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COMPARATIVE ORGANIC NUTRITION OF
AN OBLIGATELY AND A FACULTATIVELY
AUTOTROPHIC THIOBACILLUS.

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requirements for the degree of
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ABSTRACT.

The organic nutrition of a facultatively autotrophic Thiobacillus (T. novellus) and an obligately autotrophic Thiobacillus (T. thioparus) were compared. A wide range of organic compounds as potential growth substrates and as effectors of growth were tested in agar plates and in liquid culture. The uptake and metabolism of ^{14}C -labelled glucose, glutamate and acetate was examined in greater detail.

(i) T. novellus used a wide range of organic compounds, including amino-acids, sugars, organic acids and alcohols, as substrates for heterotrophic growth. T. thioparus used no organic compounds as a sole energy or carbon source.

(ii) Of the tested organic compounds most, but not all, were inhibitory to T. thioparus, but very few to T. novellus. Some similarities in the shape of the growth curves for the two species were evident in the presence of inhibitors, and possible mechanisms for this are discussed. The compounds capable of reversing threonine inhibition of T. thioparus are biosynthetically related.

(iii) A very low rate of uptake of ^{14}C -labelled glucose and glutamate (supplied at μ Molar levels) was found in T. thioparus, compared to the high rate of uptake in T. novellus. Acetate was rapidly taken up by both species. The assimilated compounds were largely oxidized to CO_2 by T. novellus, both heterotrophically- and autotrophically-grown, but in T. thioparus most of the ^{14}C taken up was incorporated into cell constituents.

(iv) The metabolism of both acetate and glutamate by T. thioparus was restricted by an incomplete TCA cycle: the resultant distribution of ^{14}C can be correlated with a lack of α -ketoglutarate dehydrogenase. The TCA cycle was apparently complete in T. novellus.

(v) The inhibitory effect of 16 mM succinate on T. thioparus was investigated. In liquid culture growth and consequent thiosulphate consumption and pH changes were inhibited. In cell suspensions the rate of acid production and of $^{14}\text{CO}_2$ assimilation was unaffected. The uptake and metabolism of acetate was unaffected. The uptake of ^{14}C -glucose was markedly depressed, and possible mechanisms for this are discussed. No basis for the inhibitory effect of succinate on growth was found in these experiments.

The findings are discussed in relation to the current theories of obligate autotrophy.

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ABBREVIATIONS USED IN THIS TEXT

ADP	Adenosine DiPhosphate
AMP	Adenosine MonoPhosphate
APS	Adenosine-5'-Phospho-Sulphate
ATP	Adenosine TriPhosphate
CoA	Coenzyme A
Co Q	Coenzyme Q
Cyt a ₃	Cytochrome a ₃
Cyt b	Cytochrome b
Cyt c	Cytochrome c
Cyt o	Cytochrome o
D.A.P.	DiAmino Pimelic Acid
DNA	DeoxyRiboNucleic Acid
E.D.T.A.	Ethylene Diamine TetraAcetic Acid
Gm	Gram (stained)
gm	gramme
KN/m ²	KiloNewtons per square Metre
M	Molar (concentration)
mM	milliMolar (concentration)
mls	millilitres
NADH ₂	reduced Nicotinamide Adenine Dinucleotide
NADPH ₂	reduced Nicotinamide Adenine Dinucleotide Phosphate
P	Phosphate (in compounds)
P _i	inorganic Phosphate
P.P.O.	2,5-diPhenyl Oxazole
P.O.P.O.P.	1,4-di(2-(5-PhenylOxazolyl))benzene
TCA	TriCarboxylicAcid
μm	micrometre

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PREFACE

"The characteristics of the chemosynthetic organism are as follows:

- (1) Development occurs in a purely mineral milieu using an oxidizable inorganic substance.
- (2) Vital processes depend on the presence of this substance, which is ammonia in the case of denitrification.
- (3) Oxidation of this substance is the sole source of energy.
- (4) Inability to use any organic compound as a source of energy.
- (5) Inability to decompose organic compounds, which in fact prevents development.
- (6) Assimilation of Carbonic acid as the sole source of carbon, for chemosynthesis."

S. Winogradsky (1890) Annals Instiut de Pasteur. 4. 213. Quoted in S.C. Rittenberg (1970a).

"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 achronism on earth as it now exists."

S.C. Rittenberg (1972). The obligate autotroph - the demise of a concept. Antonie van Leeuwenhoek Journal of Microbiology and Serology. 38 457.

1. INTRODUCTION

1.1. OBLIGATE AUTOTROPHS.

The problem of obligate autotrophy has intrigued biologists since the concept of "chemosynthesis" was introduced by Winogradsky (1890) (see Preface). During the ensuing years the changing understanding of metabolism and a wider knowledge of the different nutritional types found amongst living organisms, has led to a changing meaning of the terms "facultative autotroph" and "obligate autotroph".

"Autotrophy" indicates an independence from the products of other living organisms i.e. organic compounds, for both nutrients (e.g. C and N) and energy. The autotrophs are thus lithotrophs, obtaining energy from the oxidation of inorganic compounds, and also using carbon dioxide as the sole source of carbon. In biochemical terms such organisms are characterized by the existence in the cell of an active ribulose diphosphate carboxylase.

The methylotrophs use an organic source of carbon, i.e. methane, which is also the energy source. They do not possess ribulose diphosphate carboxylase; but the energy-generating mechanisms and the existence of obligate methylotrophs indicates many affinities with the autotrophs so they are considered in this study as autotrophs.

The term "obligate autotroph" is used to denote an autotroph incapable of using organic compounds as a sole carbon or sole energy source under most experimental conditions. It is a term that loses much of its effectiveness if too rigorously defined, so as to be incapable of accommodating exceptions under certain experimental conditions.

"Facultative autotroph" denotes an autotroph also able to exist as a heterotroph.

1.2. THE OCCURRENCE OF OBLIGATE AUTOTROPHS IN THE MICROBIAL WORLD.

Although several claims have been made for the occurrence of obligate autotrophs among the motile and unicellular algae and protozoa, these claims have not been investigated sufficiently thoroughly over a wide range of nutrient conditions. Smiley (1964) found evidence to suggest that the "obligate autotrophic" nature of the diatoms was not absolute and that marine pennate diatoms are photo-heterotrophic (i.e. use an organic carbon source and light as an energy source).

