# Chris Anthony


## HOME

**NEWS & WEATHER**

**UNIVERSITY**

**SCIENCE** ***General***
***My research***

**INDIA** ***General***
***My pictures***

**MUSIC**

**FAVOURITES shopping, friends, DIY, entertainment, Southampton etc**

**BOOKS**

**TRAVEL & HOLIDAYS**

**FAMILY**

**MEDICAL**

**PHOTOGRAPHY**

**BIRDS**

**SEARCH ENGINES**
**All engines**
**Google**
**YouTube**
**Stumbleupon**

**MY NET PROVIDER**


**NOTE** 'My files' open with:
ppt Powerpoint
doc Word
xls Excel
pdf Acrobat Reader [download free]
jpg Opens direct
html Opens direct;
For ***BIGGER*** text
Click Ctl plus +
Normal text click Ctl plus 0

## Presentations of some of my Teaching and Research Topics

**Go Back to**:

My main Research Page Science Education page Methanotroph history Main Science Page India Page

There are two sets of material here:
a) A summary of most of my work on methylotrophs given in a lecture in MAY 2024

b) The slides taken from my powerpoint presentations as a guest of Prof. Colin Murrel at the University of East A
They include many pictures of relevant scientists. This can be downloaded as a Powerpoint presentation.

c) Lecture notes and slides for use of students on my Microbial Physiology course in SV University, India. This ma
can be downloaded as a Word document or as a powerpoint presentation.

Methylotroph stories: my methylotroph research summarised in about 50 slides PPT

| | |
|---|---|
| The Methylotrophs and their investigators PPT | History of Methanotrophy |
| Oxidation of methane methanol & formaldehyde PPT | The oxidation of methylated amines PP |
| The cytochromes and electron transport in methylotrophs PPT | Growth yields of methylotrophs PF |
| Carbon assimilation pathways PPT | Completion of Serine cycle PF |

Link to: Teaching page on Microbial Physiology (SV University, India)

## Methylotroph stories; dedicated to research students everywhere (May 2024)

VIDEOS can be speeded up by downloading an **accelerator**

**HOME**

# This is a power point presentation of a lecture I gave on 20th May to the Microbiology group in Biological Sciences, University of Southampton

The lecture summarised my 40 years of work on methylotroph metabolism in 40 minutes.

I have added a few extra slides to make the stories complete.

# Methylotroph stories

## Dedicated to research students everywhere

**My CV**

Born Watford in 1939, son of a brewery labourer.

1950 – 1957 Watford Grammar School

1957 – 1960 Reading University BSc Microbiology

1960 – 1963 Reading University PhD Thesis: The microbial metabolism of one-carbon compounds

1963 – 1966 Reading University Post Doc: A novel methanol dehydrogenase

1966 - 1967 Sheffield University Post Doc: glutamate fermentation in Clostridia

1967 – 2004 Southampton University: Lecturer etc

2005 – present time: Southampton University: Emeritus Professor

# Thank you to my research students (1969 – 2008)

| **Methanol dehydrogenase** | **Cytochromes and electron transport** | **Metabolic pathways** | **3D structures** |
|---|---|---|---|
| Steven Ford | Jim Elliott | Pat Dunstan | Peter James |
| Dudley Page | Steven Froud | Iain Taylor | Paul Williams |
| Darren Day | David O'Keefe | John Bolbot | Gyles Cozier |
| Jonathan Cox | Andrew Cross | | Simon Dales |
| Ian Richardson | Mathew Beardmore-Gray | | [John Read] |
| Alain Avezoux | Anthony Long | | [Chenyi] |
| Karen Amaratunga | Claude Chan | | [Leigh Felton] |
| Tosin Majekodunmi | Helen Ashworth | | |
| Paul Afolabi | | | |
| | Also Bill Keevil (Post-doc); Paul Skipp (Technician) | | |
| **Amino acid transport** | **Electron transport and nitrogen fixation** | **Neomycin biosynthesis** | **Electron transport in methylamine metabolism** |
| Jeff Robinson | Andrew Smith | Cedric Pearce | Ashley Lawton |
| Bob Cook | Nick Juty | | Kevin Auton |

# Methylotroph stories

Methylotrophs are bacteria that grow with compounds with a single carbon atom as their sole source of carbon and energy. 'Ordinary bacteria' never grow methylotrophically.

1. The rise of the methylotrophs. Only 2 species in 1960. Many hundreds now – with enormous genetic and metabolic diversity.

2. Methanol dehydrogenase (MDH); the first Quinoprotein.

3. A special electron transport chain for methanol oxidation.

4. A remarkable resurgence of interest in MDH.

**My PhD project (1960 – 1963)**: Isolate a bacterium able to grow on methanol and therefore able to oxidise it. Then find the enzyme responsible.

# 1. The rise of the methylotrophs.

Always specialised genera; never the usual types of bacteria
Many grow well on 'ordinary' substrates – succinate, lactate etc

Only 2 species in 1960. Only 4 papers in my PhD introduction

Many hundreds of species now – with enormous genetic diversity

A wealth of novel metabolic pathways for conversion of formaldehyde to cell material

Unusual energy transduction

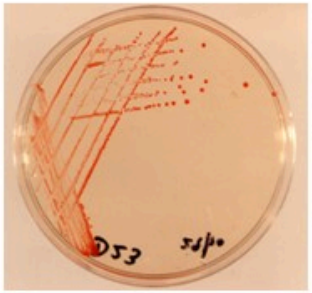
Regular international symposia (from 1970 to present day)

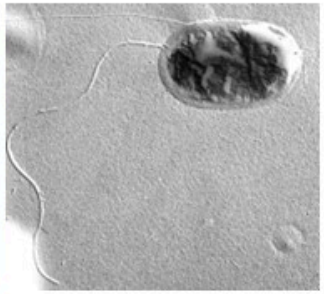
Industrial exploitation. This is because of their metabolic diversity and because their growth substrate, methanol, is inexpensive

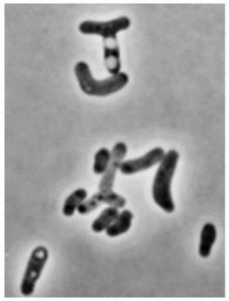
# The main 'workhorses' in the study of methylotrophs

- *Pseudomonas* AM1 (Peel & Quayle, 1961)
- *Pseudomonas* sp. M27 (Anthony & Zatman, 1964)

***Methylobacterium extorquens***. A pink facultative methylotroph





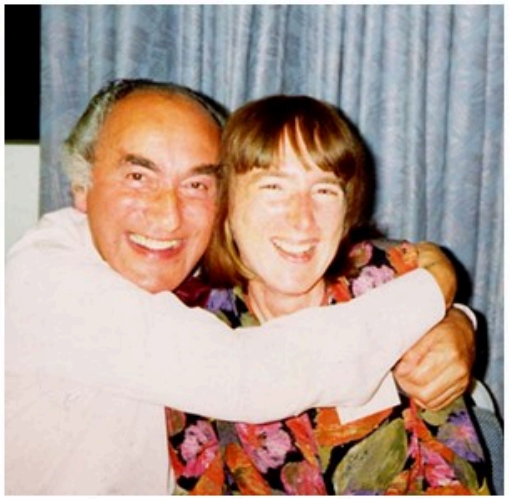




Me in 1963



J Rod Quayle

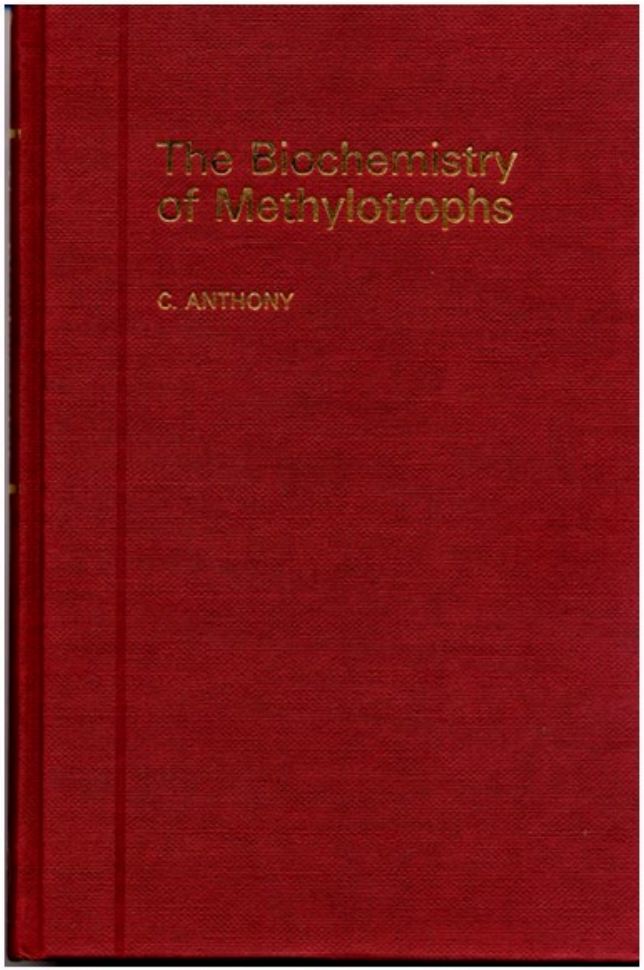


My PhD supervisor and my first research student (Len Zatman and Pat Dunstan, now Goodwin)




Howard Dalton, Rod and me

# The Biochemistry of Methylotrophs 1982




The Biochemistry of Methylotrophs

C. ANTHONY

*Department of Biochemistry*
*University of Southampton*
*England*

**Chapters 12**
**Text pages 350**
**Index 48 pages**
**Tables 60**
**Figures 57**
**References 1300**

1982

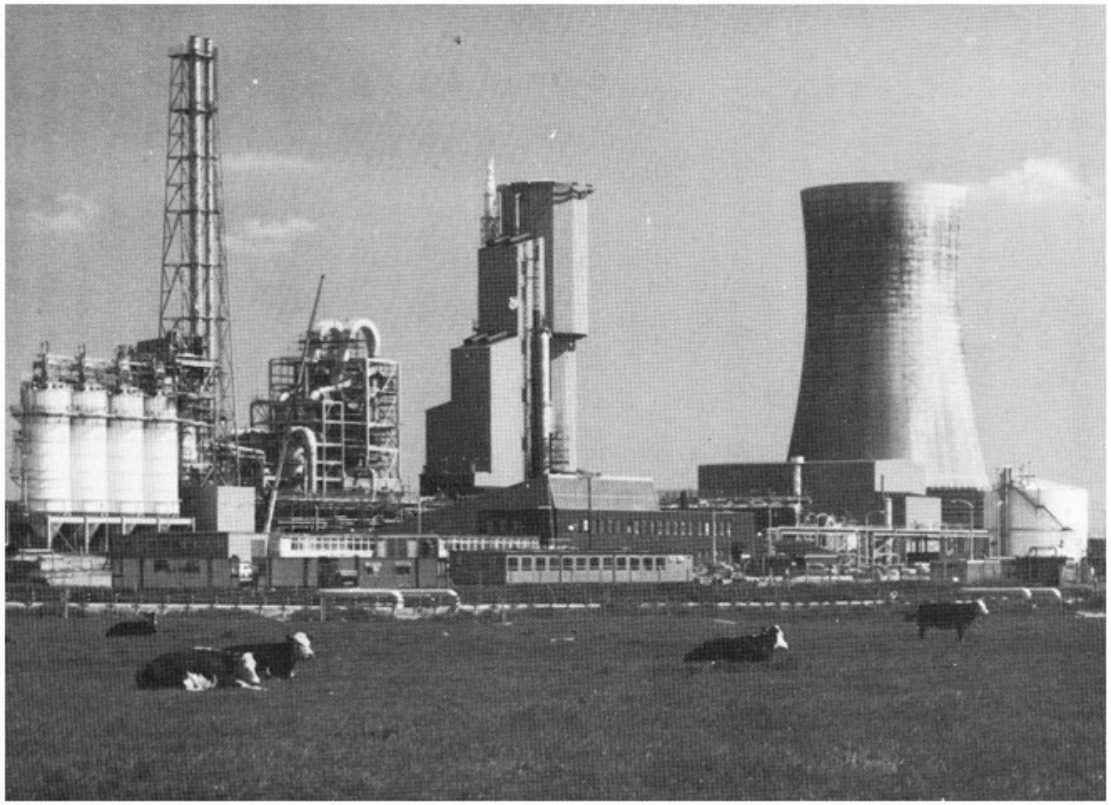
ACADEMIC PRESS
*A Subsidiary of Harcourt Brace Jovanovich, Publishers*

London · New York
Paris · San Diego · San Francisco
São Paulo · Sydney · Tokyo · Toronto

Methylotrophic bacteria
Ribulose bisphosphate pathv
Ribulose monophosphate cy
The Serine pathway
The TCA cycle and growth
on multicarbon compounc
Oxidation of methane, metha
formaldehyde and format
Oxidation of methylated amii
Electron transport and
energy transduction
Growth yields and bioenerge
Methylotrophic yeasts
Methanogens and
methanogenesis
Commercial exploitation

A CD version is available and the complete b
is on my website: chris-anthony.co.uk

## The ICI ‘Pruteen’ plant at Billingham, UK 1980
## For production of protein supplement for cattle feed



The centre tower is the 1.5 million litre fermenter

It contains about 100,000,000,000,000,000,000 methylotrophs
10% of their soluble protein is Methanol dehydrogenase

# 2nd Story: Methanol dehydrogenase

*An enzyme with a remarkable structure and novel type of prosthetic group - **PQQ** (the first Quinoprotein). The false claim that PQQ is a new vitamin*

## Oxidising enzymes.

Most common: alcohol + NAD+ → aldehyde + NAD + H+

Alternative oxidising enzymes are membrane bound and have FAD or FMN as a bound prosthetic group: succinate + Enz-FAD → fumarate + Enz-FADH2

Most living things have an NAD-linked alcohol dehydrogenase
BUT none oxidises methanol so my supervisor, Len Zatman, thought there might be an interesting dehydrogenase in bacteria able to oxidise methanol to formaldehyde.

**My project**: Isolate a bacterium able to grow on methanol and therefore able to oxidise it. Then find the enzyme responsible.
I thought this was a trivial problem but it was not.
My first paper was on a novel methanol dehydrogenase (1964)
My last paper was on the very high (atomic) resolution of this enzyme (2004)

We published 2 papers together. The first described our new methylotroph and the next slide is the second.

# Methanol dehydrogenase

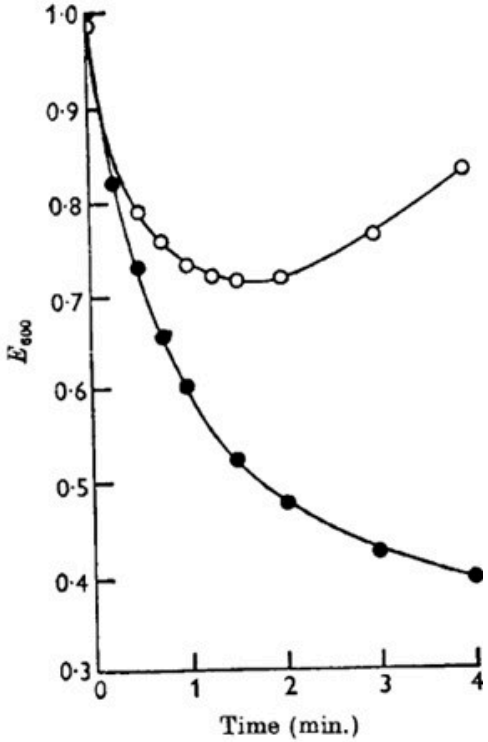
**Responsible for all bacterial methanol oxidation**

The Microbial Oxidation of Methanol

2. THE METHANOL-OXIDIZING ENZYME OF *PSEUDOMONAS* SP. M 27

By C. ANTHONY and L. J. ZATMAN
*Department of Microbiology, University of Reading*

(*Received* 21 *November* 1963)




MDH assay
with PMS/indophenol

A soluble enzyme – now known to be in the periplasm

Dye-linked assay (PMS/PIP); pH optimum 9.2; pI 8.2

Methanol + PMS ⇒ Formaldehyde + $PMSH_2$
$PMSH_2$ is oxidised by blue PIP which loses it colour

Activator is ammonia (not $NH_4$+ ion) (this is unique)

Wide substrate specificity

Unusual absorption spectrum – not FAD or FMN so novel prosthetic group??

High proportion of tryptophans

# Isolation of the prosthetic group

The prosthetic group is the part of the enzyme that is not the amino acid chain.

It is FAD or FMN in flavoproteins, or haem in cytochromes

Grow huge amounts of bacteria on methanol. Harvest and break (ultrasound)
Purify the enzyme from the soluble fraction (Ion exchange, Sephadex etc)

Precipitate the protein and release the fluorescent prosthetic group by acid treatment

Purify the prosthetic group (ion exchange etc). After 3 months I had a few red grains.

Characterised by its excitation and emission fluorescence. A beautiful green colour.

The only known compound with a maximum excitation at 360nm with this maximum occurring at low pH. It is tightly bound to the enzyme.

SO a completely new compound. Thirteen years later its structure was determined by X-ray crystallogaphy at Cambridge; then confirmed by us a further 15 years later with the X-ray structure of the whole enzyme.

# A new type of enzyme prosthetic group

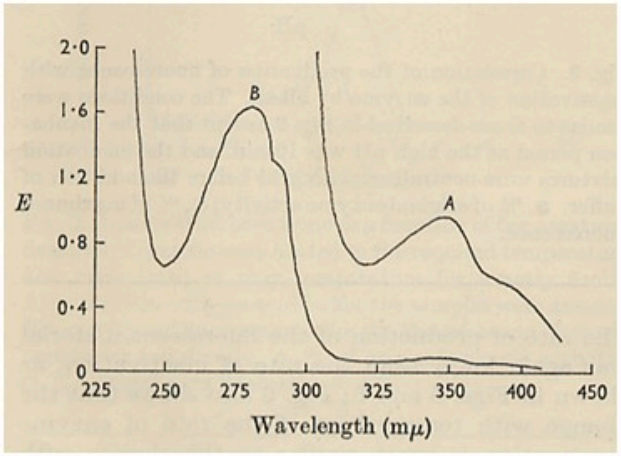
**Note: all other dehydrogenases use NAD(P) as hydrogen acceptor or they ar membrane flavoproteins that use FMN or FAD as prosthetic group**

**The Microbial Oxidation of Methanol**

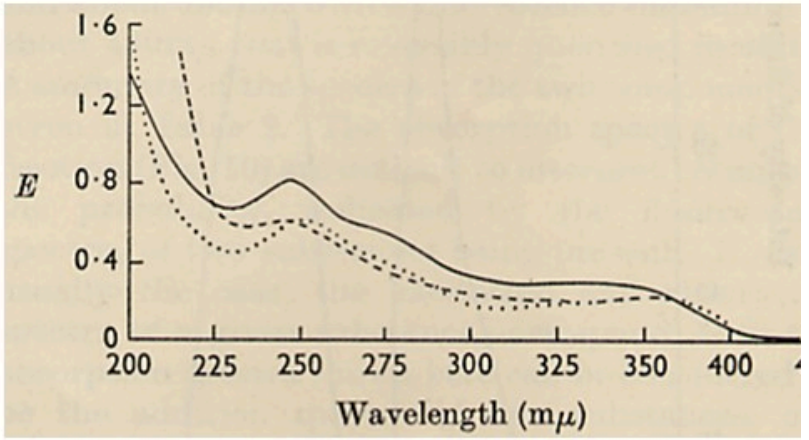
THE PROSTHETIC GROUP OF THE ALCOHOL DEHYDROGENASE OF *PSEUDOMONAS* SP. M27: A NEW OXIDOREDUCTASE PROSTHETIC GROUP

BY C. ANTHONY* AND L. J. ZATMAN
*Department of Microbiology, University of Reading*

(*Received* 30 *December* 1966)




Spectrum of MDH




Spectrum of prosthetic group

**PQQ and other quinoproteins** The novel prosthetic group of MDH was later in other dehydrogenases an Hans Duine and Hans Frank called these Quinoproteins. These include the alcohol dehydrogenase of acetic acid bacteria and the typical glucose dehydrogenase in *Pseudomonas*, *E. coli*.




Hauge described GDH and PQQ very early on




Friends from Yamaguchi, Japan:
Matsushita, Uragami (?), Ameyama, Adachi.
Found many new quinoproteins especially in Acetic acid bacteria



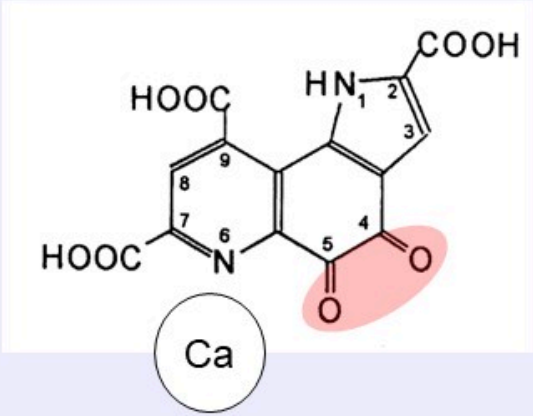


Hans Duine and Hans Frank
'invented' quinoproteins

**Note: No PQQ-containing quinoprote have been described in anything othe than bactera**

# PQQ and Quinoproteins

**Pyrroloquinoline Quinone PQQ)**




First isolated and purified by Anthony & Zatman in1967 from bacterial methanol dehydrogenase

Structure determined by Kennard's group in1979

Remains tightly bound throughout reaction cycle

**PQQ-containing Quinoproteins**

All bacterial dehydrogenases

Usually responsible for 1st step in the oxidation of growth substrate

Mainly alcohols and aldoses

eg ethanol oxidation to acetic acid in acetic acid bacteria

Reaction occurs in the periplasm

Electron acceptors are cytochromes, blue copper proteins or ubiquinone

May be soluble or membrane-bound

May also have contain haem

**None has been described in eukaryotic organisms**

# From 1988 onwards there have been International Symposia on PQQ and Quinoproteins The 5th was in Southampton in 2002



**3rd International Symposium on Vitamin $B_6$, PQQ, Carbonyl Catalysis and Quinoproteins**

**University of Southampton, UK**

**April 14th-19th 2002**

Comprising the
11th International Symposium on Vitamin $B_6$ and Carbonyl Catalysis
and the 5th International Symposium on PQQ and Quinoproteins



Boldrewood conference Centre

**Programme book and Abstracts**

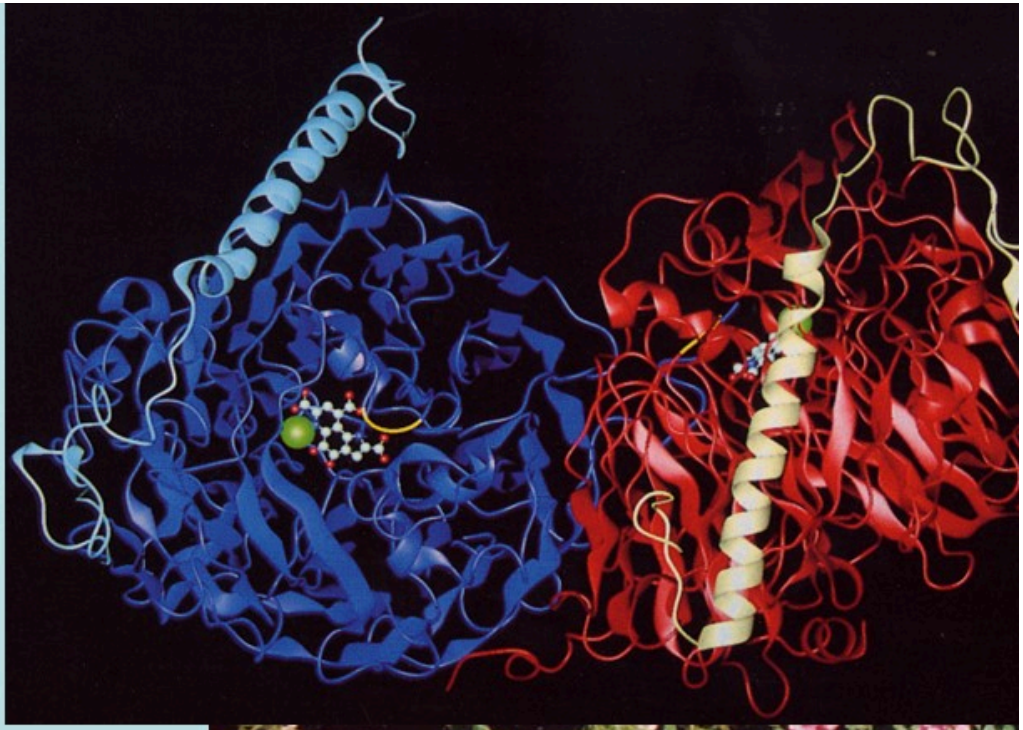
The following slide was included to show what was lost when Boldrewood was demolished. This was my room, complete with coffee machine and comfort. Murali with back showing was a 4th year medical student solv a problem with his project and the other is a research student friend Leigh Felton.
Staff rooms were always availble for students to see members of staf.



**My office with Murali and Leigh**

The foolowing section is describes our work with MDH which was done some years later in collaboration with Meenakshi Ghosh who came to Oxford from Calcutta to learn how to do X-ray crystallography of proteins. She had previously had experience only with inorganic crystals. She needed a project so i provided it.

# The X-ray structure of MDH 1992-2004



Southampton:
Chris Anthony
Alain Avezoux

Oxford:
Meenakshi Ghosh,
Colin Blake

MDH is a dimer
Large subunit is 60k
Small subunit is 9k




A wonderful collaboration; Meenakshi knew no biochemistry and i knew no crystallography and its interpretation

# The ß-propeller structure of MDH




Eight 4-stranded ß sheets (W1-8); these are 'propeller blades'. The 'propeller fold' forms a superbarrel with active site inside

Active site contains PQQ and calcium (green sphere)

The ß-sheet 'propeller blades' are held together by 'tryptophan-docking motifs'

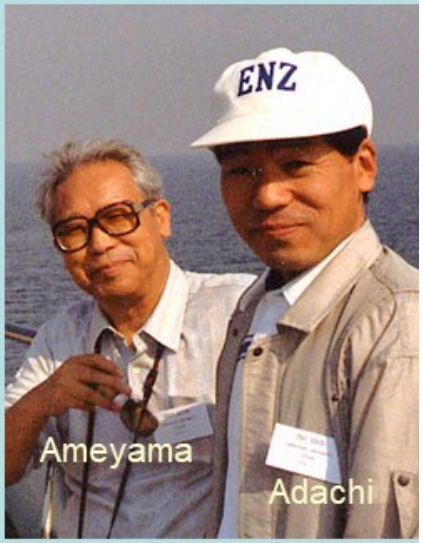
We made the mistake of relying on an 'expert' to asssy our enzyme for metal ion content and he found none. After our Japanes friends showed that their Ethanol dehydrogenase contained calcium we found it in MDH. Our work with a mutant lacking calcium enabled us to reconstitute it with strontium or barium.

# The calcium ion in MDH

1965-1989 ‘ MDH contains no metal ion’ [Anthony & Zatman]

1986 MDH from mutants (*mxaA, K & L*) was inactive and had unusual spectrum [Nunn & Lidstrom]

**1990 MDH contains Calcium (or strontium) [*Adachi et al.*]**




1992 Other MDHs contain calcium.
MDH from *mxaA, K & L* lacked Calcium;
incubation with Ca produced active enzyme & normal spectrum [Ian Richardson]

1992 MDH isolated from marine *Methylophaga Marina is red and inactive;*
incubation with Ca produced
normal green active enzyme [Claude Chan]



# The calcium ion in MDH

1994 Kinetics of MDH containing strontium *[Harris & Victor Davidson]*



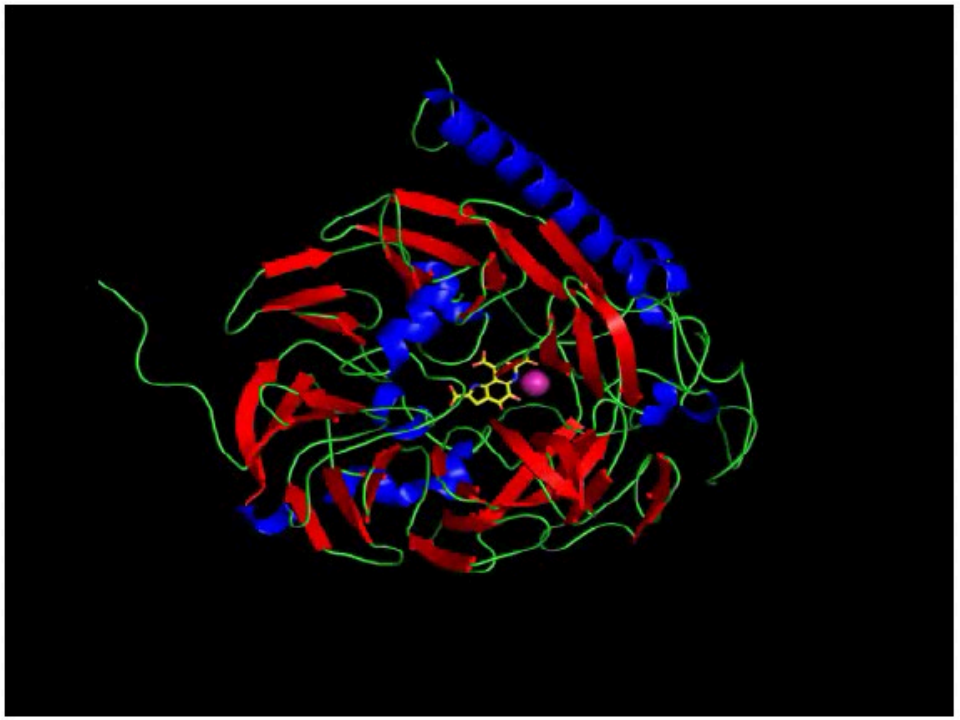

1996 Incubation with Barium produced MDH which was more active than wild-type MDH [Matt Goodwin, Alain Avezoux & Simon Dales]







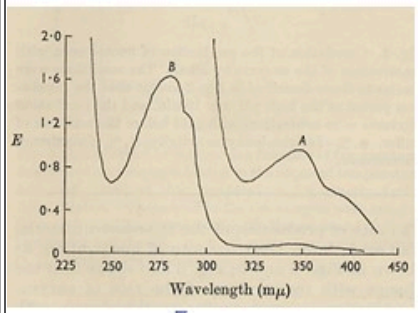


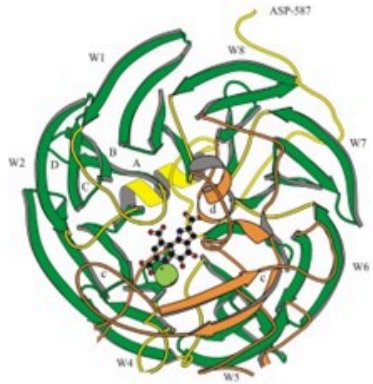


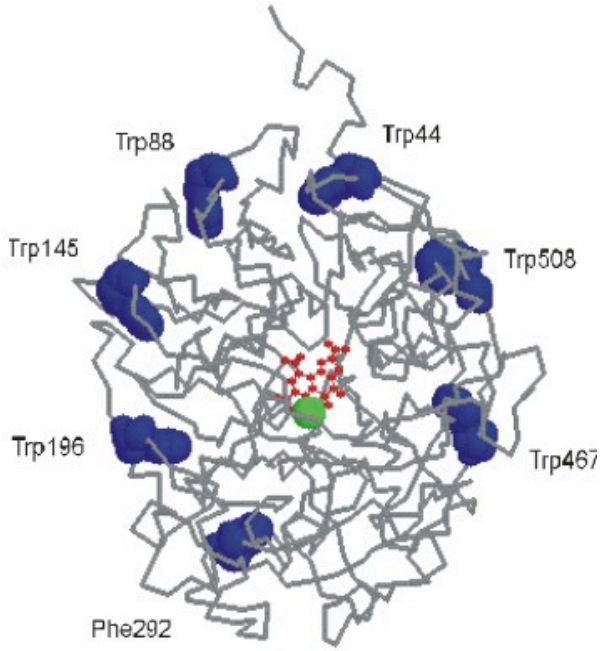
# Tryptophan docking system

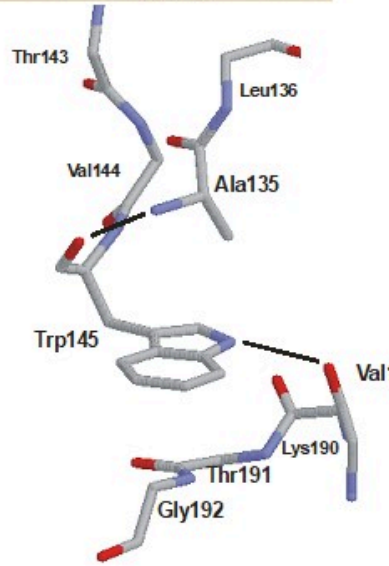
High proportion of tryptophans;
19 per α subunit

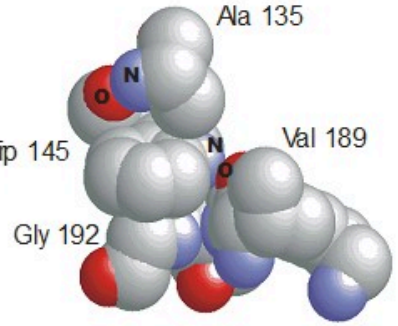
The ß-sheet 'propeller blades' are held together by 'tryptophan-docking motifs'



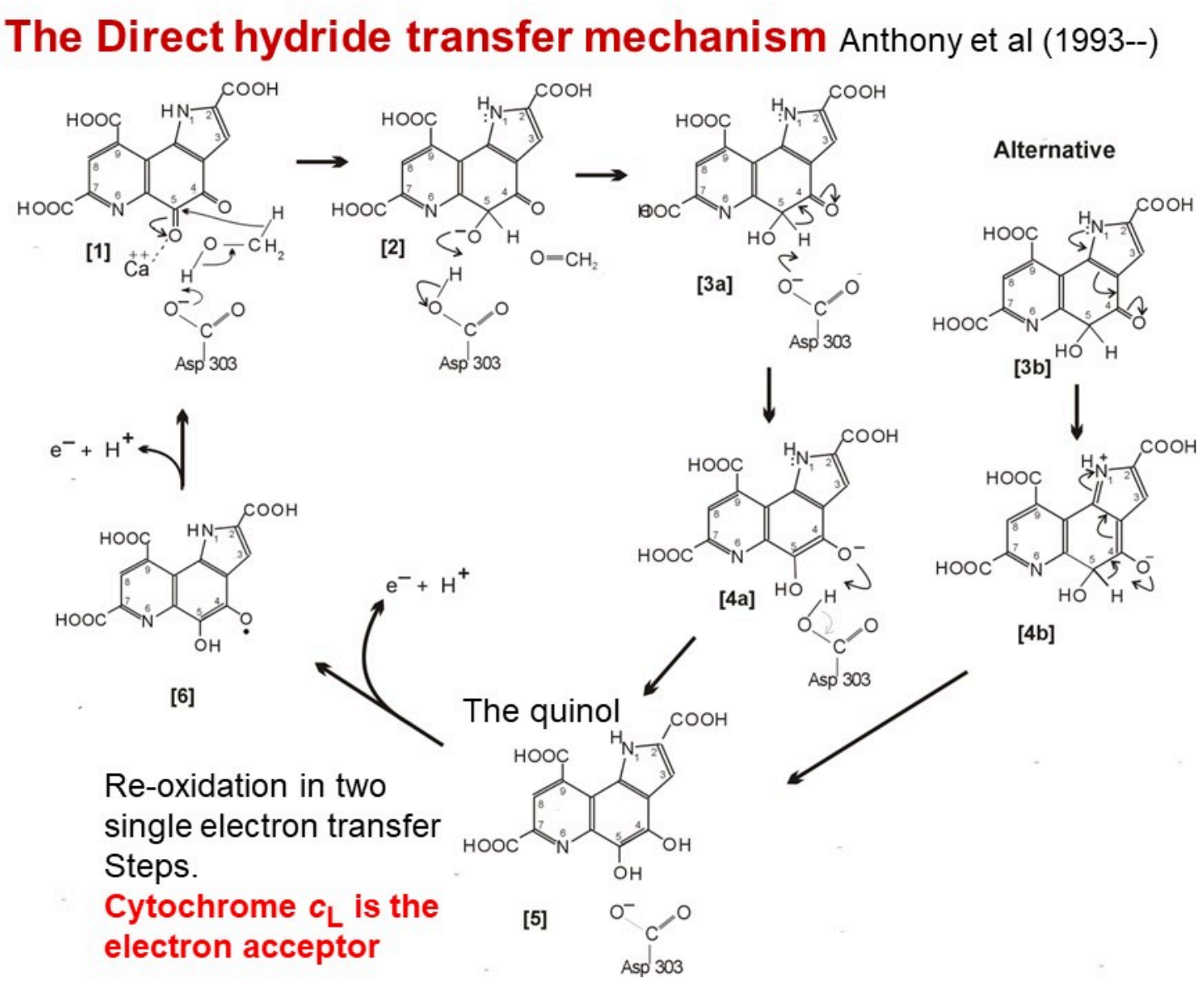

The following slide shows the consensus sequence involved in the docking motifs which demonstrated to Kashahara and Kato that they had a propeller structure which they wrongly interpreted as evidence for a quinopı

# The consensus sequence in the tryptophan-docking motifs

Propeller blades W1-W8

| Position | 1 | 2 | 3 | 4 | 5 | - | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Motif | C | C | C | | | | D | D | D | D | D | D |
| W1 | Ala 77 | Leu | Gly | Leu | Asp | Asp | Pro | Gly | Thr | Ile | Leu | Trp 88 |
| W2 | Ala135 | Leu | Asn | Ala | Glu | - | Thr | Gly | Glu | Thr | Val | Trp145 |
| W3 | Ala 186 | Tyr | Asp | Val | Lys | - | Thr191-Gly192 | | Glu | Gln | Val | Trp 196 |
| W4 | Gly 282 | Arg | Asp | Ala | Asp | - | Thr | Gly | Glu | Ala | Lys | Phe 292 |
| W5 | Thr 337 | Leu | Asp | Arg | Thr | - | Asp | Gly | Ala | Glu | Val | Ser 347 |
| W6 | Ala 457 | Tyr | Asn | Ala | Ile | - | Thr | Gly | Asp | Tyr | Lys | Trp 467 |
| W7 | Ala 489 | Arg | Asp | Ser | Asp | - | Thr | Gly | Asp | Leu | Leu | Trp 508 |
| W8 | Val 577 | Phe | Ser | Leu | Asp 581 | - | Gln 39 | Leu | Arg | Pro | Ala | Trp 44 |
| MDH Consensus | Ala | X | Asp/ Asn | X | X | - | Thr | Gly | Asp/ Glu | X | X | Trp |
| GDH/ADH Consensus | Ala | X | Asp/ Asn | X | X | - | Thr | Gly | Lys | X | X | Trp |

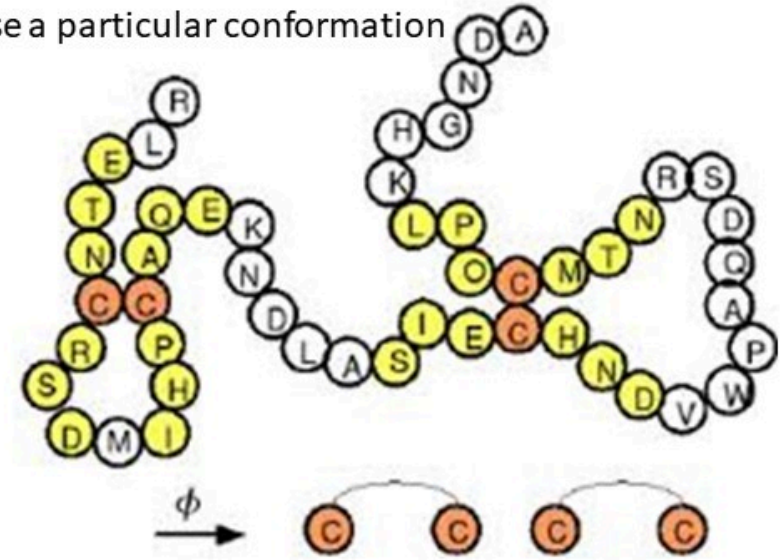
For example: **Trp145** of strand D in W2 is stacked between **Ala135** in strand C in W2 and **Thr-Gly** at position **6-7** in W3




The mechanism involves an initial proton abstraction (by Asp 303) and a hydride transfer from the methyl group of methanol. The quinol then passes its electrons one at a time to the specific cytochrome electron acceptor. After a single electron has been transferred the free radical PQQH is stable, and MDH is usuall isolated with PQQ in this form (Shown by Frank and Duine)

# The unique disulphide ring structure in the MDH active site

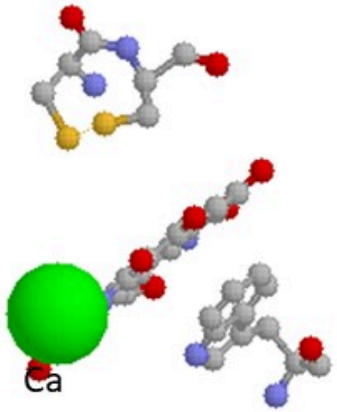
Two distant Cysteine residues in proteins can be oxidised to form a disulphide bridge that is used to stabilise a particular conformation



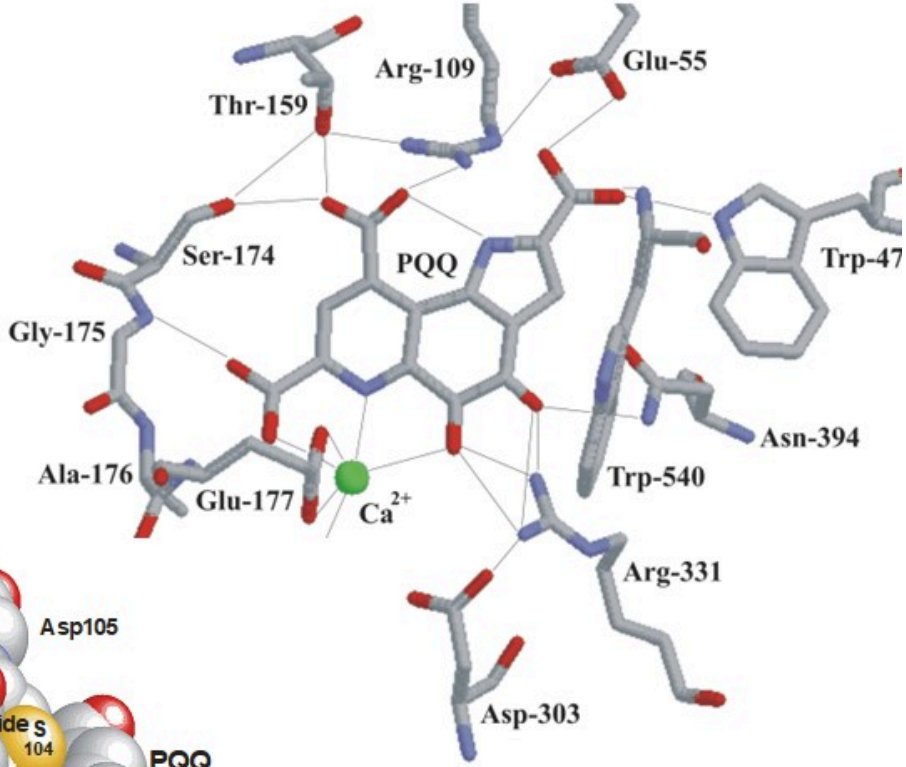

Ramachandran stated that 2 adjacent cysteines would never form a bridge. BUT if they did then they would form an 8-membered ring structure with a 'strained' peptide bond.

