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

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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 $^{14}CO_2$ 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 mileu 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 catagorized, 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 lead 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.

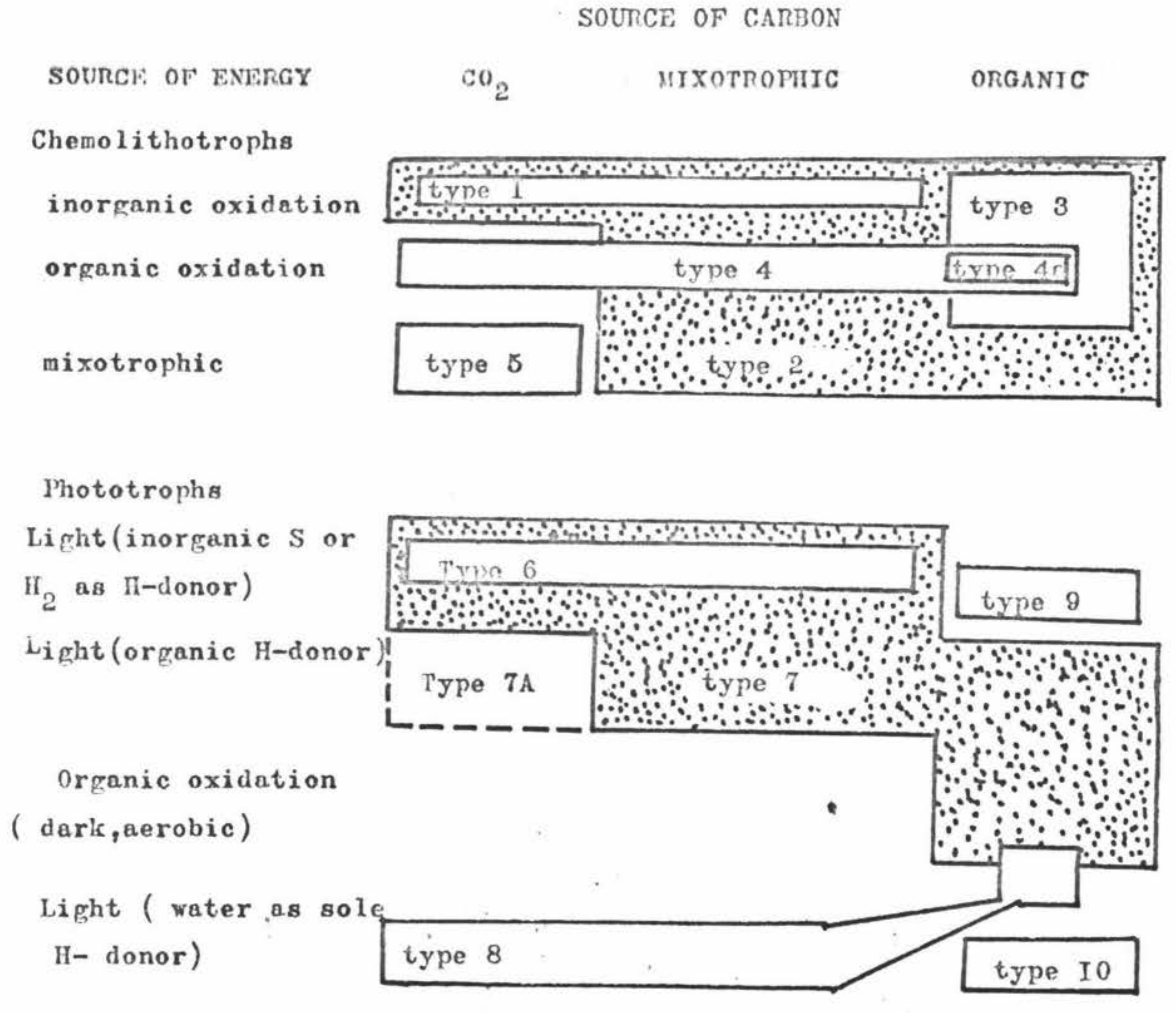
Types 1 and 7 are the obligate autotrophs - metabolically conservative - while types 2 and 7 are facultative autotrophs.

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 noteable 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 I ; 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)

Type7 Many Thiorhodaceae, the Athiorhodaceae

Type 7a Possibly some Athiorhodaceae on some substrates

Type 8 Cyanophyta(blue green algae)

Type 9. None clearly shown. Type I0 . None clearly shown.

AFTER KELLY I97Ia. Terms as defined by RITTENBERG I969.

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 or 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.

1.42. 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 dehydrogenose	Type I Methylotrophs	(1)		
	Chromatium	(3)		
	Nitrosomonas	(3)		
	T.neapolitanus	(3)		
	Anacystis nidulans	(2)		
	Cocco-chloris paviocystis	(2)		
Containing α-ketoglutarate dehydrogenose	Type II Methylotrophs	(5)	Nitrobacter agilis	(3)
			Hydrogenomonas agilis	(3)
			Hydrogenomonas eutropha	(2)
	Gleocapsa alpicola	(2)		
	T.thio-oxidans	(2)	T.novellus	(6)
	T.thioparus	(2)	T.intermedius	(2) (7)
	T.denitrificans	(4)	T. A2	(4)
			T.ferro-oxidans	(8)

References

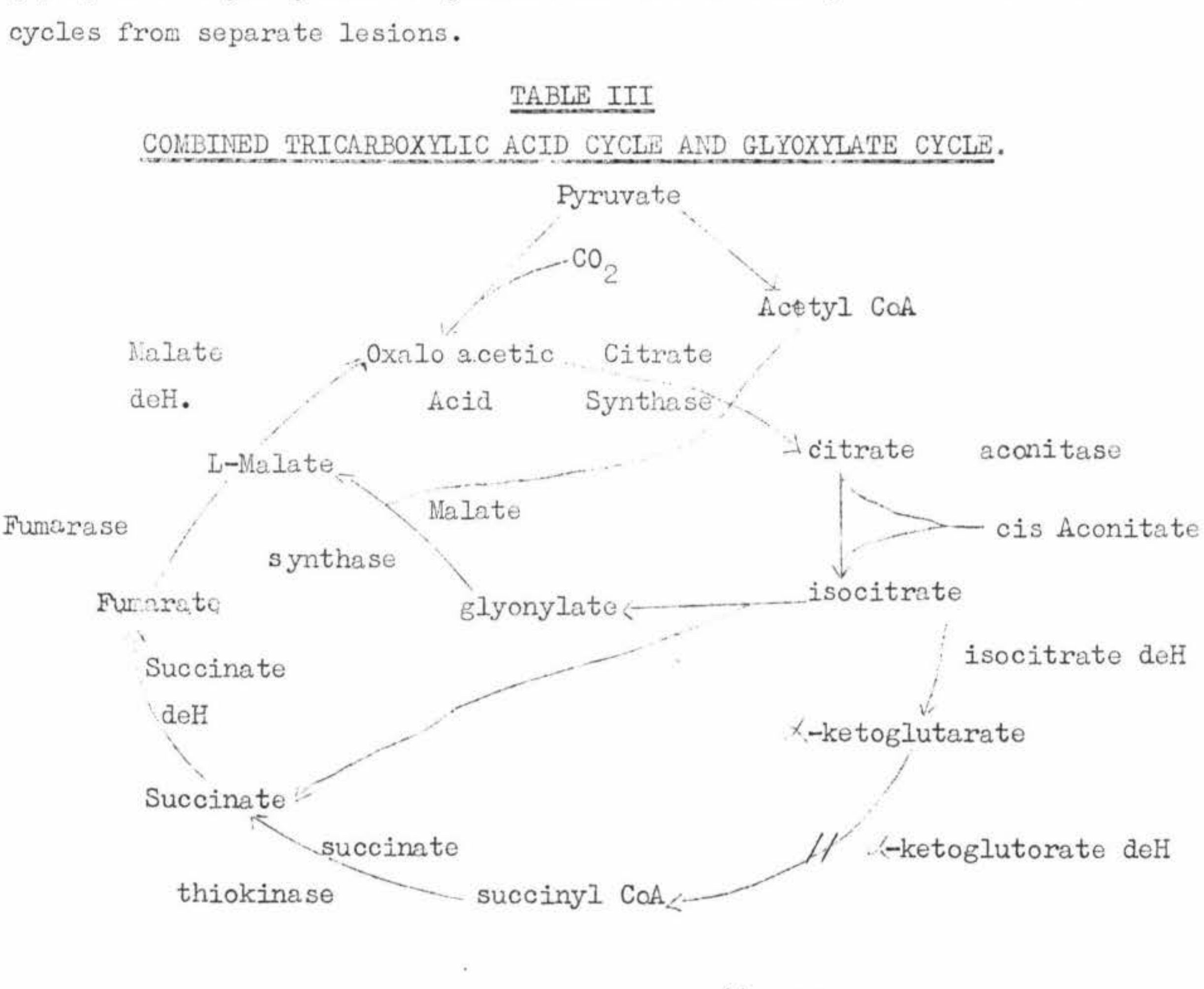
(1) Davey, Whithenberg and Wilkinson 1972
(2) Smith, London and Stanier 1967
(3) Kelly 1971a
(4) Taylor and Moore 1971a
(5) Wadzinski and Ribbons 1972
(6) Charles 1971a
(7) Matin and Rittenberg 1970b
(8) Tabita and Lundgren 1971b.

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

From the data of Table IV it would appear that only in *T. thioparus* and *Nitrobacter*, and possibly *T. neapolitanus* are there lesions such as to prevent the effective cycling of compounds to yield CO_2 and energy.

Other lesions exist so as to prevent the operation of various carbohydrate pathways. Thus the absence of hexokinase in *Methylococcus capsulatus* was considered to be a sufficient explanation of this organism's inability to metabolise glucose (Amemiya 1972).

TABLE IV

OCCURRENCE OF TCA AND GLYOXYLATE CYCLE LESIONS IN OBLIGATE AUTOTROPHS.

TCA Cycle enzyme absent. Any of.	Glyoxylate Cycle Enzymes	
	Malate Synthase or Isocitrase present.	Malate Synthase or Isocitrase absent.
Citrate Synthase Aconitase Malate deH.	*T.neapolitanus* (co) * *Nitrobacter* (co)	
α-ketoglutarate deH Isocitrate deH Succinate thiokinase Succinate deH Fumarase	*T.thio-oxidans* (co) *T.denitrificans* *Nitro-somonas* *Chromatium* **	*T.thioparus* *T.neapolitanus* *

From (Kelly 1971a) and (Cooper 1964)

(co) = Contradictory Reports.

Although there is no complete lack of isocitrate dehydrogenase some organisms may lack either the NAD or the NADP specific enzyme.

* = No cycling possible

** = Cycling possible.

Type II Methylotrophs (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 phospherylation, 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

NADH$_2$-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 NADH$_2$-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 NADH$_2$-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.

	S_2O_3'' + 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"	Anabaena variabilis		Anacystis nidulans	
		"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 THIOBACILLI

	T. neapolitanus		T. thio-oxidans	
	190 S_2O_3''	190 S_2O_3'' + 0.8% Glucose	190 S_2O_3''	190 S_2O_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 dehydrase	2	2	2	2
phosphogluconate deH	74	97	42	44
Fructose-di Paldolase	16	20		
Phosphoglycerate deH	1320	1380	13	14

From Matin 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
T.thio-oxidans	Glucose	- ve (60 days)	Levin 1971
	"	+ ve	Borichowski & Umbreit 1969a.
	Acetate	- ve (60 days)	Levin 1971
T. neapolitanus	Glucose	+ ve	Pan & Umbreit 1972a
	"	- ve (60 days)	Matin & Rittenberg 1971a
T. denitrificans	Glucose (aerobic)	+ ve	Pan & Umbreit 1972a
	" (an ")	+ ve	"
T. thioparus	"	+ ve	"
Nitrobacter agilis	Glucose	+ ve	Pan 1971a
	"	+ ve	Pan & Umbreit 1972a
Nitrosomonas europaea	Glucose	+ ve	"
Methanomonas methano-oxidans	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 Caeseele 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 CO_2 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. denitrications, 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	
Obligate Autotrophs		
Facultative anaerobes		
T. denitrificans	~pH 7.0	Nitrate Anaerobic Respiration
Obligate aerobes		
T. thiparus	~pH 7.0	
T. neapolitanus	~pH 6.0	Resistant to osmotic stress.
T. thio-oxidans	~pH 3.0	
Able to oxidize Fe		Some strains facultative autotrphs
T. ferro-oxidans	~pH 3.0	also utilizes reduced S compds.
Spore-former		
T. thermophilica		Obligate thermophile
Facultative Autotrophs		
T. novellus	~pH 8.0	Low Autotrophic ability
T. perometabolis		Cannot grow autotrophically *
T. intermedius	~pH3-pH4	Autotrophic. Chemoheterotroph
T. A_2		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 THIOBACILLUS BY MULTIVARIATE ANALYSIS AND DNA COMPOSITION.

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

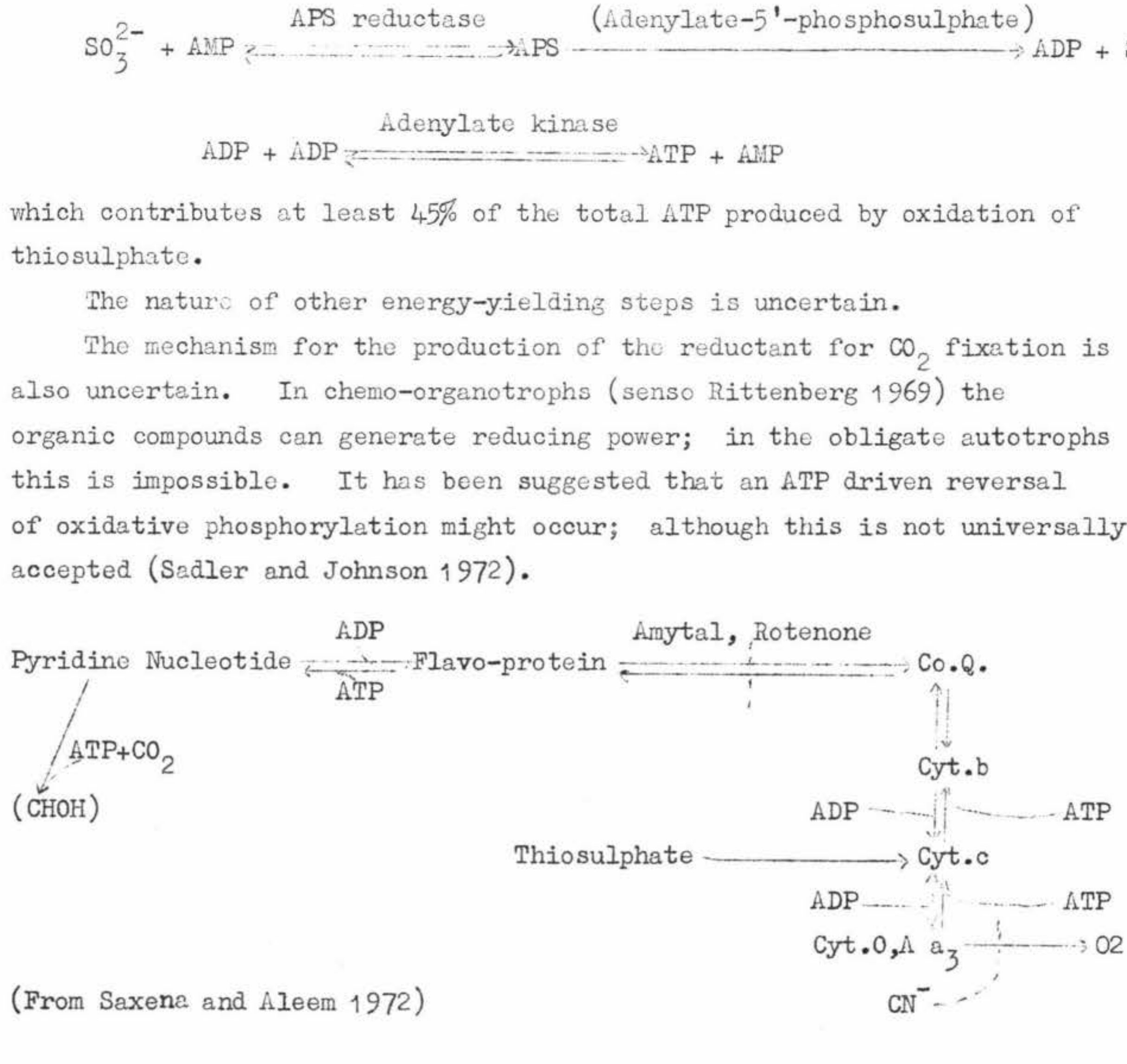
$$SO_3^{2-} + AMP \xrightleftharpoons{\text{APS reductase}} APS \xrightarrow{\text{(Adenylate-5'-phosphosulphate)}} ADP + SO_4^{2-}$$

$$ADP + ADP \xrightleftharpoons{\text{Adenylate kinase}} ATP + AMP$$

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 (senso 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.

2. PHYSIOLOGICAL CHARACTERISTICS OF THIOBACILLUS NOVELLUS AND OF T. THIOPARUS

2.1. INTRODUCTION.

The general characteristics of the selected pair of autotrophs, one facultative and one obligate, were first investigated. Besides confirming the identity of each culture a preliminary study of suitable standard media for the two species and their growth behaviour on these media was required for planning further experiments, on the growth and the metabolism of isotopically labelled compounds.

2.2. PHYSIOLOGICAL CHARACTERIZATION OF T. NOVELLUS.

2.21. SOURCE AND RECONSITUTION OF THE FREEZE-DRIED CULTURE.

A freeze-dried ampoule of *T. novellus* ATCC 8093 was opened and reconstituted as recommended by the source, the American Type Culture Collection (Rockville, Maryland) (ATCC Catalogue).

The reconstituted organism consisted of Gm -ve plump rods, 0.8 x 1.5µm. in large clumps held together by a ropey material. Growth was largely restricted to the surfaces on nutrient broth and agar. This culture was maintained on nutrient agar at 25°C, until adapted to autotrophy when the organism was maintained on thiosulphate agar and samples freeze-dried.

2.22. ADAPTATION TO AUTOTROPHY.

The reconstituted organism was unable to grow on Beijerinck's Thiosulphate Agar (Sokolova and Karavaiko 1964). It was therefore necessary to adapt the culture to autotrophy through a series of plates of increasing thiosulphate concentration and decreasing nutrient broth concentration until able to grow on Beijerinck's Thiosulphate Agar. This is the method used by Charles and Suzuki (1965). Some workers, e.g. Santer, Boyer and Santer (1959) also using Starkey's strain of *T. novellus* do not appear to have needed the adaptation sequence at all.

2.22.1. Media.

		Final Concentration.
(i)	Nutrient Broth.	
	Difco Nutrient Broth (Catalogue No..00301)	1.6% w/v
(ii)	Nutrient Agar	
	Difco Nutrient Broth	0.8% "
	Agar	1.5% "
(iii)	Beijerinck's Thiosulphate Agar (Modified)	
(a)	$MgCl_2$	0.1% "
	Na_2HPO_4	0.2% "
	$FeSO_4$	trace
	Trace Metals solution (Vishniac & Santer 1967)	1.03 v/v
	Agar	1.5% w/v
	Water	980 mls.
(b)	$Na_2S_2O_3.5H_2O$	.25% w/v
	Water	100 mls.
(c)	0.1M Potassium Phosphate Buffer pH 7.0	.05M
	Water	900 mls.

All solutions were autoclaved at 15 lbs / sq.in. (121 °C or $72 KN/M^2$) for fifteen minutes.

Beijerinck's Thiosulphate agar (iii) (a) + (b) + (c) were mixed aseptically and plates poured.

2.22.2. Training Agar Series.

The following protocol was set up and the plates poured.

No.	Sterile Nutrient Broth Mls(ii)	 Conc.% w/v	Sterile Thiosulphate Broth Mls(iii)(b)	 Conc.% w/v	Agar Base (iii)(a) mls.
1	10	.80	0	0	10
2	10	.80	0	0	10
3	9	.72	1	.25	10
4	8	.64	2	.50	10
5	7	.56	3	.75	10
6	6	.48	4	1.0	10
7	2	.16	8	2.0	10
8	1	.08	9	2.25	10
9	0	0	10	2.5	10

A heavy inoculum was streaked across the surface of the first plate in the series and the plate incubated at 30°C for fourteen days. Colonies were harvested into 2.0 mls. of sterile 0.2M potassium phosphate buffer pH8.0 and the heavy suspension used to inoculate the next plate in the series. At each transfer the ability to grow on nutrient agar and on Beijerinck's Thiosulphate agar was tested. Nutrient agar plates were incubated at 30°C for four days; all other plates for up to a month.

Training Agar Series No.	Ability of harvested colonies to grow on: Nutrient agar	 Beijerinck's Thiosulphate agar
1	+ve	-ve
2	+ve	-ve
3	+ve	-ve
4	+ve	+ve

As the fourth plate in the series gave an autotrophically capable culture the remaining plates in the series were not used.

The harvest from this first successful autotrophic plate was able to produce, on Beijerinck's Thiosulphate Broth at 25°C, a film of deposited sulphur; an uninoculated control remained clear.

The mechanism for such an adaptation is not clear. Some workers found it unnecessary. The selection of mutants was rendered unlikely by the mass harvesting technique used, and the appearance of the culture microscopically was unchanged.

2.23. DEVELOPMENT OF SATISFACTORY AUTOTROPHIC MEDIA.

Examination of the literature reveals that a wide variety of media have been used for growth of *T. novellus*. The effect of medium composition on growth was therefore investigated to find a satisfactory medium for the present study. The constituents to be investigated and their concentration ranges were selected after some consideration of the literature. Each major constituent was omitted and varied, independently, and the effect on growth determined.

2.23.1. Methods.

A modified Vishniac and Santer (1967) Medium was prepared;

(a)	NH_4Cl	8.0 gm.
	$MgSO_4 \cdot 7H_2O$	1.6 "
	$CaCl_2$	0.1 "
	NaCl	0.1 "
	Trace metals solution	20 mls.
	Water	800 "
(b)	$Na_2S_2O_3 \cdot 5H_2O$	40 gm.
	Water	200 mls.

(c) 0.1M potassium phosphate buffer pH 7.0

These solutions were autoclaved at 15lbs./ sq.in. (121 °C or $72KN/M^2$) for fifteen minutes and then mixed according to the following protocols, in sterile nephelometer flasks. These were innoculated with cold stored autotrophically-grown *T. novellus*. The flasks were incubated at 25°C and aerated with water-saturated air. Growth was followed turbidometrically in a Klett-Summerson colorimeter with a green filter (no.54) set to zero with a water blank.

2.23.2. Effect of Thiosulphate Concentration.

Media of varying thiosulphate concentration were prepared according to the following protocol.

Mineral base (a)	Thiosulphate (b)	Phosphate Buffer (c)	Thiosulphate Concentration
20 mls.	10 mls	0 mls	6.6%
20 "	8 "	2 "	5.3%
20 "	6 "	4 "	4.0%
20 "	4 "	6 "	2.7%
20 "	2 "	8 "	1.3%
20 "	0	10 "	0

Growth curves are shown in Figure 1.

There is a minor increase in turbidity in the medium of zero thiosulphate concentration. As this does not occur in the 6.6% thiosulphate medium it may represent a genuine growth rather than a non-biological increase in turbidity. There is no growth in 6.6% thiosulphate.

2.23.3. Effect of Omitting Other Constituents of the Basal Medium.

The individual components of the mineral base (a) were omitted in turn. The volume was brought to 20 mls. with water and 10 mls. of sterile 10% w/v Thiosulphate and 10 mls. of sterile .01M potassium phosphate buffer added to the sterile nephelometer flask.

Growth was followed turbidometrically; growth curves are shown in Figure 2.

The absence of $CaCl_2$ or NaCl was without effect. The absence of $MgSO_4$ or of trace metals solution reduced growth and increased the lag-phase. The absence of NH_4Cl gave almost normal growth in the early stages but the maximum yield was markedly reduced.

$CaCl_2$ and NaCl can apparently be omitted without detriment. NH_4Cl is necessary for continued growth. $MgSO_4$ and trace metals solution were also necessary for rapid growth and high yields.

High concentrations of ammonium chloride (greater than 1.0% w/v) and of magnesium sulphate (higher than 0.3% w/v) gave crystalline precipitates which obscured growth measurements.

2.23.4. Effect of Initial pH.

The initial pH was varied by using various buffers in the place of solution (c). A pH maximum of pH 9.4 and a minimum of pH 5.7 was found.

With buffers consisting of varying proportions of the two potassium phosphate salts, the optimum pH was pH 8.0; with tris-HCl buffers the optimum initial pH was also pH 8.0.

Figure I : KEY

Thiosulphate Concentration

○ I.3%   △ 0%
□ 2.0%
○ 5.3%
▲ 6.6%

Figure 1

EFFECT OF THIOSULPHATE CONCENTRATION ON GROWTH OF *T.NOVELLUS*

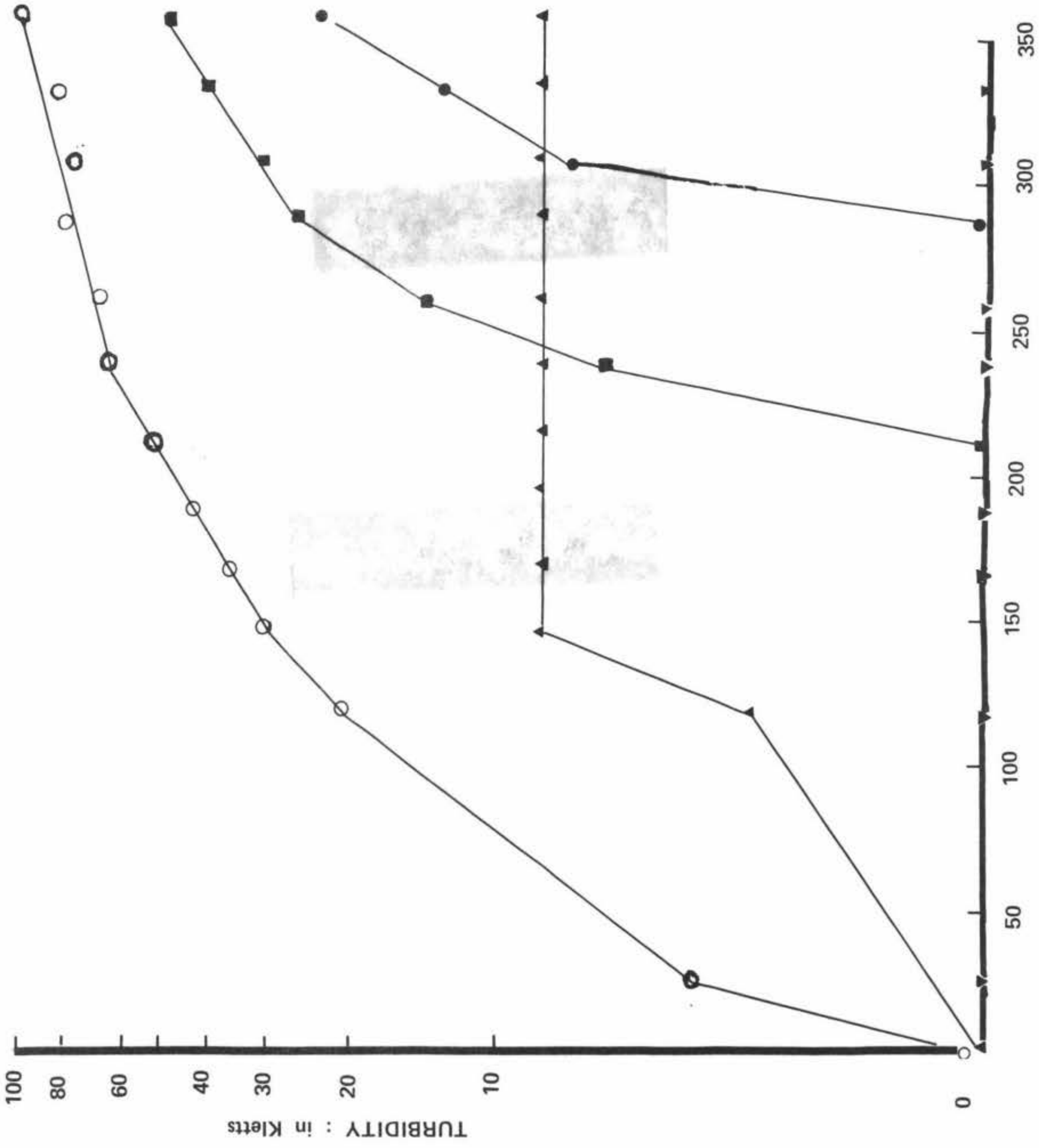


Figure 2 : KEY

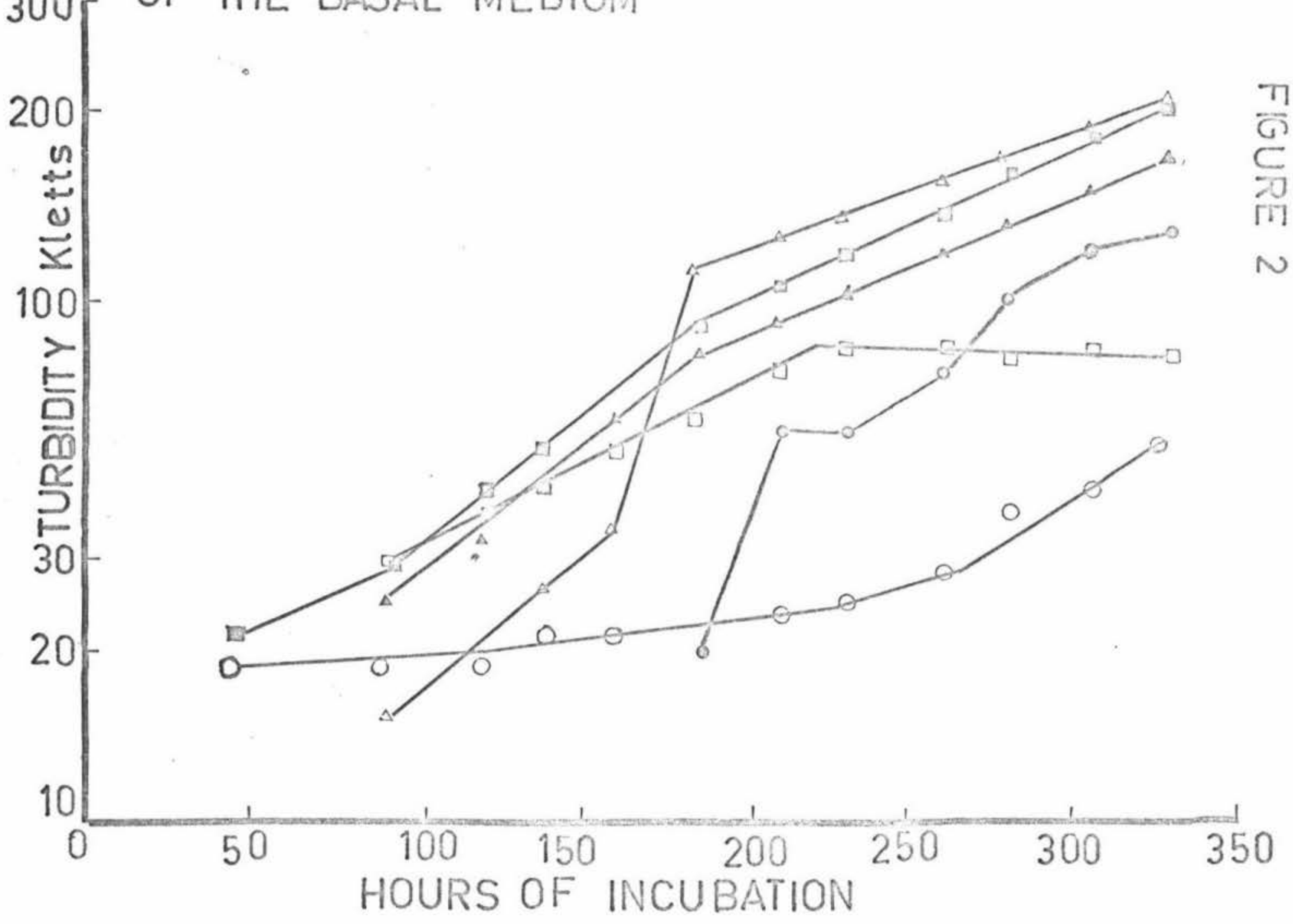
Component **Omitted**

▲ NaCl
○ Trace Metals
□ NH_4Cl
● $MgSO_4$
▨ $CaCl_2$

△ **None**

FIGURE 2

EFFECT OF OMITTING OTHER CONSTITUENTS
OF THE BASAL MEDIUM



2.23.5. Comparison with Other Thiosulphate Media.

Because of the very great variety of media reported in the literature and the rather cursory examination of optimum conditions in this survey, a comparative study of growth rates in various media was made. The media were those of Trudinger and Kelly (1968); Taylor and Hoare (1969a); Santer, Boyer and Santer (1959); Le John, van Caeseele and Lees (1967); modified Matin and Rittenberg (1970b) and the medium arrived at as a result of the experiments described in the preceeding sections.

The solutions were autoclaved at 15lbs/sq.in. (121 °C or 72KN/M^2) for fifteen minutes, then mixed aseptically in sterile nephelometer flasks. After inoculation with cold stored, autotrophically-grown T. novellus the flasks were incubated at 25°C and aerated with water-saturated air. Growth was followed turbidometrically. Uninoculated controls were also incubated simultaneously.

The medium of Trudinger and Kelly (1968) gave the crystalline precipitate and no increase in turbidity was evident after prolonged incubation. The best and only marked increases in turbidity were noted in the medium developed in this study and that of Santer, Boyer and Santer (1959). Little or no growth was evident on the remaining media.

2.23.6. Crystal Formation in Thiosulphate Agar.

When thiosulphate-containing media were used for the preparation of agar plates, long thin needle-like crystals developed and made spreading and streaking of cultures difficult. Various modifications were made in an attempt to eliminate these crystals.

The crystals did not react as thiosulphate and nor was their formation affected by either thiosulphate or agar concentration. Single component elimination showed that magnesium and ammonium chlorides and phosphate buffer were needed for crystal formation.

If the solutions were autoclaved separately and mixed while the agar was molten and then incubated for about an hour at 45°C crystal formation was largely prevented, although small needles might still appear in the track of the inoculating loop.

2.23.7. Medium Adopted for Subsequent Studies. ((modified Vishniac and Santer (1967))

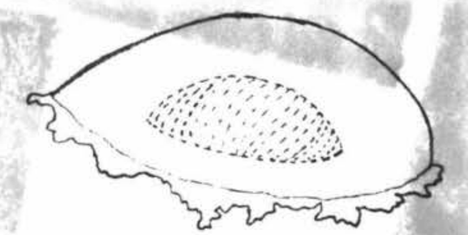
		Weight	Final Concentration
(a)	NH_4Cl	1.0 gm.	0.1% w/v
	$MgCl_2$	0.5 "	.05% "
	Trace metals solution	10 mls.	1.03 "
	Water	890 "	
	Agar	15	1.5% "
(b)	$Na_2S_2O_3.5H_2O$	15.0	1.5% "
	0.2M potassium phosphate buffer pH 8.0	20 mls.	4 mM
	Water	80 "	

These were autoclaved at 15lbs/sq.in. (121 °C or 72KN/M^2) for fifteen minutes then mixed, and for solid media, the molten mixture was incubated at 45°C for at least an hour before pouring.

2.24. EXISTENCE OF DISTINCT COLONY FORMS.

Immediately after the culture was adapted to autotrophy it became evident that diverse colony forms existed.

Two extremes can be distinguished; both are facultative autotrophs; a round glistening colony with smooth edges (Illustration a) and a rough [illegible] raised wrinkled form cohesive with irregular edges (Illustration b).



COLONY FORM A

COLONY FORM B

These forms, depending on the substrate may vary in colour. Plate A.

Various intermediate forms can intergrade between the two extremes but occur in much lower numbers. Plates B and C.

The proportions of the two forms, when these are evident, varies between substrates and individual plates.

Examination of gram-stains of the various forms showed no differences.

Continued single colony isolations failed to separate the two forms, although the proportions tended to favour the form from which the isolation was made. A liquid culture of any single colony form yielded all the forms on solid media.

It is possible that the distinct colony forms represent a colony arising from a single cell (round form) or from several cells (intermediate and rough forms).

Alternatively the colony forms could be physiological variants of the common culture with a high rate of formation and of reversion.

Santer, Boyer and Santer (1959) mention the occurrence, at high rates, of fast and slow strains; indistinguishable, on their media, other than by colony size, of T. novellus. Kocur et al. (1968) found two cell forms in electron photomicrographs.

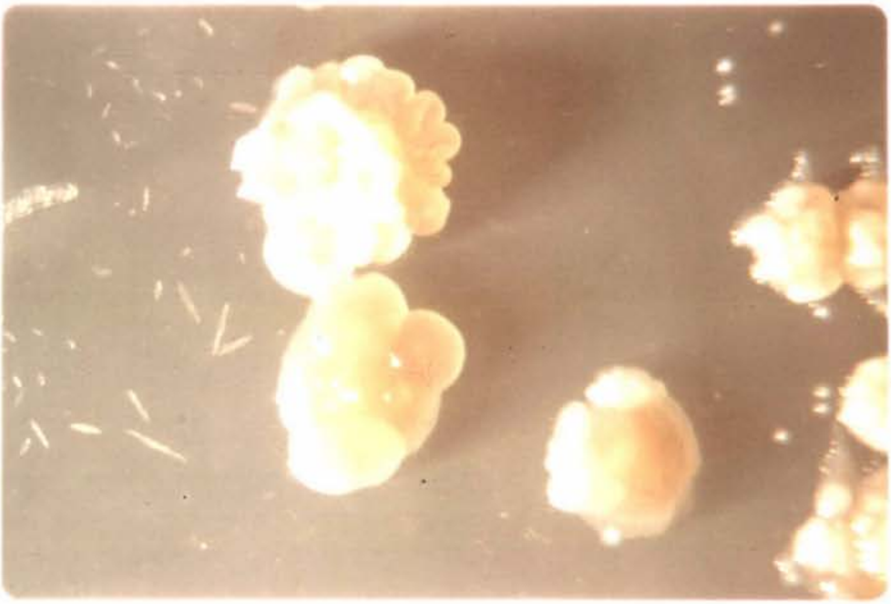
2.25. FACULTATIVE AUTOTROPHIC ABILITY.

The autogrophic ability of the culture was established by the distinctive appearance of the colonies and the production of acid on thiosulphate agar, the increase of turbidity relative to uninoculated controls in liquid culture, and the increase in counts as the thiosulphate disappears in liquid media.

The existence of distinctive colony forms and the necessity for adaptation of the culture to autotrophy made it necessary to establish that all cells were facultatively autotrophic. This had been investigated by Santer, Boyer and Santer (1959) who concluded that distinct physiological types were not selected for under the differing conditions of heterotrophic and autotrophic growth. The confirmation of individual facultative ability was done in two ways.

Plates A.

T. Novellus Colony forms
Differences in Colour.



X120: Glutamate Grown: background flooded with Methylene blue to improve contrast.

T. novellus Thiosulphate Grown.

X500: Smooth and Intermediate forms.

Plates B.

T. novellus Colony Forms

Glutamate - Grown

X150:

Rough Form. Side Lit Oblique-angle, Black background.

X100:

Smooth Form

X100:

Rough Form.

COLONY FORMS OF T. NOVELLUS GROWN ON THIOSULPHATE AGAR.

X100:
Smooth and
Rough Forms
Showing
Crystal
Formation.

X300:

Intermediate

X100:

Rough Form

(a) Statistical analysis of plate counts on different media. A suitably-diluted culture of autotrophically-grown T. novellus was spread on a number of nutrient agar, glutamate agar and thiosulphate agar plates and these were incubated at 30°C. After several days counts were made:

Medium	No. of plates	No. of Colonies x 10^{-5}	
		Average	Standard Deviation
Nutrient Agar	10	42.8	7.47
Glutamate Agar	10	67.1	6.77
ThiosulphateAgar	10	64.9	7.02

Results show that the counts on nutrient agar are significantly lower than the numbers on the other two media. The probability that the glutamate and thiosulphate counts arose from separate populations is negligible (considerably less than .01).

Viable counts are commonly considered to be normally distributed, and the hypothesis that the cells are individually facultatively autotrophic means that the same variances occur for the various media; then Student's "t" test can be applied. (Paradine and Rivett, Arkin and Colton.)

(b) Replica plating. Several plates were spread with a dilution of autotrophically grown T. novellus such as to give a calculated viable count of 100. After incubation at 30°C for two to five days the plates were pressed on to a sterile velvet replicator. Thiosulphate and nutrient agar plates were then replicated and incubated at 30°C.

Plates replicated from nutrient agar showed nearly all the colonies present on the original; the colonies present on the replicate nutrient agar or glutamate agar plates were also present on replicate thiosulphate agar plates.

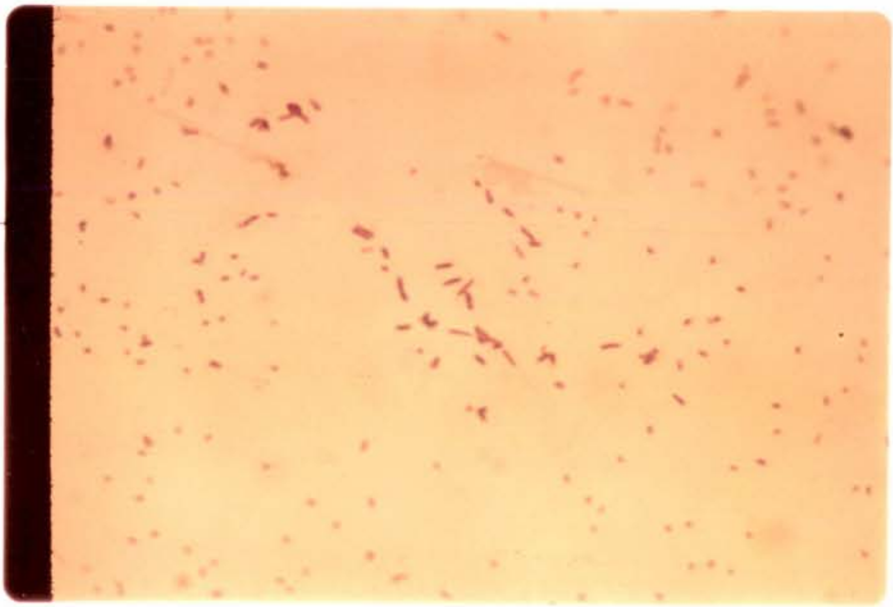
The pile of the velvet was not sufficiently rigid to break the sulphur-sulphate crust of the thiosulphate grown colonies so there was only irregular growth on the replicates from the thiosulphate plates.

Colonies from nutrient agar were capable of both heterotrophic and autotrophic growth. Autotrophically grown cells gave identical viable counts on both heterotrophic and autotrophic agars. It may reasonably be concluded that each cell in the culture is facultatively autotrophic.

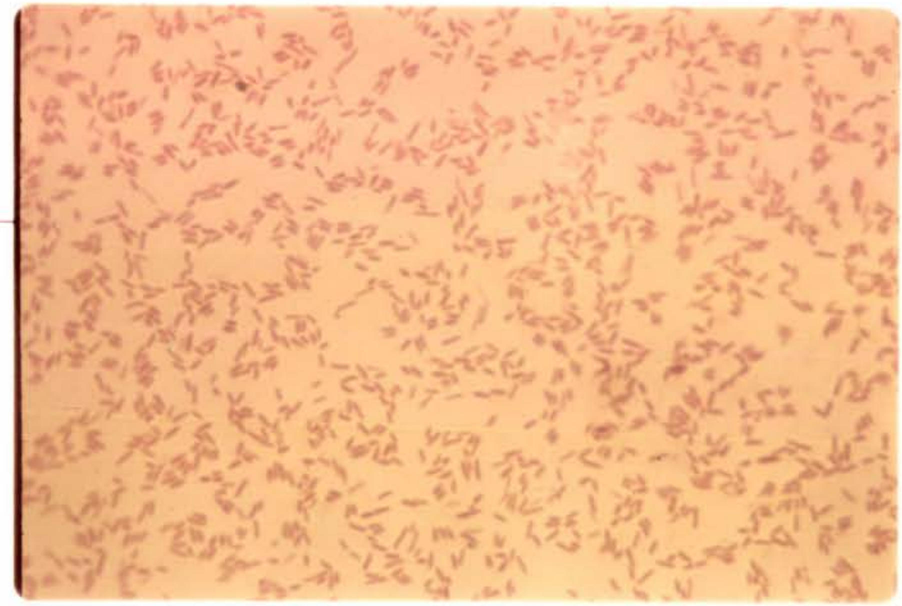
Plates D.

THIOBACILLUS NOVELLUS

GRAM STAINS X3000



Autotrophically Grown



Heterotrophically Grown

APPENDIX I

TRACE METALS SOLUTION

E.D.T.A.	50.0 gms
$ZnSO_4.7H_2O$	22.0 "
$CaCl_2$	5.54 "
$MnCl_2.4H_2O$	5.06 "
$FeSO_4.7H_2O$	4.99 "
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.10 "
$CuSO_4.5H_2O$	1.57 "
$CoCl_2.6H_2O$	1.61 "
H_2O	to 1 litre

The salts were suspended in about 800 mls of water, well shaken and KOH pellets added until the pH was pH 6.0, when the solution was green and most of the salts had dissolved. The solution was brought to 1 litre in a volumetric flask and the pH adjusted to pH 6.0 with KOH. The green solution was stored in the cold and the solution checked occasionally for pH. The colour changed to red with time.

From Vishniac and Santer 1957.

2.25. COMPARISON WITH PUBLISHED DESCRIPTIONS OF T. NOVELLUS.

The following is a description of the organism used in this present study:

A gram-ve to slightly gram variable (autotrophically) short fat rod, occasionally in clumps or in pairs. 0.8 x 1.5µm. Plates D show the gram stains.