The blue-green algae (Schizophyceae) have generally been classed as obligate autotrophs, i.e. obligate photo-lithotrophs (Van Baalen, Hoare and Brandt 1971). However a report by Khoja and Whitton (1971) in which 17 out of 24 strains of blue-green algae were capable of growth, albeit slowly, on 0.01M sucrose in the dark, suggests that the heterotrophic potential of more strains on more substrates should be examined. It is unfortunate that the organisms widely used as typical examples, in research, of blue-green algae, do not appear, as yet, to have been shown to use organic sources of carbon.

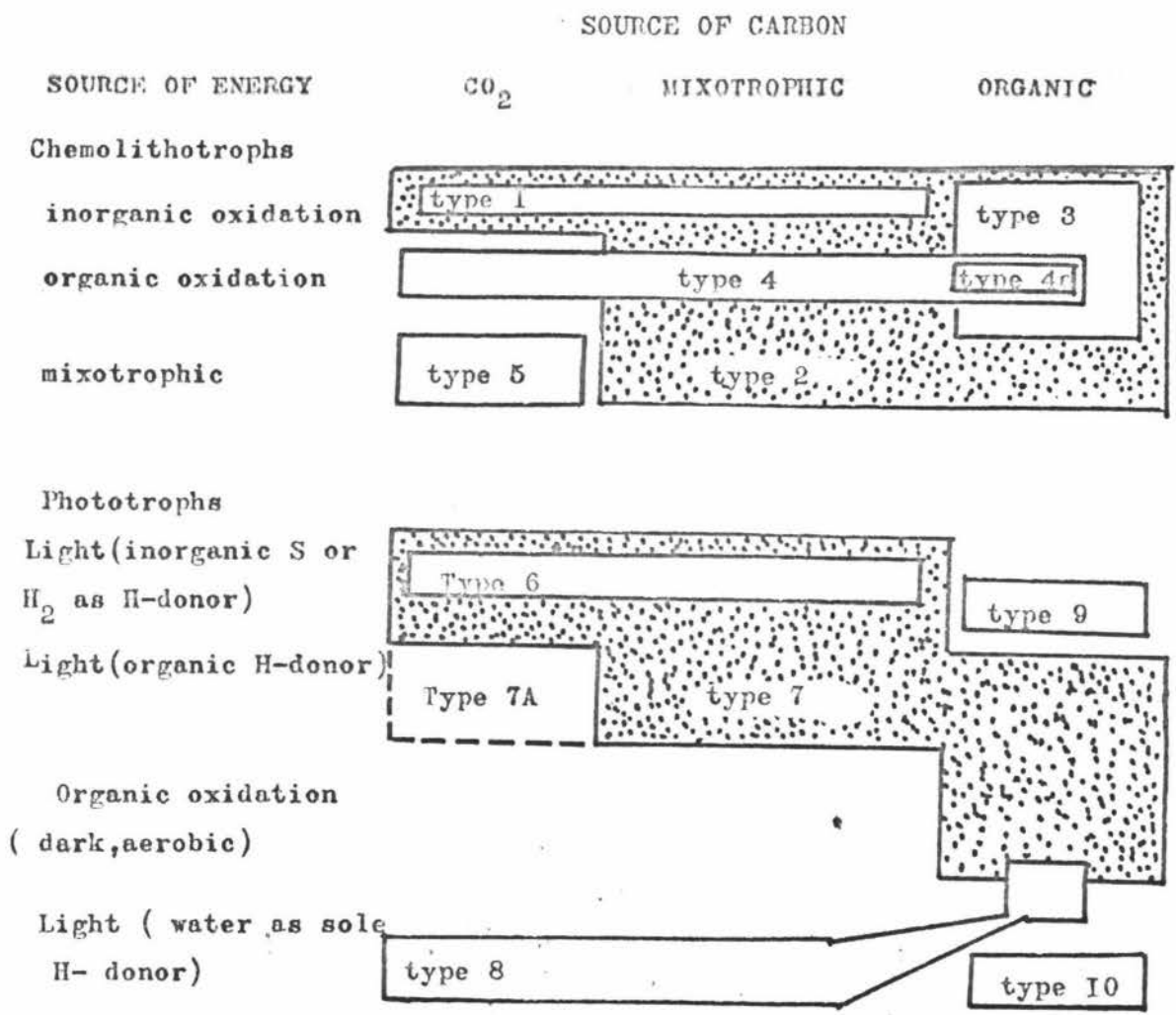
The distribution of autotrophy and obligate autotrophy amongst the bacteria is complicated by conceptual difficulties as to the nature of lithotrophy (Kelly 1971a). Obligate autotrophs are found among chemolithotrophs (such as species of thiobacilli and nitrifying bacteria) and among the photolithotrophs (such as Chlorobium spp). Certain methylotrophs may also be regarded as being obligate autotrophs because of their dependence on the presence of one specific form of reduced carbon as an energy source. However increasing knowledge of the physiology of these groups has led to the need to qualify the classification of many organisms as obligate autotrophs. Kelly (1971a) recognises a number of physiological types which he has classified as shown in Table 1, in which the classification is by reductant source as well as the more traditional carbon and energy source. One organism can, according to conditions, adapt to life over a spectrum of types so that it belongs to several or many of these groups; at least one organism exists to exemplify each of these types.

Types 1 and 7 are the obligate autotrophs - metabolically conservative - while types 2 and 7 are facultative autotrophs.

Certain supposed autotrophs in the bacteria have not been sufficiently studied to establish their status as autotrophs let alone as obligate autotrophs. The quite high organic content of most mineral-salt solutions, even in distilled water, often hampers such characterization; as it did for Desulphovibrio (Rittenberg 1969).

It is notable that obligate autotrophy is confined to unicellular organisms and quite probably entirely a property of prokaryotes.

TABLE I
CLASSIFICATION OF PHYSIOLOGICAL TYPES.