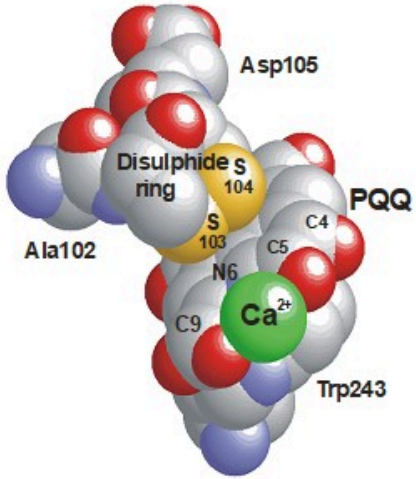
**None has ever been seen except in the active site of MDH.**
**It is very easily reduced, so breaking the bond and inactivating the enzyme.**

# The active site of MDH




Novel disulphide ring structure from adjacent cysteines



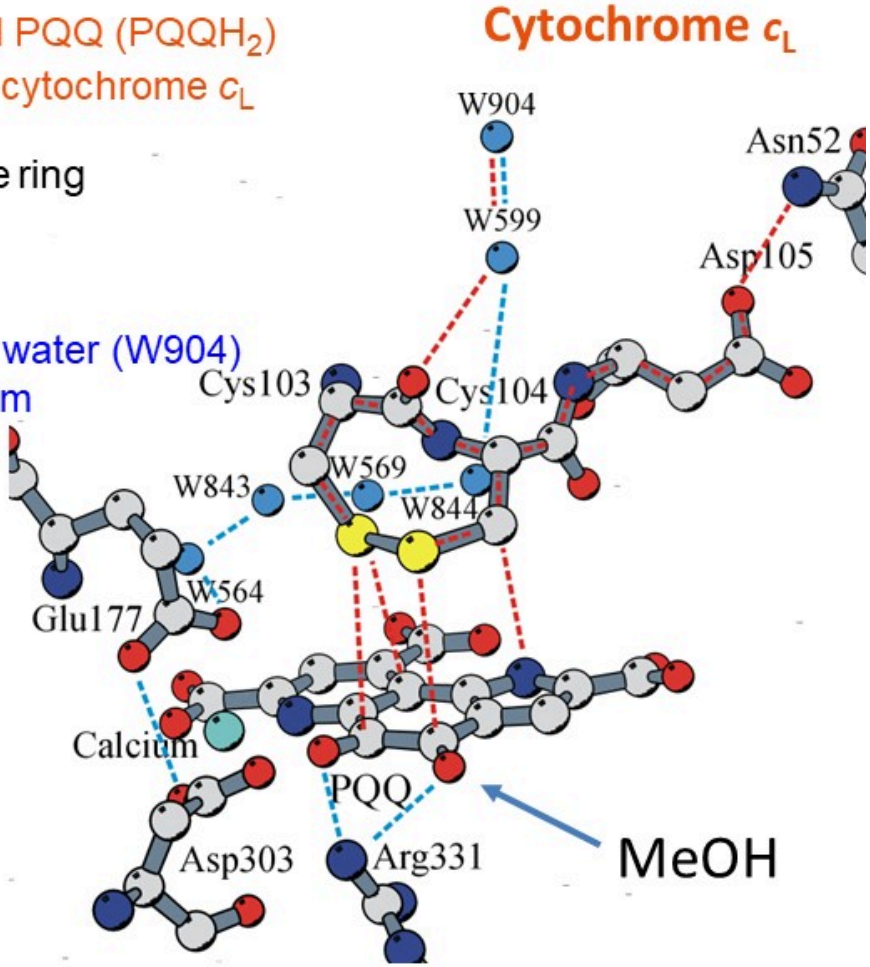
# The path of electrons and protons during the oxidative part of the reaction mechanism of MDH

Electrons must pass from reduced PQQ ($PQQH_2$) to a unique type of cytochrome - cytochrome $c_L$

Note involvement of the disulphide ring

Protons must pass from PQQH2 by way of its hydroxyls, and the hydrogen-bonding network, to the water (W904) which is accessible to the periplasm

The pathways are based on those proposed for the quinohaemoprotein alcohol dehydrogenase by *Oubrie, Dijkstra et al, and Chen, Matsushita, Toyama, Scott Mathews et al.*



# False claim that PQQ is vitamin

Most enzyme cofactors cannot be made in humans so have to be provided as their precursors – vitamins. Eg: NAD (Niacin), FAD (riboflavin)

If mammals do have quinoprotein enzymes then PQQ is almost certainly a vitamin.

In 2003 Kashara and Kato published a paper in Nature claiming to have found a gene coding for a quinoprotein and announcing that this meant that PQQ is "The first new vitamin for 55 years".
This was welcomed with a great fanfare

Their argument was so obviously false so I ignored it.

BUT two years later Mitsubishi announced that they were investing 4 million dollars in producing this new vitamin. So I contacted them and published a paper in Nature explaining why it is not. But Mitsubishi went ahead and now it is on the market as a growth nutrient!

# Premature announcement that PQQ is a vitamin for mammals

**brief communications**

Nutritional biochemistry

## A new redox-cofactor vitamin for mammals

**Takaoki Kasahara, Tadafumi Kato**
*Laboratory for Molecular Dynamics of Mental Disorders, Brain Science Institute, RIKEN, Japan*




NATURE | VOL 422 | 24 APRIL 2003 | www.nature.com/nature

Scientists Add New Member to Vitamin B Family
---Sarah Graham, Scientific American, April 24, 2003.

A new page may need to be added to the vitamin B family album. According to a report published today in the journal Nature, researcher have determined that the compound known as pyrroloquinoline quinon (PQQ) should be classified as a vitamin in the same class as vitamins B and B3. Scientists first isolated PQQ from bacteria in 1979.

MITSUBISHI GAS CHEMICAL COMPANY, INC.

Biological Products

September 24, 2003

**Mitsubishi Gas Chemical Company Inc. speeds the development of application of coenzyme PQQ-vitamin.**

IN THE NEWS

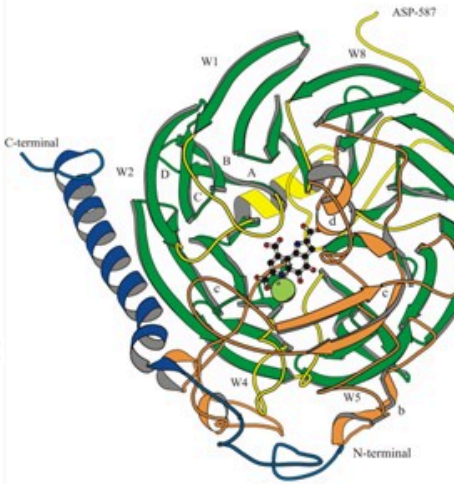
**Japanese Discover First New Vitamin in 55 Years**

April 24, 2003
By REUTERS

**Mind your PQQ - properties of pyrroloquinoline quinine, nutrient - vitamins and supplements in a bodybuilder's di**

# The argument by Kasahara and Kato

**Search engines 'identified' PQQ-binding sites in a protein in mice [predicted from a gene sequence]**




## Our argument against

**We cannot identify PQQ-binding sites in a database**

**In fact, the search engine found 7 'propeller blades' that typically occur in PQQ-containing quinoproteins**

**The 'propeller blades' – wrongly identified by the search engine as 'PQQ-binding sites' are not directly involved in binding PQQ; they are purely structural**

| | |
|---|---|
| All trees are green | All PQQ enzymes have propeller structure |
| My coat is green | My protein has a propeller structure |
| SO: My coat is a tree | SO: My protein is a PQQ enzyme |

***Therefore*** **PQQ is a vitamin**

**brief communications**

Nutritional biochemistry

**A new redox-cofactor vitamin for mammals**

# Premature announcement that PQQ is a vitamin for mammals

Leigh M. Felton and Chris Anthony:
Nature; Communication Arising

The only evidence that the proposed dehydrogenase is PQQ-dependent is that the Smart and Pfam databases 'identified' '7 PQQ-binding sites' in the C-terminal regio

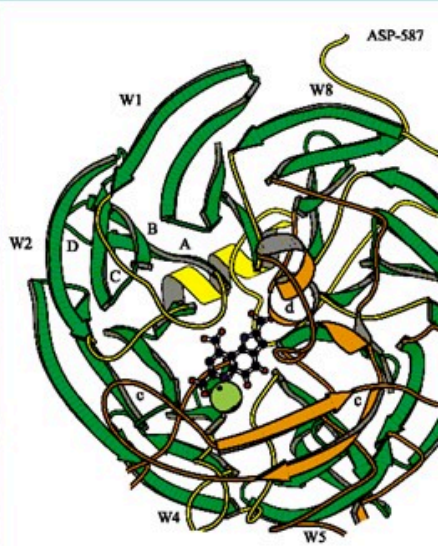
In fact, the 'sites' are sequences predicted to form a series of ß-sheet 'blades' in a 'propeller fold' as seen in PQQ-dependent dehydrogenases.
This fold has nothing to do with PQQ binding and is found in many other proteins

The proposed enzyme has no sequence similarity to any PQQ-dependent dehydrogenase

There is still no evidence that PQQ
Is a cofactor for a eukaryotic enzyme
Or that it is a vitamin

Thank you,
Goodbye and Good luck






# Triple Strength PQQ (Pyrroloquinoline Quinone) 30 x 60 Veggie Capsules

★★★★★ 11 reviews  Delivery in 1-2 days  Free D

Get a daily dose of this all new super potent anti-oxidant, regarded by many as Supercharged CoQ10. Our super strength PQQ has a potent 30mg of the finest-quality Pyrroloquinoline Quinone, triple the strength of most PQQ supplements.




Available options (please select one)

1 Bottle - £27.99 Per Bottle | 2 Bottles £22.39 Per Bottle

4 Bottles £20.99 Per Bottle | 6 Bottles £18.19 Per Bottle

1

£27.9

3rd Story. Before we knew the structure of MDH we started working on how this enzyme with a novel prosthetic group interacts with an electron transport chain so that the oxidation of methanol will be coupled to ATP production.

# 3rd Story: A special electron transport chain for methanol oxidation involves a novel type of cytochrome c

Methylobacterium has a 'normal' system for NADH and succinate oxidation

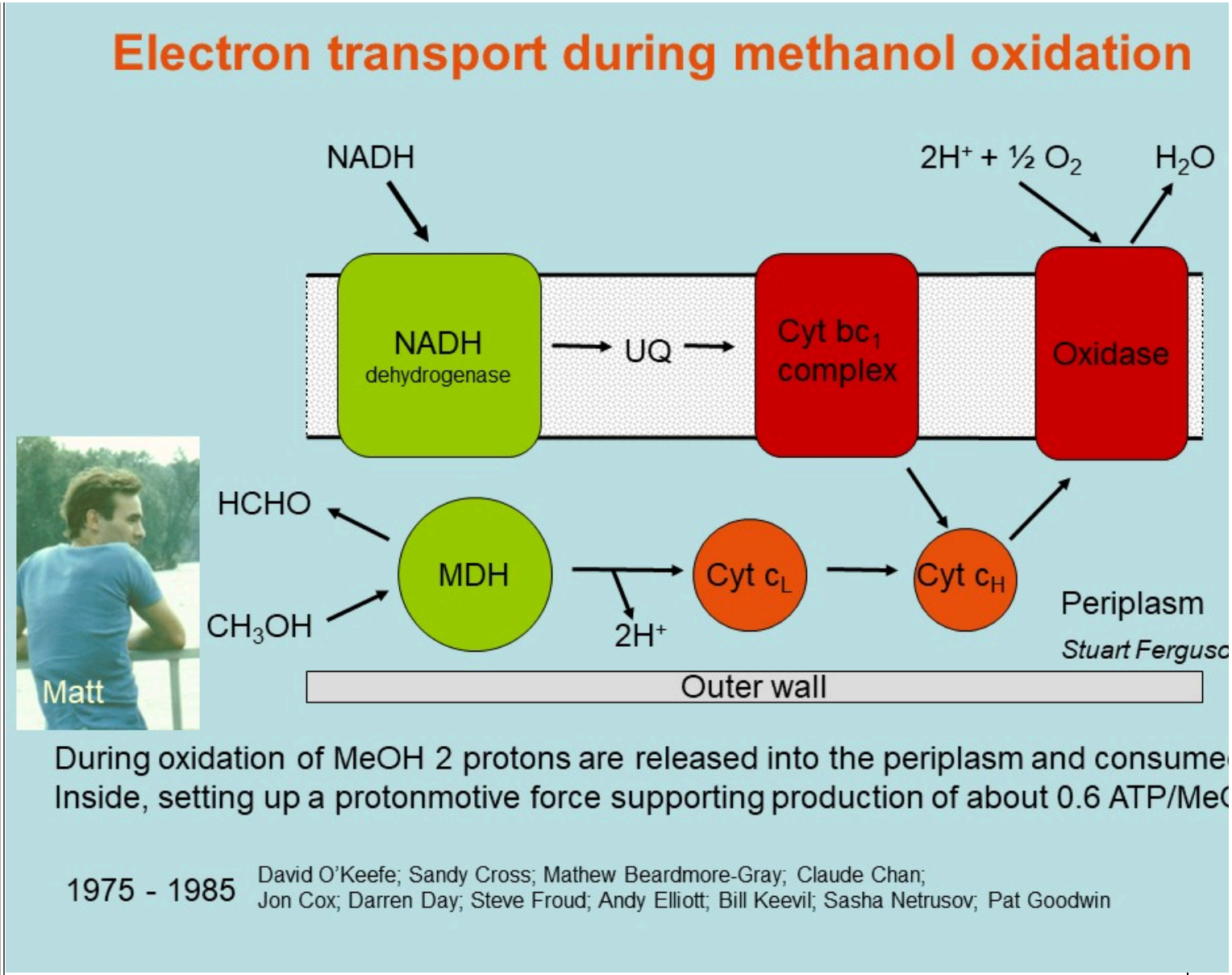
Inhibitors of NADH and succinate oxidation did not inhibit methanol oxidation
Inhibitors of the cytochrome oxidase did inhibit methanol oxidation

Addition of lactate (giving NADH) or succinate reduced cytochromes b, c and oxidase

BUT: Addition of methanol only reduced cytochromes c and oxidase

Methylobacterium and all methylotrophs have an extra cytochrome c called cytochrome $c_L$ This is the specific electron acceptor for MDH. They react in the periplasm.

A student David O'Keefe boldly went against my advice, staying up most of the night and found it after months of my failure.

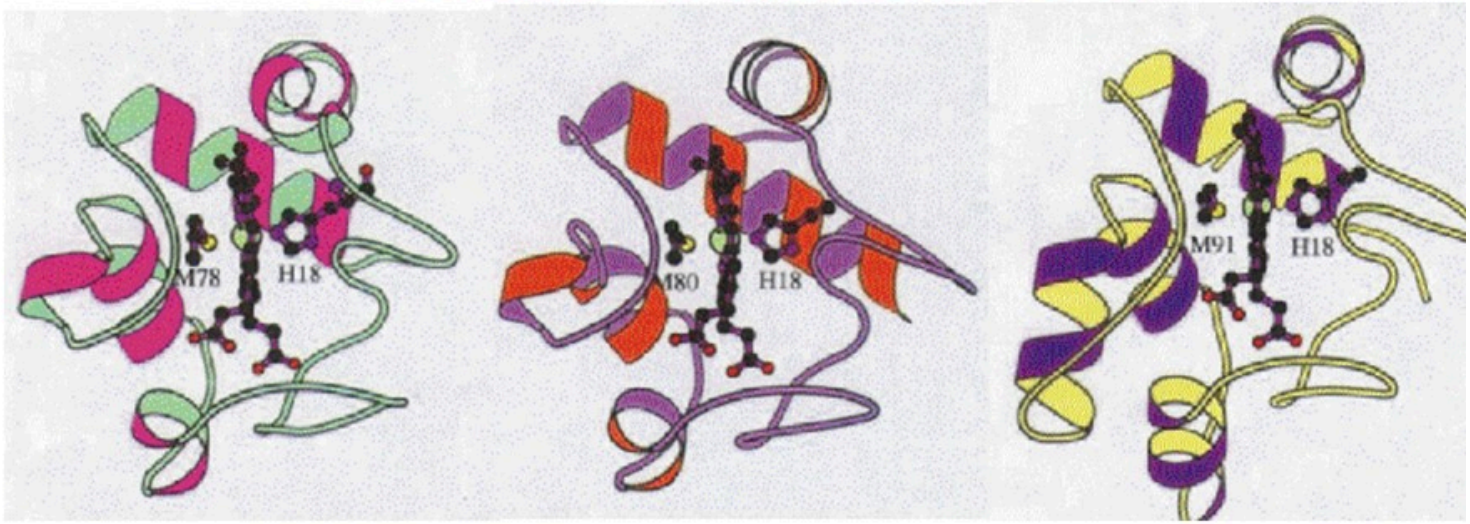



NOTE: The ATP yield from methanol is very low but this does not matter because almost every atom of carbon in these bacteria comes by way of this oxidation process so there is ATP to spare. Cell yields are not limited (as in other bacteria) by ATP but by avaialbility of NADH. Matt Beardmore-Gray calculated that the large amount of cytochrome and MDH in these bacteria is sufficient to cover the surface in a mono-molecular layer.

The solution of the unusual MDH structure helped us get an X-ray crystallogrqphy unit set up in the Department,

with the appointment of John Cooper and Steve Wood, and they helped solve the structure of the two methylotroph cytochromes.

# Structure of cytochrome $c_H$




*M. extorquens* cytochrome $c_H$

Mammalian cytochrome *c*

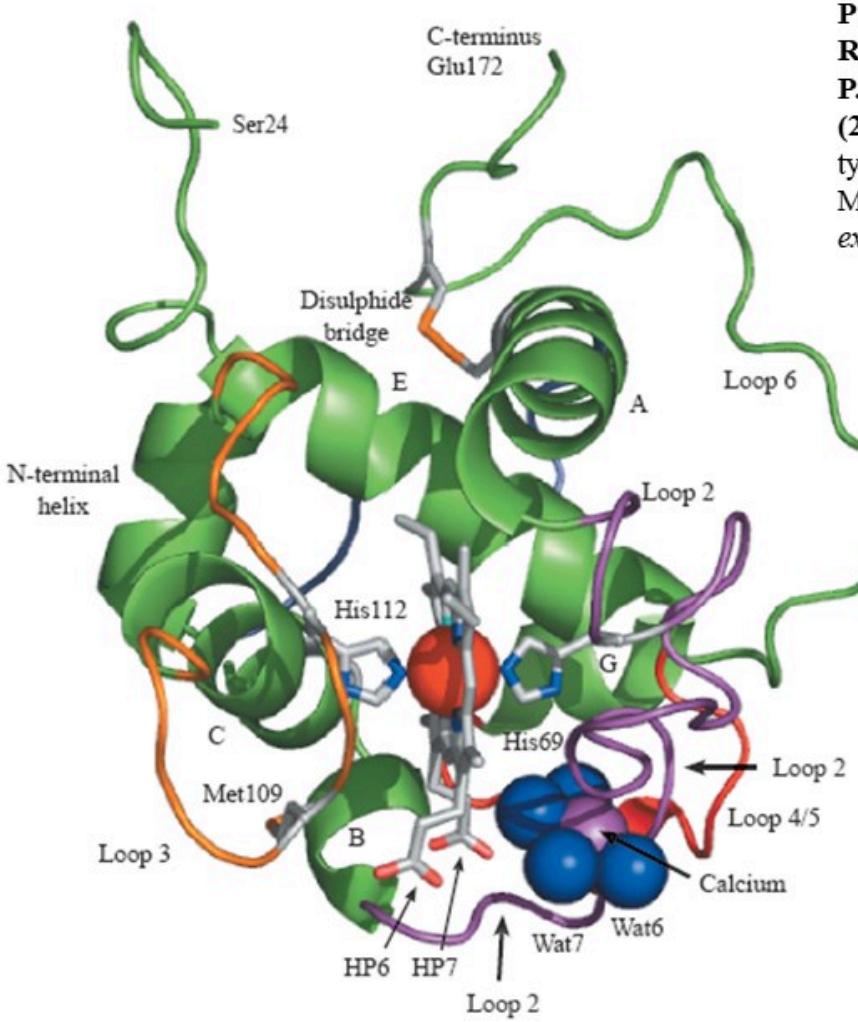
Bacterial cytochrome $c_2$

Remarkably, cytochrome $c_H$ is unique for a bacterial cytochrome in being similar in structure to mammalian cytochrome c

Its internal structure 'disproves' a key proposal for cytochrome function:
All other cytochromes c have a tyrosine bonded to water in the haem cleft:
Cytochrome cH has tryptophan and no water. So the water has no function.

# The X-ray structure of cytochrome $c_L$, the electron acceptor from methanol dehydrogenase

Paul Williams and Southampton X-ray structure group




**Paul Williams, Leighton Coates, Fiyaz Mohamm Raj Gill Peter Erskine, Dominique Bourgeois, St P. Wood, Chris Anthony and Jonathan B. Coope (2006).** The 1.6 A° X-ray Structure of the Unusual c type Cytochrome, Cytochrome cL, from the Methylotrophic Bacterium *Methylobacterium extorquens*. *J. Mol. Biol*. 357, 151–162

The amino acid sequence is unique for cytochromes

The core structure is exactly as see in other cytochromes c

Two cytochromes c have evolved ir the same organism, functioning in t same electron transport chain, havi similar core structure but no sequer identity

NOTE: the unique calcium ion and the exposed haem propionates

## 4. A resurgence of interest in MDH

In 1986 Mary Lidstrom and David Nunn published the methanol operon.

David came to work in my lab and he sequenced the genes for MDH and cytochrome $c_L$

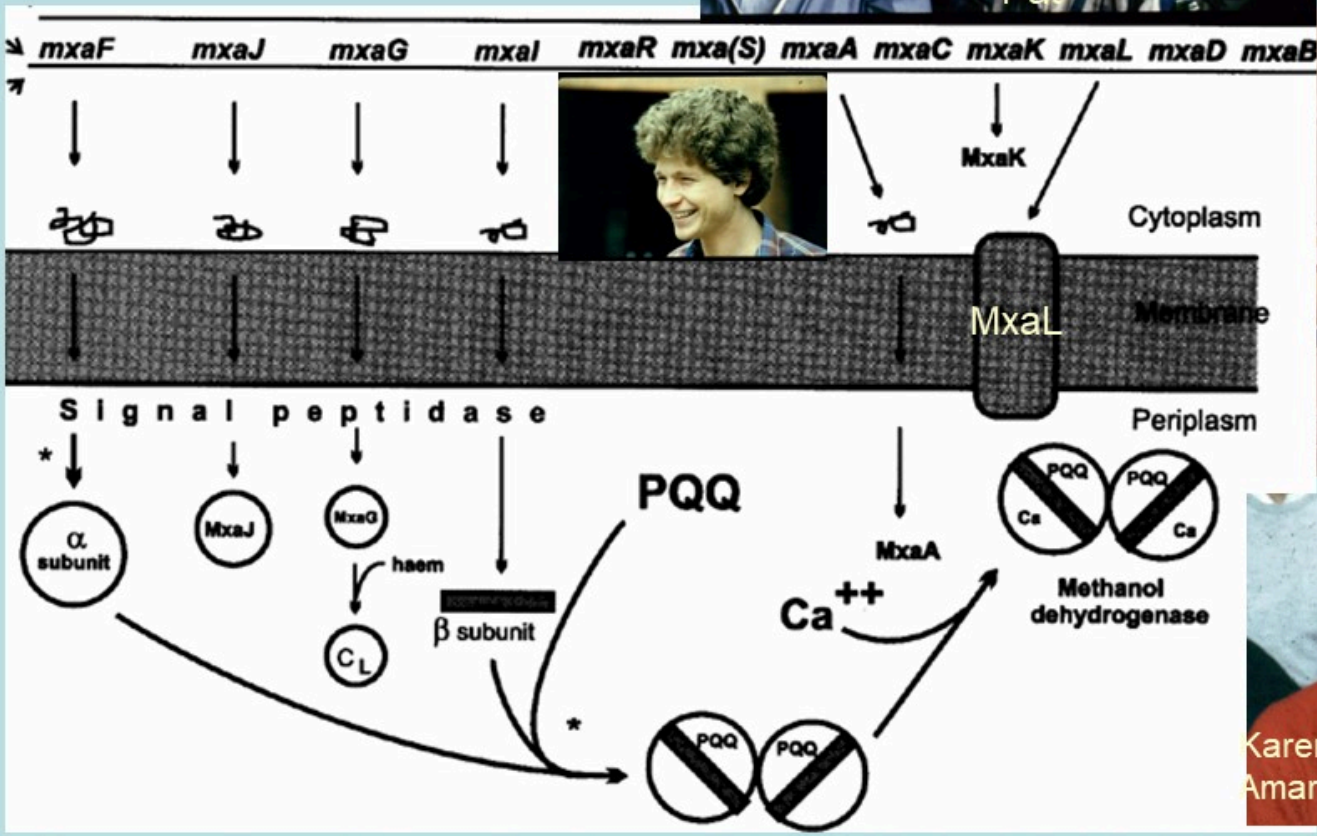
## A very remarkable observation

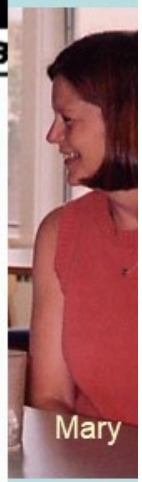
Mary Lidstrom showed that all methylotrophs have two methanol oxidation operons.

The 2nd (Mox2) is never expressed. Mutations in Mox2 do not affect growth o methanol.

# Expression of the mxa operon







The genes: Nunn, Lidstrom, Amaratunga, Anderson, Anthony, Goodwin, Morris, O'Connor

NOTE: the immense amount of difficult work on the genetics of our *Methylobacterium* by Karen Amaratunga and Simon Dales led them to provide important evidence for all out ideas about structure, mechanism and electron transport by a programme of site-directed mutagenesis.

## A novel methanotroph (Methanotrophs grow on methane which is oxidised to methanol which is then oxidised by MDH)

In 2017 a novel methanotroph (*Methylacidiphilum fumariolicu* ) was isolated from acidic volcanic mud. It grows at high temperature and low pH.
It requires a rare earth element for growth (eg Lanthanum, Cerium, Neodymium).

It only uses the 2nd methanol oxidation operon to produce MDH which uses one of the lanthanides instead of calcium at the active site. As a result during the last few years I have weekly reports on ResearchGate of my 1964 paper being cited!

## A final strange thing

In 2018 Colin Murrell's group at UEA described a novel methylotroph isolated from seawater using a methanol medium containing lanthanum salts.

"We propose the name *Marinibacterium anthonyii* (in honour of the British microbiologis Professor Christopher Anthony)". Note: I was Colin Murrell's tutor here and he played second violin in my string quartet.

**Marinibacterium anthonyii is the only methylotroph that does not have 'my' MDH); it only has the 2nd methanol oxidation operon.**

# Our last publication on MDH 40 years after my first

Acta Crystallographica Section D
**Biological Crystallography**
ISSN 0907-4449

## The atomic resolution structure of methanol dehydrogenase from *Methylobacterium extorquens*

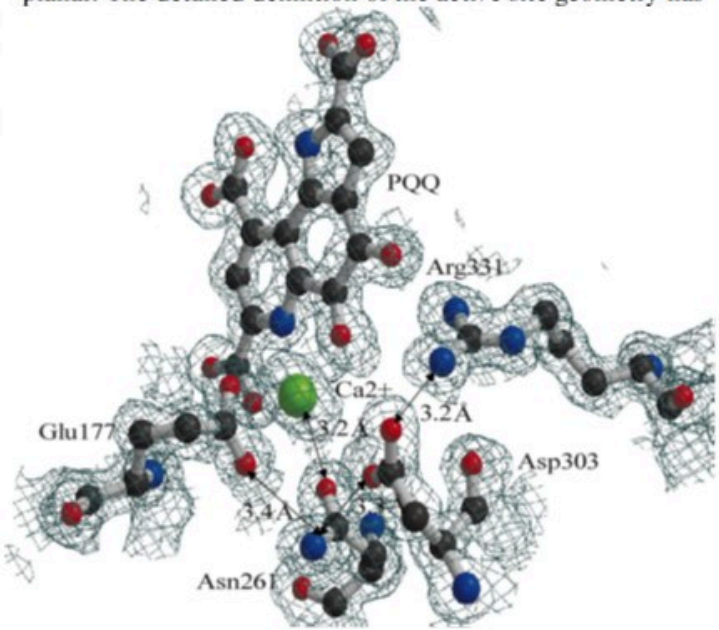
**P. A. Williams, L. Coates, F. Mohammed, R. Gill, P. T. Erskine, A. Coker, S. P. Wood, C. Anthony and J. B. Cooper***

School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, England

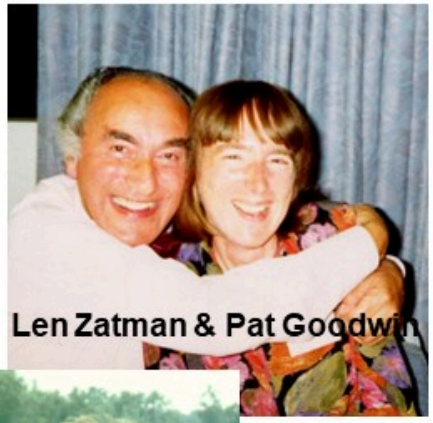
The crystal structure of methanol dehydrogenase (MDH) from *Methylobacterium extorquens* has been refined without stereochemical restraints at a resolution of 1.2 Å. The high-resolution data have defined the conformation of the tricyclic pyrroloquinoline quinone (PQQ) cofactor ring as entirely planar. The detailed definition of the active-site geometry has

Received 1 September 2004
Accepted 23 October 2004

**PDB Reference:** methanol dehydrogenase, 1w6s, r1w6ssf.



## Acknowledgements



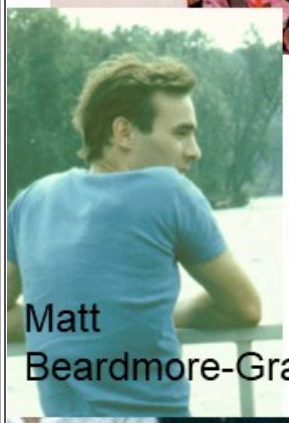


















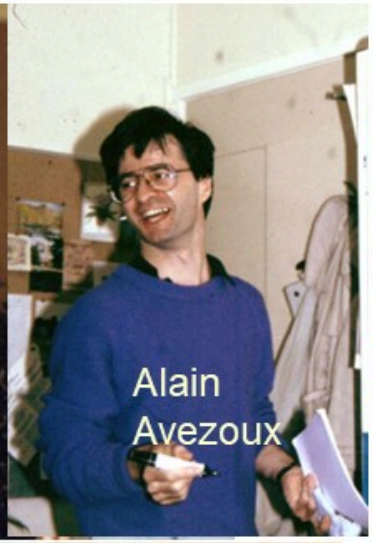






## Acknowledgements









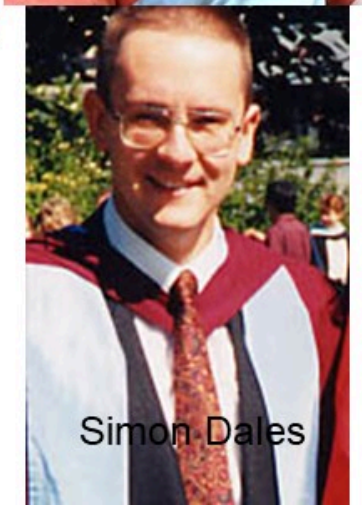












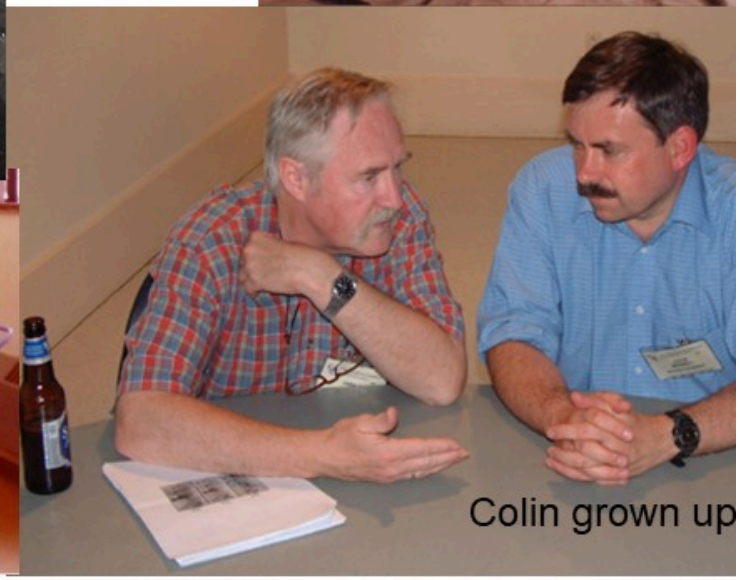



# A few more pictures of methylotroph friends


Chris
1959

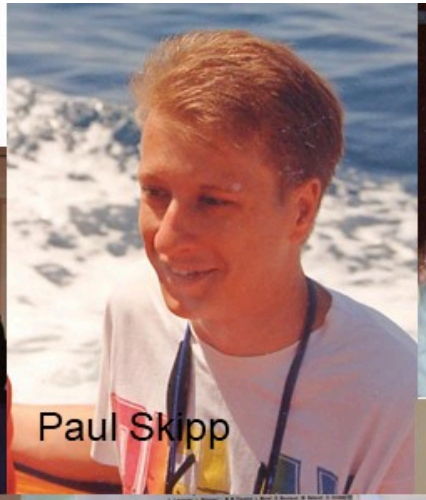

A BioCellist


Colin Murrell
2nd fiddle
1977


Colin grown up

# More friends



















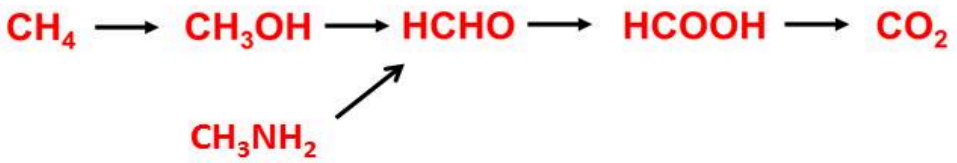
**Oxidation methane, methanol and formaldehyde**

PPT Top of Summaries
Return to Methanotroph History

## The oxidative metabolism of methylotrophic bacteria

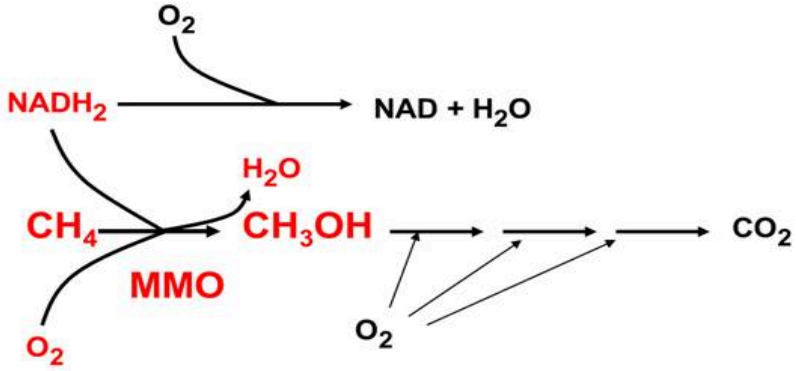
These slides were to support (very) informal seminars at UEA as guest of Professor Colin Murrell.
There are separate presentation on cytochromes etc

Almost all the enzymes for the initial oxidation steps to formaldehyde are unique to methylotrophs. These unique enzymes are linked to unusual energy-yielding electron transport chains.




a) Hydroxylation of methane to methanol [Dalton, Murrell etc]
b) Oxidation of methanol to formaldehyde [Main topic of this seminar]
c) Oxidation of methylated amines to formaldehyde [Separate seminar]
d) Oxidation of formaldehyde to formate [Next slide]
e) Oxidation of formate to carbon dioxide (typical NAD-linked enzyme)
f) Electron transport chains for energy production

**Hydroxylation of methane to methanol**




**Catalysed by Methane mono-oxygenase (MMO) (Two types)**

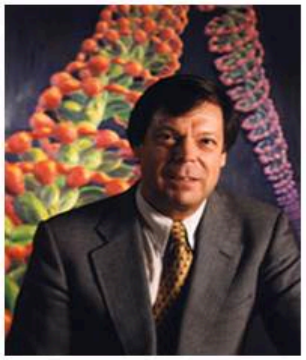
Soluble (sMMO)

Membrane bounds (particulate or pMMO)
Mainly solved by Howard Dalton; genetics (etc) by Colin Murrell at University of Warwick

More than half oxygen is used by initial hydroxylation in a reaction that requires input of energy (as NADH)

# Important people in study of methane oxidation

The late Sir Howard Dalton FRS. Professor Colin Murrell, Mary Lidstrom, Ed Bellion





My second fiddle (juvenile form of Murrell), when I was his tutor at University of Southampton







Nearly adult form

## Oxidation of formaldehyde by methylotrophs

Dye-linked dehydrogenases; so possibly flavoproteins or quinoproteins
NAD-linked dehydrogenases. Absolutely essential during growth on methane with NADH as donor to the hydroxylase
Methanol dehydrogenase. Good substrate but not likely to be relevant.
Complex route involving methanopterin and folates [next slide] (NAD-linked)
Cyclic route in bacteria with RuMP pathway

Note: There must always be routes for removal of toxic formaldehyde. And many aldehyde dehydrogenases are non-specific

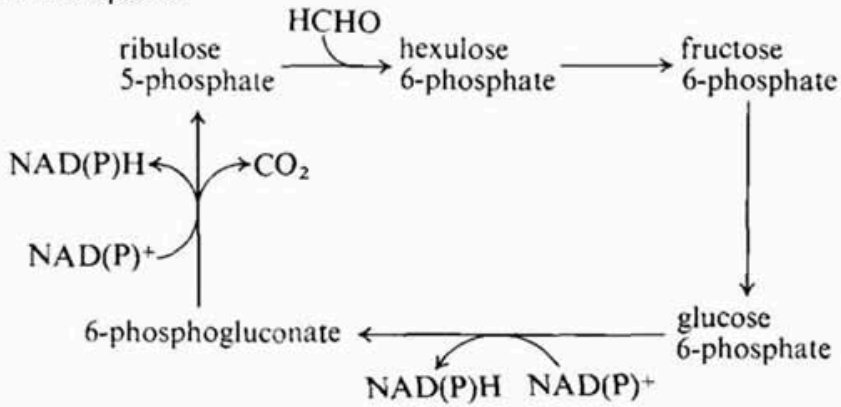
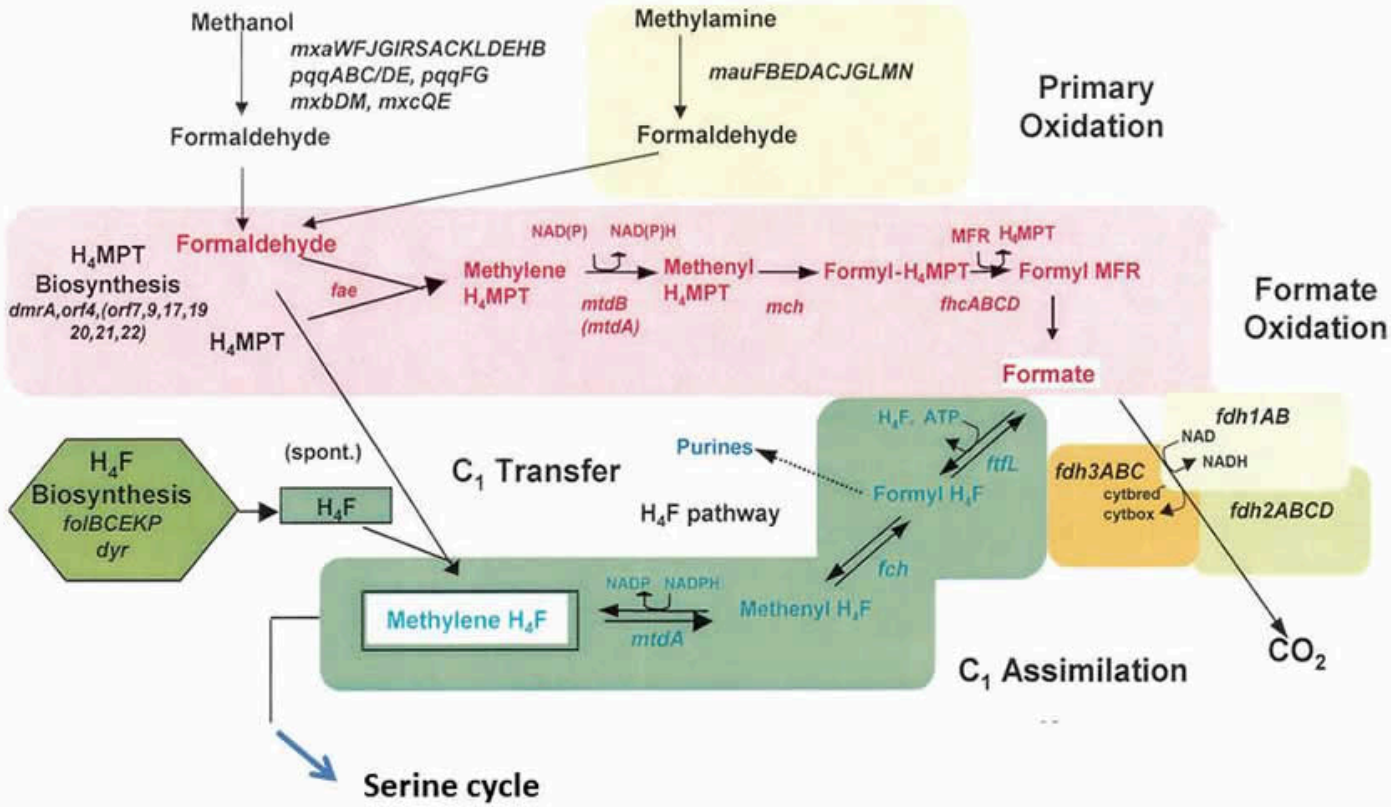



Fig. 25. The cyclic route for oxidation of formaldehyde. This is probably the main route for oxidation of formaldehyde in *Pseudomonas* C, *Methylophilus methylotrophus* and *Pseudomonas oleovorans*. This cyclic pathway is discussed further in Chapter 3.

## Formaldehyde oxidation in Methylobacterium extorquens

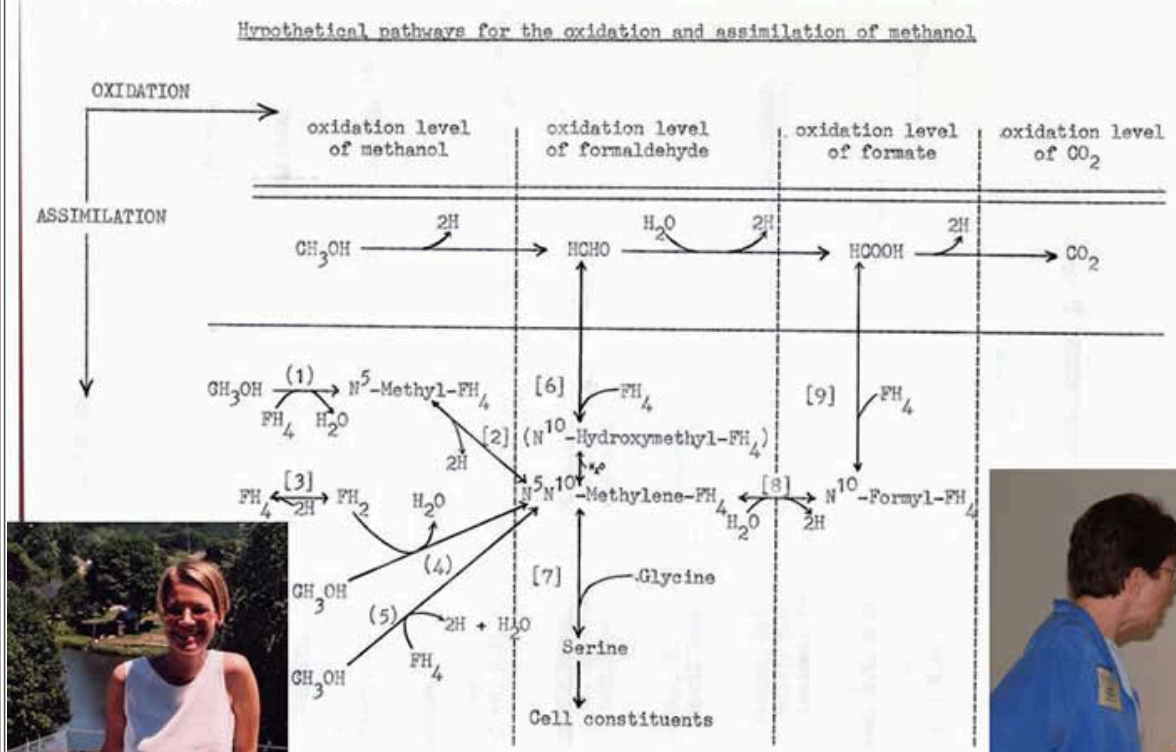
Methanopterin was previously only known as carrier in methanogens. This work is by Lidstrom and colleagues. Much is based on genetic/mutant studies.

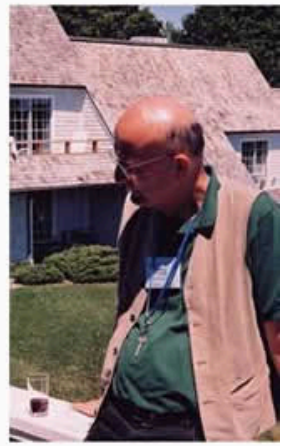


# Last page of my PhD Thesis (submitted 50 years ago; May 1963) suggesting folate involvement in formaldehyde oxidation

Fig.40.

Hypothetical pathways for the oxidation and assimilation of methanol







## The oxidation of methanol in Gram negative methylotrophs

This was my PhD project with Len Zatman (1960-1963). He thought the enzyme for methanol oxidation would be unusual as no NAD-linked alcohol dehydrogenase is able to oxidise methanol.