On glutamate media grew with the production of ammonia; colonies were orange-shaded; bulk cells were pink to orange.

On nutrient broth cells grew in ropey clumps.

Two colony forms with intermediate types are evident on some media.

Grew very slowly on thiosulphate with the production of some acid and much sulphur.

Optimum initial pH was 8.0 (minimum pH 5.7, maximum pH 9.2) for both autotrophic and heterotrophic growth.

Maximum thiosulphate concentration permiting growth was 4.6%.

A wide variety of heterotrophic substrates is utilised. (see Section three)

Non motile facultative autotroph.

The following are descriptions published by other workers:

(i) Sokolova and Karavaiko (1964): Facultative autotroph. Short, non-motile ellipsoidal or almost coccoidal cells (0.4 - 0.6 x 0.6 - 1.8) µm. sometimes paired.

Slow growth on thiosulphate to oxidize a maximum of 1/3 thiosulphate, to produce only sulphates.

Optimum pH 8.0 - pH 9.0; minimum pH 5 - pH 6

On thiosulphate agar; slow growth to small fluid white colonies containing sulphur.

On nutrient agar white to cream colonies, brown in reflected light. Grows best on glutamate and aspartate agars. Aerobic.

(ii) Vishniac and Santer (1967). Gm-ve, non-sporulating (0.5 x 1 - 3)µm. Non-motile. Facultative autotroph. Slowly oxidizes thiosulphate.

(iii) Santer, Boyer and Santer (1959). Gm-ve (0.5 - 1.0) x (1 - 4)µm. single, occasionally paired rods, grows on nutrient and thiosulphate agars.

(iv) Bergey's Manual of Determinative Bacteriology (Breed et al. 1957). Facultative autotroph not forming tetrathionate as an intermediate.

Aerobe, not oxidizing free sulphur.

Short rods or ellipsoidal cells (0,4 - 0.8) x (0.6 - 1.8)μm.

Non-motile Gm -ve.

Slow growth on thiosulphate agar plates; small white circular colonies with crystals of sulphate.

Grows on nutrient agar broth to form a ropey pellicle.

On thiosulphate forms acid.

Optimum pH around pH 7.0; minimum pH 5.0; maximum pH 9.0.

Apart from the observed diversity of colony forms, the descriptions agree quite well.

The slightly gram variable nature of the autotrophically grown cells may be due to the change in cell wall structure reported. (Le John, Caesaelle and Lees 1967)

2.27. GROWTH CHARACTERISTICS IN LIQUID MEDIA.

2.27.1. In 1.0% Glutamate.

On the basis of a survey of organic compounds supporting growth (Section 3.3) glutamate was selected as the most suitable carbon and energy source for heterotrophic growth. This has been used by other workers (Santer, Boyer and Santer 1959; Charles 1970; Matin and Rittenberg 1970b). The growth characteristics for growth on glutamate medium are described in this section.

Preliminary work showed that the pH optimum for growth on glutamate was pH 8.0: at lower pH values the lag phase was extended and the growth rate was maintained until the stationary phase was reached. Final turbidity was less at pH values less than pH 8.0.

2.27.11. Media and Methods. The same medium as in Section 2.26.8 with the substitution of 10 gms of glutamate (1.0%) for 15 gms. of thiosulphate.

A known inoculum of cold-stored autotrophically-grown T. novellus, to give a calculated viable count of 10^3 viable cells per ml. was added to 1 litre of the medium. The culture was incubated at 25°C and aerated with water-saturated air while shaken at 12 cycles/min. With the baffle-shaped aerater this agitation gave very good aeration throughout the two litre flask.

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Figure 3

GROWTH OF *T.NOVELLUS* ON 1.0% GLUTAMATE : pH and Viable Count.

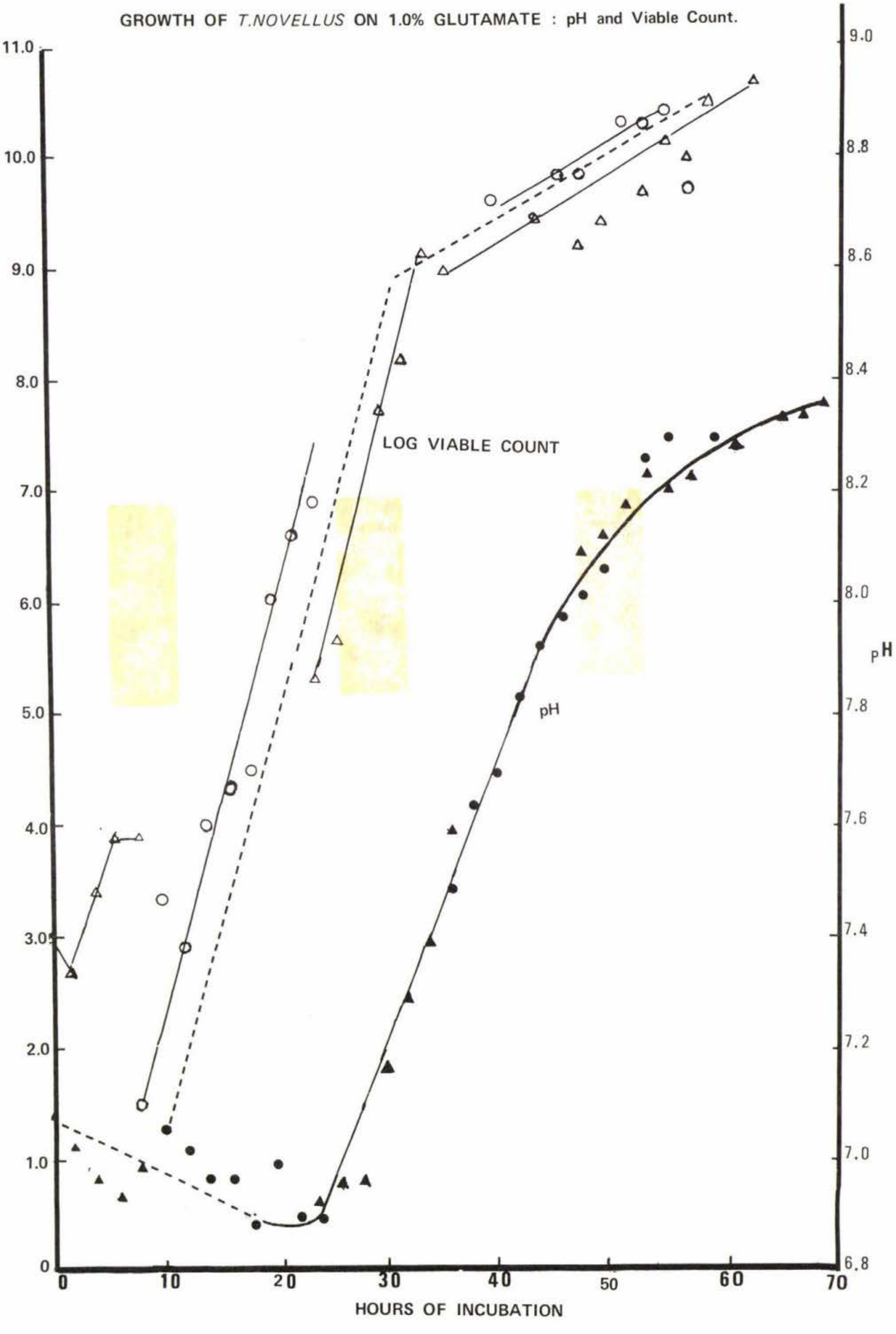
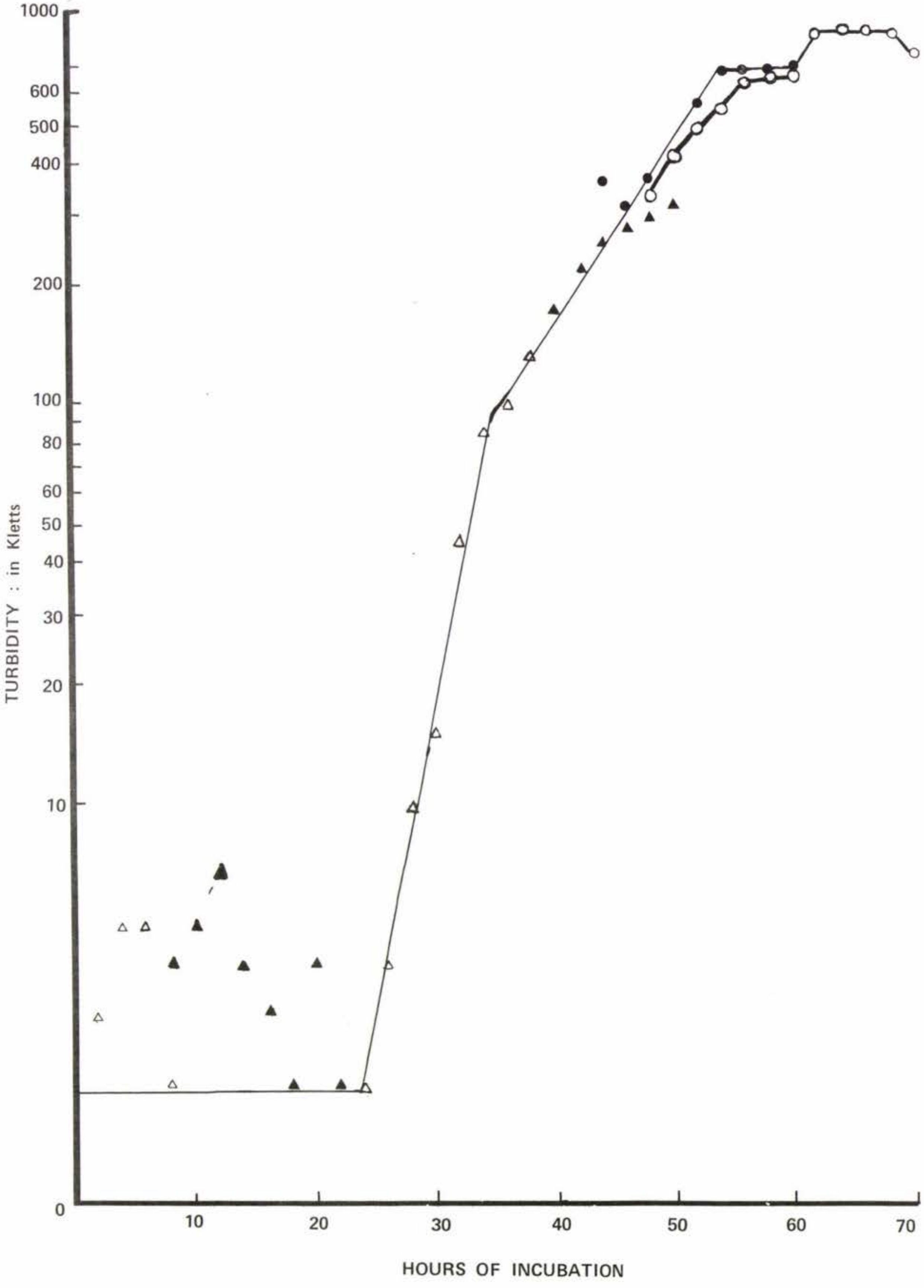


Figure 4

GROWTH OF *T.NOVELLUS* on 1.0% GLUTAMATE : Turbidity



5.0 ml. samples were removed at two-hour intervals into sterile Klett tubes by pipette. Viable counts were determined by dilution in .004M potassium phosphate buffer pH 8.0-mineral salts solution and spreading on to thiosulphate, glutamate and nutrient agar plates at appropriate dilutions. Plates were incubated at 30°C. Turbidity was determined in a Klett-Summerson Colorimeter with a green filter (no.54), set to zero with a water blank. pH was determined by a Radiometer pH meter.

Two flasks were inoculated; one twelve hours after the other, to give all day coverage.

2.27.12. <u>Results and Discussion</u>. Spot tests for ammonia (Russell:Conway 1947) and glutamic acid (Ninhydrin:Jacob 1959) showed that ammonia was produced as the pH rose and glutamate disappeared to become zero at sixty hours.

An uninoculated control aerated at 25°C showed no change in turbidity or pH.

From the Figures 3 and 4 several phases can be distinguished:

(a) Lag Phase. A reproducible lag-phase lasting up to 16 hours occurs, during which there is an erratic variation, up to ten-fold, in viable counts.

(b) Log Phase. From ten to thirty-six hours there is an exponential increase in viable counts and turbidity, with a parallel increase of lesser duration in pH. There is a period in which counts increase but turbidity has failed to register. Then follows an exponential increase in viable counts and turbidity at a slower rate, while the rate of increase of pH steadily decreases. This decreasing rate of increase may be due to the sparging out of ammonia as the pH rises and ammonia becomes more volatile. The Klett measurement becomes inaccurate at about Klett 300 so 1/10 dilutions were made to give a more accurate picture. This second phase lasts until sixty hours when glutamate has become exhausted.

		First Phase	Second Phase
Generation Time in Hours.	From Turbidity	1.85	6.70
	From Viable Counts	0.80	3.32
	Ratio	.50	.43

Turbidity is a measure of cell mass so the ratio would appear to suggest that the cells decrease in mass by 1/2 every generation. This is clearly not possible but no explanation could usefully be proferred without further investigation.

Charles (1971) at 30°C found a generation time, turbidomotrically, of 1.2 hours.

(c) Stationary Phase. A stationary phase is reached at about sixty hours, with the disappearance of glutamate, after which viable counts became erratic and may decline (not shown) and turbidity may also decline.

The step wise increments in turbidity at about this time may not be a true reflection of events, as more steps occur in the undiluted turbidity curve than in the diluted correct turbidity curve.

During the initial increase in viable counts, before turbidity registers, there is a slight decrease in pH which may represent the formation of α-keto-glutaric acid by deamination of glutamate, the other product ammonia being removed by the vigorous aeration, but later accumulating to give a pH rise.

2.27.2. On 1.5% Thiosulphate.

The medium, described earlier in Section 2.26.3. was used in attempts to obtain comparable data on turbidity, viable counts and pH changes during growth on a thiosulphate medium.

Long term attempts showed an initial increase in pH, followed by an equal drop with an initial increase in viable counts and the disappearance of a small amount of thiosulphate. However the very slow growth rates and the complicating effects of sulphur and other inorganic precipitates and components made it very difficult to obtain reproducible and valid measurements of growth.

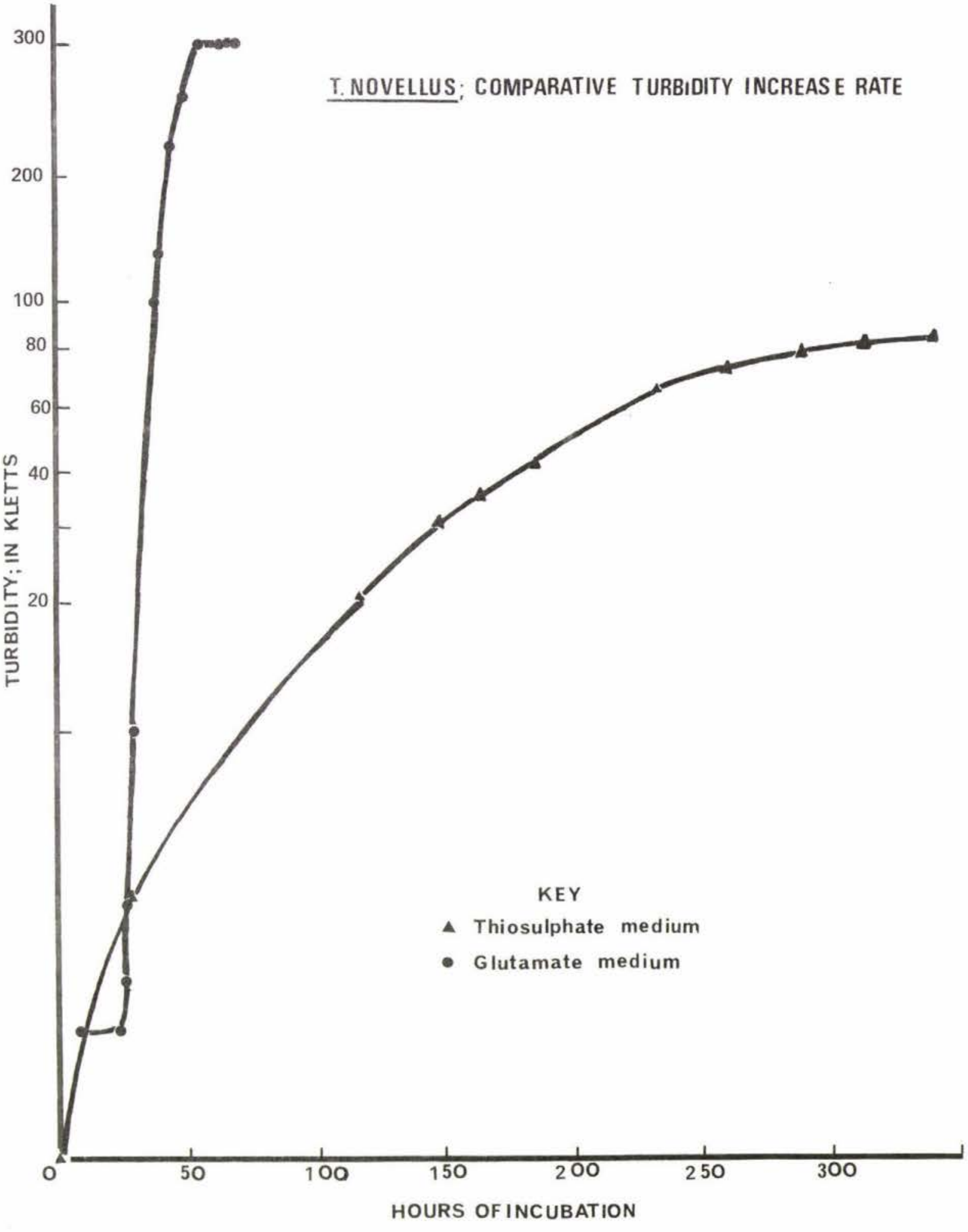
A short term experiment using a heavy initial inoculum failed as the initial pH rise, previously noted, gave a pH higher than the maximum for growth.

The failure to achieve a satisfactory autotrophic growth curve is not unique. (Parker and Prisk 1953; Starkey 1934; Sokolova and Karavaiko 1964). All results show a strictly limited oxidation of thiosulphate and low bacterial activity, with a minor decrease in pH.

Generation times reported by other workers were obtained using large inocula of heterotrophically grown cells; and the media often contained traces of yeast extract.

An approximate comparison of the rate of turbidity increase on glutamate and thiosulphate is shown in Figure 5. The turbidity increase for

FIGURE 5


300
200
100
80
60
40
20
TURBIDITY; IN KLETTS
T. NOVELLUS; COMPARATIVE TURBIDITY INCREASE RATE
KEY
▲ Thiosulphate medium
● Glutamate medium
0
50
100
150
200
250
300
HOURS OF INCUBATION

thiosulphate medium, though valid for comparative purposes, also represents inorganic products of both biological and non-biological origin, so is not suitable for the estimation of generation times.

2.3. PHYSIOLOGICAL CHARACTERIZATION OF THIOBACILLUS THIOPARUS.

2.31. SOURCE AND RECONSTITUTION OF FREEZE-DRIED CULTURES.

Some difficulty was experienced in successfully reconstituting and maintaining cultures from a variety of sources, either because of contamination or from inexperience in handling these cultures.

A freeze-dried culture of T. thioparus NCIB 8349, was obtained from the National Collection of Industrial Bacteria (Edinburgh), reconstituted in sterile water and aliquots distributed to a variety of media. Successful cultures were subcultured on the same medium (described in Section 2.32.).

Later, samples were freeze-dried for long term maintenance. Plates and harvested bulk cells were stored in the cold. Frequent checks were made for heterotrophic contaminants; when contamination was detected the contaminated culture was rejected.

2.32. COMPARISON OF DIFFERENT THIOSULPHATE MEDIA.

As with T. novellus examination of the literature indicates a large variety of media suggested for growth of T. thioparus. The media of Matin and Rittenberg (1971a); Johnson and Abraham (1959); "The American Type Culture Collection", Kelly and Syrett (1964) and the modified T. novellus medium described later, were made up, autoclaved at 15lbs./sq.in. (121 $^{\circ}$C or 72KN/M^{2}) for fifteen minutes and then the component solutions were mixed. Solid media were incubated molten at 45°C for some hours, in a procedure that reduces crystal formation, and plates poured and allowed to cool.

The plates and liquid media were inoculated with T. thioparus and incubated at 30°C.

The media that showed growth were also the media that allowed recovery of viable T. thioparus from the aliquots of freeze-dried suspension dispensed after reconstitution.

The media prepared according to the directions of the American Type Culture Collection showed no growth. Although the first of these two media showed crystals, no explanation for this failure to support growth was evident. The medium of Kelly and Syrett (1964) gave a heavy inorganic precipitate.

The modified <u>T. novellus</u> medium proved to be the most suitable growth medium.

<u>MEDIUM</u>. Modified <u>T. novellus</u> medium, after Vishniac and Santer (1967).

		Weight in Grams	Final Concentration
(a)	as in 2.23.6.		
	NH_4Cl	1.0	0.1% w/v
	$MgCl_2$	0,5	.05% "
	Trace Metals Solution	10 mls.	1.0% v/v
	Water	890 "	
	Agar (for solid media)	15	1.5% w/v
(b)	$Na_2S_2O_3.5H_2O$	15.0	1.5% "
	$KH2PO_4$	4.0	0.4% "
	K_2HPO_4	4.0	0.4% "
	Water	100 mls.	

These solutions were autoclaved for fifteen minutes at 15lbs./sq.in. (121°C or $72KN/M^2$). Solid media were incubated molten at 45°C after mixing, for some time before being poured.

Liquid solutions, after mixing, were used within a week or an inorganic precipitate developed.

It was found that the addition of filter-sterilised pH 8.0 $NaHCO_3$ (to final concentrations of 2mM $NaHCO_3$) markedly increased growth. Concentrations above 12mM prevented growth.

Aeration of liquid media with water-saturated 5% CO_2: 95% Air rather than water-saturated air improved growth rates and reduced the lag-phase markedly.

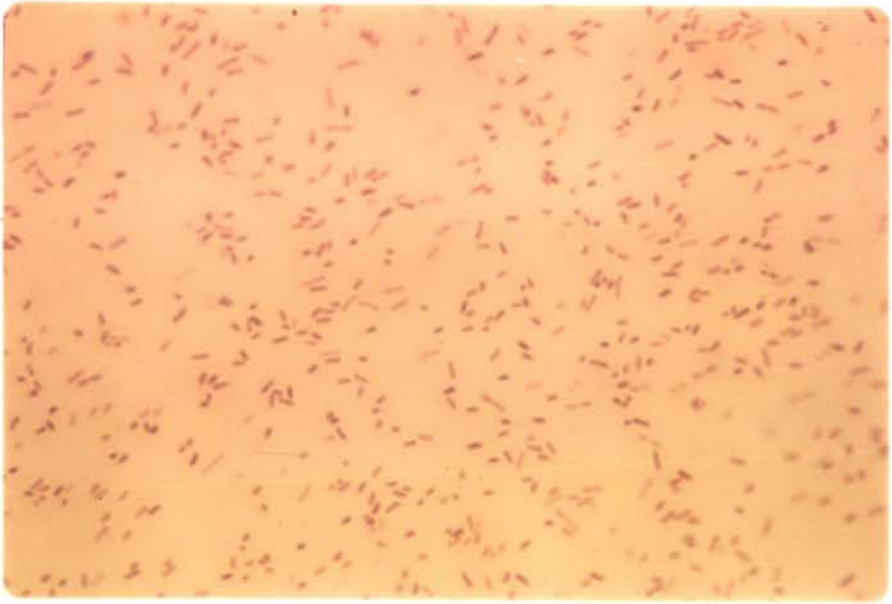
A temperature of 30°C was found to give much more rapid growth than a temperature of 25°C.

Plates E.

THIOBACILLUS THIOPARUS

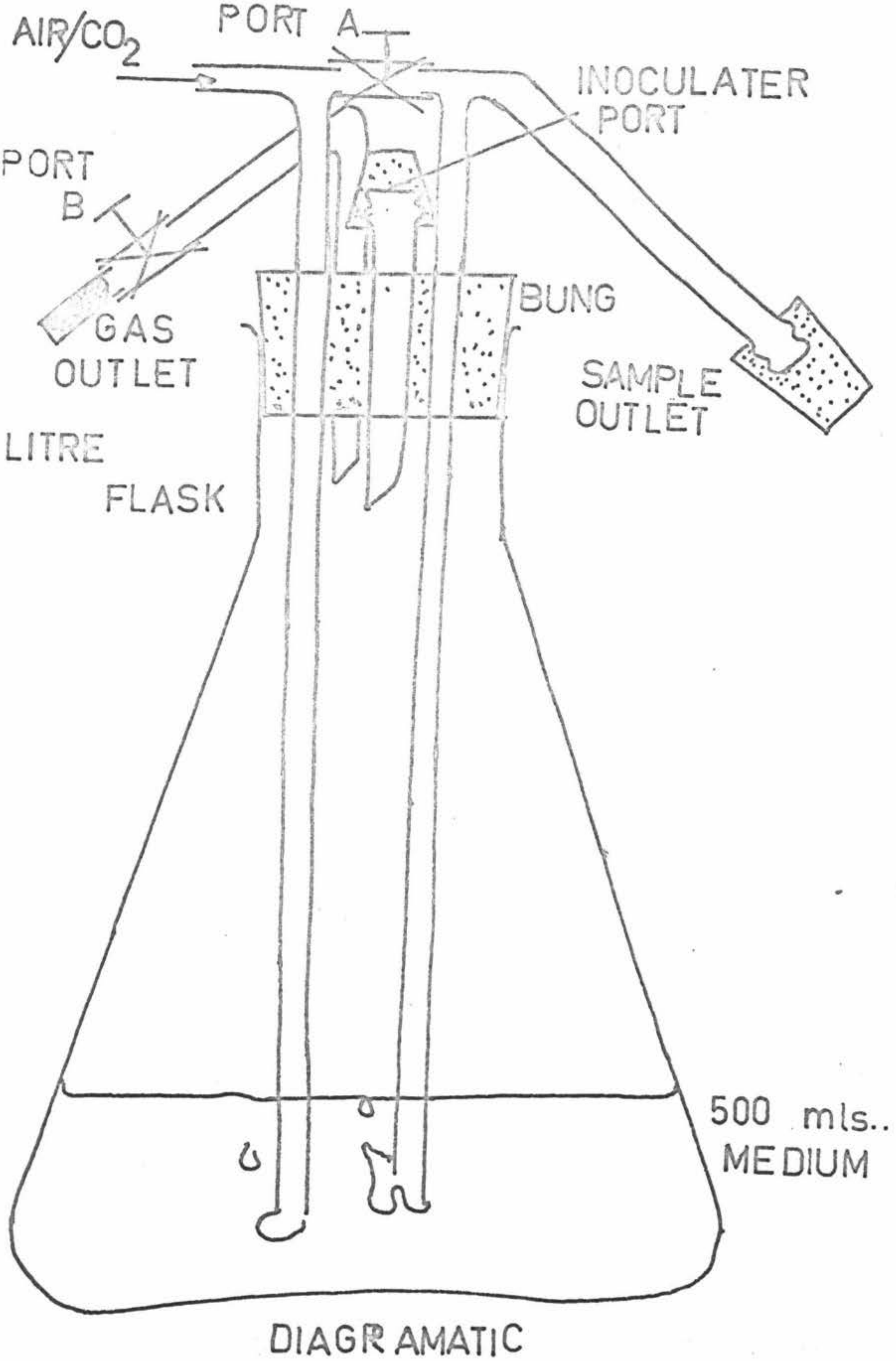
THIOSULPHATE GROWN

T. THIOPARUS Side Lit. X300.



Gram Stain: X3000

FIGURE 6



DIAGRAMATIC
INCUBATION APPARATUS FOR T. THIOPARUS

2.32. DESCRIPTION OF MAIN CHARACTERISTICS.

The results to be described in Section 2.34. show that the culture oxidizes thiosulphate with a concomittant increase in viable and total counts and in cell mass and production of acid after an initial pH increase with some sulphur production. This indicates autotrophic growth. Later data (Section 3.5.) shows that the organism is unable to grow on organic media and is inhibited by a wide variety of organic compounds.

The initial pH optimum of the culture is around pH 7.0 and a pH of pH 4.0 is reached and not exceeded while oxidizing thiosulphate.

Temperature optimum was around 30°C.

Small fat gm-ve rods singly or in pairs. Very actively motile, especially when young. Gram stain is shown in Plates E.

On thiosulphate-agar small white colonies encrusted with sulphur are produced; as is shown in Plates E. Cells en masse are pink.

Actively incorporate ^{14}C from $^{14}CO_2$.

This description agrees with the descriptions of Vishniac and Santer (1967); Matin and Rittenberg (1970); Bergey's Manual of Determinative Bacteriology, Breed et al (1957); Sokolova and Karavaiko (1964), except that Sokolova and Karavaiko (1964) give a more alkaline pH optimum (pH 8.0 - pH 9.0). There is considerable disagreement as to the intermediate formation of tetrathionates (which would give a pH rise).

2.34. NORMAL GROWTH CURVE ON THIOSULPHATE.

2.34.1. Media and Methods.

500 mls. of the medium, as described in Section 2.32. in the apparatus in Figure 6, was inoculated with 1.0 ml. of a cold-stored culture of T. thioparus known to be free of heterotrophic contaminants, diluted so as to give an estimated final viable count of 10^3 viable counts/ml. An uninoculated control was also set up.

Both flasks were incubated in a 30°C water-bath while being aerated with water-saturated 5% CO_2: 95% Air mixture. Aeration was increased with turbidity.

At frequent intervals samples were taken. The outlet was unplugged and flamed with alcohol, then ports A and B were closed and the gas flow forced the sample out the outlet and into a sterile Klett tube. When pH

- △ Flask A
- ▲ Flask B
- - - - Composite

Turbidity

- □ Flask A
- ■ Flask B

Figure 7

GROWTH OF *T.THIOPARUS* ON 1.5% THIOSULPHATE : Turbidity and pH

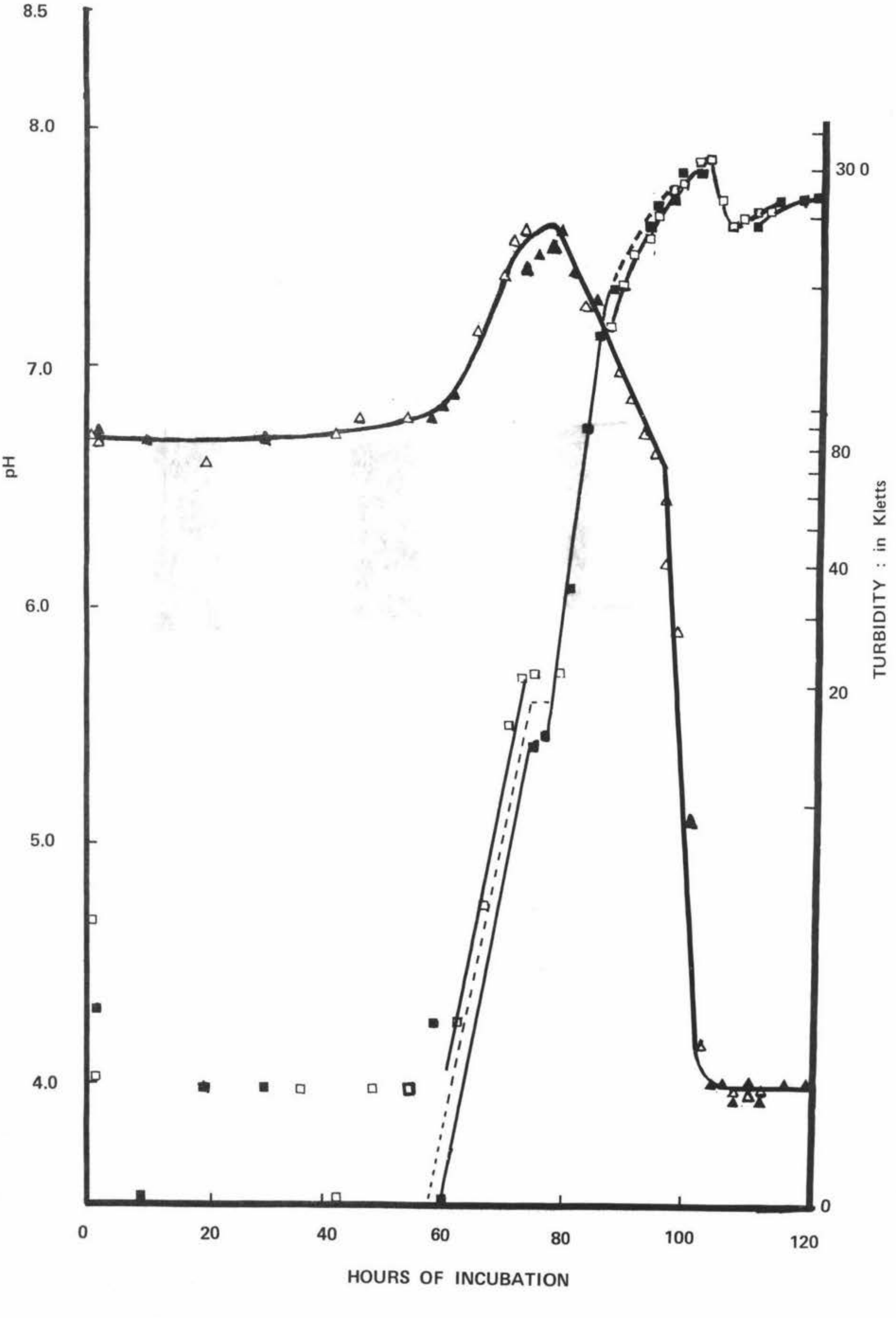


Figure 8 : KEY

△ Flask A

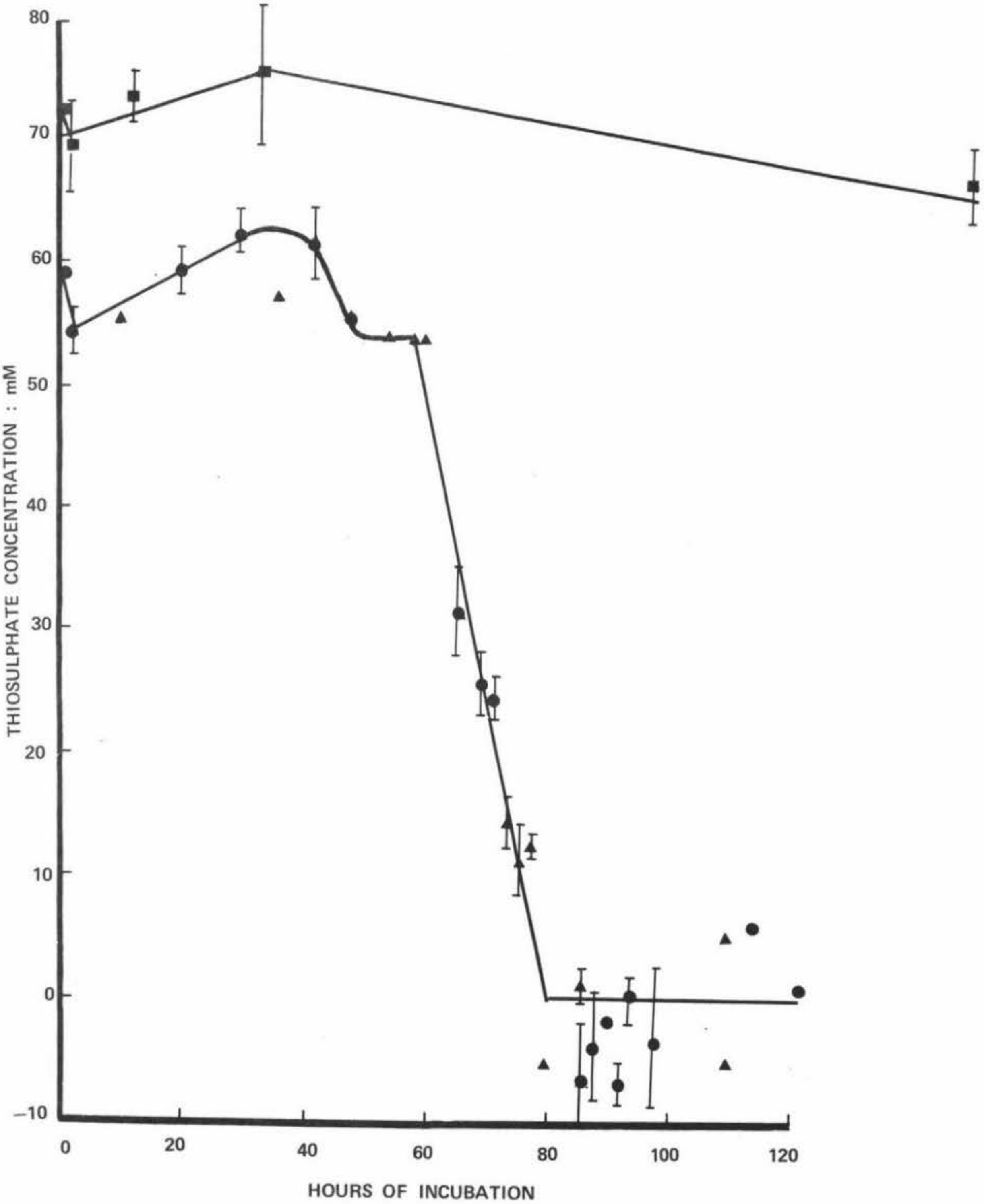
○ Flask B

□ Zero

Figure 7 : KEY

Figure 8

GROWTH OF *T.THIOPARUS* ON 1.5% THIOSULPHATE : Thiosulphate Concentration



sufficient had accumulated, port B and then port A were opened; after the outlet was plugged aeration continued normally.

In samples from the inoculated flask bacterial numbers were determined. Dilutions were made in sterile media and appropriate quantities spread on thiosulphate agar plates and incubated at 30°C. At visible turbidities aliquots were counted directly, after settling, in a Thoma cell at phase contrast magnifications of 400 diameters. In both the inoculated flask and the control (less often) samples were taken for determination of turbidity, in a Klett-Summerson Colorimeter with a green filter (no.54). pH was determined by a Radiometer pH meter. Duplicate aliquots were titrated for thiosulphate (see Appendix II).

Samples used to determine dry-weight were insufficient in size.

2.34.2. Results and Discussion.

There were no heterotrophic contaminants detectable on nutrient and glutamate agars.

The results are shown in Figures 7 and 8.

There was no pH change or increase in turbidity in the uninoculated control. There was a slow disappearance of thiosulphate, to a maximum of 10% after 170 hours.

Over the first 50 - 60 hours after inoculation little change is apparent in any of the parameters. The titration values for thiosulphate are somewhat erratic over this period, but appear to indicate an increase in thiosulphate. This initial increase of titrable thiosulphate occurs after an immediate drop in titration values: this occurs in both the inoculated and the uninoculated flasks and is thus not of physiological significance. This portion of the titration curve may represent the transient precipitation of insoluble non-alkali-metal thiosulphates, soon after the component solutions of the medium were mixed, followed by their resolution.

The commencement of the disappearance of the thiosulphate coincides with the increase in pH and indicates the log-phase of growth. This rise in pH probably represents the formation of a mixture of poly-thionates (Trudinger 1964). The poly-thionates are not titratable but support growth while being oxidized to sulphate.

The thiosulphate is exhausted at the same time the pH peaks. The transient inflexion in the turbidity curve at around Klett 20 may be an artefact. The drop in the turbidity at 100 hours at maximum turbidity is a reproducible feature of the curve. This drop coincides with the change of rate of pH decrease. This could reflect the lysis of sensitive cells by the acid conditions; viable cells can be recovered but after prolonged incubation viability is lost. However the turbidity drop does not continue as would be expected from this. The sulphur produced earlier in metabolism might now be metabolised, when the preferred substrate tetra-thionate is exhausted. The turbidity would then drop until the sulphur was exhausted or, as in this case, an unfavourable pH was reached. The change in the rate of pH decrease as the turbidity drops is in accord with this.

Viable count results were erratic and never reached a very high percentage of the direct counts. There was an increase in direct counts from about 10^3 to at least 5×10^8. The low viable counts may represent a low percentage of viable cells in the population or may be due to an effect of dilution. The same low counts were obtained in dilution series in phosphate-mineral salts solutions as in full media-solutions, from an actively growing culture with a direct count of around 10^8. Thus the explanation is not substrate poisoning and is not likely to be dilution shock.

Generation Time in hours:

From turbidity	1.3
From Thiosulphate decrease	1.8

As turbidity measures sulphur which is transiently produced, while thiosulphate is oxidized in several separate steps, neither generation time is an accurate measure of the growth rate.

2.34.3. Comparison with Published Results.

The formation of tetrathionate with increase in pH as the initial step of autotrophic metabolism, is now generally accepted. (Trudinger 1964; Parker and Prisk 1953)

Old cells, according to Sokolova and Karavaiko (1964) are prone to autolysis, which may account for the presence of many organic compounds in spent media.

The final pH is generally in the range pH 3.5 - pH 5.0 (Bergey's Manual of Determinative Bacteriology; Sokolova and Karavaiko 1964;. Parker and Prisk 1953.)

Parker and Prisk (1953) found that there was only a small increase (from 10^6 to 10^7) in viable counts during the period of consumption of thiosulphate; growth lagged behind thiosulphate consumption and the viable count declined at pH values less than pH 5.0. The pH did not increase and showed a drop of only 0.3 pH units over the period of consumption of 43mM thiosulphate. After this the pH dropped from pH 6.3 to pH 4.7. From the Parker and Prisk (1953) data a generation time of 22.8 hours was calculated from the thiosulphate consumption rate.

No other reports of generation times could be found.

APPENDIX II

DETERMINATION OF THIOSULPHATE.

The Sörbo colorimetric method of thiosulphate determination (Sörbo 1957) was tested and found to be sensitive to interference from a number of possible products of thiosulphate oxidation such as sulphide, sulphite and tetrathionate ions, as well as some organic compounds. Most interfering compounds can be removed by precipitation with cadmium sulphate but polythionates remain. The usable range of thiosulphate concentrations is narrow. Because of this and the amount of processing required, an iodate-thiosulphate titration system was preferred.

After some difficulties a method using an iodate titration and free from interference, except by strong oxidizing agents, was developed. This system is pH independent, unaffected by turbidity and does not measure polythionates. The system was developed from that of Svehia et al. (1963).

System.

Reagents.

Indicator:	NaCl	125 gms.
	2.6. para dichlorophenolindophenol	.125 gms.

About 0.1 KI.

About .025N Ascorbic Acid freshly prepared.

Exactly 0.IN KIO_3

1.0 ml. of each of the standard iodate and iodide solutions was mixed with 1.0 ml. of the thiosulphate solution. This mixture can be stored without deterioration if frozen.

Several drops of glacial acetic acid were added. The resultant iodine-coloured solution was titrated immediately with ascorbic acid solution until straw coloured. Solid potassium bicarbonate was then added until frothing ceased. A heaped knife-point of the indicator was added. If the solution was red more bicarbonate was added. The blue solution was titrated until clear.

The redox indicator indicates the point at which the ascorbate is present in a standard excess concentration at which the redox potential is that of the colourless form of the indicator. The dilution of the indicator in NaCl is such that variations in the "knife point" have a minimal effect on the required redox potential.

The ascorbic acid solution must be freshly prepared and standardised against the iodide and iodate mixture within an hour, preferably less, of the determination.

The system covers the range of thiosulphate concentrations of interest. There are two colour stages on the way to the end point so the chances of overshoot and thence of error are reduced.

All determinations were in duplicate and were averaged.

The titration is a simple back titration with a freshly standardised solution.

3. EXAMINATION OF THE EFFECT OF VARIOUS ORGANIC COMPOUNDS ON GROWTH OF THIOBACILLUS NOVELLUS AND T. THIOPARUS.

3.1. INTRODUCTION.

The work to be described here concerns the effect of various exogenous organic compounds on the growth, and various related functions, of the facultative autotroph T. novellus and of the obligate autotroph T. thioparus. The organic compounds were selected on availability from the list of substrates given by Skerman (1969). Compounds were tested at several concentrations to reduce the possibility of a concentration effect, such as a growth inhibition at higher substrate concentrations which commonly occurs in the thiobacilli (Rittenberg 1969), obscuring a genuine substrate effect.

The compounds were first screened as possible substrates for heterotrophic growth of T. novellus, as there are contradictory reports in the literature on the heterotrophic abilities of this species (Taylor and Hoare 1969a; Charles 1971; Santer, Boyer and Santer 1959).

The same range of organic compounds at similar concentrations was examined again, to detect inhibitory or stimulatory effects on autotrophic growth of T. thioparus and on both autotrophic and heterotrophic growth of T. novellus.

After the initial survey of the compounds in solid media, selected organic compounds were examined in equivalent liquid media.

The ability of certain compounds to reverse the inhibitory effect, of one particular inhibitory amino acid, on growth of T. thioparus was also examined.

3.2. GENERAL METHODS.

3.21. PREPARATION OF STERILE SOLUTIONS OF ORGANIC COMPOUNDS.

Appropriate quantities of the compounds were dissolved in water and 10 ml. quantities were filter sterilised through 0.22μm. membrane filters, unless the solutions were of known stability, in which case they were autoclaved at 121 °C (15lbs./sq.in. or $72KN/M^2$) for fifteen minutes.

Successive tenfold dilutions were made in sterile distilled water. All sterile solutions were stored at $4^{\circ}C$: after which some solutions required warming to redissolve.

In most cases the pH was adjusted to pH 7.8 ± 0.5 before sterilisation.