Sources a of energy and carbon in lithotrophic bacteria

- Type 1 ; Obligate chemolithotrophs(some thiobacilli, possibly Nitrosomonas, Spirillum sp, Gallionella)
- Type 2; "facultative autotrophs " and mixotrophs (Hydrogenomonas, T.novellus, T.A2, Beggiatoa, N.agilis , T.ferro-oxidans, T.intermedius)
- Type 3; Chemolithotrophic heterotrophs (T.perometabolis, Desulfovibrio)
- Type 4 Pseudomonas oxaliticus Type 4a Obligate methylotrophs
- Type 5 None clearly shown Type 6 Obligate photolithotrophs (Chlorobium spp, Prosthecochloris , some Thiorhodaceae, Pelodictyon)
- Type 7 Many Thiorhodaceae, the Athiorhodaceae
- Type 7a Possibly some Athiorhodaceae on some substrates
- Type 8 Cyanophyta(blue green algae)
- Type 9. None clearly shown. Type 10 . None clearly shown.

AFTER KELLY 1971a. Terms as defined by RITTENBERG 1969.

1.3. EVOLUTIONARY SIGNIFICANCE OF OBLIGATE AUTOTROPHS.

The earlier view of Breed et al. (1957) that photosynthetic and chemosynthetic organisms represented a primitive form was based on the idea that their independence of preformed organic media, i.e. their autotrophy, enabled them to live in the totally inorganic *primaeval* environment. Thus the obligate autotrophs, unable to use preformed organic matter, would have been the most primitive; not yet having gained the ability to utilise the organic compounds synthesized by other organisms.

This view has given place to that of Haldane and Oparin (Oparin 1968) who suggest that "life" originated in a "*primaeval*" soup of organic compounds formed by various energy sources from an atmosphere considerably more reduced (as against oxidized) than the contemporary atmosphere. The succession of metabolic forms that follows from this is almost the reverse of that suggested by Breed et al. (1957).

The chemosynthetic organisms would be regarded as highly evolved, being both oxidative and independent of the presence of organic compounds, in some cases. The loss of the primitive feature of heterotrophy would suggest that the "obligate autotrophs" have become even more specialised.

1.4. MECHANISMS FOR OBLIGATE AUTOTROPHY.

The basis for obligate autotrophy must lie in some specific difference between obligate autotrophs, on the one hand, and facultative autotrophs and heterotrophs, on the other.

The explanations, beside explaining the inability to utilise organic compounds must also explain the general but not total sensitivity to organic compounds.

An early suggestion that obligate autotrophs were a totally different life-form seems to have been based on the claims of Winogradsky concerning the general toxicity of organic compounds to obligate autotrophs (Winogradsky 1890, 1922). The discovery of T. novellus by Starkey (1934), a facultative autotroph in an otherwise obligately autotrophic genus, rendered this view very unlikely. An analysis by Umbreit (le Page and Umbreit 1943) indicating the presence of 3'-ATP rather than 5'-ATP temporarily revived this idea. Subsequent reinvestigation of this finding has failed to substantiate the result.

A number of different hypotheses attempting to explain obligate autotrophy in terms of specific differences in the physiology and biochemistry of these organisms has been put forward over the last fifteen years. The explanation may well only be valid in the presence of the organic compound and/or the absence of the energy source.

1.41. RESTRICTED PERMEABILITY TO ORGANIC COMPOUNDS.

Umbreit (1951) suggested, in relation to the extreme acid tolerance of T. thio-oxidans, that the cell membrane was impermeable, even to the small hydrogen ion (which is variously hydrated in aqueous solution). This was soon discounted but the basic idea of the obligate autotroph as a biological submarine (Umbreit 1951) was revived by Dugan and Lundgren (quoted by Rittenberg 1969) to explain the inability of T. ferroxidans to utilise organic compounds. The inhibitory properties of certain organic compounds, it was suggested, arose from their effect on the membrane, which was such as to decrease the permeability to ferrous ions and hence inhibited growth.

There is however a wealth of data to indicate that organic compounds can penetrate cell membranes of obligate autotrophs. Assimilation of organic compounds is amply documented by Rittenberg (1969).

It is, however, possible that certain compounds inhibit the uptake of the autotrophic energy source in chemolithotrophs or the inorganic reductant in photolithotrophs. It has been suggested that the metabolism of the inorganic energy source in some chemolithotrophs, at least in the initial stages, takes place at the membrane surface (Trudinger 1965). If this is the case then although permeability changes may not account for the inhibition, membrane changes affecting this surface step may.

Even if the organic compounds are taken up, they must be taken up in sufficient quantities and at sufficient rates to act as energy source for growth. The rate of energy production for detectable growth must exceed the maintenance energy requirement. This maintenance energy requirement is often seriously underestimated and can vary with substrate and nature of the growth-limiting components (Stouthammer and Bethenhausen 1973). Thus permeases may be so rate-limiting as to prevent growth.

Pelroy et al. (1972) concluded that the absence of a glucose permease is responsible for the low glucose metabolism rates of the two obligately autotrophic blue-green algae, Synechococcus 6301 and Aphanocapsa 6308, in

comparison to the high rate in the facultative photo-autotroph Aphanocapsa 6714, which is freely permeable to glucose.

1.4.2. METABOLIC DEFICIENCIES.

Assuming the organic compounds can penetrate the cell membrane they must be metabolised to be effective.

The organic compounds that are utilised as energy sources by heterotrophs are those which can be readily transformed into intermediates of "central metabolic pathways" (i.e. those pathways such as the EMP, Hexosemonophosphate, Entner-Doudoroff and TCA cycles which serve to provide the cellular requirement for ATP and reducing equivalents such as NADPH_2) or are intermediates already.

For those compounds that require one or more enzymic steps for conversion to intermediates of central metabolism, the presence or absence of the specific converting enzymes determines the ability to utilise these compounds. This, in many cases, is sufficient to explain the range of utilisable substrates available to various heterotrophs. The absence of these enzymes in obligate autotrophs would not be a unique feature.

