ASPECTS OF ONE-CARBON METABOLISM IN MICRO-ORGANISMS

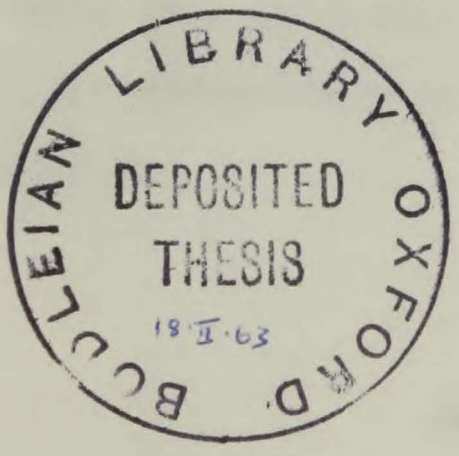
A THESIS

submitted by

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"Crawling at your feet," said the Gnat (Alice drew her feet back in some alarm), "you may observe a Bread-and-butter-fly. Its wings are thin slices of bread-and-butter, its body is a crust, and its head is a lump of sugar."

"And what does it live on?"

"Weak tea with cream in it."

A new difficulty came into Alice's head. "Supposing it couldn't find any?" she suggested.

"Then it would die, of course."

"But that must happen very often," Alice remarked thoughtfully.

Carroll (1872).

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SECTION I.

INTRODUCTION AND METHODS

Chapter I

INTRODUCTION

Living organisms must obtain from their environment all the nutrient materials necessary for their growth and reproduction. The process of growth may be regarded as having two aspects: (a) the generation of energy in a form utilisable for the biosynthesis of the cellular constituents of the organism (Krebs & Kornberg, 1957), and (b) the transformation of the carbon sources on which the organism is growing to a compound which can act as a direct precursor of cellular constituents.

Two sources of the energy for the growth of living organisms are known: light and nonphoto-chemical oxidoreduction reactions. Chemically useful energy for biosynthesis is regarded (Lipmann, 1941) as being stored in "energy-rich bonds" of certain organic compounds, of which adenosine-5^1-triphosphate (ATP) is the most important. Chemical syntheses by living cells have been found always to involve the fission of the "energy-rich" terminal or penultimate pyrophosphate bond of this compound. The production of ATP (Racker, 1961) involves the phosphorylation of adenosine diphosphate, (a) at substrate

level, as in anaerobic fermentative processes, (b) by oxidative phosphorylation or (c) by photosynthetic phosphorylation.

It has been recognised that from C_3 compounds such as pyruvate or 3-phosphoglycerate (PGA), established metabolic routes can lead to the carbon skeletons of all cell constituents (Bassham & Calvin, 1960; Kornberg & Elsden, 1961). The initial metabolic transformations undergone by a carbon source can thus be regarded as routes for the conversion of the substrate to PGA or pyruvate.

The problem of the synthesis of cell constituents from C_2 compounds by micro-organisms has been studied in detail over the last five years (Kornberg, 1959a; Kornberg & Elsden, 1961), and the reactions necessary to enable micro-organisms to grow on C_2 compounds as sole sources of carbon and energy are by now in most cases well-established. Similar studies on the growth of micro-organisms on C_1 compounds, however, have only recently been undertaken. It should be noted that with the exception of carbon dioxide, synthesis of all cell constituents from C_1 compounds is a property confined to micro-organisms.

GROWTH ON C_1 COMPOUNDS

Growth on carbon dioxide.

Since carbon dioxide represents the ultimate degradation product of all organic compounds, it cannot act as a simultaneous source of carbon and energy. It can only be utilised as a growth substrate in the presence of an ancillary energy source. This energy may be derived either from light, as in the green plants and photosynthetic bacteria, or from the oxidation of inorganic compounds, as in the chemosynthetic autotrophs. The possibility has also always been recognised (though it has not been demonstrated directly until recently, see below) that organisms might exist which synthesised all their cellular constituents from carbon dioxide utilising for this process energy derived from the oxidation of organic compounds.

The pathway by which green photosynthetic organisms synthesise their cellular constituents from carbon dioxide has been elucidated principally by Calvin and co-workers. This mechanism has been described variously as the photosynthetic carbon cycle, the ribulose diphosphate (RuDP) cycle and the reductive pentose phosphate cycle. Details of the pathway are set out in reviews (Bassham & Calvin,

1960; Krebs & Kornberg, 1957).

Evidence of varying degrees of thoroughness has also been obtained that the RuDP cycle operates in the chemosynthetic autotrophs, which synthesise their cell constituents from carbon dioxide using energy obtained by the oxidation of inorganic compounds such as hydrogen, sulphides, thiosulphates, nitrites and ammonia (Quayle, 1961).

Similar evidence has recently been obtained for the green photosynthetic bacteria (Smillie, Rigopoulos & Kelly, 1962), the purple sulphur photosynthetic bacteria (Fuller, Smillie, Sisler & Kornberg, 1961) and the purple non-sulphur photosynthetic bacteria (Glover, Kamen & van Genderen, 1952; Stoppani, Fuller & Calvin, 1955); when these organisms are growing autotrophically in the light utilising inorganic electron donors, and carbon dioxide as sole carbon source.

Growth on organic C_1 compounds.

A number of organisms have been described which are capable of growth on organic C_1 compounds, such as formate, methanol or methane, as sole sources of carbon and energy, either aerobically or anaerobically. A detailed list of the organisms whose ability to grow on C_1 substrates has

TABLE I. 1.