3.22. MEDIA.

		Standard Solution	Final Plate Conc.	Volume per Plate
(a)	NH_4Cl	2.5 gm.	0.1% w/v	10.0 mls.
	$MgCl_2$	1.25 "	.05% "	
	Trace Metals Solution (Vishniac and Santer 1967)	25 mls.	1.0% v/v	
	Agar (solid media)	38 gm.	1.5% "	
	Water to	1 litre		
(b)	*T. novellus* 0.2M Potassium Phosphate buffer pH 8.0	100 mls.	4mM	5.0 mls.
	Zero			
or	$Na_2S_2O_3.5H_2O$	75 gms.	1.5% w/v	
or	NaH glutamate	50 "	1.0% "	
	Water	900 mls.		
for	*T. thioparus*			
	$Na_2S_2O_3.5H_2O$	75 gms.	1.5% w/v	5.0 mls.
	KH_2PO_4	20 "	0.4% "	
	K_2HPO_4	20 "	0.4% "	
	Water to one litre			
(c)	Organic compound solution and water.		According to Tables	10 mls.

Solutions (a) and (b) were sterilised by autoclaving at $121^{\circ}C$ (15lbs./sq.in. or $72KN/M^2$), solution (c) was sterilised as in Section 3.21. These were held at $45^{\circ}C$ and mixed in the Petri dish.

3.23. MEASUREMENT OF GROWTH.

Growth on solid media was measured with an eyepiece grating in a dissecting microscope. Autotrophic colonies were measured against a black matt background and side-lit. Heterotrophic colonies were measured by transmitted light. The colonies were sized arbitrarily; the rate of increase in these classes was compared to the rate in standard media, and further arbitrary growth rate classes set up. Because of these arbitrary classes, the classes were neither sharp nor definitive.

Growth in liquid media cultures was measured turbidometrically in a Klett-Summerson colorimeter, using a green filter (no.54), set to zero with a water blank.

3.24. MISCELLANEOUS.

pH was determined in most cultures. On solid media moistened Whatman-BDH close range pH papers were used to determine pH to within .5 pH. In liquid cultures a Radiometer pH meter was used.

Contamination checks were by inspection of colony shape and form.

3.3. POTENTIAL GROWTH SUBSTRATES FOR *T. NOVELLUS*.

3.31. SURVEY OF POTENTIAL GROWTH SUBSTRATES USING SOLID MEDIA.

Medium components as specified in Section 3.22. were poured together with a standard inoculum of thiosulphate grown *T. novellus*, sufficient to produce ca. 80 -150 colonies, mixed thoroughly and allowed to cool. This gave well separated colonies, both submerged and on the surface. Comparison of colony counts with the known viable count would reveal gross contamination.

Plates were incubated at 30°C in several batches and examined at frequent intervals for growth. At the end of growth colonies were checked for form and large colonies were streaked on to thiosulphate agar, to detect any loss of autotrophic ability. The pH was then determined.

From the size class data the substrates were placed in order or suitability as substrates.

Small indefinite pale colonies of visible size appeared in both normal Davis agar and washed Davis agar, lacking added organic compounds. Consequently substrates giving colonies no different in size or appearance from those on "non-substrate zero plates" were scored as not supporting growth. Confusion between low and no growth scorings can also occur; so these scorings were grouped together.

Surface colonies were generally much larger than submerged colonies.

The diversity of colony forms was generally evident (See Section 2.2.4.); but not on all substrates. Most colonies were shades of orange, from extremes of almost pink to brown.

Subcultures of large surface colonies from various substrates were all capable of growth on standard Thiosulphate T. novellus Agar.

Substrates at concentrations of 0.1 mM and 0.01 mM where tested, gave scorings indicating little or no growth. The results for higher concentrations are shown in Table XI; good growth was obtained on a wide range of substrates.

Tyrosine, originally gave a white powdery agar which oxidized in air to give a brown transparent compound (probably a phenol) most noticeably around the "non-substrate zero" colonies. Hence an artificial good growth was scored by the naked eye; very high back lighting gave a true score.

The pH of the organic acid plates rose.

The ethanol and glycerol substrate colonies were coloured but the colonies on the other alcohols were pale or white.

3.32. GROWTH OF T. NOVELLUS ON SELECTED ORGANIC SUBSTRATES IN LIQUID CULTURE.

The following organic compounds were selected from the range used in the solid media survey, and examined as growth substrates in liquid media; All at 100mM.

Asparagine
Ornithine
Pyruvate
Formate
Glutamate
Arginine
Acetate
Alanine
Glucose
Methanol
Glutamine
Fructose
Ethanol
10mM Phenylalanine
1mM Formate
5.0mM Tyrosine.

A standard large inoculum of cold-stored thiosulphate-grown T.novellus was pipetted into each nephelometer flask of medium (as in Section 3.22.). The flasks were incubated in a 25°C water-bath while aerated with water-saturated air. Flasks were stored occasionally at 4°C during the

TABLE XI

GROWTH OF T. NOVELLUS ON VARIOUS ORGANIC COMPOUNDS AT SEVERAL CONCENTRATIONS.

Amino Acids	100mM	10mM	1mM
D/L Alanine	3	2	0
Arginine	3	2	0
Asparagine	2	0	0
Aspartic Acid			2
Cystine		2	1
L-Glutamic Acid	3	2	1
L-Glutamine	2	2	0
Histidine		2	1
Methionine		2	1
L-Ornithine	3	2	1
L-Phenylalanine		3	2
L-Proline		2(2.1)	1(.21)
L-Serine		3	1
Tyrosine			
L-Valine		3	3

Little or no growth ((at even the highest concentration (given) tested))

Citrulline	100	Norleucine	10	D/L Tryptophane	10
Glycine	100	Lysine	100	L-Tryptophane	10
Isoleucine	100	D/L Serine	10	Tyrosine	5.0
Leucine	100	L-Threonine	100		

Organic Acids	100mM	10mM	1mM
Acetate Na	3	2	0
Formate Na	2	1	0
Lactate Li	3(0.5%)	1(.05%)	0(.005%)
Propionate Na	2	2	1
Pyruvate Na	3	2	0
Succinate Na	2		0

Little or no growth

Oxalate Na	100	Tartrate KNa	100

TABLE XI (Continued)

Bases and Related Compounds	100mM	10mM	1mM
Adenine		3	0

Little or no growth

Creatine	100	Thymine	10	Xanthine	10
Indole	10	Uracil	10		

Sugars	100mM	10mM	1mM
Arabinose	2	0	0
Glucose	3	3	1
Maltose	2	2	1
Mannose	2	1	0
Starch	2(1.0%)	0(.1%)	0(.01%)
Sucrose	2(50)	2 (5)	2(.5)
Xylose	2		

Little or no growth

Cellobiose	50	D Lactose	100	D Rhamnose	100
Fructose	100	Mellobiose	25	Salicin	10
Galactose	100	D Raffinose	10	Trehalose	100

Alcohols	100mM	10mM	1mM
Ethanol	3	1	0
Glycerol	2(71)	2(7.1)	1(.71)
Mannitol	2	1	1
Methanol	3	1	0
Sorbitol	2(25)	0(2.5)	0(.25)

Little or no growth

Dulcitol	100	Isopropanol	100

Figures in brackets are concentrations other than at the column head: in mM unless otherwise given as %.

Code:
3. Very good growth; comparable to that for glutamate
2. Moderate growth
1. Small colonies could be confused with 0.
0. Colonies are the same size as for non-substrate "zero" plates.

Figures after substrates for little or no growth are the highest concentrations of the tenfold dilutions tested.

incubation, so that turbidity measurements were made at convenient times. The effects of this storage, a slight increase in turbidity and a short period of slower growth on the return to the higher temperature, were corrected for where possible, and smoothed out in the curves.

Growth was followed turbidometrically.

At the end of the incubation, samples were streaked on to thiosulphate agar, and on to nutrient agar to test for contaminants. The pH was also determined.

The results are shown in Figures 9, 10 and 11.

There was considerable variation in the duration of the lag-phase, The sulphur present in the inoculum sank in the pipette and carried cells with it. Thus successive inocula varied considerably and so did the lag-phase before turbidity became apparent. This lag-phase has been omitted in the graphs; the zero time in the graphs represents the beginning of the log-phase not the time of inoculation.

There was, where determined, a small drop in pH to pH 6.5 ± 0.5.

100mM Methanol and 100mM ethanol showed no growth, presumably because the aeration had removed these volatile compounds.

The milky-white 5.0mM tyrosine solution became brown and cleared during aeration, but never became turbid; the brown clear solution was obviously due to a non-biological oxidation of tyrosine.

1mM Cystine showed no detectable growth; the substrate concentration was probably too low for detectable growth.

100mM Formate showed no detectable growth.

There was, where determined, a small pH drop to pH 6.5 ± 0.3.

The graphs have been grouped into similar curves.

(i) Glutamate, Glutamine, Alanine, Asparagine; all 100mM. All show a fairly rapid initial growth rate, with some decrease in mid log-phase in the case of glutamate (see also Section 2.27.). See Figure 9.

The curves for alanine and asparagine are equivalent until late log-phase when asparagine shows a substantial decrease in turbidity.

(ii) Arginine, Ornithine both 100mM, Phenylalanine 10mM. All curves show a steadily decreasing growth rate. See Figure 10.

(iii) Acetate, Pyruvate, Fructose, Glucose all at 100mM. Acetate, pyruvate and glucose show very rapid intial growth rates. Pyruvate and fructose show parallel growth-curves as do acetate and glucose. The most interesting feature is the low yield for glucose. See Figure 11.

Figure 9 : KEY

- ▲ I00 mM Asparagine
- ■ I00 mM Glutamate
- △ I00 mM Alanine
- ● I00 mM Glutamine

Figure 9

GROWTH OF T.NOVELLUS IN LIQUID CULTURE

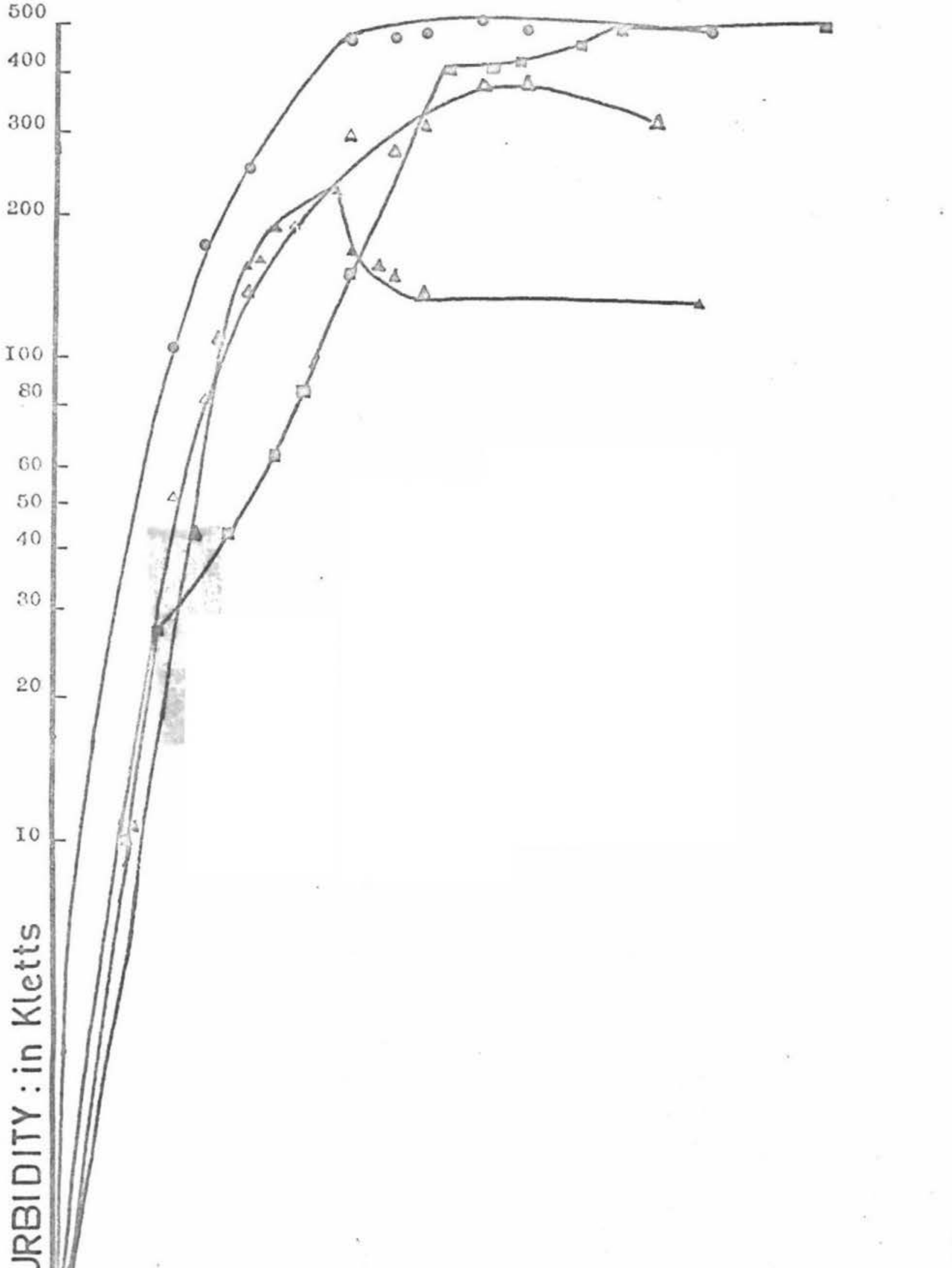


Figure I0: KEY

○ I00 mM Phenylalanine
▲ I00 mM Ornithine
■ I00 mM Glutamate
● I00 mM Arginine

Figure I0

GROWTH OF T.NOVELLUS IN LIQUID CULTURE.

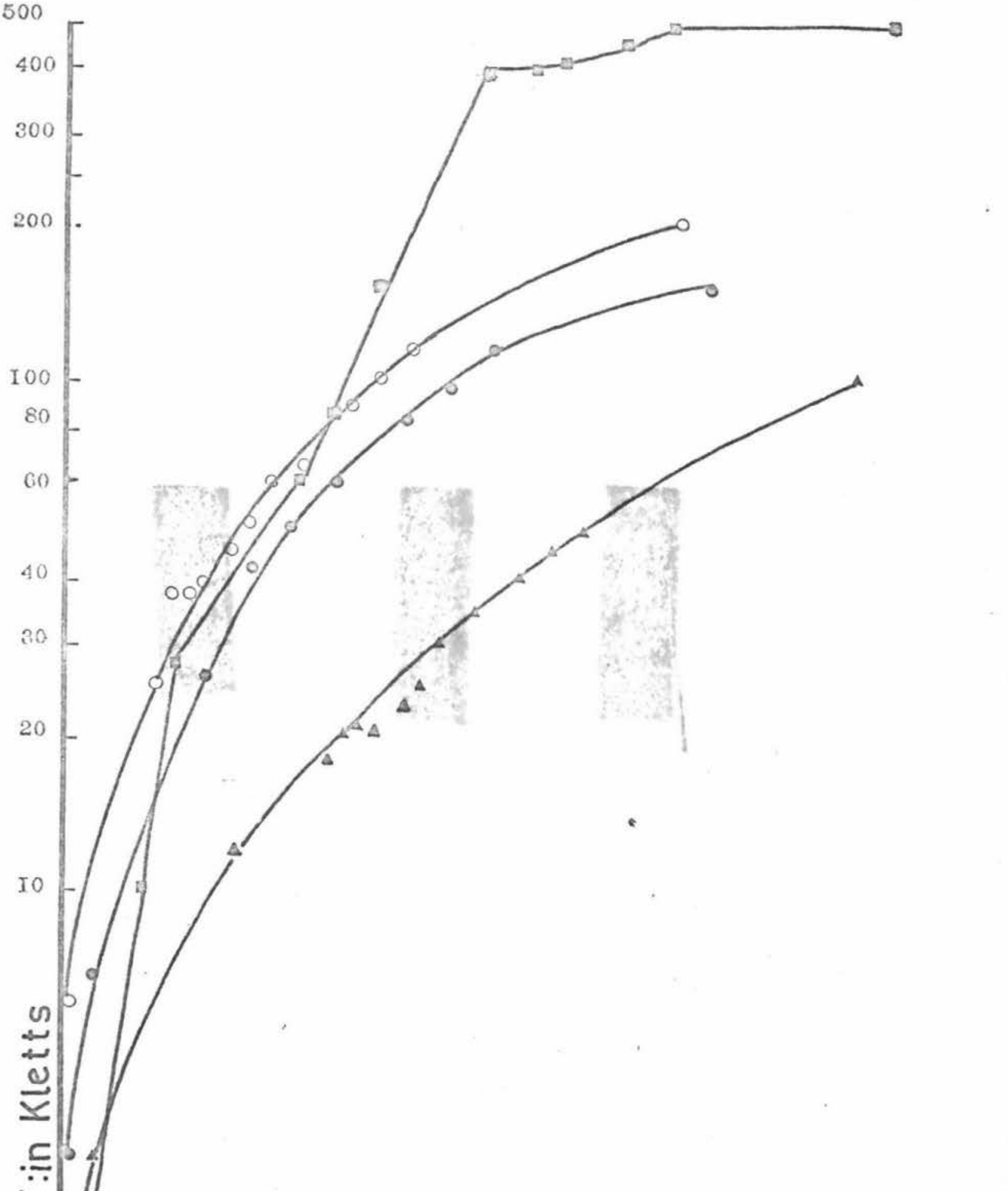
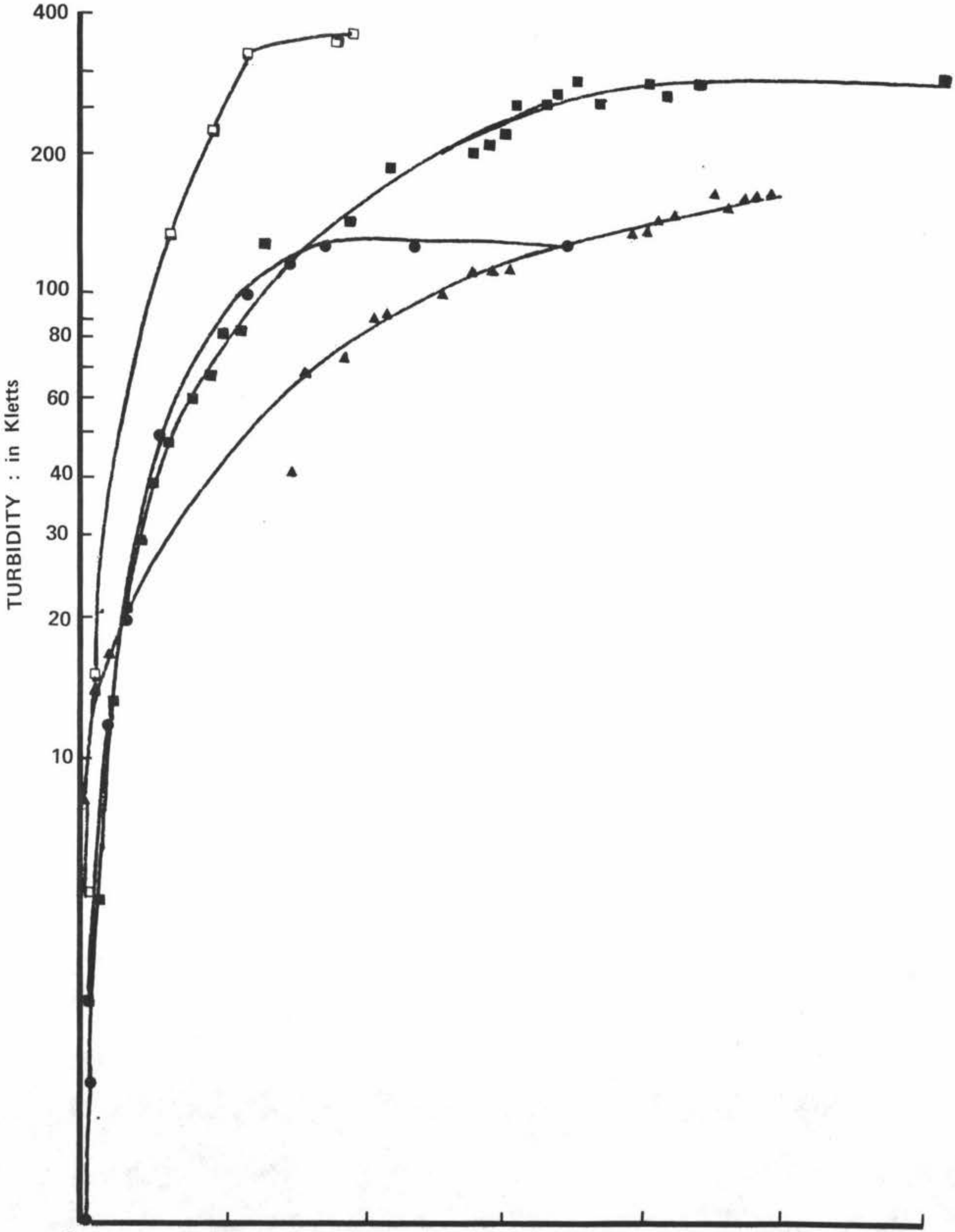


Figure 11

GROWTH OF *T.NOVELLUS* IN LIQUID CULTURE



The pH rise during growth on amino acids (groups (i) and (ii)) is presumably due to the liberation of ammonia during deamination, although a considerable loss of ammonia occurs due to the vigorous aeration.

3.33. DISCUSSION.

3.33.1. Comparison with Results on Solid Media.

In general those substrates supporting good growth on agar (scores of 2 or 3) also give high growth rates and growth yields in liquid media. Two exceptions are fructose, which gave little or no growth on agar but did support slow growth in the liquid medium, and sodium formate, which did not support growth on a liquid medium but gave good colonies on agar.

3.33.2. Comparison with Published Results.

The only extensive survey of growth substrates for T. novellus is that by Taylor and Hoare (1969a). There is considerable conflict between the results of these workers and the results of this survey and of other workers (see Table XII).

A striking example is glutamate. This was reported by Taylor and Hoare (1969a) not to support growth, although it is a substrate commonly used for heterotrophic growth by many workers, and gave high growth rates and yields in this survey.

No explanation can be offered for these differences as all the workers have used the same strain; Starkey's or subcultures thereof (ATCC 8093). Differences could arise during the adaption to autotrophic growth; even this could not sufficiently explain the discrepancies. Chance selection of a "slow" or "fast" strain, as reported by Santer, Boyer and Santer (1959) would give apparent differences in substrate utilisation or non-utilisation, particularly if the comparison was also between solid and liquid media. This would not serve as an explanation for the apparent failure of Taylor and Hoare (1969a) and of Santer, Boyer and Santer (1959) to detect growth on glutamate, glucose and acetate, which in this survey and in others were substrates for rapid, high yield growth.

TABLE XII

GROWTH SUBSTRATES OF T. NOVELLUS: COMPARISON WITH PUBLISHED RESULTS.

	Temperature of Incubation	This Survey 25°C	Taylor & Hoare (1969a) 27°C	Charles (1971a) 30°C	Santer, Boyer & Santer (1959) 30°C	Various
Amino Acids	D/L alanine	3	Weak			
	Aspartic-Acid	2	-		+citrate +++	
	L-Glutamic "	3	-	1.2 hrs	+citrate ++++	
	Histioline	2	+			
	L-Proline	2	+			
	Glycine	0				
	Isoleucine	0	-			
	Leucine	0	-			
	D/L Serine	1	-			
	L-Tryptoptan	1	-			
Other amino acids		mainly +ve			all -ve	
Organic Acids	Acetate	3	+	2.2 hrs	-	+ve (Charles 1969)
	Formate	2	+			
	Propionate	2	Weak			
	Pyruvate	3	+	1.25hrs		
	Succinate	2	-	1.8 hrs	-	
Sugars	Glucose	3	+	2.0 hrs	-	+ve (L.van Caeseele & Lees 1969)
	Maltose	2	+			
	Sucrose	2	-		-	
	Fractose	1	+			
	Lactose	1	-		-	
Alcohols	Ethanol	3	+			
	Glycerol	2	+			
	Mannitol	2	+			
	Methanol	3	+			
Not Determined	Malate		-	2.0hrs	-	
	Citrate		-	2.2hrs	+++	

Considerate difficulties exist in comparison because of the difference in incubation conditions and substrate concentration. An attempt to compare the different results with those obtained in the present survey is given in the Table above.

3.4. POTENTIAL EFFECTORS OF GROWTH OF T. NOVELLUS.

3.41. EFFECT OF VARIOUS ORGANIC COMPOUNDS ON HETEROTROPHIC GROWTH OF T. NOVELLUS ON GLUTAMATE.

3.41.1. Survey of the Effect of Various Organic Compounds on Heterotrophic Growth of T. Novellus, Using Solid Media.

The same organic compounds, at the same concentrations, as in Section 3.31. were mixed to give sodium glutamate agar plates, including the same standardinoculum of autotrophically-grown T. novellus. Plates were incubated at 30 °C in several batches and examined at frequent intervals for growth. At the end of growth colonies were checked for form and the pH determined.

The rate of increase in size class was compared with glutamate only plates; deviations indicated effectors of growth.

Growth in all cases resulted in a large pH increase, generally to pH 10 - 11, with an accompanying smell of ammonia, presumably due to deamination of the preferred substrate, glutamate.

As before colonies were shades of orange in colour, and showed the normal variation of colony form.

The higher concentrations (50mM and 100mM) of most added compounds were slightly inhibitory, but this is probably a non-specific concentration effect. At concentrations of 10mM or less very few of the organic compounds tested, showed any effects. The D-amino acids, and norleucine and citrulline were inhibitory.

All compounds which showed apparently inhibitory or stimulatory effects were tested in liquid culture.

3.41.2. Effect of Selected Organic Compounds on Growth of T. novellus on Glutamate in Liquid Culture.

The following compounds were tested as liquid culture effectors:

100mM:	Leucine	Rhamnose	Succinate	Threonine
10mM:	L-Tryptophane	Threonine	D/L Serine	Raffinose
	Ethanol			
1mM:	Norleucine	Methionine	Thymine	
8.0mM:	Tartrate			
2mM:	Sorbitol			
0.5%	LiLactate			

Lysine: 100, 80, 60, 50, 30 mM

Isoleucine: 10, 20, 50 mM.

Nephelometer flasks containing twenty-five mls. of sterile glutamate medium (Section 3.31) were inoculated with cold-stored autotrophically grown T. novellus and incubated at 25°C while aerated with water-saturated air. Flasks were on occasion, stored at 4°C during incubation; the minor effects on growth resulting from this storage were corrected for, as before.

Growth was followed turbidometrically, and at the end of each run streaks were made for contaminants on both nutrient and thiosulphate agars, and the pH determined.

There was considerable variation in the duration of log-phase. This was due to difficulties in standardising the inoculum as the heavy sulphur sank in the pipette and carried cells downward. The curves are started at zero, as a matter of convenience, while actually there has been at least twenty to forty hours incubation, before this.

25mM Sorbitol gives an acid pH 4.5; all others were within pH 0.5 of the pH at the same turbidity, in glutamate alone.

The following compounds were without effect, in liquid culture:

Leucine	100mM	Isoleucine	10, 20, 50 mM
Norleucine	1mM	Lysine	30, 50, 60, 80, 100 mM
Methionine	1mM	Succinate	100mM
Li.Lactate	0.5%	Thymine	1mM
Rhamnose	100mM	Ethanol	10mM (see Note)

Note: Ethanol was probably removed by sparging-out during aeration and so was completely without effect.

The inhibitory effect of Sorbitol may have been a pH effect: the acid produced by metabolism of sorbitol then preventing further growth.

There are no published results, to act as comparisons, except that Santer, Boyer and Santer (1959) found that citrate stimulated growth on glutamate, probably acting as a complexing agent. In the media used in this survey EDTA from the trace metals solutions acted as a complexing agent, so citrate was not stimulatory.

100mM Threonine was completely inhibitory.

Very few compounds were effective on growth as either stimulators or as inhibitors, and the pH indicates that in most cases glutamate was used as the preferred substrate. The compounds that did effect growth gave curves shown in Figures 12 and 13.

Figure I2 : Key

□ I.0% Glutamate
● " + IO mM D/L Serine
■ " + 8.0 mM Tartrate
▲ " + IO mM Raffinose

Figure 12

EFFECT OF SELECTED ORGANIC COMPOUNDS ON HETEROTROPHIC GROWTH OF *T.NOVELLUS*

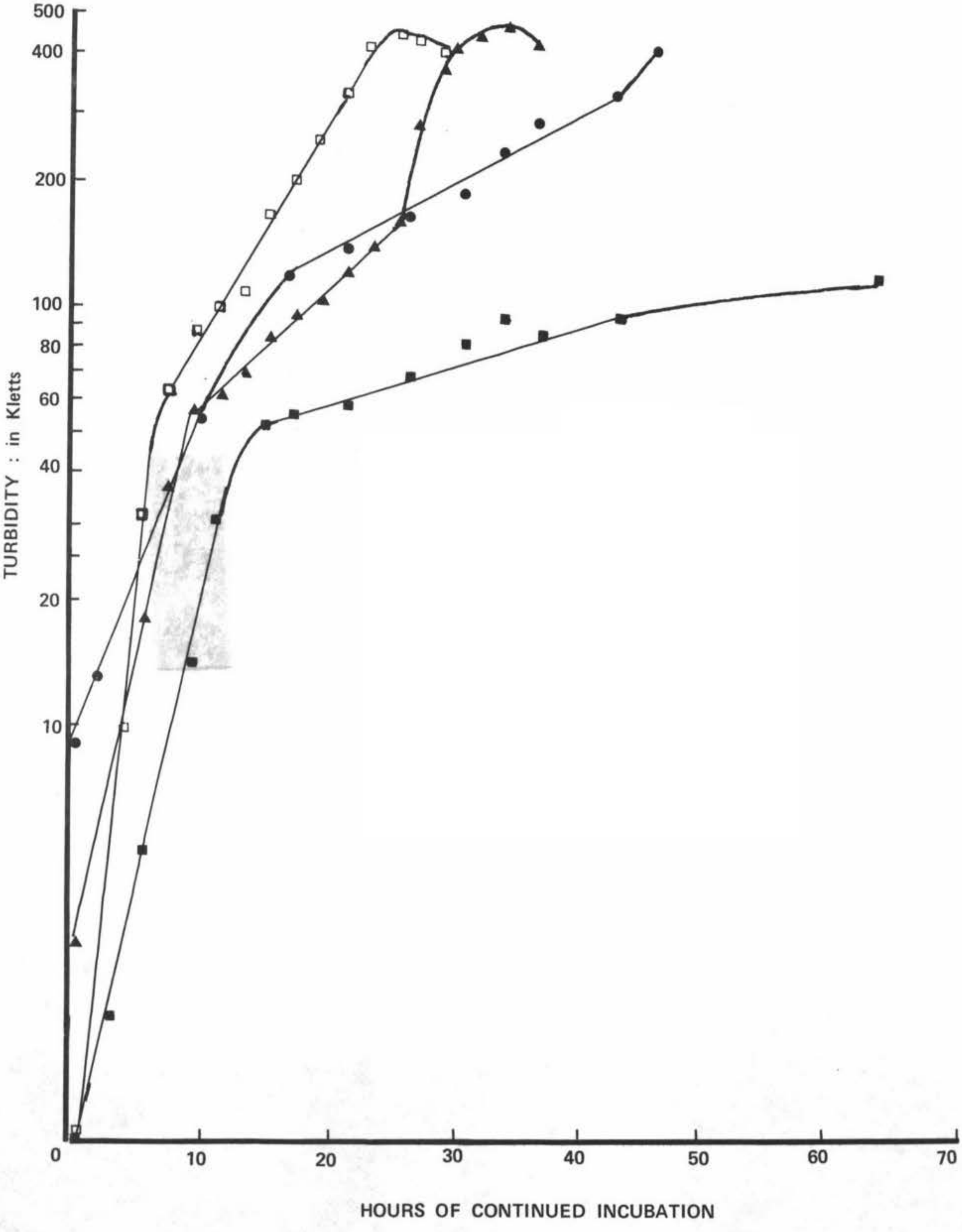
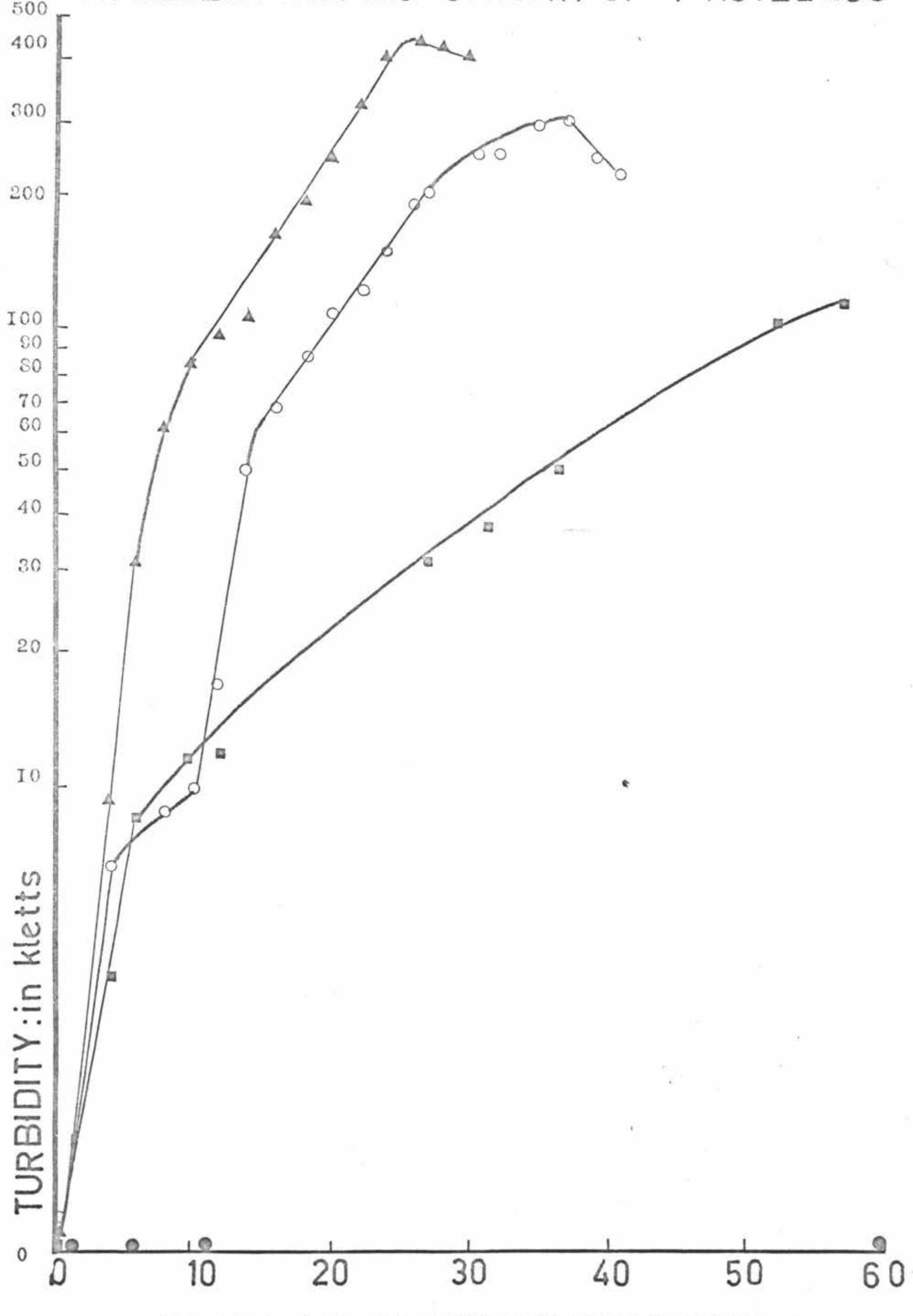


Figure I3 : KEY

▲ I.0 % Glutamate
■ " + I0 mM L-Tryptophane
○ " + I0 mM L-Threonine
● " + I00mM L-Threonine

FIGURE 13
EFFECT OF SELECTED ORGANIC COMPOUNDS ON HETEROTROPHIC GROWTH OF T NOVELLUS



D/L Serine and tryptophane resulted in a marked inhibition of growth in the early log-phase, after a period of growth at the normal growth rate.

Threonine at 10mM gave a similar effect, which was also noted with T. thioparus (Section 3.52.). Again there was little effect in the early log-phase, then a short period of reduced growth rate, and then a resumption of the normal growth rate.

3.42. EFFECT OF VARIOUS ORGANIC COMPOUNDS ON AUTOTROPHIC GROWTH OF T. NOVELLUS ON THIOSULPHATE.

3.42.1. Survey of the Effect of Various Organic Compounds on Autotrophic Growth of T. Novellus Using Solid Media.

The same organic compounds as in Section 3.31. were used, mixed to give T. novellus thiosulphate plates containing the standard inoculum of autotrophically-grown T. novellus. Plates were incubated at 30°C in several batches and examined at frequent intervals for growth. At the end of growth colonies were checked for form and the pH determined. The rate of increase of size class was compared with thiosulphate - only plates and deviations noted.

Autotrophic cultures though small were clearly distinguishable from the small "non-substrate zero" colonies:

(a) They grew only on the surface,

(b) They have a dark, almost black centre with a fringe of blunt-ended long needle-like crystals as against a very pale uncentred colony without fringes

(c) They often, depending on agar cloudiness, have a halo of acid cleared transparent agar.

No compounds were effective on growth at concentrations of 1mM or less.

100mM Threonine was the only completely inhibitory compound, i.e. no growth at all evident on the plate.

Some compounds supressed autotrophic growth.

Some of these supressors did not act as heterotrophic substrates but gave small "non-substrate zero" colonies and so were not completely inhibitory for all growth. These compounds were:

Pyruvate 100mM Xylose 100mM Tyrosine 5.0mM Starch 1.0%

Some of these supressors act as heterotrophic substrates to give substantial sized colonies, or normal heterotrophic appearance. These compounds were:

Acetate	100mM	Glutamine	100mM	Arginine	100mM
Fructose	100mM	Glucose	100mM	Glycerol	70mM
Glutamate	82mM	Maltose	100mM		

The low growth rate meant that the system was too insensitive to detect minor effects; threonine was the only inhibitor.

Pyruvate suppressed autotrophic growth, was not inhibitory, but in the presence of thiosulphate, was not the good heterotrophic substrate it is alone.

The autotrophic supressors that acted as substrates for heterotrophic growth were all good (score 2 or 3) heterotrophic substrates on their own. The converse did not apply. Le John, van Caeseele and Lees (1967) found that the fermentable substrates glucose, glycerol, lactate, lactose, ribose and pyruvate repressed thiosulphate oxidation while the aerobically-utilizable amono acids and organic acids were not repressive.

Threonine was the only total inhibitory of both, or either of, autotrophic and heterotrophic growth.

DISCUSSION.

The main conclusions from this survey of the effect of organic compounds on the growth of the facultative autotroph *T. novellus* are:

(a) that a large number of organic compounds can be used as substrates for growth

(b) that very few organic compounds inhibit growth on glutamate. When supplied in the presence of thiosulphate some acted as normal heterotrophic substrates, whereas others were without effect. The only compound of the wide range tested which inhibited both heterotrophic and autotrophic growth of *T. novellus*, was threonine and even this compound was only inhibitory at relatively high concentrations (10mM or greater cf *T. thioparus*, Section 3.52.)

3.5. EFFECT OF EXOGENOUS ORGANIC COMPOUNDS ON AUTOTROPHIC GROWTH OF T. THIOPARUS ON THIOSULPHATE.

3.51. SURVEY OF THE EFFECT OF ADDED ORGANIC COMPOUNDS ON AUTOTROPHIC GROWTH OF T. THIOPARUS ON SOLID MEDIA.

No extensive survey has been made of the effect of organic compounds on the growth of T. thioparus. Such a survey would form the basis for further investigation of individual compounds, and serve as a comparison with the similar survey already made for T. novellus.

T. thioparus media, as in Section 3.22. was mixed with filter sterilised $NaHCO_3$ at pH 8.0 to a final concentration of 2mM. When set, a standard inoculum of 60 - 150 colonies, of cold-stored T. thioparus, grown on thiosulphate and checked on nutrient and glutamate agars for freedom from heterotrophic contaminants, was spread over the surface of the plate. After drying the plates were inverted and incubated at 30°C.

Plates were incubated in several batches and examined at several intervals for growth. At the end of the run colonies were examined and then streaked on to T. thioparus thiosulphate agar. The pH was also determined.

From comparison with the thiosulphate-only agar the colonies were classed as normal, partially or completely inhibited, or as stimulated. The results are shown in Table XIII.

There was no evident pH change within the limits detectable by pH papers.

Colonies were the usual autotrophic form, although on some partially inhibitory substrates the colonies were clearly abnormal:

α-ketoglutaric acid	10mM	D-lactose	10mM
L-proline	2.1mM		

3.52. EFFECT OF SELECTED ORGANIC COMPOUNDS ON AUTOTROPHIC GROWTH OF T. THIOPARUS IN LIQUID MEDIA.

The following compounds selected from Table XIII were tested as effectors in liquid culture:

10mM	Isoleucine	Succinate	Citrulline		Trehalose
1mM	Histidine	Raffinose	D-Galactose		
0.1mM	Thymine	Benzoate	Uracil		
0.01mM	Hypoxanthine	.05mM	Tyrosine	8.2mM	Glutamate
11.4mM	Asparagine	.05% Li.Lactate.			

TABLE XIII

EFFECT OF ADDED ORGANIC COMPOUNDS ON GROWTH OF T. THIOPARUS ON SOLID MEDIA.

	Plate Concentrations			
Amino Acids	100mM	10mM	1mM	.1mM
Arginine	NG	pI	pI	
Asparagine	NG	pI	pI	
Citrulline	NG	pI	pI	
Glutamine	NG	pI	NE	
D/L Alanine	NG	NG	pI	
Glycine		NG	pI	
Histidine		NG	pI	NE
Norleucine		NG	pI	NE
Phenylalanine		NG	pI	NE
D/L Serine		NG	pI	pI
L-Tryptophane		NG	pI	NE
L-Valine		NG	pI	pI
Cystine			NG	NE
L-Threonine		NG	NG	pI

No Effect ((at even the highest concentration (given) tested))

Aspartic Acid	10mM	L-Proline	2.1mM	Tyrosine*	.5mM
Leucine	10mM	L-Serine	10 mM	Lysine	100 mM
Methionine	10mM	D/L Tryptophane	1 mM	Ornithine	100 mM

Organic Acids	100mM	10mM	1mM	.1mM	.01mM
Acetate		St	NE		
Benzoate			NG	pI	pI
Formate	NG	NE	NE		
Propionate	NG	NG	pI		
Succinate	NG	pI	pI		
K tartrate	NG(80)	St(8)	O(0.8)		

No Effect

Li.Lactate	.0%	Pyruvate	10mM

TABLE XIII (Continued)

Bases and Related Compounds	100mM	10mM	1mM	.1mM	.01mM
		Plate Concentrations			
Adenine			NG	pI	
Hypoxanthine		NG	pI	pI	
Guanine			NG	NE	
Xanthine			NG	pI	pI

No Effect

Creatine	10mM	Thymine	1mM	Uracil	1mM

Sugars	100mM	10mM	1mM	.1mM
Arabinose	NG	NE	pI	
Fructose	NG	pI	pI	
D-Glucose	NG	pI	NE	
D-Lactose	NG	pI	NE	
Mellobiose	NG(25)	pI(2.5)	NE(0.25)	
D-Raffinose		NG	pI	NE
D-Rhamnose		NG	pI	pI
Sucrose	NG	pI	pI	
Starch	NG(1.0%)	NE(0.1%)	pI(.01%)	
Trehalose	NG	pI	pI	
D-Xylose	NG	pI	pI	

No Effect

Maltose	10mM	Mannose	10mM

Alcohols	100mM	10mM	1mM	.1mM
Glycerol	NG	pI	pI	
Salicin		NG	pI	pI
Sorbitol		NG(2.5)	pI(.25)	pI(.025)

No Effect

Dulcitol	100mM	Mannitol	100mM	Methanol	100mM
Isopropanol	100mM				

NG: No Growth   pI: Partially inhibited, reduced growth

NE: No effect   St: Stimulated

* Tyrosine agar was purple; probably, as previously mentioned, with oxidation products. Figures in brackets are concentrations other than those at column heads: in mM unless given as percentage. The concentrations after the substrate in the No Effect tables are the highest concentrations tested.

Selected organic compounds and the required components, as in Section 3.31. were mixed in sterile nephelometer tubes and inoculated with cold-stored. T. thioparus known to be free of heterotrophic contaminants. Flasks were incubated in a stream of water-saturated 5% CO_2 : 95% air. One batch was incubated at the normal 30°C; another batch was incubated at 25°C, in an attempt to increase sensitivity of measurement by decreasing the growth rate.

Turbidity was followed turbidometrically. At the end of growth the pH was determined and streaks made on nutrient and glutamate agars to test for heterotrophic contaminants.

In most cases there was only a minor variation in log-phase duration, but the variability in readings of less than ten Kletts meant that curves below this figure may easily not be true representations. Curves are therefore drawn from the same zero time on the graph, although the flasks had been incubated for some time in the solution.

Of the compounds tested the following compounds were without effect:

Hypoxanthine	.01mM	D-Galactose	1mM	Raffinose	1mM
Asparagine	11.4mM	Trehalose	10mM		

0.05% Lactate was completely inhibitory.

A complex range of growth curves was obtained, of which a selection of representative types is shown in Figures 14, 15 and 16. Accepting the wide variations in the low turbidity readings as not true, examination of the curves shows that the same two phase effect found for T. novellus is present here. An increased or decreased final yield may also occur.

There is a period of greatly reduced growth rate, which occurs at some stage during the log-phase; in many cases the organism resumes rapid growth again after a period which varies greatly from one organic compound to another.

This effect is duscussed later.

3.53. DETAILED EXAMINATION OF THE GROWTH CURVE OF T. THIOPARUS ON THIOSULPHATE IN THE PRESENCE OF SUCCINATE.

Succinate was chosen as the inhibitor for a detailed study of the effect of an inhibitor on thiosulphate utilisation, pH change, and growth. The effect of the same inhibitor on the distribution of label from various radioactive compounds is discussed later (Section 4).