Since the central metabolism pathways provide all the energy generating mechanisms for heterotrophic growth, either by substrate-level phosphorylation or by providing NADH_2 for oxidative phosphorylation, a biochemical "lesion" in these pathways such as that suggested by Smith, London and Stanier (1967) for α -keto-glutarate dehydrogenase or NADH_2 -oxidase could, if appropriately located, prevent energy generation from organic sources.

A number of investigations of label distributions and enzymes occurrences has, with some contradictions, shown that if there are lesions, there are no common lesions to explain all situations of obligate autotrophy.

The first lesion to be detected was in the tricarboxylic acid cycle at α -keto glutarate dehydrogenase which would, it was suggested, convert a cyclic degradative pathway producing CO_2 , and ATP, to a solely biosynthetic system.

A survey of published evidence from enzymic studies (Table II) supported by labelling patterns, does indeed confirm the low level or absence of α -ketoglutarate dehydrogenase in obligate autotrophs and facultative autotrophs growing autotrophically. The occurrence of exceptions amongst the obligate autotrophs indicates that this is not a universal feature, as shown in Table II.

TABLE II
OCCURRENCE OF α -KETOGLUTARATE DEHYDROGENASE IN
OBLIGATE AND FACULTATIVE AUTOTROPHS.

	Obligate Autotrophs	Facultative Autotrophs
Lacking α -ketoglutarate dehydrogenase	Type I Methylo t rophs (1)	
	<u>Chromatium</u> (3)	
	<u>Nitrosomonas</u> (3)	
	<u>T.neapolitanus</u> (3)	
	<u>Anacystis nidulans</u> (2)	
	<u>Cocco-chloris</u>	
	<u>paviocystis</u> (2)	
Containing α -ketoglutarate dehydrogenase	Type II Methylo t rophs (5)	<u>Nitrobacter a gilis</u> (3)
		<u>Hydrogenomonas a gilis</u> (3)
		<u>Hydrogenomonas eutropha</u> (2)
	<u>Gleocapsa alpicola</u> (2)	
	<u>T.thio-oxidans</u> (2)	<u>T.novellus</u> (6)
	<u>T.thioparus</u> (2)	<u>T.intermedius</u> (2) (7)
	<u>T.denitrificans</u> (4)	<u>T. A2</u> (4)
		<u>T.ferro-oxidans</u> (8)

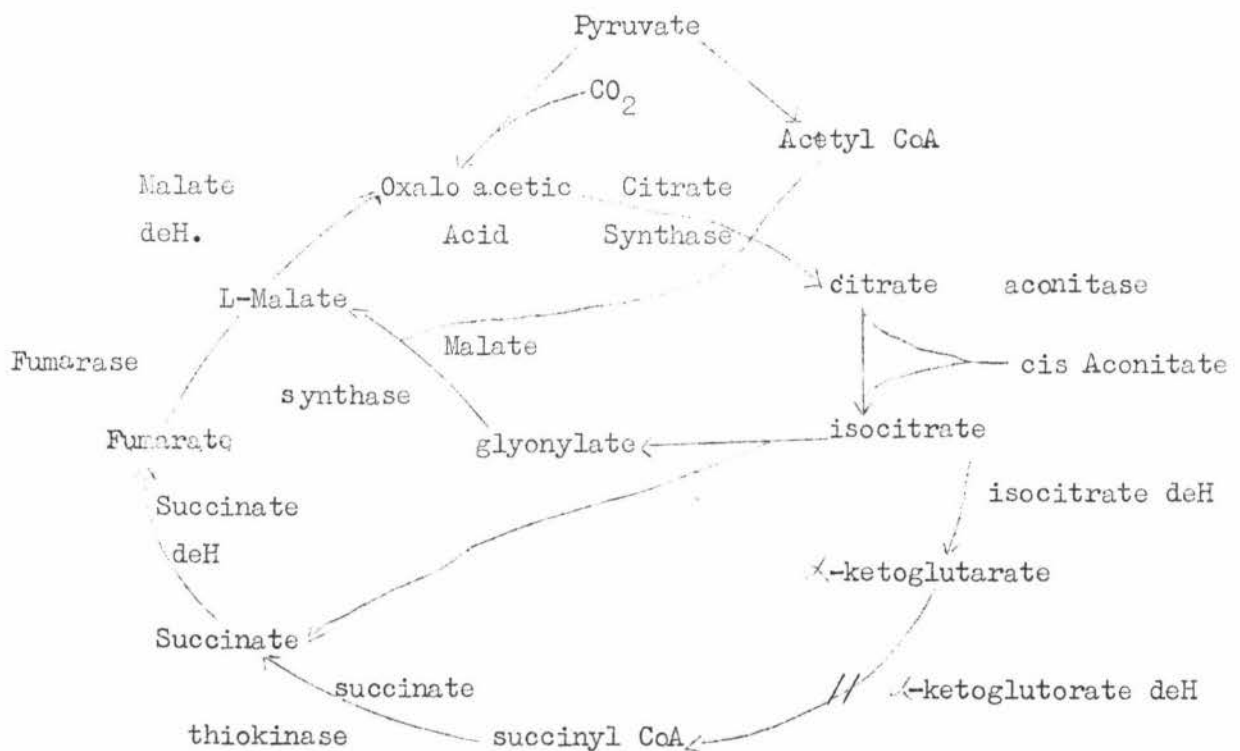
References

- (1) Davey, Whithenberg and Wilkinson 1972
- (2) Smith, London and Stanier 1967
- (3) Kelly 1971a
- (4) Taylor and Moore 1971a
- (5) Wadzinski and Ribbons 1972
- (6) Charles 1971a
- (7) Matin and Rittenberg 1970b
- (8) Tabita and Lundgren 1971b.

This lesion could be circumvented by the operation of the glyoxylate cycle as shown in Table III.

Such an effectively cyclic system could then enable the organism to oxidise the products of catabolism such as pyruvate and so generate ATP. In order to prevent the operation of both the TCA cycle and the Glyoxylate cycle from functioning other lesions, in addition to that at α -ketoglutarate dehydrogenase, must exist. A single lesion at isocitrate dehydrogenase, α -ketoglutarate dehydrogenase, succinate thiokinase, succinate dehydrogenase or fumarase disables the TCA cycle but still allows it to function biosynthetically. The simultaneous absence of the glyoxylate enzymes, malate synthase and isocitratase, will disable both cycles from separate lesions.