ORGANISMS CAPABLE OF GROWTH ON C_1 COMPOUNDS AS SOLE SOURCE OF CARBON AND ENERGY

ORGANISM	REFERENCE	DESCRIPTION	COMPOUNDS SUPPORTING GROWTH
A. AEROBES			
1. Pink group.			
Bacillus methylicus	Loew (1892)	Pink-pigmented rods, 1 x 2 μ. Non-spore-forming.	Methanol Formaldehyde Formate Methylamine
Pseudomonas extorquens (Syn. Bacillus e., Vibrio e., (Bergey, 1957), Flavobacterium e.).	Bassalik (1913) Janota (1950, 1956, 1957) Bassalik, Janota-Bassalik & Brisou (1960)	Pink-pigmented rods, 1.5 x 3 μ. Non-spore-forming. Single polar flagellum	Methanol Formate Methylamine * Oxalate and others
Protaminobacter ruber	den Dooren de Jong (1926, 1927)	Pink-pigmented rods, non-spore forming. Single polar flagellum (Weaver, Samuels and Sherago, 1938).	Methanol * Formate * Methylamine and others.

TABLE I. 1 (continued)

Pseudomonas methanica (Syn. Methanomonas m., Bergey, 1957).	Söhngen (1906) Dworkin & Foster (1956) Leadbetter & Foster (1958)	27 strains isolated by Leadbetter & Foster. Most pink, but red, brown, yellow and colourless varieties recognised. Single polar flagellum, 0.4 x 1.5 μ	Methane Methanol Methylamine No others	*
	Harrington & Kallio (1960)	Red-pigmented rods 0.3 x 0.7 μ	Methanol No others	
Pseudomonas PRL-W4	Kaneda & Roxburgh (1959 a,b,c)	Pink-pigmented rods 0.5 x 1.2μ Non-spore-forming. Single polar flagellum. Requires biotin for growth on methanol	Methanol and others	
Pseudomonas AM1	Peel & Quayle (1961) Large, Peel & Quayle (1961)	Pink-pigmented rods 0.8 x 2 μ Non-spore-forming. Single polar flagellum.	Methanol Formate Methylamine and others	

TABLE I. 1 (continued)

2. Colourless group

Pseudomonas aminovorans	den Dooren de Jong (1926, 1927)	7 strains isolated. Colourless or yellow. Single polar flagellum. Non-spore-forming.	Methanol Formate Methylamine Formamide and others.
Pseudomonas oxalaticus	Khambata & Bhat (1953) Quayle & Keech (1959 a, b)	Colourless rods, 0.3 x 0.9 μ. Single polar flagellum. Non-spore-forming.	Formate Oxalate and others.
Hyphomicrobium vulgare	Mevius (1953) Näveke & Engel (1955) Large, Peel & Quayle (1961)	Oval cells producing fine threads, at the end of which a bud is formed, developing into a new cell. Cells remain together to form fibrous colonies.	Methanol Formate Methylamine Oxalate and others.
Hydrogenomonas carboxydovorans	Kistner (1953, 1954)	Colourless, Gram-negative rods. Single polar flagellum.	Carbon monoxide and others.
Methanomonas methanooxidans	Strawinski & Brown (1957) Brown & Strawinski (1958)	-	Methane
Vibrio oxaliticus	Bhat & Barker (1948)	Colourless, curved rods, 0.4 x 1.3 μ. Single polar flagellum	Formate (requires yeast extract in addition) Oxalate and others

TABLE I. 1 (continued)

Bacillus sphaericus	Iyer & Kallio (1958)	Gram positive Spore-forming rod.	N-methyl-urea and others
B. ANAEROBES			
Methanosarcina barkerii	Schnellen (1947)	Colourless spherical cells, arranged in cubicle sarcina packets	Carbon Monoxide Methanol Acetate
Methanococcus vannielii	Stadtman & Barker (1951)	Colourless, spherical cells, aggregating in chains.	Formate
Methanobacterium formicicum	Schnellen (1947) Kluyver & Schnellen (1947)	Colourless rods, non-spore-forming	Formate Carbon monoxide

* Unpublished observations recorded in chapter XI.

been conclusively established is given in table I.1.

For many years, there was speculation that these organisms might be autotrophic, i.e. might utilise the energy obtained by oxidation of their one-carbon growth substrate to carbon dioxide to synthesise their cell constituents from the latter. This is exemplified by Stephenson (1930) who, without explicit comment, included bacteria which can grow aerobically on methane, methanol, formaldehyde, formate and carbon monoxide as sole carbon and energy sources in a chapter on chemosynthetic autotrophs. This speculation was first explicitly stated by Bhat & Barker (1948) and van Niel (1954). Much of the early work on these organisms consisted of attempts, by indirect biochemical methods, to find out whether these organisms could derive all or most of their cellular material from carbon dioxide.

Hyphomicrobium vulgare.

Studies have been made by Mevius (1953), Näveke & Engel (1955) and Näveke (1957) on the stalked bacterium Hyphomicrobium vulgare, which can grow on methanol or formate as sole carbon and energy sources. After extensive studies on formate oxidation by this organism, these workers

concluded that H. vulgare couples the oxidation of formate to carbon dioxide with the reduction of the latter to cellular material, and hence behaves as an autotroph. The indirect evidence for this conclusion is summarised by Näveke & Engel (1957) as follows: (a) slow growth (Mevius, 1953); (b) no growth on glucose, sucrose, mannose or raffinose (Mevius, 1953); (c) no growth on removal of carbon dioxide; (d) the presence of $^{14}CO_2$ in the growth phase leads to the incorporation of radio-activity into the amino acids of the cell protein, particularly into glutamate, glycine and aspartate (Mevius, 1953; Näveke, 1957); (e) the organism oxidises formate and oxalate to carbon dioxide and water and releases 85-95% of the energy as heat (Korte & Engel, 1955). Since the nitrifying bacteria oxidise nitrite to nitrate with the release of 93-95% of the available energy as heat, the authors state that the similarity in formate or oxalate oxidation by H. vulgare leads to the probability that these substrates serve only as energy sources and not as carbon sources. This evidence is very indirect, and has been criticised (Quayle, 1961; Large, Peel & Quayle, 1961). Later work indicates that the organism is probably heterotrophic (see below).