Figure I4 : KEY

△ Standard
▲ " + 0.ImM Thymine
○ " + 0.I mM Benzoate
■ " + 0.I mM Uracil
● " + 0.05 mM Tyrosine

Figure 14

EFFECT OF ADDED ORGANIC COMPOUNDS ON GROWTH OF *T.THIOPARUS* : at 30°C

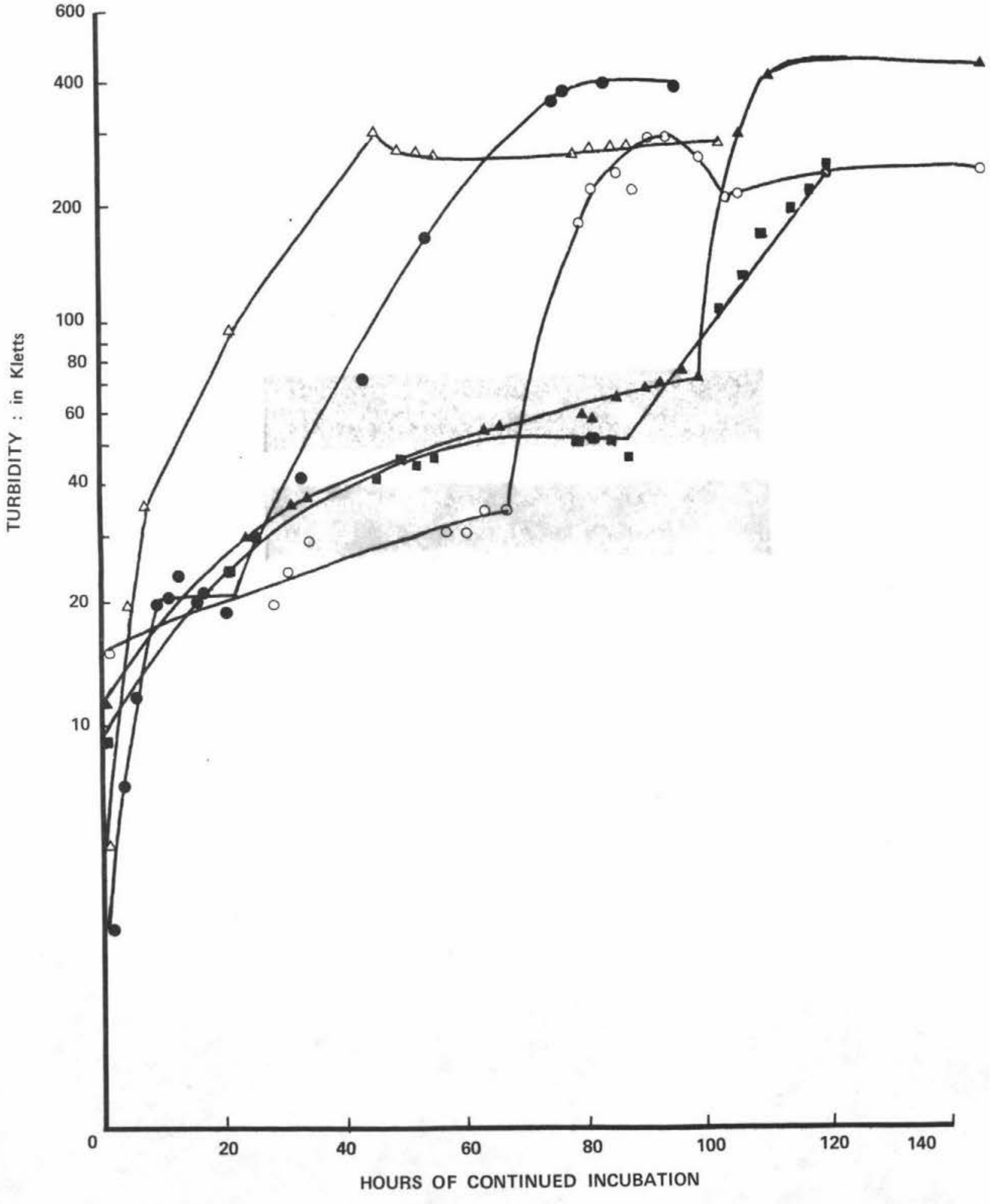


Figure I5 :KEY

▲ Standard

□ " + I0 mM Isoleucine

● " + I0 mM Succinate

figure 15

EFFECT OF ADDED ORGANIC COMPOUNDS ON GROWTH OF T.THIOPARUS:at 25°C

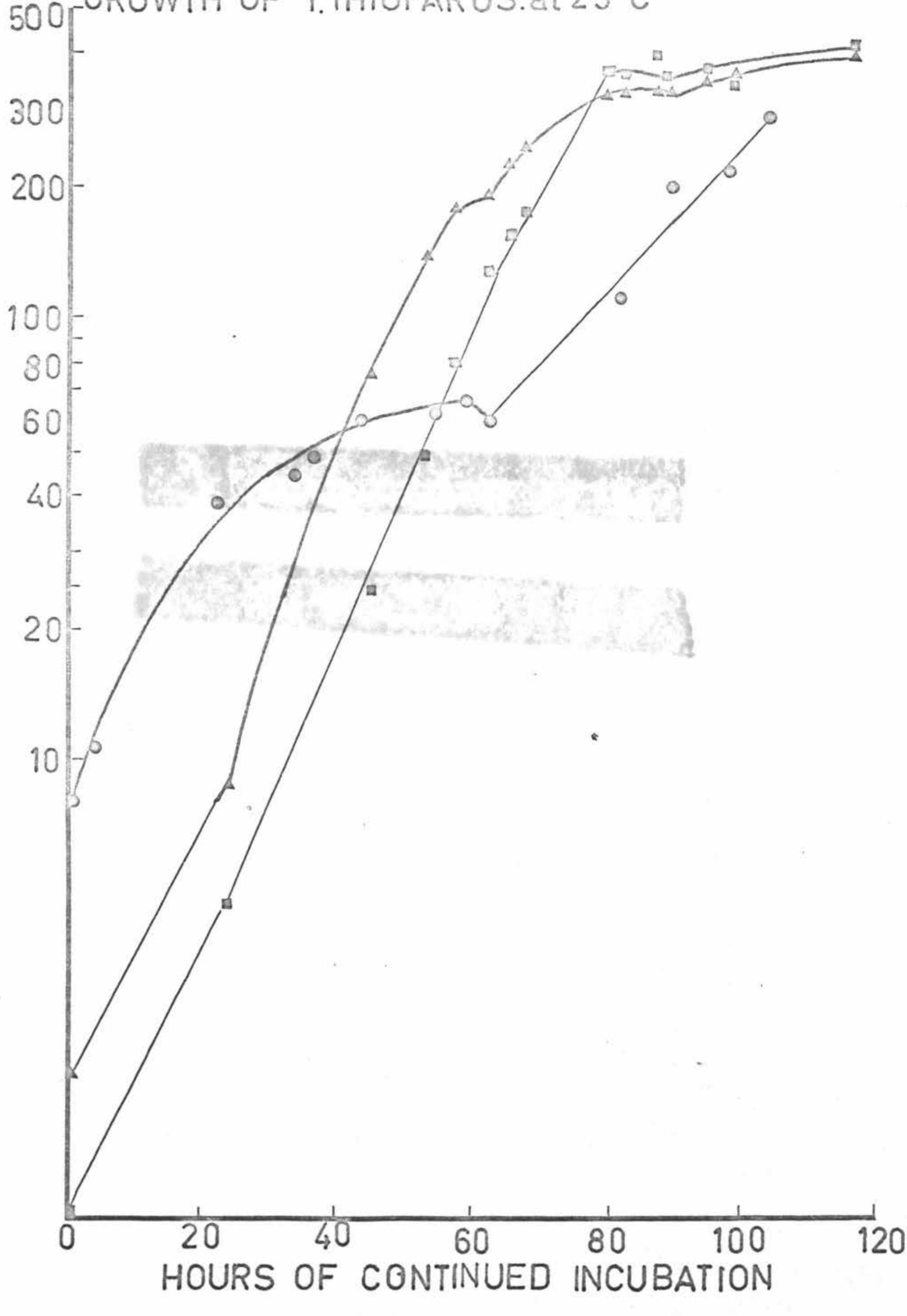
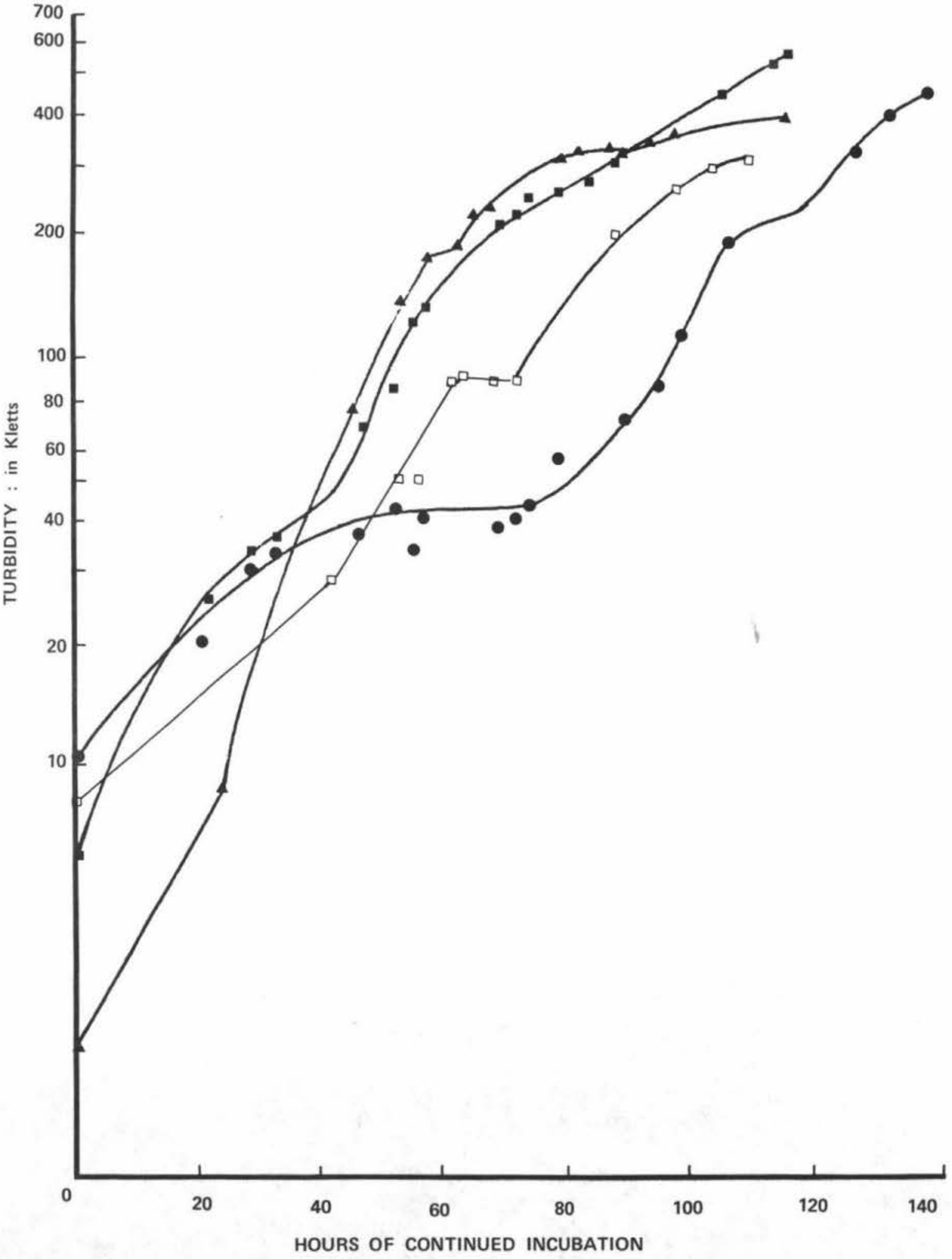


Figure I6 : KEY

▲ Standard
□ " + I mM Histidine
● " + I0 mM Citrulline
■ " + 8.2 mM Glutamate.

Figure 16

EFFECT OF ADDED ORGANIC COMPOUNDS ON GROWTH OF *T.THIOPARUS* : at 25°C



Succinic acid was dissolved in minerals salts solution to give a final concentration of 1.87% w/v (16mM), in the same medium as in Section 2.34. and similarly autoclaved. The incubation conditions sampling and analysis methods are the same as in Section 2.34. The experiment was not run to completion.

As in normal growth of T. thioparus, viable counts were erratic and never exceeded a low percentage of direct counts.

Results are shown in Figures 17 and 18.

That an inhibition exists is quite clear. Turbidity increase, pH change, and thiosulphate consumption occur much more slowly in the presence of succinate.

The relationship between pH and thiosulphate consumption noted for normal growth does not occur: there is still considerable thiosulphate remaining when the pH peaks. Despite the lower initial thiosulphate concentration the pH rise is much the same as for uninhibited growth (Section 2.34.). The initial increase in titratable thiosulphate observed earlier was absent.

This would suggest that the utilisation of thiosulphate does not produce asmuch acid as in the uninhibited system.

3.54. DISCUSSION.

3.54.1. Comparison with Published Results.

Some of the obvious differences shown in Table XIV could be due to differences between strains (Kelly 1971a), as each worker has used a different strain. Other differences could arise from different experimental techniques. The survey of Lu, Matin and Rittenberg (1971) was performed using liquid cultures at high cell concentrations, so there may be an actual qualitative difference.

The most obvious difference is with L-threonine which only this study found to be inhibitory. Minor differences occur with cystine, glycine and phenylalanine.

Figure I7 : KEY

Succinate Inhibited

■ Thiosulphate

● pH

Uninhibited

□ Thiosulphate

○ pH

Figure 17

EFFECT OF SUCCINATE ON GROWTH OF *T.THIOPARUS* : pH and Thiosulphate Concentration

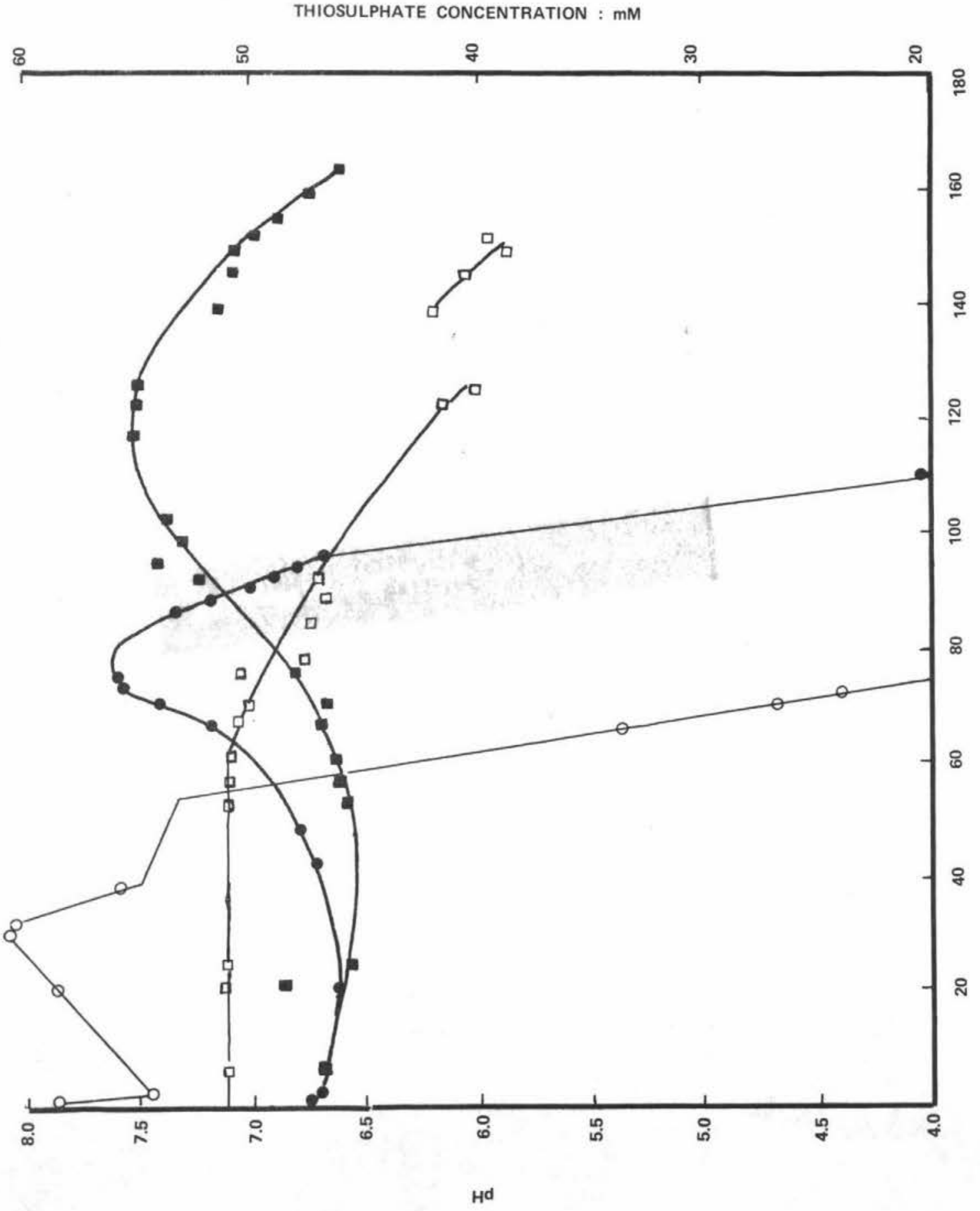


Figure I8 : KEY

- ● Uninhibited
- ▣ Inhibited

figure 18
EFFECT OF SUCCINATE ON GROWTH
OF T. THIOPARUS: Turbidity

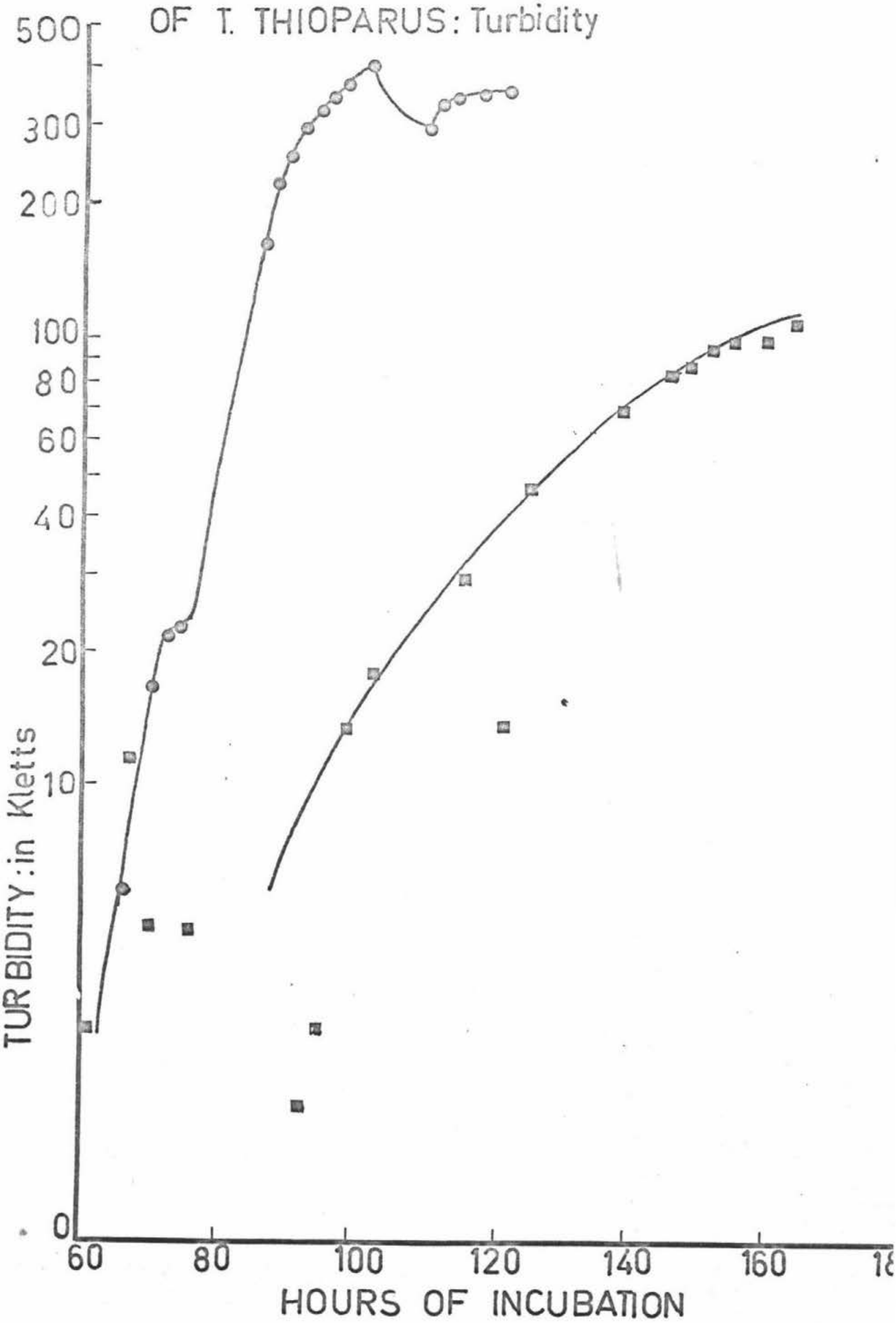


TABLE XIV

EFFECT OF ADDED ORGANIC COMPOUNDS ON GROWTH OF T. THIOPARUS: COMPARISON WITH PUBLISHED RESULTS.

		References			This Survey			
		Rittenberg (1969)	Lu, Matin & Rittenberg	(1971)	Solid Media		Liquid	
	Compound	10mM	10mM	1mM	10mM	1mM	mM Con.	
Amino	Alanine		pI	NE	* NG	pI	1.0	pI
Acids	Arginine	NE	NE	NE	pI	pI		
	Asparagine		NE	NE	pI	pI	11.4	NE
	Aspartic Acid	NE	NE	NE	NE	NE		
	Citrulline				pI	pI	10	pI
	Cystine		I	NE		NG	.5	pI
	Glycine		I	NE	NG	pI	1	pI
	L-Glutamate	pI	pI	pI	ND	ND	8.2	pI
	L-Glutamine		NE	NE	pI	NE		
	Histidine	NE	NE	NE	ND	ND	1	pI
	Isoleucine		NE	St	ND	ND	10	NE
	Leucine		NE	NE	NE	NE		
	Norleucine				NG	pI	1	pI
	Lysine	NE	pI	NE	NE	NE		
	Methionine	NE	pI	NE	NE	NE		
	Ornithine				pI	NE		
	Phenylalanine	NG	NG	NG	NG	pI	.1	pI
	L-Proline		NE	NE		NE(2.1)		
	L-Serine		NE	NE	NE	NE		
	D/L Serine				NG	pI	.1	pI
	L-Threonine	NE	pI	NE	NG	NG	.1	pI
	D/L-Tryplophone					NE		
	L-Tryptophane			NE	ND	ND		
	Tyrosine			pI		NE(.5)	.05	pI
	Valine	NG	St	NE	NG	pI		

TABLE XIV (Continued)

		Rittenberg (1969)		Pan and Umbreit 1972(a) 1 mM	This Study Solid Media 100mM
Organic Acids	α-Ketoglutarate			NE	
	Malate	(100)St			
	Pyruvate	(5.0)NE		NE	
	p-OH Phenyl-Pyruvate			pI	
	Succinate	(100)St			NG

* For this survey D/L Alanine was used.

NG: No growth, totally inhibitory

I: Inhibitory

pI: Partly inhibitory

NE: No Effect

ND: Not Determined

St: Stimulatory

Figures in brackets are concentrations in mM, not those at the table head.

3.54.2. Comparison with Results for T. Novellus.

Heterotrophically there were only a few minor effectors of T. novellus growth; and threonine was the only inhibitor. With autotrophic growth only threonine was completely inhibitory. Some compounds were supressors of autotrophic growth. Most of these were very good heterotrophic substrates and with the exception of pyruvate gave good heterotrophic growth in the presence of thiosulphate.

T. thioparus is inhibited by a wide variety of compounds; although a sufficient number of organic compounds were without effect, to make the generalisation that all are toxic (Winogradsky 1890) invalid. This insensitivity of the facultative autotroph and the sensitivity of the obligate autotroph is the most obvious distinction between the responses of the two species to exogenous organic compounds.

3.6. REVERSAL OF THREONINE INHIBITION OF AUTOTROPHIC GROWTH OF T. THIOPARUS.

Threonine which caused inhibition of T. novellus and T. thioparus was selected for further study. If, as suggested in the Introduction, many amino-acids affect the obligate autotroph via the same mechanisms by which they inhibit heterotrophs, then the inhibition may well be reserved by a variety of metabolically related compounds.

Using a concentration of threonine known to be inhibitory in solid media, plates were spread so as to give a lawn of T. thioparus if growth occurred. Various compounds were placed in the plates so as to give a series of pair-wise interactions. Single compound effects would be shown by a ring of growth around the application spot. Pairwise interactions, depending on their nature, would be revealed either by an arc joining the spots or a line between the spots, at right angles to the common axis.

Plates containing threonine to a final concentration of 0.5mM were set up as in Section 3.22. for T. thioparus. A suspension of T. thioparus sufficient to form a lawn on the agar, and free of heterotrophic contaminants, was spread on the plate surfaces and allowed to dry. When dried, holes were cut in the agar with an alcohol sterilised cork-borer; aliquots of selected sterile organic compounds, prepared as in Section 3.21. were placed in the wells, according to a pattern drawn to give all possible pairwise interactions.

Plates were incubated at 30°C, until growth was evident. During this period the well contents (0.2 to 0.4 mls. of solution) were completely absorbed.

Controls were also run and showed that 0.5mM threonine was completely inhibitory.

Photographs were taken to record the results. An example is shown in Plates F.

Reversal of the inhibition is shown in the table:

Releasor.	Conc.	Effect	Score	Total
Methionine	2.5mM	Permits growth	2 +ve; 1 doubtful	3
Isoleucine	250 "	" "	5 +ve	5
Casamino Acids	.25%	" "	2 +ve; 2 doubtful	4
Valine	2.5mM	Doubtful	4 Doubtful	4
Aspartic Acid	"	"	3 "	3
Cystine	"	No Effect	1 No Effect 3 Doubtful	4
Asparagine	2.9mM	" "	2 " " 2 "	4
D.A.P.	25 "	" "	4 " " 1 "	5
Arginine	"	" "	3 " " 2 "	5
Lysine	250mM	" "	3 "	3

Two selected plates, illustrating the effects, are shown in Plates F. .

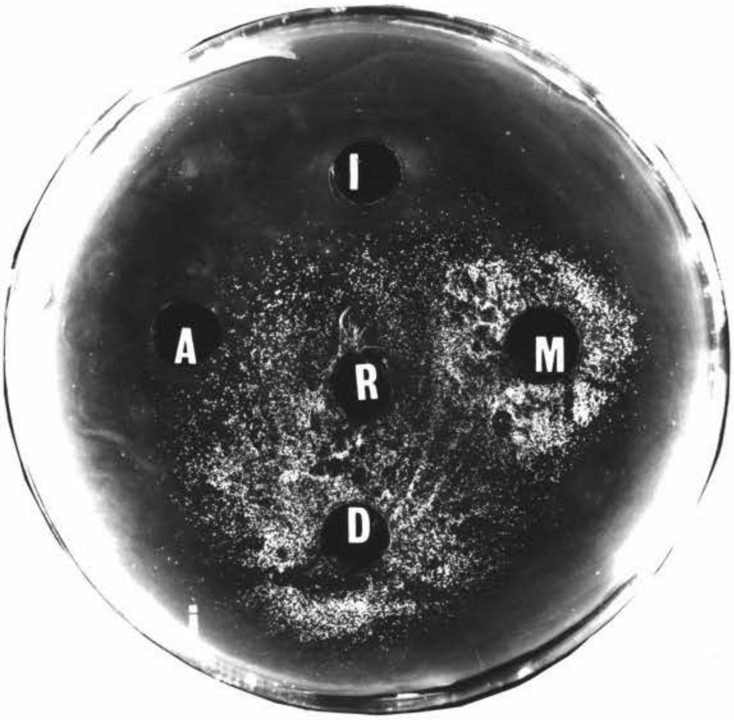
The close packed nature of the wells and the efficiency of reversal of some of the effectors, by themselves, may have obscured some interaction.

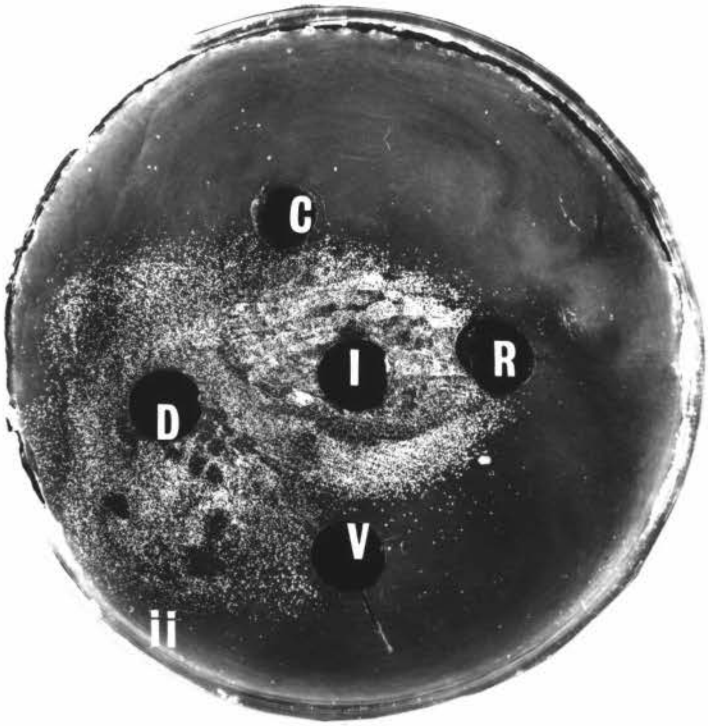
Lu, Matin and Rittenberg (1971) have also investigated the mechanism of inhibition by amino acids of T. thioparus. They found that casein hydrolysate (i.e. casamino acids) reversed completely or partially the inhibitory effects of amino acids, and that the L-serine inhibition of T. thioparus was partially reversed by L-threonine and completely reversed by either L-valine or L-isoleucine.

It is also noteworthy that the inhibition-reversors, methionine, isoleucine, and valine, belong with threonine to the postulated Group ; a set of amino acids with a common transport mechanism in T. neapolitanus (Kelly 1969b). Other members of the same group were not tested. Members of Group II, a separate transport mechanism, were without effect.

Plates F.

REVERSAL OF THREONINE INHIBITION OF T. THIOPARUS: TYPICAL EXAMPLES





KEY

C: Casamino acids
V: L-valine
I: isoleucine
A: aspartic acid
M: methionine
R: arginine
D: D.A.P.

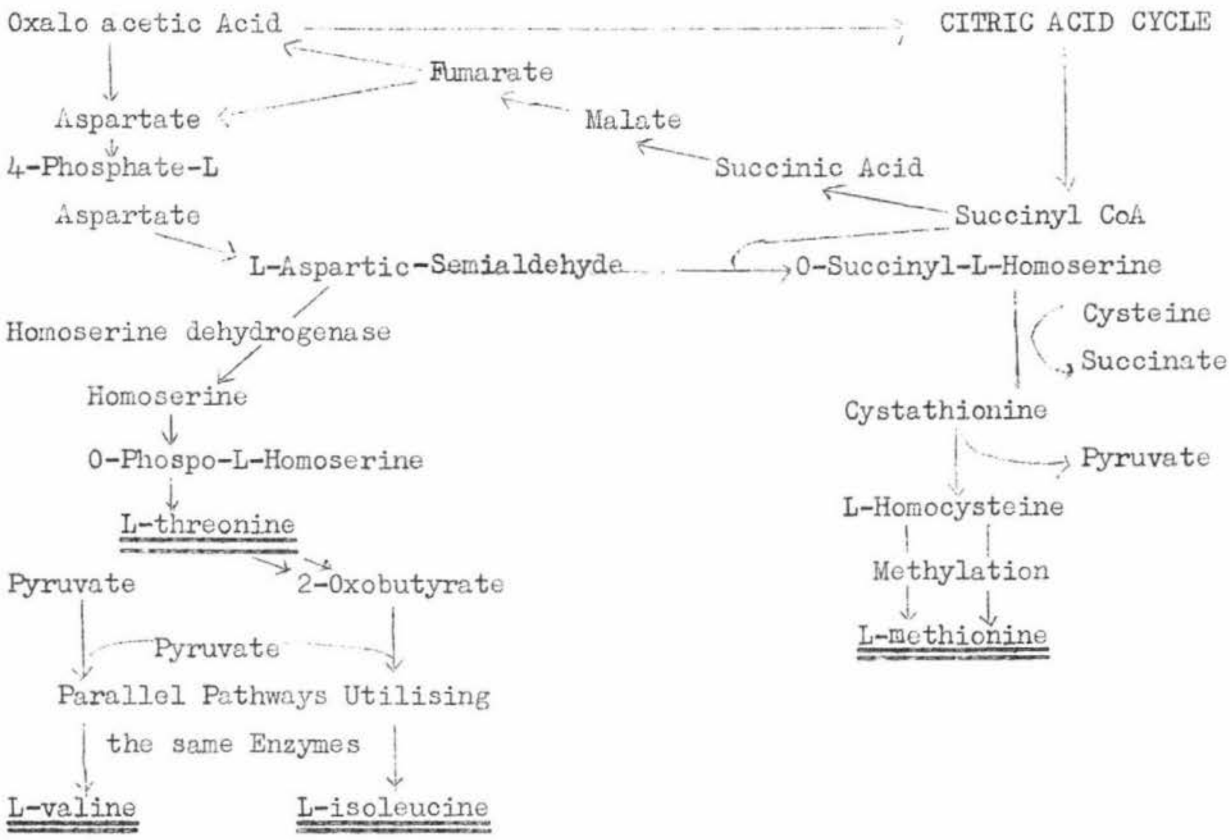
In the obligate Methylotroph, Methylococcus capsulatus, Eccleston and Kelly (1972a) found that the inhibition by 1.0mM threonine was reversed by L-methionine, L-alanine and L-valine but not L-lysine, D.A.P. or L-arginine; this was suggested to be related to the effect on homoserine dehydrogenase.

Figure 19 shows the biochemical relationships of the compounds that reverse L-threonine inhibition. The fact that these act singly with full effect rather than only in concert or in some combination is difficult to explain in terms of this relationship.

Further investigation is needed to establish the mechanism but sufficient has been shown to establish that the inhibition effect of threonine is fully compatible with the normal heterotrophic metabolism. As suggested by Lu, Matin and Rittenberg (1971) and Kelly (1971), the inhibition by amino acids of obligate autotrophs is analogous to that of heterotrophs and requires no special or unique mechanism.

FIGURE 19.

BIOCHEMICAL RELATIONSHIPS OF AMINO ACIDS, METHIONINE, ISOLEUCINE, L-VALINE, ETC.



4. UPTAKE AND METABOLISM OF ^{14}C-LABELLED ORGANIC COMPOUNDS BY T. NOVELLUS AND T. THIOPARUS.

4.1. INTRODUCTION

Although there is a certain amount of information on the metabolism of both T. thioparus and T. novellus, most of this has been derived from studies on enzyme activities in cell extracts. There have been few comparative studies using labelled organic compounds, apart from that of Smith, London and Stanier (1967). Some studies of the uptake and metabolism of labelled compounds have been made with other autotrophs.

In the study of Smith et al. (1967) autotrophically-grown T. thioparus and T. thio-oxidans were compared with heterotrophically-grown T. intermedius. In the present study it was proposed to make three types of comparison:

(a) between autotrophically and heterotrophically-grown T. novellus

(b) between autotrophically-grown T. novellus and T. thioparus

(c) between T. thioparus grown autotrophically in the presence and absence of 16mM succinate, an inhibitor.

Isotopically-labelled compounds used were ^{14}C- acetate, -glutamate, -glucose and CO_2: very low levels of tracer, without carrier, were used in order to avoid inhibitory effects and the induction of metabolic adaptations.

4.2. METHODS.

4.21. CULTIVATION AND HARVESTING.

4.21.1. T. novellus.

Heterotrophically-grown Cells. Autotrophically-grown T. novellus was inoculated into 1.0% glutamate mineral-salts solution (Section 2.23.7.) and aerated with water-saturated air while shaken at 12 cycles per minute at 25°C. The heterotrophically-grown cells were harvested in mid-log phase (20-40 hours after the first appearance of detectable turbidity).

Autotrophically-grown Cells. Autotrophically-grown T. novellus was inoculated into 1.5% thiosulphate mineral-salts solution (Section 2.23.7.) and aerated with water-saturated air at 25°C for 14-28 days.

In both cases the resultant suspensions were centrifuged at $4x10^3$ g for 20 minutes. Cells were washed in fresh sterile medium and recentrifuged.

The cells were finally resuspended in fresh sterile medium.

4.21.2. T. thioparus.

A T. thioparus 1.5% thiosulphate mineral-salts solution (Section 2.3.) was inoculated with cold stored T. thioparus, known to be free of heterotrophic contaminants, and incubated in the apparatus shown in Figure 6, at 30°C while aerated with water-saturated 5% CO_2: 95% Air. Cells were harvested in mid-log phase (20-40 hours after turbidity first became apparent).

Succinate-inhibited cells were harvested from a similar medium containing 16mM succinate incubated similarly, also harvested in mid-log phase (60-100 hours after turbidity first became apparent).

The cell-suspensions were centrifuged at $4x10^3$ g for twenty minutes. The sulphur present formed a hard cake at the lower edge of the centrifuge bottle and was overlaid with a pink layer of cells. The cell layer was suspended in fresh sterile medium pipetted actively at the cells. The cell-suspension was transferred to sterile centrifuge tubes and centrifuged at $2x10^3$ g for ten minutes. Again the cells were separated from the sulphur, and were resuspended in fresh sterile medium by agitating with a sterile wire loop. The transfer, centrifugation and suspension were repeated.

4.22. INCUBATION CONDITIONS.

4.22.1. Incubation Apparatus.

The apparatus used is shown in Figure 20. A glass ultrasonicator cup (100ml, 25x100mm) containing between 15 and 70 mls of cell suspension, depending on the number and size of samples to be taken, was fitted with the bung, and the acid/alkali feed and aerater adjusted so as to be as close to the bottom as possible. The alcohol-sterilised combination pH probe was inserted and adjusted similarly. The aeration rate was adjusted to give a vigorous rate so that mixing was good. Exhaust gas passes through two successive 5.0 ml quantities of 18% v/v ethanolamine: water to trap CO_2 (which it does with high efficiency). The acid/alkali feed was adjusted so that the magnetic valve released the minimum addition of acid or alkali at each adjustment of pH. When this was done, all joints were sealed with finger-nail polish and the bung wired down. The pH probe and the magnetic valve were connected to a Radiometer 101 pH Stat set at the appropriate pH and to minimal adjustment time and closest pH range.

4.22.2. Experimental Procedure for Heterotrophic T. novellus.

Preliminary experiments showed that suspensions of T. novellus in 1.0% glutamate media, where aerated at 25°C, did not show a turbidity increase for some time (6-8 hours) although the pH dropped slightly during this period. The growth of the culture was accompanied by a large increase in pH.

As the pH increase affected the uptake of label in preliminary experiments the pH must be controlled; the drop in pH during the lag-phase means that a preincubation is necessary.

The pH at which the optimum growth rate was obtained was with phosphate buffer pH 8.0 (Section 2.23.4.) so the pH was controlled at pH 7.8 with acid addition by the pH-Stat.

A suspension of washed T. novellus glutamate-grown cells in glutamate media was adjusted to a turbidity of around Klett 100, and then incubated at 25°C, while aerated with water-saturated CO_2-free air, for six to eight hours.

An appropriate quantity of incubated suspension was placed in the cup of the incubation apparatus, the pH-Stat was set to pH 7.8, acid was placed in the reservoir and the pH of the incubator contents adjusted to pH 7.8. A squirt of aerosol Antifoam A (Dow-Corning) was added to prevent frothing.

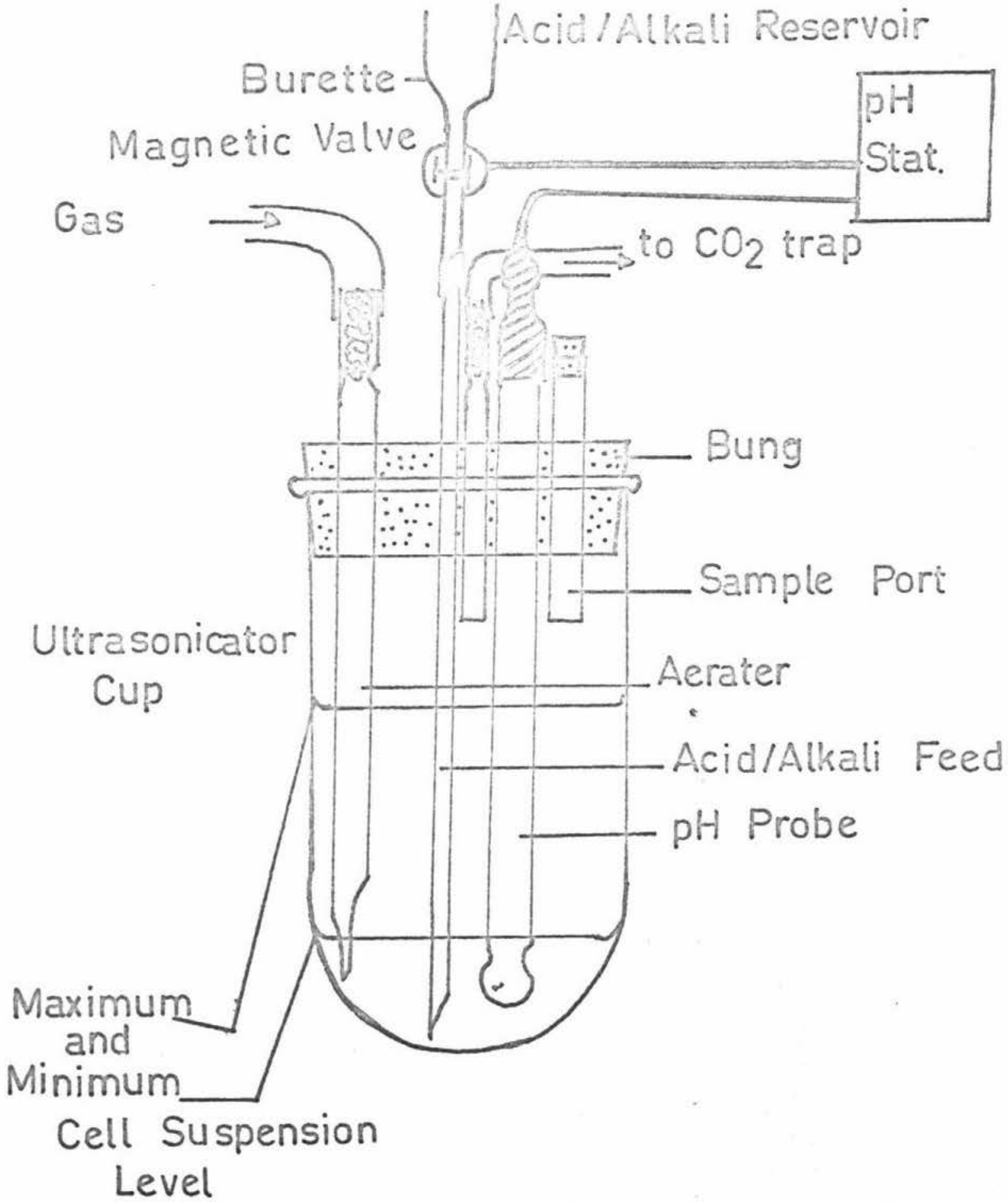
4.22.3. Experimental Procedure for Autotrophic T. novellus.

A suspension of autotrophically-grown T. novellus in autotrophic medium was placed in the cup. After aeration for about an hour, with water-saturated air, at 25°C, the experiment was started. The pH was recorded but not controlled as only a minor increase in pH occurred during the course of the experiment. Aeration was continued during the experiment.

4.22.4. Experimental Procedure for T. thioparus.

The suspensions of T. thioparus in the appropriate medium were incubated overnight at 30°C while aerated with water-saturated 5% CO_2:95% Air. This was required by the prolonged lag-phase and the initial pH increase that occurred, somewhat similar to the situation during growth (see Section 2.34.2.). With an initial cell density giving a turbidity of around Klett 100, the lag period, until the pH began to drop, was 12-14 hours for normal T. thioparus and 18-20 hours for succinate inhibited T. thioparus. After this the appropriate quantity was placed in the cup. The pH-Stat was set to pH 6.8 (and to the most rapid response and most narrow pH limits)

FIGURE 20
INCUBATION APPARATUS: for ^{14}C
Radioactive Compounds Experiments



and alkali was placed in the reservoir. When continued incubation brought the pH down to pH 6.8 it was maintained at this level and the experiment started.

4.23. SAMPLING AND EXTRACTION PROCEDURES.

All experiments with radiochemicals, save for those involving $^{14}CO_2$ uptake by T. thioparus, were run for six hours. At zero time (i.e. time of addition of the ^{14}C-labelled compound) and at subsequent fifteen minute intervals 0.2 ml samples were removed, by pipette, through the sample port of the apparatus (Figure 20).

The zero-time 0.2 ml sample was placed into toluene, to kill cells, to give a total label when counted. Other samples were filtered on to 0.22 µm membrane filters, under suction, and washed with about 1.0 ml of appropriate sterile medium, also under suction. The process was very rapid: from sample to finish of wash took about one minute. The membrane filters were removed to a ring holder, several drops of toluene added to kill the cells, and the whole was then dried under a U.V. lamp.

