}C-glutamic acid (0.1 μCi/ml)	326	3,626	289
^{14}C-leucine (1.0 μCi/ml)	1,084	16,083	
^{14}C-acetate (0.1 μCi/ml)	564	6,847	71
^{14}C-bicarbonate (1.0 μCi/ml)	337	1,341	301
^{14}C-lactate (0.12 μCi/ml)	956	3,234	15
^{3}H-thymidine (1 μCi/ml)	5,196	24,712	671
^{3}H-phenylalanine (0.06 μCi/ml)	3,012	3,868	214

taken from a hot spring in Yellowstone National Park enhances incorporation of organic compounds into the cell material of the bacterial population indigenous to the water (Table 17). This, perhaps, is a demonstration of mixotrophy in the natural environment. It is unlikely, however, that the organisms involved are classical thiobacilli.

At the moment, it is not known whether the nitrifying organisms functioning in the activated sludge tanks or the sulfide oxidizing bacteria studied by Brock derive a significant percent of their cell carbon from the organic compounds available in the environment. I would guess, however, that they do. This conjecture is based on the proposition that it is more efficient to utilize preformed organic materials than it is to synthesize such materials *de novo*. In general we expect that Nature will follow the path of minimum energy expenditure. Let me illustrate this point with data (Table 18) compiled from various sources. The

Table 18. Energy efficiency and growth yields. Y_{ATP} values calculated from primary data from indicated sources

Organism and medium	Y_{ATP}	Y_{O_2}
Thiobacillus neapolitanus[1]; Thiosulfate–mineral salts	3.0(?)	10
Pseudomonas sp.[2]; Glucose–mineral salts	5.3(?)	32
Streptococcus faecalis[3]; Glucose–peptone	10	–
Bdellovibrio bacteriovorus[4]; growing on *E. coli*	30(?)	180

[1] Hempfling and Vishniac, 1967.
[2] MacKechnie and Dawes, 1969.
[3] Bauchop and Elsden, 1960.
[4] Hespell and Rittenberg, unpublished.

recent data of Hempfling and Vishniac (1967) are probably the best available for growth yields of chemolithotrophic bacteria growing autotrophically. Calculations from their data show some ten grams dry weight of cells produced per mole of oxygen consumed when *T. neapolitanus* is grown in a thiosulfate–mineral salts medium. Based on a variety of assumptions as to ATP yields, this translates into a Y_{ATP} of approximately three grams dry weight of cells. Data of MacKechnie and Dawes (1969), indicate that per mole of oxygen consumed, *Pseudomonas aeruginosa* growing in a glucose–mineral salts medium forms 32 grams dry weight of cells. Making the assumption that electron transport to oxygen results in the production of 3 ATP per atom of oxygen, this translates into a Y_{ATP} of 5.3 grams dry weight. The pseudomonad, having the carbon skeleton of glucose and its degradation products available for biosynthesis, grows with an efficiency of almost twice that of *T. neapolitanus* which must make all cell material from CO_2.

Streptococcus faecalis growing anaerobically in a glucose–peptone medium has even fewer biosynthetic tasks and has a Y_{ATP} of 10 (Bauchop and Elsden, 1960). To complete the list, I have included some data we have obtained with *Bdellovibrio bacteriovorus* (Hespell and Rittenberg, unpublished) growing on *Escherichia coli* as its sole source of food. Under these conditions, with apparently a complete supply of monomers available, 180 grams of new cell material are produced for each mole of oxygen consumed. Based on an assumed ATP yield of 6 per mole of O_2, the Y_{ATP} is 30.

Frankly, I would not like to be forced into a detailed defence of all the assumptions that went into these calculations. In a general way, however, the data show that the less biosynthetic work an organism must do, the greater its reproductive power per unit of energy expenditure. Considering these data and the nature of the environment, it seems unlikely that an organism living in the midst of organic molecules would ignore these molecules and make them *de novo* from carbon dioxide.

Finally, it makes little sense from an evolutionary standpoint that organisms completely restricted to carbon dioxide as their carbon source would survive in competition with those not bound by such restrictions. Compare, for example, *T. intermedius* and *T. neapolitanus*. We have shown that the first is capable of using energy derived from thiosulfate for the assimilation of organic carbon compounds. Growth yield data show that this organism functioning as a chemolithotrophic heterotroph produces more cell material per unit of thiosulfate metabolized and grows more rapidly than does *T. neapolitanus* growing as a chemolithotrophic autotroph. The question is, then, which organism would predominate and be selected for in an environment in which both thiosulfate (or another reduced sulfur compound) and organic matter were present? In

other words, which would be selected for in a typical, natural environment? The answer is, unfortunately, we do not know. I for one, would not wager on *T. neapolitanus*, unless it also has a significant mixotrophic potential.

In summary, examples of bacteria corresponding to Winogradsky's original concept of the "inorgoxidant" or "obligate autotroph" are not known. That such bacteria exist and remain to be discovered seems unlikely although admittedly one could not prove this negative assertion. We could amend Winogradsky's concept to redefine the term "obligate autotroph" and make it fit known organisms of a particular physiology. After all, as the Queen (*Alice in Wonderland*, Lewis Carrol) stated, "a word means what I want it to mean." I think, however, that the concept, fruitful as it has been, has outlived its usefulness. In accepting its demise we would in no way detract from Winogradsky's brilliance in conceiving it and from its historical significance in the development of our science.

The work reported from the author's laboratory was supported by grants from the National Science Foundation.

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