Pseudomonas methanica.

The next organism to be studied was Pseudomonas methanica. This organism, originally isolated by Söhngen (1906) and named Methanomonas methanica by Orla-Jensen (1909) grows aerobically in a simple mineral salt medium with methane gas as sole carbon and energy source. The organism was reisolated by Dworkin & Foster (1956) from pond water and renamed by them Pseudomonas methanica. The organism had the pink pigmentation characteristic of many strains of C_1-utilising organisms (see table I.1). Its most unusual property was that, of a large range of carbon sources tested, only two, methane and methanol, were found to be utilised as growth substrates. Only 18% of the methane utilised was converted to carbon dioxide, the rest appeared as cellular material and extracellular slime. Methane-grown cells readily oxidised methane and also (after a 20 min. lag period) methanol, formaldehyde and sodium formate. Subsequent work in the same laboratory (Leadbetter & Foster, 1958) led to the isolation of over thirty pure cultures of obligate methane-utilising organisms, and these were all identified as differently pigmented strains of P. methanica. The pink strain was shown to be the parent strain, the others were described as varieties.

Leadbetter and Foster (1958) also reported a series of experiments designed to determine what fraction of the cell material of methane-grown cells was derived from carbon dioxide, and how much from reduced C_1 units. This is obviously a very important approach to determining whether the organism is an autotroph, which would derive all its cellular carbon from carbon dioxide, or a heterotroph which assimilates at least some of its cellular carbon at a more reduced level than that of carbon dioxide. The cells were grown in closed flasks on methane-air in the presence of $^{14}CO_2$. Two heterotrophic bacteria were included as controls, both growing on a glucose-salts medium in air containing $^{14}CO_2$. It was found that with P. methanica the specific activity of the cellular carbon was 20 to 70% of the exogenous $^{14}CO_2$, varying with the strain of organism used, while with the glucose-grown heterotrophs the figures were 23% for Escherichia coli and 30% for P. fluorescens. These figures are almost certainly too high, since later in the incubation, the $^{14}CO_2$ will be diluted with unlabelled carbon dioxide derived from the methane. However, they do serve to indicate that a substantial proportion of the cellular material is derived from a reduced C_1 unit arising from methane and not in equilibrium with exogenous carbon

dioxide. This shows that the organisms are heterotrophic, though the contribution of carbon dioxide to cellular material is probably greater than in glucose-grown heterotrophs, at least with some strains of P. methanica.

Bacillus sphaericus.

In 1958, Iyer & Kallio isolated a strain of Bacillus sphaericus which could grow on N-methylurea, $CH_3.NH.CO.NH_2$, as sole carbon and nitrogen source. Since the two carbon atoms of methylurea are separated by a nitrogen atom, this represents a case of growth at the expense of C_1 compounds. The degradation of methylurea by whole cells and extracts was found to involve hydrolysis to methylamine, ammonia and carbon dioxide. The methylamine was then oxidised to formaldehyde and ammonia, and the formaldehyde was oxidised to carbon dioxide. No experiments on the pathway of carbon assimilation were reported.

Pseudomonas oxalaticus.

This organism, isolated by Khambata & Bhat (1953) from earthworm intestine, can grow on formate or oxalate as sole source of carbon and energy. The pathway of formate assimilation was studied in detail by Quayle & Keech (1958, 1959a,b) and shown to be of the autotrophic type long

postulated for C_1 organisms (see above). Evidence for this was as follows:

1. When [^{14}C]formate or [^{14}C]bicarbonate was administered to cells growing on formate and the pathway of assimilation followed by the short term kinetic techniques described in chapter III, the first radioactive product formed was PGA. The only other compounds found at early times were sugar phosphates and malate, and they contained relatively little radioactivity.

2. When two cultures of P. oxalaticus were grown on [^{14}C]formate as sole carbon source, one aerated with air, and the other with air - CO_2 (95 : 5 v/v), the specific activity of the cellular material derived from the latter culture was 17-fold lower than that derived from the former, indicating that at least 94% of the cellular carbon passes through a stage in equilibrium with exogenous carbon dioxide.

3. Cell-free extracts of formate-grown P. oxalaticus were shown to contain carboxydismutase and phosphoribulokinase, the two enzymes unique to the RuDP cycle. The latter enzymes were not present if the organism was grown on any substrate other than formate. This evidence shows beyond all doubt that the growth of P. oxalaticus on formate involves an autotrophic pathway of an analogous type to that observed

in the hydrogen - or nitrite - oxidising chemosynthetic autotrophs.

This autotrophic growth on C_1 compounds, has only been demonstrated conclusively in formate-grown P. oxalaticus (see chapter XII). It might be expected that growth on C_1 compounds more reduced than formate might involve a heterotrophic pathway in which some at least of the cellular carbon is derived directly from a reduced C_1 unit, as indicated by the experiments of Leadbetter & Foster (1958).

Pseudomonas PRL-W4.