I started by isolating a soil methylotroph, *Pseudomonas* sp M27. It was the same as Quayle's *Pseudomonas* AM1 (published at end of my first year); now called *Methylobacterium extorquens* AM1

**Whole cells** oxidised methanol and a very wide range of primary alcohols
Inhibited by p-nitro- phenylhydrazine. (50% inhibition by 1.0 µM). *This has never been explained or investigated further.*

Inhibited by EDTA. Not due to chelation of metal ions. Unique to alcohol oxidation. Later shown to inhibit competitively the binding MDH to its cytochrome electron acceptor

**Extracts** did not catalyse reduction by methanol of NAD but it was possible to get reduction of a blue indophenol dye in the presence of an intermediate phenazine methosulphate using whole cells in anaerobic conditions.
We then found that this assay could be used with sonic extracts: this is similar to flavoprotein dehydrogenases like succinate dehydrogenase.
The enzyme is **Methanol dehydrogenase (MDH).**

**Problems with the dye linked system for methanol dehydrogenase**

The rate was not proportional to protein concentration.
No dye reduction could be seen with lower concentrations of extract.
When water in the assay was replaced with salts growth medium the rate became proportional to protein concentration.
The active constituent was ammonium chloride $NH_4Cl$.
The activator species is Ammonia base **$NH_3$** not the ammonium **ion $NH_4^+$**

This is unique. In some enzymes ammonium ions replace potassium ions. But with MDH ammonium chloride could only be replaced with methylammonium chloride **$CH_3NH_3^+$**

At limiting concentrations of activator 10 times more **$CH_3NH_3^+$** was required than with **$NH_4^+$**. This is because its pK value is 10 times higher than that of ammonium chloride.

AND 10 times more ammonium chloride was required at pH 8, when the proportion of **$NH_3$** was one tenth of the proportion at pH 9.

$$NH_4^+ \longrightarrow NH_3 + H^+$$

# Methanol dehydrogenase

**Responsible for all bacterial methanol oxidation**

The Microbial Oxidation of Methanol

2. THE METHANOL-OXIDIZING ENZYME OF *PSEUDOMONAS* SP. M 27

By C. ANTHONY and L. J. ZATMAN
*Department of Microbiology, University of Reading*

(*Received* 21 *November* 1963)

**Dye-linked assay (PMS/PIP); pH optimum 9.2; pI 8.2**

**Activator- ammonia (not $NH_4$+ ion)**

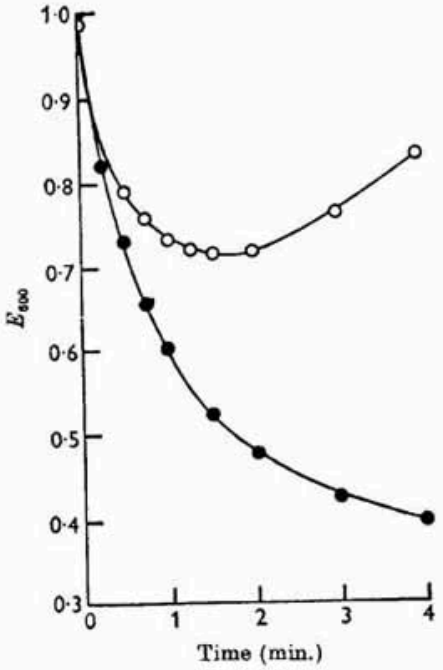
**Wide substrate specificity**

**Unusual absorption spectrum – novel prosthetic group??**

**High proportion of tryptophans**

**A homodimer (two 60 kDa subunits); later shown to have two 66kDa subunits and two 8kDa subunits**

**No metal ion. [Later shown to have Calcium ion]**




**MDH assay with PMS/indophenol**

# A new type of enzyme prosthetic group

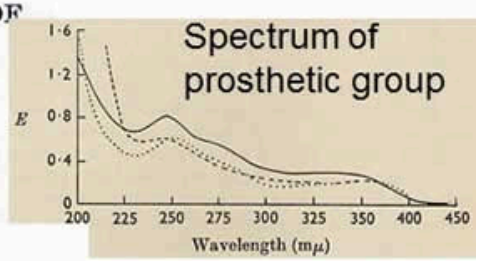
**Note: all other dehydrogenases use NAD(P) as hydrogen acceptor or they are membrane flavoproteins that use FMN or FAD as prosthetic group**

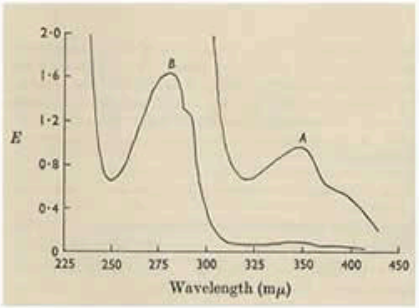
## The Microbial Oxidation of Methanol

THE PROSTHETIC GROUP OF THE ALCOHOL DEHYDROGENASE OF *PSEUDOMONAS* SP. M27: A NEW OXIDOREDUCTASE PROSTHETIC GROUP

By C. ANTHONY* and L. J. ZATMAN
*Department of Microbiology, University of Reading*

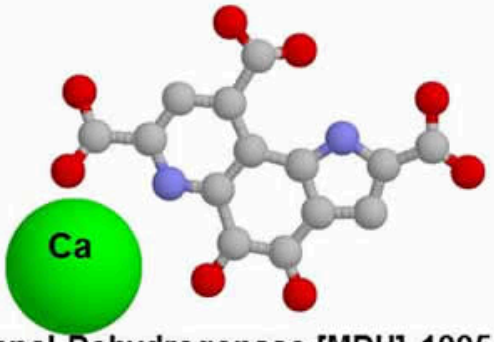
(*Received* 30 *December* 1966)




Spectrum of MDH

**PQQ** 1979 Kennard's lab



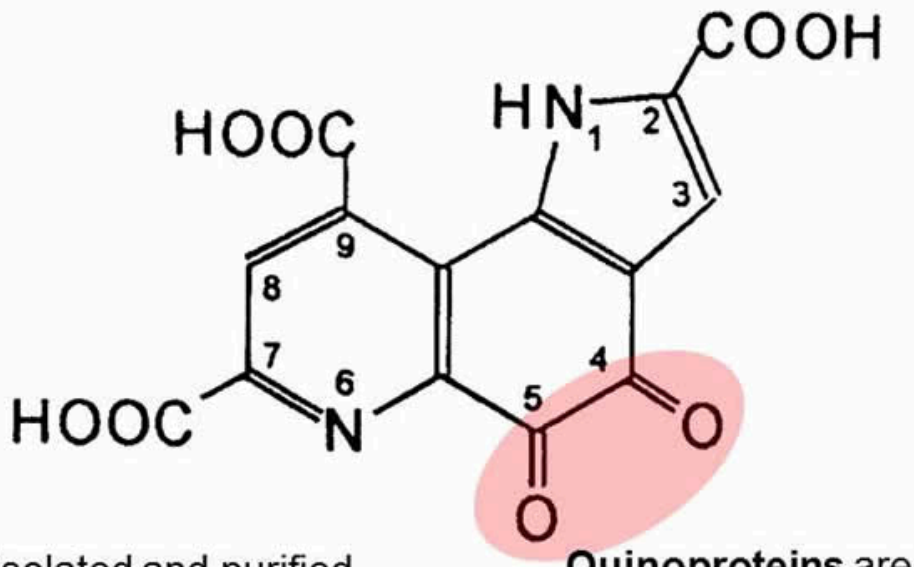

**Methanol Dehydrogenase [MDH] 1995**

**New prosthetic group is PQQ**

**Pyrrolo-Quinoline Quinone**

**MDH is now known as a Quinoprotein**

# Pyrroloquinoline Quinone (PQQ)




First isolated and purified by Anthony & Zatman in1967 from bacterial methanol dehydrogenase

Structure determined by Kennard's group in1979

**Quinoproteins** are enzymes with an *ortho*-quinone prosthetic group

They are equivalent to flavoproteins which has riboflavin derivatives FMN and FAD as prosthetic group

**PQQ and other quinoproteins** The novel prosthetic group of MDH was later in other dehydrogenases and Hans Duine and Hans Frank called these Quinoproteins. These include the alcohol dehydrogenase of acetic acid bacteria and the typical glucose dehydrogenase in *Pseudomonas*, *E. coli*.




Hauge described GDH and PQQ very early on





Hans Duine and Hans Frank 'invented' quinoproteins




Friends from Yamaguchi, Japan:
Matsushita, Uragami (?), Ameyama, Adachi.
Found many new quinoproteins especially in Acetic acid bacteria

**Note: No PQQ-containing quinoproteins have been described in anything other than bactera**


2nd International Symposium on PQQ and Quinoproteins
November 19 - 22. 1991 Yamaguchi University. Yamaguchi

# Substrate specificity of methanol dehydrogenase

Substrates oxidised

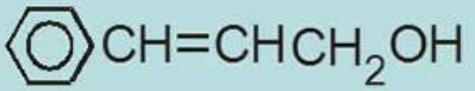
$CH_3OH$ $HOCH_2OH$

Hydrated formaldehyde

Only primary alcohols

R- $CH_2CH_2OH$

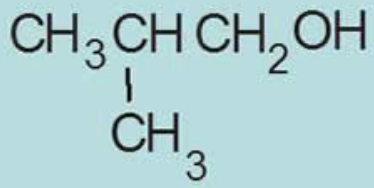
R = H, Cl, Br, OH, $(CH_2)_n$



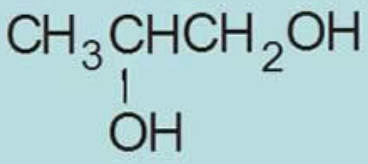
Substrates not oxidised

$NH_2CH_2CH_2OH$

Alcohols with C2 substituent are not oxidised



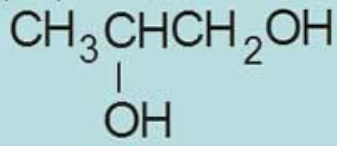
Surprisingly, propane 1,2-diol is oxidised
But only in crude preparations



# Modifier protein for methanol dehydrogenase

This was found by accident when investigating growth on propane 1,2-diol

This is oxidised by MDH but only in crude extracts;
it does not obey the substrate rules for MDH

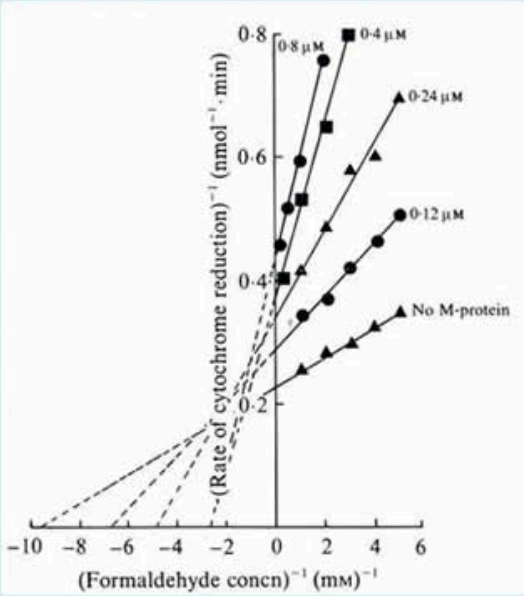



The periplasmic Modifier protein increases affinity for propanediol

Surprisingly, it is also present in obligate methylotrophs that do not grow on propanediol

M-protein decreases the Vmax,
and decreases affinity, for formaldehyde

This prevents the wasteful oxidation of methanol through formaldehyde to formate




1980-1990:John Bolbot; Steve Ford; Dudley Page; Tony Long

## Energy production from methanol dehydrogenase

**For energy production the MDH must be linked to the electron transport chain**

### The cytochromes of M. extorquens AM1

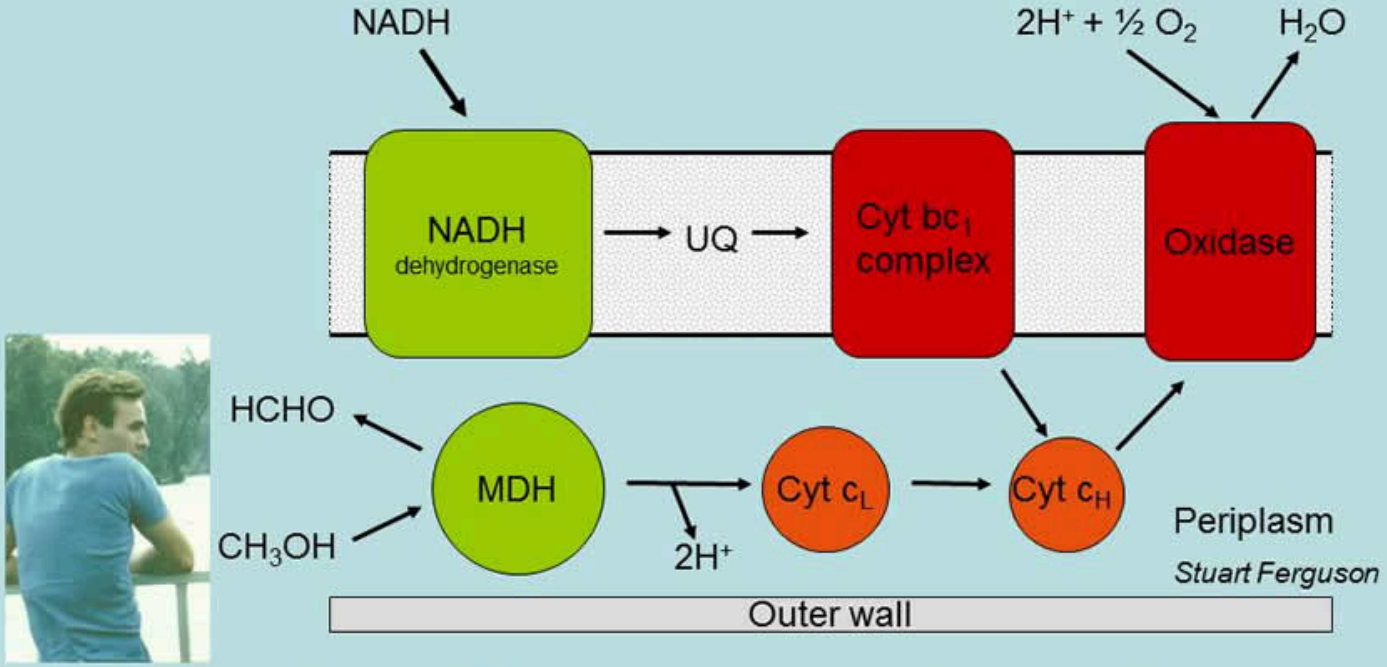
**Membranes** have typical b-type cytochromes and a cytochrome $aa_3$ able to oxidise typical soluble mitochondrial cytochrome c.

**The Soluble fraction** has 2 cytochromes c; one is small typical high pI (cytochrome $c_H$) which is oxidised by membrane fractions. And a much larger one with low pI (cytochrome $c_L$) which is not oxidised by the membrane oxidase.
**Cytochrome $c_L$** is a unique cytochrome. It is the electron acceptor for MDH. It is then able to pass the electrons on to cytochrome $c_H$ which is oxidised by the membrane oxidase. [There is a separate presentation on these cytochromes]

**Proton translocation during methanol oxidation**
Measured by typical Peter Mitchell method.
The ratio of protons pumped with pyruvate (NADH), succinate and methanol are 3:2:1.

**Conclusion**: methanol is oxidised by a unique electron transport chain that is similar to those for oxidation of inorganic ions by chemolithotrophs, where ubiquinone and the cytochrome $bc_1$ complex are not involved.

# Electron transport during methanol oxidation






During oxidation of MeOH 2 protons are 'translocated' from inside to outside
Setting up a protonmotive force supporting production of about 0.6 ATP/MeOH

1975 - 1985 David O'Keefe; Sandy Cross; Mathew Beardmore-Gray; Claude Chan; Jon Cox; Darren Day; Steve Froud; Andy Elliott; Bill Keevil; Sasha Netrusov; Pat Goodwin


Research students
(Graduated 1990)
Darren Day
Claude Chan
Ian Richardson
Tony Long
Darren
Jon Cox

# The second subunit of MDH

1965 - 1989 MDH is a homodimer (2 x 60 kDa); 'confirmed' by others for > 20 enzymes

1989 Jim Elliott insisted that his 8.5 kDa 'contaminant' must be a subunit



1986: The key papers starting genetics of the methanol oxidation system
Nunn & Lidstrom (1986a,b) *J. Bacteriol*. **166**

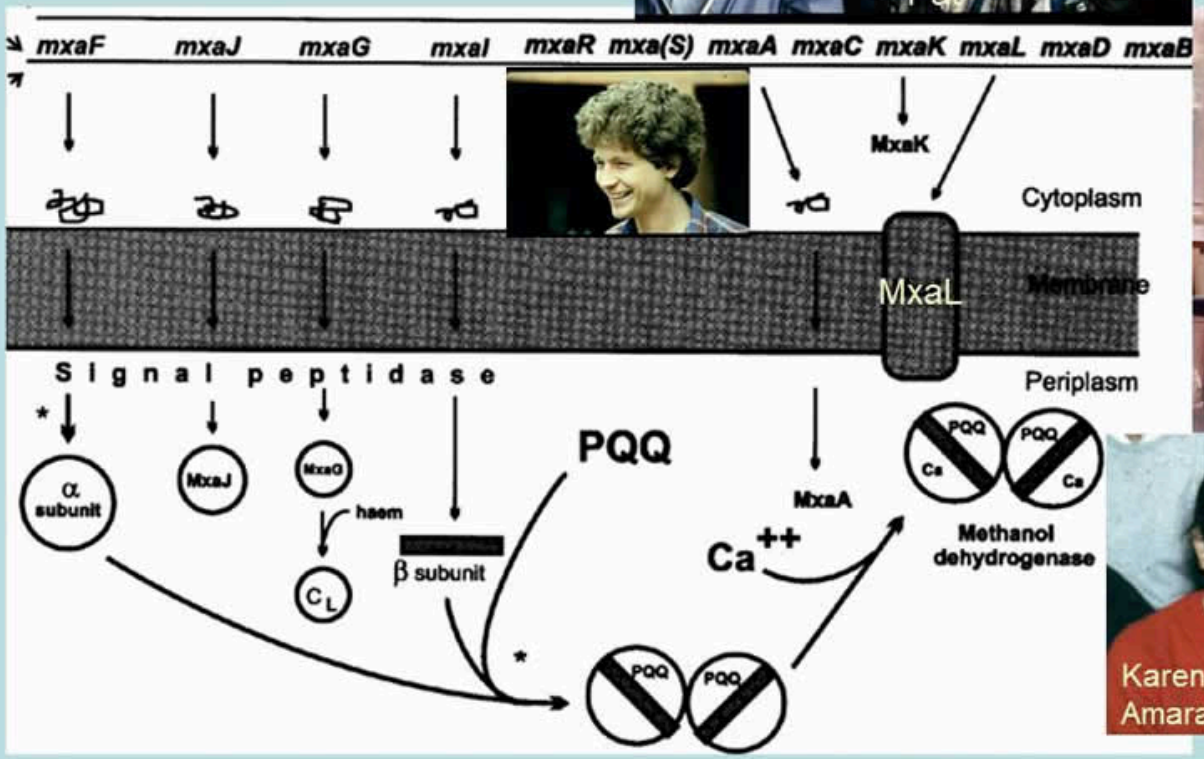


1989 We (David Nunn) sequenced the gene for cytochrome $c_L$ (*mxaG);* and showed that the downstream gene (*mxaI)* in the methanol oxidation operon coded for the 'contaminant' which thus became the 8.5kDa ß subunit

1989 Darren Day confirmed that MDH is an $\alpha_2ß_2$ tetramer

# Expression of the mxa operon



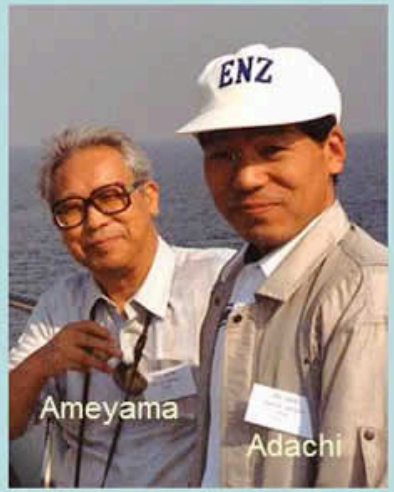

The genes: Nunn, Lidstrom, Amaratunga, Anderson, Anthony, Goodwin, Morris, O'Connor

# The calcium ion in MDH

1965-1989 ' MDH contains no metal ion' [Anthony & Zatman]

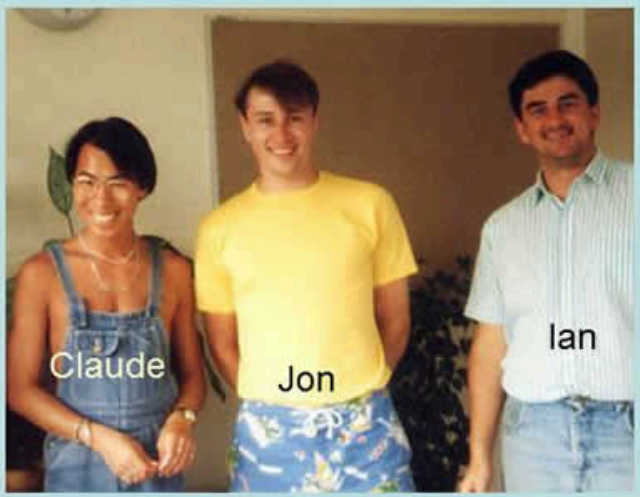
1986 MDH from mutants (*mxaA, K & L*) was inactive and had unusual spectrum [Nunn & Lidstrom]

**1990 MDH contains Calcium (or strontium) [*Adachi et al.*]**




1992 Other MDHs contain calcium.
MDH from *mxaA, K & L* lacked Calcium;
incubation with Ca produced active enzyme & normal spectrum [Ian Richardson]

1992 MDH isolated from marine *Methylophaga Marina is red and inactive;*
incubation with Ca produced normal green active enzyme [Claude Chan]



# The calcium ion in MDH

1994 Kinetics of MDH containing strontium *[Harris & Victor Davidson]*




1996 Incubation with Barium produced MDH which was more active than wild-type MDH [Matt Goodwin, Alain Avezoux & Simon Dales]









# The Mechanism of MDH

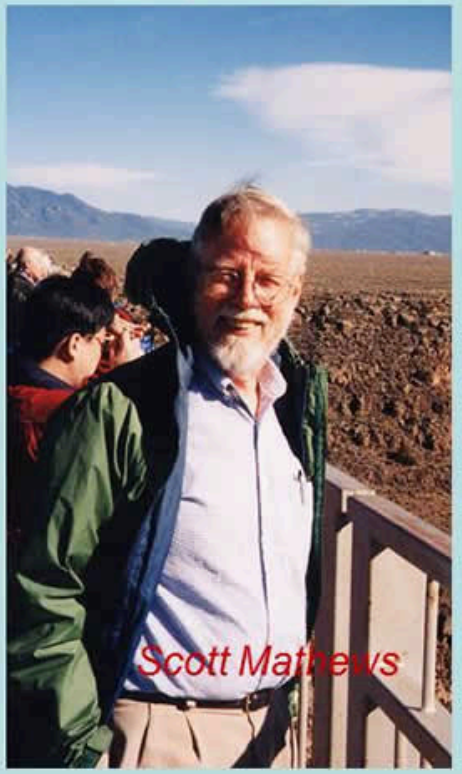
1979: X-ray structure of PQQ; *Salisbury, Forrest, Cruse, Kennard*

1978 – 1991: Main work on the mechanism; *Hans Frank and Hans Duine*
Ping-pong reaction with PQQ quinol produced from quinone
Reaction is catalysed by an active site base;
Addition/elimination reaction with a covalently attached methanol






# The X-ray structure of MDH (1992 – 2004)





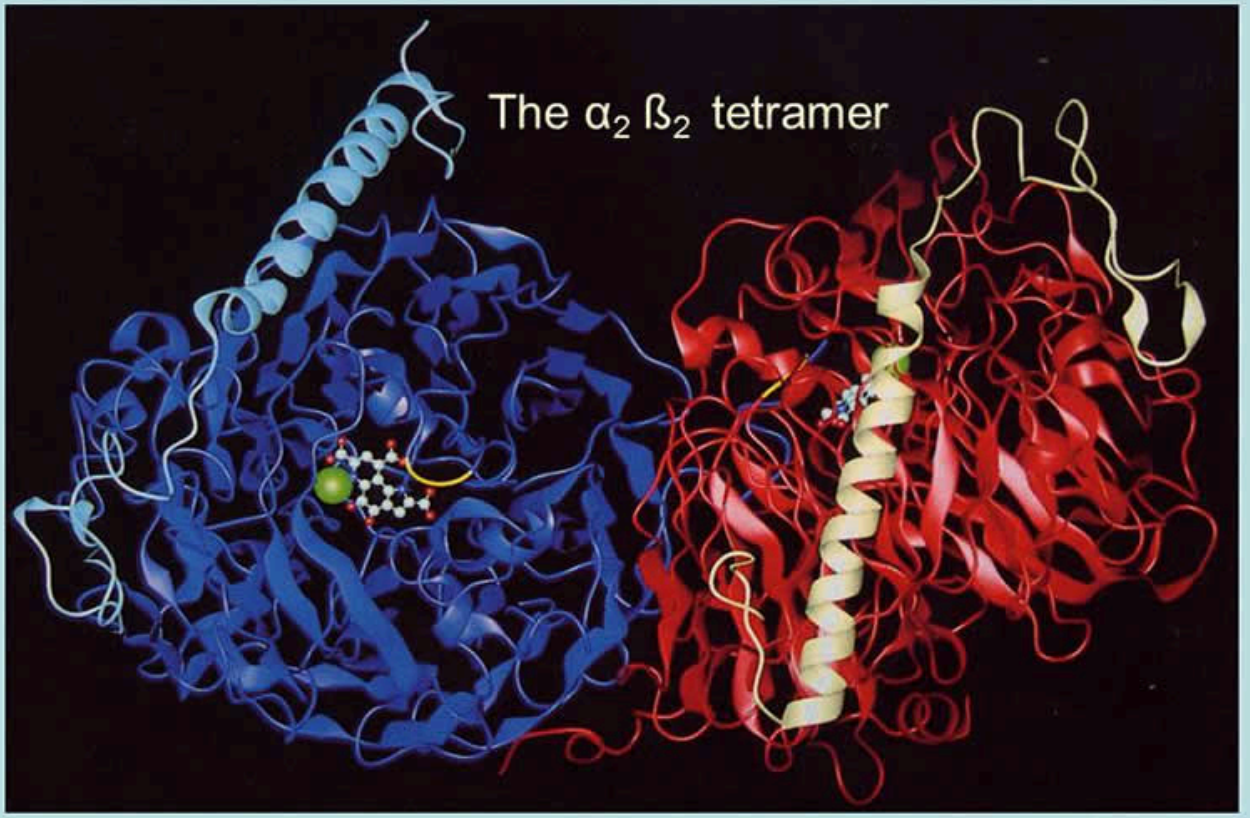




Meenakshi Ghosh, Chris Anthony; Colin Blake, Alain Avezoux, Matt Goodwin

*Scott Mathews, Z-X Xia, Victor Davidson, Scott White et al.*

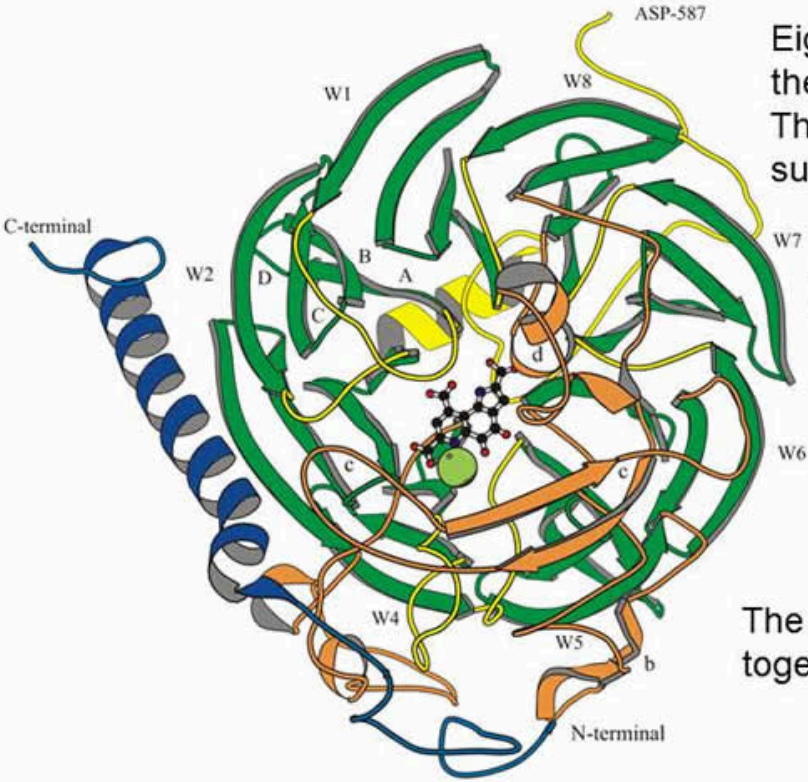
# The structure of MDH of *M. extorquens*




Meenakshi Ghosh, Chris Anthony; Colin Blake, Alain Avezoux, Matt Goodwin

*Scott Mathews, Z-X Xia, Victor Davidson, Scott White et al.* 1992 - 2003

# The ß-propeller structure of MDH




Eight 4-stranded ß sheets (W1-8); these are 'propeller blades'. The 'propeller fold' forms a superbarrel with active site inside

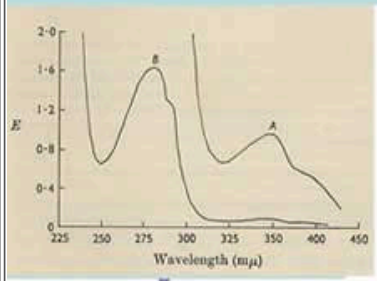
Active site contains PQQ and calcium (green sphere)

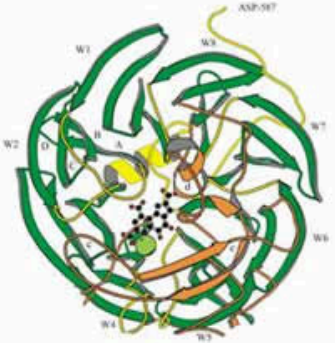
The ß-sheet 'propeller blades' are held together by 'tryptophan-docking motifs'

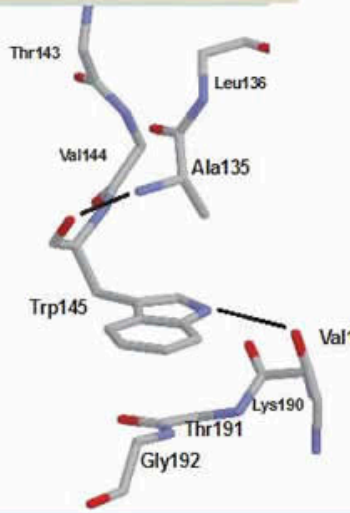
# Tryptophan docking system

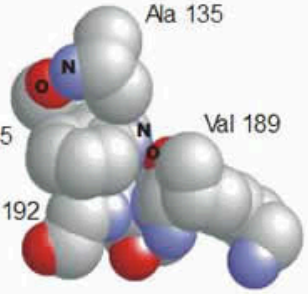
High proportion of tryptophans;
19 per α subunit

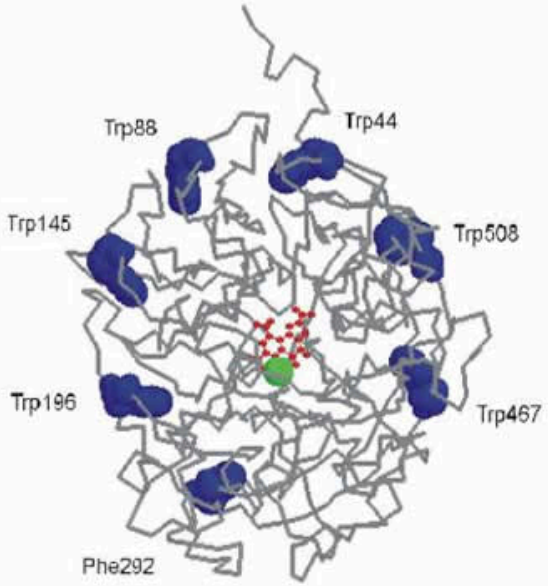
The ß-sheet 'propeller blades' are held together by 'tryptophan-docking motifs'



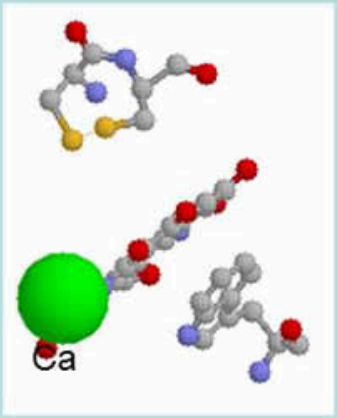





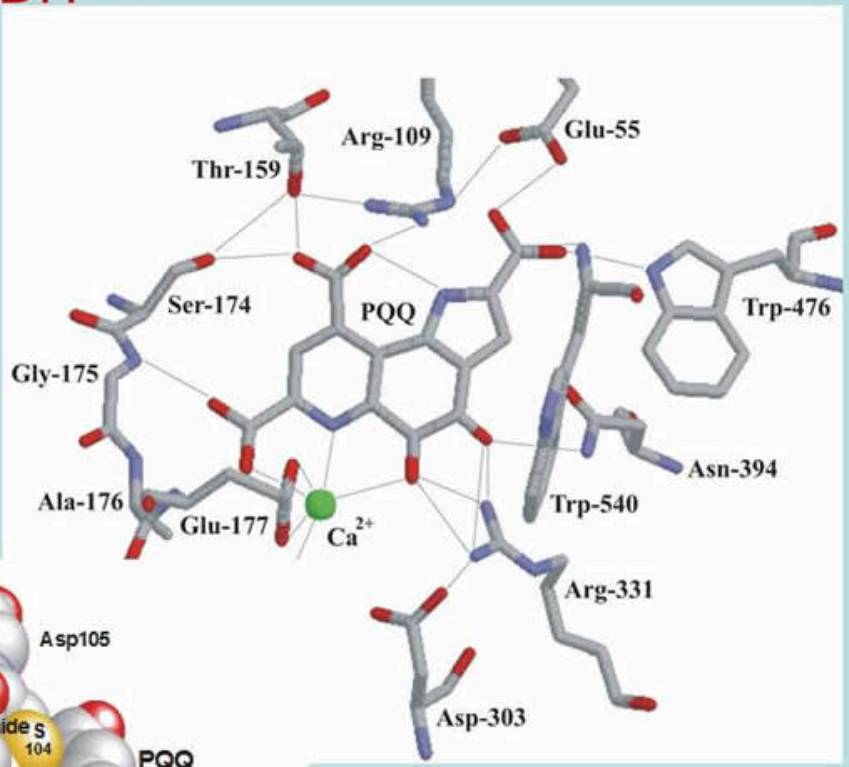



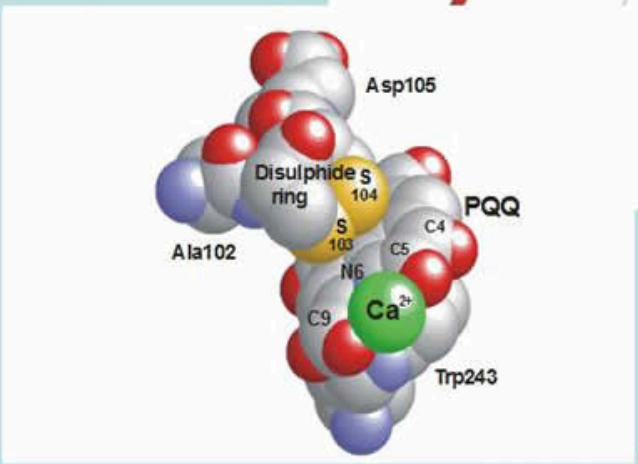



# The active site of MDH




Novel disulphide ring structure from adjacent cysteines



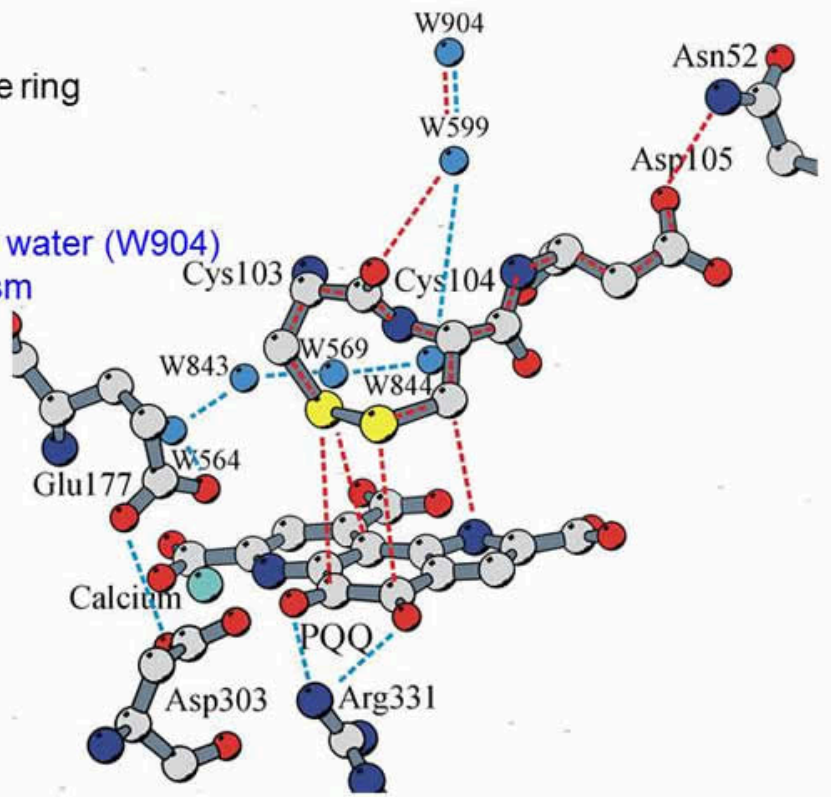
## The path of electrons and protons during the oxidative part of the reaction mechanism of MDH

Electrons must pass from reduced PQQ ($PQQH_2$) to cytochrome $c_L$

Note involvement of the disulphide ring

Protons must pass from PQQH2 by way of its hydroxyls, and the hydrogen-bonding network, to the water (W904) which is accessible to the periplasm

The pathways are based on those proposed for the quinohaemoprotein alcohol dehydrogenase by *Oubrie, Dijkstra et al, and Chen, Matsushita, Toyama, Scott Mathews et al.*




**Movie of MDH** Made by Stuart Findlow, University of Southampton, form the X-ray diffraction coordinates of MDH. If you canno movie when you click on arrrow then Go to YouTube clip

There is a separate set of slides for the special cytochromes of methylotrophs

Top of Oxidation

Top of Summaries Return to History

**The oxidation of methylated amines in methylotrophs** PPT Top of Summaries

# Oxidation of methylated amines

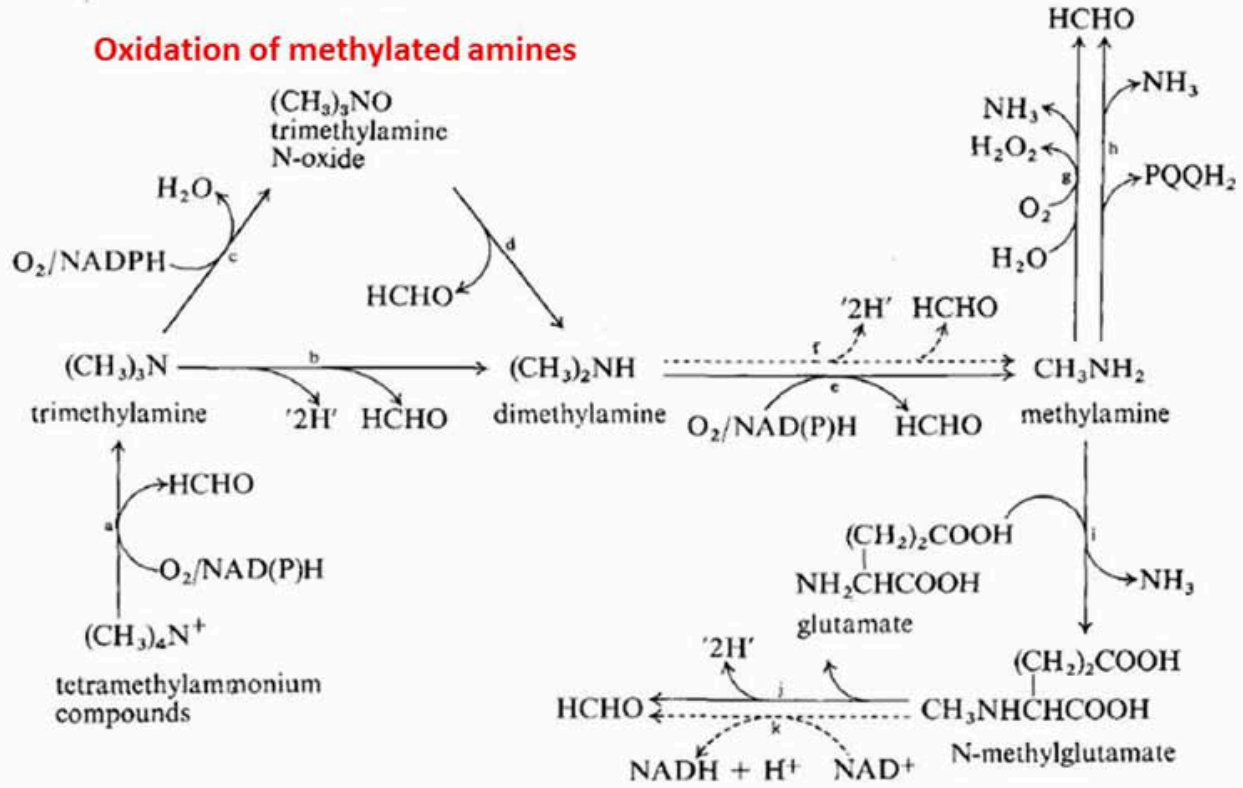
| $(CH_3)_4N^+$ | $(CH_3)_3N$ | $(CH_3)_2NH$ | $CH_3NH_2$ | $NH_3$ | HCHO |
|---|---|---|---|---|---|
| Tetramethylammonium | Trimethylamine | Dimethylamine | Methylamine | Ammonia | Formaldehyde |

Metabolism involves demethylation which always occurs at the same time as an oxidation. One methyl group is removed at a time, the C1 unit being removed as formaldehyde.

**Methylated amines in the marine environment:** Quote from Yin Chen,[1] Nisha A. Patel, Andrew Crombie, James H. Scrivens, and J. Colin Murrell[1]

Methylated amine compounds, including TMA, are ubiquitous in the environment—for example, as end products of protein putrefaction (18). In the marine environment, methylated amines are released as a result of degradation of quaternary amine osmoregulators, such as glycine betaine, which are used by marine organisms to counteract water stress (19–21). Once released into the environment, methylated amines can be used by microorganisms as a C or N source. In fact, in the oceans, methylated amines represent a significant pool of C and N, and standing concentrations up to hundreds of nanomolar and micromolar have been reported in the water column (22, 23) and sediment pore water (24, 25), respectively. In addition to being involved in biogeochemical cycles of C and N, recent studies also suggest that methylated amines have the potential to affect global climate, being precursors of aerosol formation in the upper atmosphere (26–28

## Oxidation of methylated amines




(a) Tetramethylammonium monooxygenase; (b) trimethylamine dehydrogenase (in obligate methylotrophs); (c) trimethylamine monooxygenase (in facultative methylotrophs); (d) trimethylamine TV-oxide demethylase (aldolase); (e) dimethylamine monooxygenase (all methylotrophs); (f) dimethylamine dehydrogenase (anaerobic *Hyphomicrobia);* (g) amine oxidase (in *Arthrobacter* and methazotrophic yeasts); (h) methylamine dehydrogenase; (i) N-methylglutamate synthase; (j) and (k) N-methylglutamate dehydrogenases.

## Bacterial oxidation of methylated amines

Much of the earlier work on this was done by Bob Eady in the lab of Peter Large in Hull in *Pseudomonas* AM1 (now *Methylobacterium extorquens* AM1) which was first isolated on methylamine (AM1 means Airborne Methylamine 1). 1968 -

He showed that oxidation is catalysed by a dye-linked methylamine dehydrogenase, assayed in a reaction system like methanol dehydrogenase.

$$CH_3NH_2 + A + H_2O \rightarrow NH_3 + AH_2 + HCHO$$

The prosthetic group of methylamine dehydrogenase is TTQ, tryptophan tryptophyl quinone [McIntire et al 1991]. It is covalently bound, being a modified part of the protein backbone.

**Remarkable fact:** the first 2 types of quinoprotein to be described were both isolated in *Methylobacterium extorquens* AM1.