5.0 ml samples were removed after 0.25, 0.5, 1.0, 1.5, 2.0, 3.0 and 6.0 hours from the commencement of the experiment. These were removed with a bulb-pipette, also through the sample port, into sterile Klett tubes in which the turbidity was determined. At the same time the amount of acid/alkali removed from the reservoir during the course of the experiment, or the pH in the case of autotrophically-grown T. novellus, was recorded.

The two ethanolamine/water CO_2 traps were also replaced at the same time.

4.23.1. Procedure for Determining Distribution of ^{14}C in Various Cell Fractions.

The 5.0 ml aliquots of radio-active cell suspension were spun at 4×10^3 g for ten minutes, resuspended in fresh sterile chilled medium and respun. The supernatant was stored frozen, after a 0.2 ml sample had been removed for counting. The second supernatant was discarded and the cells drained, before being resuspended in boiling 70% ethanol. This concentration of alcohol was found to be the most efficient for extraction.

A 0.5 ml sample of this cell suspension in ethanol was counted to give the TOTAL CELL FRACTION and the remainder was spun at $2x10^3$ g for ten minutes. The supernatant was removed: 0.5 mls counted as CELL EXTRACT FRACTION and the remnant stored in the freezer. The ethanol-insoluble cell residue was resuspended in 1.2 mls of water. A 0.2 ml sample of this was counted as the CELI RESIDUE FRACTION and the remnant frozen.

Where calculations indicated that the labelling level was sufficiently high to give detectable spots on radio-chromatograms, the CELL RESIDUE FRACTION was thawed and transferred with 2.0 mls of concentrated HCl (pronalys-BDH) (to give a final concentration of 8N HCl) to a vial. This was sealed under suction and autoclaved overnight (for at least twelve hours) at 121°C (15 lbs./sq.in. or $72KN/M^2$) (Smith, London and Stanier 1967) to give an acid cell hydrolysate in which lipid components and amino-acids are the major constituents. A 0.2 ml sample of this was counted as the CELL HYDROLYSATE FRACTION. 30µl was spotted on to chromatography plates.

The stored alcohol extract was dried in an air stream in a warm oven (40-60°C) and the dry film redissolved in 0.4 mls of water. 0.2 mls of this was counted as RECONSTITUTED EXTRACT: 40µl of this was spotted on to chromatography plates.

Extraction of Poly-β-Hydroxybutyrate. A method adopted from Williamson and Wilkinson (1958) was used.

Reagents. Alkaline Hypochlorite. 200 gms of bleaching powder was ground with distilled water and the volume brought to 1 litre. One litre of 30% w/v Na_2CO_3 was then added with stirring. After standing for 2-3 hours, shaken at intervals, the mixture was filtered. The filtrate was adjusted to pH 9.8 with conc. HCl, warmed to 37°C and refiltered. This solution was stored at 4°C.

Method. The 1.0 ml remnant of CELL RESIDUE FRACTION was thawed at 37°C and mixed with 5.0 mls of alkaline hypochlorite and then incubated at 37°C for ninety minutes. The mixture which had cleared considerably as most cell components dissolved, was spun at $2x10^3$ g for ten minutes. The supernatant was counted as ALKALINE HYPOCHLORITE FRACTION and contains most of the cell consituents apart from poly-β-hydroxybutyrate. To the drained precipitate 0.4 ml of boiling chloroform was added. This was spun at $2x10^3$ g for 15 minutes and 0.2 mls counted as CHLOROFORM EXTRACT; this represents the poly-β-hydroxybutyrate. There may be some loss of ^{14}C-compounds as the residue from the second centrifugation.

While not entirely specific this method has the advantage of ease.

Calculations. The dilution due to the acid/alkali added was corrected for in presenting the figures for the various fractions determined.

A further allowance was made in the dpm errors for pipetting errors. These were estimated as being equivalent to 1/2 the smallest division on the pipette.

Radio-active Compounds. All radio-active compounds were obtained from the Radiochemical Centre (Amersham).

L-Glutamic acid (uniformally labelled and chromatographically pure); 1µCi was used in each experiment. The specific activity was 10.0µCi/µmol. Hence the final concentrations were between 3 and 5µM.

Na acetate-^{14}C (uniformally labelled and chemically and chromatographically pure); 10µCi was used in each experiment. The specific activity was 57µCi/µmol. Hence the final concentration was between 2.2 and 9.8µM.

D-Glucose (uniformally labelled and chromatographically pure); approximately 10µCi was used in each experiment. The specific activity was 311µCi/µmol. Hence the final concentration was between .28 and 1.0µM.

4.24. RADIO-ISOTOPE METHODS.

4.24.1. Counting Techniques.

Scintillation Fluid.

Redistilled S-free Toluene	1 Litre
P.O.P.O.P. (Koch-Light)	.3500 gm.
P.P.O. (Koch-Light)	3.000 gm.
Triton X-100 (Rohm and Haas)	500 mls.

Solution of Substances to be Counted.

The following orders of addition and the given quantities of additives must be followed to give clear solutions of acceptable quenching levels (0.4 or higher).

(a) For 18% v/v Ethanolamine:water from CO_2 trap.

9.0 ml of scintillation fluid then 0.5 ml water, shaken and then 0.5 ml. of the ethanolamine solution is added, then shaken vigorously.

(b) For Aqueous solutions.

1.0 ml of solution then 9.0 ml of scintillation fluid, then shaken sufficiently to mix.

(c) For Alcoholic extracts (TOTAL CELL FRACTION or CELL EXTRACT FRACTION).

0.5 ml 70% Ethanolic extract, then 0.5 ml water, shaken then 9.0 ml of scintillation fluid added, then shaken sufficiently to mix.

(d) For Acid hydrolysate.

0.2 ml 8N HCl (CELL HYDROLYSATE FRACTION) 0.9 ml 2N NaOH, mixed to allow neutralization. Then 9.0 ml of scintillation fluid was added and shaken sufficiently to mix.

(e) For ALKALINE HYPOCHLORITE FRACTION.

0.5 ml ALKALINE HYPOCHLORITE FRACTION and 0.5 ml 2N HCl added and mixed to allow neutralization. After aerating out any chlorine vapour left, 9.0 ml of scintillation fluid was added and the vial shaken sufficiently to mix.

(f) For CHLOROFORM EXTRACT FRACTION

0.1 ml CHLOROFORM EXTRACT FRACTION and 9.0 ml scintillation fluid added and shaken sufficiently to mix.

(g) For Membrane filters etc.

9.0 ml scintiallation fluid and membrane filter (dried under the U.V. lamp to the point where it is just able to crack when flexed so that the water present does not give an opaque film in the fluid) or the radio-chromatogram "spot".

Any solutions that failed to clear could generally be cleared by adding 0.2 ml water, 0.2 ml toluene, 0.2 ml ethanolamine or finally 3.5 ml of scintillation fluid. After each addition the mixture was shaken vigorously and allowed to stand for some minutes.

Scintillation Counting. A Packard 3375 Tricarb Scintillation Spectrometer (Packard) on standard preset ^{3}H and ^{14}C channels, at 50% and 5.8% amplifications respectively was used.

All vials were counted twice, each time for ten minutes. If the readings, after conversion to absolute counts did not agree within the limits of error, a further count was made. This was not necessary for 90-95% of the vials.

A channel-ratio method of correction for quenching was used to give absolute counts of ^{14}C (Peng 1965; Herberg 1965).

A known activity of ^{14}C-hexadecane was placed in a scintillation vial and 9.0 mls of scintillation fluid added. A series of such vials had quantities of quenching agents added so that the whole range of channel-ratios of interest was covered in a series of overlapping ranges for each quenching agent. These generally correspond to the normal solvents used in the determinations: water, chloroform and ethanol. Methyl-ethyl ketone was also used.

From the ^{14}C -standard the counting efficienty (determined cpm/expected cpm as a percentage) was determined: the channel-ratio is the ratio of the cpm in the ^{14}C channel and the ^{3}H channel and is a measure of quenching effects. From this a curve relating channel-ratios to % counting efficiency was constructed and was found to be absolutely congruent with the same curve constructed using the manufacturer's quenched standards.

The accuracy of the curve was assumed to be such that using it added 1.0% to the error of the absolute counts that resulted. The accuracy is much less at low channel-ratios (less than 0.1) at which the background counts are a significant contribution. The actual accuracy was determined as a standard deviation of 1.3% at a channel-ratio of around 0.75.

Although the determined counts in the ^{14}C channel for successive determinations of the same vial may vary considerably, the absolute counts obtained from the conversion curve using channel-ratio agree within the expected error limits.

The absolute counts include background counts which were then deducted to give dpm (disintegrations per minute). Background was 53.9 ± 1.6 dpm.

4.24.2. Chromatography and Autoradiography.

Aliquots of extracts and cell hydrolysates were spotted on to 0.1 mm thick 20 cm x 20 cm plates spread with cellulose powder (M N 300: Macherey and Nagel). These were run first in phenol:water (100:40 w/v) (the phenol was redistilled according to Draper and Pollard 1949). After drying the plate was run, at right angles to the phenol direction, in n-butanol: n-propionic acid: water (47:23:30, v/v/v) (Bassham and Calvin 1957).

When run and dried, two spots of radio-active ink, to correlate the orientation of the plates and the developed film, were applied to the plates above the solvent fronts. The two spots were asymmetric. These plates were then applied face-down to the film surface of 8 in. x 10 in. Osray-M (Agfa-Gevaert) X-ray film and stored in the original film packet, at room temperature, for periods of up to twenty weeks.

When developed, films were traced on to clear sheets of plastic then on to parchment (imitation greaseproof). When this tracing and the plate orientated using the ink-spots, a needle punched through the tracing into the plate outlined the radio-active spots.

The most efficient method of transfer from the plates to vials was found to be spraying the plate with 0.8% Celloidion (Gurr) dissolved in 50% ether:ethanol, spots were scribed with a hot needle and excised with a razor-blade.

Counts as low as a 50% excess of background could be detected.

Spots were identified by reference to standards similarly run, by the reactions of spots with various identifying sprays and by reference to standard maps (Bassham and Calvin 1957).

Some difficulty was experienced in obtaining accurate and complete reproductions of the developed radiochromatogram films. Photographic representations of high contrast failed to detect the fainter spots. Tracings fail to show the differing densities that were developed.

4.25. EXAMINATION OF T. THIOPARUS CULTURES FOR HETEROTROPHIC CONTAMINATION.

Before the experiment was started a 5.0 ml sample was removed from the cup of the apparatus and spun at 2×10^3 g for ten minutes. The pellet of cells was stored wet in the cold for up to two weeks.

Plates containing 0.25%, 0.5% and 1.0% of acetate, glutamate, glucose and succinate, with and without added thiosulphate (1.5%) in the normal mineral salts solution, were prepared. Small quantities of a suspension of the cells in water were spread on these plates and on thiosulphate-mineral-salts agar and nutrient agar plates. These were incubated for up to one week. Growth on any plate other than the thiosulphate-only plate indicated contamination.

No contamination of <u>T. thioparus</u> cultures was noted.

4.3. RESULTS.

The results obtained using each of the ^{14}C-labelled compounds will be presented in turn. The most complete set of results were obtained from experiments in which ^{14}C-glucose and ^{14}C-acetate were used. Much less complete data are available from the series of experiments using ^{14}C-glutamate. Finally a comparison of $^{14}CO_2$ metabolism by T. thioparus in the presence and absence of the inhibitor, succinate, will be presented.

In each case, data will be presented for total uptake, $^{14}CO_2$ evolution, ^{14}C incorporated into ethanol-soluble (CELL EXTRACT) and insoluble fractions (CELL RESIDUE FRACTION) and also, in some cases, for incorporation into specific amino acids of the CELL HYDROLYSATE FRACTION.

4.31. GENERAL CONSIDERATIONS.

The following general considerations need to be borne in mind in examining these data.

(a) Total Uptake. The following values represent equivalent determinations of the same function:

(i) Cells trapped by the membrane filter (CELL);

(ii) TOTAL CELL FRACTION, i.e. from cell-suspension in 70% ethanol - see Section 4.23.1.;

(iii) The sum of the CELL EXTRACT and CELL RESIDUE FRACTIONS; These with the addition of the counts in $^{14}CO_2$ should be equivalent to

(iv) the decrease in SUPERNATANT FRACTION from total zero label; and the totals (i), (ii) and (iii) with the addition of $^{14}CO_2$ and the SUPERNATANT FRACTION should give similar figures (the "budget"), equal to the "zero-time total" label.

The correlations between the equivalent numbers and the budgets is a fair indicator of accuracy.

The total uptake presented in the figures is the sum of $^{14}CO_2$ and CELL dpm figures.

The membrane filter figures (CELL) were frequently higher than the other two equivalent figures (ii) and (iii). The cellulose material of the membrane may trap some of the label from the supernatant, depending on the ionic strength and pH of the solution as well as on the organic compound. An approximate maximum measure of this for ^{14}C-glucose may be gauged from the T. thioparus succinate-inhibited ^{14}C-glucose uptake. This shows a maximum of 0.6% uptake which, even if this is all ^{14}C-glucose trapped in the membrane, is a very low figure. Further label may be absorbed on to cell-wall and capsular material, or on to sulphur and not be removed by the membrane-washing material. The washing of cells before extraction into ethanol may permit the leakage of label from the cell into the wash medium; the subsequent centrifugation might remove absorbed material from the cells. This variation will vary with the compound and conditions; but will be largest for heterotrophically-grown T. novellus, which forms a mucilaginous capsule.

The TOTAL CELL FRACTION and CELL RESIDUE FRACTIONS are perhaps more variable than the errors indicate. The boiling ethanol used in the extraction causes any sulphur present, formed in the autotrophic experiments, to become "tacky" and form clumps with the cells. This extraction also forms ropey-clumps in the glutamate-grown T. novellus. In all these cases the clumping can prevent correct distribution of the fraction in the suspension, that is then sampled.

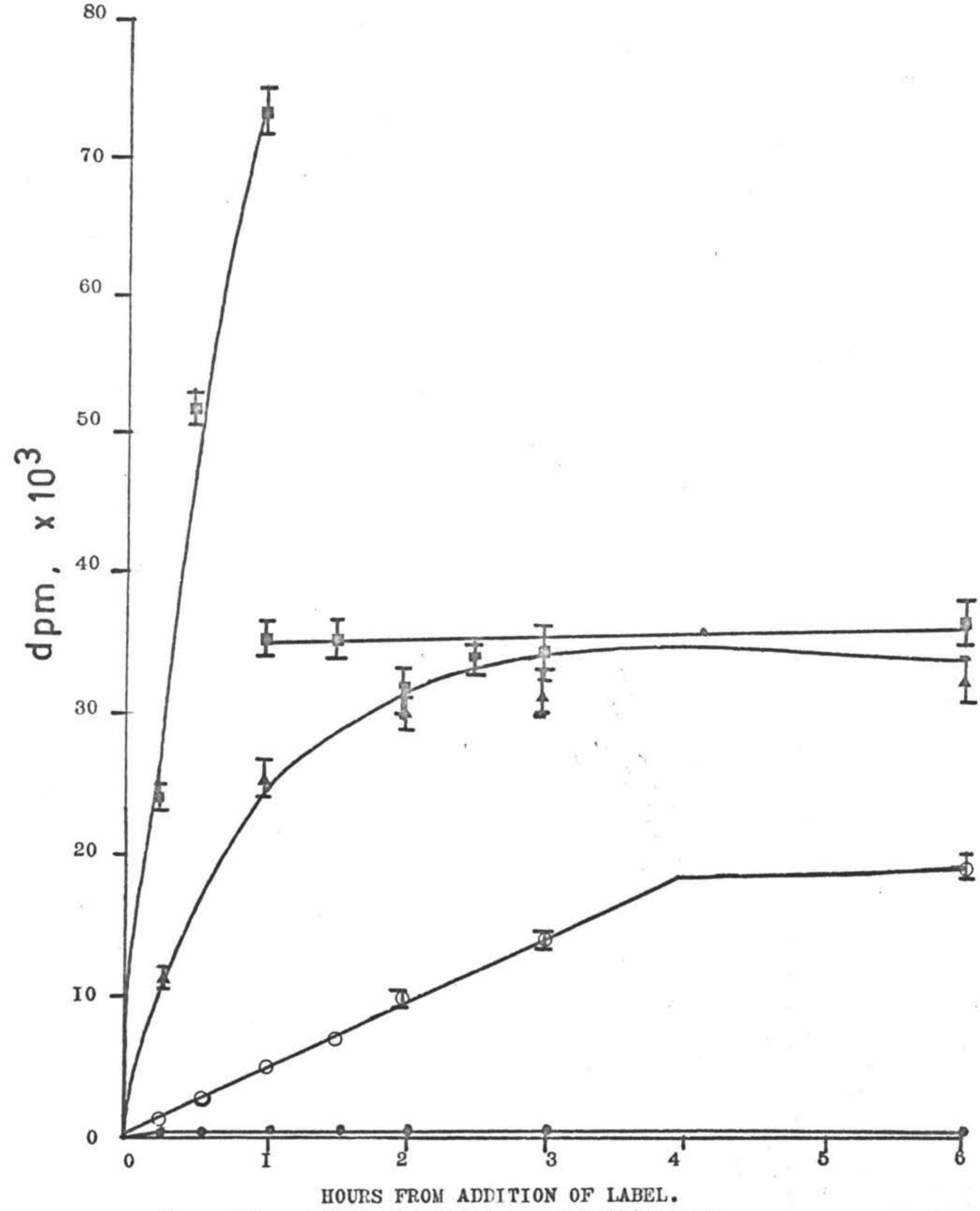
(b) $^{14}CO_2$ Values. It is assumed that the ^{14}C trapped by the ethanolamine solutions represents CO_2. The efficiency of CO_2 capture is very high. Even in a fast stream of 5% CO_2:95% Air for three hours, the first five mls of 18% ethanolamine solution trapped most of the issuing $^{14}CO_2$ (90-95% in most cases). However the back pressure is such that any leakages result in a fairly heavy loss of $^{14}CO_2$: for this reason the joints were all wired and sealed with finger-nail polish, and the bung wired down. The gas flow was such that the loss during sampling was small; and a "dead-volume" correction was not necessary.

(c) The presence of sulphur in the T. novellus autotrophically-grown runs often gives quite variable Klett readings and uptake readings as the amount of sulphur, and sometimes of absorbed cells, varies as the amount of sulphur in the sample varies with settling. This problem does not arise to the same extent in T. thioparus, where sulphur occurs to a lesser proportion of turbidity and is also finer and less prone to precipitation.

(d) The alkali required to maintain the pH in T. thioparus is added at similar rates in both the presence and absence of succinate. The appears to contradict the data of Section 3.53. in which succinate inhibited the utilisation of thiosulphate and pH changes to the same extent to which it inhibited the increase in turbidity. However this is a growth situation in which very low initial cell inocula are used. In the uptake experiments heavy suspensions are used and grown to the same pH (i.e. same pH changes) and similar turbidities. Thus it is an indication that succinate, in fact has no effect on the rate of thiosulphate utilisation per se. As will be evident from the results, it is markedly inhibitory to organic metabolism even at these high cell densities.

Figure 2I

TOTAL UPTAKE OF ^{14}C-GLUCOSE-(U) .



HOURS FROM ADDITION OF LABEL.

■ *T.novellus*, glutamate grown ○ *T.thioparus*

▲ *T.novellus*, thiosulphate grown ● *T.thioparus*, succinate-inhibited

4.4. RESULTS AND DISCUSSION FOR ^{14}C-GLUCOSE-(U) EXPERIMENTS.

The total assimilation of ^{14}C-glucose-(U) by the two species is shown in Figure 21. This is the sum of CELL and CO_2 dpm figures. Since somewhat different concentrations of labelled compounds were used these absolute rates are not strictly comparable. The total uptake curve for glutamate-grown T. novellus is in two parts as two separate experiments, with different label levels, were run.

The distribution of label, that is taken up, amongst $^{14}CO_2$, CELL RESIDUE and CELL EXTRACT FRACTIONs as a percentage of the total of these fractions is shown in Table XV. There is no label distribution data available for thiosulphate-grown T. novellus.

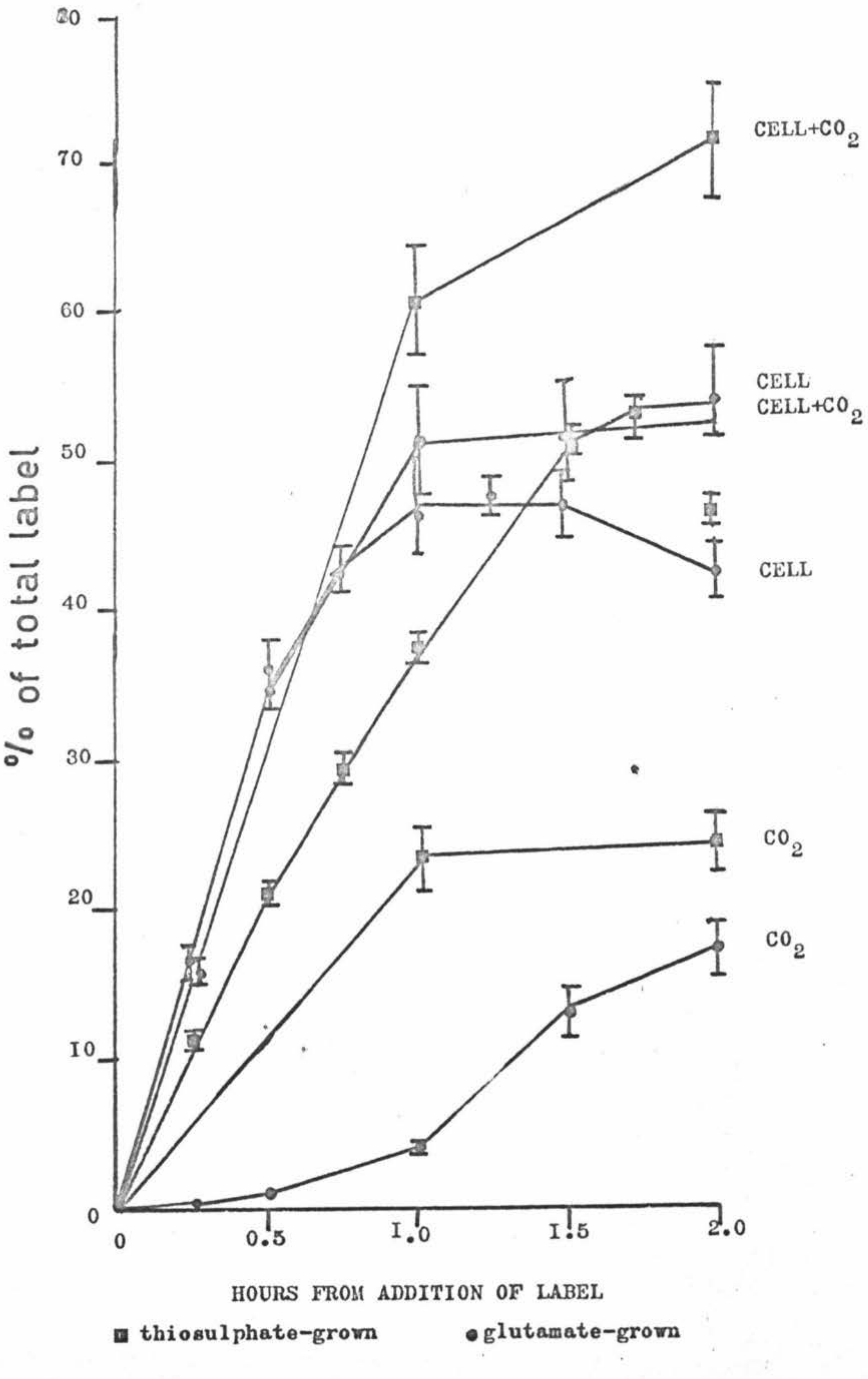
TABLE XV

PERCENTAGE DISTRIBUTION OF ASSIMILATED ^{14}C FROM ^{14}C-GLUCOSE-(U) AMONGST VARIOUS CELL FRACTIONS.

Time	Cell Fractions		
Hours	Gas	Extract	Cell Residue
	T. novellus - Heterotrophically-Grown		
0.25	3.4 ± .3	54.3 ± 6.3	38.9 ± 5.4
0.5	5.7 ± .5	41.6 ± 4.8	52.6 ± 6.7
1.0	17.4 ± 1.4	41.9 ± 5.1	41.6 ± 5.8
1.0	37.0 ± 6.4	16.9 ± 2.7	46.1 ± 8.2
2.0	64.1 ± 9.5	11.0 ± 1.5	24.9 ± 3.9
	T. thioparus - Normal		
0.25	8.0 ± 2.2	61.0 ± 25.7	31.1 ± 8.3
0.5	21.5 ± 5.9	59.9 ± 25.8	27.4 ± 7.9
1.0	19.1 ± 4.7	64.5 ± 26.3	31.3 ± 7.8
1.5	32.7 ± 8.7	62.9 ± 26.4	23.1 ± 6.2
2.0	22.9 ± 5.4	61.9 ± 24.5	14.6 ± 3.5
	T. thioparus - Succinate Inhibited		
0.25	4.0 ± 4.0	82.3 ± 37.3	13.8 ± 10.5
0.5	6.0 ± 5.4	93.0 ± 33.4	6.7 ± 4.1
1.0	12.7 ± 14.8	78.2 ± 44.1	9.1 ± 10.6
1.5	13.2 ± 15.8	80.8 ± 38.9	6.0 ± 7.9
2.0	29.6 ± 16.5	64.9 ± 21.9	5.5 ± 4.2

Figure 22

PERCENTAGE UPTAKE OF ^{14}C-GLUCOSE-(U) BY T.NOVELLUS



4.41. COMPARISON OF T. NOVELLUS: HETEROTROPHICALLY- AND AUTOTROPHICALLY- GROWN.

The total assimilation as a percentage of the total ^{14}C-glucose added is shown in Figure 22. The CO_2 production for the autotrophically-grown T. novellus was calculated from the difference between the activity present in an 0.2 ml sample taken and placed in toluene at the appropriate sampling times, and the initial total activity in a 0.2 ml sample taken at zero-time. This estimate will not be as accurate as that obtained from the ethanolamine CO_2 trap, the data for which are not available in this case.

There is an increased production of $^{14}CO_2$ by autotrophic T. novellus, in comparison to the heterotrophically-grown T. novellus, so that although the rate of increase in CELLs is similar, the total rate of assimilation is higher in the autotrophic form.

The total uptake in the heterotrophic form ceases when about 50% of the available ^{14}C-glucose has been assimilated; continued $^{14}CO_2$ production reduces the level in CELLs until a lower stable level is reached (not shown).

There are some minor differences in distribution of label amongst CELL HYDROLYSATE FRACTION components in thiosulphate-grown and glutamate-grown T. novellus, as shown in radiochromatograms; these minor differences are shown in detail in Table XVI.

TABLE XVI

DISTRIBUTION OF ^{14}C FROM ^{14}C-GLUCOSE-(U) AMONGST COMPOUNDS IN CELL HYDROLYSATE FRACTIONS

	T. novellus	
	Autotrophically-grown	Heterotrophically-grown
Lipid Components	18.5 ± 4.3	16.1 ± 3.6
Leucine Isoleucine	5.6 ± 1.6	7.6 ± 2.0
Phenylalanine	5.0 ± 1.4	7.8 ± 2.1
Unknown 1	5.5 ± 1.6	absent
" 2	5.2 ± 1.5	"
Proline	4.9 ± 1.4	4.8 ± 1.3
Valine	4.3 ± 1.3	5.1 ± 1.6
Unknown 3	4.2 ± 1.3	4.3 ± 1.3
Tyrosine	9.6 ± 2.1	8.6 ± 2.6
Alanine	5.1 ± 1.6	6.3 ± 1.8
Unknown 4	6.7 ± 1.9	4.8 ± 1.4
" 5	absent	4.2 ± 1.3
Glutamic Acid	5.7 ± 1.8	7.3 ± 2.2
Aspartic Acid	5.0 ± 1.6	4.4 ± 1.4
Others near origin and origin	14.4 ± 4.2	17.6 ± 5.9

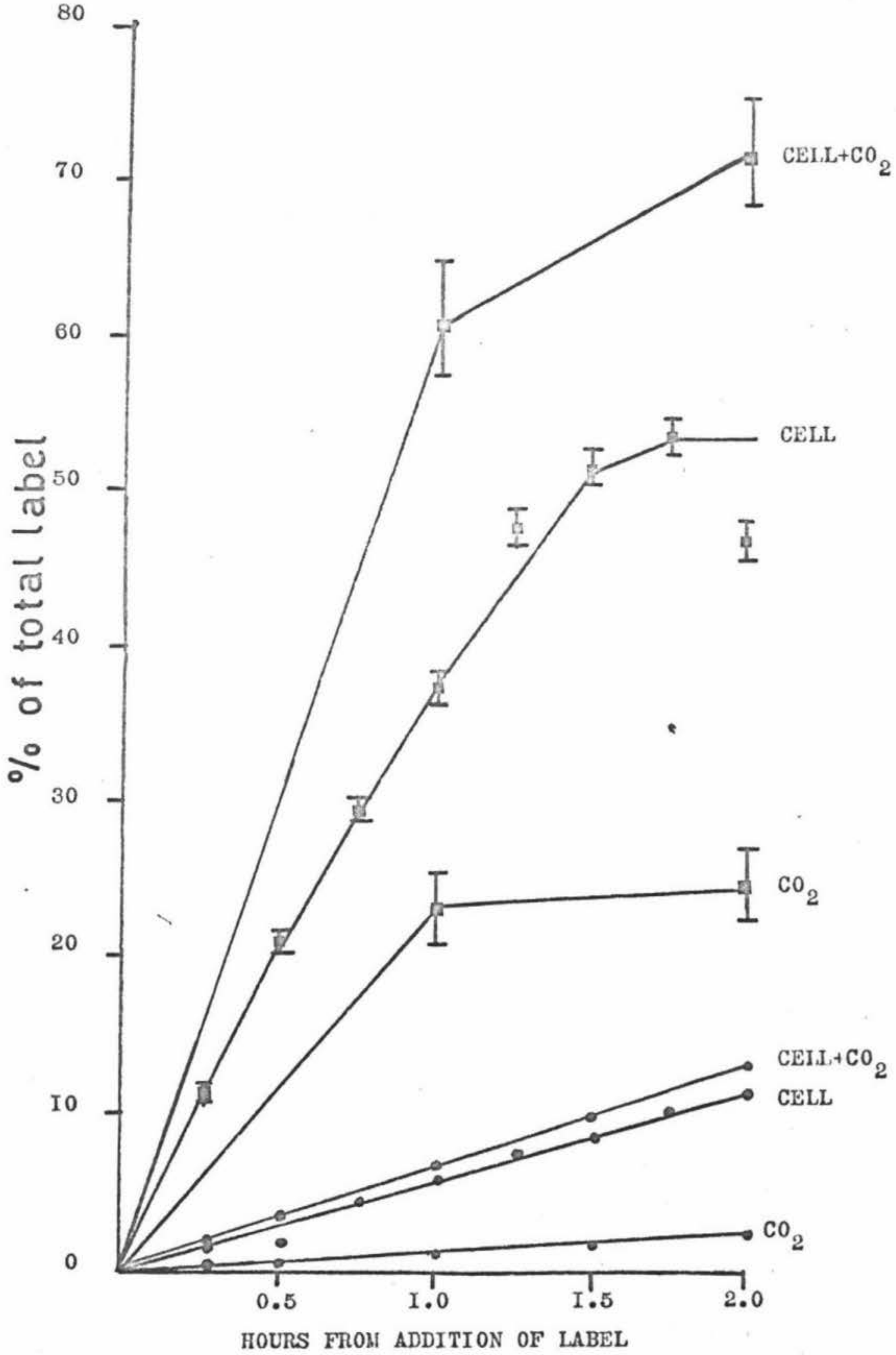
Figures are % of total radio-activity recovered.

Figure 23 : KEY

- ■ **autotrophically-grown <u>T.novellus</u>**
- ● **<u>T.thioparus</u>**

Figure 23

PERCENTAGE UPTAKE OF ^{14}C-GLUCOSE-(U) BY AUTOTROPHICALLY-GROWN <u>T.NOVELLUS</u> AND <u>T.THIOPARUS</u>.



4.42. COMPARISON OF AUTOTROPHICALLY-GROWN T. NOVELLUS AND T. THIOPARUS.

The uptake, as a percentage of the ^{14}C-glucose added, is shown in Figure 23, for these two situations.

The rate of production of $^{14}CO_2$ and of uptake is far higher in T. novellus than in T. thioparus. In T. novellus maximum uptake is reached at 1.5 hours, while in T. thioparus the uptake of ^{14}C-glucose continues at a steady, though much lower, rate for up to four hours, as shown in Figures 21 and 22. This suggests that the T. thioparus glucose uptake system may be saturated, even at these very low concentrations. Furthermore since a linear rate of uptake was maintained for four hours, it seems unlikely that this is due to passive diffusion, as the uptake ceased abruptly after four hours, when less than 20% of the available total ^{14}C-glucose had been taken up.

It appears that the major limitation of glucose utilization in T. thioparus is the uptake system. Once taken up the metabolism of glucose in the two organisms is apparently similar, although less oxidation of ^{14}C-glucose to CO_2 occurs in T. thioparus. In the case of autotrophically-growing cells any $^{14}CO_2$ produced may well be refixed by CO_2 assimilation, although refixation of $^{14}CO_2$ released from the cells will be reduced by the rapid gas flow used. Thus, as T. thioparus is the more active with respect to autotrophic CO_2 fixation, it will effectively release less $^{14}CO_2$, if this is an important factor, than T. novellus.

Radiochromatograms of CELL EXTRACT FRACTIONs show fewer labelled compounds in T. novellus, but both organisms exhibit the same major components. Since the labelled compounds in CELL EXTRACT FRACTION have not been identified, further discussion of the radiochromatogram's patterns is not justified.

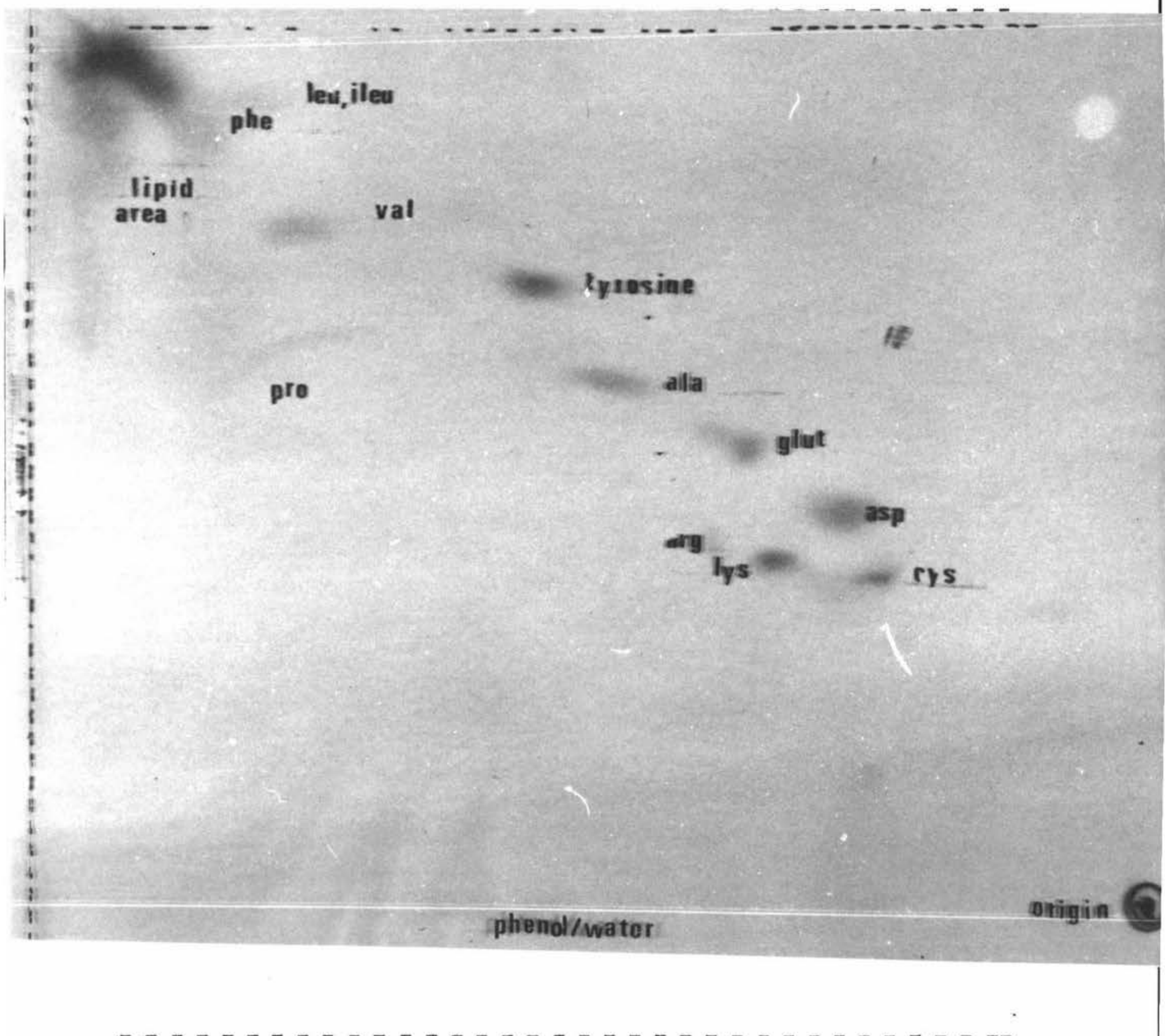
The distribution of label in CELL HYDROLYSATE FRACTION compounds, for T. thioparus, as shown in Figure 24, is very similar to that of autotrophically-grown and heterotrophically-grown T. novellus.

The incorporation of label into poly-β-hydroxybutyrate has been reliably shown only in T. novellus, where it initially forms some 10% of the CELL RESIDUE FRACTION.

The linear uptake of ^{14}C-glucose by T. thioparus suggests that there is no adaptation of metabolism, such as induced or repressed synthesis of enzymes. However the concentration of glucose used (about 0.5µm) is probably too low to induce such changes in any case. The distribution

FIGURE 24.

DISTRIBUTION OF CELL RESIDUE ^{14}C FROM ^{14}C-GLUCOSE-(U) AMONGST COMPOUNDS IN T. THIOPARUS AT 2 HOURS.



Dotted lines represent solvent fronts

leu	L-leucine	ileu	L-isoleucine
phe	L-phenylalanine	val	L-valine
pro	L-proline	ala	L-alanine
glut	L-glutamic acid	asp	L-aspartic acid
arg	L-arginine	lys	L-lysine
cys	L-cysteine		

of label as revealed by the radiochromatograms of both CELL EXTRACT and CELL HYDROLYSATE FRACTIONS, does not alter with time for T. thioparus, again indicating no adaptive changes in metabolism.

4.43. COMPARISON OF T. THIOPARUS IN THE PRESENCE AND ABSENCE OF THE INHIBITOR, SUCCINATE.

The actual uptake figures are shown in Figure 21; the uptake as a percentage of the ^{14}C initially present is shown in Figure 25. The distribution of label after assimilation is shown in Table XV.

The uptake of ^{14}C-glucose in T. thioparus is almost completely suppressed by 16 mM succinate. This is despite the fact that at the similar turbidities used the alkali-addition rates are substantially identical in the two situations, as shown in Figure 36.

Because of this low level of uptake in the presence of succinate, the distribution figures (Table XV) show large errors. The appearance of ^{14}C in CO_2 indicates that some metabolism is occurring. The proportion of label in the CELL RESIDUE FRACTION remains very low (4-14%) whereas the proportion in CO_2 is increased towards the end of the experiment (not shown) in the presence of succinate. Thus succinate appears to affect the metabolism of glucose as well as its uptake. The specific effects on ^{14}C-distribution are unknown as the low levels of label assimilated precluded any radiochromatographic examination.

Figure 25

PERCENTAGE UPTAKE OF ^{14}C-GLUCOSE -(U) BY *T.THIOPARUS*, IN THE PRESENCE AND ABSENCE OF SUCCINATE

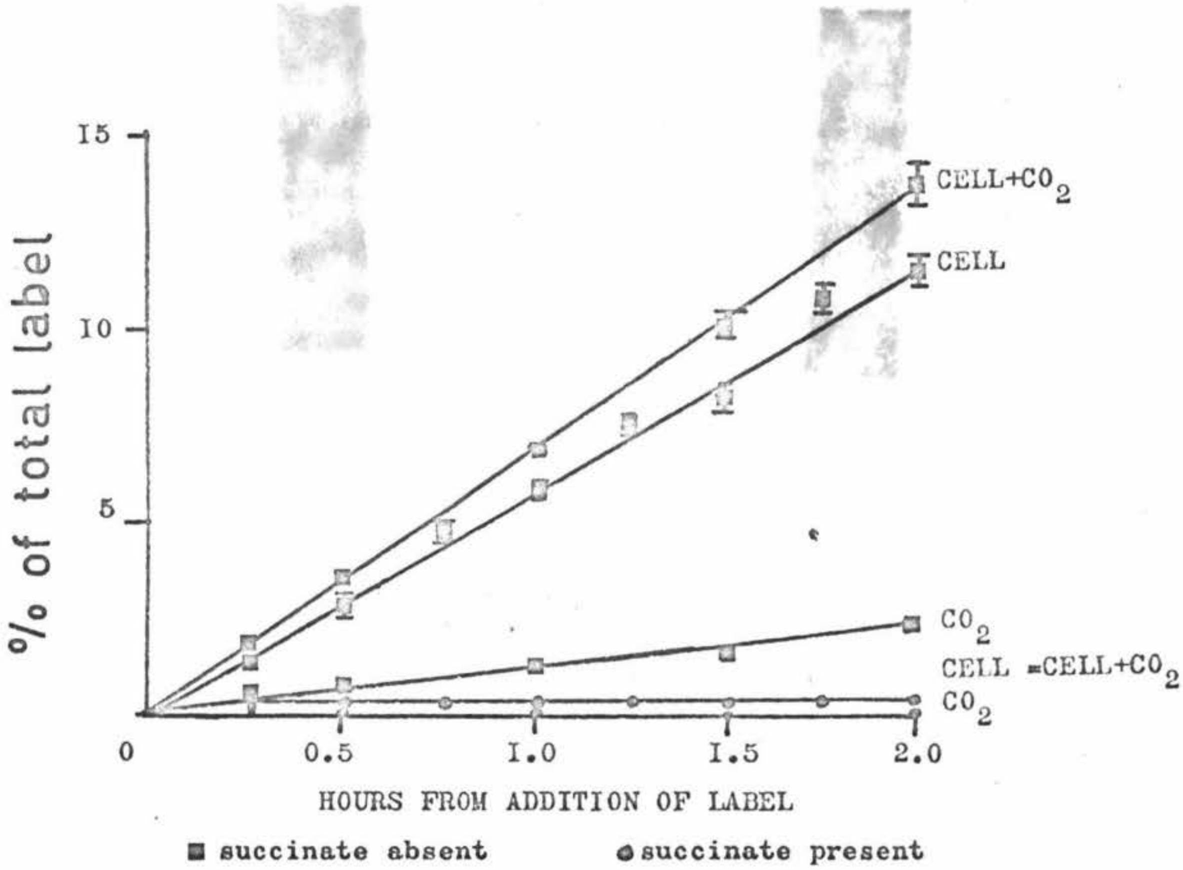


Figure 26 : KEY

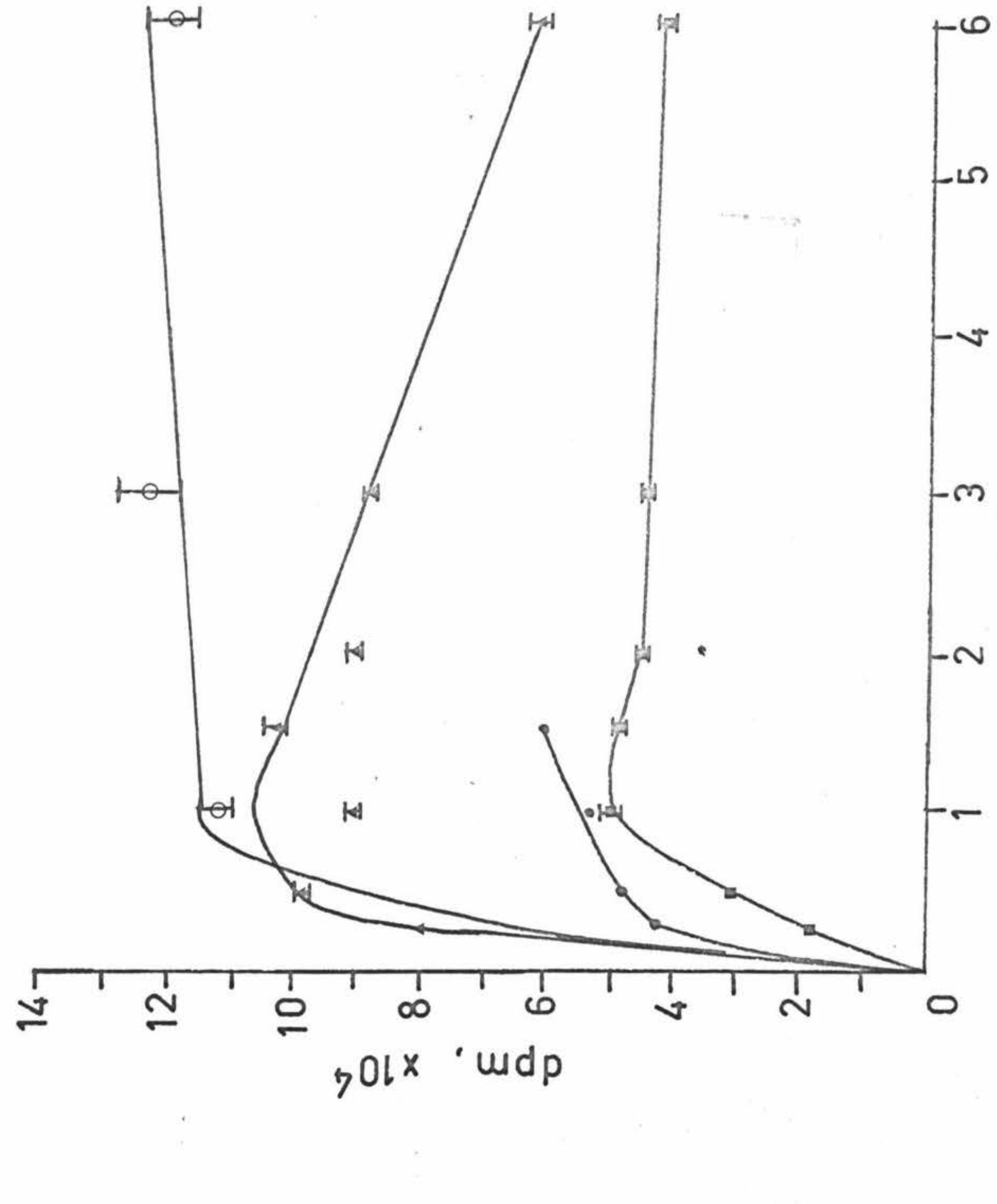
■ *T.thioparus* in the presence of succinate

▲ *T.thioparus*.(figures halved)

● glutamate-grown *T.novellus*

○ thiosulphate-grown *T.novellus*

Figure 26
TOTAL UPTAKE OF ^{14}C-ACETATE-(U)



4.5. RESULTS AND DISCUSSION FOR ^{14}C-ACETATE-(U) EXPERIMENTS.

The total uptake figures in dpm are shown in Figure 26. The differences arise from differences in initial label levels. The total uptake presented is the sum of the ^{14}C in cells retained on membrane filters (CELL) and $^{14}CO_2$ figures. The distribution of label amongst various cell fractions, as a percentage of the sum of these fractions, is shown in Tables XVII (a) and (b).