The first study of growth on reduced C_1 compounds in which early products of assimilation were identified was the work of Kaneda & Roxburgh (1959a,b,c) with P. PRL-W4. This organism, isolated from soil, could grow on methanol as sole carbon and energy source. In addition it could grow on many other substrates. Formate, formaldehyde and methane were among the substrates not utilised for growth. Growth on methanol was slow (mean generation time, 72 hr.), and biotin was required as a growth factor. Methanol-grown cells would oxidise methanol and formate, whereas cells grown on aspartate or succinate would oxidise only formate.

When cell suspensions were incubated with [^{14}C]methanol or [^{14}C]bicarbonate for short periods of time (up to 80 sec.), the only compound to acquire radioactivity in the first 50 sec. was identified as serine. Two other compounds were detected at later times, but were not identified. Thus in this organism growth on methanol cannot be an autotrophic process, or PGA would have been detected as the first labelled intermediate. The authors suggested that the port of entry of methanol into cellular material is via serine transhydroxymethylase:

$$CH_3OH \longrightarrow \text{"Active HCHO"} \qquad \text{(i)}$$

$$\text{"Active HCHO"} + \text{Glycine} \longrightarrow \text{Serine} \qquad \text{(ii)}$$

Kaneda & Roxburgh (1959c) also performed a series of experiments in which radioactive methanol was incubated with growing cells, resting cells and extracts in the presence of unlabelled formaldehyde, formate and carbonate. They measured the specific activity of the cellular material and respiratory carbon dioxide or else the overall fixation of radioactivity by the culture. In addition, they showed that while fixation of [^{14}C]methanol by sonic extracts of P. PRL-W4 was not stimulated by the addition of glycine or serine alone, it was stimulated when both were added together. Extracts were found to contain NAD-linked dehydrogenases for formate and methanol, but not for formaldehyde. On the

basis of these findings, they proposed a scheme for carbon assimilation in which serine, the precursor of cellular carbon, could be formed from both formate (derived from carbon dioxide by reduction) and formaldehyde (derived from methanol by oxidation). The energy required for carbon dioxide fixation by way of formate was supposed to be derived from the oxidation of methanol or formate, but not formaldehyde.

This work provides a direct demonstration of the nature of the first product of methanol assimilation and a clear demonstration that growth on methanol is not autotrophic.

Other organisms.

Recently, another pink C_1 organism has been isolated. This organism, isolated from Iowa soil by Harrington & Kallio (1960), grows on methanol as sole source of carbon and energy. Since it would not grow on any other carbon source tested, these authors suggested that it was a strain of Pseudomonas methanica. However, it would not grow on methane, and since obligate methane dependence has been emphasised repeatedly (Dworkin & Foster, 1956; Leadbetter & Foster, 1958) as an essential taxonomic criterion for

P. methanica, it is difficult to justify this description. Like P. methanica and P. AM1 (see below), the organism exhibited "clumped" growth on liquid media. Whole cells oxidised methanol, formaldehyde and formate readily. Formaldehyde was demonstrated as an intermediate of methanol oxidation by use of bisulphite as a trapping agent. Methanol oxidation was 100% inhibited by 5mM-ethylene-diaminetetraacetate (EDTA) and by 10mM -$\alpha\alpha'$-dipyridyl, but neither compound had any appreciable effect on formaldehyde oxidation.

A study of methanol and formaldehyde oxidation in extracts was made by these authors, details of which are given in chapter VI below, but no study of the pathway of carbon assimilation was undertaken. The same workers (Kallio & Harrington, 1960) also demonstrated that sudanophilic granules observed in P. methanica are largely composed of poly-β-hydroxybutyrate, a storage compound now known to be widely distributed in bacteria.

Pseudomonas AM1.

The organism used in most of the work reported in this thesis, Pseudomonas AM1, was isolated by Peel (Peel, 1961 p.17; Peel & Quayle, 1961) as a contaminant in a methylamine hydrochloride growth medium. After repeated subculture

the organism was obtained in pure culture and found to be a Gram-negative rod 0.8μ x 2.0μ, motile with a single polar flagellum. It had thus all the morphological characteristics of the genus Pseudomonas.

The organism grew readily over the pH range 6.0 - 8.5 at an optimum temperature of 30°. Many compounds would serve as growth substrates, the best of which were methanol (mean generation time, 4 - 10 hr.), methylamine hydrochloride, sodium formate, ethanol, lactate, succinate and malate. The organism would not grow on oxalate, formaldehyde, amino acids, methane-air (1 : 1 by vol.), or CO_2-hydrogen-air (1 : 4 : 15 by vol.). Unshaken cultures produced a thin pellicle, and shaken cultures had a tendency to aggregate in clumps, particularly during growth on formate and methylamine hydrochloride.

The organism produced a red intracellular pigment which has recently been shown (Goodwin, personal communication) to be a carotenoid similar in properties to the pigment spirilloxanthin found in the purple photo-synthetic bacteria. The organism, like P. methanica, contained poly-β-hydroxybutyrate comprising 6% of the dry weight of methanol-grown cells.

Pseudomonas AM1 thus closely resembles many of the pink organisms listed in table I.1. All are Gram-negative rods, motile with a single polar flagellum, and which can grow aerobically on C_1 compounds as sole source of carbon and energy. The taxonomy of P. AM1 has been discussed by Peel (1961, p.20) and Peel & Quayle (1961).