TABLE III
COMBINED TRICARBOXYLIC ACID CYCLE AND GLYOXYLATE CYCLE.



// = Site of deficiency

Type II Methylo-trophs (Methylococcus trichosporium, Methylosinus sporium, Methylocystis parris (Davey, Whittenberg and Wilkinson 1972) lack glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the oxidative pentose-phosphate cycle, although the TCA cycle is complete.

6-phosphogluconate dehydrase is low or absent in T. neapolitanus, T. thio-oxidans, and T. thioparus, all obligate autotrophs, as well as in T. novellus but present in T. perometabolis (Matin and Rittenberg 1971a). In T. ferro-oxidans this and other Entner-Doudoroff pathway enzymes are present (Anderson and Lundgren 1969; Gale and Beck 1967; Tabita and Lundgren 1971b).

The absence of phosphofructokinase in T. neapolitanus and T. thioparus would prevent the operation of the Embden-Meyerhof-Parnas pathway (Johnson and Abraham 1969).

Even in the absence of a clearly evident lesion there may be no utilisation of the substrate: the blue-green algae Anabaena variabilis, Anacystis nidulans and Chlorogloea fritshii are enzymatically fully capable of assimilating and converting acetate to necessary cell components and yet are obligate autotrophs.

Thus although there are many reported instances of specific metabolic deficiencies or lesions of central metabolism among obligate autotrophs, there are few cases where it has been shown that the lesion(s) are such that they cannot be circumvented by some alternative pathway. In the case of lesions that would prevent ATP synthesis via oxidative phosphorylation, substrate level phosphorylation could provide an adequate substitute. However, in this case, toxic or repressive end products might accumulate, e.g. pyruvate, in the absence of functional terminal pathways such as the TCA cycle. However, continued ATP synthesis by substrate level phosphorylation would require some mechanism of re-oxidation of NADH_2 . The absence of fermentative enzymes such as lactate dehydrogenase in obligate autotrophs would mean a reliance on mechanisms coupling NADH_2 re-oxidation to an external electron acceptor such as oxygen.

Smith, London and Stanier (1967) on finding an absence of NADH_2 oxidase in obligate autotrophs, concluded that an inability to utilize organic compounds as an energy source arose from an inability to utilize NADH_2 as an electron donor for oxidative phosphorylation. The occurrence of

NADH₂-oxidase in most obligate autotrophs has since been established (Davey, Whittenburg and Wilkinson 1972; Kelly 1971a; Pearce and Carr 1967; Smith, London and Stanier 1967; Matin and Rittenberg 1971a) but only in a few cases has the enzyme's ability to catalyse reduction of cytochromes concurrently been investigated and shown; nor has the coupling of NADH₂-oxidation to ADP phosphorylation been shown. This is, no doubt, partly due to the fact that in vitro bacterial electron transport systems give low P/O ratios. However the NADH₂-oxidation coupled production of ATP has been shown in the blue-green algal obligate autotroph Anabaena variabilis (Leach and Carr 1970).

1.43. DISTINCTIVE CONTROL MECHANISMS.

As the existence of metabolic lesions is generally an insufficient explanation, it has been suggested that the autotrophic levels of central metabolism enzymes are fixed i.e. permanently repressed. A comparison of the enzyme levels in facultative autotrophs (as in Table V) under autotrophic and heterotrophic, conditions, indicates a change in enzyme levels such that the energy-yielding steps are favoured and the "carbon distribution" is to all metabolic areas.

TABLE V
COMPARISON OF LEVELS OF CERTAIN CENTRAL METABOLISM
ENZYMES IN A FACULTATIVE AUTOTROPH ON VARIOUS SUBSTRATES.

	3.0% + CO ₂	Acetate	Pyruvate	Succinate	Glucose
Citrate Synthase	8.5	62	46	58.9	24
Aconitase	143	367	131	128	99
Isocitrate deH	451	689	141	452	43.4
α-ketoglutarate	0.5	3.5	1.8	2.4	1.1
Succinate deH	10.6	136	82	84	5.2
Fumarase	55	169	124	114	128
Malic deH	124	224	121	127	69
Isocitrate lyase	97	460	166	115	214
Malate Synthase	13	177	49	58	33.7
NADH-Oxidase	25	67.5	72	7.1	45

From Charles 1971a

n moles/min/mg protein

If such a shift is not possible, in obligate autotrophs, then the organism will be unable to adapt its metabolism to allow it to utilise organic compounds at a rate sufficient to sustain growth. It is noteworthy in this context that the differential rate of incorporation of organic compounds by obligate autotrophs does not increase with time during autotrophic growth, which suggests that there is no induction or derepression of enzymes to favour such incorporation (Kelly 1971a).

This permanent repression was first suggested by Pearce and Carr (1967) for Anabaena variabilis as shown in Table VI.

TABLE VI
EFFECT OF ACETATE ON CENTRAL METABOLISM ENZYMES
LEVELS IN OBLIGATELY AUTOTROPHIC BLUE-GREEN ALGAE

Photosynthetically grown in presence 20m Acetate absence "M"	<u>Anabaena</u> <u>variabilis</u>		<u>Anacystis</u> <u>nidulans</u>	
	"M"		"M"	
Acetate kinase	4.5	4.1	0	0
Acetyl synthetase	0	0	3.0	3.1
Phosphotransacetylase	1.7	1.4	nt	nt
Isocitrate lyase	.38	.39	.45	.43
Malate synthase	.82	.80	1.07	1.00
Isocitrate deHydrogenase	5.0	4.7	116.7	16.5
Citrate synthase	6.1	5.7	.67	.70

From Pearce and Carr 1967 n moles/min/mg protein

A comparison of enzyme's levels in Thiobacillus neapolitanus and T. thio-oxidans in the presence and absence of glucose (Table VII) shows that there is a small but possibly significant increase in isocitrate dehydrogenase and glucokinase for T. thio-oxidans and in glucokinase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and fructose di-phosphate aldolase for T. neapolitanus.