TABLE XVII (a)

PERCENTAGE DISTRIBUTION OF ASSIMILATED ^{14}C FROM ^{14}C-ACETATE-(U) AMONGST VARIOUS CELL FRACTIONS.

Time	Cell Fractions		
Hours	Gas	Extract	Cell Residue
	T. novellus - Heterotrophically-Grown		
0.25	5.8 ± .9	44.4 ± 7.8	49.7 ± 9.3
0.5	10.2 ± 1.8	43.7 ± 7.9	45.6 ± 8.7
1.0	18.2 ± 3.5	35.9 ± 6.4	45.9 ± 8.7
1.5	23.0 ± 4.8	22.8 ± 4.0	54.2 ± 10.2
	T. novellus - Autotrophically-Grown		
0.25	13.6 ± 1.4	84.8 ± 8.8	1.6 ± .1[*]
0.5	25.8 ± 2.4	71.7 ± 7.7	2.4 ± .2[*]
1.0	67.4 ± 7.1	22.7 ± 2.4	9.8 ± 1.0
	T. thioparus - Normal		
0.25	1.0 ± .1	49.0 ± 4.6	50.0 ± 6.7
0.5	2.2 ± .2	57.5 ± 4.5	40.2 ± 4.8
1.0	3.0 ± .3	51.1 ± 7.6	45.9 ± 8.6
1.5	6.6 ± .7	70.4 ± 6.2	23.1 ± 3.0
	T. Thioparus - Succinate Inhibited		
0.25	1.6 ± .2	76.3 ± 8.7	22.2 ± 3.1
0.5	1.7 ± .2	77.9 ± 8.7	18.6 ± 2.5
1.0	2.9 ± .3	73.4 ± 8.4	23.5 ± 3.3
1.5	2.4 ± .3	58.7 ± 6.8	38.9 ± 5.2

[*] These two figures were derived from the poly-β-hydroxybutyrate ALKALINE HYPOCHLORITE FRACTION rather than the CELL RESIDUE FRACTION, since the latter figures were obviously anamolous. Therefore this whole set of CELL RESIDUE figures may be a considerable under-estimate, in which case the CO_2 and CELL EXTRACT proportions would be artificially inflated.

Figure 27 : KEY

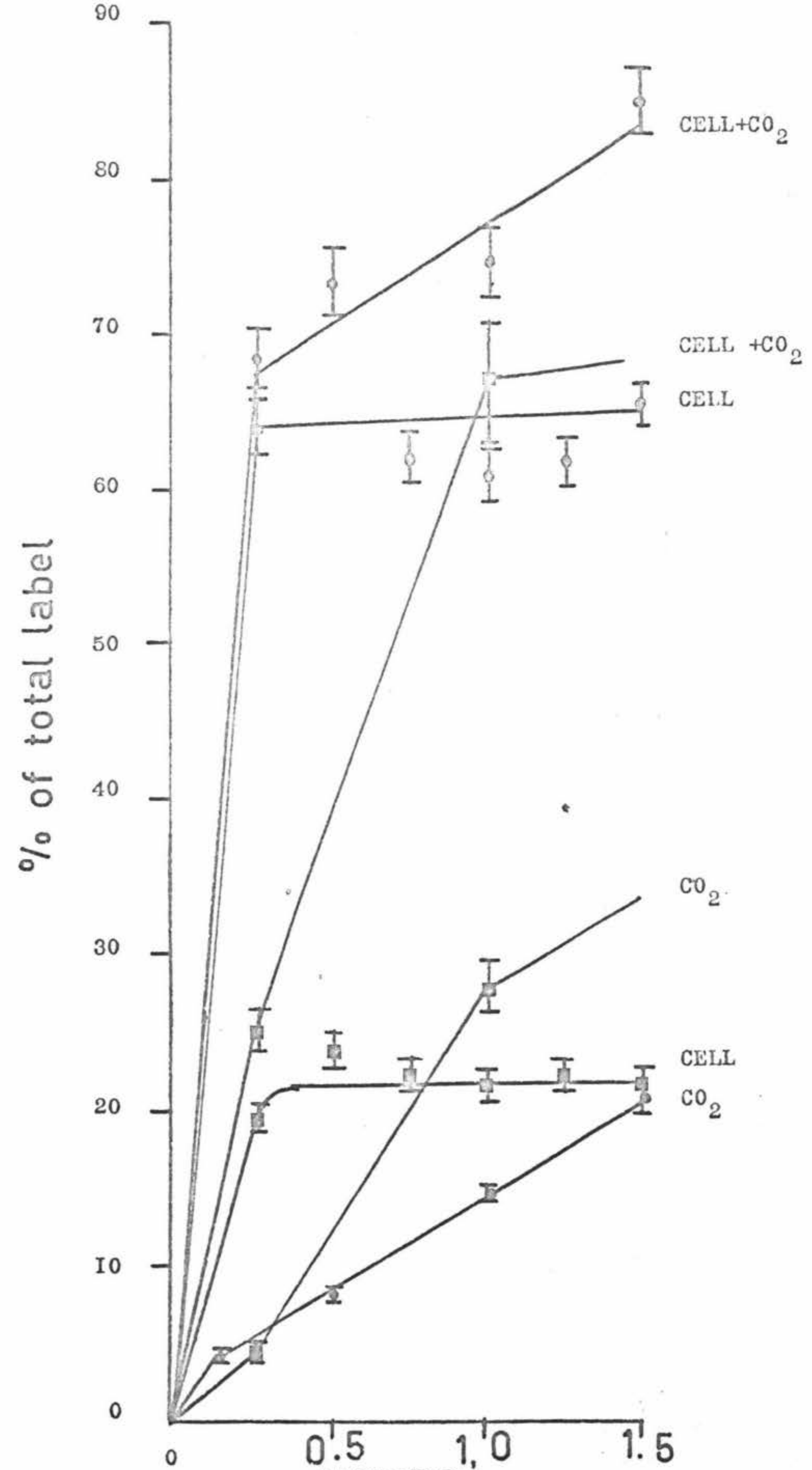
● glutamate-grown *T.novellus*

■ thiosulphate-grown *T.novellus*

Figure 27

PERCENTAGE UPTAKE OF ^{14}C-ACETATE-(U) BY

T.NOVELLUS



4.51. COMPARISON OF T. NOVELLUS: HETEROTROPHICALLY- AND AUTOTROPHICALLY- GROWN.

In both autotrophically- and heterotrophically- grown T. novellus about 70-80% of the total ^{14}C-acetate is taken up in the first hour. The initial rate of uptake is about half as great again in the glutamate-grown cells, than in the thiosulphate-grown cells. However a greater proportion of total uptake is oxidized to $^{14}CO_2$ by the thiosulphate-grown cells after the first half-hour; this is evident from Table XVII (a) and from Figure 27,

Both sets of CELLs figures reach a maximum after 0.25 hours then remain at a constant level.

In the autotroph the proportion of ^{14}C incorporated into CELL RESIDUE is very much less (see, however, footnote to Table XVII (a)) and rises many-fold while in the heterotrophically-grown cells it is substantially constant.

In the thiosulphate-grown T. novellus an increasing proportion of label in CELL RESIDUE is incorporated into the storage compound poly-β-hydroxybutyrate to at least 25%. There is no comparable data for glutamate-grown T. novellus.

Although the labelled compounds present in the CELL EXTRACT FRACTION, shown by the radiochromatograms, have not been identified some conclusions can be drawn from the general region in which the label lies. In the heterotrophically-grown cells the distribution of label is limited to a few compounds. After 30 minutes exposure to ^{14}C-acetate most ^{14}C is in the lipid components; only four other components were evident. A few more compounds were labelled at 0.25 hours, but longer exposures than 0.5 hours do not give additional labelled compounds. In autotrophically-grown T. novellus the CELL EXTRACT FRACTION radiochromatograms show far more compounds, although most of these are only present in the early samples and disappear in later radiochromatograms. Those that linger are present initially and remain in similar proportions.

In the CELL HYDROLYSATE FRACTION radiochromatograms there is again evident a much larger number of labelled compounds in the autotrophically-grown T. novellus samples than in the heterotrophically-grown T. novellus. This is despite the lower proportion of assimilated label incorporated into the CELL RESIDUE FRACTION in the autotroph, which is compensated for by the higher labelling leverl used (see Figure 26).

4.52. COMPARISON OF AUTOTROPHICALLY-GROWN *T. NOVELLUS* AND *T. THIOPARUS*.

The maximum uptake for the two systems species is similar (70 and 80% of the ^{14}C-acetate present) but the rate of uptake is much more rapid with *T. thioparus*. There are also major differences in $^{14}CO_2$ production. The production of $^{14}CO_2$ by *T. thioparus* is only 3% of total assimilated label at 1.0 hours, while in *T. novellus* it is 67% of total assimilated ^{14}C at the same time; this is shown in Table XVII (a) and in Figure 28.

This may reflect the differences in relative activities of CO_2 fixation in the two organisms, with the $^{14}CO_2$ produced being refixed more actively in *T. thioparus*; but the distributions in the radiochromatograms indicate that this is not so. There are few labelled compounds in *T. thioparus*; many in *T. novellus*. The $^{14}CO_2$ production by *T. thioparus* from ^{14}C-glucose is therefore also genuine. The $^{14}CO_2$ production thus indicates that acetate can be readily oxidized by autotrophically-grown *T. novellus*, whereas *T. thioparus* largely lacks the ability to oxidize acetate to CO_2.

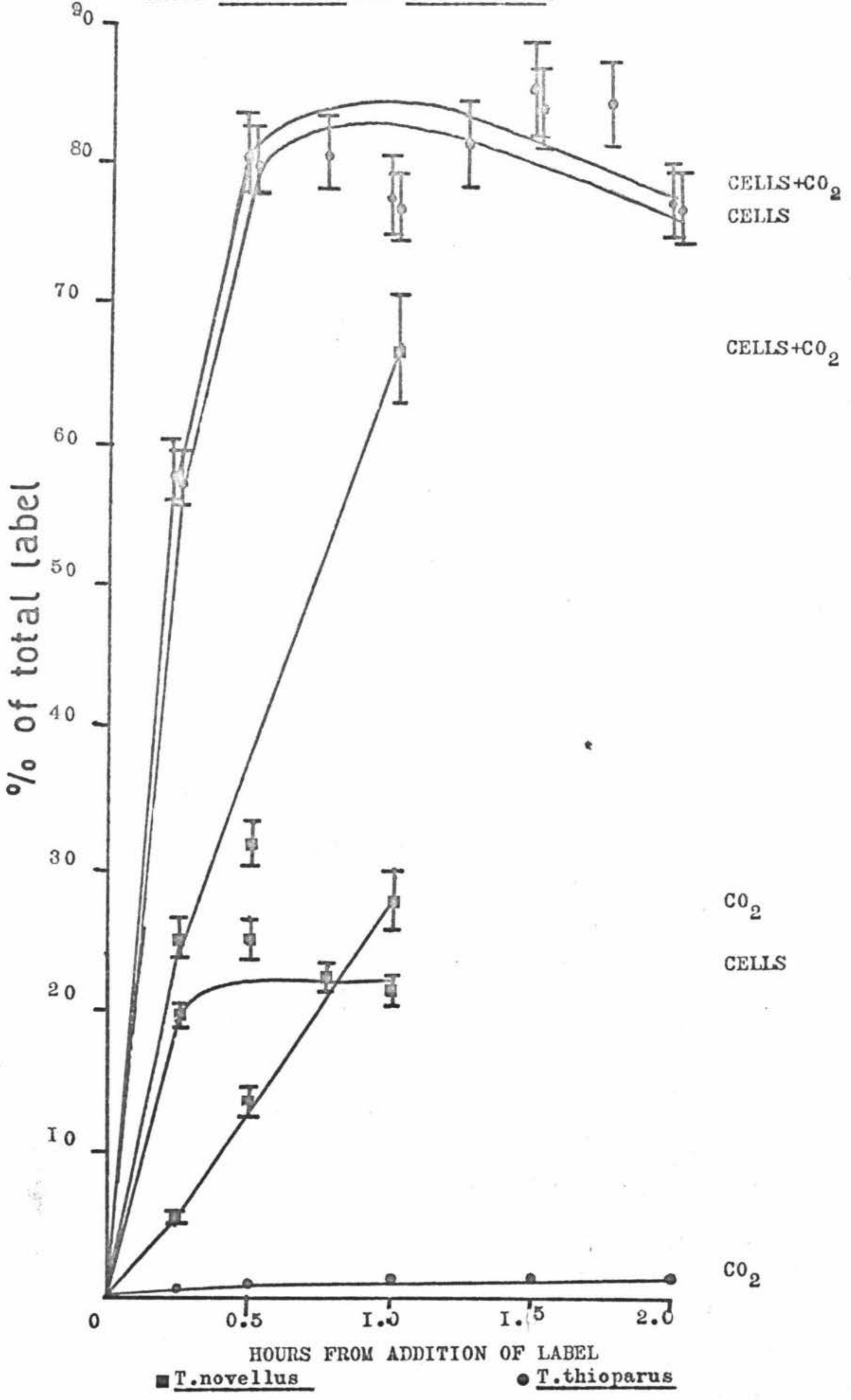
The radiochromatograms of the CELL EXTRACT FRACTION show a much greater variety of compounds for *T. novellus* than for *T. thioparus*. The number of compounds in each decreases with time: although the relative proportions of those that linger are fairly constant throughout the exposure time. In neither organism have the labelled compounds present in CELL EXTRACT FRACTIONs been identified. However the spots in the areas characteristic for organic acids, sugars and sugar-phosphates are reduced in intensity in *T. thioparus*.

The difference in distribution of ^{14}C is even more evident in the radiochromatograms of CELL HYDROLYSATE FRACTIONs. The pattern of distribution amongst the amino acids does not alter with time for *T. thioparus* (at 0.25, 0.5, 2.0 and 6.0 hours). There is a much larger number of amino acids in the *T. novellus* distributions. These spots can, in the main, be identified. The radiochromatograms are shown in Figures 29 and 30. The distribution of label in the *T. thioparus* CELL HYDROLYSATE FRACTION radiochromatograms is shown in Table XVIII. Only a few amino acids have a significant level of incorporation.

Incorporation of label into poly-β-hydroxybutyrate in *T. thioparus* does not exceed 4% of the CELL RESIDUE FRACTION and is poorly characterized; in *T. novellus* it reaches 25% of the CELL RESIDUE FRACTION and has increased with time.

Figure 28

PERCENTAGE UPTAKE OF ^{14}C-ACETATE-(U):AUTOTROPHICALLY-GROWN T.NOVELLUS AND T.THIOPARUS.



4.53. COMPARISON OF T. THIOPARUS IN THE PRESENCE AND ABSENCE OF THE INHIBITOR, SUCCINATE.

A comparison of the uptake for T. thioparus in the presence and absence of succinate, is shown in Figure 26 and, in more detail over a shorter period, in Figure 31, as a percentage of total available label added. The slower initial rate of uptake for the succinate-inhibited system is probably a result of the lower cell density used (initial Klett 264 in the presence of succinate, as against Klett 580 in the uninhibited system). The subsequent decrease in the presence of succinate is less than in its absence (Figure 26: not shown in Figure 31). There is an apparent increase in supernatant label with this decrease in assimilated ^{14}C but there are equivalent errors in "budget" figures so it is difficult to attach much significance to these decreases.

TABLE XVII (b)

PERCENTAGE DISTRIBUTION OF ASSIMILATED ^{14}C FROM ^{14}C-ACETATE (U) AMONGST VARIOUS CELL FRACTIONS.

Time	Cell Fractions		
Hours	Gas	Extract	Cell Residue
	T. Thioparus - Normal		
0.25	1.0 ± .1	49.0 ± 4.6	50.0 ± 6.7
0.5	2.2 ± .2	57.5 ± 4.5	40.2 ± 4.8
1.0	3.0 ± .3	51.1 ± 7.6	45.9 ± 8.6
1.5	6.6 ± .7	70.4 ± 6.2	23.1 ± 3.0
2.0	5.3 ± .6	51.9 ± 5.0	42.8 ± 5.8
3.0	4.0 ± .5	59.4 ± 5.3	36.6 ± 4.8
6.0	11.6 ± 1.4	55.7 ± 5.2	52.7 ± 7.0
	T. Thioparus - Succinate-Inhibited		
0.25	1.6 ± .2	76.3 ± 8.7	22.2 ± 3.1
0.5	1.7 ± .2	77.9 ± 8.7	18.6 ± 2.5
1.0	2.9 ± .3	73.4 ± 8.4	23.5 ± 3.3
1.5	2.4 ± .3	58.7 ± 6.8	38.9 ± 5.2
2.0	3.2 ± .4	68.7 ± 7.9	28.1 ± 3.9
3.0	3.6 ± .5	79.2 ± 8.9	17.2 ± 2.4
6.0	5.6 ± .9	70.8 ± 10.2	23.6 ± 4.1

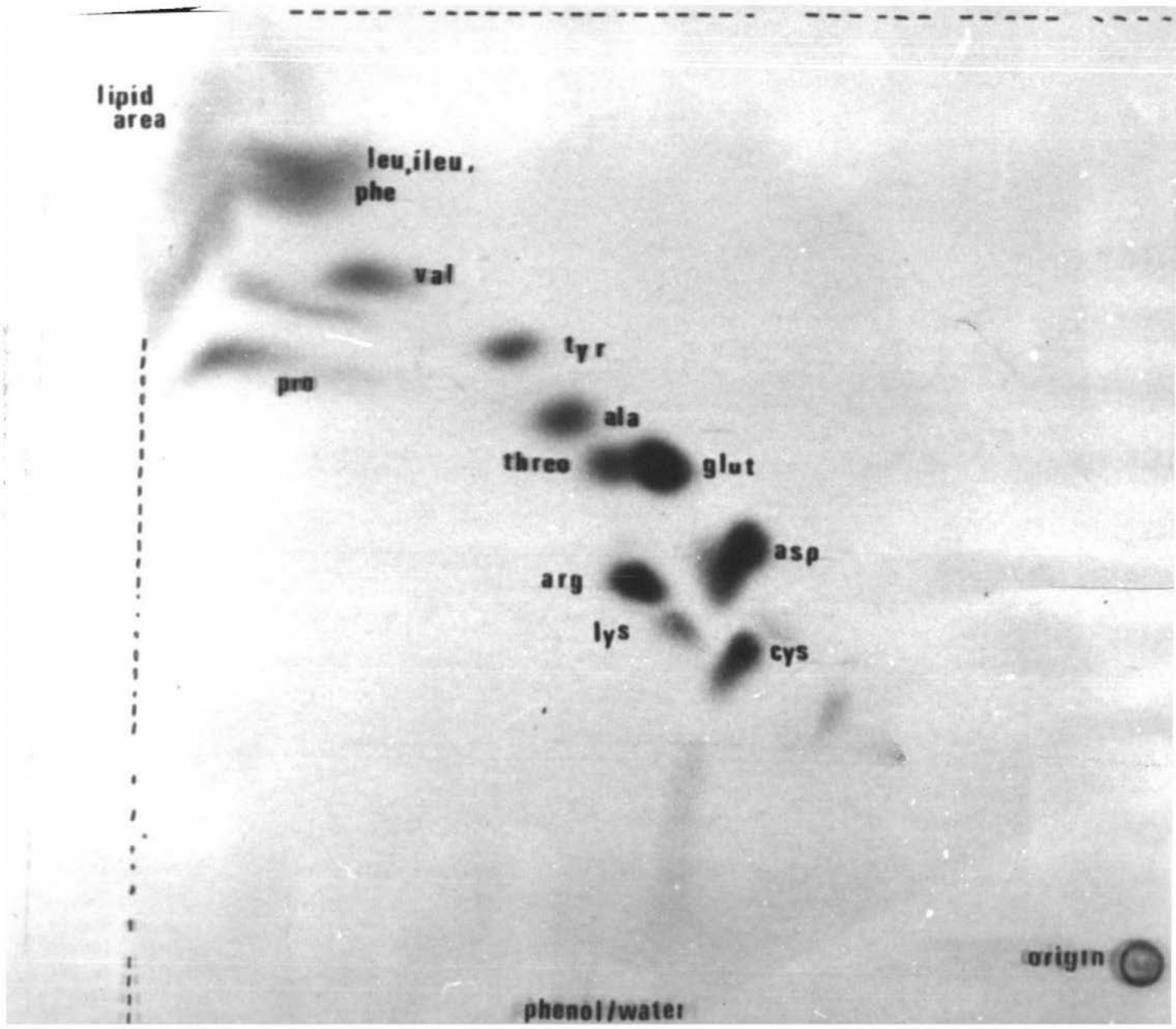

lipid area
leu,ileu, phe
val
tyr
pro
ala
threo
glut
asp
arg
lys
cys
origin
phenol/water

FIGURE 29.

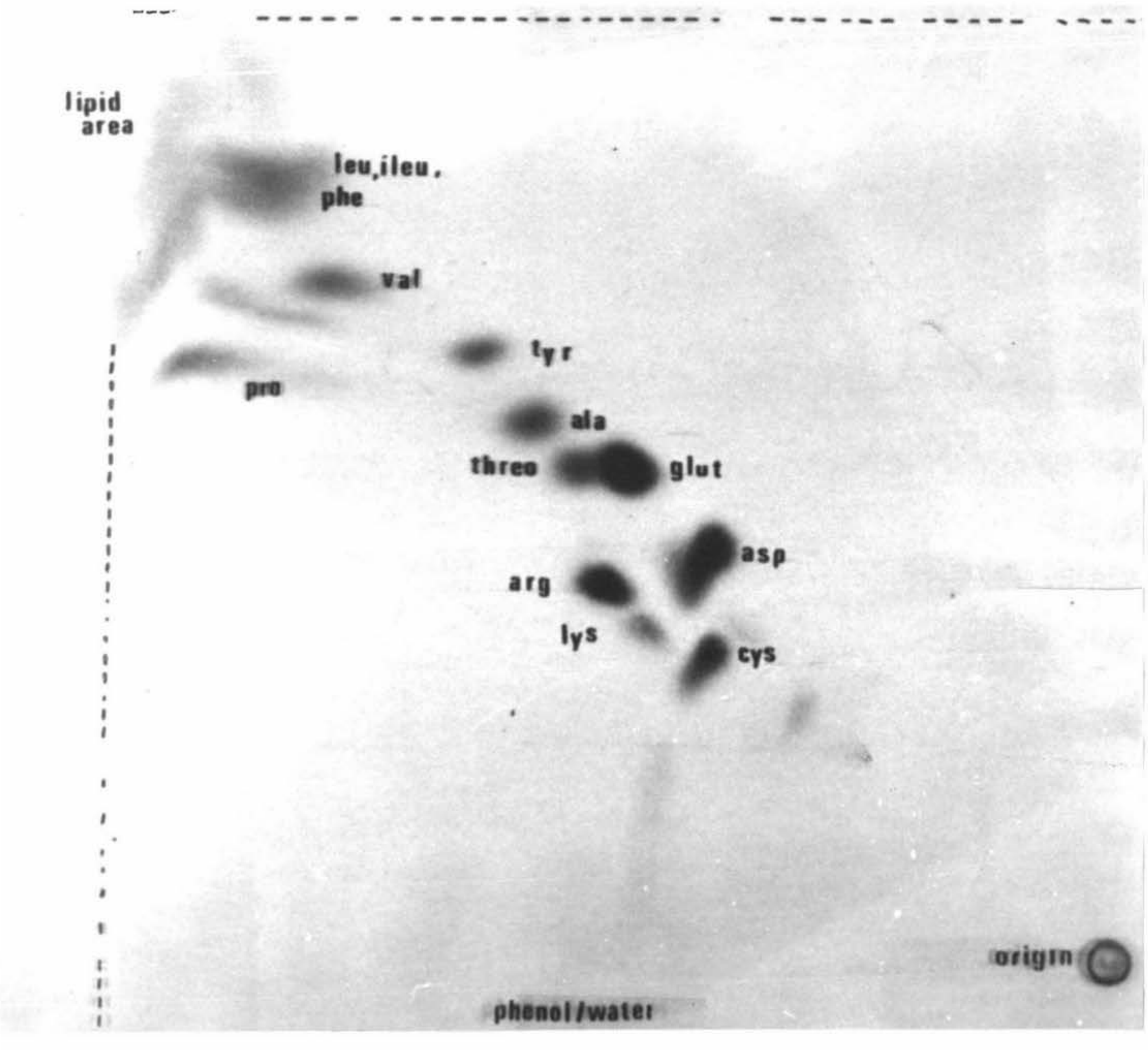
DISTRIBUTION OF CELL RESIDUE ^{14}C FROM ^{14}C-ACETATE-(U) IN COMPOUNDS IN AUTOTROPHIC T. NOVELLUS.

Dotted lines represent solvent fronts

leu	L-leucine	ileu	L-isoleucine
phe	L-phenylalanine	val	L-valine
pro	L-proline	tyr	L-tyrosine
ala	L-alanine	threo	L-threonine
glut	L-glutamic acid	asp	L-aspartic acid
arg	L-arginine	lys	L-lysine
cys	L-cysteine		

FIGURE 29.

DISTRIBUTION OF CELL RESIDUE ^{14}C FROM ^{14}C-ACETATE-(U) IN COMPOUNDS IN AUTOTROPHIC T. NOVELLUS.



Dotted lines represent solvent fronts

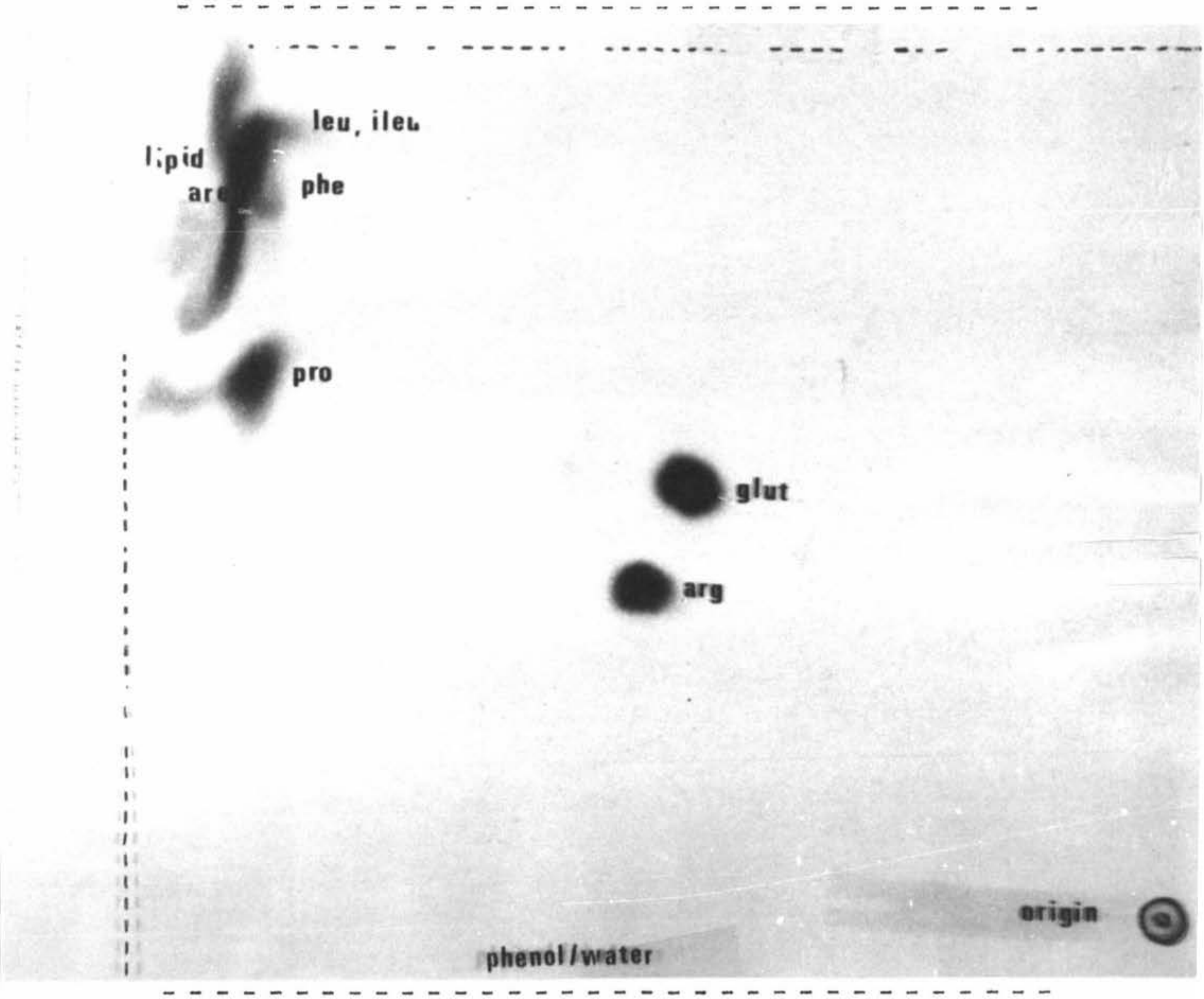
leu	L-leucine	ileu	L-isoleucine
phe	L-phenylalanine	val	L-valine
pro	L-proline	tyr	L-tyrosine
ala	L-alanine	threo	L-threonine
glut	L-glutamic acid	asp	L-aspartic acid
arg	L-arginine	lys	L-lysine
cys	L-cysteine		

FIGURE 30.

DISTRIBUTION OF CELL RESIDUE ^{14}C FROM

^{14}C-ACETATE-(U) AMONGST COMPOUNDS IN

T. THIOPARUS.



leu	L-leucine	ileu	L-isoleucine
phe	L-phenylalanine	pro	L-proline
glut	L-glutamic acid	arg	L-arginine

Radiochromatograms of SUPERNATANT FRACTIONs were badly affected by salt-induced streaking but the number of separated compounds present is greater in the absence of succinate than in its presence.

In both cases $^{14}CO_2$ production is limited. Table XVII (b) shows the distribution of assimilated label for a longer period than Table XVII (a).

From this it can be seen that while $^{14}CO_2$ production is limited in both cases it is more restricted in the presence of succinate. The proportion of label in CELL EXTRACT and CELL RESIDUE FRACTIONs is fairly constant in both cases, although there are some erratic variations around this mean, but the proportion in the CELL RESIDUE FRACTION is reduced in the presence of succinate, and that in CELL EXTRACT correspondingly increased (55% to 73%). This reduced incorporation in to CELL RESIDUE may reflect the lower growth rate in the presence of succinate.

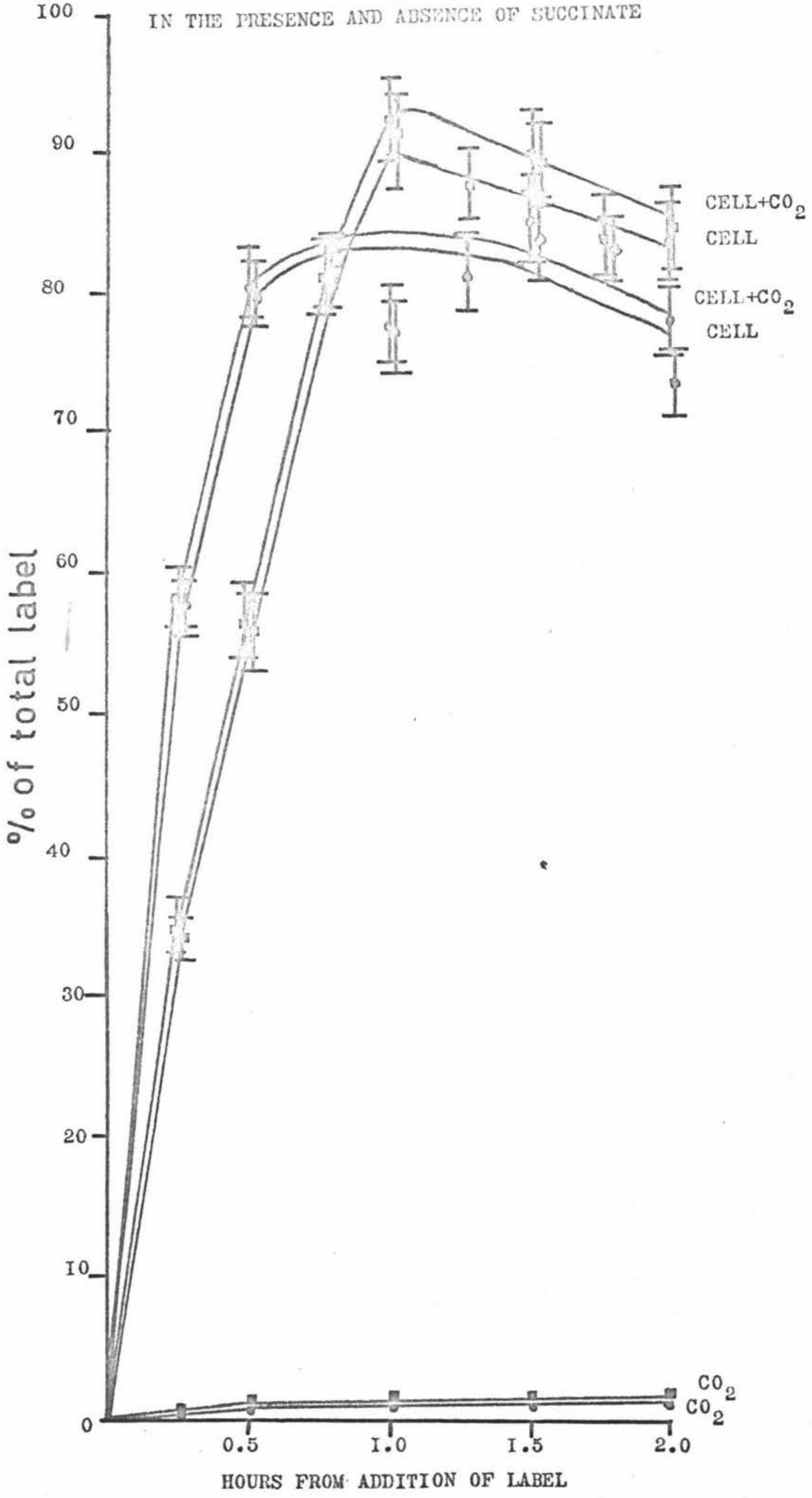
The CELL HYDROLYSATE FRACTION radiochromatograms show the same patterns of labelled compounds, in the presence as in the absence of the inhibitor, succinate.

Figure 3I : KEY

- ■ presence of I6mM succinate
- ● absence of succinate

Figure 31

PERCENTAGE UPTAKE OF ^{14}C-ACETATE-(U) BY T.THIOPARUS ; IN THE PRESENCE AND ABSENCE OF SUCCINATE



4.54. COMPARISON WITH PUBLISHED RESULTS OF DIRECT RELEVANCE.

TABLE XVIII

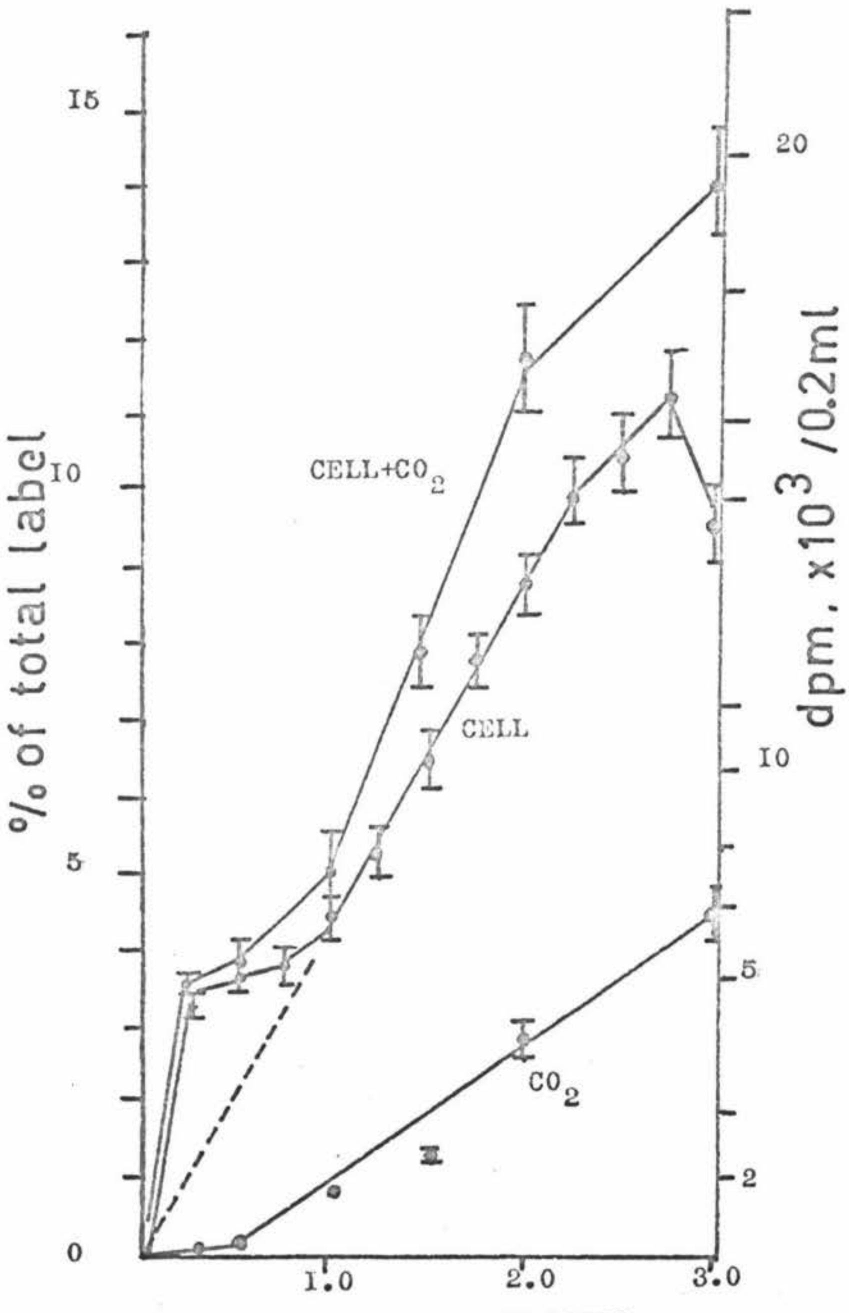
DISTRIBUTION OF ^{14}C FROM ^{14}C-ACETATE AMONGST AMINO-ACIDS IN CELL HYDROLYSATE FRACTIONS.

	This study	Smith, London and Stanier 1967.		
	T. thioparus			*T. intermedius*
	dpm	%	%	%
Origin	77.2 ± 23.1	2.3 ± .9		
Glutamic Acid	1120.2 ± 48.6	33.8 ± 4.2	42	11
Arginine	562.6 ± 38.6	17.0 ± 2.6	15	7
Proline	308.8 ± 23.4	9.3 ± 1.5	14	5
Leucine-Isoleucine-Phenylalanine	797.7 ± 49.6	24.1 ± 3.5	29	43
Methionine				
Valine				6
Tyrosine				1
Alanine				6
Threonine				6
Aspartate				8
Cystine				1
Serine-Glycine				6
Spot 1	39.1 ± 10.9	1.2 ± .4		
2	24.6 ± 11.4	.7 ± .4		
3	63.0 ± 13.3	1.9 ± .6		
4	30.6 ± 10.1	.9 ± .4		
5	5.7 ± 11.2	.2 ± .4		
Background	177.4 ± 15.5	5.3 ± .9		

% are of total label recovered from radiochromatograms.

Table XVIII shows the distribution, found in this study, of ^{14}C-acetate in *T. thioparus* CELL HYDROLYSATE FRACTION at 3.0 hours, and compares this with the similar distribution found by Smith, London and Stanier (1967) for *T. thioparus*. The distribution for the facultative autotroph *T. intermedius* resembles that in *T. novellus*, and although this study used

Figure 32
UPTAKE OF ^{14}C-GLUTAMATE-(U) BY
GLUTAMATE-GROWN <u>T.NOVELLUS</u> .



trace levels, while Smith et al. used 2 mM acetate. The recovery of label found in this study was:

Label placed at origin:	5592.7 ± 78.7
Recovered Label:	3317.9 ± 270.9
therefore % Recovery:	59.3 ± 5.7%

Johnson and Abraham (1969) found considerable incorporation of ^{14}C-acetate, into a limited range of compounds, with only trace production of $^{14}CO_2$.

4.6. ^{14}C-GLUTAMATE-(U) UPTAKE AND METABOLISM IN GLUTAMATE-GROWN T. NOVELLUS.

In this case glutamate is present as the sole substrate in the medium and consequently will considerably dilute the specific activity of the ^{14}C-glutamate. The large difference in external glutamate concentration and specific activity makes inclusion in the standard set of comparisons of little value.

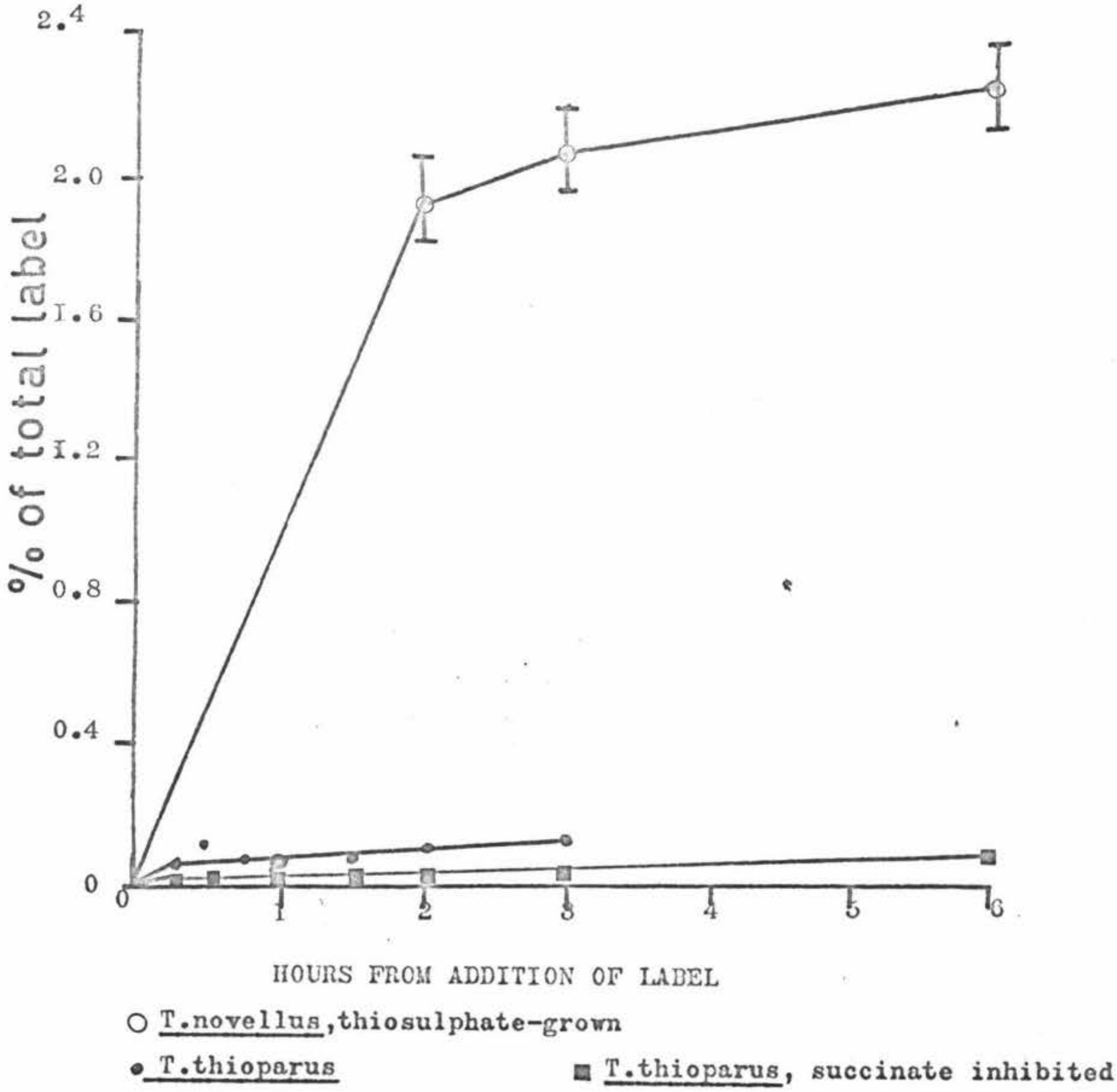
From Figure 32 it can be seen that CELL and $^{14}CO_2$ dpm, after an initial period of about one hour, increase linearly. The CELL dpm figures increase linearly after one hour and this can be extrapolated back through zero. This rate of incorporation is closely correlated with the rate of acid addition to maintain the pH, which is a measure of the rate of glutamate metabolism.