The relative ease with which P. AM1 grows on several C_1 compounds makes it a valuable organism for the study of microbial growth on such compounds, and at the beginning of the present investigation, studies on the biosynthesis of cell constituents from methanol had been initiated by Peel (1961). When [^{14}C]methanol was administered in short term kinetic incubation experiments to P. AM1 growing on unlabelled methanol, the first detectable labelled product was serine. When [^{14}C]bicarbonate was administered, the product was malate. This indicated that, in P. AM1, growth on methanol proceeds via a similar pathway to that reported by Kaneda & Roxburgh (1959a) for growth of P. PRL-W4 on methanol. When P. AM1 was grown on [^{14}C]methanol aerated with air containing 1% CO_2 (v/v), the specific radioactivity of the cells so obtained was only half that of cells grown under the same conditions but aerated with CO_2-free air. This experiment indicated that during growth on methanol,

at least 50% of the cellular carbon is assimilated at a level exchangeable with exogenous carbon dioxide. When P AM1 was grown on [^{14}C]succinate under the same conditions, Peel (1961, p. 26; Large et al., 1961) found that flushing with air-carbon dioxide reduced the cellular specific radioactivity by only 7%.

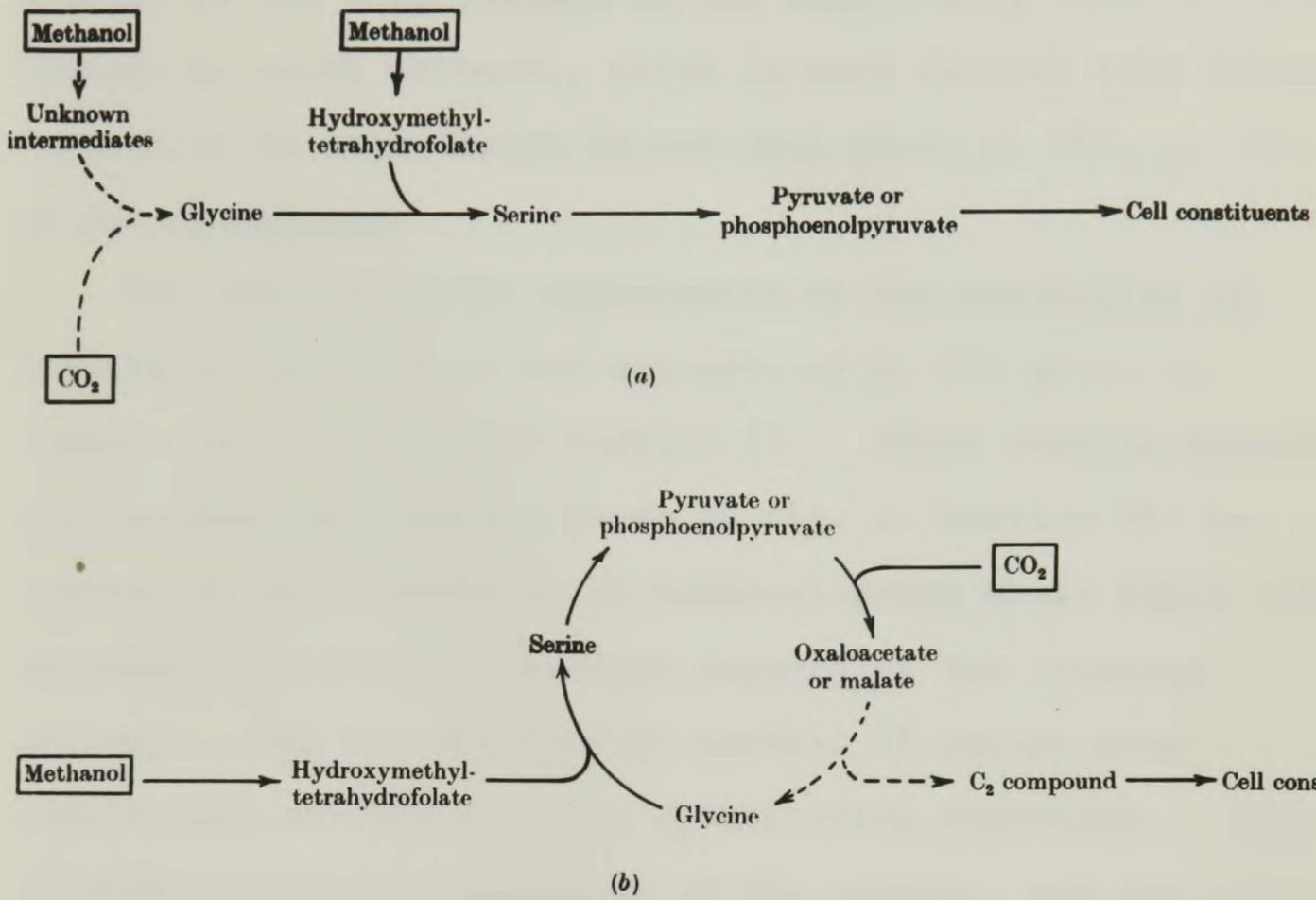
Similar kinetic experiments by Dr. J.R. Quayle using [^{14}C]methanol and [^{14}C]bicarbonate were also performed on Hyphomicrobium vulgare (Large et al., 1961), and essentially similar results were obtained, indicating that, contrary to the views of Näveke & Engel (1955), this organism is heterotrophic.

On the basis of these observations, a scheme was proposed (Peel, 1961, p. 36; Large et al., 1962; see chapter III) for the biosynthesis of cell constituents from methanol, in which half the cellular carbon is assimilated at the oxidation level of carbon dioxide, the remainder from a reduced C_1 unit. The formation of serine from a reduced C_1 unit is an essential part of this scheme, which is shown in fig. 1.

The present investigation.