It can be seen that the presence of an organic compound may, but need not, affect enzyme levels in the obligate autotroph; it yet remains to be shown that this can happen in a co-ordinate manner, so as to give the enzyme levels required to utilise the substrate at an adequate rate.

TABLE VII
EFFECT OF GLUCOSE ON CENTRAL METABOLISM ENZYME
LEVELS IN OBLIGATE AUTOTROPHIC THIOPACILLI

	<u>T. neapolitanus</u>		<u>T. thio-oxidans</u>	
	190 S ₂ O ₃ "	190 S ₂ O ₃ " + 0.8% Glucose	190 S ₂ O ₃ "	190 S ₂ O ₃ " + 0.8% Glucose
Isocitrate deH	1672	1605	1167	1330
NADH Oxidase	59	65	42	40
Glucokinase	490	590	40	40
Glucose-6-P deH	110	140	68	162
phosphogluconate de- hydrogenase	2	2	2	2
phosphogluconate deH	74	97	42	44
Fructose-di Paldolase	16	20		
Phosphoglycerate deH	1320	1380	13	14

From Marin and Rittenberg 1971a.

10^{-4} enzyme units/mg protein

In an alternative theory to "permanent repression", Borichewski and Umbreit (1966a) suggested that organic compounds were toxic because their metabolic products repressed essential central metabolism enzymes; and that such products formed during autotrophic growth could limit autotrophic growth. If this was the case then removal of the toxic products, which might accumulate in the absence of a specific disposal mechanism, might well permit heterotrophic growth. In attempts to achieve this, organisms have been grown in dialysis sacs with continuous flow removal of dialysing fluid. Such results as have been reported have been contradictory: as can be seen from Table VIII.

TABLE VIII
GROWTH OF OBLIGATE AUTOTROPHS ON ORGANIC SUBSTRATES
UNDER DIALYSIS CONDITIONS

Organism	Substrate	Result	Reference
<u>T. thio-oxidans</u>	Glucose	- ve (60 days)	Levin 1971
	"	+ ve	Borichowski & Umbreit 1969a.
	Acetate	- ve (60 days)	Levin 1971
<u>T. neapolitanus</u>	Glucose	+ ve	Pan & Umbreit 1972a
	"	- ve (60 days)	Matin & Rittenberg 1971a
<u>T. denitrificans</u>	Glucose (aerobic)	+ ve	Pan & Umbreit 1972a
	" (an ")	+ ve	"
<u>T. thioparus</u>	"	+ ve	"
<u>Nitrobacter agilis</u>	Glucose	+ ve	Pan 1971a
	"	+ ve	Pan & Umbreit 1972a
<u>Nitrosomonas europaea</u>	Glucose	+ ve	"
<u>Methanomonas methano-oxidans</u>	Glucose	- ve (3 days)	Ameniya 1972

The results of Borichewski (1966b) show an increase in growth rate in a dialysing system that contained an "inhibitory" concentration of pyruvate.

The results must be confirmed and contradictions resolved before the theory of Borichewski and Umbreit can be accepted as an explanation. The changes in enzyme levels in the presence of an organic substrate or the absence of the lithotrophic energy source may be such as to permit accumulation of toxic products: the inhibitory compounds removed by dialysis from autotrophic growth commonly appear to be keto-acids, especially pyruvate.

It has been suggested that a situation resembling catabolite repression might occur: compounds capable of producing or of affecting the production of a general metabolic effector, such as cyclic AMP might so effect metabolism, via this effector as to prevent or inhibit growth. Such a situation may well explain the inhibition of autotrophic growth of

T. novellus by glucose, lactate, glycerol, lactose, ribose and pyruvate (Le John, van Caesele and Lees 1967).

There need not be a single general effector through which many inhibitors operate. It is possible that in the obligate autotrophs the presence of an organic compound represses the formation or activity of enzymes not, as in heterotrophs, only in metabolically-related pathways but also in distant metabolic areas. Thus there may be superimposed on the normal compact heterotrophic control patterns, a wide "super-control" pattern in which the presence of, for example, pyruvate, might repress enzymes involved in the biosynthesis of various amino acids. This control could be direct, not through the intermediary of such an effector as cyclic AMP.

In the case of many inhibitory compounds it is not necessary to invoke special mechanisms. Thus the "building block" organic compounds such as amino-acids and nucleotides which are end-products of long branched biosynthetic pathways, may be inhibitory because they exert feed-back repression or inhibition on early steps in biosynthetic pathways and so block the synthesis of other amino-acids derived from the same pathway (Kelly 1969b,c,d). Similar inhibitory effects are not uncommon among heterotrophs.

1.44. OBLIGATE LINKS BETWEEN PATHWAYS OF OXIDATION OF INORGANIC ENERGY SOURCE AND OTHER AREAS OF METABOLISM.

Some type of obligately link may occur between autotrophic functions such as to the energy-generating mechanism or the CO₂ fixation or methane fixation pathway, and some other essential facet of metabolism.

The uptake of organic compounds is an energy-requiring step and hence could be linked to the energy-generating mechanism. If this link involved, not ATP, but some intermediate of the substrate oxidation pathway, e.g. APS (Adenosine-5'-phospho-sulphate) then the uptake requires autotrophic metabolism. In support of this suggestion it has been shown that in T. denitrifications, acetate uptake requires both an oxidizable inorganic substrate and bicarbonate and was proportional to the inorganic substrate oxidized (Taylor and Hoare 1971a).

The formation of some essential compounds may depend on autotrophic functions. Sulphur oxidizing phototrophs such as Chlorobium, Chromatium, Thiopedia require sulphide as a sulphur-source as sulphate is not

assimilated (Kelly 1971a); T. neapolitanus also cannot assimilate sulphate (Kelly 1971a). Nitrosomonas could require ammonium ion as a nitrogen source, as well as an energy-source; Baalsrud and Baalsrud (1965) found NH_4^+ necessary for isolation of T. denitrificans.

The formation of vitamins etc. may be linked to autotrophy. Certain hydrogen bacteria (Rittenberg 1969) and some Athiorhodaceae require vitamins for autotrophic growth; the reverse may well be true. In liquid culture the growth rate of T. novellus on liquid media is enhanced by low concentrations of yeast extract (Matin and Rittenberg 1971a).