The initial faster uptake of ^{14}C during which $^{14}CO_2$ is produced only very slowly, as followed by a period in which there is little further increase in the ^{14}C-content of the cells but a normal rate of production of $^{14}CO_2$. It can be assumed that this early proportion represents exchange with a large cellular pool of initially unlabelled glutamate. The lag period before a steady rate of $^{14}CO_2$ production is reached presumably represents the time required for this large pool to reach isotopic equilibrium. The delay means that formation of the pool does not involve CO_2 production, which occurs later as the pool contents reach isotopic equilibrium with intermediates of the $^{14}CO_2$-producing metabolic pathways.

The distribution of ^{14}C revealed by the radiochromatograms of both CELL HYDROLYSATE **and** CELL EXTRACT FRACTIONs show little change with time. ^{14}C enters the majority of the amino-acids present in proteins.

Figure 33

TOTAL UPTAKE OF ^{14}C-GLUTAMATE-(U)



4.7. COMPARISON OF ^{14}C-GLUTAMATE UPTAKE AND METABOLISM BY AUTOTROPHICALLY-GROWN T. NOVELLUS AND T. THIOPARUS.

The uptake of ^{14}C-glutamate-(U) in terms of dpm is shown in Figure 33. These figures are the sum of dpms for cells trapped on membrane filters (CELL) and for CO_2. The differences due to differences in the concentration of ^{14}C-glutamate used are allowed for in Figure 34 by using the % of the total zero-time label.

The amount of $^{14}CO_2$ produced by the autotrophically-grown T. novellus was calculated from the difference between label present in total 0.2 ml samples taken at various times and the total zero-time label.

The very great difference between the two species is evident in Figure 34: T. novellus takes up nearly all the available label and oxidizes a large proportion of the ^{14}C-glutamate to $^{14}CO_2$; T. thioparus accumulates ^{14}C-glutamate at a very much slower rate, in parallel with the alkali required to maintain the pH, and produces negligible carbon-dioxide.

The early drop in CELL dpm for T. novellus is assumed to be due to the production of $^{14}CO_2$ becoming more evident: after this most further metabolism is to $^{14}CO_2$ while the cellular content of ^{14}C remains constant. It can be presumed that the metabolism of ^{14}C-glutamate by T. novellus is largely by way of the TCA cycle via glutamate dehydrogenase. The distribution of ^{14}C differs from that in the glutamate-grown T. novellus: the CELL HYDROLYSATE radiochromatograms differ substantially, but the identification of the labelled compounds has not proved possible.

The thiosulphate-grown T. novellus CELL EXTRACT radiochromatograms, as shown in Figure 35, show a large number of compounds, mainly organic acids and amino-acids.

In T. thioparus there was very little production of carbon-dioxide. The low level of uptake precluded any radiochromatograms as spots would not reach detectable levels of label.

Figure 34

PERCENTAGE UPTAKE OF ^{14}C-GLUTAMATE(U) BY AUTOTROPHICALLY-GROWN <u>T.NOVELLUS</u> AND <u>T.THIOPARUS</u>

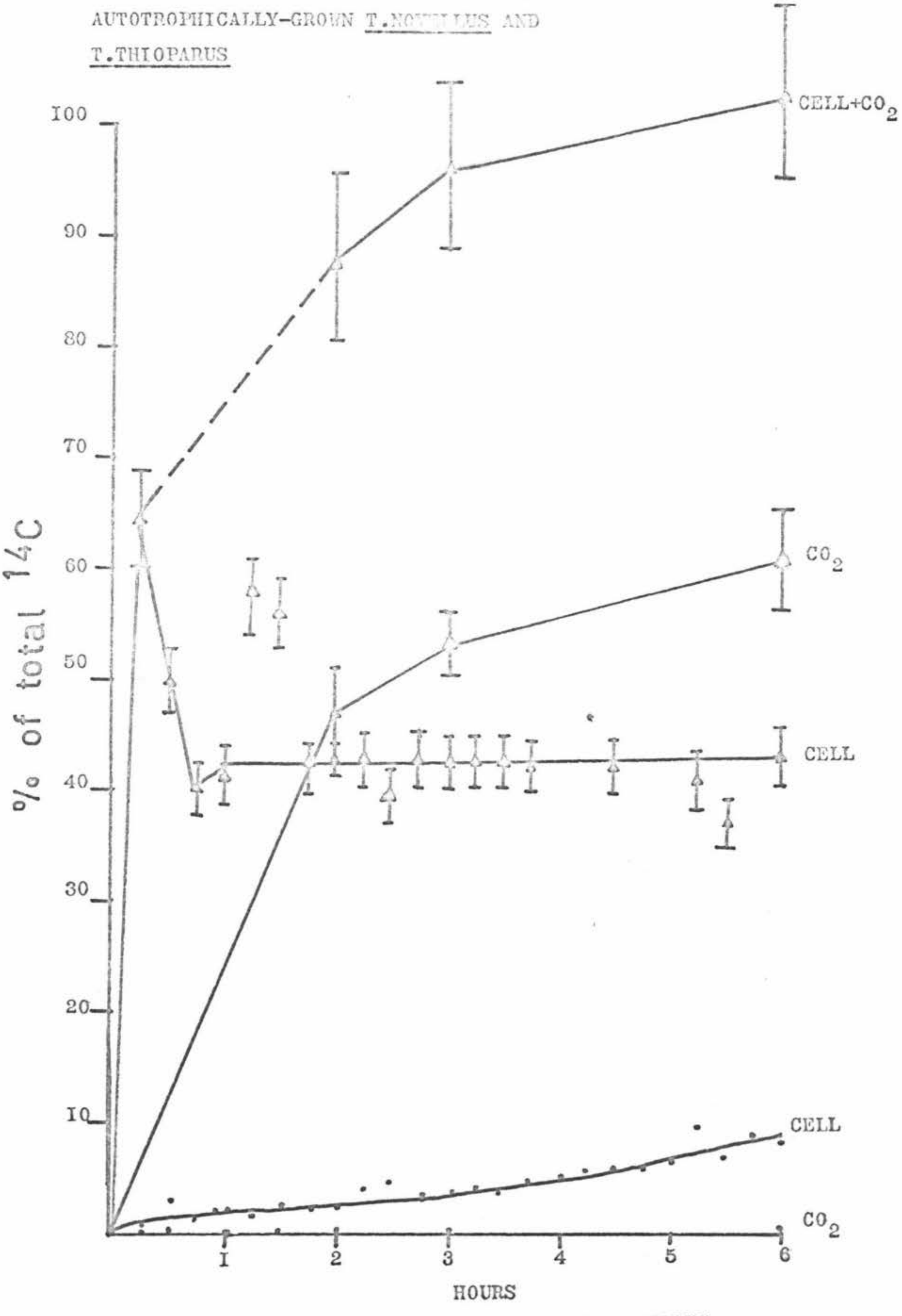
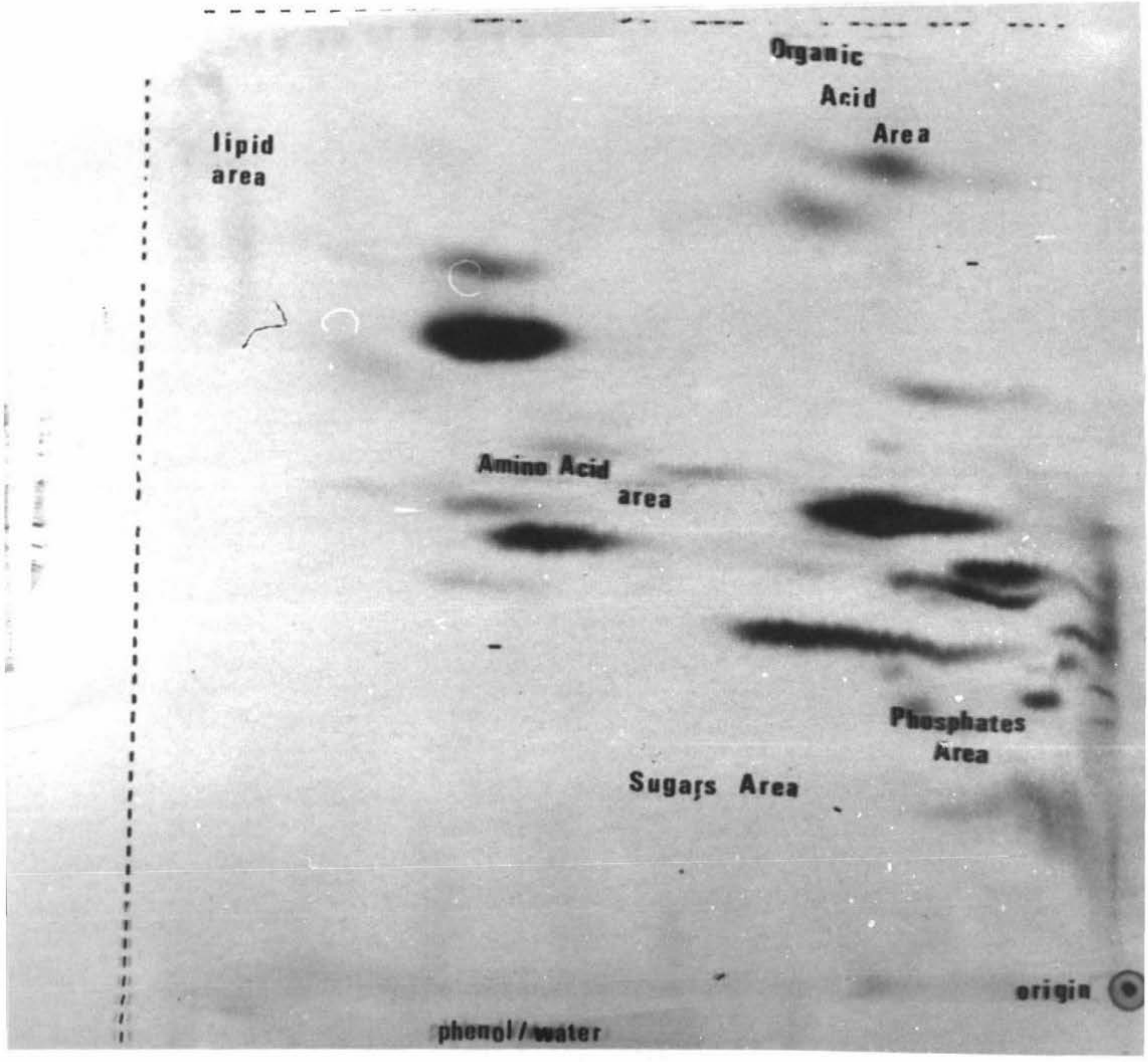


FIGURE 35.

DISTRIBUTION OF CELL EXTRACT ^{14}C
FROM ^{14}C-GLUTAMATE-(U) IN T. NOVELLUS,
AMONGST COMPOUNDS



Dotted lines represent solvent fronts.

4.8. EFFECT OF SUCCINATE ON $^{14}CO_2$ UPTAKE BY *T. THIOPARUS*.

Since the rates of alkali addition required to maintain the pH at pH 6.8 are independent of the presence of 16 mM succinate this suggests that succinate is not inhibiting thiosulphate oxidation (this is shown in Figure 36). However this concentration of succinate severely inhibits glucose uptake so it is still effectively inhibiting metabolism. In order to examine the effects of succinate on autotrophic CO_2 assimilation by *T. thioparus* an experiment with $^{14}CO_2$ was carried out.

An uninhibited culture of *T. thioparus* was harvested as in Section 4.21.2. Equal aliquots were placed in medium containing only half the usual concentration of thiosulphate (i.e. it was now 0.75%) with and without 10 mM succinate. These media were incubated until pH 6.8 ± .2 was reached. In the case of the succinate-containing culture a longer preincubation period was required. When this point was reached 7.5 mls of the medium was transferred to a modified Klett tube, gassed out with the 5% CO_2 : 95% Air mixture, $^{14}CO_2$ added and the tube sealed. 0.2 ml samples were removed at various time intervals. The first 0.2 ml sample was removed at zero-time, immediately after the label was added, and placed in alkaline toluene. Later 0.2 ml samples were filtered, dried and counted as in Section 4.23.

A 5.0 ml sample was removed and processed as in Section 4.23.1. after 25 minutes.

The effect of succinate on the % uptake of $^{14}CO_2$ by *T. thioparus* is shown in Figure 37. There is no alteration in the uptake of $^{14}CO_2$ in the presence of succinate, save at the very last sample, which may be an artefactual end-effect.

The percentage distribution of the assimilated carbon amongst cell fraction, as shown in the table below, is not affected at all.

Percentage Distribution of Assimilated $^{14}CO_2$ Amongst Cell Fractions.

	CELL EXTRACT	CELL RESIDUE
NORMAL	56.0 ± 7.5	44.0 ± 6.9
SUCCINATE-INHIBITED	56.2 ± 7.1	43.8 ± 6.4

CELL HYDROLYSATE FRACTION radiochromatograms of the two situations at the same time show the same pattern of spots in similar proportions (visually).

So the effect of succinate is not on the uptake of $^{14}CO_2$ and its subsequent incorporation into amino-acids. The resultant distribution is shown in Figure 38.

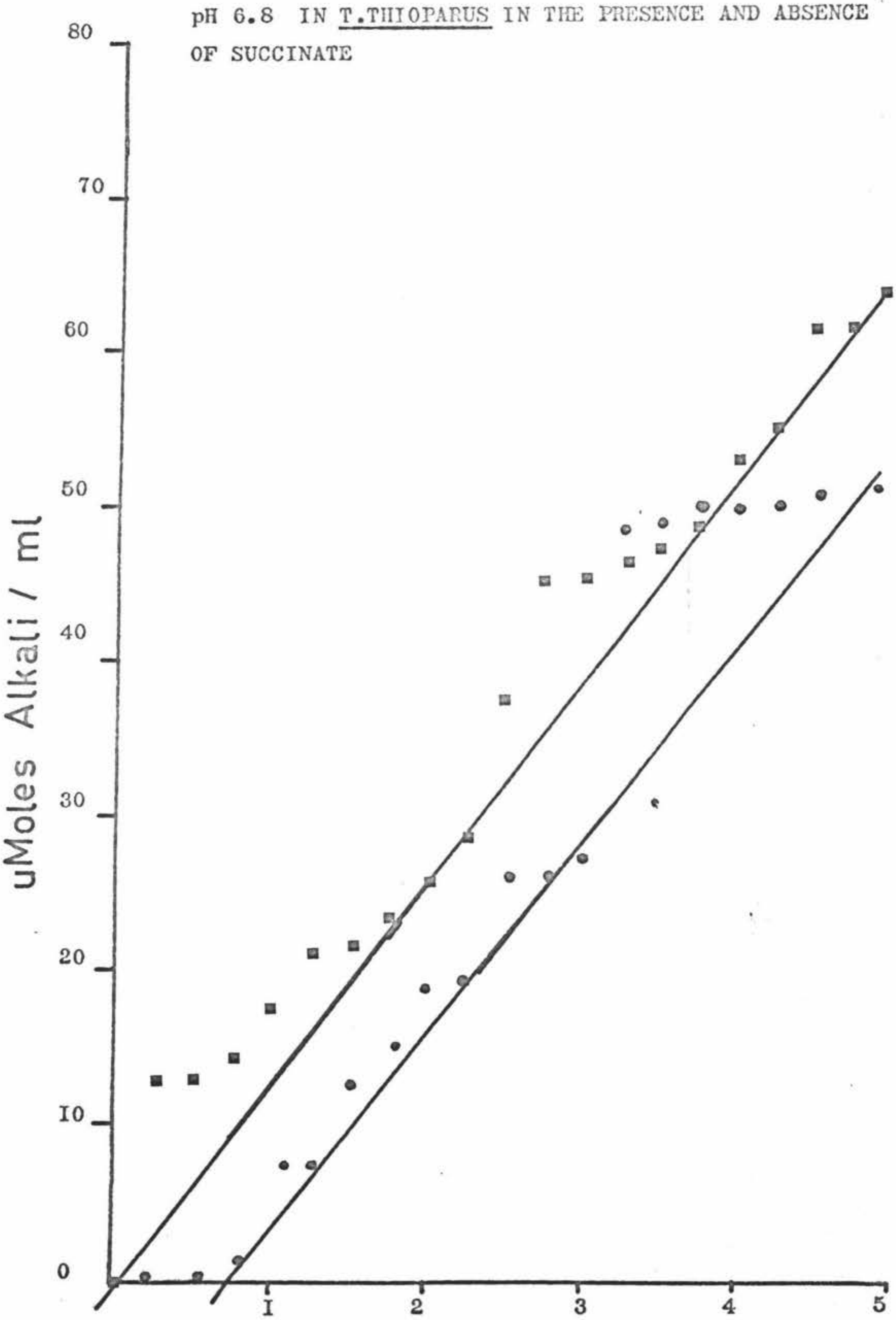
Figure 36 : KEY

■ absence of I6mM succinate
Klett 305

● presence of I6mM succinate
Klett 337

Figure 36

RATES OF ALKALI-ADDITION REQUIRED TO MAINTAIN pH 6.8 IN T.THIOPARUS IN THE PRESENCE AND ABSENCE OF SUCCINATE



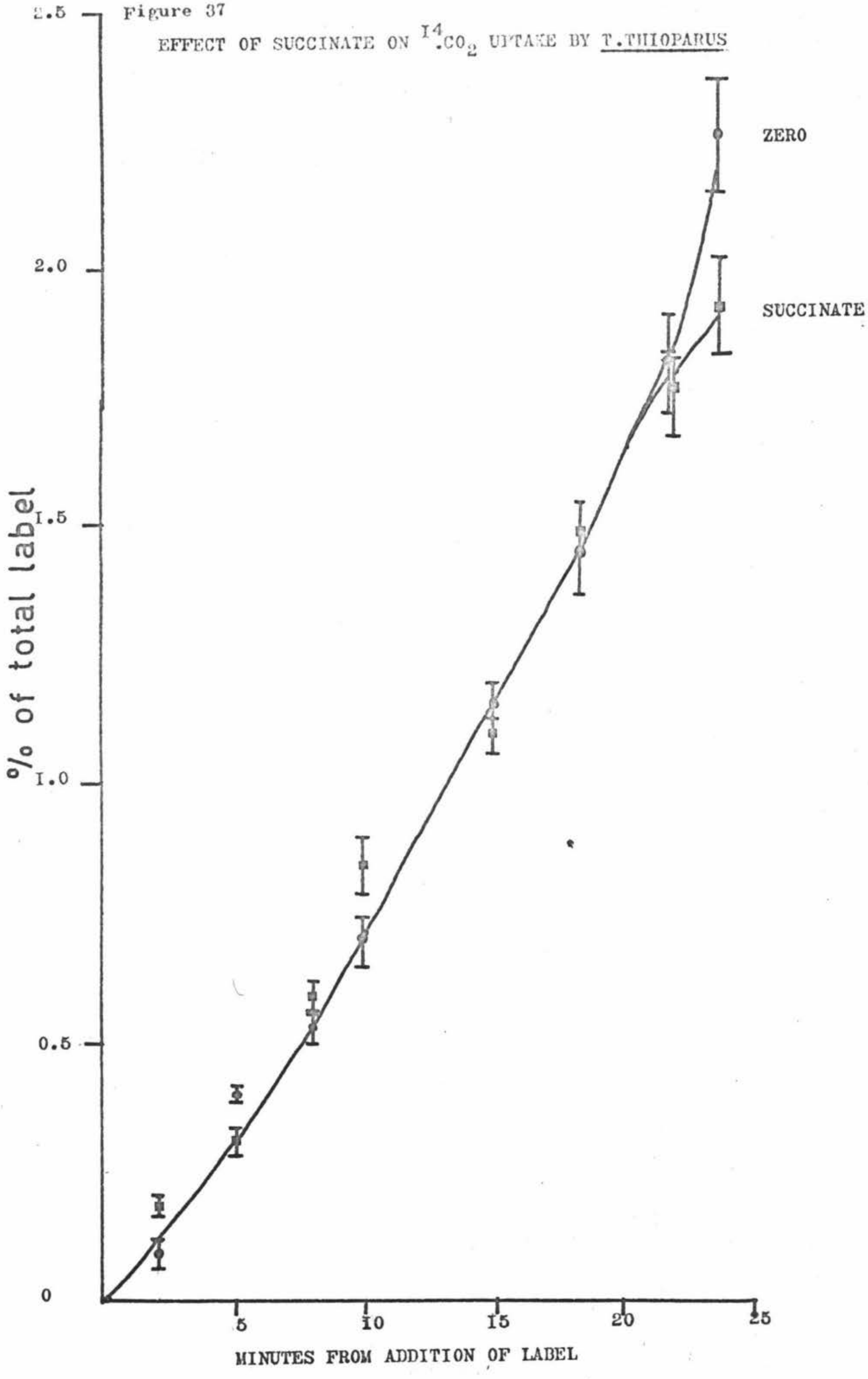


Figure 37

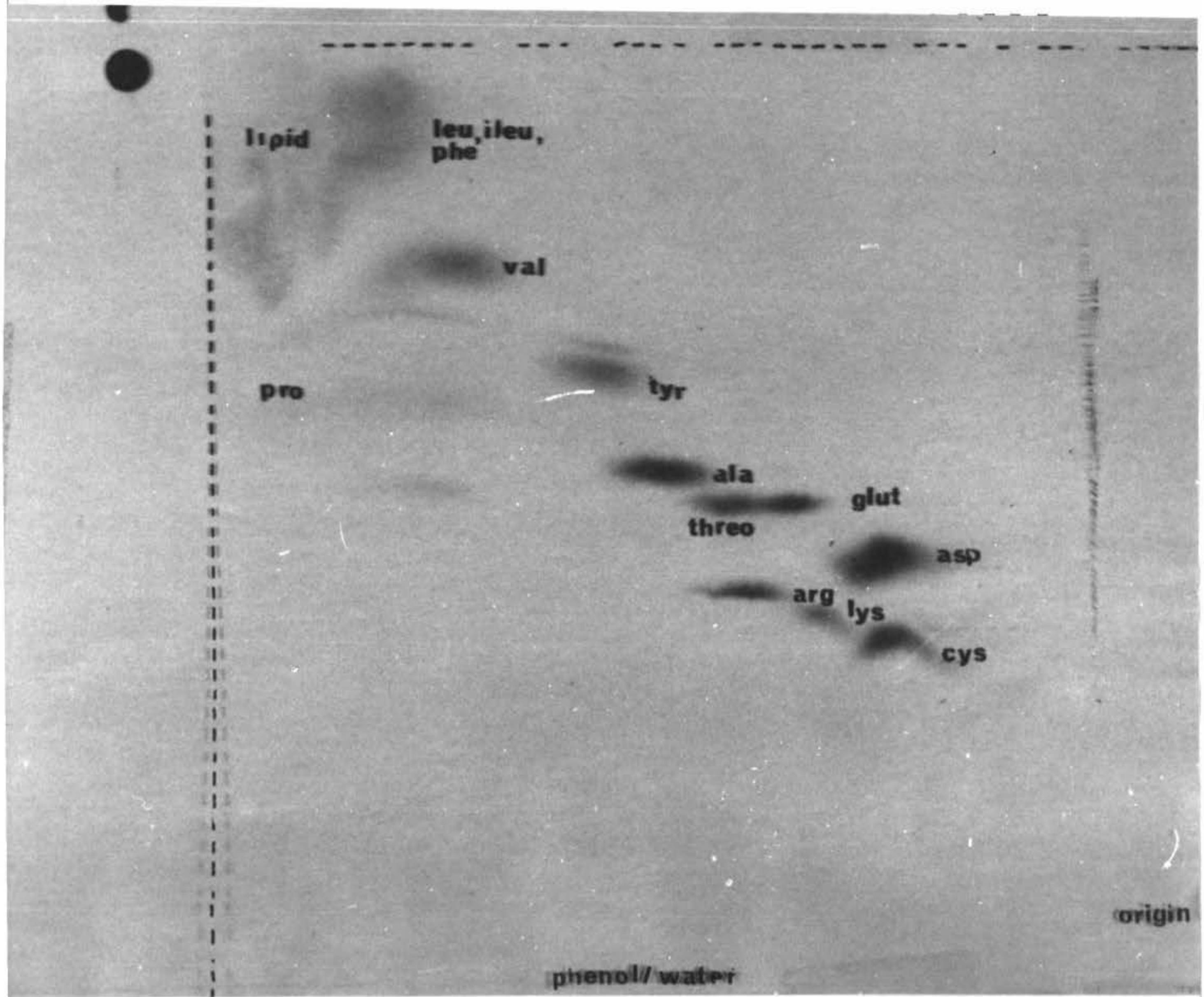
EFFECT OF SUCCINATE ON $^{14}.CO_2$ UPTAKE BY <u>T.THIOPARUS</u>

FIGURE 38.

DISTRIBUTION OF CELL RESIDUE ^{14}C

FROM $^{14}CO_2$ IN T. THIOPARUS

SOLVENT FRONT



Dotted lines represent solvent fronts

leu	L-leucine	ileu	L-isoleucine
phe	L-phenylalanine	val	L-valine
tyr	L-tyrosine	pro	L-proline
ala	L-alanine	glut	L-glutamic acid
threo	L-threonine	asp	L-aspartic acid
arg	L-arginine	lys	L-lysine
cys	cysteine		

5. DISCUSSION

The results presented in Sections 3 and 4 will now be discussed in relation to each other and to comparable results from other autotrophic species and to the current theories of obligate autotrophy.

5.1. ABILITY TO UTILISE ORGANIC COMPOUNDS AS GROWTH SUBSTRATES

T. novellus has been shown (Section 3.3.) to use a wide range of organic compounds as substrates for heterotrophic growth. These include many amino-acids, sugars, alcohols, organic acids and nucleic acid bases. Such a wide range of substrates indicates a versatility of enzymatic capability and inducible enzyme alterations to covert the compounds to intermediates of central metabolic pathways and thence to distribute carbon to other essential areas of metabolism.

There is insufficient information to decide on mechanisms and relationships of metabolism. What is clearly evident is the metabolic adaptability of T. novellus.

This can be compared to T. thioparus which is clearly unable to subsist on any organic compounds under the same conditions. This may reflect the complete absence of the necessary enzymes or an inability to adapt metabolism to the new carbon source. Other explanations are possible (see Section 1.).

5.2. INHIBITORY EFFECTS OF ORGANIC COMPOUNDS ON GROWTH.

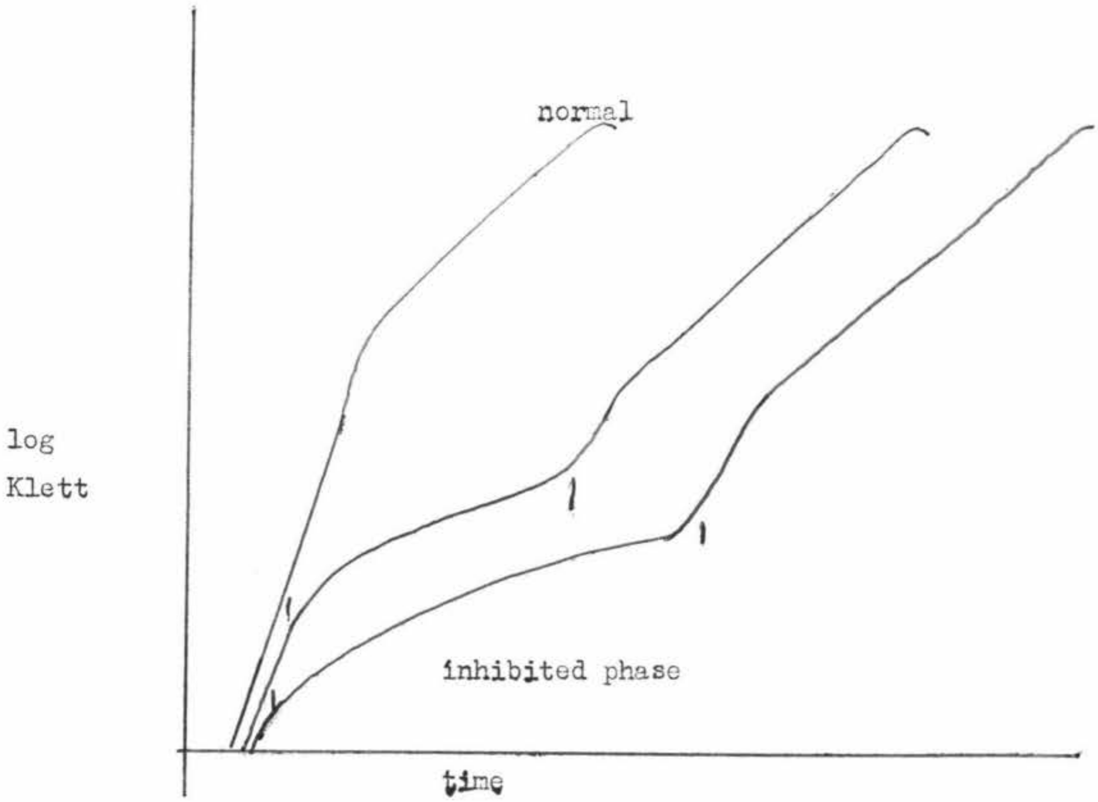
T. novellus is markedly resistant to inhibition. Even threonine, the only totally effective inhibitor found in this study, only gave complete inhibition at 100 mM and was only partially inhibitory at 10 mM (Figure 13). Some explanation must exist for this.

The wide range of substrates utilized shows that the cell is permeable to most organic compounds. However, the presence of glutamate, which was used as the substrate in testing the effects of added organic compounds on growth, may inhibit or repress the uptake of organic compounds which are readily taken up in its absence. The general insensitivity to inhibitors was also noted when thiosulphate was the substrate, although some of the organic compounds acted as substrates in the presence of thiosulphate, so it is unlikely that impermeability in the presence of glutamate or thiosulphate is the explanation.

It is possible that the inhibitors, in many cases, do not act because they are detoxified by metabolism, by the enzymes that would be induced by these same organic compounds acting in their substrate capacity.

When the effect of organic compounds on growth in liquid culture was examined there was found to be considerable similarity in the shape of the growth curves for the two species in the presence of many organic compounds (Sections 3.41.1. and 3.52.): an effect referred to as the "two phase effect".

There is an initial period of normal growth rate, then a period of reduced growth rate, then a resumption of the normal growth rate. There may be an independent effect on final yield. The second period of normal growth rate may not have been reached when the experiment was terminated, as the duration of the inhibited phase varies enormously, although it was generally much longer for T. thioparus than for T. novellus. The period of reduced growth rate can occur at any point in the growth curve.



This occurs in all cases where an added organic compound affected the growth of T. novellus growing on glutamate, with a generally short inhibited phase.

There is some inaccuracy in the turbidities of T. thioparus determined at Klett values of less than 10. However, if it is accepted that the inhibited phase may begin before the growth is evident, turbidometrically, then the same "two phase effect" is evident. The inhibited phase can be initiated quite rapidly or can be attained more slowly (lower curve in the figure).

Lu et al. (1971) appear not to have detected any such effect during inhibited growth of T. thioparus, T. neapolitanus and T. thio-oxidans at either of the two concentrations (10^{-2}, 10^{-3} M) of amino acids tested. They noted either inhibited utilization rates or completely inhibited growth as the only two effects. However these workers monitered growth by measuring thiosulphate consumption and noted that this need not parallel protein increase.

This study used turbidity, which measured cell density and precipitated sulphur, as the growth criterion; which may be one of the reasons for the discrepancy. In the studies of the effect of amino-acids on growth in Methylococcus capsulatus (Eccleston and Kelly 1972a), T. neapolitanus (Johnson and Vishniac 1970, Kelly 1969b, c, d) Anacystis nidulans (Hoare, Hoare and Moore 1967) and T. concretivorus (Kelly 1969c) the inhibitor was added to actively growing cells and turbidities showed an inhibited growth rate immediately or after a short interval with no "two phase effect".

It is quite possible that the "two phase effect" is an effect in thiobacilli revealed in growth curves of turbidity against time, only when the inhibitor is added to cultures along with the small inoculum at the start of growth. Such a difference in the action of the inhibitor at different points in the growth curve was considered possible by Rittenberg (1969).

Two possible mechanisms can be suggested to account for this "two phase effect".

(1) The organic compound may be present initially at only a slightly or non-inhibitory concentration and if the same or a similar compound is formed during metabolism a threshold inhibitory concentration

may be exceeded. This formation will increase in parallel with growth. The attainment of the inhibited phase will be aided by a small difference between non-inhibitory and totally-inhibitory concentrations. Thus in Anacystis nidulans an increase in acetate concentration from 10^{-2} to $4x10^{-2}$ M increases the inhibitory effect from a minor effect to almost total inhibition (Hoare, Hoare and Moore 1967).

(2) If the compound is itself not inhibitory but can undergo metabolism to some toxic product then inhibition may become evident only when metabolism has formed sufficient of the toxic metabolite. Such an effect has been suggested by Borichewski and Umbreit (1966a) (see Section 1.43.).

The formation of exogenous organic compounds is well documented (Schwaitman and Lundgren 1968; Johnson and Abraham 1969; Borichewski and Umbreit 1966a; Borichewski 1966b; Butler and Umbreit 1969; Pan and Umbreit 1972a; Kelly 1969a).

The resumption of normal growth rate may be due to detoxification by either assimilation or further metabolism, or by some adaptation phenomenon.

As well as an effect on growth rate an effect on final yield is also possible. If the inhibited growth phase is prolonged then the phase resembles, to some extent, a resting cell suspension. During this period the substrate, thiosulphate or glutamate, is depleted to a greater extent than the increase in cell density would suggest at normal growth rates. When normal growth rate is resumed there is less substrate than at the equivalent turbidity in uninhibited cultures so the final yield is reduced. Alternatively the inhibitor by its incorporation may have a carbon- and energy- saving effect so that the final yield may be increased.

Both these effects are evident in Figures 12, 13, 14, 15, 16, 17 and 18.

5.21. SUCCINATE INHIBITION OF T. THIOPARUS.

The succinate-inhibited growth of T. thioparus was investigated in more detail. The pH rise and fall and thiosulphate consumption were delayed in duration but this is undoubtedly a consequence of the inhibited growth in the presence of succinate (Section 3.53.). In the radioactive uptake experiments it was noted that succinate did not affect the rate of acid production (Figure 36) or of $^{14}CO_2$ fixation (Figure 37) at comparable turbidities. As these cells had been harvested from succinate-containing media it was possible that some adaptation to succinate had occurred.

However a cell suspension of T. thioparus harvested from a succinate-containing medium took considerably longer to reach comparable pH values and turbidity values in the presence of succinate than T. thioparus harvested from normal medium did in the absence of succinate. This indicates that the succinate effect persists. The succinate was still effective on ^{14}C-glucose uptake so it could not have been detoxified during incubation.

5.22. AUTOTROPHIC SUPPRESSORS

Certain compounds, not all of which were capable of acting as substrates for heterotrophic growth, suppressed autotrophic growth of T. novellus. A similar effect in T. thioparus would be inhibitory. One substance, pyruvate, suppressed autotrophic growth but did not act as a heterotrophic substrate; in the absence of thiosulphate pyruvate was a growth substrate for T. novellus. A similar effect in T. thioparus might explain, in some cases, an inability to utilize potential substrates as carbon or energy sources.

5.3. THE EFFECT OF THREONINE ON T. THIOPARUS.

The reversal of the threonine inhibition of T. thioparus by the biosynthetically-related amino-acids, isoleucine and methionine (Section 3.6.) indicates a close relationship of amino-acid biosynthetic control mechanisms in heterotrophs and obligate autotrophs.

The reversal of inhibition may have occurred at the level of the transport system, thus preventing the accumulation of inhibitory levels of threonine. In T. neapolitanus Johnson and Vishniac (1970) found that only histidine, methionine, phenylalanine and threonine of the amino-acids tested at 3×10^{-4} M were inhibitory: ^{14}C-phenylalanine impermeable mutants were simultaneously resistant to histidine, phenylalanine and threonine inhibition. In Agmenellum quadrupliticum (an obligately photo-autotrophic blue-green alga) the uptake of inhibition concentrations of L-phenylalanine was prevented by many amino-acids but once accumulated the effect was reversed only by tyrosine; phenylalanine inhibited this organism by conversion to phenyl-pyruvate which, along with shikimic acid, is a feed-back inhibitor of L-tyrosine formation. Similarly in T. neapolitanus

Kelly (1969b, c, d) found that the phenylalanine inhibition of DAHP synthase (7-phospho-2-oxo-3 deoxy D-arabino heptonate D-erythrose-4-phosphate lyase E.C.4.1.2.15) was prevented at the transport level by some amino-acids but not others (Kelly 1969a, b, c, d) (Section 3.6.).

The metabolism and control functions in the biosynthesis of an amino-acid in T. neapolitanus (Kelly 1969a, b, c, 1967a: Lu et al. 1971), T. thioparus (Lu et al. 1971), T. thio-oxidans (Lu et al. 1971), Agmenellum quadrupliticum (Ingram and Jensen 1973), and Methylococcus capsulatus (Eccleston and Kelly 1972a) can be explained in similar terms to those in heterotrophic organisms (Cohen 1968).

Despite this similarity the high sensitivity of most obligate autotrophs to inhibition by such a wide range of "building block" compounds, such as amino-acids and nucleic acid bases, makes this an unusual, though not necessarily unique or distinctive, feature of obligate autotrophs. T. novellus, a facultative autotroph, was largely unaffected while T. thioparus, an obligate autotroph, was generally sensitive.

The effect of mixtures of amino-acids in reversing individual amino-acid inhibitions might be at the transport level, at the level of biosynthetic control or by providing required amino-acids. As the single components of the mixtures might well be at inhibitory concentrations the inhibition of growth, but not the effect on biosynthetic mechanisms, is dependent on the individual occurrence of the amino-acid.

As noted by Rittenberg (1972) such a sensitivity, which results in the cessation of biosynthesis of an exogenously-supplied compound, permits a higher cell yield from a given energy supply. Thus in the organically rich environments in which both heterotrophs and obligate autotrophs occur, in high numbers and presumably some activity, this cessation will provide higher cell yields for the same autotrophic energy source utilization. In the organically-impoverished environments in which growth and activity of an obligate autotroph has been shown, cell yields will be much lower. There is thus an ecological advantage, with no ecological disadvantage, in such sensitivity arising from quite usual control mechanisms.

Such a feed-back repression and inhibition explanation for the effect of amino-acids etc. is not valid for the central metabolism compounds such as glucose, pyruvate and citrate which have only limited biosynthetic functions in terms of direct incorporation.

5.4. ASSIMILATION OF EXOGENOUS ORGANIC COMPOUNDS.

The incorporation of exogenous organic compounds by obligate autotrophs has been well documented by Rittenberg (1969). However, as shown by Stouthammer and Bethenhausen (1973) a rate of assimilation such that subsequent metabolism provides energy in excess of maintenance energy requirements is necessary for growth to become evident (Section 1.41.).

In the present study, the time-course of uptake of trace amounts (0.5 - 10μM) of ^{14}C-labelled compounds (acetate, glucose, and glutamate) was followed for the facultative autotroph T. novellus, grown on glutamate and on thiosulphate, and the obligate autotroph T. thioparus, grown in the presence and absence of the inhibitor, succinate.

The uptake of the compounds by T. novellus, either heterotrophically- or autotrophically-grown was fairly rapid and over 70% of the ^{14}C-labelled compound supplied was assimilated within an hour (Section 4.3.). In T. thioparus, however, only the uptake of ^{14}C-acetate was rapid and complete. Uptake of ^{14}C-glucose and ^{14}C-glutamate was low and occurred at a slow rate proportional to the rate of thiosulphate metabolism (as indicated by the rate of alkali addition). The linear rates of glucose and glutamate uptake, maintained for some hours, indicated that no adaptation had occurred at the concentrations used. It is tempting to assume further that the low linear rates of uptake for glucose and glutamate in T. thioparus are a consequence of the low level of the transport system present. This may not be true; there is insufficient evidence in this study for a firm conclusion to be drawn.

In the obligate photo-autotroph Anacystis nidulans Hoare, Hoare and Moore (1967) found that assimilation of exogenous organic compounds varied considerably: after 5 hours the % of added ^{14}C in membrane-filter retained cells was:

	In the light	In the dark
For Acetate	66.5	13.4
Succinate	1.2	1.2
Glutamate	0.2	0.1
Citrate	0.2	0.1

In the same organism the uptake of 10 mM acetate was linear with time for twelve hours (Hoare and Moore 1965).

Taylor and Hoare (1971a) found the extent of uptake of ^{14}C-acetate by T. denitrificans to be independent of acetate concentration (from 0.75 to 18.6 mM).

The uptake per μgm dry weight for acetate was higher than for any other compound, at the same concentrations, in the obligate autotrophs A. nidulans, Gloeocapsa alpicola, Cocciochloris peniocystis, T. thioparus, and Thiobacillus thio-oxidans (Smith, London and Stanier 1967). It is, perhaps, of significance that only acetate and succinate, of a range of compounds tested, were able to increase cell yields in continuous cultures of Thiomicrospira pelophila, Thiobacillus thioparus and T. neapolitanus, (Kuenen and Veldkamp 1973).

Incorporation that parallels growth is very common, almost characteristic, of obligate autotrophs (Kelly 1967c, 1969b, 1970a; Eccleston and Kelly 1972a; Johnson and Vishniac 1970; Smith and Hoare 1968; Butler and Umbreit 1966; Smith, London and Stanier 1967; Hoare, Hoare and Moore 1967) with only one exception, for an inhibitory concentration of pyruvate assimilated by T. neapolitanus x (Kelly 1970a). This does not imply that the system is saturated - as shown by glucose uptake in Anacystis nidulans (Smith, London and Stanier 1967):

Glucose Conc.	Uptake rate (linear) mM/gm Cells
5 mM	4×10^{-2}
25 mM	20×10^{-2}
50 mM	59×10^{-2}

The linear differential rate does however indicate that no induction or adaptation is occurring to increase the uptake rate. This implies that the system for uptake and subsequent metabolism cannot be induced (consistent with the theory of permanent repression, Section 1.43.), that such systems are fully induced, that the uptake system is saturated at these very low levels, or that internally effective levels of the compound cannot accumulate since the compound is used more rapidly than it is taken up.

The existence of uptake systems saturated at very low levels of organic compounds poses the problem of the manner in which the inhibition is effected at concentrations higher than the saturating concentrations, which are not inhibitory. The additional concentration required to reach the inhibitory concentrations, internally, probably accumulates by passive diffusion at high external concentrations.

The uptake of acetate by Anacystis nidulans (Smith, London and Stanier 1967) is independent of the concentration of acetate:

Acetate Conc.	Uptake rate: mM/gm cells Formed
1.0 mM	2.16
5.0 mM	2.15
25 mM	2.62

this organism is totally inhibited by 40mM acetate (Hoare, Hoare and Moore 1969).

Similarly in T. neapolitanus (Kelly 1967c):

Acetate Conc.	Differential Uptake Rate mM/gm Cells
15 μM	0.5
110 μM	0.5
510 μM	0.5
1000 μM	.55

It is possible that in certain cases there is some limitation other than the uptake system. The trace amounts used in this study make it unlikely that competition for energy is a limitation on "transport" operation. The inability of many organic compounds to be taken up in the absence of the autotrophic energy source probably reflects their inability to act as an alternative energy source rather than some obligate link to autotrophic energy-generation mechanisms. Once taken up the compounds must be metabolised; if the metabolism of these compounds requires some acceptor then the rate of supply of the acceptor limits the rate of metabolism of the compound; so that in the absence of the acceptor feed-back inhibitory concentrations accumulate and further uptake ceases.

For compounds such as acetate the acceptors will be compounds capable of reacting with acetyl-CoA. Acetate uptake by T. denitrificans required bicarbonate, which could only be replaced by oxalo-acetate (Taylor and Hoare 1971); in T. neapolitanus bicarbonate was required (Kelly 1967c).

As these acceptors are provided by metabolic activity then the rate of uptake is a linear function of growth; as the taken-up label enters a pool already present even very low concentrations will be assimilated linearly. Furthermore the size of the acceptor pool and not only the transport-system activity, may decide the "saturation level" for uptake.

5.5. DISTRIBUTION OF ASSIMILATED LABEL.

The trace amounts of labelled compounds were used in this study so that the assimilation of these compounds would not distort the normal internal metabolism by affecting enzyme levels or activities or by markedly altering pool sizes. The label thus provides an outline of metabolic pathways. The CELL HYDROLYSATE amino-acids can be assumed to become labelled by the well recognized biosynthetic pathways, via intermediates of central metabolism (see Figure 40).

The $^{14}CO_2$ produced is a measure of the oxidative degradation of the labelled compounds.

The distribution of ^{14}C from each of the substrates will first be discussed for each organism and the distribution pattern will then be compared with data available from other workers.

5.51. GLUTAMATE-GROWN T. NOVELLUS.

Label from ^{14}C-glucose appears in most amino-acids of the CELL HYDROLYSATE FRACTION and also in CO_2, indicating substantial metabolism of glucose. The presence of exogenous glutamate has not greatly affected the incorporation of ^{14}C from ^{14}C-glucose into the glutamate family amino-acids (glutamate, proline and arginine) since there is a similar distribution of ^{14}C from ^{14}C-glucose in thiosulphate-grown T. novellus. Labelled carbon-dioxide can arise from glucose by the reactions catalyzed by phosphogluconate dehydrogenase, pyruvate dehydrogenase and the enzymes of the TCA cycle (see Figure 41).