At the outset of this work, it was important to study the pathway of biosynthesis of cell constituents from

Fig. 1



Possible routes for synthesis of cell constituents from methanol by *Pseudomonas* AM1. The broken lines represent the hypothetical schemes for the crucial step in the biosynthesis, namely the net synthesis of glycine from C_1 units. (*a*), Direct synthesis of glycine; (*b*), cyclic synthesis of glycine.

formate by P. AM1, to determine whether it followed the same autotrophic pathway as formate in P. oxalaticus, or whether it was incorporated by the essentially heterotrophic pathway by which methanol, which is more reduced than formate, appears to be assimilated in methanol-grown P. AM1, P. PRL-W4 and H. vulgare.

The results of the experiments on the metabolism of formate by whole cells and extracts of P. AM1 grown on formate are described in section II. These results supported the hypothetical pathway shown in fig. 1. Section III is devoted to an examination of methanol-grown whole cells and extracts, for further evidence supporting the proposed pathway. The two chapters in section IV report some preliminary studies on other C_1-utilising organisms. Each chapter includes a discussion of the results, and the final chapter (XIII) presents a brief general discussion of the work as a whole.

Some of the work described in this thesis has already been published (see appendix IV).

A list of abbreviations used in the thesis will be found in appendix I.

Chapter II

METHODS

The techniques described in this chapter are general methods which were used throughout the work. Techniques used in particular cases are described in the appropriate chapter.

General techniques.

The usual sterile precautions were observed in all microbiological work. All glassware was acid-cleaned and washed repeatedly in tap water and distilled water. A.R. purity chemicals were used wherever possible. The sources of special chemicals are listed in appendices II and III.

Maintenance and growth of the organism.

Pseudomonas AM1 was grown in aerobic cultures at 30°. All growth media contained the same concentrations of inorganic salts as those used by Jayasuriya (1955) for the growth of Pseudomonas OD1. Stock solutions of phosphate buffer and mineral salts were diluted 1 to 20 for use in the medium where the final concentrations were: (all figures

are mg./100ml.) KH_2PO_4 136, anhydrous Na_2HPO_4 213, $(NH_4)_2SO_4$ 50, $MgSO_4.7H_2O$ 20, $CaCl_2.2H_2O$ 1, $FeSO_4.7H_2O$ 0.5, $MnSO_4.5H_2O$ 0.25, $Na_2MoO_4.2H_2O$ 0.25. The carbon sources for liquid culture were either sodium formate 0.1M or methanol (A.R.) 0.5% v/v. The latter substrate was added by sterile pipette after the medium had been autoclaved and immediately before inoculation. Stock cultures were grown on slopes of 0.1M -methylammonium chloride (methylamine hydrochloride) solidified with 1% w/v agar-agar, stored at 2° and subcultured each month. To grow the organism in liquid culture, a small inoculum was transferred from a slope or from another liquid culture into 10 ml. of liquid medium. This was incubated at 30° for 24-48 hr., and was then introduced into either a 1 l. conical culture flask (Fernbach flask) containing 200 ml. of medium, or a 2 l. Carrel culture flask containing 400 ml. of medium, and the cells were then grown on a reciprocal shaker for 24-48 hr. Large quantities of the organism were grown in 5, 10 or 20 l. of medium in a large stoppered bottle inoculated with 400 ml. of an exponentially growing culture, vigorously aerated by blowing a stream of sterile air through a block of pumice fixed to the end of a submerged inlet tube. The bacteria were harvested whilst

in the logarithmic phase of growth at cell densities of 0.2 - 0.4 mg. dry wt./ml. by centrifuging at 1500 x g for 5 - 10 min. at 4°. Larger quantities were harvested at room temperature in a Sharples continuous-flow centrifuge. Cells to be used for the preparation of extracts or for manometric studies were washed once with 0.9% w/v K Cl.

Growth on methanol was followed by measuring the turbidity of cultures with a nephelometer (Evans Electro-selenium Ltd., Essex), previously calibrated in terms of dry wt. of cells/ml. of suspension. The calibration curve was linear at the cell densities used. The growth of cultures on formate could only be followed nephelometrically at low cell densities. At higher densities, the cells agreggated to form clumps which tended to settle to the bottom of the flask as soon as aeration ceased.

Succinate-grown cells (see chapters IV and V) were grown on the Jayasuriya salt medium containing as carbon source 0.05M-sodium succinate.

Preparation of cell-free extracts.

Formate-grown cells were disintegrated by crushing the frozen bacteria in the Hughes press (Hughes, 1951). 0.4 - 1.0 g. wet wt. of cells were suspended in 1 - 2 ml. of

40mM-tris hydrochloride pH 7.5 and crushed in a Hughes press which had been cooled to -25°. After crushing, the press was opened, the frozen contents rapidly removed with a cold spatula, about 200 µg each of crystalline ribonuclease and deoxyribonuclease added, and the extract allowed to warm up to 0°.

Methanol-grown cells were usually disrupted by ultrasonic oscillation. 1 g. wet wt. of cells were suspended in 5 ml. 40 mM-tris hydrochloride pH7.5, containing 50 µmoles of glutathione or mercaptoethanol. The suspension was cooled in ice and subjected for 2 - 5 min. to the full output at 25 Kcyc./sec. of a 600 W Mullard ultrasonic oscillator. The magneto-strictor was brazed to the bottom of a stainless steel conical flask containing the suspension, and the whole assembly was cooled in a rapidly-flowing stream of tap water.

Extracts prepared by both methods were either used as such, or more usually centrifuged at 25000 x g for 10 min. in the high speed angle head of an International refrigerated centrifuge (model PR - 2). The extracts contained 2 - 20 mg. of protein/ml.

Protein determinations.

Protein was estimated by the Folin-Ciocalteu method,

as described by Lowry, Rosebrough, Farr & Randall (1951).