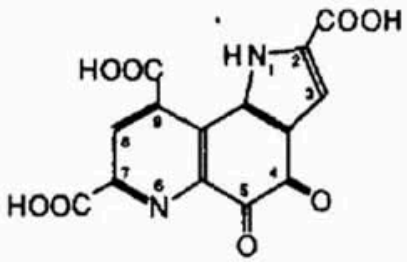
The electron acceptor for methylamine dehydrogenase was shown in 1981 by Tobari and Harada to be a blue copper protein called Amicyanin. This is a *single electron* acceptor.

## The prosthetic groups of some Quinoproteins

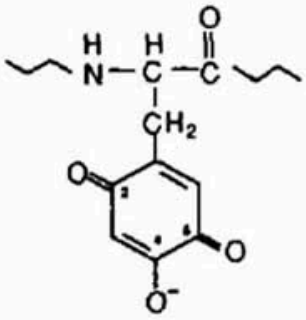
The prosthetic group of methanol dehydrogenase is PQQ, Pyrrolo-quinoline quinone [Kennard's group 1979]

The prosthetic group of methylamine dehydrogenase is TTQ, tryptophan tryptophyl quinone [McIntire et al 1991

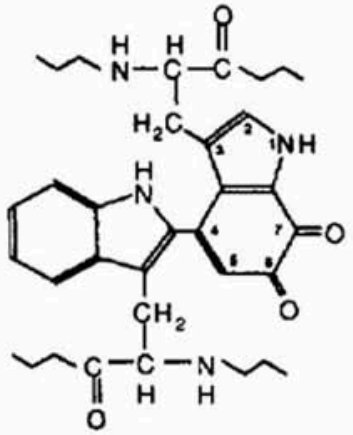
The prosthetic group of the copper containing amine oxidases is TPQ, topa-quinone [Klinman's group 1990]



PQQ



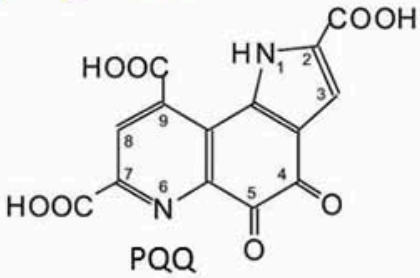
TPQ



TTQ

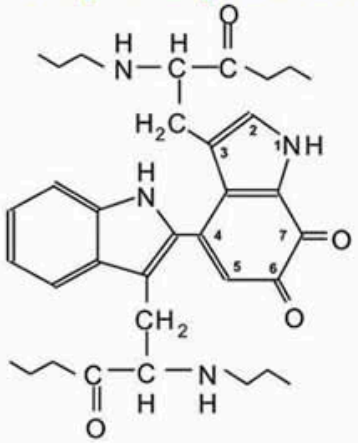
# Prosthetic groups of quinoproteins

## Dehydrogenases

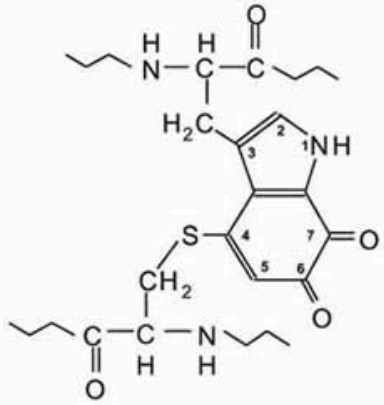



PQQ

**Not covalently bound**



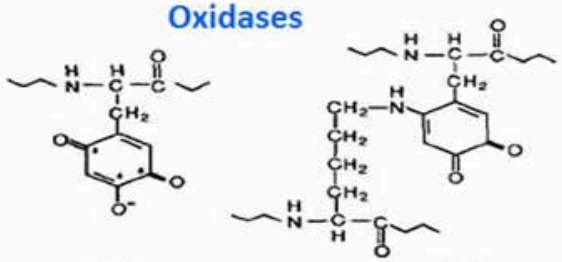

TTQ




CTQ

**[Derived from tryptophan]**

## Oxidases

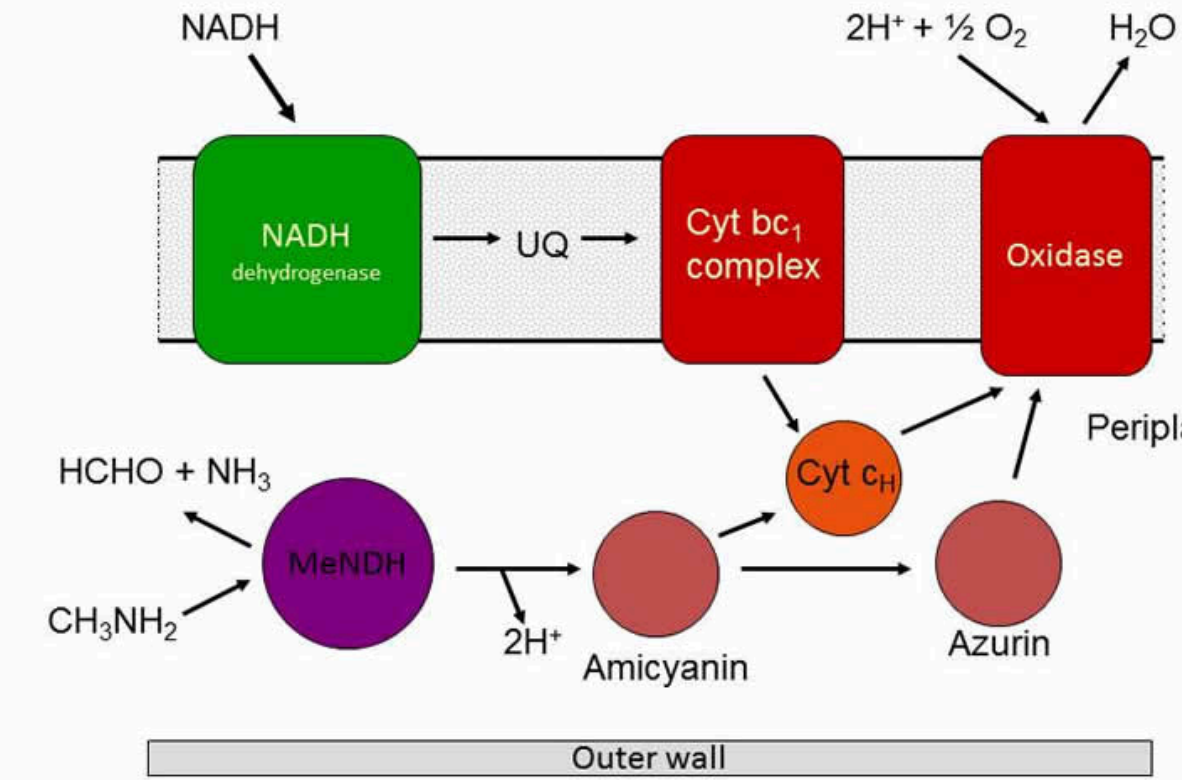


TPQ LTQ

**[derived from tyrosine]**

PQQ, pyrroloquinoline quinone
TTQ, Tryptophan tryptophylquinone
CTQ, cysteine tryptophylquinone
TPQ, Topaquinone
LTQ, Lysine tyrosylquinone

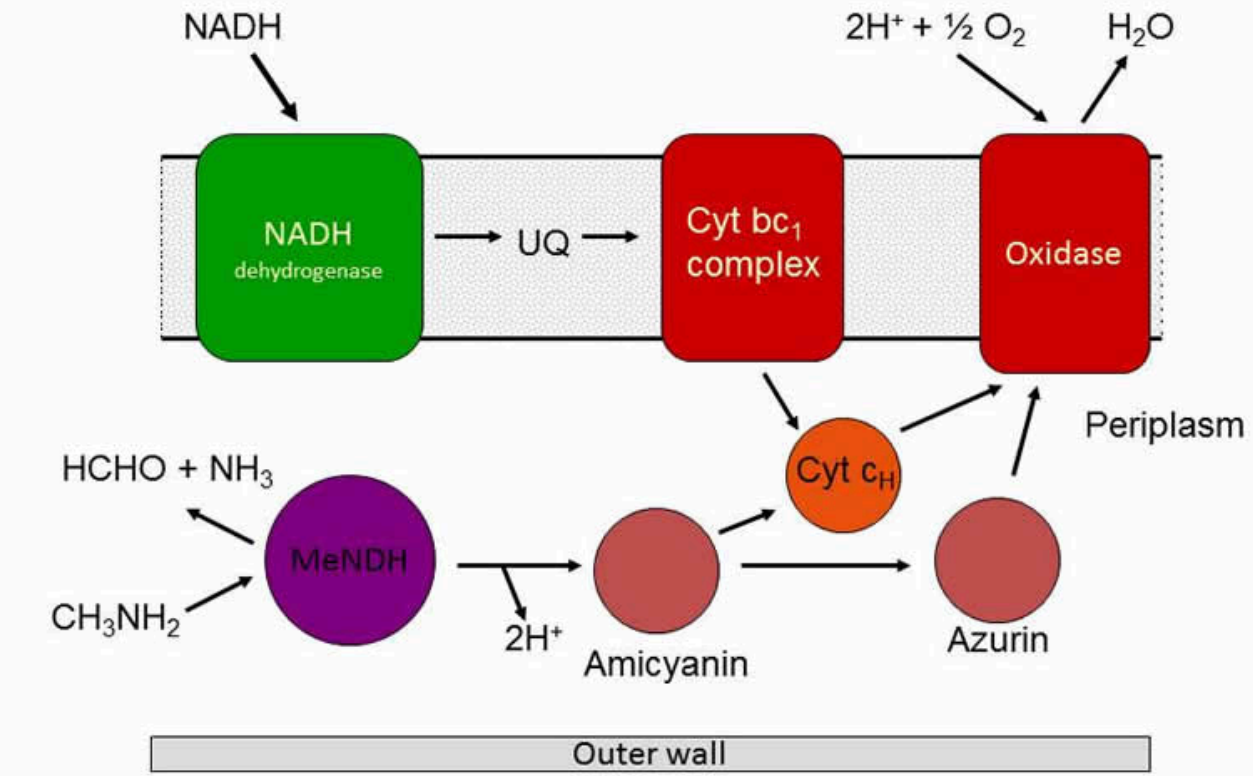
# Electron transport during methylamine oxidation




1985 - 1990 Ashley Lawton and Kevin Auton [Amicyanin – *Tobari*]

*Bob Eady & Peter Large [MeNDH; 1968-1971]*

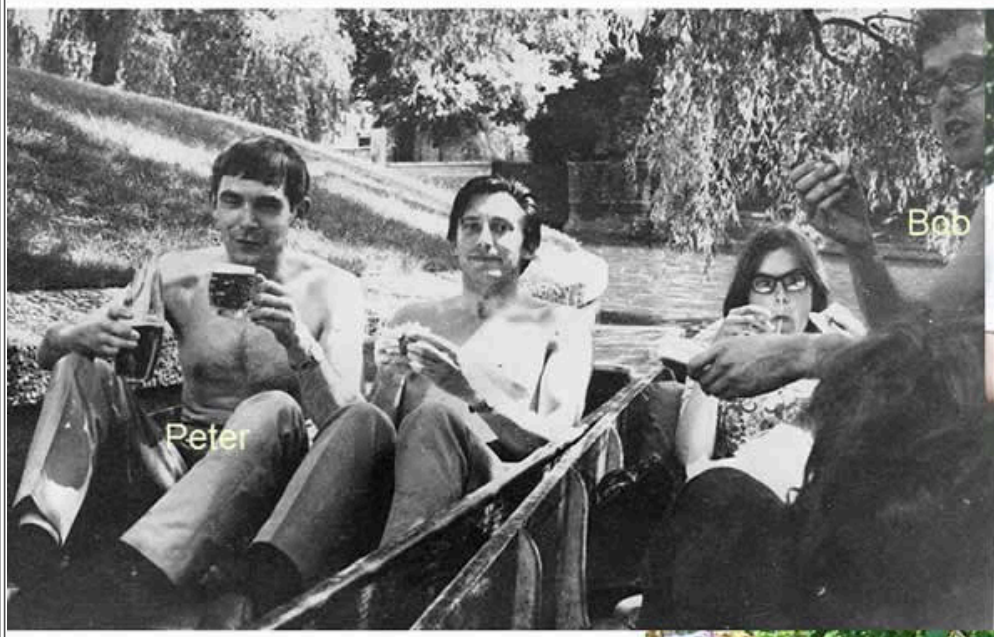
# Electron transport during methylamine oxidation




1985 - 1990 Ashley Lawton and Kevin Auton [Amicyanin – *Tobari*]

*Bob Eady & Peter Large [MeNDH; 1968-1971]*

## Methylamine dehydrogenase [Bob Eady and Peter Large, 1968-1971]







**Electron transport chain [Ashley Lawton & Kevin Auton, 1983-1989]**






Kevin Auton
Founder and CEO
of NextGen

Much of the subsequent study of the mechanism of methylamine dehydrogenase has been done by Victor Davidson and the genetics in Mary Lidstrom's group (with Mila Chistaserdova)



## Other amine oxidising enzymes

**Methylamine oxidase** (Amine oxidase) In *Arthrobacter* P1

$$CH_3NH_2 + O_2 + H_2O \longrightarrow HCHO + NH_3 + H_2O_2$$

The peroxide is removed by catalase. No useable energy from this reaction.
Its prosthetic group is probably TPQ as in other copper-containing amine oxidases.

### Systems involving methylated amino acids

In 'non-pigmented pseudomonads': *Ps. aminovorans*; *Pseudomonas* MA, & *Hyphomicrobium*

**Two step system**

Methylamine + glutamate ⟶ N-methylglutamate + ammonia (synthase)

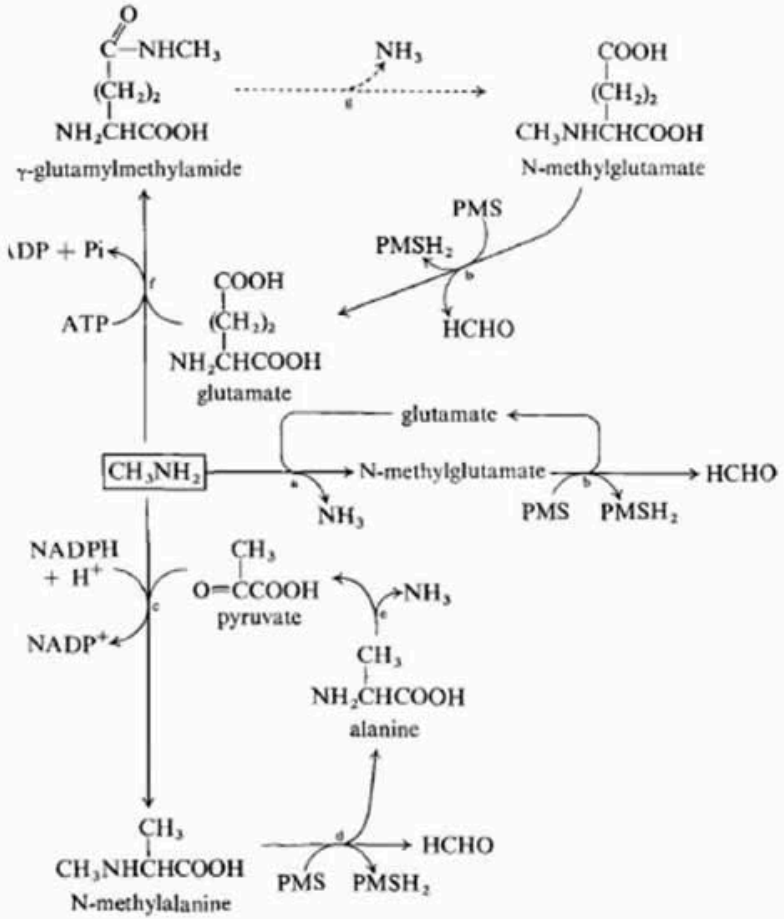
N-methylglutamate + PMS + $H_2O$ ⟶ glutamate + $PMSH_2$ + HCHO (dehydrogenase)

The dehydrogenase is usually a flavoprotein that interacts like other flavoproteins with the electron transport chain at the level of ubiquinone and cytochrome b.
(There are some reports of NAD-linked dehydrogenases in some *Pseudomonas* and *Hyphomicrobia* Netrusov)

The next slide shows some alternative two-part systems

## Some alternative two-part systems

Alternative systems for production of N-methylglutamate are in *Pseudomonas* MS and Hyphomicrobium but they may not be involved in growth. Kung & Wagner, Loginova & Trotsenko, Meiberg & Harder



**Oxidation of tetramethylammonium salts to trimethylamine and formaldehyde**

In Organism 5H2. Hampton & Zatman 1973. A non-pigmented Gram-negative, non-motile facultative methylotroph. No energy is available from this reaction

Mono-oxygenase

Tetramethylammonia + $O_2$ + NADH ⟶ trimethylamine + HCHO + $H_2O$

**Oxidation of trimethylamine to dimethylamine and formaldehyde**

In obligate methylotrophs; organism W3A1, & 4B6 (like *Methylophilus*), and *Hyphomicrobium* Colby & Zatman 1973, 1974

Trimethylamine dehydrogenase

$(CH_3)_3N + PMS + H_2O \longrightarrow (CH_3)_2NH + HCHO + PMSH_2$

The enzyme is an unusual flavoprotein, interacting with the electron transport chain by way of a second flavoprotein, cytochrome b etc. So provides energy

## Indirect route for trimethylamine oxidation

A trimethylamine mono-oxygenase produces the N-oxide which is then demethylated to formaldehyde plus dimethylamine. During growth on trimethylamine. In *Pseudomonas aminovorans* (a typical Pseudomonas sp.) and *Bacillus* PM6. Boulton & Large; Myers and Zatman.

a) Trimethylamine mono-oxygenase. No energy available

$$(CH_3)_3N + NADPH + H^+ + O_2 \longrightarrow (CH_3)_3NO + H_2O + NADP^+$$

b) Trimethylamine N-oxide demethylase No energy available

It is the 2nd enzyme for oxidation of trimethylamine and also the 1st enzyme for growth of Bacillus PM6 on trimethylamine N-oxide

$$(CH_3)_3NO \longrightarrow (CH_3)_2NH + HCHO$$

## The oxidation of dimethylamine to methylamine and formaldehyde

There are two types of enzyme; the same one operates when dimethylamine is a growth substrate as when it is an intermediate in the oxidation of higher methylated amines.

**Dimethylamine mono-oxygenase** No energy is available from this reaction
In *Pseudomonas aminovorans* (a typical pseudomonad). Bob Eady and Peter Large.
Also in Hyphomicrobium in aerobic conditions

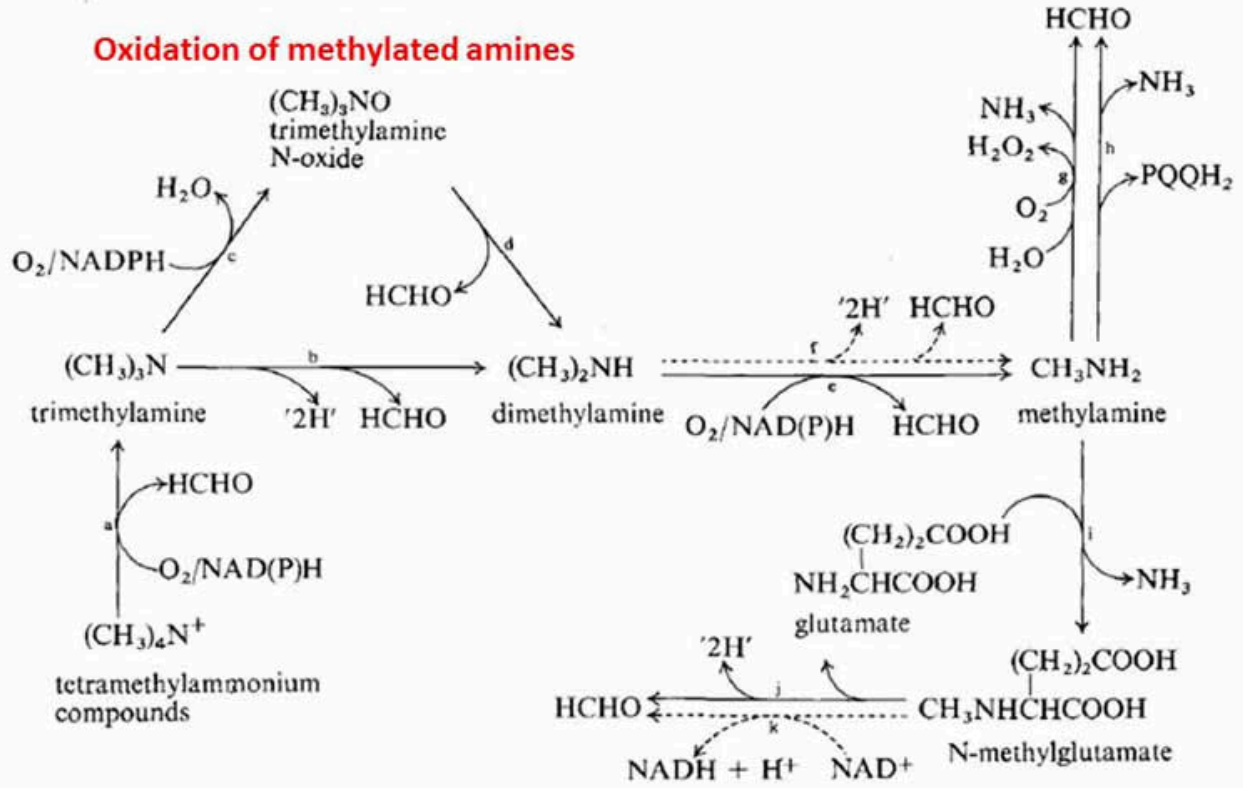
$$(CH_3)_2NH + NAD(P)H + H^+ + O_2 \longrightarrow CH_3NH_2 + HCHO + NAD(P)^+ + H_2O$$

**Dimethylamine dehydrogenase** Energy is available. Meiberg and Harder 1978.
In *Hyphomicrobium* during anaerobic growth with nitrate as electron acceptor.

$$(CH_3)_2NH + PMS + H_2O \longrightarrow CH_3NH_2 + HCHO + PMSH_2$$

Similar to trimethylamine dehydrogenase. An unusual flavoprotein that interacts with electron transport chain before cytochrome b.

## Oxidation of methylated amines




(a) Tetramethylammonium monooxygenase; (b) trimethylamine dehydrogenase (in obligate methylotrophs); (c) trimethylamine monooxygenase (in facultative methylotrophs); (d) trimethylamine TV-oxide demethylase (aldolase); (e) dimethylamine monooxygenase (all methylotrophs); (f) dimethylamine dehydrogenase (anaerobic *Hyphomicrobia);* (g) amine oxidase (in *Arthrobacter* and methazotrophic yeasts); (h) methylamine dehydrogenase; (i) N-methylglutamate synthase; (j) and (k) N-methylglutamate dehydrogenases.

## Distribution of routes for oxidation of methylated amines

### Trimethylamine

NAD-independent TMN dehydrogenase: Obligate methylotrophs (eg *Methylophilus*) and *Hyphomicrobium*
Mono-oxygenase system: All other methylotrophs including *Bacillus*

### Dimethylamine

Mono-oxygenase: All aerobic methylotrophs including *Hyphomicrobium* growing aerobically
Dimethylamine dehydrogenase: *Hyphomicrobium* growing anaerobically

### Methylamine

There are three types of system.
Methylamine dehydrogenase (MD); Methylamine oxidase (MO); N-methylglutamate-linked (NMG)

| | |
|---|---|
| Most obligate methylotrophs | MD |
| Facultative autotrophs | MD |
| Pink facultative methylotrophs | MD or NAD-dependent or NAD-independent NMG dehydrogenase |
| Non-pigmented pseudomonads | NAD-independent NMG dehydrogenase |
| *Hyphomicrobia* | NAD-dependent or NAD-independent NMG dehydrogenase |
| *Arthrobacter* and yeasts | MO |

NOTE: Some bacteria may have more than one system but this has rarely been tested.
There is no correlation between the system for methyamine oxidation and assimilation pathway

Top of oxidation of methylated amines

Top of summaries

**The unusual cytochromes and electron transport in methylotrophs** PPT Return to History Top of summaries

# The strange structures of special cytochromes *c* from methylotrophic bacteria

The molecular structure of an unusual cytochrome $c_2$ determined at 2.0 Å; the cytochrome $c_H$ from *Methylobacterium extorquens*

Jon Read, Raj Gill, Simon L. Dales, Jon B. Cooper, Steve P. Wood, and Chris Anthony *Protein Sci.* 1999 8: 1232-1240

The 1.6 A° X-ray structure of the unusual c-type cytochrome, cytochrome $c_L$, from the methylotrophic bacterium *Methylobacterium extorquens*

Paul Williams, Leighton Coates, Fiyaz Mohammed, Raj Gill Peter Erskine, Dominique Bourgeois, Steve P. Wood, Chris Anthony and Jonathan B. Cooper*
*J. Mol. Biol.* (2006) 357, 151–162

INTRODUCTION:
cytochrome c: methylotrophs: methanol oxidation: electron transport chains
methylotroph cytochromes c (earlier work)

# Cytochrome c (Class I)

Mitochondrial cytochrome c and the equivalent bacterial cytochrome $c_2$.

Small water soluble protein; about 100 amino acids

Hundreds of sequences and many structures are known.

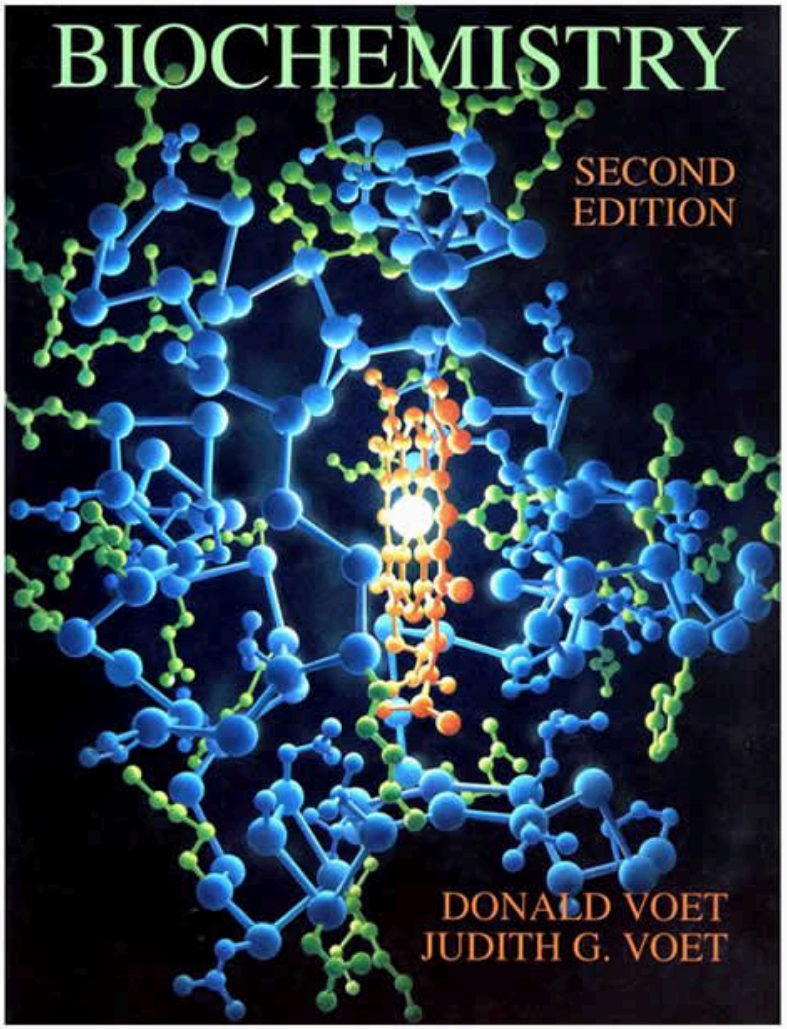
Haem prosthetic group covalently bonded by thioether bridges to protein

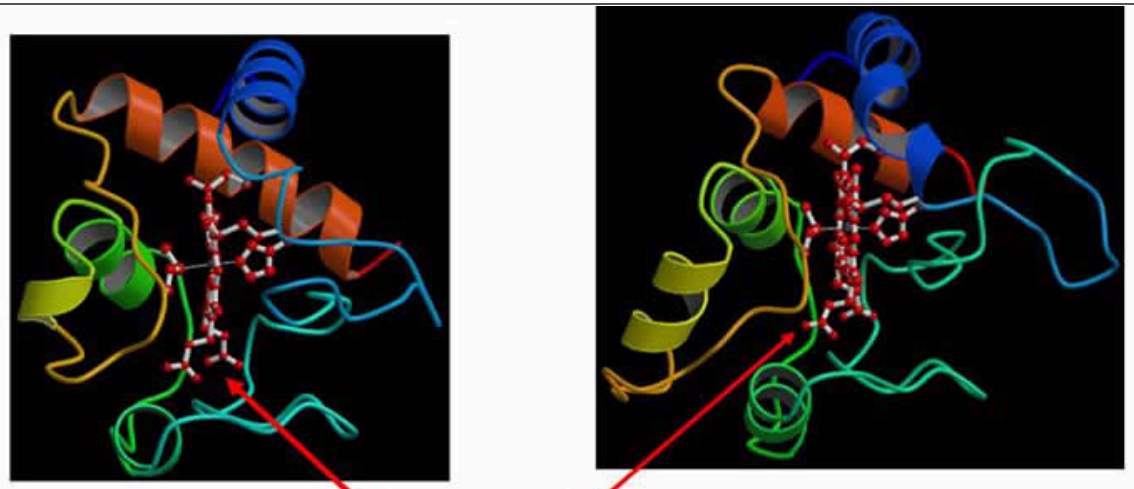
The iron atom forms ligands with the 4 pyrrole N of the haem; the 5th and 6th ligands are histidine and METHIONINE

Electron carriers $Fe^{+++} + e^- \longleftrightarrow Fe^{++}$

They function in electron transport chains, mediating electron transfer between membrane cytochrome bc1 complex and the oxidase [or between membrane proteins in photosynthetic electron transport in bacteria]

They are located between the inner and outer membranes of mitochondria and in the equivalent periplasmic space in bacteria

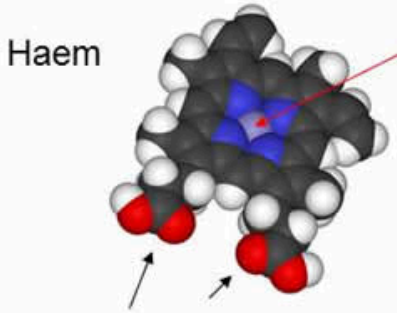

BIOCHEMISTRY
SECOND EDITION
DONALD VOET
JUDITH G. VOET



Cytochrome c

Propionates

Bacterial cytochrome $c_2$



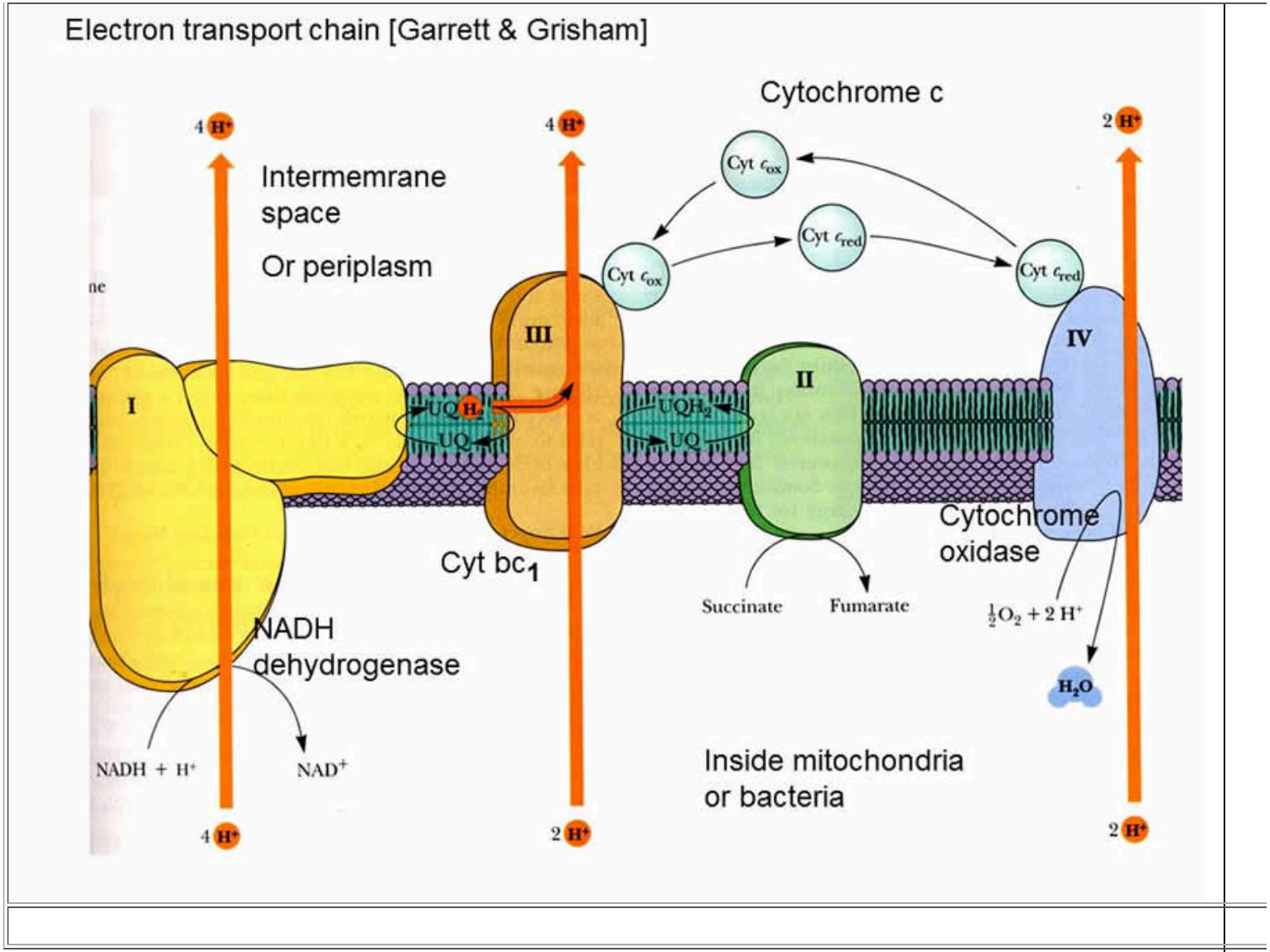
Haem

Iron

Propionates

The planar haem lies enclosed in the haem cleft; electrons enter and leave the haem at its exposed edge

In all structures there is a water molecule in the LHS of the haem cleft; the movement of this molecule is said to be essential during the oxidation and reduction processes


Electron transport chain [Garrett & Grisham]
Cytochrome c
4 H+
4 H+
2 H+
Intermemrane space
Or periplasm
Cyt $c_{ox}$
Cyt $c_{red}$
Cyt $c_{ox}$
Cyt $c_{red}$
III
IV
I
II
UQ $H_2$
UQ
$UQH_2$
UQ
Cytochrome oxidase
Cyt $bc_1$
Succinate
Fumarate
$\frac{1}{2}O_2 + 2\,H^+$
NADH dehydrogenase
$H_2O$
NADH + H+
$NAD^+$
Inside mitochondria or bacteria
4 H+
2 H+
2 H+

# Electron transport chain in mitochondria

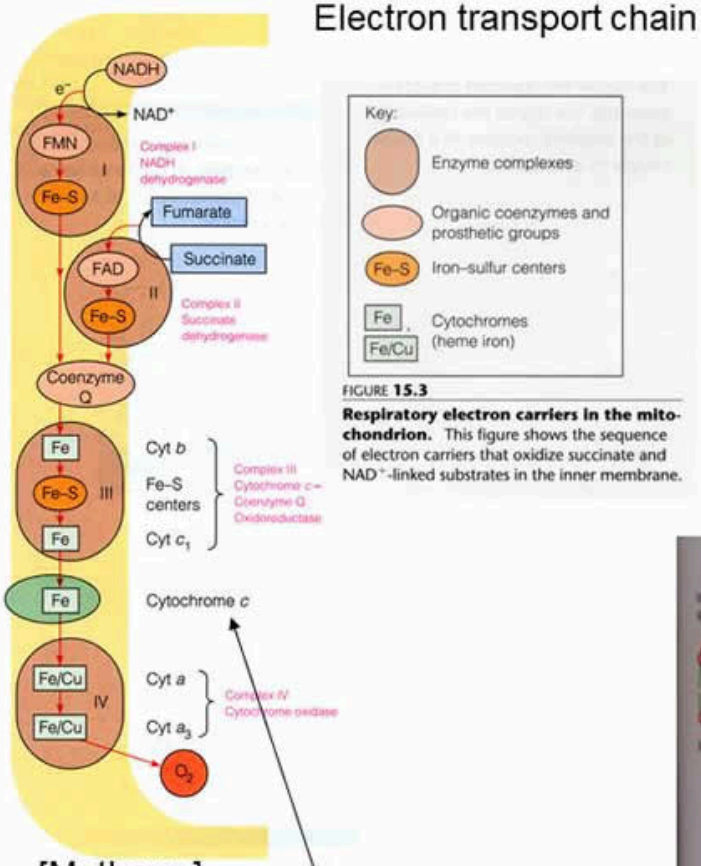
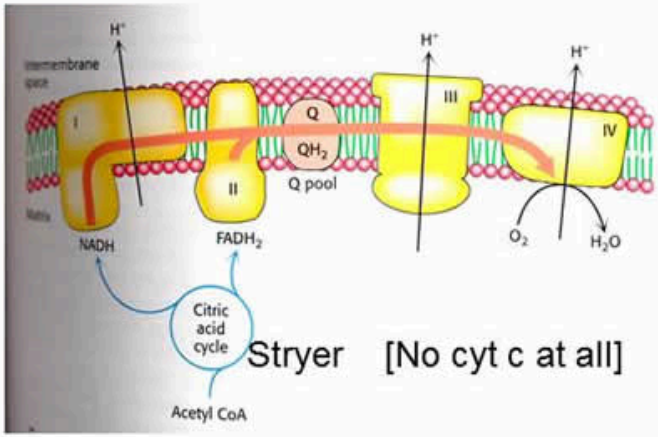



FIGURE **15.3**

**Respiratory electron carriers in the mitochondrion.** This figure shows the sequence of electron carriers that oxidize succinate and $NAD^+$-linked substrates in the inner membrane.

Text books are nearly all wrong




[Mathews]
Cyt c is a membrane protein!

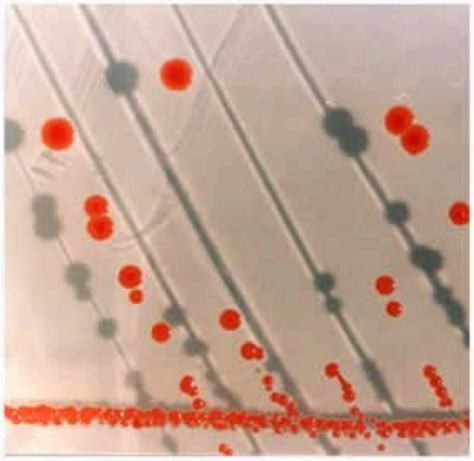
Stryer [No cyt c at all]

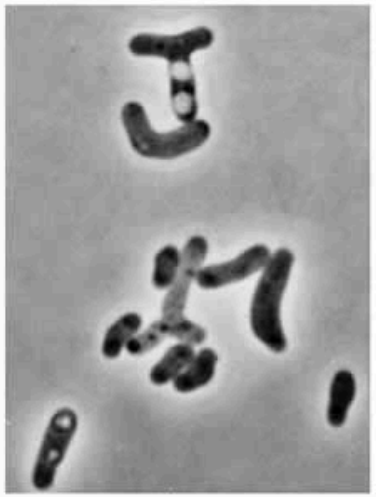
# *Methylobacterium extorquens*

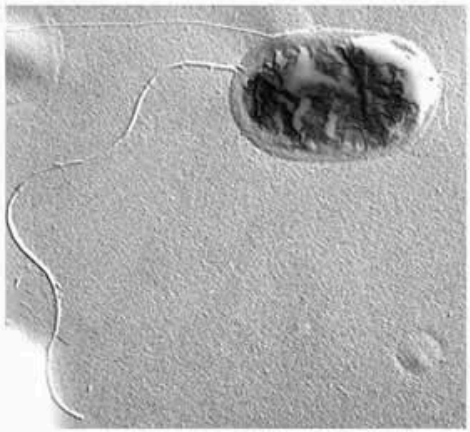
- *Pseudomonas* AM1 (Peel & Quayle, 1961)
- *Pseudomonas* sp. M27 (Anthony & Zatman, 1964)

$CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$







# The oxidation of methanol

Catalysed by soluble Methanol dehydrogenase [MDH] in the periplasm. NAD is not involved.

MDH has a 'novel' prosthetic group called PQQ

MDH is equivalent to a soluble flavoprotein but with PQQ instead of FMN or FAD

The question: how is energy obtained from this oxidation of methanol; how does MDH interact with the electron transport chain?

Cytochrome c is particularly important in methanol oxidation [kinetic and mutant studies].