The continued production of $^{14}CO_2$ after glucose uptake has ceased is presumably due to the continued oxidation of internal metabolic pools or, less likely in the presence of the substrate glutamate, stored reserves such as poly-β-hydroxybutyrate, containing ^{14}C. This $^{14}CO_2$ would arise from the TCA cycle reactions, and constitutes the larger part of $^{14}CO_2$ production from ^{14}C-glucose.

^{14}C-acetate is taken up rapidly and completely and much of it is metabolized to CO_2. Most of the label, in CELL EXTRACT and CELL HYDROLYSATE FRACTIONs, appears in lipid compounds. Thus a major fate of acetyl-CoA is incorporation into lipid. That proportion that does enter the TCA cycle appears in most of the amino-acids and in CO_2.

In the case of ^{14}C-glutamate about 40% of the assimilated glutamate becomes CO_2, presumably by way of the TCA cycle which glutamate enters via the degradative glutamate dehydrogenase (Charles 1971).

The distributions indicate that an active, complete TCA cycle is operating as is at least one of the pathways capable of converting glucose to pyruvate.

5,52, THIOSULPHATE-GROWN T. NOVELLUS.

Again ^{14}C from ^{14}C-glucose appears in a wide range of amino-acids distributed similarly to that in glutamate-grown T. novellus, so a functional, complete TCA cycle and at least one pathway for the conversion of glucose to pyruvate must be operating.

^{14}C-acetate is largely metabolized to $^{14}CO_2$, presumably by way of the TCA cycle, to a greater extent than for glutamate-grown T. novellus. The much lower incorporation into CELL RESIDUE, despite the only minor differences in distribution of ^{14}C, may be related to the much lower growth rate of the autotrophic T. novellus (i.e. to a much lower absolute rate of biosynthetic activity).

The distribution of ^{14}C from ^{14}C-glutamate into $^{14}CO_2$, ethanol-soluble (CELL EXTRACT FRACTION) compounds and amongst amino-acids indicates that glutamate is entering a complete, functional TCA cycle, presumably via a degradative glutamate-dehydrogenase, which is present but at very much lower levels than in glutamate-grown T. novellus (Charles 1971).

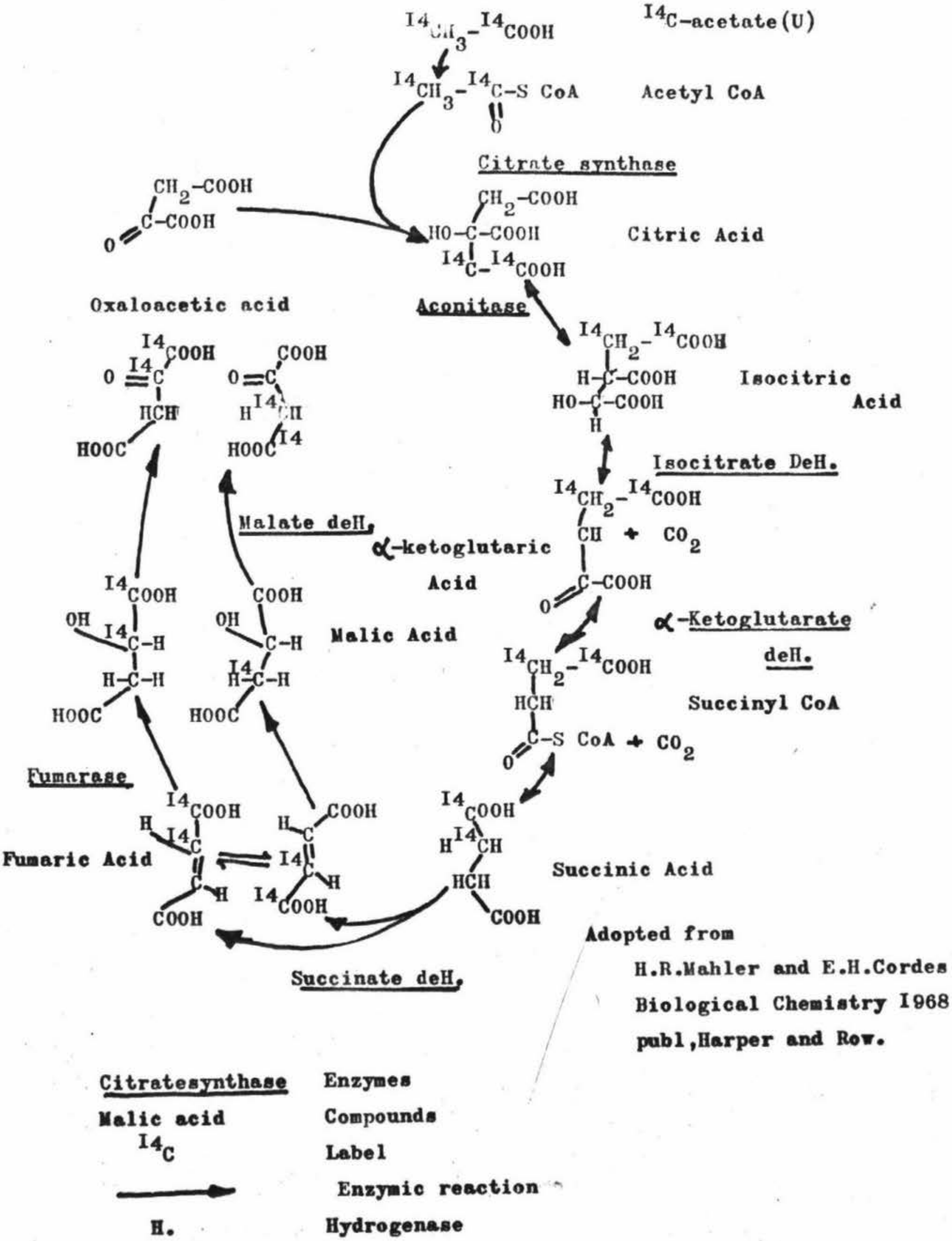
5.53. T. THIOPARUS.

The label from ^{14}C-glucose appears in most of the amino-acids, but the distribution, by inspection, is not identical with that for T. novellus. The production of $^{14}CO_2$ from ^{14}C-glucose is less than for T. novellus, indicating that less of the glucose assimilated is oxidized. The distribution of ^{14}C among amino-acids resembles that obtained using $^{14}CO_2$. This partial similarity may be due to the similarity of the pathways involved in CO_2 fixation and in glucose metabolism (see Figure 41).

^{14}C-acetate is not oxidized and there are few labelled compounds in CELL EXTRACT and CELL HYDROLYSATE FRACTIONs so that the metabolism of acetate is quite restricted.

Figure 39

DISTRIBUTION OF ^{14}C FROM ^{14}C-ACETATE IN COMPOUNDS OF AN INCOMPLETE TCA CYCLE .

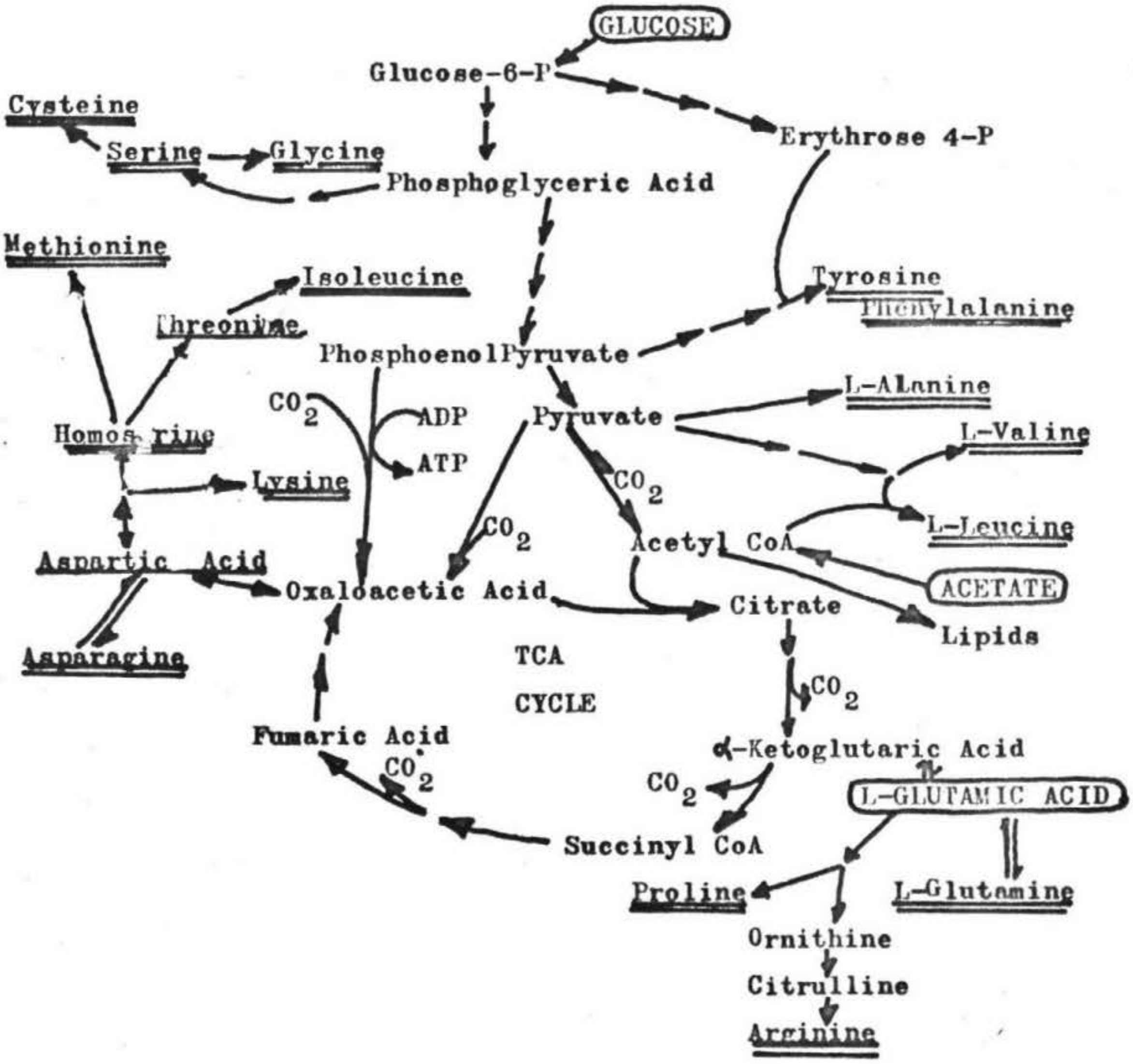


Adopted from
H.R.Mahler and E.H.Cordes
Biological Chemistry 1968
publ,Harper and Row.

Citratesynthase	Enzymes
Malic acid	Compounds
^{14}C	Label
⟶	Enzymic reaction
H.	Hydrogenase

Figure 40

ORIGIN OF CARBON SKELETONS IN AMINO-ACID BIOSYNTHESES



Adopted from

H.R.Mahler and E.H.Cordes p54I
Biological Chemistry I968
publ. Harper and Row.

⬭ Labelled Compounds
→ Enzymic conversions
→ → Multistep pathways
↔ Reversible processes
Serine Amino acids

The cell hydrolysate amino-acids (Figure 30) are those of the glutamate family and an amino-acid in the isoleucine/leucine/phenylalanine/methionine region of the radiochromatograms, presumably leucine. Very low proportions of other amino-acids (0 - 10%) may represent refixed $^{14}CO_2$ or exchange into other metabolic areas. As there is no incorporation into aspartate family amino-acids the TCA cycle can be presumed to be incomplete; this is consistent with the low $^{14}CO_2$ production.

As glutamate family amino-acids but not aspartate family amino-acids are formed (Figure 40) the lesion in the TCA cycle must lie between α-ketoglutarate dehydrogenase and malate dehydrogenase (inclusive) (Figures 39 and 40).

In none of these reactions, in the incomplete cycle, is $^{14}CO_2$ formed from acetate, nor does it arise in the biosynthesis of glutamate family amino-acids and of leucine. In these biosyntheses there is similarly no opportunity for exchange of ^{14}C into other amino-acids, even by transamination reactions, save possibly valine. In fact the only possible origin of the label in the amino-acids at low levels is by formation of pyruvic acid, by the formation of other compounds during lipid metabolism, or by recycling of the amino-acids by catabolic pathways.

^{14}C-glutamate is assimilated and incorporated in parallel with growth: there is some $^{14}CO_2$ production but this is at such low absolute values it may not, in fact, be $^{14}CO_2$ but some other ^{14}C-compounds, or can be assumed to arise from exchange into the TCA cycle and subsequent formation as for acetate. The ^{14}C in CELL RESIDUE is presumably due to the glutamate family amino-acids (glutamate, arginine and proline) in proteins.

As the TCA cycle is incomplete the $^{14}CO_2$ from ^{14}C-glucose must arise from those decarboxylations in the TCA cycle, i.e. those which produce $^{14}CO_2$, which means those compounds arising from ^{14}C-pyruvate and not from ^{14}C-acetate (see Figures 39 and 41), and also from the decarboxylations by 6-phosphogluconate dehydrogenase and pyruvate dehydrogenase (see Figures 39 and 41). The label that goes through pyruvate dehydrogenase, and hence through acetyl-CoA, will appear in the same compounds as acetate did. No estimate of these compounds is available for ^{14}C-glucose.

This diagram shows the various reactions that are considered to be involved in "central metabolism". Some rearrangements and some single enzymes that are not of widespread occurrence have been omitted for clarity as have those reactions that form some of the products formed in fermentative metabolism. The combined TCA and glyoxylate cycles have been shown in more detail in Table III on Page 9.

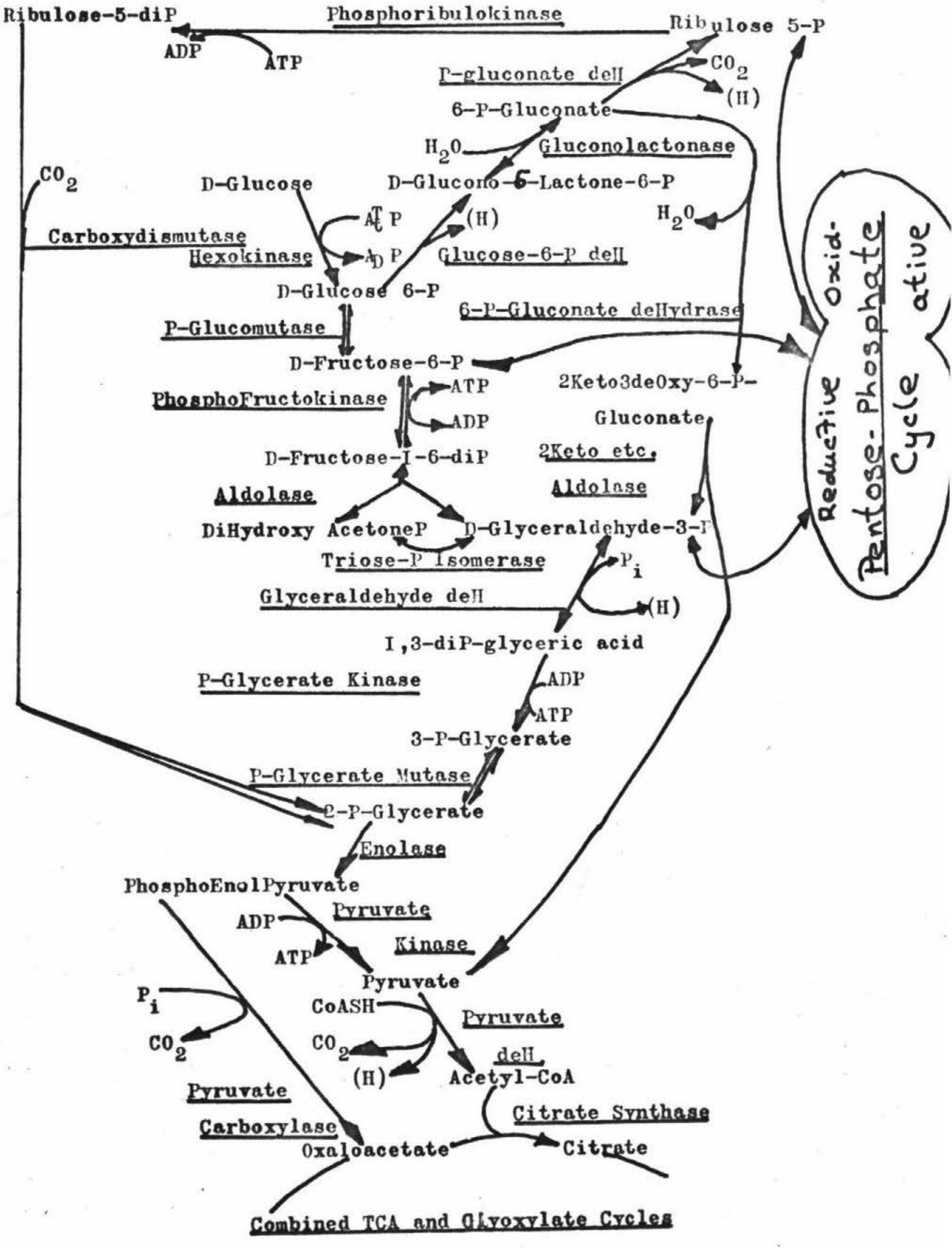
The various enzymes are underlined.

(H) represents the products of dehydrogenations, either $NADH_2$ or $NADPH_2$.

The complex of rearrangements that occur in the pentose phosphate cycles are not shown for clarity, but the enzymes can be assumed to exist in both the thiobacilli under the conditions used.

Figure 4I

CENTRAL METABOLISM



5.54. T. THIOPARUS IN THE PRESENCE OF 16 mM SUCCINATE.

Both the uptake and the subsequent metabolism of glucose are affected by the presence of succinate. Uptake is very low and the proportion of assimilated ^{14}C in $^{14}CO_2$ is decreased when succinate is present.

However succinate has no effect on ^{14}C-acetate uptake and metabolism; although there may be a reduction in the release of radio-active substances from the cell.

The rate of $^{14}CO_2$ uptake and the distribution of ^{14}C from $^{14}CO_2$ among cellular constituents is also unaffected by succinate. It can be concluded that succinate is not inhibitory by affecting amino-acid metabolism as CELL HYDROLYSATE radiochromatograms, using either $^{14}CO_2$ or ^{14}C-acetate, are identical in the presence and absence of succinate. There is, however, an effect on glucose metabolism.

5.55. COMPARISON OF DISTRIBUTIONS OF $^{14}CO_2$ AMONGST AMINO-ACIDS OBTAINED IN THIS STUDY WITH THOSE OBTAINED IN OTHER STUDIES.

Two types of pattern of distribution for the label from ^{14}C-acetate can be distinguished:

(1) a "T. thioparus type" in which CELL RESIDUE label appears in lipids, and the amino-acids of the glutamate family and leucine alone.

(2) a "T. novellus type" in which the CELL RESIDUE label appears in most amino-acids and there is considerable oxidation with consequent production of $^{14}CO_2$.

The type (1) distribution is found only in obligate autotrophs, and type (2) only in facultative autotrophs and heterotrophs (Table XIX) and these patterns can be correlated with a lack of α-ketoglutarate dehydrogenase in type (1) (Table XX; see also Table II).

The distribution of label using ^{14}C-glutamate can again be classified as one of two types, which can be correlated with the occurrence of α-ketoglutarate dehydrogenase. The T. thioparus type in which ^{14}C appears in only a very few amino-acids, of the glutamate family, and only a very low proportion in CO_2, has also been found in Anacystis nidulans and T. thio-oxidans (Smith, London and Stanier 1967) and in T. neapolitanus (Kelly 1967a).

TABLE XIX

DISTRIBUTION OF ^{14}C-ACETATE IN AMINO-ACIDS; OCCURRENCE OF TYPES.

T. thioparus: Type (1)		T. novellus: Type (2)	
T. neapolitanus c	(1) (3)	T. intermedius	(4)
T. neapolitanus x	(1)	Nitrobacter agilis	(6)
Anabaena variabilis	(2)	Chlorogloea fritschii	(7)
Anacystis nidulans	(2) (5)	T. A_2	(9)
Nostoc muscorum	(2)		
Anabaena flos-aquae	(2)		
Coccochloris peniocystis	(4)		
Gloeocapsa alpicola	(4)		
T. thio-oxidans	(4)		
T. thioparus	(4)		
Methylococcus capsulatus	(8)		

TABLE XX

DISTRIBUTION OF α-KETOGLUTARATE DEHYDROGENASE IN TYPE (1) AND (2) ORGANISMS.

α- Ketoglutarate Dehydrogenase			
Absent		Present	
Anacystis nidulans	(4)	T. intermedius	(4)
Coccochloris peniocystis	(4)	T. thio-oxidans	(12)
Gloeocapsa alpicola	(4)	Nitrobacter agilis	(6)
T. thio-oxidans	(4)	T. A_2	(13)
T. thioparus	(4)	T. novellus	(14)
T. A_2	(10)		
Methylococcus capsulatus	(11)		

(1) Kelly 1970a
(2) Hoare, Hoare and Moore 1967
(3) Kelly 1967b
(4) Smith, London and Stanier 1967
(5) Hoare and Moore 1965
(6) Smith and Hoare 1968
(7) Miller and Allen 1962
(8) Eccleston and Kelly 1973
(9) Williams and Hoare 1972
(10) Peeters, Liu and Aleem 1970
(11) Davey *et al.* 1972
(12) Butler and Umbreit 1969
(13) Taylor and Hoare 1965
(14) Charles 1971

Thus the fate of acetate depends upon the completeness of the TCA cycle; if it is incomplete, due to the absence of α-ketoglutarate dehydrogenase, then acetate carbon is assimilated entirely into lipids and a few amino-acids, without CO_2 production. If however the cycle is complete the acetate is incorporated into most amino-acids and a large proportion appears as CO_2. Under such conditions energy-generation is possible from acetate so long as oxidative phosphorylation can occur; acetate can serve as a carbon source for most amino-acids. In the absence of a complete TCA cycle a functional glyoxylate cycle can fulfil an assimilatory role so that ^{14}C-acetate label would appear in most amino-acids and eventually in CO_2, but not to the same extent as for the dissimilatory TCA cycle. T. thioparus has neither a functional TCA cycle nor a functional glyoxylate cycle; α-ketoglutarate dehydrogenase (Smith, London and Stanier 1967) and isocitrate lyase (Cooper 1964) are both absent.

The fates of glutamate also depend upon a functional TCA cycle. In an incomplete TCA cycle (as in T. thioparus) glutamate serves only a biosynthetic function and none can be oxidized. With a complete TCA cycle the glutamate that does enter the TCA cycle via glutamate dehydrogenase, can be dissimilated with extensive production of carbon dioxide. In the presence of thiosulphate the fate of glutamate in T. novellus, as a biosynthetic precursor after its entry into the TCA cycle, is different from that in the presence of glutamate; possibly due to a difference in the ability of TCA cycle intermediates to enter carbohydrate pathways, e.g. via the glyoxylate cycle.

That such lesions are a sufficient explanation of obligate autotrophy for certain substrates is shown by the study of Neilson et al. (1972) on a mutant of the green alga Chlamydomonas dysosmos which thence became an obligate autotroph towards acetate, a normal heterotrophic substrate. Although the TCA cycle is complete the glyoxylate cycle, which serves an anaplerotic function in the metabolism of acetate, was incomplete as isocitrate lyase was absent. The production of ATP in the short term was possible, but diminished later.

In T. thioparus the only pathway available for glucose metabolism is the oxidative and reductive pentose phosphate cycle, as the absence of phosphofructokinase (Johnson and Abraham 1969) and 6-phosphogluconate dehydrase (Matin and Rittenberg 1971a) prevents the operation of the

Embden-Meyerhoff-Parnas and Entner-Doudoroff pathways respectively. Thus glucose ^{14}C will enter intermediates of the Hexose Monophosphate pathway (oxidative and reductive pentose phosphate cycle or the Calvin cycle) to give a similar distribution of ^{14}C amongst amino-acids, to $^{14}CO_2$. The absence of phosphofructokinase would be an advantage, autotrophically, as carbon dioxide fixed through the Calvin cycle would not be recycled uselessly through glycolysis (see Figure 41) (Embden-Meyerhoff-Parnas pathway). This enzyme is also absent in Nitrosocystis oceanus (Williams and Watson 1968) and T. neapolitanus (Johnson and Abraham 1969). Its presence or absence in other autotrophs does not appear to have been examined.

The mechanism of the inhibitory effect of succinate on glucose metabolism is not evident from the data of this study. This could be at the level of uptake; or at any of the enzymes exclusive to glucose metabolism, and not in the reductive pentose phosphate cycle:

i.e. hexokinase, phosphoglucoisomerase, glucose-6-P-dehydrogenase, gluconolactonase, phosphogluconate dehydrogenase, as the Entner-Doudoroff and Embden-Meyerhoff-Parnas pathways are absent.

Although the uptake mechanism for glucose is unknown in Thiobacilli, the uptake could well involve hexokinase, which is hence a likely candidate for succinate-inhibition of glucose metabolism. This would effectively inhibit glucose metabolism whereas single-enzyme inhibition of any of the other enzymes (above) could simply divert glucose from entering the Calvin cycle by one route to the other available route (glucose-6-P-dehydrogenase, gluconolactonase and phosphogluconate dehydrogenase or phosphoglucomutase series of enzymes to different intermediates of the Calvin cycle)(see Figure 41) and this would not explain the very large drop in glucose metabolism in the presence of succinate.

Hence phosphoglucoisomerase and at least one of the other enzymes, besides hexokinase, must be inhibited if hexokinase is not. However in normal autotrophic metabolism glucose-phosphate and not glucose is the metabolic intermediary so an inhibition of hexokinase by succinate would not explain the inhibition of growth. Whatever the basis, it is surprising to find succinate inhibiting an area of metabolism so far removed from its own metabolism (see Figure 41).

As previously mentioned there was no effect by succinate on $^{14}CO_2$ fixation by T. thioparus. Succinate also inhibits the growth of T. thio-oxidans but Rao and Berger (1970) found no initial effect on $^{14}CO_2$ fixation in this species. Other organic compounds do affect CO_2 fixation in obligate autotrophs, e.g. acetate and pyruvate at 1 mM markedly depresses the $^{14}CO_2$ fixation in T. neapolitanus (Kelly 1967c) and T. denitrificans (Taylor and Hoare 1971). Such effects also occur in facultative autotrophs e.g. acetate depresses $^{14}CO_2$ fixation in Nitrobacter agilis (Smith and Hoare 1968), T. A2 (Taylor and Hoare 1971) and Hydrogenomonas facilis (Stukus and De. Cicco 1970). Such depressions of CO_2 fixation may be due to direct inhibition or repression, as is generally the case in the facultative organisms, or by "carbon-saving". This increased yield for the same energy-utilization has definitely been shown by Kuenen and Veldkamp (1973) in continuous cultures of Thiomicrospira pelophila, T. thioparus and T. neapolitanus for acetate and succinate. These compounds and no others gave increased yields, the increase in yield being to the amount of the substance assimilated: the increment due to acetate reached a maximum at 1 mM acetate. These effects were additive for the two compounds. The other compounds were not effective either because they were not taken up or because they were not metabolised.

The limits of "carbon-saving" probably represent the extent of assimilation of these compounds of which the organism is capable. It is possible, as suggested by Rittenberg (1969) that some special combination e.g. succinate, acetate and glucose-6-P for T. thioparus, of compounds might act as a "sole" carbon source for obligate autotrophs.

In this light the statement of Kuenen and Veldkamp (1973) is of interest. "The metabolic machinery of the obligately chemolithotrophic thiobacilli and T. pelophila appears to be much less flexible than that of the mixotrophic thiobacilli (Rittenberg 1969 and Kelly 1971). However, the maximum specific growth rate of the obligate chemolithotrophs in inorganic growth media is much higher than that of the mixotrophs ($0.3\ hr^{-1}$ versus $0.05\ hr^{-1}$). Apparently the price to be paid for this advantage is the loss of the heterotrophic potential. The ubiquitious presence of large numbers of obligately chemolithotrophic thiobacilli in natural environments indicates that this loss is worthwhile."

BIBLIOGRAPHY

1. American Type Culture Collection Catalogue 1970. 9th Edition. Publ. American Type Culture Collection, Rockville, Maryland.

2. K. Ameniya 1972. Absence of Glucokinase in Methanomonas sp as a Cause for their Inability to Grow on Glucose. Canadian Journal of Microbiology 18 1907-1913.

3. K.J. Anderson and D.G. Lundgren 1969. Enzymatic Studies of the Iron-oxidizing Bacterium Ferrobacillus ferro-oxidans: Evidence for a Glycolytic Pathway and Krebs Cycle. Canadian Journal of Microbiology 15 73-79.

4. H. Arkin and R.R. Colton. Tables for Statisticians. Publ. Barnes and Noble.

5. K. Baalsrud and K.S. Baalsrud.1954. Studies on T. denitrificans. Archiv fur Mikrobiologie 20 34-62.

6. J.K. Barridge and J.M. Shively 1968. Phospholipids of the Thiobacilli. Journal of Bacteriology 95 2182-2185.

7. J.A. Bassham and M. Calvin 1957. The Path of Carbon in Photosynthesis. Publ. Prentice-Hall.

8. R.M. Borichewski 1965. Effect of Sucrose on Glucose Utilization by Thiobacillus thio-oxidans. Bacteriological Proceedings 1965 p77.

9. R.M. Borischowski 1966b. Keto-Acids as Growth-Limiting Factors in Autotrophic Growth of T. thio-oxidans. Journal of Bacteriology 93 597-599.

10. R.M. Borichewski and W.W. Umbreit 1966a. Growth of T. thio-oxidans on Glucose. Archives of Biochemistry and Biophysics 116 97-102.

11. Breed, Murray and Smith 1957. Bergey's Manual of Determinative Bacteriology. Publ. Williams and Wilkins.

12. R.G. Butler and W.W. Umbreit 1966. Absorption and Utilization of Organic Matter by the Strict Autotroph, T. thio-oxidans, with Special Reference to Aspartic Acid. Journal of Bacteriology 91 662-666.

13. R.G. Butler and W.W. Umbreit 1969. $NADH_2$ Oxidase and α-Ketoglutarate Dehydrogenase Activity in T. thio-oxidans. Journal of Bacteriology 102 966-967.

14. A.M. Charles 1970. Properties of an NADP-Specific Isocitric Dehydrogenase from Thiobacillus novellus. Canadian Journal of Biochemistry 48 95-103.

15, A.M. Charles 1971. Effect of Growth Substrates on Enzymes of the Citric and Glyoxylate Acid Cycles in T. novellus. Canadian Journal of Microbiology 17 617-624.

16. A.M. Charles and I. Suzuki 1965. Sulphite Oxidase of a Facultative Autotroph Thiobacillus novellus. Biochemical and Biophysical Research Communications 19 686-690.

17. G.N. Cohen 1968. The regulation of Cell Metabolism. Publ. Holt, Rhinehart and Winston.

18. E.J. Conway 1947. Microdiffussion Analysis and Volumetric Analysis. Publ. Crosby, Lockwood and Son.

19. R.C. Cooper 1964. Evidence for the Presence of Certain TCA Cycle Enzymes in T. thioparus. Journal of Bacteriology 88 624-629.

20. J.F. Davey, R. Whittenberg and J.F. Wilkinson 1972. The Distribution in the Methylobacteria of some Key Enzymes Concerned with Intermediary Metabolism. Archiv fur Mikrobiologie 87 359-366.

21. O.J. Draper and A.L. Polard 1949. The Purification of Phenol for Paper Partition Chromatography. Science 109 448.

22. M. Eccleston and D.P. Kelly 1972a. Assimilation and Toxicity of Exogenous Amino-Acids in the Methane-Oxidizing Bacterium Methylococcus capsulatus. Journal of General Microbiology 71 541-554.

23. M. Eccleston and D.P. Kelly 1973. Assimilation and Toxicity of Some Exogenous C_1 Compounds, Alcohols, Sugars and Acetate in the Methane-oxidizing Bacterium Methylococcus capsulatus. Journal of General Microbiology 75 211-221.

24. N.L. Gale and J.V. Beck 1967. Evidence for the Calvin Cycle and Hexose Monophosphate Pathway in *Thiobacillus ferro-oxidans*. *Journal of Bacteriology* 94 1052-1059.

25. R.J. Herberg 1965. Channels-ratio Method of Quench Correction in Liquid Scintillation Counting. Packard Technical Bulletin No. 15.

26. D.S. Hoare, S.L. Hoare and R.B. Moore 1967. The Photo-assimilation of Organic Compounds by Autotrophic Blue-green Algae. *Journal of General Microbiology* 49 351-370.

27. D.S. Hoare and R.B. Moore 1965. Photoassimilation of Organic Compounds by Autotrophic Blue-green Algae. *Biochimica et Biophysica Acta* 109 622-625.

28. M. Hutchinson, K.I. Johnstone and D. White 1969. Taxonomy of the Genus *Thiobacillus*: the Outcome of Numerical Taxonomy Applied to the Group as a Whole. *Journal of General Microbiology* 57 397-410.

29. O. Ingram and R.A. Jenson 1973. Growth Inhibition by L-Phenylalanine in *Agmenellum quadrupliticum*. A Clue to Some Amino-Acid Interrelationships. *Archiv fur Mikrobiologie* 91 221-233.

30. J.F. Jackson, D.J.W. Moriaty and D.J.D. Nicholas 1968. Deoxyribonucleic Acid Base Composition and Taxonomy of Thiobacilli and Some Nitrifying Bacteria. *Journal of General Microbiology* 53 53-60.

31. S. Jacob 1959. Determination of Nitrogen in Protein by Means of Indanetrione Hydrate. *Nature* 183 262.

32. E.J. Johnson and S. Abraham 1969. Enzymes of Intermediary Carbohydrate Metabolism in the Obligate Autotrophs *T. thioparus* and *T. neapolitanus*. *Journal of Bacteriology* 100 962-968.

33. C.L. Johnson and W. Vishniac 19 . Growth Inhibition in *T. neapolitanus* by Histidine, Methionine, Phenylalanine and Threonine. *Journal of Bacteriology* 104 1145-1150.

34. D.P. Kelly 1967a. Problems of the Autotrophic Micro-organisms. *Science Progress Oxford* 55 35-51.

35. D.P. Kelly 1967b. Influence of Amino-Acids and Organic Antimetabolites on Growth and Biosynthesis of the Chemo-autotroph T. neapolitanus strain C. Archiv fur Mikrobiologie 56 91-105.

36. D.P. Kelly 1967c. The Incorporation of Acetate by the Chemoautotroph T. neapolitanus strain C. Archiv fur Mikrobiologie 58 99-116.

37. D.P. Kelly 1969a. Regulation of Chemo-autotrophic Metabolism. I. Toxicity of Phenylalanine to Thiobacilli. Archiv fur Mikrobiologie 69 330-342.

38. D.P. Kelly 1969b. Regulation of Chemolithotrophic Metabolism. II. Competition Between Amino-Acids for Incorporation into Thiobacillus. Archiv fur Mikrobiologie 69 343-359.

39. D.P. Kelly 1969d. Regulation of Chemo-autotrophic Metabolism. III. D.A.H.P. Synthetase in T. neapolitanus. Archiv fur Mikrobiologie 69 360-369.

40. D.P. Kelly 1970a. Metabolism of Organic Acids by T. neapolitanus. Archiv fur Mikrobiologie 73 177-192.

41. D.P. Kelly 1971a. Autotrophy: Concepts of Lithotrophic Bacteria and their Organic Metabolism. Annual Review of Microbiology 25 177-213.

42. D.P. Kelly and P.J. Syrett 1964. The Effect of Uncoupling Agents on CO_2 Fixation by a Thiobacillus. Journal of General Microbiology 34 304-317.

43. T. Khoja and B.A. Whitton 1971. Heterotrophic Growth of Blue-green Algae. Archiv fur Mikrobiologie 79 280-282.

44. M. Kocur, T. Martinec and K. Mazenec 1968. Fine Structure of T. novellus. Journal of General Microbiology 52 343-345.

45. J.G. Kuenen and H. Veldkamp 1973. Effects of Organic Compounds on Growth of Chemostat Cultures of Thiomicrospora pelophila Thiobacillus thioparus and T. neapolitanus. Archiv fur Mikrobiologie 94 173-190.

46. C.K. Leach and N.G. Carr 1970. Electron Transport and Oxidative Phosphorylation in the Blue-green Algae Anabaena Variabilis. Journal of General Microbiology 64 55-70.

47. H.B. Le John, L. van Caeseele and M. Lees 1967. Catabolite Repression in the Facultative Chemo-autotroph T. novellus. Journal of Bacteriology 94 1484-1491.

48. G.A. Le Page and W.W. Umbreit 1943. The Occurrence of 3'-ATP in Autotrophic Bacteria. The Journal of Biological Chemistry 148 255-260.

49. R.A. Lewin 1971. Fatty Acids of Thiobacillus thio-oxidans. Journal of Bacteriology 108 992-995.

50. J. London 1963. Thiobacillus intermedius. nov.sp. A Novel Type of Facultative Autotroph. Archiv fur Mikrobiologie 46 329-337.

51. J. London and S.C. Rittenberg 1967. Thiobacillus perometabolis, nov.sp., a Non-autotrophic Thiobacillus. Archiv fur Mikrobiologie 59 218-225.

52. M.C. Lu, A. Matin and S.C. Rittenberg.1971. Inhibition of Obligately Chemolithotrophic Thiobacilli by Amino-Acids. Archiv fur Mikrobiologie 79 354-366.

53. A. Matin and S.C. Rittenberg 1970b. Regulation of Glucose Metabolism in T. intermedius. Journal of Bacteriology 104 239-246.

54. A. Matin and S.C. Rittenberg 1971a. Enzymes of Carbohydrate Metabolism in Thiobacillus species. Journal of Bacteriology 107 179-186,

55. I.R. Miller and M.M. Allen 1972. Carbon Utilization Patterns in the Heterotrophic Blue-green Alga Chlorogloea fritschii. Archiv fur Mikrobiologie 86 1-12.

56. A.H. Neilson, O. Holm-Hansen and R.A. Lewin 1972. An Obligately Autotrophic Mutant of Chlamydomonas dysosmos: A Biochemical Elucidation. Journal of General Microbiology 71 141-148.

57. A.I. Oparin 1968. Genesis and Evolutionary Development of Life. Publ. Academic Press.

58. P.H.C. Pan 1971a. Lack of Distinction Between Nitrobacter agilis and Nitrobacter winogradskyi. Journal of Bacteriology 108 1416-1418

59. P. Pan and W.W. Umbreit 1972a. Growth of Obligate Autotrophic Bacteria on Glucose in a Continuous Flow-through Apparatus. Journal of Bacteriology 109 1149-1155.

60. C.G. Paradine and B.H.P. Rivett. Statistical Methods for Technologists. Publ. English Universities Press.

61. C.D. Parker and J. Prisk 1953. The Oxidation of Inorganic Compounds of Sulphur by Various Sulphur Bacteria. Journal of General Microbiology 8 344-364.

62. J. Pearce and N.G. Carr 1967. The Metabolism of Acetate by the Blue-green Algae, Anabaena variabilis and Anacystis nidulans. Journal of General Microbiology 49 301-313.

63. T.L. Peeters, M.S. Liu and M.I.H. Aleem 1970. The Tricarboxylic Acid Cycle in T. denitrificans and Thiobacillus A2. Journal of General Microbiology 64 29-32.

64. R.A. Pelroy, R. Rippka and R.Y. Stanier 1972. Metabolism of Glucose by Unicellular Blue-green Algae. Archiv fur Mikrobiologie 87 303-322.

65. C.T. Peng 1965. Quenching Correction in Liquid Scintillation Counting. Atomlight 44.

66. G.S. Rao and L.R. Berger 1970. Basis of Pyruvate Inhibition in T. thiooxidans. Journal of Bacteriology 102 462-466.

67. S.C. Rittenberg 1969. The Roles of Exogenous Organic Matter in the Physiology of Chemolithotrophic Bacteria. Advances in Microbial Physiology 3 159-196.

68. S.C. Rittenberg 1972. The Obligate Autotroph - the Demise of a Concept. Antonie van Leeuwenhoek Journal of Microbiology and Serology 38 457-478.

69. M.M. Sadler and E.J. Johnson 1972. A Comparison of the NADH Oxidase - Electron Transport System of Two Obligately Chemo-lithotrophic Bacteria. Biochemica et Biophysica Acta 282 167-179.

70. M. Santer, J. Boyer and U. Santer 1959. Thiobacillus novellus I. Growth on Organic and Inorganic Media. Journal of Bacteriology 74 197-202.

71. J. Saxena and M.I.H. Aleem 1972. Generation of Reducing Power in Chemosynthesis VII: Mechanism of Pyruvate Nucleotide Reduction by Thiosulphate in the Chemo-autotroph Thiobacillus neapolitanus. Archiv fur Mikrobiologie 84 317-326.

72. A.G. Schwaitman and D.G. Lundgren 1968. Organic Compounds in the Spent Medium of Ferrobacillus ferro-oxidans. Canadian Journal of Microbiology 11 23.

73. J.M. Shively, G.L. Decker and J.V. Greenawalt 1970. Comparative Ultra-structure of the Thiobacilli. Journal of Bacteriology 101 618-627.

74. V.B.D. Skerman 1969. Ed. Abstracts of Microbiological Methods. Publ. Wiley-Interscience.

75. R. Smiley 1964. Photoheterotrophy in Marine Pennate Diatoms. Journal of Physiology 8 p.15.

76. A.J. Smith and D.S. Hoare 1968. Acetate Assimilation by Nitrobacter agilis in Relation to it's "Obligate Autotrophy". Journal of Bacteriology 95 841-855.

77. A.J. Smith, J. London and R.Y. Stanier 1967. Biochemical Basis of Obligate Autotrophy in Blue-green Algae and Thiobacilli. Journal of Bacteriology 94 972-983.

78. G.A. Sokolova and G.I. Karavaiko 1964. Physiology and Geochemical Activity of Thiobacilli. Publ. Israel Program for Scientific Translations.

79. B. Sörbo 1957. A Colorimetric Method for the Determination of Thiosulphate. Biochemica et Biophysica Acta 23 412-414.

80. R.L. Starkey 1934. Cultivation of Organisms Concerned in the Oxidation of Thiosulphate. Journal of Bacteriology 28 365-386.

81. A.H. Stouthammer and C. Bethenhausen 1973. Utilization of Energy for Growth and Maintenance in Continuous and Batch Cultures of Microorganisms. A Re-evaluation of the Method for the Determination of ATP Production by Measuring Molar Growth Yields. Biochemica et Biophysica Acta 301 53-70.

82. P.E. Stukus and B.T. DeCicco 1970. Autotrophic and Heterotrophic Metabolism of Hydrogenomonas: Regulation of Autotrophic Growth by Organic Substrates. Journal of Bacteriology 101 339-345.

83. G. Svehia, L. Kolzai and L. Edey 1963. The Use of 2.6.Dichlorophenol Idophenol as Indicator in Iodometric Titrations with Ascorbic Acid. *Analytica Chemica Acta* 29 442-447.

84. R. Tabita and D.G. Lundgren 1971b. Heterotrophic Metabolism of the Chemolithotroph *T. ferro-oxidans*. *Journal of Bacteriology* 108 334-342.

85. B.F. Taylor and D.S. Hoare 1969a. New Facultative *Thiobacillus* and a Re-evaluation of the Heterotrophic Potential of *Thiobacillus novellus*. *Journal of Bacteriology* 100 487-497.

86. B.F. Taylor and D.S. Hoare 1971a. *T. denitrificans* as an Obligate Chemolithotroph. II. Cell Suspension and Enzymic Studies. *Archiv fur Mikrobiologie* 80 262-276.

87. P.A. Trudinger 1964. The Effect of Thiosulphate and O_2 Concentration on Tetrathionate Oxidation by *Thiobacillus X* and *T. thioparus*. *The Biochemical Journal* 90 640-646.

88. P.A. Trudinger 1965. On the Permeability of *T. neapolitanus* to Thiosulphate. *Australian Journal of Biological Science* 18 563-568.

89. P.A. Trudinger and D.P. Kelly 1968. $NADH_2$ Oxidation by *T. neapolitanus* and *T.* strain C. *Journal of Bacteriology* 95 1962-1963.

90. W.W. Umbreit 1951. Significance of Autotrophy for Comparative Physiology in "Bacterial Physiology" pp. 566-576 Ed. C.H. Werkman and P.W. Wilson. Publ. Academic Press.

91. C. van Baalen, D.S. Hoare and E. Brandt 1971. Heterotrophic Growth of Blue-green Algae in Dim Light. *Journal of Bacteriology* 105 685-689.

92. L. van Caeseele and H. Lees 1969. The Ultrastructures of Autotrophically and Heterotrophically Grown *Thiobacillus novellus*. *Canadian Journal of Microbiology* 15 651-654.

93. W. Vishniac and M. Santer 1957. The Thiobacilli. *Bacteriological Reviews* 21 195-213.

94. A.M. Wadzinski and D.W. Ribbons 1972. TCA Cycle Enzymes in the Methylotroph *Methanomonas methano-oxidans*. *Abstracts 72nd Annual Meeting of the American Society for Microbiology* 166.

95. R.A.D. Williams and D.S. Hoare 1972. Physiology of a New Facultative Autotrophic Thermophilic Thiobacillus. Journal of General Microbiology 70 555-566,

96. P.J. Le B. Williams and S.W. Watson 1968. Autotrophy in Nitrosocystis oceanus. Journal of Bacteriology 96 1640-1648.

97. D.H. Williamson and J.F. Wilkinson 1958. The Isolation and Estimation of the Poly-β-hydroxybutyrate Inclusions of Bacillus Species. Journal of General Microbiology 19 198-209.

98. S. Winogradsky. Annals Institut de Pasteur 4 213 1890 Quoted Rittenberg 1970a.

99. S. Winogradsky 1922. Sur le Prétendue Transformation du Ferment Nitrique en Espece Saprophyte. Academie des Sciences 175 301-304.