Radioactive chemicals.

Carbon-14-labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks. with the exception of DL-[2 - ^{14}C]malic acid, which was a gift from Dr. J.A. Bassham.

[^{14}C]methanol was supplied in a sealed tube under vacuum. One end of the tube was cooled in liquid air, and the other end was then opened. Water was added to the methanol whilst the tube was still in liquid air. After slow thawing, the solution of [^{14}C]methanol was transferred to a stoppered tube and stored at -10°.

$Ba^{14}CO_3$ was decomposed with an excess of 2N-$HClO_4$ and the liberated $^{14}CO_2$ was trapped in the theoretical quantity of NaOH using a transfer unit similar to that described by Aronoff (1956).

Sodium [^{14}C]formate was chromatographed on Whatman No. 4 filter paper in ethanol-ammonia-water (see below). After development, the papers were sprayed with 0.5M-K_2HPO_4 and dried in air. After location of the formate area by radioautography, it was eluted with water into one arm of a transfer unit (Aronoff, 1956) and the formic acid was liberated with 5N-H_2SO_4 and distilled *in vacuo* into the

theoretical quantity of NaOH solution.

[2,3-$^{14}C_2$]succinic acid, [2,3-$^{14}C_2$]fumaric acid and DL-[2-^{14}C]malic acid were chromatographed on paper in butan-1-ol-propionic acid-water (see below) and the radioactive area eluted.

[^{14}C]glycine and [^{14}C]serine were used without further purification.

Assay of radioactive materials.

Radioactivity was assayed with either a mica end-window Geiger-Müller (GM) tube connected to a suitable scaler; or a gas-flow counter with automatic sample changer (Model D-47, Nuclear Instrument and Chemical Corpn., Chicago, U.S.A.) with a thin window between the sample and the gas. Under these conditions, the counting efficiency was 15%. The general principles of counting radioactive materials on chromatograms, planchets and as $Ba^{14}CO_3$ were as follows:

Paper chromatograms. After location of the spots by radioautography, the radioactive areas were outlined in pencil on the chromatograms and counted on one side of the paper with a mica end window GM tube (type 2B2, General Electric Co.) standing on a Perspex mask out of which a

suitable square had been cut. Each area was counted for a total of at least 1000 counts which gives a standard error of radioassay of about ±3%. The counts recorded were corrected for background radioactivity.

Non-volatile radioactive material. The material was suspended in a known volume of liquid, and aliquots were applied to planchets (3 cm^2 area) on a rotating turntable and dried in a stream of warm air. The planchets were either of ground glass or thin aluminium discs 1 inch in diameter, lightly sandblasted on the upper surace. Volatile tracer ($[^{14}C]$carbonate, $[^{14}C]$methanol or $[^{14}C]$formate), if present, was removed by irrigating the planchets with 0.1 ml of conc. formic acid (90% w/v A.R.), and drying. In the case of $[^{14}C]$formate repeated applications of formic acid were necessary to remove the tracer completely. Blank determinations showed that this treatment removed all the volatile starting material. The samples were infinitely thin (<0.2 mg. dry wt./cm^2). They were assayed either with the gas flow counter or with a lead shielded GM tube and corrected for background activity. The count of a radioactive sample on paper after chromatography and on a glass planchet counted with a GM tube or with the gas flow

counter was in the ratio 2 : 3 : 12 (Peel, 1961, p.11). Duplicates agreed well with one another.

Plating of $Ba^{14}CO_3$. $^{14}CO_2$ collected from combustion or degradation of organic compounds (see chapter VII) or from respiring cells in manometer flasks was trapped as $Na_2{}^{14}CO_3$ in 2.5N- or 2N-NaOH (freed from CO_2 by being made from saturated NaOH and distilled water which had been boiled and allowed to cool under soda-lime). It was then converted to $Ba^{14}CO_3$ by precipitation with aqueous CO_2-free 5% $BaCl_2$ as described in detail by Sakami (1955, p.5). The precipitated $Ba^{14}CO_3$ was collected on weighed filter paper discs (2.69 cm^2 area), dried under an infra-red lamp, weighed, and assayed for radioactivity either in the gas-flow counter or with the GM tube. The results were corrected for self-absorption and are expressed as counts/min. at infinite thinness/mg. $BaCO_3$.

Chromatographic analysis and radioautography of labelled compounds.

Incubations of whole cells or extracts with ^{14}C-tracer were usually stopped by the addition of 3 vol. of hot ethanol. The resulting precipitated protein was removed by centri-

fugation and washed with 0.5 ml. of cold aqueous 20% v/v ethanol. After recentrifugation, the combined supernatants and washings were evaporated to dryness at 60° under a stream of nitrogen gas, and redissolved in aqueous 20% v/v ethanol. Aliquots of this were applied to a spot 3 in. from either edge of sheets of Whatman No. 4 chromatography paper (18 in. x 22 in.). The chromatograms were then developed in a descending fashion, using firstly along the short direction of the paper the solvent system of Kornberg (1958): phenol-formic acid (90% w/v A.R.) - water (500 : 13 : 167 w/v/v). After the papers had been dried at room temperature for 24-48 hr., they were equilibrated and developed along the long axis of the paper with butan-1-ol-propionic acid-water (47 : 22 : 31 by vol.; Benson et al., 1950). Both solvent systems were allowed to run to the edge of the paper. Peel (1961 fig. 1) has produced a map giving the location of many important compounds in this two-dimensional system. Glycine, serine and glutamate were not usually resolved on these chromatograms. Accordingly the radioactive area containing them was cut out, eluted with water, and the amino acids separated by further chromatography (see below). The proportion of each amino acid in the radioactive area in the original chromatogram was assumed to be that found after

separation, even though losses of total count occurred during the overall procedure.