*Biochem. J.* (1980) **192**, 411–419
*Printed in Great Britain*

# The two cytochromes *c* in the facultative methylotroph *Pseudomonas* AM1

David T. O'KEEFFE and Christopher ANTHONY
*Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.*

**Cytochrome $c_H$** High isoelectric point [basic, +ve charge at pH 7; *M*r = c 10,000
Does not react with MDH; **rapidly oxidised by the oxidase**.
It is also involved in oxidation of NADH
This cytochrome appears to be a typical bacterial cytochrome $c_2$

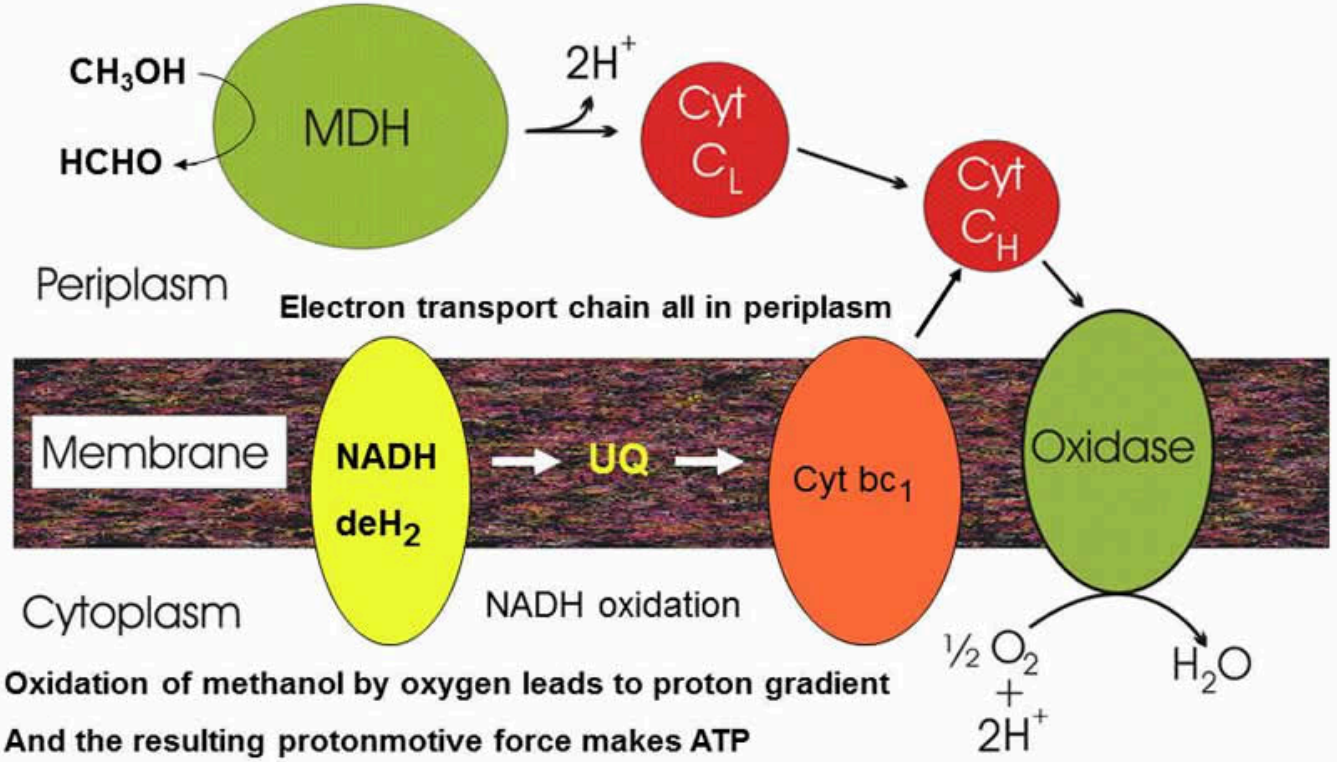
**Cytochrome $c_L$** Low isoelectric point [acidic, –ve charge at pH 7; *M*r = c 20,000
**Reacts rapidly with MDH; not oxidised by the oxidase**.
Not involved in NADH oxidation

Reacts slowly with CO. This displaces the 6th Methionine ligand.
This is very rare in cytochromes c and indicates an exceptionally flexible structure in the LHS of the haem cleft

**The two cytochromes c** are able to react very rapidly with each other

**MeOH → MDH → Cyt $c_L$ → Cyt $c_H$ → oxidase → O2**

# The oxidation of methanol in methylotrophs




**Oxidation of methanol by oxygen leads to proton gradient**

**And the resulting protonmotive force makes ATP**

**Project: Solve structure of all the soluble proteins**

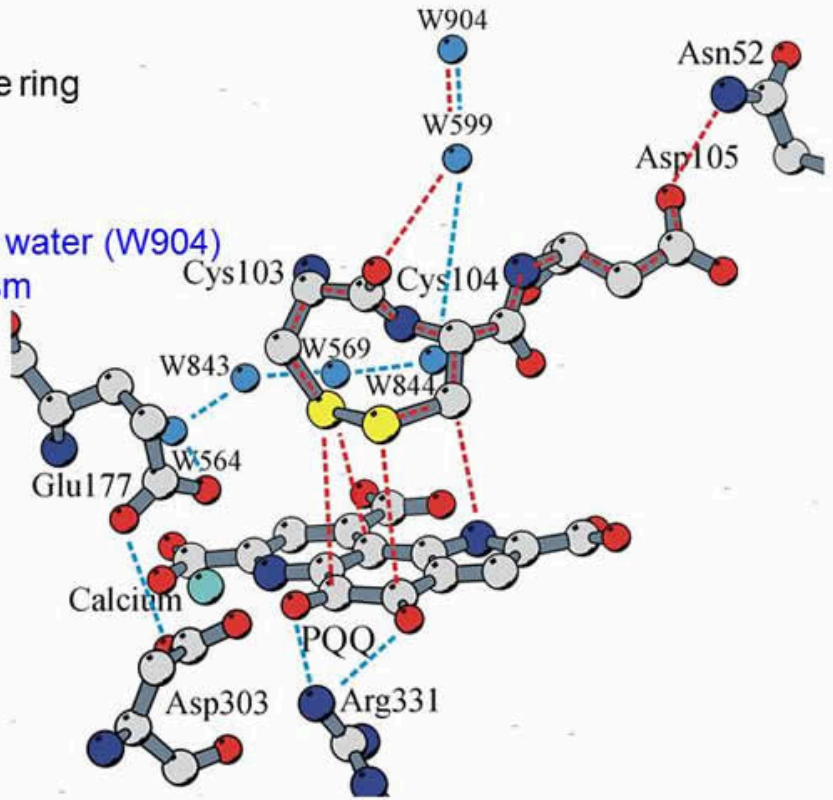
**Study electron transport within the proteins and between them**

# The path of electrons and protons during the oxidative part of the reaction mechanism of MDH

Electrons must pass from reduced PQQ ($PQQH_2$) to cytochrome $c_L$

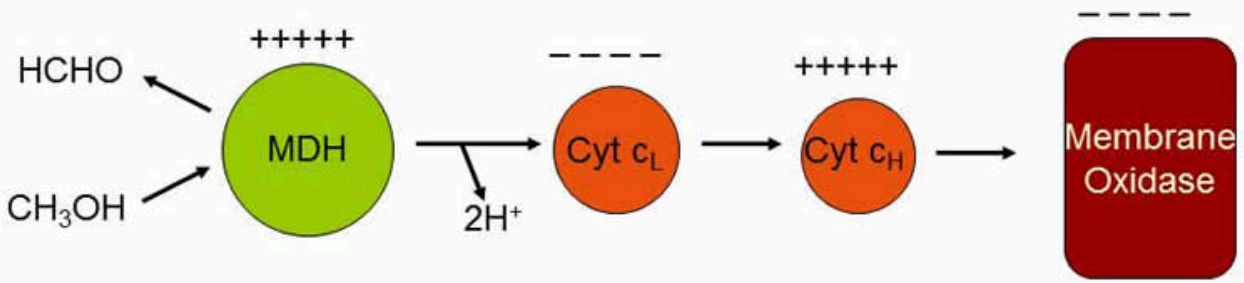
Note involvement of the disulphide ring

Protons must pass from PQQH2 by way of its hydroxyls, and the hydrogen-bonding network, to the water (W904) which is accessible to the periplasm




The pathways are based on those proposed for the quinohaemoprotein alcohol dehydrogenase by *Oubrie, Dijkstra et al, and Chen, Matsushita, Toyama, Scott Mathews et al.*

# Interaction of the electron transport proteins



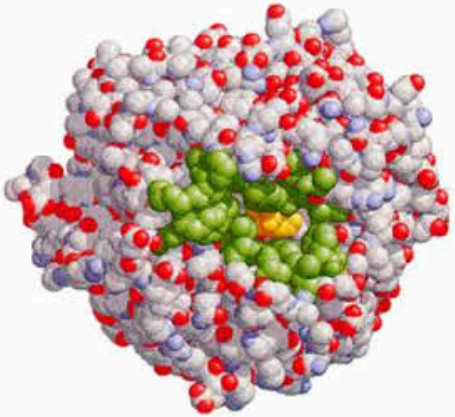

**MDH and cytochrome $c_L$ 'dock' together by ionic interactions between lysines on MDH and carboxylates on cytochrome $c_L$.**

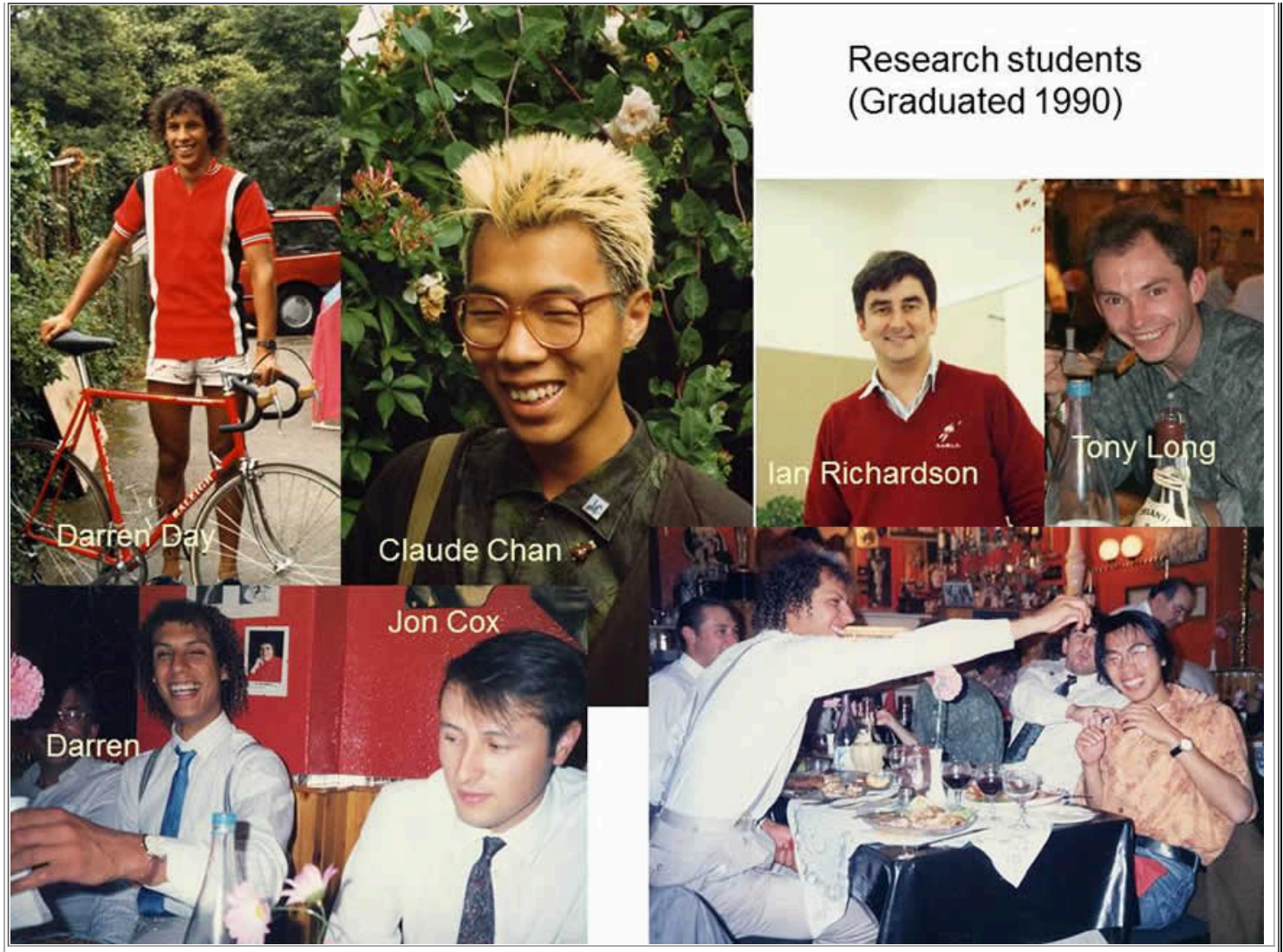
**The electron transfer complex may involve interaction of the cytochrome with the hydrophobic funnel on the surface of MDH.**

**Cytochrome $c_L$ dissociates, and then passes electrons to cytochrome $c_H$.**

**Cytochrome $c_H$ is almost a typical cytochrome c, passing its electrons to the oxidase**

**Claude Chan, Darren Day, Jon Cox, Jim Elliott, Simon Dales**




Research students
(Graduated 1990)
Darren Day
Claude Chan
Ian Richardson
Tony Long
Jon Cox
Darren

*Biochem. J.* (1980) **192**, 411–419
*Printed in Great Britain*

# The two cytochromes *c* in the facultative methylotroph *Pseudomonas* AM1

David T. O'KEEFFE and Christopher ANTHONY
*Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.*

Cytochrome $c_H$ High isoelectric point [basic, +ve charge at pH 7; *Mr* = c 10,000

Cytochrome $c_L$ Low isoelectric point [acidic –ve charge at pH 7; *Mr* = c 20,000

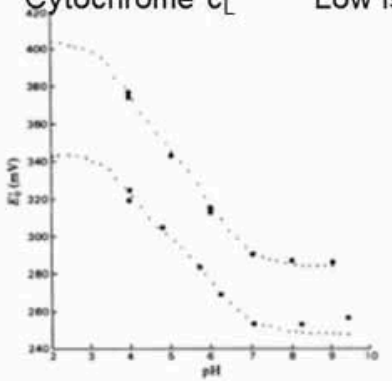



Fig. 5. *pH-dependence of the midpoint redox potentials of cytochromes $c_H$ and $c_L$*

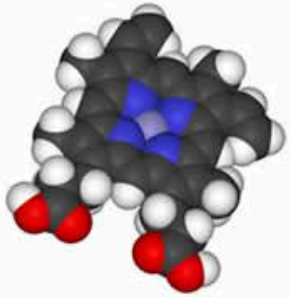
Details of the methods are given in the Materials and methods section. Oxidative and reductive titrations gave identical results. ●, Midpoint potentials of cytochrome $c_H$; ■, midpoint potentials of cytochrome $c_L$. The dotted lines are theoretical curves calculated from the equation:

$$E_h' = E_0 + 59\log\left(\frac{[H^+]^2 + K_{o1}[H^+] + K_{o1}K_{o2}}{[H^+]^2 + K_{r1}[H^+] + K_{r1}K_{r2}}\right)$$

where $K_{o1}$ and $K_{o2}$ are the dissociation constants for the first and second ionizations in the oxidized species and $K_{r1}$ and $K_{r2}$ are the equivalent dissociation constants for the reduced species. The p$K'$ (−log $K$) and $E_0$ values assumed for calculation of the theoretical curves are given in Table 3.

There were four ionizations affecting the redox potentials in the pH range studied (pH 4.0-9.5), two in the oxidized form (pK values about 3.5 and 5.5) and two in the reduced form (pK values about 4.5 and 6.5),

suggesting that the ionizing groups involved may be the two propionate side chains of the haem.



This suggests the propionates are very unusual in being exposed to solvent and not buried within the cytochrome structure as with other cytochromes

**Fig. 1.** The sequence of cytochrome $c_H$ of *M. extorquens*.

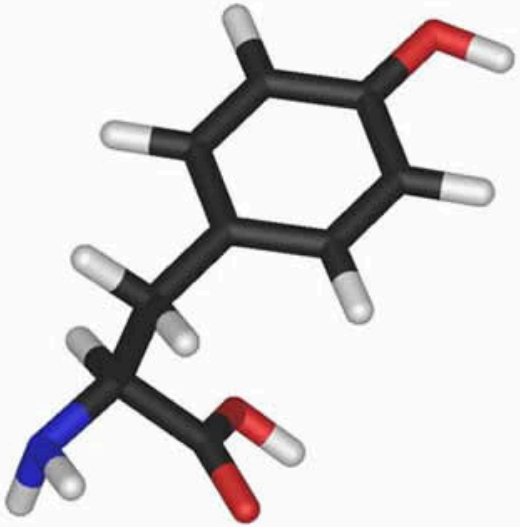
```
                    -5    1         10        20        30        40          50
Yeast                TEFKAGSAKKGATLFKTRCLQCHTVEKGGPHKVGPNLHGIFGRHSGQAE-GYSTYTDA
Horse                     GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAP-GF-TYTDA
Tuna                      GDVAKGKKTFVQKCAQCHTVENGGKHKVGPNLWGLFGRKTGQAE-GY-SYTDA
                        1         10        20        30        40
Methylobacterium          EGDAAAGEKAFA-PCKACHNFEKNG---VGPTLKGVVGAKAGEGADGYA-FSDA

                                  60        70                  80        90          112
Yeast                NIK---KNVLWDENNMSEYLTNP-KYIPG--------TKMAFAGLKKEKDRNDITYLKKACE
Horse                NKN---KGITWKEETLMEYLENPKKYIPG--------TKMIFAGIKKKTEREDLIAYLKKATNE
Tuna                 NKS---KGIVWNENTLMEYLENPKKYIPG--------TKMIFAGIKKKGERQDLVAYLKSATS
                     50            60        70                  80        90          100
Methylobacterium     LKK---SGLTWDQADLKQWLADPKKKVPG--------TKMVFPGISDPKKVDDIIAYLKTKS
```

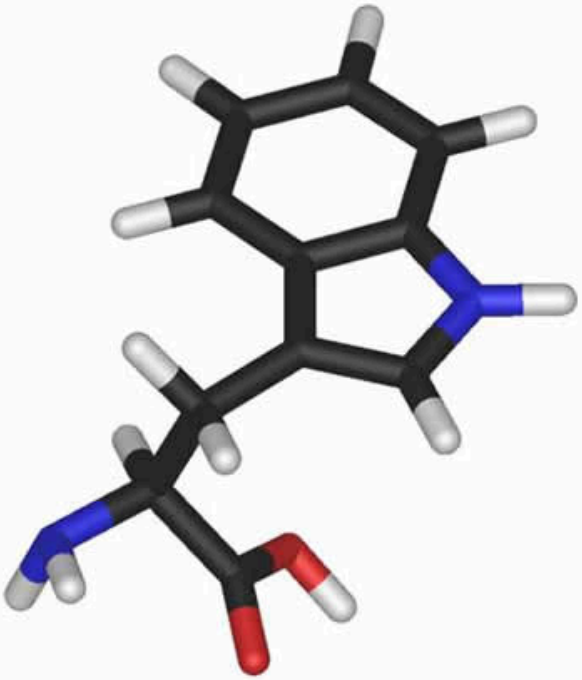
About 50% identity with sequence of typical cytochromes c

Almost all 'conserved residues' are present

Residues in red box are always Tyrosine
(100% conservation in 160 sequences).
But replaced by tryptophan in cytochrome $c_H$



Tyrosine



Tryptophan

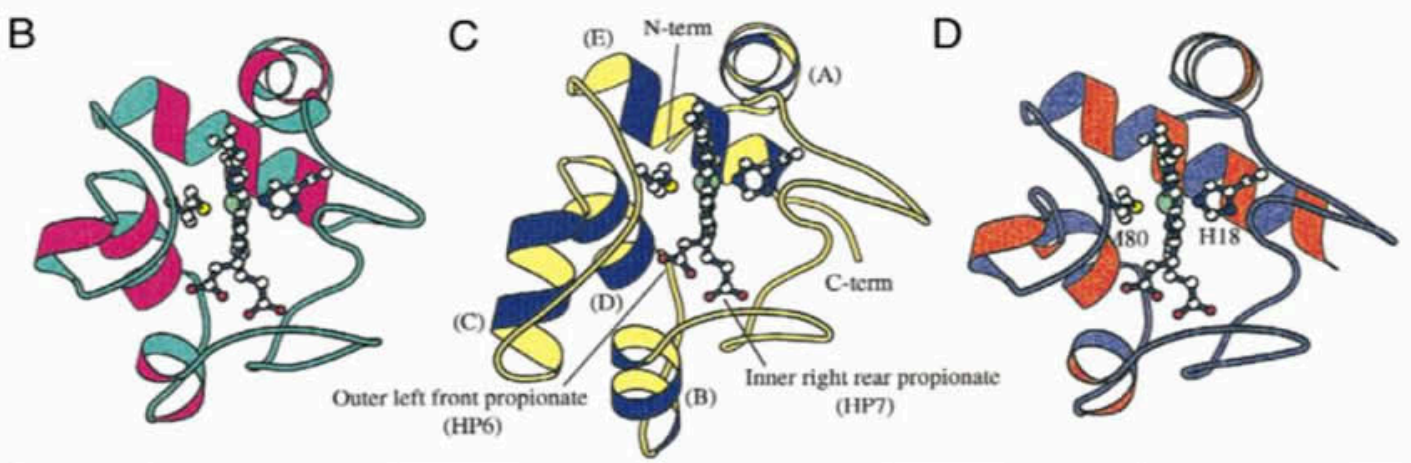
# The molecular structure of an unusual cytochrome $c_2$ determined at 2.0 Å; the cytochrome $c_H$ from *Methylobacterium extorquens*

JON READ, RAJ GILL, SIMON L. DALES, JON B. COOPER, STEVE P. WOOD, AND CHRIS ANTHONY

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, United Kingdom

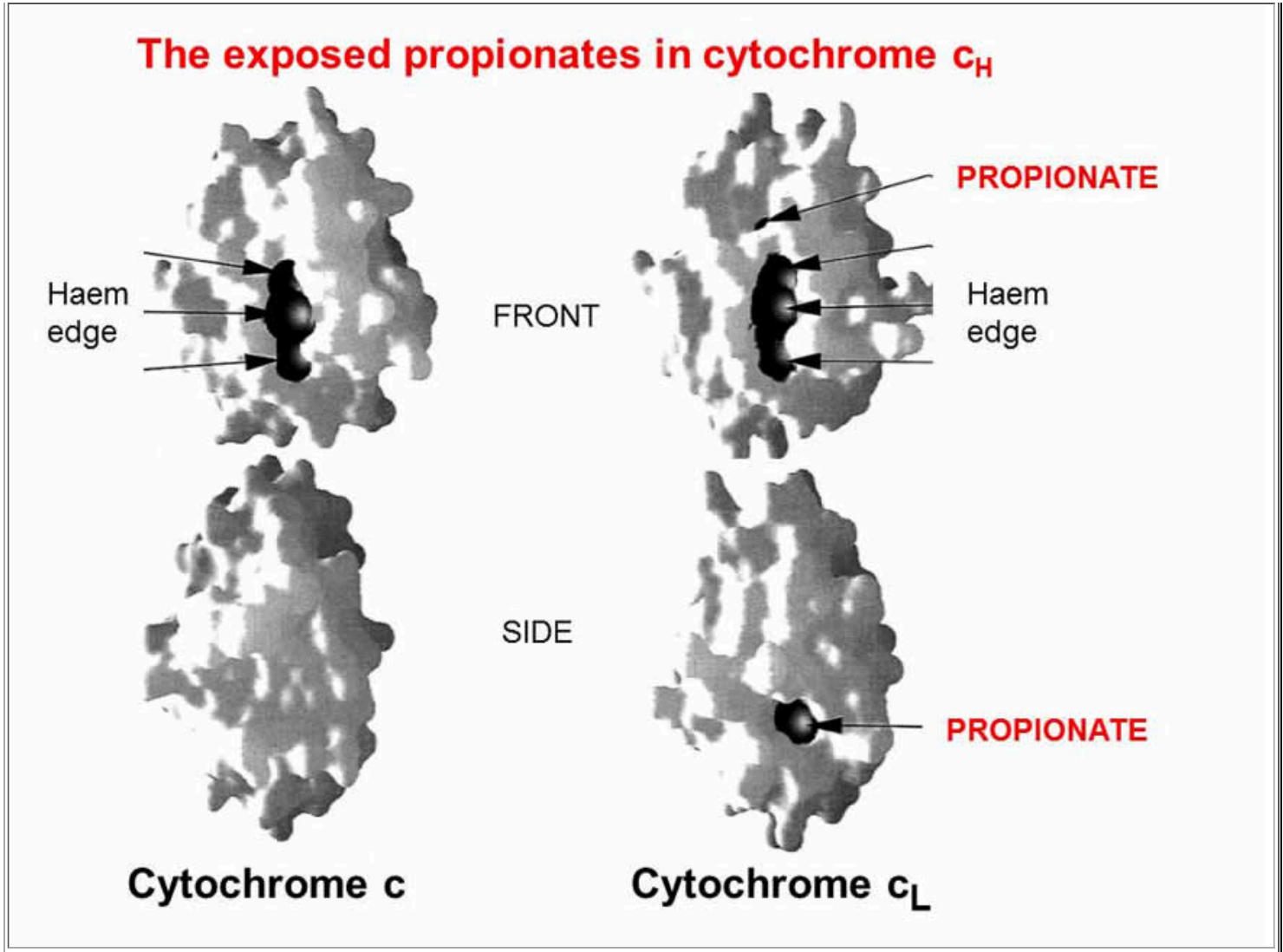
*Protein Science* (1999), *8*:1232–1240.

The overall folds of cytochrome $c_H$, cytochrome $c$, and cytochrome $c_2$.

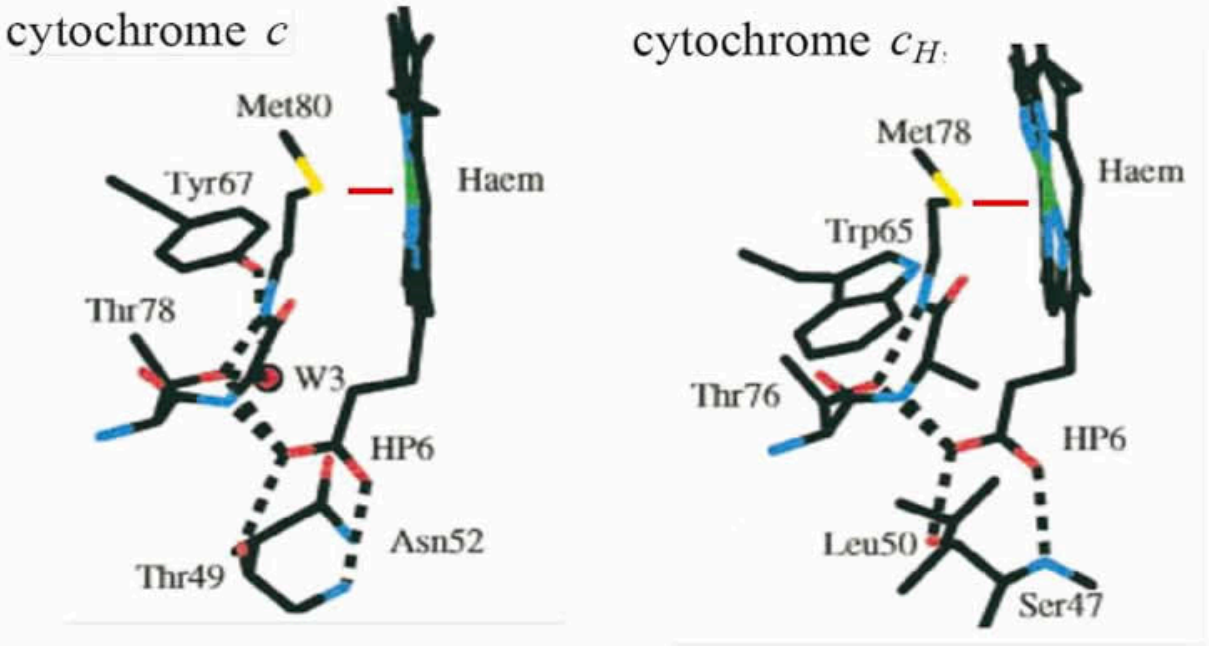



cytochrome $c_H$ cytochrome $c_2$ cytochrome $c$

**Cytochrome $c_H$ is unique in being a bacterial cytochrome c that is folded into a mitochondrial cytochrome c structure**


The exposed propionates in cytochrome $c_H$
Haem edge
FRONT
PROPIONATE
Haem edge
SIDE
PROPIONATE
Cytochrome c
Cytochrome $c_L$

## The LHS of the haem cleft in the cytochromes c




The'absolutely conserved' Tyr67 is replaced by tryptophan

The 'conserved' water is absent; the LHS of haem cleft is hydrophobic

**This shows that the previously proposed mechanism of cytochrome c, involving the' essential water' is no longer valid.**

# The 1.6 Å X-ray Structure of the Unusual *c*-type Cytochrome, Cytochrome $c_L$, from the Methylotrophic Bacterium *Methylobacterium extorquens*

**Paul Williams[1], Leighton Coates[1], Fiyaz Mohammed[1], Raj Gill[1]
Peter Erskine[1], Dominique Bourgeois[2], Steve P. Wood[1]
Chris Anthony[1] and Jonathan B. Cooper[1]***

[1]*School of Biological Sciences
University of Southampton
Bassett Crescent East
Southampton SO16 7PX*

*J. Mol. Biol.* (2006) **357**, 151–162

172 residues instead of usual 100. Has 40-50 extra residues at N terminus

and a C-terminal extension

Negligible sequence identity to other cytochromes c.

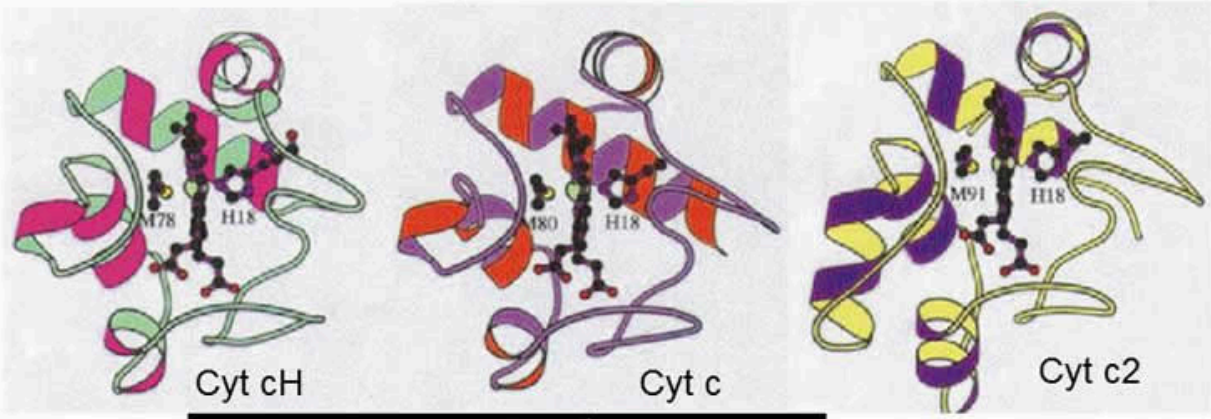
## Predictions:

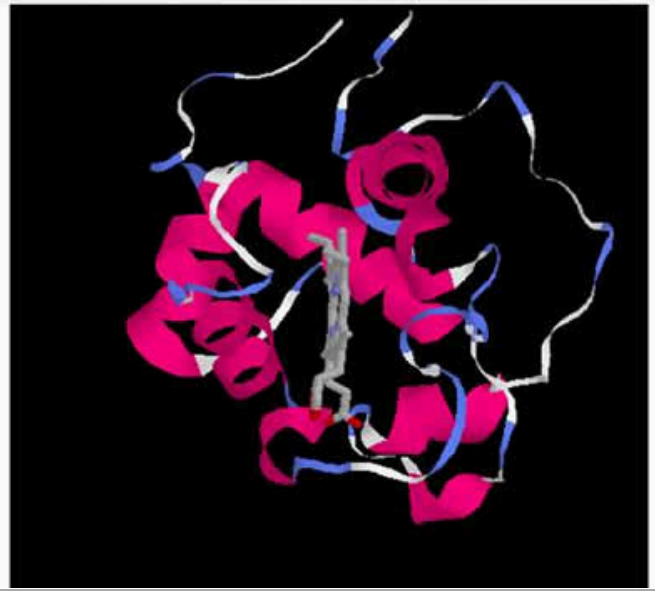
The haem propionates would be exposed to solvent

The loop bearing the 6th ligand methionine might be unusually flexible

The two very unusual cysteines would be involved in a disulphide bridge to hold the long loops together in the structure

# The structure of cytochrome $c_L$



172 amino acids

No sequence similarity

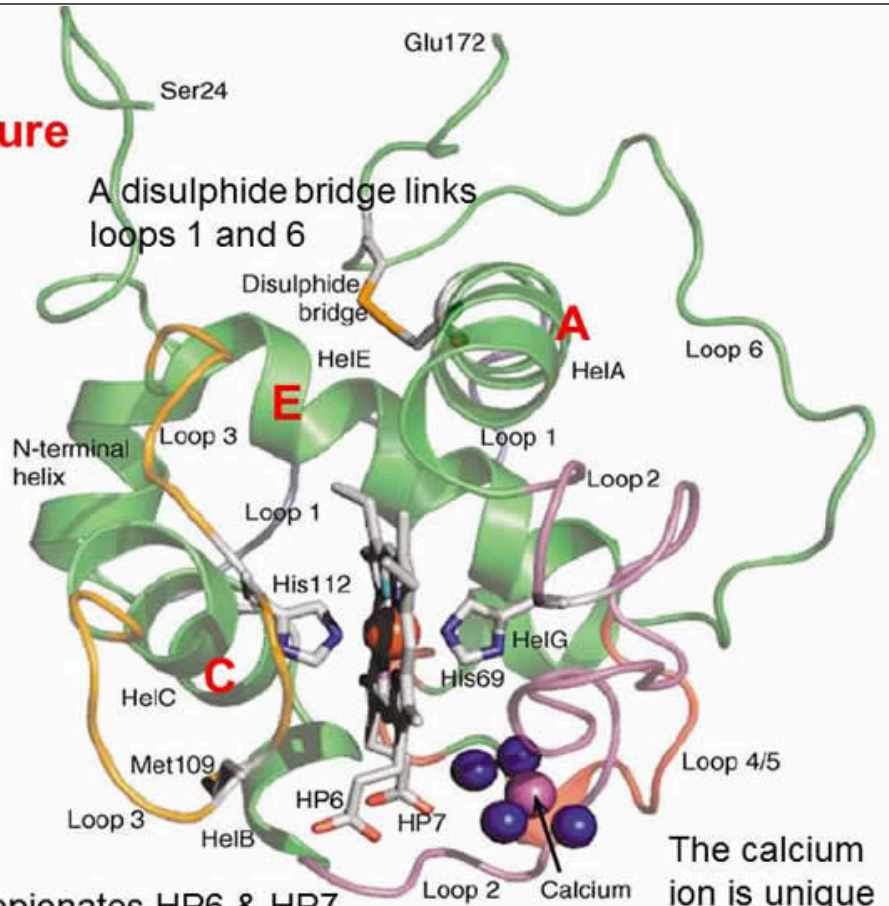
Similar basic structure with additional features

Paul Williams

# Cytochrome $c_L$ structure

**Helices A, C and E are usual haem-enclosing helices**

His112 has replaced Met 109 as ligand to haem iron; Loop 3 is relatively flexible in this cytochrome leading to reaction with CO



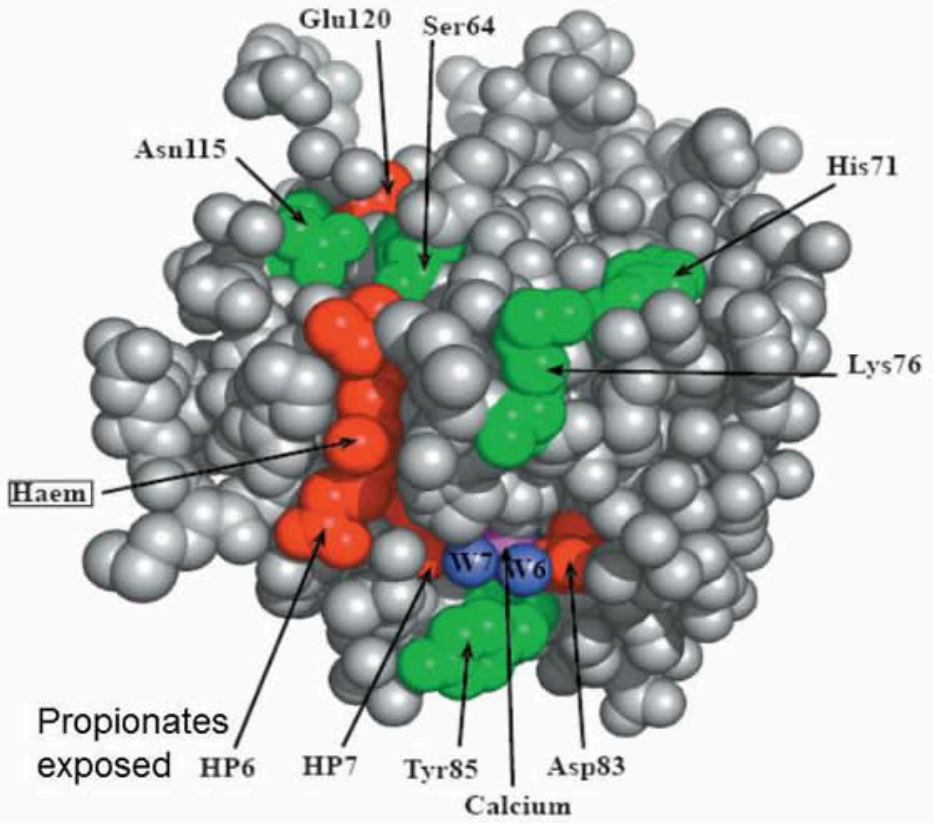
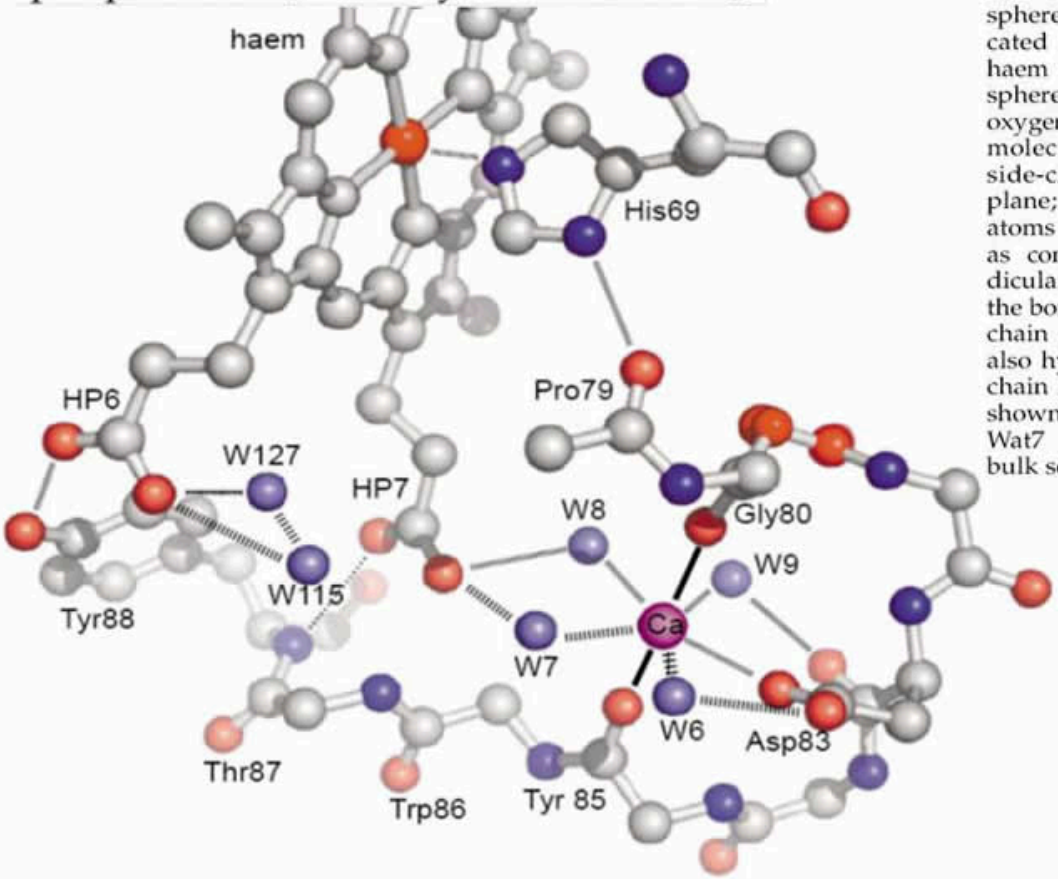



Figure 4. Surface view of cytochrome cL. The outer haem propionate (HP6) is clearly exposed to solvent as are the two waters (W6 and W7). W7 is bonded to the inner haem propionate (HP7). Asp83, one of the ligands to the Ca2+ ion, has been proposed to be involved in interaction with methanol dehydrogenase together with Glu120, the outer haem propionate (HP6) and those residues coloured green20.

**Figure 4.** The calcium ion and the interactions of the two haem propionates, in cytochrome $c_L$.




HP6 is the outer haem propionate and HP7 is the inner haem propionate. The lines indicate hydrogen bonds or coordinations. Water molecules (Wat6–Wat9, Wat115, and Wat127) are indicated by blue spheres; the calcium ion is indicated by a magenta sphere; the haem iron is indicated by a red sphere. The calcium ion and the oxygen atoms of the four water molecules (Wat6–Wat9) and the side-chain of Asp83 lie in one plane; the bonds to the oxygen atoms of Gly80 and Tyr85 (shown as continuous lines) are perpendicular to this plane. In addition to the bonds indicated here, the main-chain carbonyl group of Asp83 is also hydrogen bonded to the side-chain nitrogen atom of Trp86, (not shown). The water molecules Wat6, Wat7 and Wat115 are exposed to bulk solvent (Figure 7).

Two unusual c-type cytochromes are involved in methanol oxidation pathway

## Cytochrome $c_H$

Electron donor to the oxidase; Same sequence and function as typical cytochrome c

Typical cytochrome c structure except the folds are like mitochondrial (not bacterial) cytochrome

The 'conserved' tyrosine and water are absent; the hydrophobic haem cleft shows the water is not important in cytochrome mechanism

The propionates are exposed to solvent

## Cytochrome $c_L$

Completely novel cytochrome. Although the 3-helix core is same as other cytochromes, there is negligible sequence identity and it is unique in lacking one of the 7 conserved positions found in all families of cytochromes c [haem binding and residues that hold 3 helices together in position]

A calcium ion replaces the 'conserved' arginine in balancing propionate charge

As predicted the haem propionates are exposed and there is a novel disulphide bridge

The two cytochromes, in the same organism and the same electron transport chain seem to have evolved completely independently

Acknowledgements:

David O'Keeffe, Mathew Beardmore-Gray, Sandy Cross, Jim Elliott, Bill Keevil, Sasha Netrusov, Claude Chan, Darren Day, Jon Cox, Simon Dales, Karen Amaratunga, Paul Skipp, Tosin Majekodunmi, Remi Afolabi,

Jon Read, Raj Gill, Jon B. Cooper, Steve P. Wood,
Paul Williams, Leighton Coates, Fiyaz Mohammed, Raj Gill, Peter Erskine, Dominique Bourgeois,

Prof Akhtar for encouragement ("We are not interested in cytochromes, are we?")

Top of cytochromes

Top of summaries

The Methylotrophs PPT Top of summaries Return to History

# The Methylotrophs

They use compounds with one or more carbon atoms but no C-C bonds as their sole source of carbon and energy: Methane, Methanol , methylamine, dimethylamine.

Those that use methane are also called **Methanotrophs** (eg. *Methylococcus, Methylosinus*)

**Obligate methylotrophs** cannot grow on multicarbon compounds (include most methanotrophs)
**Facultative methylotrophs** also grow on multicarbon compounds (eg. *Methylobacterium extorquens* = *Pseudomonas* AM1
**Restricted facultative methylotrophs** grow on C1 or C2 compounds (eg. *Hyphomicrobium*)
**Facultative autotrophs** grow on carbon dioxide, plus hydrogen or methanol by way of RuBP path (eg Paracoccus)

**There is a huge number of different types of methylotroph. Very few are typical bacteria that had been described previously. Although their oxidative metabolism is basically similar, there are many different carbon assimilation pathways. Most are Gram-negative. The few Gram positive methylotrophs (Bacillus sp) have different oxidation enzymes**

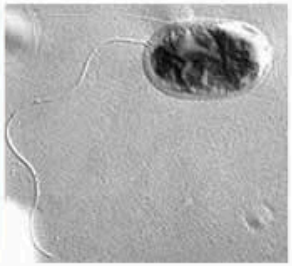
1906 Sohngen. Bacillus methanicus growing on methane
1961 Peel and Quayle. *Pseudomonas* AM1 (cited only 6 papers) A pink facultative methyltroph
1964 Anthony and Zatman. Description of Methanol dehydrogenase in Pseudomonas M27 (=AM1)
1964 Hirsch and Conti. *Hyphomicrobium*. A stalked facultative methylotroph
1970 Whittenbury (with Wilkinson). 100 new methanotrophs (2 Types)
1971 Ribbons Early work on methane oxidation
1970- Ogata, Sahm, Hazeu, van Dijken, Harder. Methylotrophic yeast.
1980 Dalton Definitive work on methane oxidation
1982 Anthony book. Cited about 1200 methylotroph references

## The main 'workhorses' in the study of methylotrophs

- *Pseudomonas* AM1 (Peel & Quayle, 1961)
- *Pseudomonas* sp. M27 (Anthony & Zatman, 1964)

***Methylobacterium extorquens***. A pink facultative methylotroph





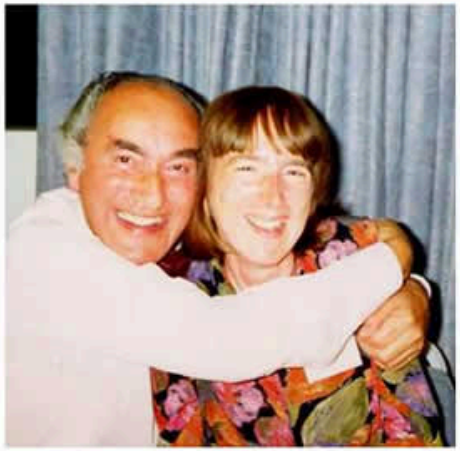




Me in 1963



J Rod Quayle



My PhD supervisor and my first research student (Len Zatman and Pat Dunstan, now Goodwin)




Howard Dalton, Rod and me

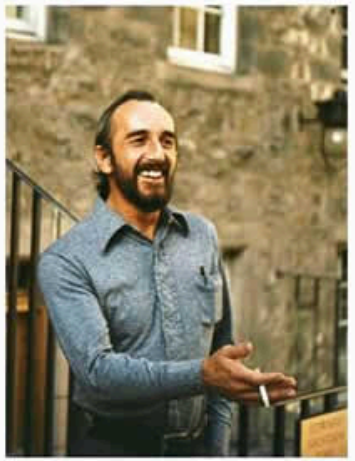
***Methylococcus capsulatum*** (Type I obligate methanotroph)
***Methylosinus trichosporium*** (Type II obligate methanotroph)





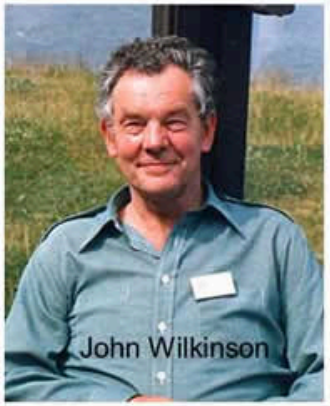
Type I membranes

Type II membranes

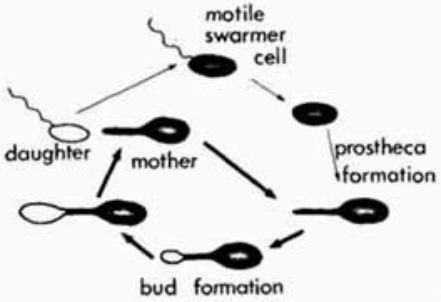


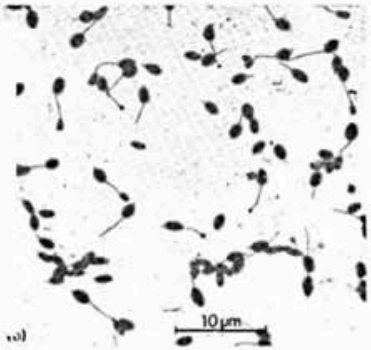
Doug Ribbons 1973



## *Hyphomicrobium*






Hans van Dijken and Wim Harder 1973 *Hyphomicrobium,* Yeast and Growth yields of methylotrophs

**Sticks to surfaces; grows only on C1 and C2 compounds. Reproduce by budding**

**Grow on methanol and methylated amines but not methane**

Hirsch & Conti, Harder, Dow, Attwood.
Quayle (assimilation pathways)

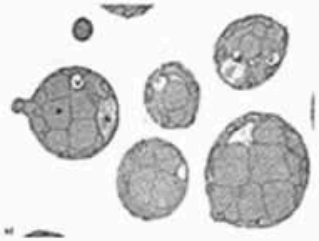
## Methylotrophic Yeasts

By contrast with bacteria, the methylotrophic yeasts are not a special group; they are in the usual genera of yeasts [*Hansenula, Candida, Pichia and Torulopsis*]. Only 7/39 genera grow methylotrophically. They grow only on methanol (not methylamines or methane). Again, by contrast with bacteria they all share the same assimilation pathways.