Poly- β -hydroxybutyrate can act as an energy-source during cell division and normal turnover in resting cells, so there is no obligate link between autotrophy and the functions that result in growth and division. However even if this is true some autotrophically-linked function may be rate limiting; heterotrophic growth of Nitrobacter agilis on acetate is very, very slow (Pan and Umbreit 1972a).

The over-rigorous exclusion of carbon-dioxide in experiments designed to test for photo- or chemo- organotrophy and also in experiments designed to test for heterotrophy may be responsible for the inability to grow: many many heterotrophs require small amounts of CO_2 to start growth and some require CO_2 for continued growth (Kelly 1967a).

The absence of the autotrophic energy source or the presence of some inhibitory organic compound may alter the permeability of the membrane or even induce active secretion, so that an essential metabolite is actively or passively secreted. The cell thus is metabolite-limited for continued growth. Borichewski (1965) obtained results to suggest that glucose gave abnormal morphologically-distorted T. thio-oxidans which were stabilized by 1-5% sucrose so that at least some substrates give osmotically fragile cells: the consequent leakage may well be growth-limiting. Pyruvate and oxaloacetate accumulate in cultures of T. thioparus on sulphur - so this leakage could be a normal end effect in growth.

1.45. SUMMARY.

It is possible that there is no single mechanism which accounts for the general inability of obligate autotrophs to utilise organic compounds, and for the toxicity of many organic compounds towards these organisms. Different mechanisms may be involved for different organic compounds and the

effect of the organic compound may depend on the presence or absence of the autotrophic energy source. However the possible mechanisms can be summarised as follows.

The compound, unless it affects membrane-associated autotrophic functions, must penetrate the cell membrane to be of effect either as a substrate or as an inhibitor (in most cases, at least). It must, if a substrate, penetrate at a sufficient rate so that subsequent metabolism, if it occurs, produces sufficient energy and reducing power, at a rate exceeding the maintenance energy requirement. Even if the enzymes exist to perform energy-yielding steps, the levels of these enzymes may be insufficient and remain so, or products toxic to the organism may accumulate.

The compound, which may not be utilisable by available enzymes, may affect control patterns so as to be inhibitory by mechanisms; analogous to catabolite repression, by "super control" or by normal heterotrophic control patterns of feed-back inhibition and repression in biosynthetic pathways. An obligate link between autotrophic functions and some essential metabolic facet may exist.

It is possible that some combination of lesions, inhibitions and links may mean that a single compound cannot fulfil all the required biosynthetic and energy-yielding functions so some combination of compounds is necessary.

To test the theories a comparative study of a closely related obligate and facultative autotroph would be of maximum benefit as physiological differences other than those responsible for obligate autotrophy would be minimised. The thiobacilli contain such types and representative species of each type were chosen for this study.

1.5. THE THIOBACILLI.

The currently accepted definition of the genus Thiobacillus is; "those bacteria capable of obtaining metabolically useful energy from the oxidation of reduced sulphur compounds".

As a genus containing both obligate and facultative autotrophs, obtainable in reasonable yields, the thiobacilli were a natural choice for study of obligate autotrophy.

1.51. CLASSIFICATION OF THE THIOBACILLI.

The classification of the thiobacilli of Breed et al. (1957) was based on characteristics, such as the formation of tetrathionate during thiosulphate oxidation (no longer regarded as definitive), and included only T. novellus and T. trautweinii as facultative autotrophs. Since then further facultative autotrophs have been isolated, while T. trautweinii is now regarded as a pseudomonad since it is non-autotrophic.

The major criteria for subdivision of the obligately autotrophic species are: the ability to carry out anaerobic respiration and the pH optima and limits.

The species commonly accepted are shown in the Table IX.

TABLE IX
CLASSIFICATION OF THE GENUS THIOBACILLUS

	pH optimum	
Obligately Autotrophs		
Facultative anaerobes		
<u>T. denitrificans</u>	~ pH 7.0	Nitrate Anaerobic Respiration
Obligately aerobes		
<u>T. thiparus</u>	Δ pH 7.0	Resistant to osmotic stress.
<u>T. neapolitanus</u>	Δ pH 6.0	
<u>T. thio-oxidans</u>	Δ pH 3.0	
Able to oxidize Fe		Some strains facultative autotrophs also utilizes reduced S compds.
<u>T. ferro-oxidans</u>	Δ pH 3.0	
Spore-former		
<u>T. thermophilica</u>		Obligately thermophile
Facultative Autotrophs		
<u>T. novellus</u>	Δ pH 8.0	Low Autotrophic ability
<u>T. perometabolis</u>		Cannot grow autotrophically *
<u>T. intermedius</u>	Δ pH3-pH4	Autotrophic. Chemoheterotroph
<u>T. A₂</u>		Autotrophic heterotrophic ability high.

* Can only grow mixotrophically or on two C. compounds.

From Jackson, Moriaty and Nicholas (1968)
 Hutchinson, Johnstone and White (1969)
 London and Rittenberg (1967)
 London (1963)

The DNA base composition of the Murray strains and some others was determined by Jackson et al. (1968) as shown in Table X.

TABLE X
CLASSIFICATION OF THE GENUS THIOPACILLUS BY
MULTIVARIATE ANALYSIS AND DNA COMPOSITION.

Species	Group No. *	S Range *	Mean *	DNA Comp. ** % C + C
<u>T.</u>				
<u>trautweinii</u>	0	94 - 63	71	66
<u>novellus</u>	1	49 - 46	47	66 - 68
<u>denitrificans</u>	2			64
<u>thioparus</u>	3	45 - 24	35	62 - 66
<u>thiocyano-oxidans</u>				63
<u>neapolitanus</u>	4	37 - 18	27	56
<u>ferro-oxidans</u>	6			57
<u>thio-oxidans</u>	5			51
<u>concretivorus</u>				51 - 52
<u>intermedius</u>	7			

* Hutchinson, Johnstone and White (1969)

** Jackson, Moriaty and Nicholas (1968)

A multivariate analysis by Hutchinson et al. (1969) did not confirm to strict Adansonian principles because it was not possible to find a set of common incubation conditions. This is due to the wide range of forms available in the thiobacilli; facultative and obligate autotrophs, aerobes and anaerobes, iron oxidizers, and obligate mixotrophs. However a series of co-ordinate groups was differentiated as shown in Table X.