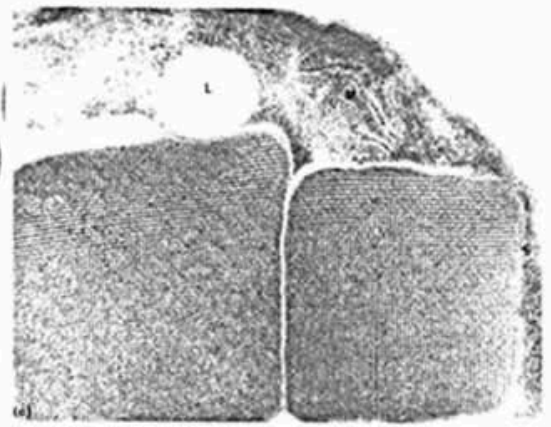
They oxidise methanol by a flavoprotein alcohol oxidase coupled to catalase which is present in crystalline form in peroxisomes and which yields no energy. Almost all bacteria use an energy-yielding quinoprotein methanol dehydrogenase. all

1969-1975
Ogata, Tani, Sahm, van Dijken, Harder, Quayle (assimilation)

**Peroxisomes in methanol-oxidising yeast**



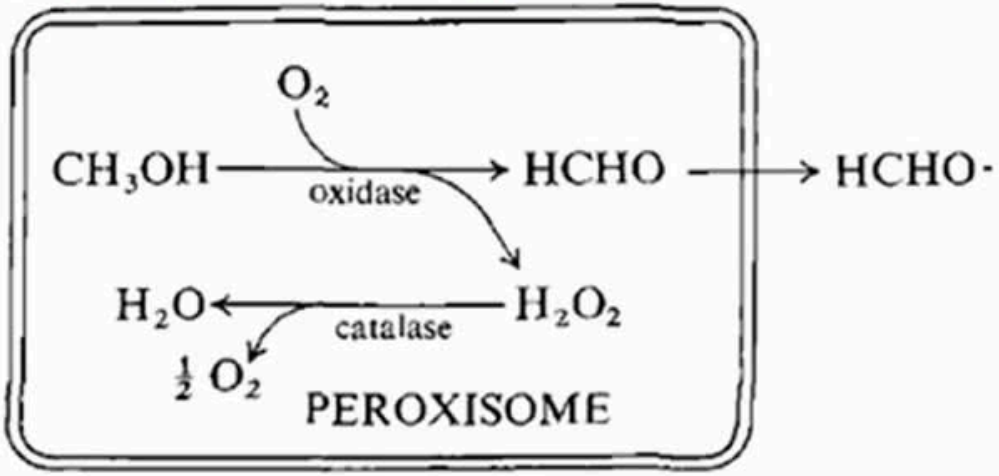
The oxidase is produced in very large amounts and transcription of the gene has a very strong promoter; it is now used in genetic engineering for production of large amounts of cloned Eucaryotic proteins.



Crystals of the flavoprotein alcohol oxidase in yeast

## Methanol oxidation in methylotrophic yeast

This is catalysed by non-specific flavoprotein called alcohol oxidase
This is in a peroxidsome with catalase




The enzyme is used as a source of hydrogen peroxide –
for wound disinfection and also used in washing powder



J. Guis Kuenen and Professor Kato (Tottori and Kyoto




Friends from Yamaguchi, Japan: Matsushita, Uragami (?), Ameyama, Adachi

# The main 'groups' of bacterial methylotrophs

**Methanotrophs.** Vast majority are obligate. (Recent important exceptions)
All have internal membranes and produce spores. Gram negative.
Type I (eg *Methylococcus, Methylomonas*) use RuMP pathway
Type II (eg *Methylosinus, Methylocystis*) use Serine pathway

**Methylotrophs not using methane.** None have internal membranes or produce spores. Vast majority are Gram negative.

Obligate methylotrophs Grow on methanol and/or methylamine. Gram negative, motile, non-sporing. Use RuMP pathway. Eg *Methylophilus*

Facultative methylotrophs. Usually grow on methanol/methylamine; use serine pathway

Pink facultative methylotrophs. Motile, single polar flagellum. Red carotenoids similar to photosynthetic bacteria. Wide range of C substrates but few carbohydrates. M*ethylobacterium*

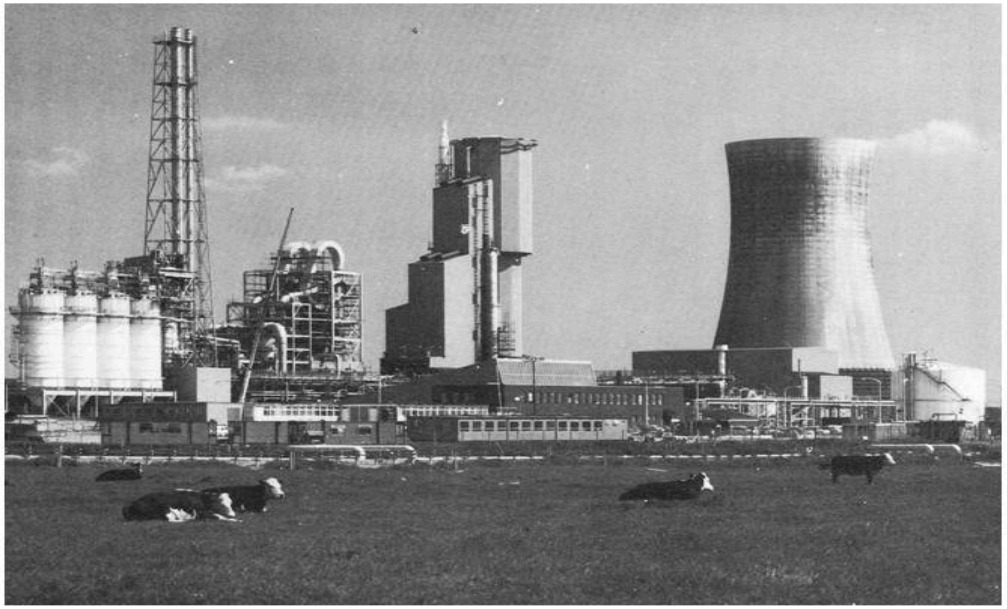
Non-pigmented facultative 'pseudomonads'. No typical pseudomonas sp use C1 compounds. *Pseudomonas aminovorans.* Use serine pathway ($icl^+$ variant)

Gram-negative or variable non-motile rods. Some only use methylated amines. A mixed group. Eg *Arthrobacter.*

Facultative autotrophs or phototrophs Use RuBP pathway (Calvin cycle). *Paracoccus denitrificans* (can use hydrogen); *Rhodopseudomonas*

Gram positive facultative methylotrophs Very rare. *Bacillus, Streptomyces*
*Hyphomicrobia* Restricted facultative methylotrophs. Stalked. Budding. Serine pathway

## The ICI ‘Pruteen’ plant at Billingham, UK 1980



The centre tower is the 1.5 million litre fermenter

It contains about 100,000,000,000,000,000,000 methylotrophs
10% of their soluble protein is Methanol dehydrogenase

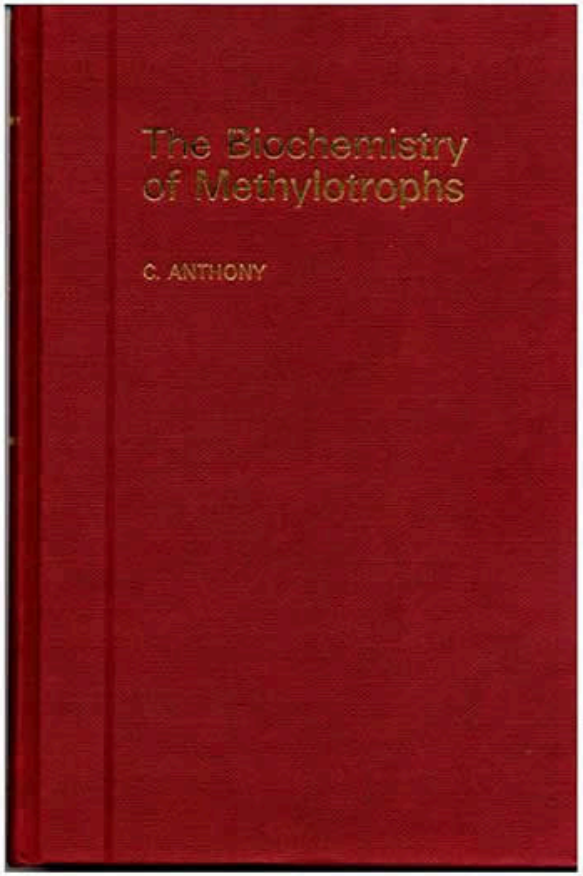
The Methylotrophs and their biochemistry as was known up to 1981 is covered comprehensively in
The Biochemistry of Methylotrophs by Cbris Anthony

It can be downloaded complete and free as a pdf file from my website

www.chris-anthony.co.uk

On this page: http://www.chris-anthony.co.uk/methylotrophs.html

# The Biochemistry of Methylotrophs 1982




The Biochemistry of Methylotrophs

C. ANTHONY

*Department of Biochemistry*
*University of Southampton*
*England*

**Chapters 12**
**Text pages 350**
**Index 48 pages**
**Tables 60**
**Figures 57**
**References 1300**

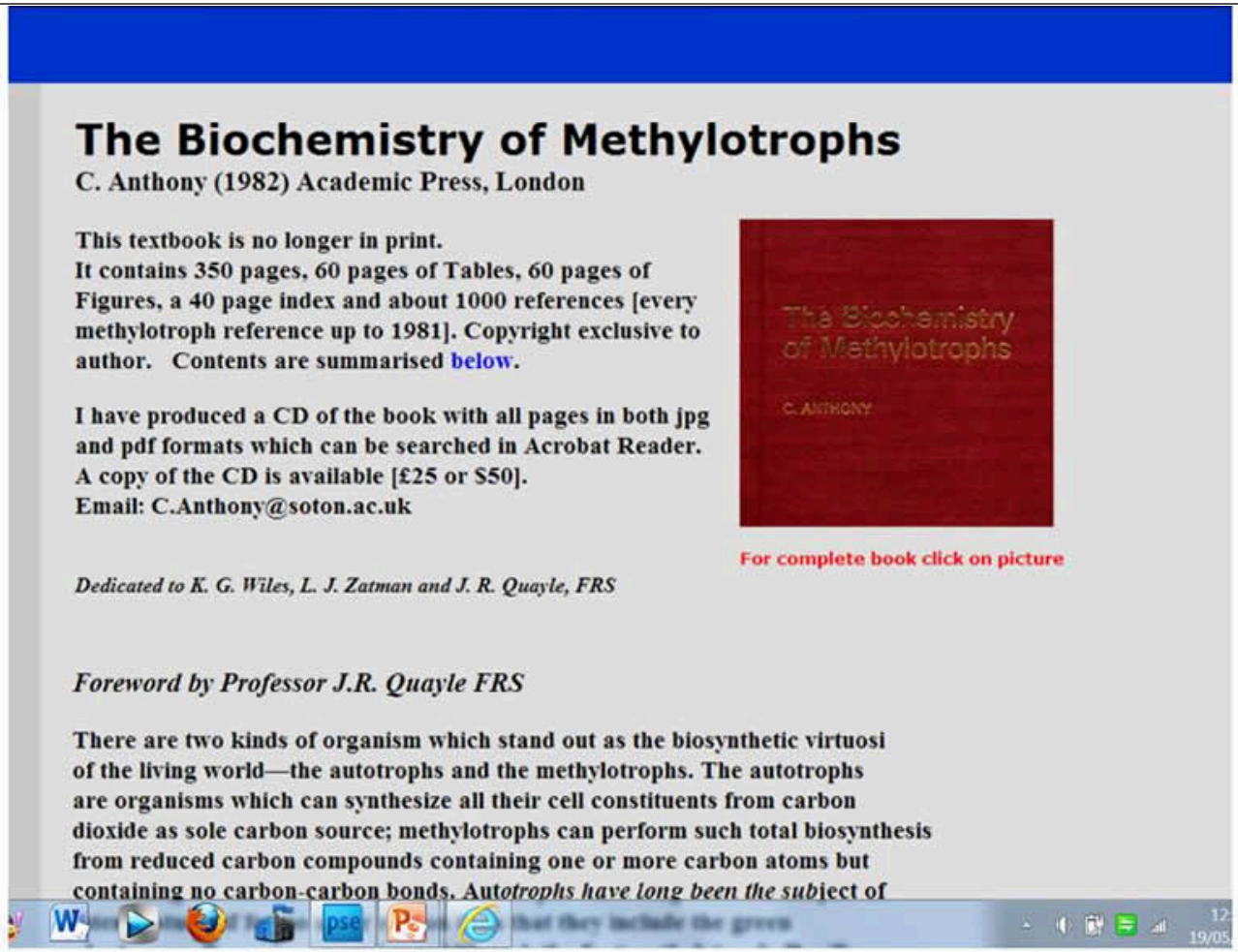
1982

ACADEMIC PRESS
*A Subsidiary of Harcourt Brace Jovanovich, Publishers*

London · New York
Paris · San Diego · San Francisco
São Paulo · Sydney · Tokyo · Toronto

Methylotrophic bacteria
Ribulose bisphosphate pathway
Ribulose monophosphate cycle
The Serine pathway
The TCA cycle and growth on multicarbon compounds
Oxidation of methane, methanol, formaldehyde and formate
Oxidation of methylated amines
Electron transport and energy transduction
Growth yields and bioenergetics
Methylotrophic yeasts
Methanogens and methanogenesis
Commercial exploitation

A CD version is available and the complete book is on my website: chris-anthony.co.uk




Click on book ot download it complete (free). Or ask for CD (free)

Top of Methylotrophs
Top of Summaries

Growth yields of Methylotrophs PPT Top of Summaries

# Factors affecting growth yields in methylotrophs

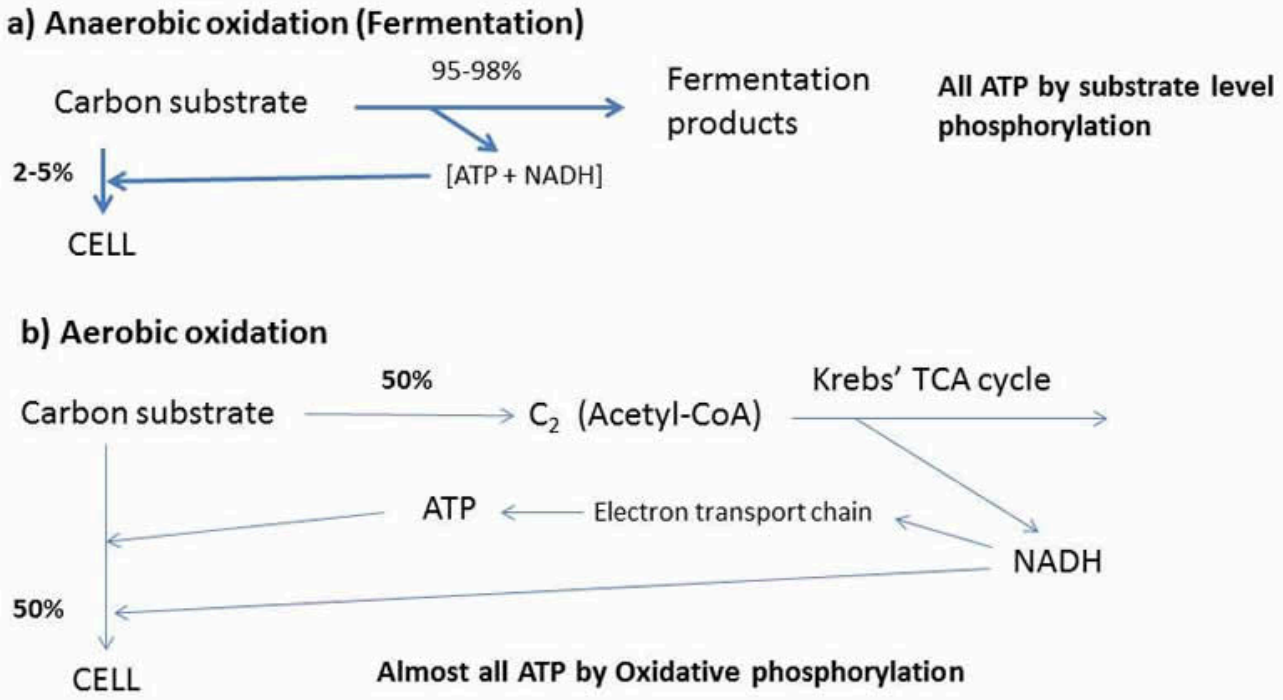
For growth on a simple defined medium with a single source of carbon:
Growth yield is Grams dry weight cells / moles of growth substrate consumed
Ys = g/mole
The substrate is consumed for 2 main purposes: production of new cells plus provision of energy (ATP and NADH) for the necessary biosynthesis.

**In anaerobes:** At least 95% of the substrate is used for energy (mainly ATP) production. This is produced by fermentation where ATP is produced by substrate level phosphorylation.
The yield is determined by the number of ATPs produced per mole of substrate.

**In conventional aerobes:** About 50% of the carbon substrate is used for energy production. This substrate is oxidised completely to $CO_2$ and the ATP is produced by oxidative phosphorylation. *******
Yields are expressed as **Ys** = g/mole substrate used and $\mathbf{Y_{O2}}$ = g/mole of $O_2$
Yields are dependent on the P/O ratio = moles ATP produced / atom of oxygen consumed during oxidative phosphorylation. = moles ATP / mole of NADH oxidised
Yield per mole of ATP consumed is $Y_{ATP}$ = g/mole ATP

This is often (justifiably) considered to be a constant.
This assumes that the ATP requirement for transport and biosynthesis is similar for all substrates.

# Oxidation of organic carbon substrates




**Note:** methylotrophs are special as the substrates are oxidised directly to $CO_2$

********

Yield per mole of ATP consumed is $Y_{ATP}$ = g/mole ATP

This is often (justifiably) considered to be a constant. It assumes that the ATP requirement for transport and biosynthesis is similar for all substrates and that the growth yield is directly proportional to the ATP available. Such microbes (the vast majority) are said to be ATP-limited

**Growth yield predictions and measurements in methylotrophs** illuminate those aspects of their biochemistry and physiology that make them special. It is important when considering C1 compounds as a substrate for methylotrophs as a source of Single Cell Protein, SCP. Also when considering biomass production in ecological studies.

Most multicarbon substrates are at the level of oxidation of $CH_2O$ [glucose, formaldehyde etc]

It might be assumed that more reduced substrates (hydrocarbons, long chain fatty acids, methane, methanol, methylamine), having more energy would give high yields. **Not true**.
To be true then all of this energy must be harnessed as NADH and ATP during oxidation.

**The Truth:** Alkanes are chemically inert and use energy (as NADH) in the initial hydroxylation step. So they are equivalent to $CH_2O$.

The oxidation of methanol and methylamine use unusual enzymes that are not NAD-linked and yield relatively little ATP in their electron transport chains.

## The prediction of growth yields

a) Substrate is converted to a central precursor (eg phosphoglycerate, PGA). This also produces some NADH and ATP

b) PGA is biosynthesised into cell material. This requires NADH and ATP

c) More substrate is oxidised to produce the NADH for this biosynthesis

d) More substrate is oxidised to produce ATP for biosynthesis

This can be expressed in equations which lead to an overall Assimilation equation. This can be used to investigate the effects of different assimilation pathways and energy production systems.

a) Calculations for a typical multicarbon substrate, glucose
b) The results of similar equations for methylotrophs

Much of the following is taken direct from a paper on prediction of growth yields and a chapter in *The Biochemistry of Methylotrophs*

*Assumptions: the constituents of most cells are similar as are the pathways for their biosynthesis. It is assumed that the nitrogen source is ammonia.*

## The Assimilation equation for glucose

The assumptions listed above lead to the following equation for assimilation of phosphoglycerate (PGA) into cell material (in these equations $H^+$, $H_2O$, $NAD^+$, ADP and Pi are omitted for clarity):

$$4\ PGA + 29\ ATP + 5{\cdot}5\ NADH + 3\ NH_3 \longrightarrow 306g\ cells\ (C_4H_8O_2N)_3$$

By taking into consideration the biosynthesis of PGA from growth substrate, an overall assimilation equation may be derived.

For example, if the formula for glucose transport and metabolism to PGA is:

$$glucose + ATP \longrightarrow 2\ PGA + 2\ NADH$$

then the assimilation equation for glucose will be:

$$2\ glucose + 31\ ATP + 1{\cdot}5\ NADH + 3NH_3 \longrightarrow 306g\ cell\ material$$

The NADH is produced by oxidising glucose to $CO_2$ ($FPH_2$ is reduced succinate dehydrogenase):

$$0{\cdot}15\ glucose \longrightarrow 0{\cdot}9\ CO_2 + 1{\cdot}5\ NADH + 0{\cdot}6\ ATP + 0{\cdot}3\ FPH_2$$

By adding the last two equations, the overall assimilation equation (simplified) for predicting the effect of varying P/O ratios on growth yields on glucose is obtained:

$$2{\cdot}15\ glucose + 30{\cdot}6\ ATP \longrightarrow 306g\ cell + 0{\cdot}9\ CO_2 + 0{\cdot}3\ FPH_2$$

## Glucose assimilation equation

$$2{\cdot}15 \text{ glucose} + 30{\cdot}6 \text{ ATP} \longrightarrow 306\text{g cell} + 0{\cdot}9\ CO_2 + 0{\cdot}3\ FPH_2$$

If glucose is metabolised by glycolysis and TCA cycle, and oxidation of NADH yields 3ATP then 1 glucose yields 38 ATP and 6 $CO_2$. So 0.8 glucose must be oxidised to give the ATP and also 4.8 $CO_2$

**SO yield equation:** 2.95 glucose gives 306 g cell + 5.7 $CO_2$ Yield = 104g/mole glucose

If the P/O ratio is only 2 instead of 3 only get 2ATP per mole NADH
For provision of 30.6 ATP need to oxidise 1.2 glucose, producing 7.2 $CO_2$
**SO yield equation:** 3.35 glucose gives 306 cell + 8.1 $CO_2$ Yield = 91 g /mole glucose

If P/O ratio is only 1 then 2.4 moles glucose is needed for ATP
**Yield equation** 4.55 glucose gives 306g cell + 15.3 $CO_2$ Yield = 67g / mole glucose

Carbon conversion efficiency if P/O ratio is 3 is 306 g/540g glucose = 57%
If P/O ratio is only 2 then CCE is 51%
If P/O ratio is only 1 then CCE is 37%

Bacteria growing on glucose are ATP- limited

## Prediction of growth yields in methylotrophs

**a) Substrate is converted to a central precursor (eg phosphoglycerate, PGA).** This **usually** produces some NADH and ATP with multicarbon substrates

In methylotrophs this first part involves initial oxidation to formaldehyde. With methane this **uses** oxygen and NADH to oxidise the methane to methanol. Oxidation of Methanol (and methylamine) to formaldehyde does not produce NADH but only reduced quinoprotein dehydrogenases that yield only 1ATP (or less) during their oxidation.

Note: The special pathways for conversion of formaldehyde to PGA sometimes consume a lot of NADH (RuBP and Serine pathways)

**b) PGA is biosynthesised into cell material.** This requires NADH and ATP as in all bacteria

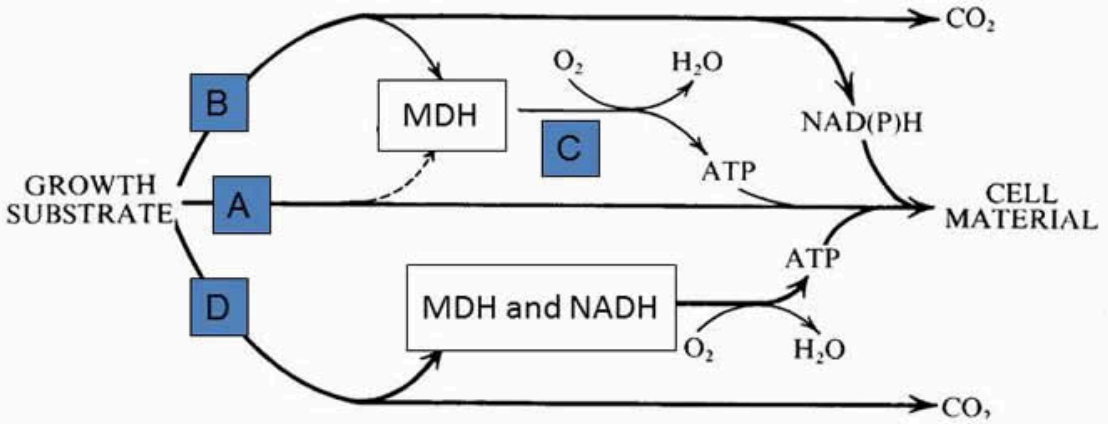
**c) More substrate is oxidised to produce the NADH** for this biosynthesis.
Again there is a problem: The first step in methane oxidation **uses** NADH. The oxidation of methanol and methylamine to formaldehyde produces no NADH. This is only produced during formaldehyde oxidation (not always) and formate oxidation (essential).

$$CH_4 \longrightarrow CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$$

**d) More substrate is oxidised to produce ATP for biosynthesis**
During oxidation of methanol or methylamine to provide NADH the reduced enzymes were re-oxidised by the electron transport chains, giving ATP. Because of this it is often the case that no substrate needs to be oxidised for this final provision of ATP

# NADH limitation and growth yields

Anthony, 1978




A: MeOH is oxidised to HCHO and assimilated into CELL using ATP and NADH.

B: MeOH is oxidised to provide NADH for assimilation pathway.

C: The reduced MDH produced in A and B is oxidised to give ATP.

D: MeOH is oxidised to produce any ATP that is still required.

During oxidation of methanol so much ATP is produced in step C that little further ATP production is required. The cells are NADH-limited instead of ATP-limited. The growth yield is not dependent on the ATP yield from methanol dehydrogenase.

On methane: the first step requires NADH and so the effect is even more marked.

## Assimilation equations for the three main methylotroph pathways

Ribulose bisphosphate pathway:

$$12CO_2 + 25{\cdot}5NADH + 61ATP + 3NH_3 \rightarrow 306\text{ g cell} + 18H_2O$$

Ribulose monophosphate pathway:

FBP variant: $12HCHO + 1{\cdot}5NADH + 29ATP + 3NH_3 \rightarrow 306\text{ g cell} + 6H_2O$

ED variant: $12HCHO + 1{\cdot}5NADH + 37ATP + 3NH_3 \rightarrow 306\text{ g cell} + 6H_2O$

Serine pathway:

$icl^+$ variant: $8HCHO + 13{\cdot}5NADH + 41ATP + 4CO_2 + 3NH_3 \rightarrow 306\text{ g cell} + 4FPH_2 + 10H_2O$

$icl^-$ variant: $8HCHO + 17{\cdot}5NADH + 41ATP + 4CO_2 + 3NH_3 + 4O_2 \rightarrow 306\text{ g cell} + 18H_2O$

RuBP and Serine pathways need a lot of NADH; RuMP pathway needs relatively little

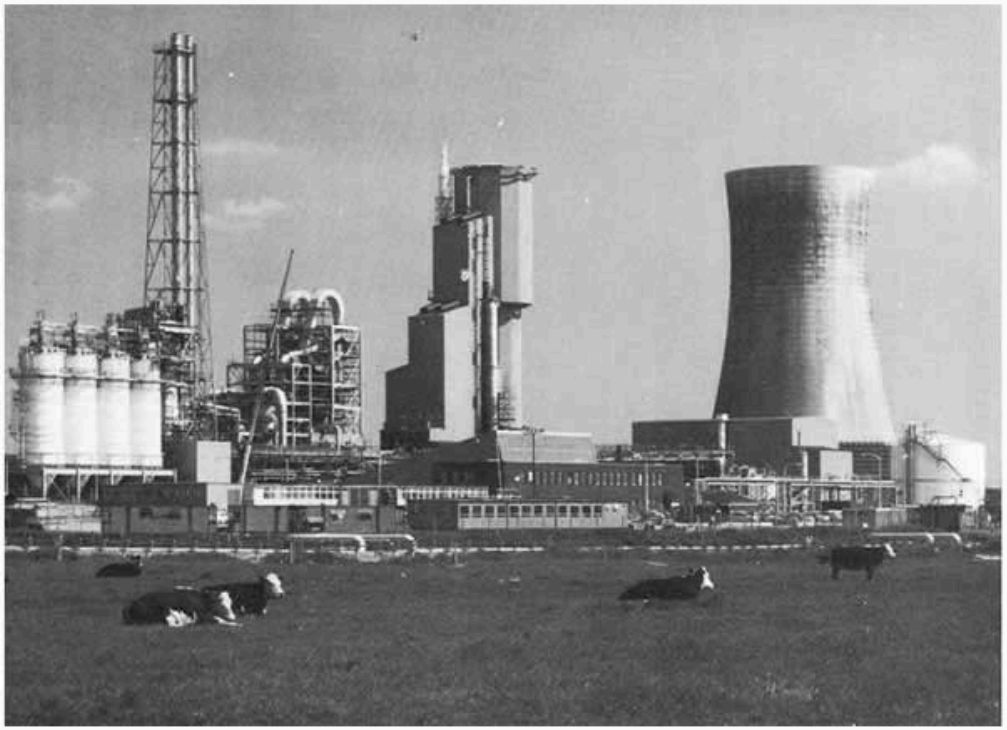
Using the equations the effects of the following characteristics on growth yields can be evaluated:

- P/O ratio
- Hydroxylation of methane and methylated amines using NADH
- The assimilation pathway
- The nature of the system for oxidation of formaldehyde (yielding NADH or not)

**Conclusions:**

Most methanotrophs are NADH-limited regardless of assimilation pathway
On methanol, bacteria with the RuMP pathway are sometimes typically ATP-limited but changing the P/O ratio will have little effect on growth yield (they tend to be carbon-limited, where the only way of increasing yield is to add oxidised substrates.
Most serine pathway bacteria will be NADH-limited where a different P/O ratio will have little effect on yield

## The ICI 'Pruteen' plant at Billingham, UK 1980



The centre tower is the 1.5 million litre fermenter

It contains about 100,000,000,000,000,000,000 methylotrophs
10% of their soluble protein is Methanol dehydrogenase

Top of Growth yields
Top of Summaries

Carbon assimilation pathways PPT Top of Summaries Return to History

# Carbon assimilation pathways

**Part one:** Brief summary of the four pathways for assimilation of C1 compounds

The elucidation of the Serine Cycle up to 1973

**Part two:** The solution of the complete Serine / Ethylmalonyl-CoA cycle

## Gordon Research Conference: Magdalen College, Oxford, 2006

## Molecular Basis of Microbial One-Carbon Metabolism

### The Biochemistry of Methylotrophs: a historical perspective

Chris Anthony, University of Southampton, UK



Dedicated to the memory of J. Rod Quayle
(1926 – 2006)

| | |
|---|---|
| 1946 – 1951 | PhD in physical-organic chemistry [University of Wales; with ED Hughes] |
| | PhD on aphid pigments [Cambridge with Alexander Todd |
| 1953 --1954 | Calvin's lab at Berkeley |
| 1955 --1963 | Krebs' MRC Unit at Oxford |
| 1963 – 1983 | University of Sheffield |
| 1983 – 1992 | Vice-Chancellor, University of Bath |

Many of my slides are from this lecture dedicated to Rod Quayle

# Carbon assimilation pathways of methylotrophs

**Ribulose bisphosphate [RuBP] pathway** [Key contribution from JRQ]
Plants, autotrophic bacteria and a few methylotrophs

Pathways first proposed by Quayle and mainly elucidated by him and his colleagues:

**Ribulose monophosphate [RuMP] pathway**

Type I methanotrophs and obligate methanol or methylamine utilisers

**Dihydroxyacetone [DHA] pathway**

Methylotrophic yeasts

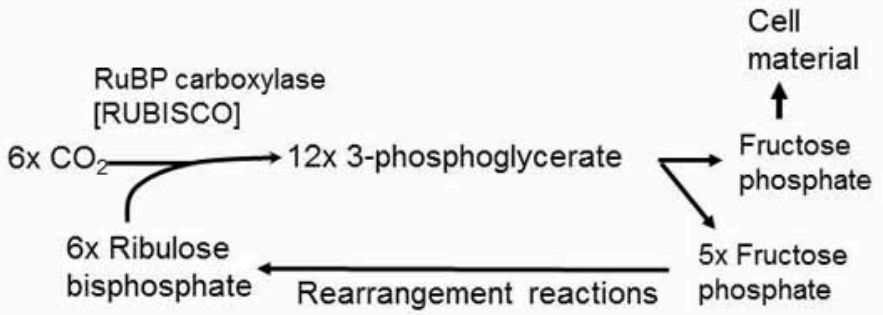
**Serine pathway**

Type II methanotrophs and facultative methanol or methylamine utilisers

I will summarise the first three and spend more time on details of serine pathway

# Calvin-Benson cycle for $CO_2$ fixation in plants [1950 – 1960]

The key demonstration of the specific RuBP carboxylase activity in extracts was published by Quayle in JACS in 1954




RADIATION LABORATORY AND
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

J. R. QUAYLE
R. C. FULLER
A. A. BENSON
M. CALVIN

RECEIVED MAY 24, 1954

This pathway was soon shown to be the path of carbon dioxide fixation in aerobic autotrophic bacteria and it was commonly assumed that methylotrophs growing on methane or methanol would assimilate their carbon by this pathway after their oxidation to $CO_2$

JRQ showed that this is the route for formate assimilation by *Pseudomonas oxalaticus* [1959].

He later showed that the facultative autotroph *Paracoccus denitrificans* assimilates methanol by this pathway.

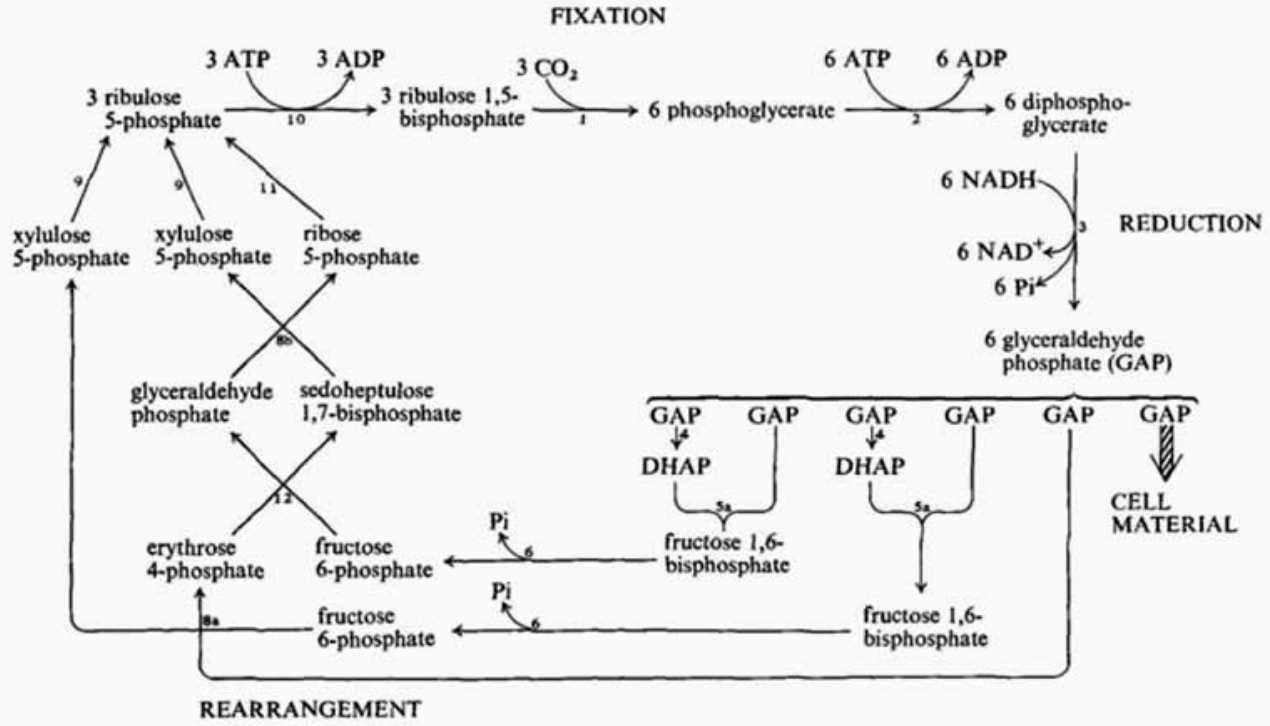



Fig. 5. The ribulose bisphosphate cycle of $CO_2$ assimilation (transaldolase variant). The enzyme numbers refer to those in the text: (1) ribulose bisphosphate carboxylase; (2) phosphoglycerate kinase; (3) glyceraldehyde phosphate dehydrogenase; (4) triose phosphate isomerase; (5a, b) aldolase; (6) fructose bisphosphatase; (8) transketolase; (9) pentose phosphate epimerase; (10) phosphoribulokinase; (11) pentose phosphate isomerase; (12) transaldolase.

**Ribulose Bisophosphate pathway in plants, autotrophs and some methylotrophs**



*Figure 1.* Some members of the photosynthesis research group (about 1953). They are in front of Old Radiation Laboratory at UCB. This building was later demolished to make space for a new Department of Chemistry building, Hildebrand Hall. *Left to right:* Clinton Fuller, Robert Norris, Hans Kornberg, Alice Holtham, Melvin Calvin, J. Rodney Quayle, Malcolm Thain, James Bassham and Jaques Mayudon. Andrew Benson agrees that he probably took this picture, accounting for his absence from the picture.

# The ribulose monophosphate pathways

Quayle, Johnson, Strom, Ferenci, Kemp, [1965 – 1974]

Occur in Type I methanotrophs and in the obligate methanol or methylamine utilisers. There are 4 variants; three of these have been demonstrated in different bacteria.

**Methods**: Short term labelling experiments; analysis of position of label in metabolites, purification and characterisation of enzymes; measurement of all enzymes of the pathway.

Similar to Ribulose bisphosphate (Calvin) cycle except for 'first reaction'

Condensation of formaldehyde with RuBP to give a novel hexulose phosphate; this is then isomerised to fructose 6 phosphate. The novel synthase and isomerase were isolated and characterised.

Subsequent reactions of the pathway are similar to the rearrangement reactions of the Calvin cycle.

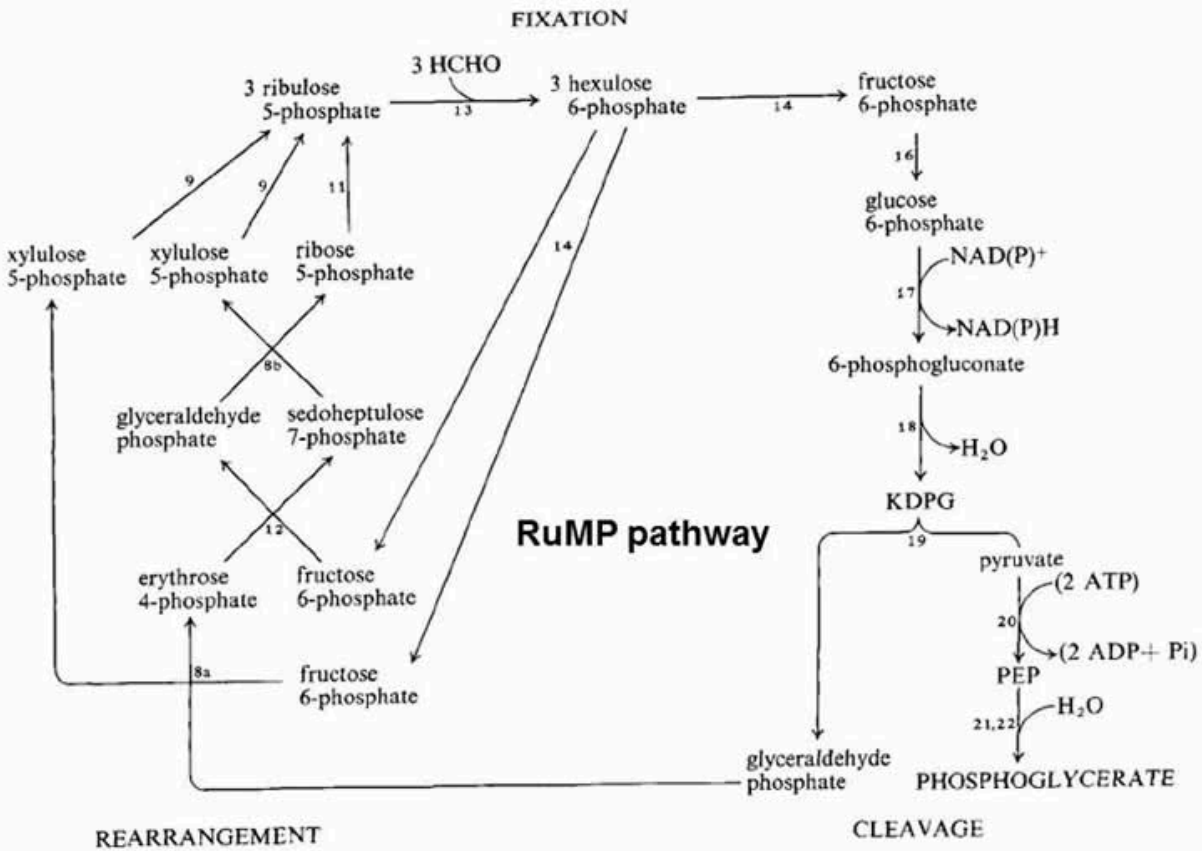



Fig. 6. The ribulose monophosphate (RuMP) cycle of formaldehyde assimilation (KDPG aldolase/transaldolase variant). This variant occurs predominantly in obligate methylotrophs. The enzyme numbers refer to those listed in the text: (8) transketolase; (9) pentose phosphate epimerase; (11) pentose phosphate isomerase; (12) transaldolase; (13) hexulose phosphate synthase; (14) hexulose phosphate isomerase; (16) glucose phosphate isomerase; (17) glucose phosphate dehydrogenase; (18) phosphogluconate dehydrase; (19) 2-keto, 3-deoxy, 6-phosphogluconate (KDPG) aldolase; (20) PEP synthetase or equivalent enzyme(s); (21) enolase; (22) phosphoglyceromutase.

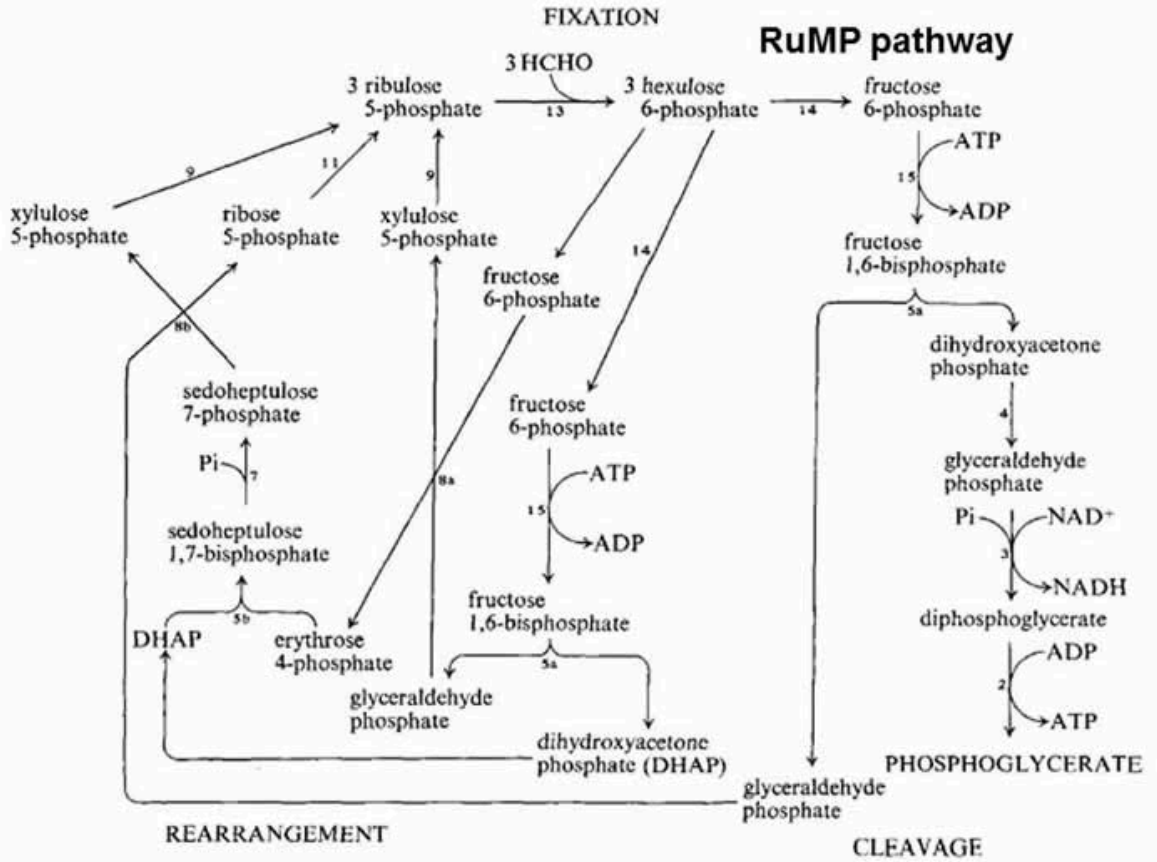



Fig. 7. The ribulose monophosphate (RuMP) cycle of formaldehyde assimilation (Fructose bisphosphate aldolase/ sedoheptulose bisphosphatase variant). This variant occurs predominantly in facultative methylotrophs. The enzyme numbers refer to those listed in the text: (2) phosphoglycerate kinase; (3) glyceraldehyde phosphate dehydrogenase; (4) triose phosphate isomerase; (5) aldolase; (7) sedoheptulose bisphosphatase; (8) transketolase; (9) pentose phosphate epimerase; (11) pentose phosphate isomerase; (13) hexulose phosphate synthase; (14) hexulose phosphate isomerase; (15) phosphofructokinase.

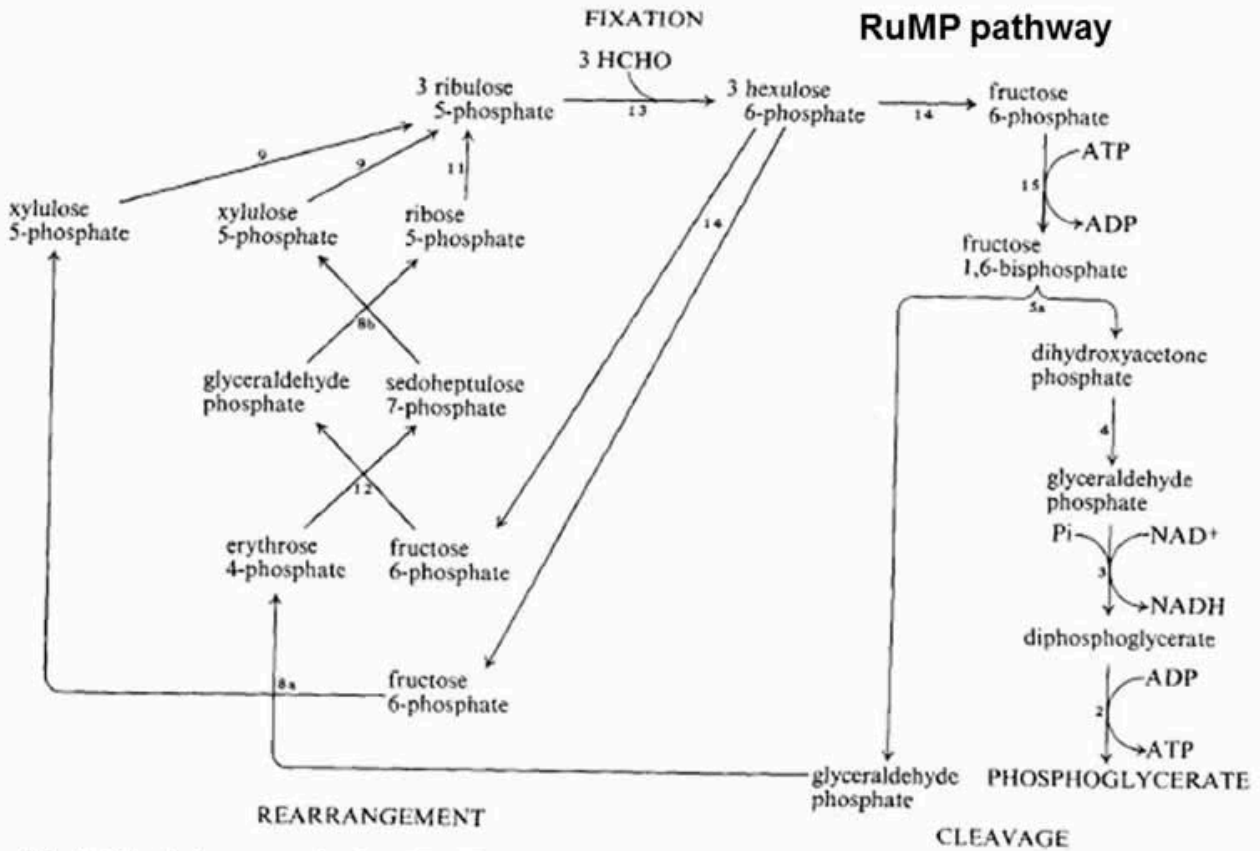



Fig. 8. The ribulose monophosphate (RuMP) cycle of formaldehyde assimilation (Fructose bisphosphate aldolase/transaldolase variant). This variant does not occur frequently in methylotrophs. The enzyme numbers refer to those listed in the text: (2) phosphoglycerate kinase; (3) glyceraldehyde phosphate dehydrogenase; (4) triose phosphate isomerase; (5) aldolase; (8) transketolase; (9) pentose phosphate epimerase; (11) pentose phosphate isomerase; (12) transaldolase; (13) hexulose phosphate synthase; (14) hexulose phosphate isomerase; (15) phosphofructokinase.

**The dihydroxyacetone [DHA] cycle of formaldehyde assimilation in yeasts**

Nobuo Kato, O'Connor (Mary Lidstrom), Sahm, Babel, van Dijken, Quayle [1977-1981]

This is similar to the RuBP and RuMP cycles

Two specific enzymes are required for formaldehyde fixation:

DHA synthase and triokinase

These were purified and characterised

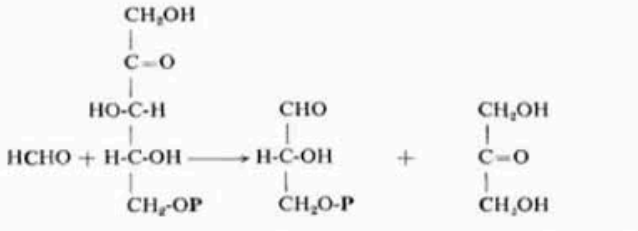
Short term labelling pattern from 14C methanol was consistent with the cycle proposed by Quayle and distribution of labelled carbon in the proposed intermediates was consistent with the cycle

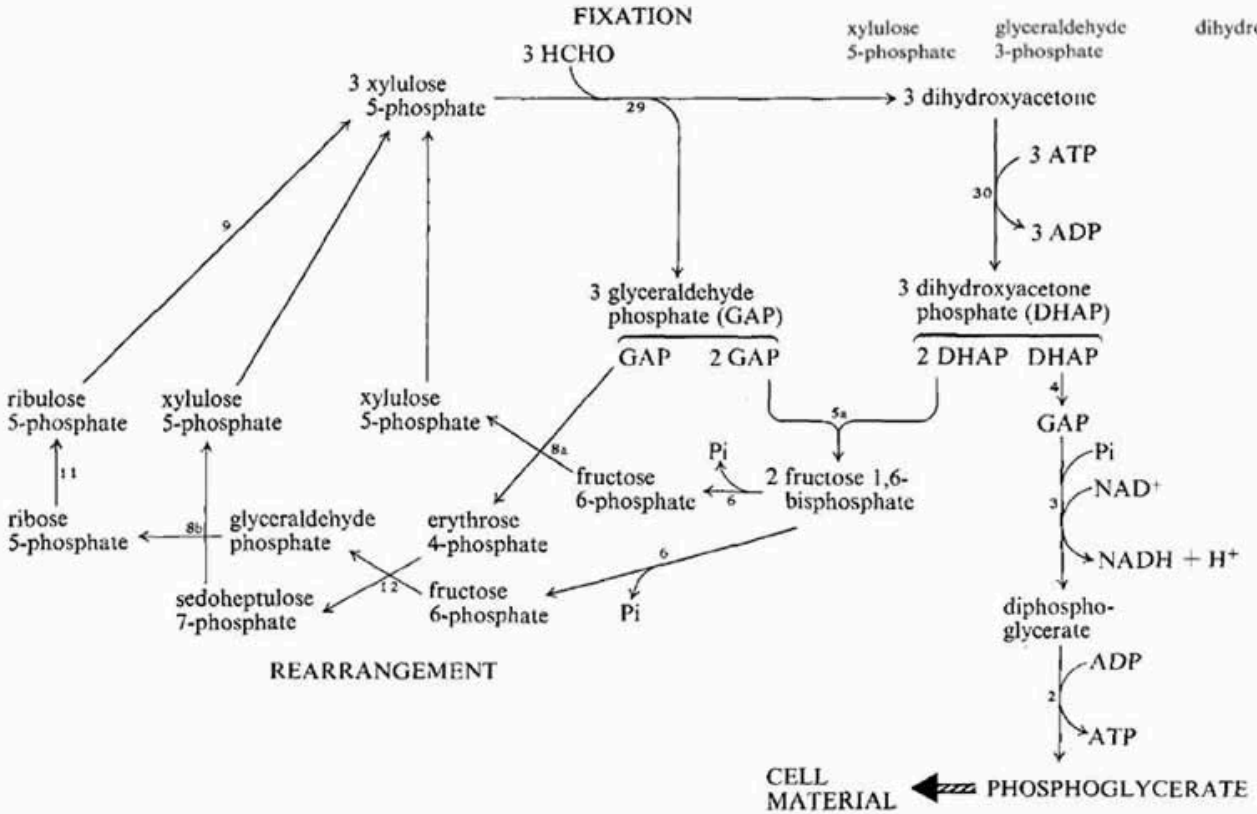
Mutants lacking the key enzymes were unable to grow on methanol

# DHA cycle in yeast

**Fixation: xylulose phosphate +HCHO ⟶**

**glyceraldehyde phosphate + dihydroxyacetone**



**The discovery of the Serine cycle in Methylobacterium extorquens AM1 (Pseudomonas AM1)**

**Part 1: J Rod Quayle, David Peel, Peter Large, Alan Salem**
**(with bits by Pat Goodwin (nee Dunstan) and Chris Anthony**



J. Rod Quayle



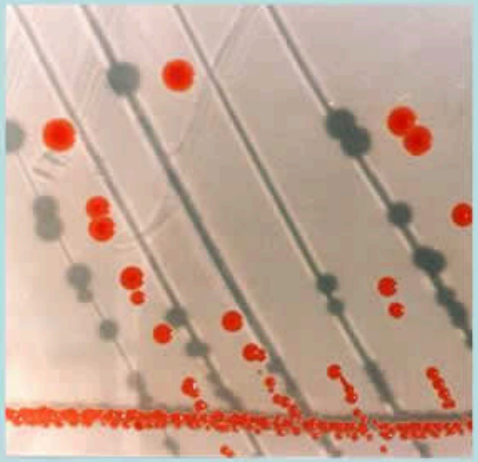

Peter Large

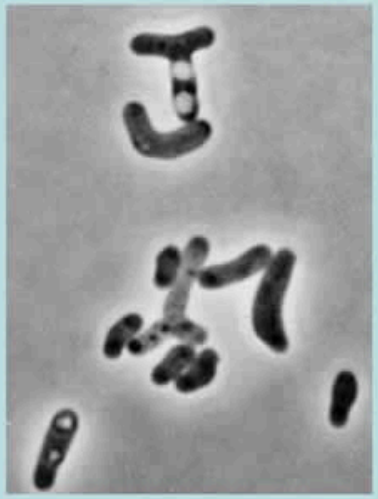
# *Methylobacterium extorquens*

- *Pseudomonas* AM1 (Peel & Quayle, 1961)
- *Pseudomonas* sp. M27 (Anthony & Zatman, 1964)

$CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$







## The Serine Pathway; Peter Large, David Peel and Rod Quayle

1961 - 1963

1. Large, P.J., Peel, D. and Quayle, J.R. *Biochemical Journal* **81** , 470-480 (1961).
Microbial growth on C1 compounds: Synthesis of cell constituents by methanol- and formate-grown *Pseudomonas* AM1 and methanol-grown *Hyphomicrobium vulgare*.

2. Large, P.J., Peel, D. and Quayle, J.R. *Biochemical Journal* **82,** 483-488 (1962).
Microbial growth on C1 compounds: Distribution of radioactivity in metabolites of methanol-grown *Pseudomonas* AM1 after incubation with [14C]methanol and [14C]bicarbonate.

3. Large, P.J., Peel, D. and Quayle, J.R. *Biochemical Journal* **85,** 243-250 (1962).
Microbial growth on C1 compounds: Carboxylation of phosphoenolpyruvate in methanol-grown *Pseudomonas* AM1.

4. Large, P.J. and Quayle, J.R. *Biochemical Journal* **87**, 386-396 (1963).
Microbial growth on C1 compounds: Enzyme activities in extracts of *Pseudomonas* AM1.

## The Elucidation of the Serine pathway in *Pseudomonas* AM1
[now *Methylobacterium extorquens* AM1]

A pink facultative methylotroph; grows on methanol, not methane

Bacteria were grown on $^{14}C$ MeOH and the label in cell material recorded. If RuBP pathway is operating then passage of 'cold' $^{14}CO_2$ would decrease the label by 95%

$$^{14}CH_3OH \longrightarrow {}^{14}CO_2 \xrightarrow{\text{RuBP carboxylase [RUBISCO]}} {}^{14}C \text{ 3- phosphoglycerate} \longrightarrow \textbf{}^{14}\textbf{C Cell material}$$

Passage of 'cold' $CO_2$ through the culture during growth on $^{14}CH_3OH$ decreased label in cell material by about 50%. This shows that half the carbon enters the biosynthetic pathway as $CO_2$ produced from the methanol

RuBP carboxylase is absent

Short term labelling experiments showed that 3- phosphoglycerate is not an early intermediate when whole cells are incubated with $^{14}CH_3OH$ or $H^{14}COOH$

## Short term label experiments to determine path of carbon

Incubate growing cells with $^{14}CH_3OH$ or $^{14}CO_2$ (bicarbonate)

Take samples into boiling ethanol at 2,4,8,20 secs etc

Separate all soluble components by 2-way paper chromatography

Identify labelled compounds by autoradiography (3 weeks)

Elute, count $^{14}C$ and confirm identity by co-chromatography with known compounds

Plot % radioactivity in each compound against time. A negative slope indicates an early intermediate.

After 1 min incubation the early intermediates were chemically analysed to determine the specific radioactivity in each carbon atom

## Distribution of label in cells incubated with labelled $CO_2$

Negative slope = earliest intermediates
Malate [reflecting oxaloacetate, OAA]
Glycine; Later - serine

Suggests typical carboxylation of a C3 to a C4 compound [OAA / malate]

And either cleavage of C4 to glycine

Or novel carboxylation to give glycine

NB: the presence of a labelled compound at 20 seconds does not indicate an early intermediate.

Coenzyme A derivatives are cannot be seen in this sort of experiment.

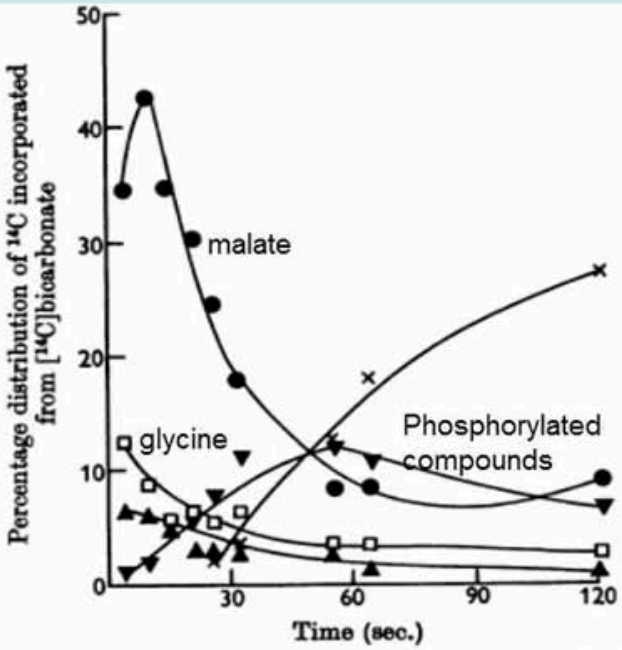



**Fig. 3.** Variation with time of the percentage distribution of $^{14}C$ incorporated from [$^{14}C$]bicarbonate into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM 1 growing on methanol. ●, Malate; ▲, serine; □, glycine; ▼, phosphorylated compounds; ×, trehalose.

Similar results were obtained using *Hyphomicrobium vulgare*

## Distribution of label in cells incubated with methanol

Negative slope = early intermediates

Serine Malate Aspartate Glycine

Suggests:

Addition of HCHO to glycine to give serine

A derivative of serine is carboxylated to OAA / malate / aspartate

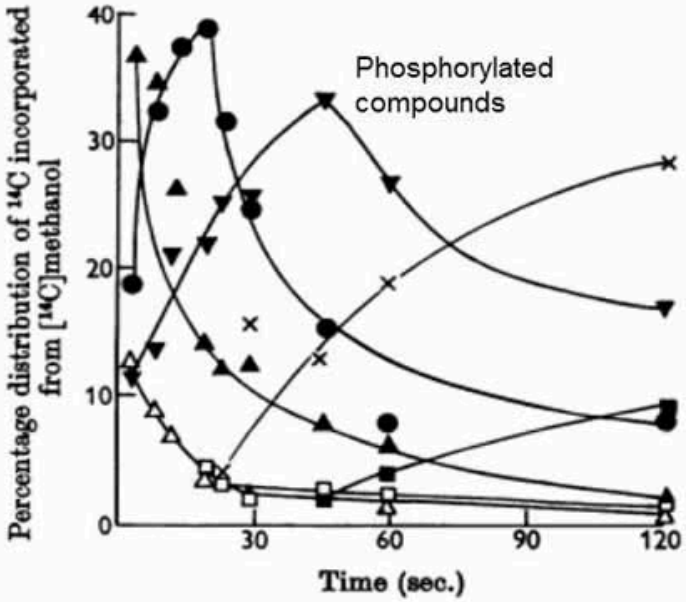



**Fig. 2. Variation with time of the percentage distribution of ¹⁴C incorporated from [¹⁴C]methanol into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM 1, growing on methanol. ●, Malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds; ×, trehalose.**

Similar results were obtained using *Hyphomicrobium vulgare*

## Distribution of $^{14}C$ in carbon atoms of early intermediates

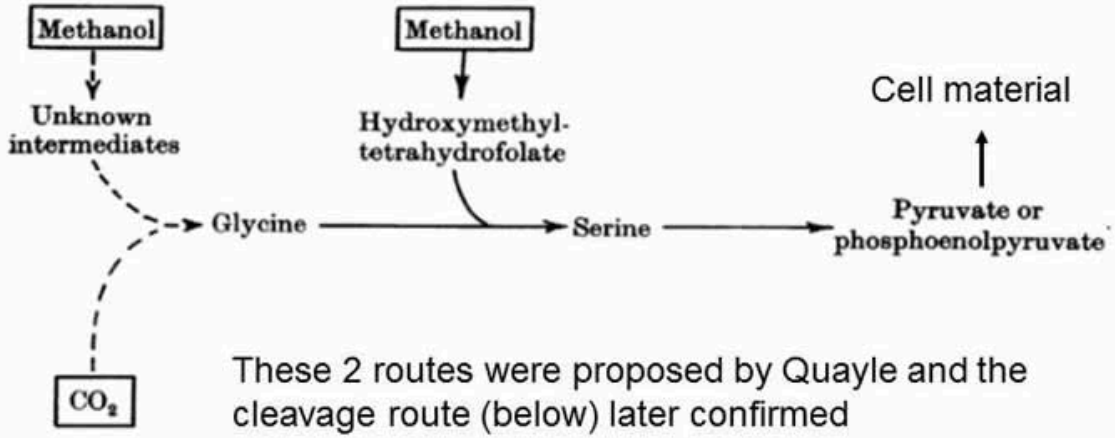
Cells were incubated for 1 minute with $^{14}C$ MeOH or $^{14}HCO_3$; Intermediates were purified, chemically degraded and $^{14}C$ in each C atom determined and expressed as % of total counts in the compound

| | From methanol | From bicarbonate |
|---|---|---|
| **Glycine** | | |
| $CH_2NH_2$ | 50 | 15 |
| COOH | 50 | 85 |
| **Serine** | | |
| $CH_2OH$ | 50 | 2 |
| $CHNH_2$ | 25 | 15 |
| COOH | 25 | 83 |

### Conclusions

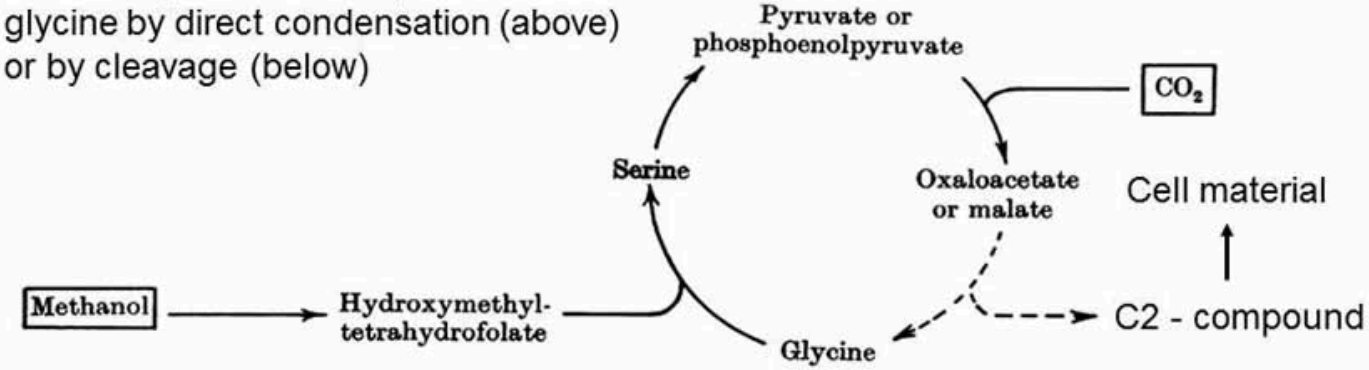
1. Carboxyl group of glycine comes from carbon dioxide; methylene carbon comes from methanol
2. Hydroxymethyl group in serine comes from methanol; the other 2 carbons mimic the distribution seen in glycine
3. Serine arises by hydroxymethylation of glycine

## Two possible routes for conversion of methanol plus $CO_2$ to cell material




These 2 routes were proposed by Quayle and the cleavage route (below) later confirmed

NOTE: key difference is production of glycine by direct condensation (above) or by cleavage (below)



## The serine cycle involves a cleavage reaction

Malyl-CoA lyase: malyl-CoA ⟶ glyoxylate + acetyl-CoA

[Salem & Quayle 1973]

glyoxylate ↓ glycine

**What happens to the acetyl-CoA?**

*In icl$^+$ bacteria:* isocitrate lyase is involved in oxidation of acetyl-CoA to glyoxylate; in these bacteria ICL is also involved during growth on ethanol or acetate

*In icl$^-$ bacteria* with no isocitrate lyase [eg *Methylobacterium extorquens*]

This route is not yet fully established.

It is also involved in metabolism of C2 compounds

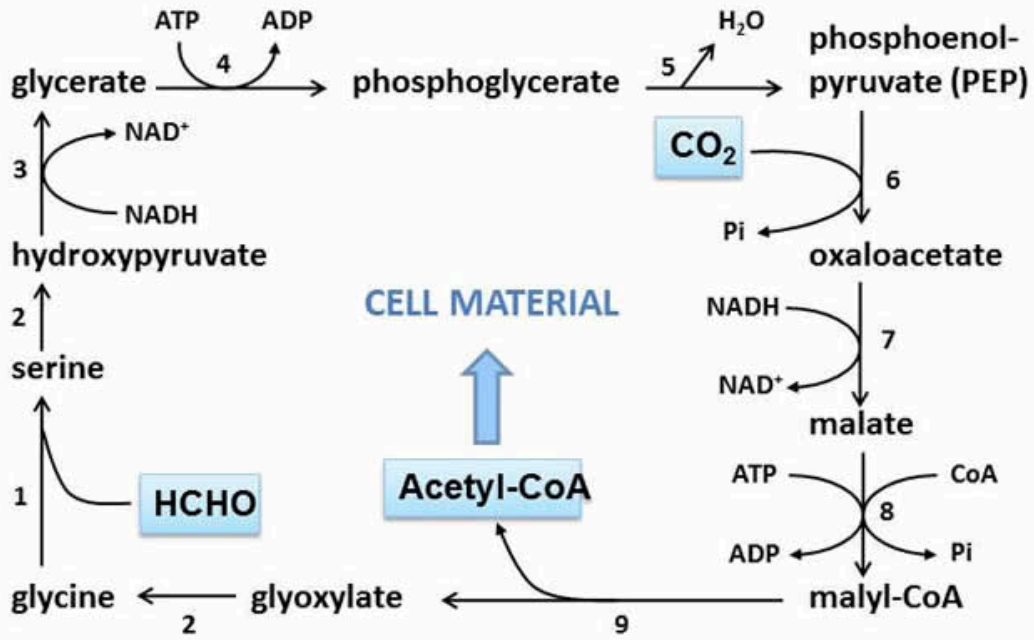



**Figure 3.** The serine cycle as proposed by Peel, Large, Salem and Quayle[9-11]. The enzymes are 1, serine transhydroxymethylase; 2, serine-glyoxylate aminotransferase; 3, hydroxypyruvate reductase; 4, glycerate kinase; 5, enolase; 6, PEP carboxylase; 7, malate dehydrogenase; 8, malate thiokinase; 9, malyl-CoA lyase. After the initial proposal much further enzymological and mutant evidence was subsequently accumulated to confirm this pathway[3]. Note that during biosynthesis of fatty acids and poly 3-hydroxybutyrate which use acetyl-CoA as their biosynthetic starting point, this pathway is sufficient for production of acetyl-CoA from formaldehyde plus carbon dioxide.

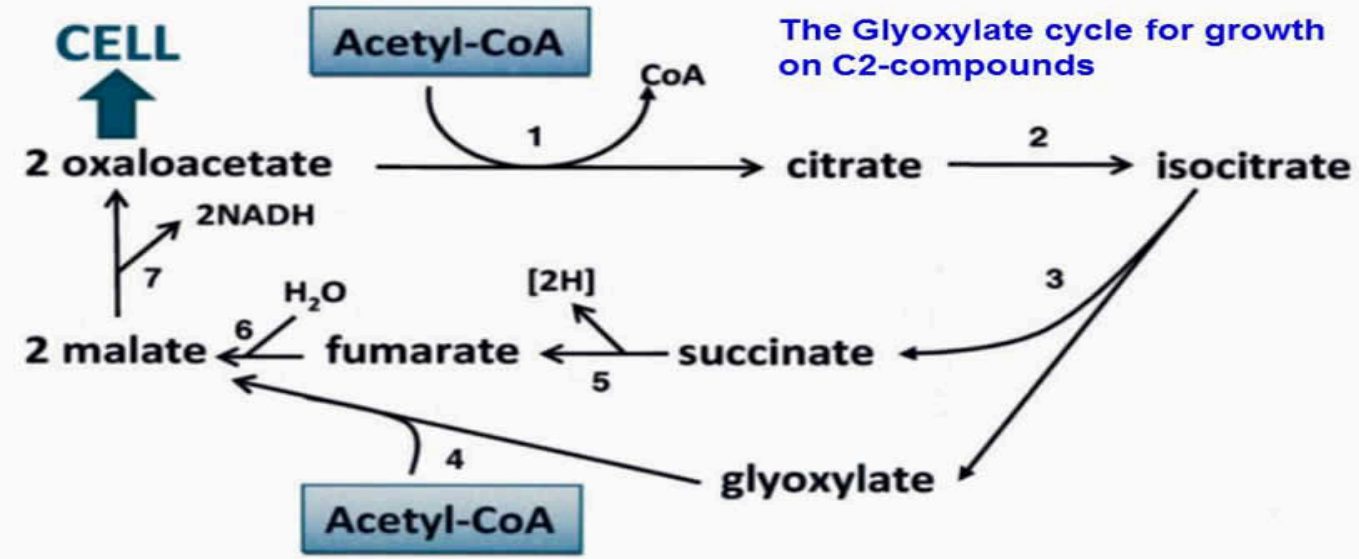



***Figure 1*** *Kornberg's glyoxylate cycle[4]. This achieves the condensation of 2 molecules of acetyl-CoA to malate. The enzymes are: 1, citrate synthase; 2, aconitase; 3, isocitrate lyase; 4, malate synthase; 5, succinate dehydrogenase; 6, fumarase; 7, malate dehydrogenase. In effect this cycle can be summarized as 2 acetyl-CoA →malate. A complete balance equation is given in Table 1 at the end of this review.*

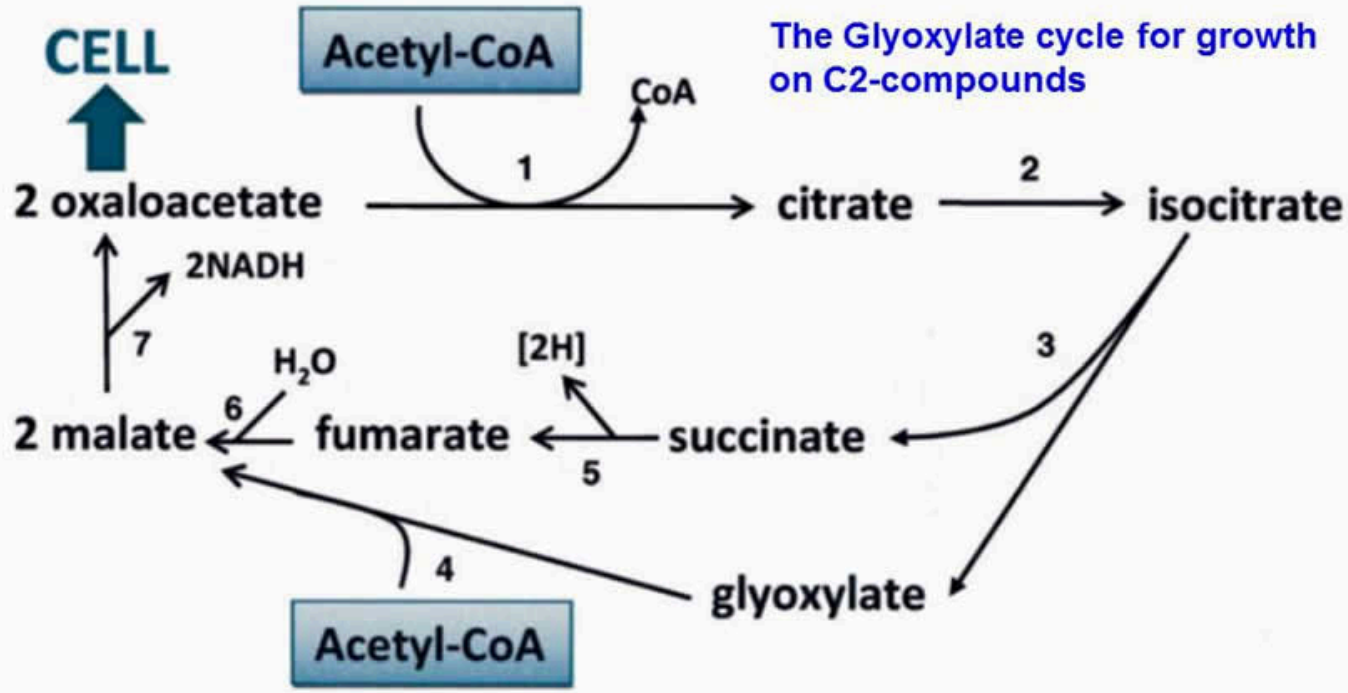
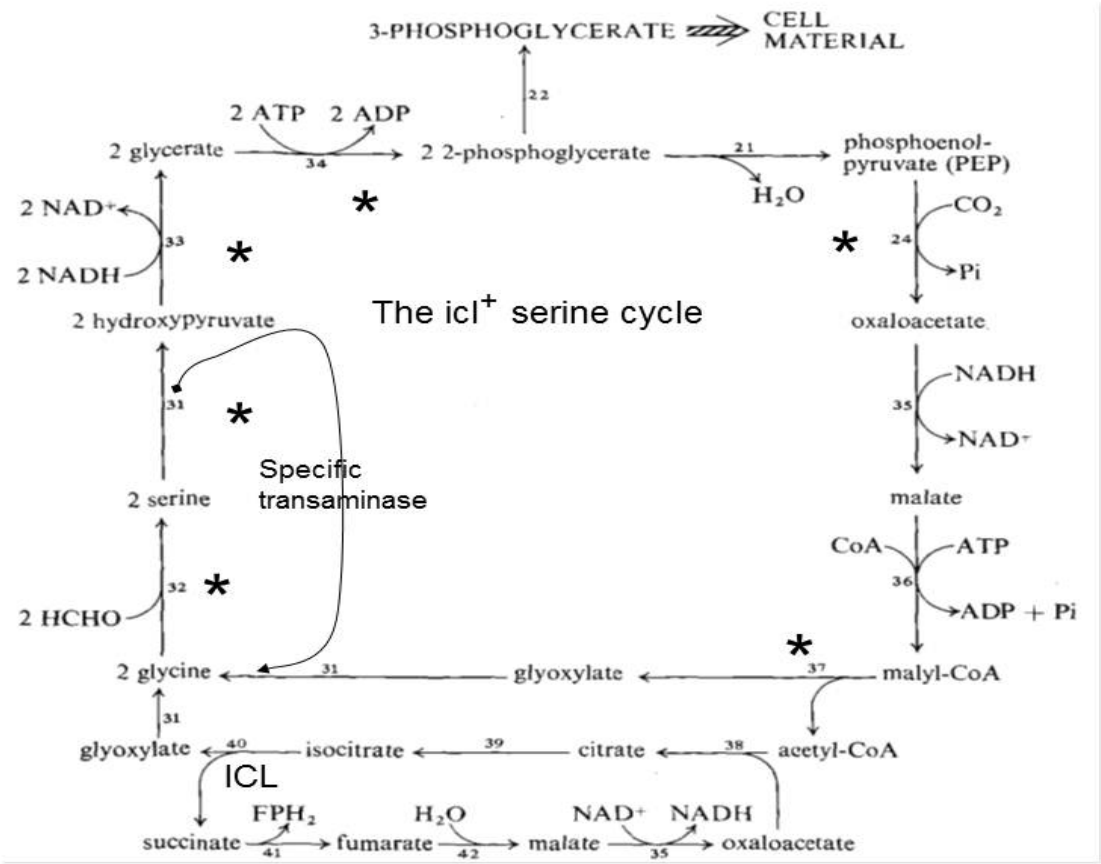



***Figure 1*** *Kornberg's glyoxylate cycle[4]. This achieves the condensation of 2 molecules of acetyl-CoA to malate. The enzymes are: 1, citrate synthase; 2, aconitase; 3, isocitrate lyase; 4, malate synthase; 5, succinate dehydrogenase; 6, fumarase; 7, malate dehydrogenase. In effect this cycle can be summarized as 2 acetyl-CoA →malate. A complete balance equation is given in Table 1 at the end of this review.*


3-PHOSPHOGLYCERATE
CELL MATERIAL
22
2 ATP
2 ADP
34
2 glycerate
2 2-phosphoglycerate
21
H2O
phosphoenol-pyruvate (PEP)
2 NAD+
2 NADH
33
The icl+ serine cycle
CO2
24
Pi
2 hydroxypyruvate
oxaloacetate
31
NADH
35
NAD+
Specific transaminase
2 serine
malate
CoA
ATP
36
ADP + Pi
2 HCHO
32
2 glycine
31
glyoxylate
37
malyl-CoA
glyoxylate
40
isocitrate
39
citrate
38
acetyl-CoA
ICL
FPH2
H2O
NAD+
NADH
succinate
41
fumarate
42
malate
35
oxaloacetate

# Confirmation of serine cycle

The proposed pathway fits the early labelled intermediates

The distribution of labelling in the intermediates fits the pathway

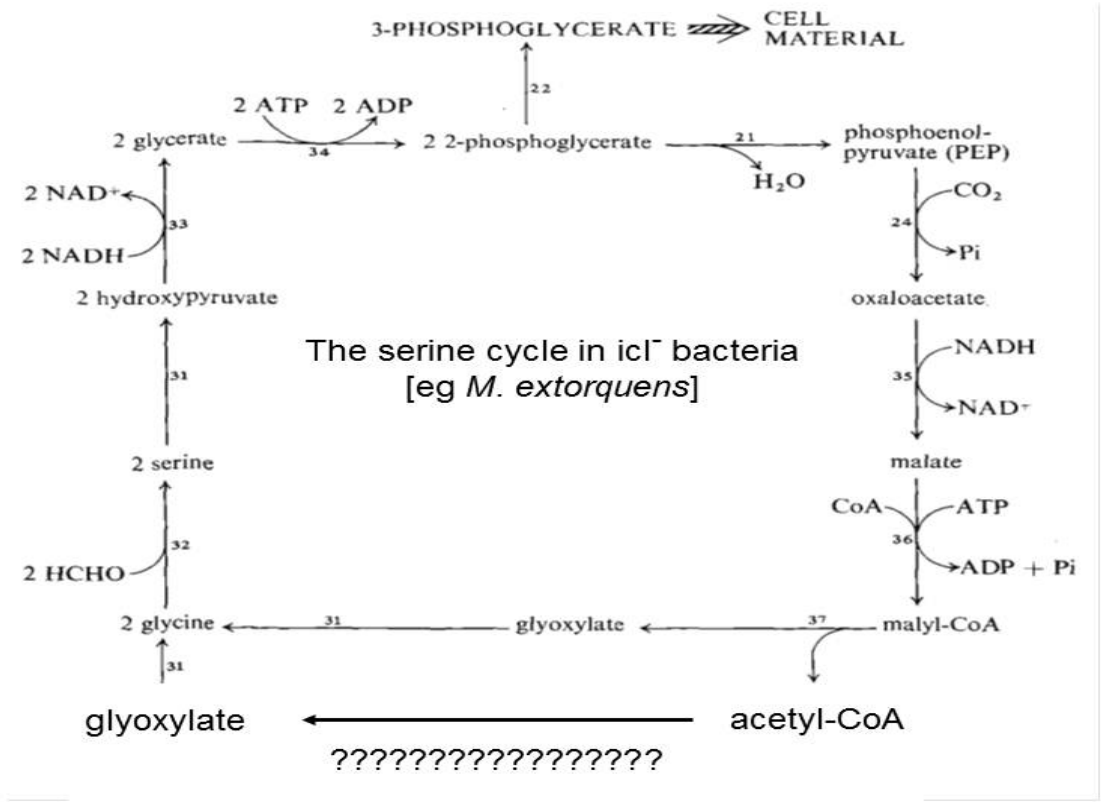
The 5 novel enzymes were purified and characterised

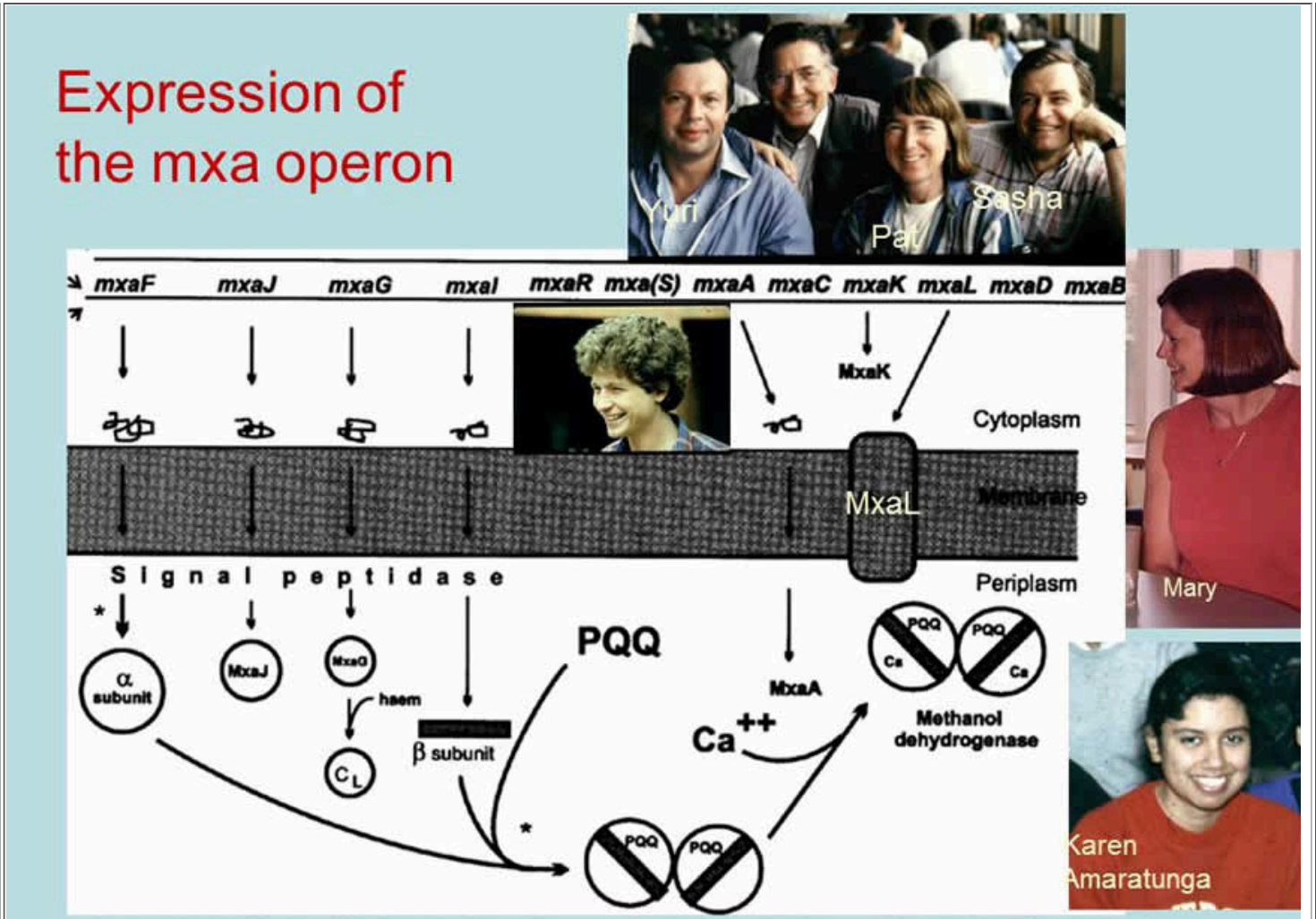
They were shown to be inducible on methanol

They were of sufficiently high specific activity to account for the growth rate on methanol

Mutants lacking them failed to grow on methanol; revertants had regained the enzyme

Later shown that key enzymes were coordinately regulated, implying the presence of an operon [Dunstan (Goodwin) & Anthony; Hanson & O'Connor (Lidstrom)]


3-PHOSPHOGLYCERATE
CELL MATERIAL
22
2 ATP
2 ADP
2 glycerate
34
2 2-phosphoglycerate
21
H2O
phosphoenol-pyruvate (PEP)
CO2
24
Pi
2 NAD+
33
2 NADH
2 hydroxypyruvate
oxaloacetate
The serine cycle in icl- bacteria
[eg M. extorquens]
NADH
35
NAD+
31
2 serine
malate
CoA
ATP
36
ADP + Pi
2 HCHO
32
2 glycine
31
glyoxylate
37
malyl-CoA
31
glyoxylate
acetyl-CoA
??????????????????




Top of carbon assimilation
Top of summaries

For completion of the Serine cycle go to Serine Cycle completion

Completion of the Serine /Ethylmalonyl-CoA Cycle PPT Top of summaries

## Solution of the Serine pathway in *Methylobacterium extorquens* (50 year project)

**Situation in 1963** There must be a route for oxidation of acetylCoA to glyoxylate. An obvious route is to use the glyoxylate cycle but the key enzyme isocitrate is absent during growth on methanol (Large and Quayle 1963).