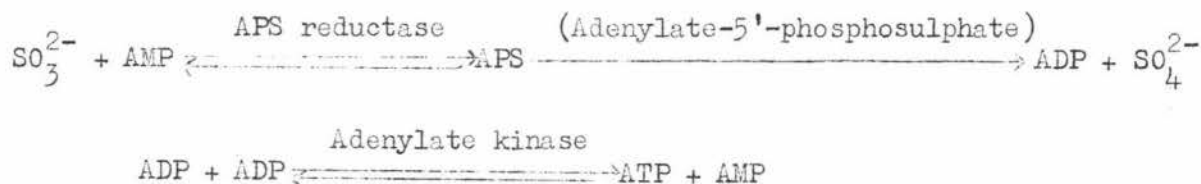
Organisms not included in this study are T. perometabolis and T. thermophilica, both somewhat unusual organisms.

The species can be differentiated by ultrastructure (Shively *et al* 1970) and by phospholipid composition (Barridge and Shively 1968): such criteria give a similar grouping to that shown in Table X.

1.52. ENERGY PRODUCTION IN THE THIOBACILLI.

The sequence of reactions and the intermediates involved in the oxidation of reduced sulphur compounds are not well characterized. This is, in part, due to the number of reactions and intermediates, the transient appearance of some intermediates and the chemical reactivity of certain intermediates, which can yield a variety of products by non-enzymatic reactions.

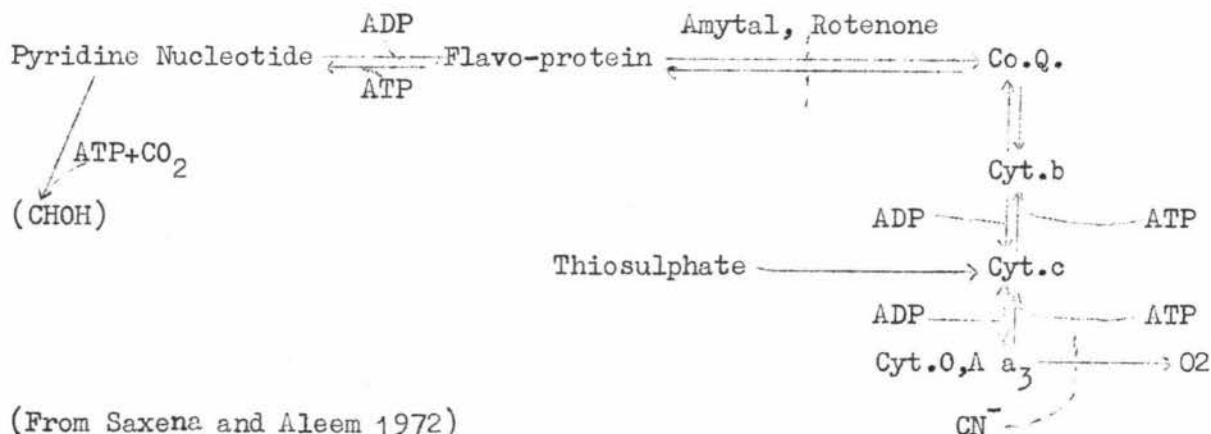
The only well characterized ATP-yielding step results from a substrate-level phosphorylation during oxidation of sulphite to sulphate



which contributes at least 45% of the total ATP produced by oxidation of thiosulphate.

The nature of other energy-yielding steps is uncertain.

The mechanism for the production of the reductant for CO_2 fixation is also uncertain. In chemo-organotrophs (*sensu* Rittenberg 1969) the organic compounds can generate reducing power; in the obligate autotrophs this is impossible. It has been suggested that an ATP driven reversal of oxidative phosphorylation might occur; although this is not universally accepted (Sadler and Johnson 1972).



(From Saxena and Aleem 1972)

Such a pathway could provide a means by which some organic compounds could influence the oxidation of thiosulphate and the production of ATP and NADH_2 .

1.6. AIMS AND EXPERIMENTAL APPROACH USED IN THE PRESENT STUDY.

The aim of the present study was to investigate the effect of a range of organic compounds on the growth of selected species of chemolithotrophic bacteria and also to study the metabolism of selected organic compounds. It was hoped that such a study would contribute to an understanding of the distinctive features of obligate autotrophs, namely their inability to utilise organic compounds as an energy-source or as a carbon-source, and their sensitivity to inhibition by a wide variety of organic compounds. In order to reveal those characteristics which are specifically related to the nature of obligate autotrophy it was proposed to compare an obligate autotroph with a closely related facultative autotroph. As mentioned earlier the genus Thiobacillus is an obvious choice for such a comparative study, since it contains apparently closely-related facultative and obligate autotrophs.

Facultative species of thiobacilli that have been clearly recognized are T. novellus and T. intermedius. Both of these, by the available criteria, have related obligate autotrophs. One of these pairs was chosen, namely that of T. novellus and T. thioparus; the other pair was T. intermedius and T. thio-oxidans.

The extent of the "facultative" and "obligate" nature of each of these species was first re-examined since a literature search reveals that, particularly in the case of T. novellus, this has not been thoroughly investigated and there are some contradictions.

A thorough examination of the effect of various organic compounds as substrates, inhibitors and stimulators on solid media was used to establish the "obligate" and "facultative" features of each organism. The effect of certain selected compounds was then examined in liquid culture to quantitate these findings, and possibly provide, from the growth curve shape, some idea of the possible nature of any inhibitory effects.

At the same time a comparison of the distribution of ^{14}C -label from various labelled compounds in the two organisms, under similar conditions was undertaken in an attempt to reveal any metabolic differences.

The alterations induced in the labelling patterns, by heterotrophic growth for T. novellus and by growth in the presence of the inhibitor for T. thioparus, might give a clue as to the differences that result in obligate autotrophy.