1970 Pat Dunstan (now Pat Goodwin). Showed that ICL is absent during growth on ethanol

***

**Isolation of unusual mutants:** A project to isolate MDH mutants.
Penicillin enrichment procedure isolated mutants unable to grow on C1 or C2 but able to grow on succinate. These should be unable to oxidise methanol and ethanol. No mutants in assimilation pathways should be selected as these pathways are different.

Three types of mutant were isolated: MDH mutants. Cytochrome c mutants [unique, indicating the special role of cytochrome c in energy transduction from MDH].
A mutants with alteration in carbon assimilation pathways (PCT48) on C1 and C2 compounds showing there must be a common step in the pathway.

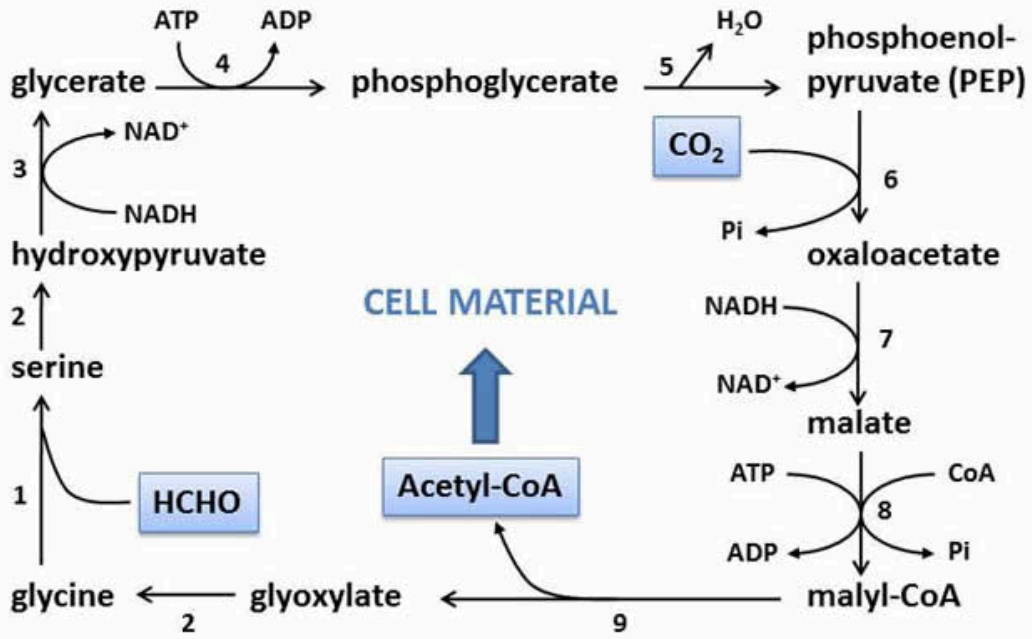



**Figure 3.** The serine cycle as proposed by Peel, Large, Salem and Quayle[9-11]. The enzymes are 1, serine transhydroxymethylase; 2, serine-glyoxylate aminotransferase; 3, hydroxypyruvate reductase; 4, glycerate kinase; 5, enolase; 6, PEP carboxylase; 7, malate dehydrogenase; 8, malate thiokinase; 9, malyl-CoA lyase. After the initial proposal much further enzymological and mutant evidence was subsequently accumulated to confirm this pathway[3]. Note that during biosynthesis of fatty acids and poly 3-hydroxybutyrate which use acetyl-CoA as their biosynthetic starting point, this pathway is sufficient for production of acetyl-CoA from formaldehyde plus carbon dioxide.

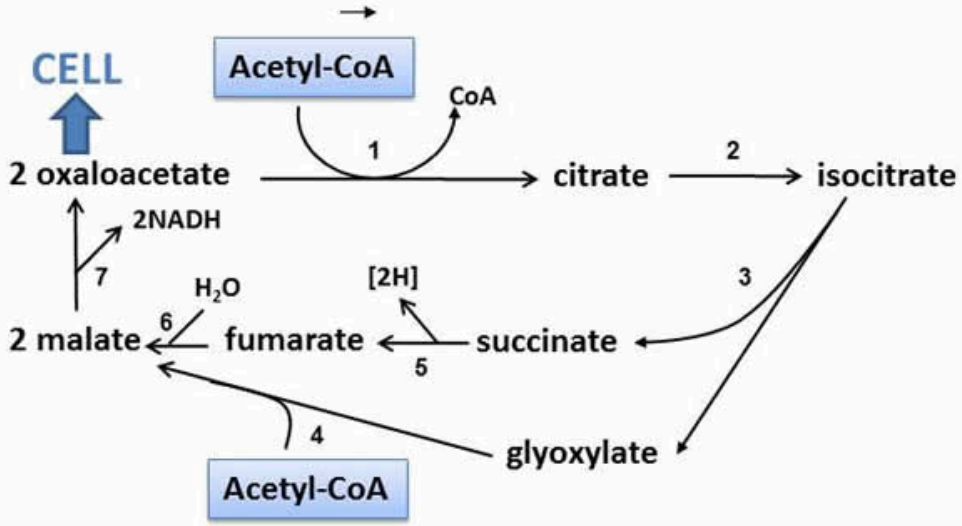



**Fig. 1.** Kornberg's glyoxylate cycle[4]. This achieves the condensation of 2 molecules of acetyl-CoA to malate. The enzymes are: 1, citrate synthase; 2, aconitase; 3, isocitrate lyase; 4, malate synthase; 5, succinate dehydrogenase; 6, fumarase; 7, malate dehydrogenase. In effect this cycle can be summarized as 2 acetyl-CoA ⟶ malate.

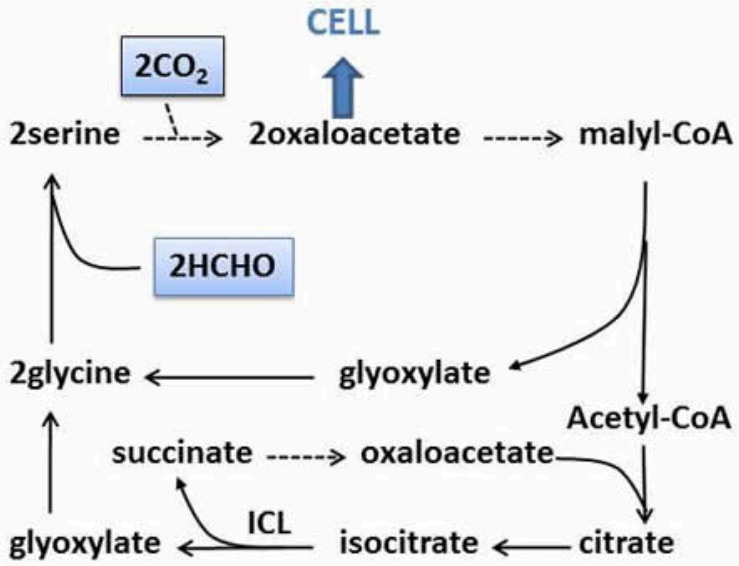



**Fig. 4.** The serine cycle in methylotrophic bacteria having isocitrate lyase [ICL][3]. The upper part of the Figure shows the serine cycle as shown on Fig 3. The lower part shows the oxidation of acetyl-CoA to glyoxylate by isocitrate lyase together with the non-decarboxylating enzymes of the TCA cycle.

## Solution of the Serine pathway in *Methylobacterium extorquens* (50 year project)

**Situation in 1963** There must be a route for oxidation of acetylCoA to glyoxylate. An obvious route is to use isocitrate lyase but this enzyme is absent during growth on methanol (Large and Quayle 1963).

1970 Pat Dunstan (now Pat Goodwin). Showed that ICL is absent during growth on ethanol

**Isolation of unusual mutants:** A project to isolate MDH mutants.
Penicillin enrichment procedure isolated mutants unable to grow on C1 or C2 but able to grow on succinate. These should be unable to oxidise methanol and ethanol. No mutants in assimilation pathways should be selected as these pathways are different.

Three types of mutant were isolated: MDH mutants. Cytochrome c mutants [unique, indicating the special role of cytochrome c in energy transduction from MDH].
A mutants with alteration in carbon assimilation pathways (PCT48) on C1 and C2 compounds showing there must be a common step in the pathway.



Yuri Me, Pat Dunstan (now Goodwin) and Sasha Netrusov in Kiev 12 days after Chernobyl

## The assimilation of ethanol in *M. extorquens* by study of 14C-acetate assimilation

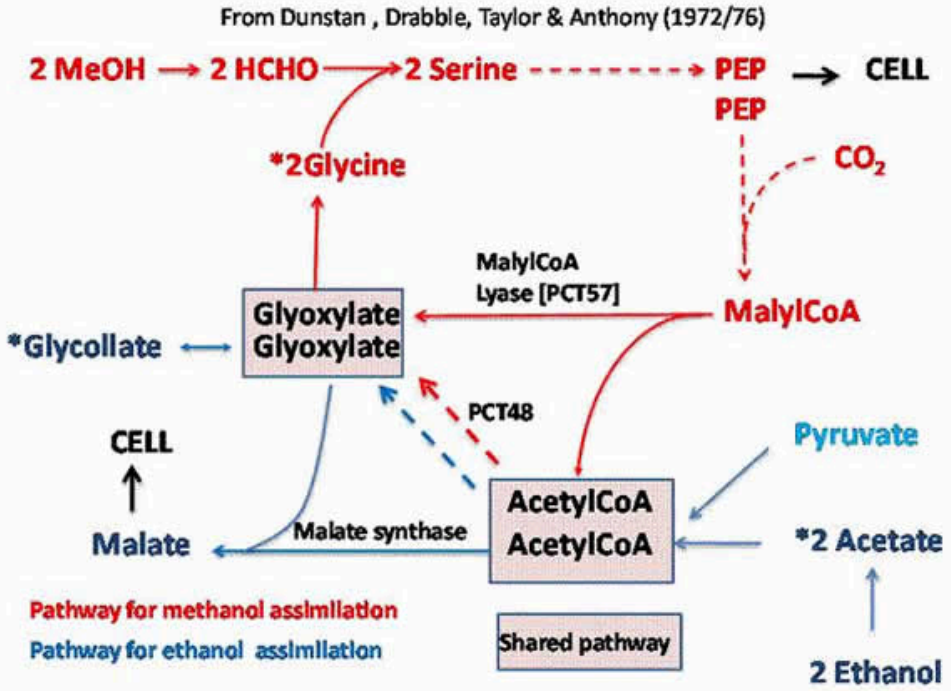
After growth on Methanol early label was in glycollate (reflects early glyoxylate label) & citrate
After growth on Ethanol early label was in glycine (reflects early glyoxylate label) & citrate

SO; there is an unknown common route for rapid metabolism of acetylCoA to glyoxylate during growth on C1 and C2 substrates.

In mutant 48 there was no rapid assimilation of acetate into glyoxylate (only citrate).

This same route was shown to operate on propanediol, 3-hydroxybutyrate and lactate

Problem: need to identify enzymes involved.




The shared pathway for methanol and ethanol assimilation

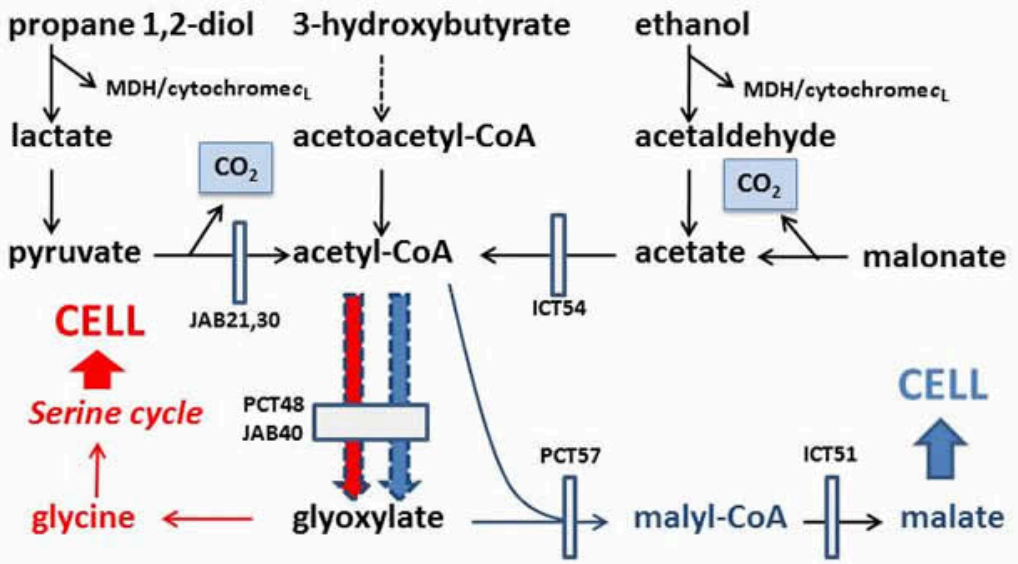


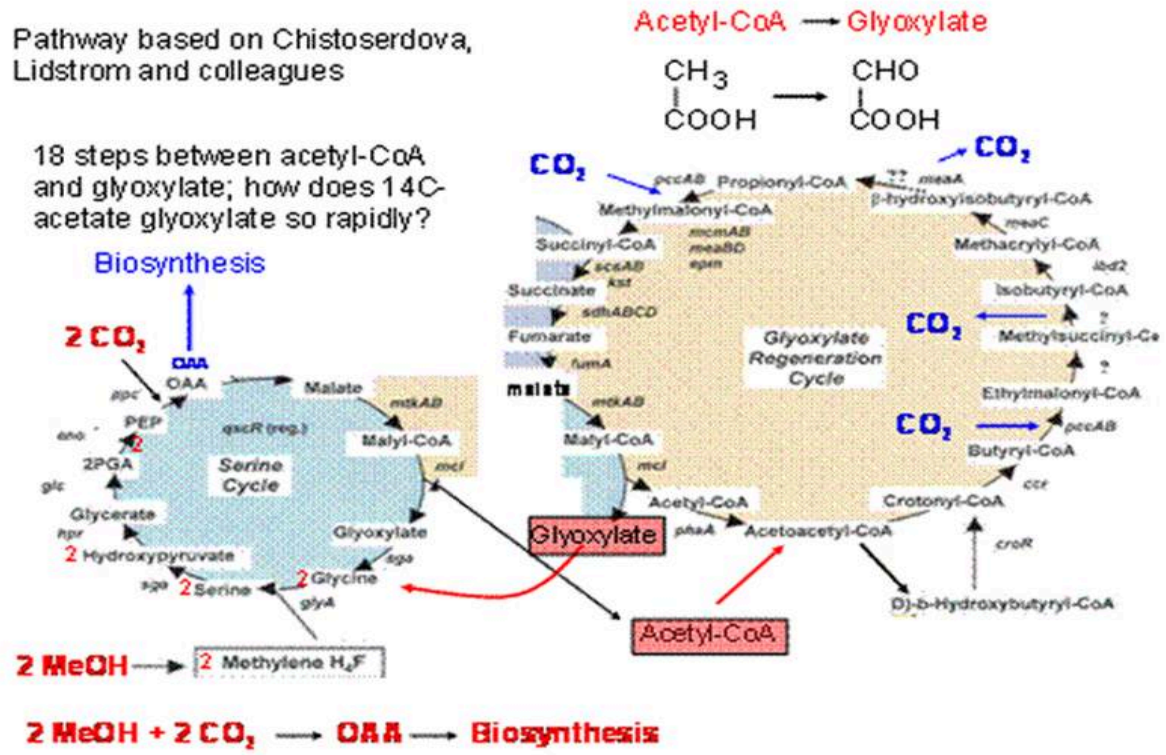

**Fig. 5. Pathways for growth of *M. extorquens* on substrates metabolized by way of acetyl-CoA**, based on the work of Pat Dunstan, John Bolbot and Ian Taylor [12, 17-19, 21, 22]. NB: only the carbon balance is illustrated. Red indicates pathway on $C_1$ compounds; blue indicates pathway on $C_2$ and related compounds. In short-term labeling experiments glycollate would arise by equilibration with glyoxylate. The growth substrates include ethanol, acetate (a poor substrate), 3-hydroxybutyrate, malonate, propanediol, lactate and pyruvate. Propanediol and ethanol are oxidized by methanol dehydrogenase (MDH) whose electron acceptor is cytochrome $c_L$ [16]; there is no growth of mutants lacking these proteins. For oxidation of propanediol by MDH an additional modifier protein is required to alter its substrate specificity[22]. Note that condensation of glyoxylate and acetyl-CoA to malate requires two enzymes: malyl-CoA lyase and malyl-CoA hydrolase.

**Glyoxylate Regeneration Cycles**

Mila Chistaserdova and Mary Lidstrom as a result of their work using mutants and some biochemistry produced many complex pathways, called Glyoxylate Regeneration cycles'
The solution was finally obtained in the lab of Georg Fuchs in Friebourg by very thorough enzymology and complex labelling techniques. Erb, Berg, Alber, Spanheimer, Ebenau-Jehle and Fuchs.

**The EthylmalonylCoA pathway (EMC pathway)**
This was done for acetate assimilation in *Rhodobacter sphaeroides* but was soon shown to be the common pathway also involved in methanol and ethanol assimilation in *M. extorquens*.

Most of the following slides are the Figures from my review:
How half a century of research was required to understand bacterial growth on C1 and C2 compounds: the story of the Serine Cycle and the Ethylmalonyl-CoA pathway. Science Progress 94, 109-138, 2011




**The Glyoxylate Regeneration Cycle Mila Chistaserdova and Mary Lidstrom**



Mary Lidstrom (right)



Mila Chistaserdova (right)

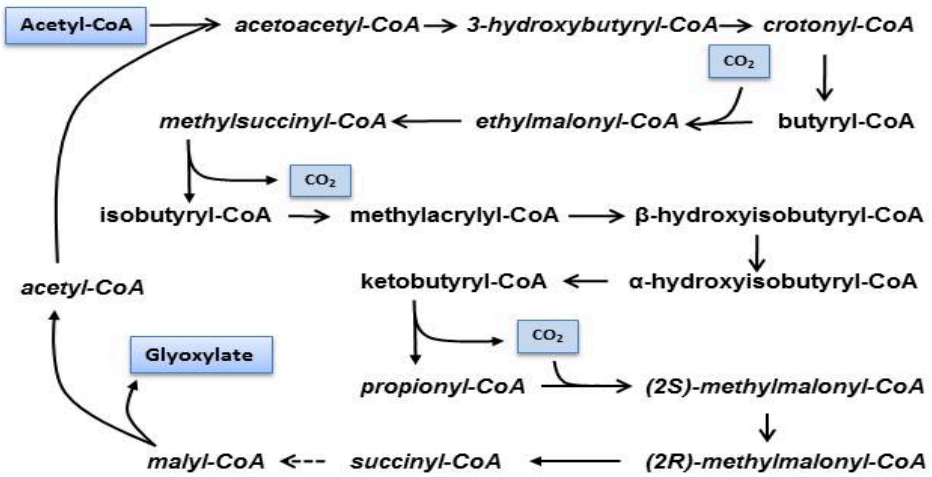



**Fig. 7. The glyoxylate regeneration cycle (GRC) for oxidation of acetyl-CoA in *M. extorquens* as proposed by Lidstrom, Chistoserdova and colleagues[27, 31-33].** Their papers should be consulted for details of the extensive experimental work, mainly using mutants and radioactive substrates that led to this [necessarily] speculative proposal. The compounds in italics were later shown to be intermediates in the ethylmalonyl-CoA (EMC) pathway. The solid arrows merely indicate proposed reactions (or series of reactions); they do not necessarily indicate that such reactions are known reactions.

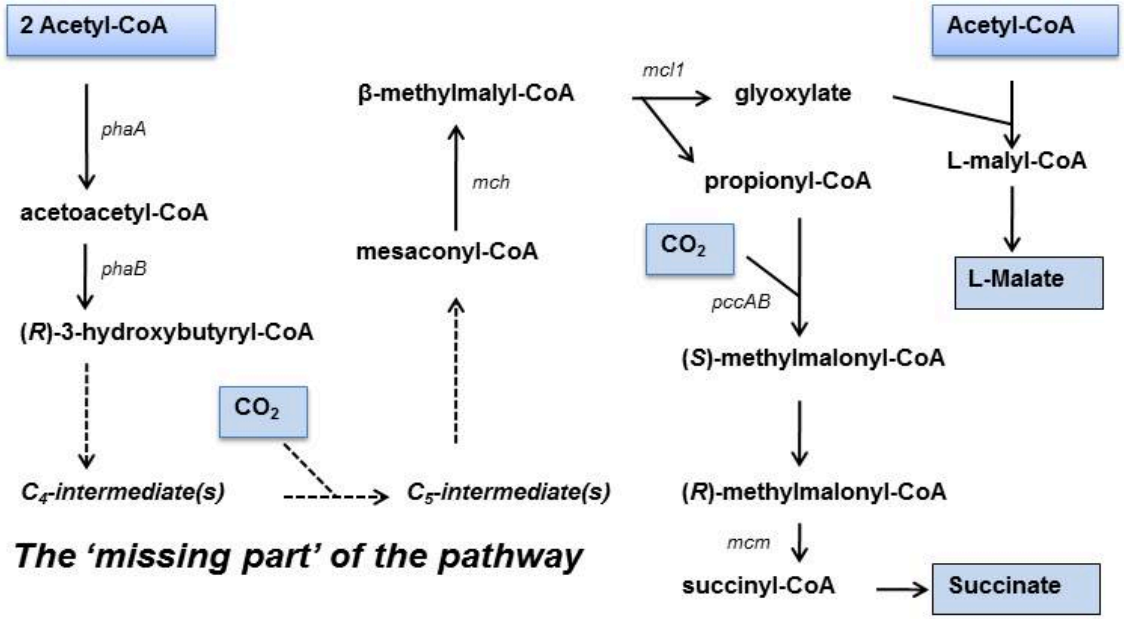



**Fig. 9. Proposed pathway for acetyl-CoA assimilation by Rhodobacter sphaeroides.** This Figure is re-drawn from the 2006 paper by Alber, Spanheimer, Ebenau-Jehle and Fuchs[43]. The gene phaA encodes β-ketothiolase; phaB, acetoacetyl-CoA reductase; mch, mesaconyl-CoA hydratase; mcl1, L-malyl-CoA/β-methylmalyl-CoA lyase; pccAB, propionyl-CoA carboxylase and mcm encodes (R)-methylmalonyl-CoA mutase. Although the enzymes catalyzing the conversion of the $C_4$ compound 3-hydroxybutyryl-CoA to the $C_5$ intermediate mesaconyl-CoA were not known at the time, it was suggested that this process probably involves a carboxylation step, as was subsequently demonstrated when the ethylmalonyl-CoA pathway was finally elucidated (Figs. 10-12).

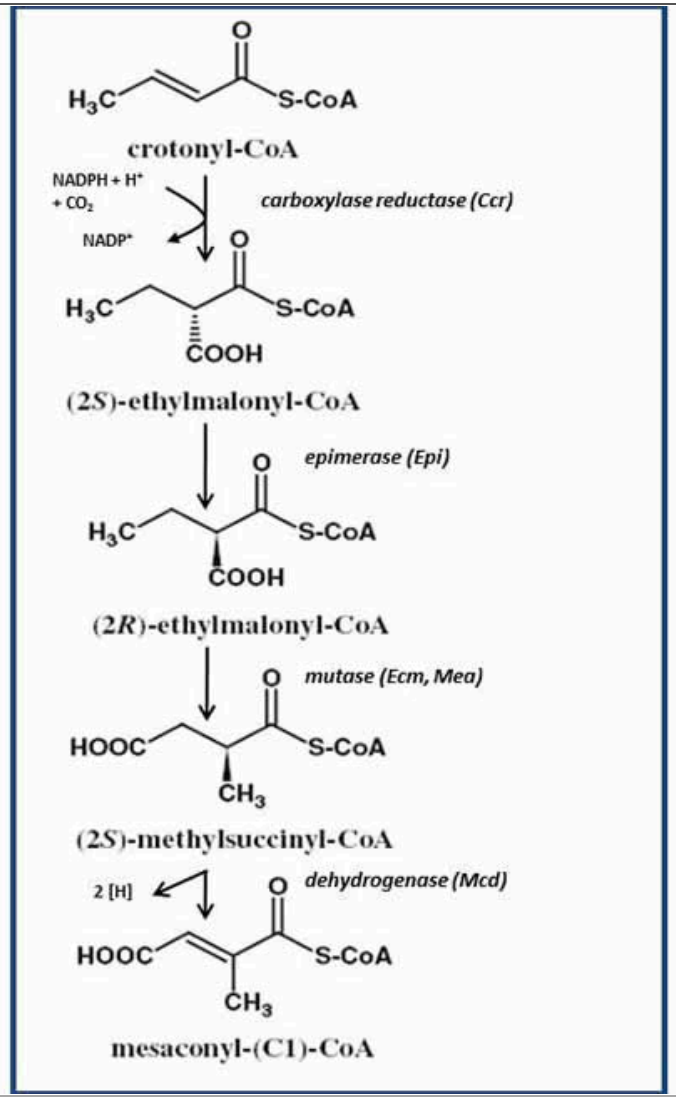
**Fig. 10**. The 'missing' part of the ethylmalonyl-CoA (EMC) pathway. The conversion of crotonyl-CoA to to mesaconyl-CoA depends on three novel enzymes:

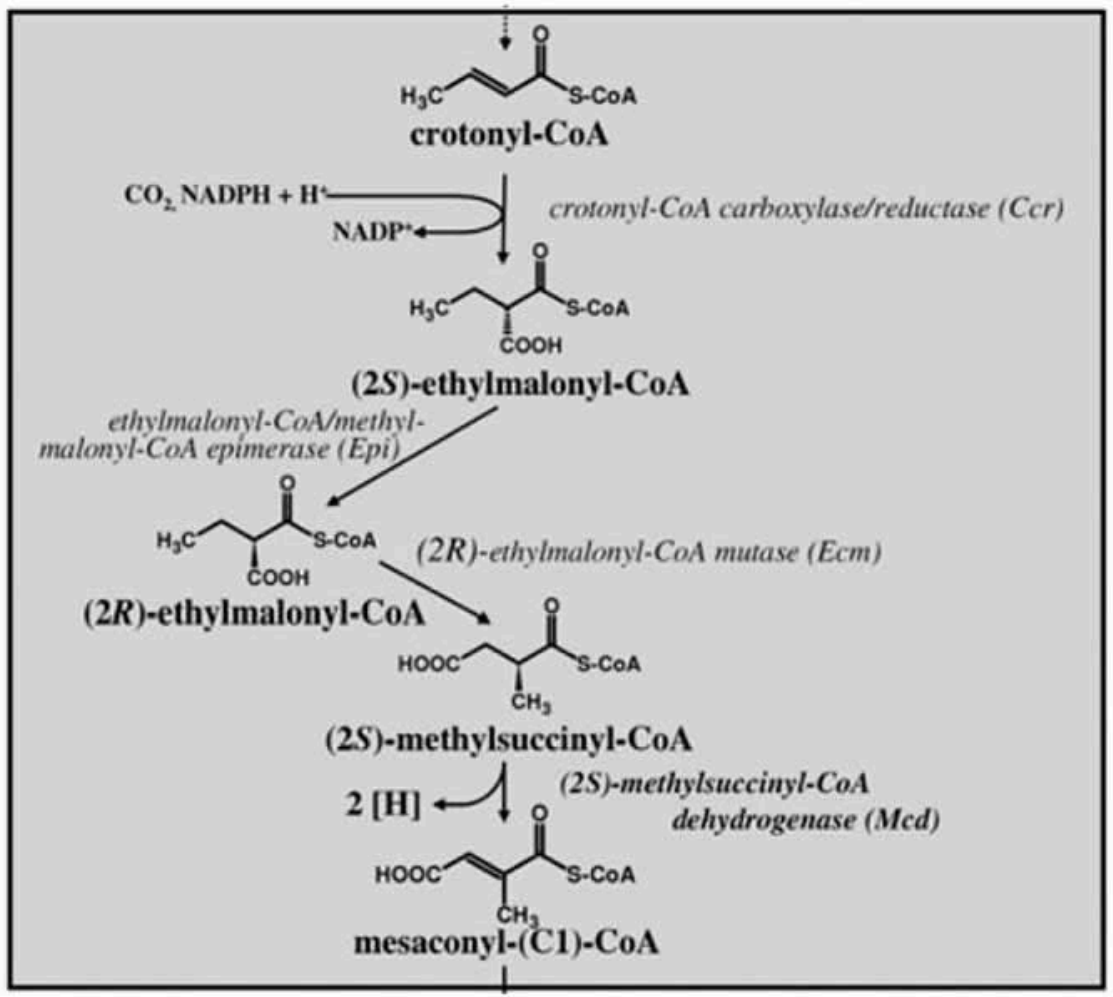
crotonyl-CoA carboxylase/reductase[44],

(2R)-ethylmalonyl-CoA mutase[46] and

(2)-methylsuccinyl-CoA dehydrogenase[47].

The two forms of ethylmalonyl-CoA are interconverted by ethylmalonyl-CoA/methylmalonyl-CoA epimerase.




crotonyl-CoA
CO2, NADPH + H+
NADP+
crotonyl-CoA carboxylase/reductase (Ccr)
(2S)-ethylmalonyl-CoA
ethylmalonyl-CoA/methyl-malonyl-CoA epimerase (Epi)
(2R)-ethylmalonyl-CoA mutase (Ecm)
(2R)-ethylmalonyl-CoA
(2S)-methylsuccinyl-CoA
2 [H]
(2S)-methylsuccinyl-CoA dehydrogenase (Mcd)
mesaconyl-(C1)-CoA

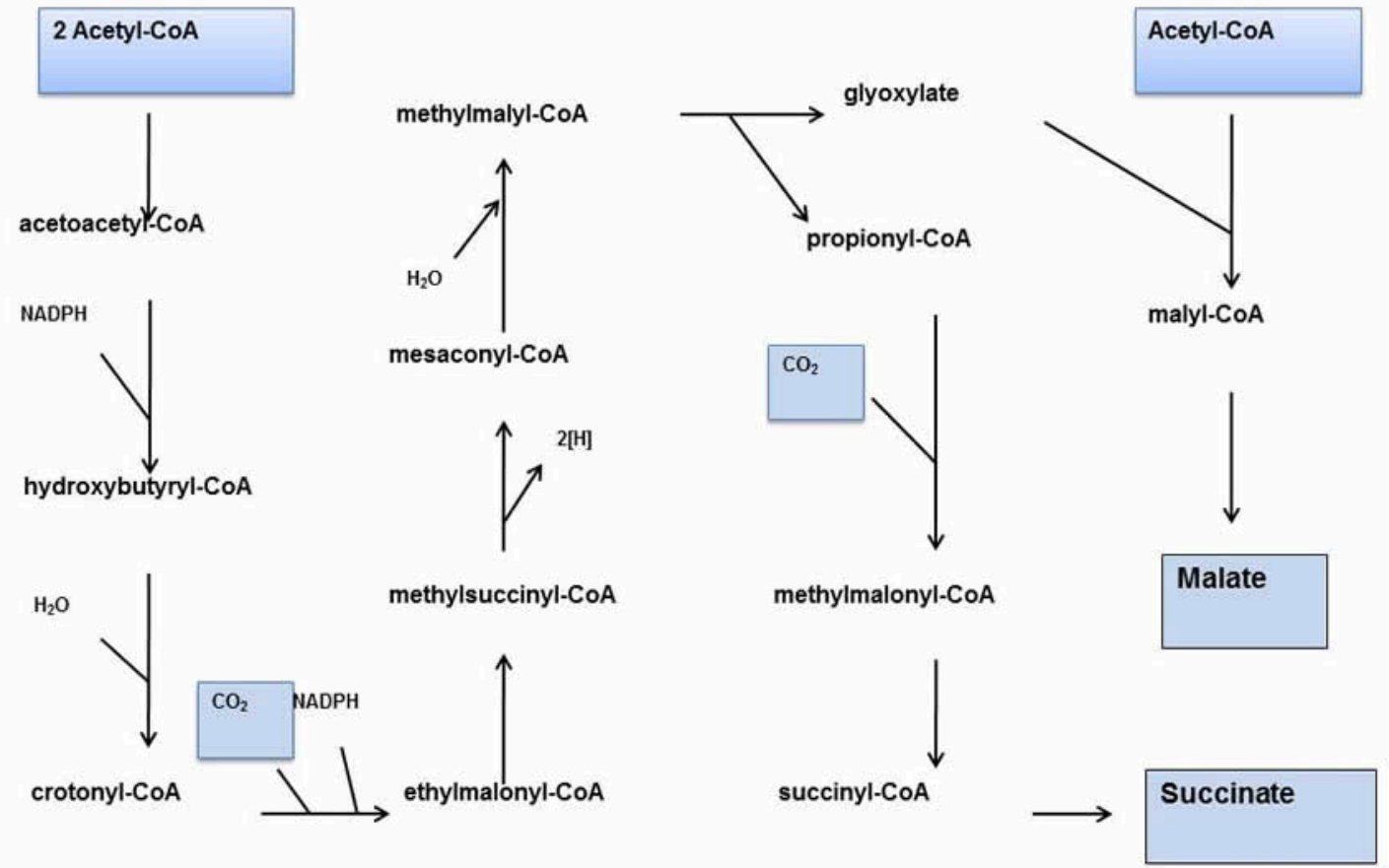



Fig. 12. **The ethylmalonyl-CoA (EMC) pathway for acetyl-CoA assimilation in Rhodobacter sphaeroides**, Note that there are two forms (R and S) of ethylmalonyl-CoA and two forms (R and S) of methylmalonyl-CoA (see Fig. 11) which are interconverted by the same epimerase.

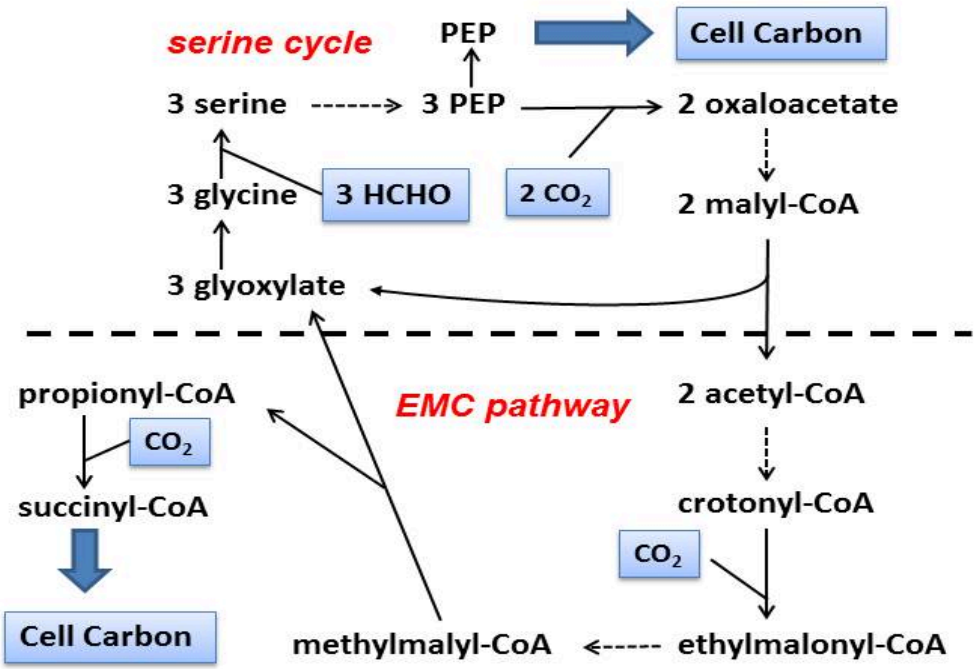



**Fig. 13. The serine/EMC cycle for assimilation of $C_1$ compounds by methylotrophs[44].** The ethylmalonyl-CoA (EMC) pathway for oxidation of acetyl-CoA to glyoxylate (lower half) (Fig. 12) is coupled to the serine cycle as shown on Fig. 3 (upper half). This is taken from the 2007 paper of Erb et al.[44] but for convenience only the carbon skeletons are shown. Dotted lines indicate that more than one reaction step is involved. Note that if acetyl-CoA is required as the biosynthetic precursor of membrane fatty acids or the storage compound poly 3-hydroxybutyrate then the EMC pathway is not required for oxidation of acetyl-CoA to glyoxylate.





Frieburg group: Georg Fuchs, Toby Erb and ? sorry



Celebrating
X, Georg Fuchs, Ivan Berg, Y, Z,
Toby

Sorry no picture of Birgit Alber

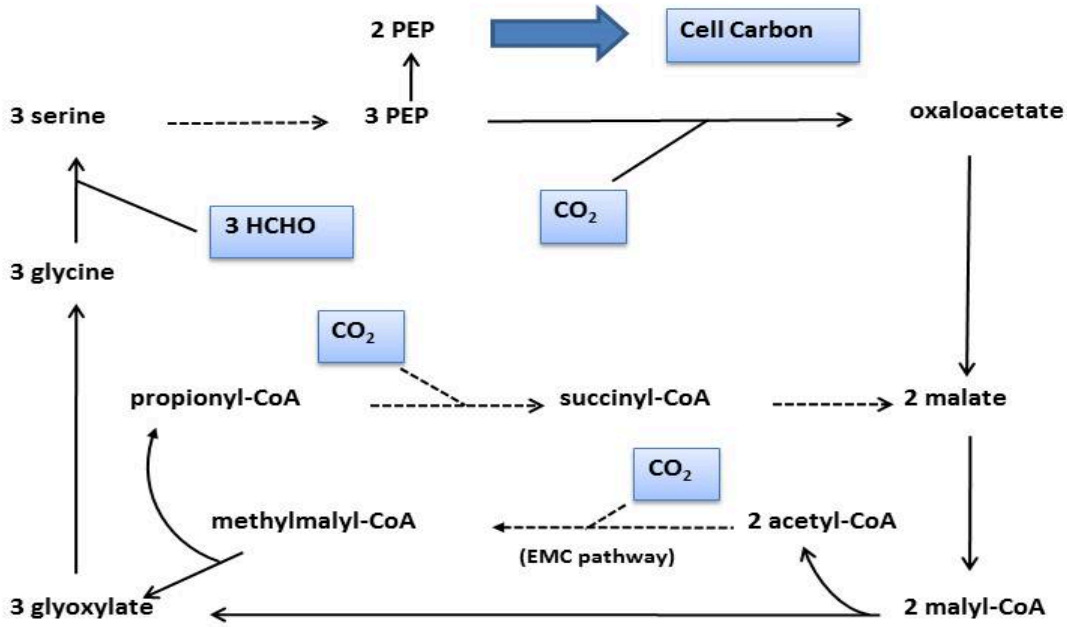
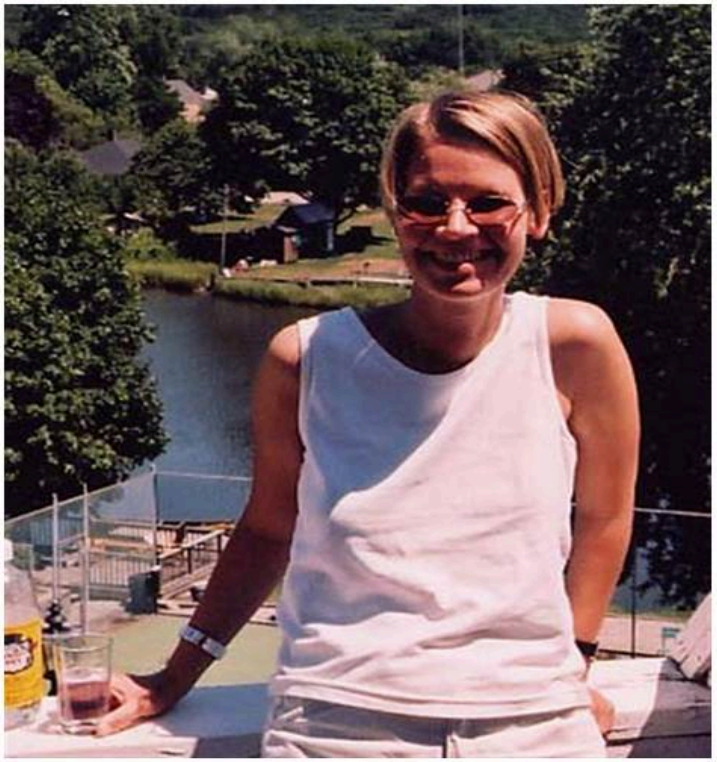



Fig. 14. **The serine/EMC cycle for assimilation of C1 compounds as it occurred during experiments described by Vorholt and colleagues[54]** (re-drawn for ease of comparison with Figs. 3 and 13). This depiction of the pathway shows the succinyl-CoA, derived from propionyl-CoA, being 'recycled' to produce a third glyoxylate.



Julia Vorholt; confirmation of the Ethylmalonyl pathway (Zurich)

Two other cycles for acetate assimilaion

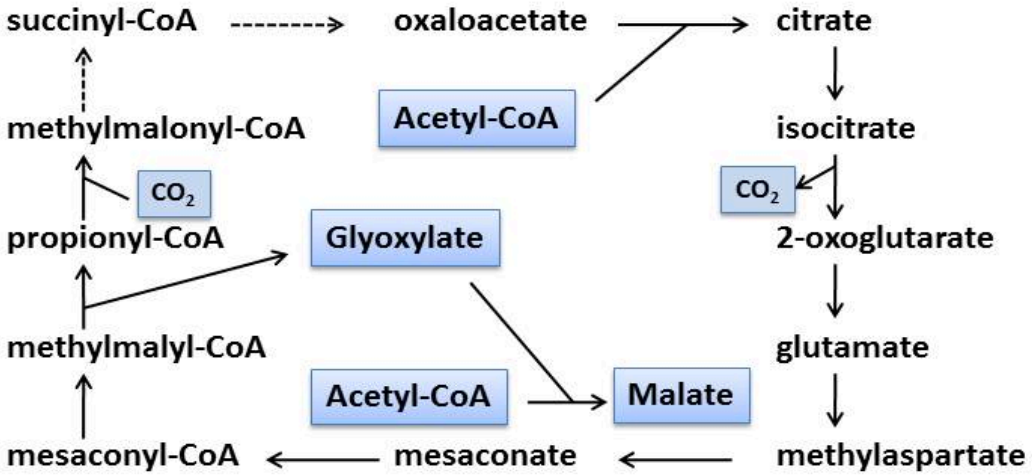



**Fig. 6. The methylaspartate cycle[24]**. This pathway for oxidation of acetyl-CoA to glyoxylate in methylotrophs was proposed in 1984 by Shimizu, Ueda and Sato[23]. Only the carbon skeletons have been included. The left hand side from mesaconyl-CoA to succinyl-CoA remains an essential part of the serine pathway as it is now understood. This cycle has recently been shown by Ivan Berg and colleagues to operate in haloarchaea for assimilation of $C_2$ compounds[24]. In the complete methylaspartate cycle the glyoxylate condenses with a second molecule of acetyl-CoA to give malate, the overall carbon balance being the same as the glyoxylate cycle (Fig. 1).

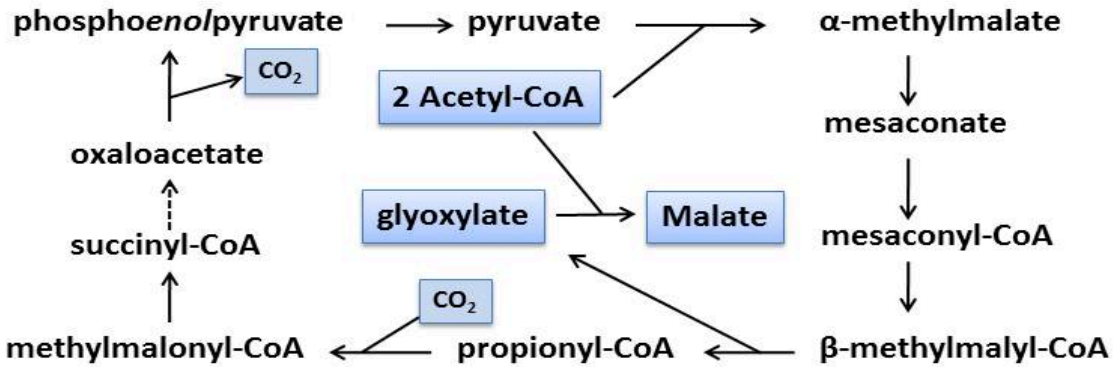



**Fig. 8.** The citramalate cycle proposed in 1977 for oxidation of acetyl-CoA to glyoxylate in Rhodospirillum rubrum by Ivanovsky's group in Moscow (note: citramalate is α-methylmalate)[39,40]. The pathway is completed by the condensation of the glyoxylate with a second acetyl-CoA to give malate.

Top of Serine/EMC cycle

Top of summaries

**HOME** **CONTACT ME**: Email **THANKS** for help to Hugh Anthony and Carl Gore