Volatile carboxylic acids were chromatographed in the solvent system ethanol - aq. NH_3 solution (sp. gr. 0.880) - water (80 : 4 : 16, by vol.; Long, Quayle & Stedman, 1951).

Amino acids were chromatographed in the solvent systems of Hardy, Holland & Nayler (1955). Butan-1-ol-acetone-water-diethylamine (10 : 10 : 5 : 2, by vol.) was particularly useful for separating glycine, serine and glutamate.

Keto-compounds in reaction mixtures were identified by adding an excess (usually 1 ml) of 2, 4-dinitrophenylhydrazine (0.1% in 2N-HCl). The resulting 2,4-dinitrophenylhydrazones (2,4-DNP-derivatives) were extracted into ethyl acetate (1 or 2 ml). Acidic 2,4-DNP-derivatives were then usually extracted into 1 ml. of M-Na_2CO_3. The aqueous alkaline layer was then acidified with HCl and the 2,4-DNP-derivatives re-extracted into ethyl acetate. Aliquots of this were then chromatographed in the solvent system butan-1-ol-ethanol-aq. NH_3 solution (sp. gr. 0.880)-water (140 : 20 : 1 : 39, by vol.; El Hawary & Thompson, 1953).

Radioautographs were made from the chromatograms by the procedures developed by Calvin and his co-workers (see Calvin, 1950-51), using Blue Brand X-ray film (Kodak, Ltd.).

Identification of labelled compounds.

The radioactive compounds were eluted from the chromatograms with water, mixed with authentic samples of the suspected compound (1-2 μmoles in the case of amino acids, 5-10 μmoles in the case of carboxylic acids) and subjected to co-chromatography. Exact coincidence of the resulting radioactive area on the radioautogram with the coloured area developed with an appropriate spray reagent was necessary for identification. Carboxylic acids were re-chromatographed either in the original solvent systems or in ethanol-ammonia-water. Amino acids were chromatographed either in the original solvent systems or in the case of glycine, serine and glutamate in butan-1-ol-acetone-water-diethylamine. Trehalose was co-chromatographed in the original solvent systems. Dephosphorylation of phosphate compounds was carried out by incubation of the eluted compounds with a preparation of Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.) according to the procedure of Benson, Bassham & Calvin (1951), and the resulting compounds were chromatographed on Whatman No. 1 paper in water-saturated phenol (first, along the long axis) and butan-1-ol-propionic acid-water (47 : 22 : 31, by vol.; Benson et al., 1950) (second, along the short axis).

Spray reagents for chromatograms.

Carboxylic acids were located on chromatograms either by spraying with a solution (0.2% w/v) of bromocresol green in 95% v/v ethanol adjusted to pH7 with aqueous NH_3 solution, or by spraying with a solution of aniline (1% v/v) and xylose (1% w/v) in 95% v/v methanol followed by heating at 105° (Wood, 1958); amino acids by spraying with 0.2% w/v ninhydrin in ethanol containing 0.1% v/v collidine, followed by heating at 100°; aldose sugars by spraying with a solution of aniline (0.9% v/v) and phthalic acid (1.7% w/v) in water-saturated butan-1-ol, followed by heating at 105° (Partridge, 1949); fructose by spraying with 1% w/v naphthoresorcinol in ethanol -2N-HCl (10 : 90 v/v; Forsyth, 1948) followed by heating to 90° for 10 min.; sedoheptulose by spraying with a solution of orcinol (0.5g.) and trichloroacetic acid (15 g) in 100 ml. of water-saturated butanol, followed by heating at 100° (Klevstrand & Nordal, 1950); trehalose by spraying first with $AgNO_3$ in aq. acetone, followed by 0.5N-NaOH in aq. ethanol (Trevelyan, Proctor & Harrison, 1950); phosphate compounds by spraying with the mixture 60% w/v $HClO_4$-HCl(N)-ammonium molybdate (4% w/v)-water (5 : 10 : 25 : 60 by vol.), followed by drying at 85° for 1 min. and then irradiating the paper with ultra-violet radiation for 30 min. (Bandurski

& Axelrod, 1951).

Manometric methods.

Consumption of oxygen was measured in conventional Warburg manometers at 30° with air as the gas phase. 1 ml. of a suspension of washed cells containing 2 - 4 mg. dry wt./ml. was added to 100 µmoles of sodium-potassium phosphate buffer pH7.5 in the main compartment of the Warburg flask. The volume of the contents of this compartment was adjusted with water to give the flask contents a total fluid volume of 2.7 ml. The centre well contained 0.2 ml. of 2N-KOH. Sufficient substrate was added from the side arm to give a total theoretical oxygen uptake of 300 - 400 µl. Flask constants were evaluated using the nomogram of Dixon (1951).

SECTION II.

FORMATE AND CARBON DIOXIDE METABOLISM IN CELLS GROWN ON FORMATE

Chapter III

INCORPORATION OF FORMATE AND CARBON DIOXIDE BY WHOLE CELLS OF FORMATE-GROWN PSEUDOMONAS AM 1

The assimilation products of radioactive formate by formate-grown Pseudomonas AM1 were investigated using the short term kinetic analysis technique developed at Berkeley by Calvin and his co-workers. This method (Calvin 1950-51) involves the administration of ^{14}C-labelled tracer to cells growing on non-radioactive substrate, followed by the rapid removal of samples (within a period of one minute) into hot ethanol. After separation of the radioactive cell constituents by two dimensional paper chromatography, the percentage of the total radioactivity of each sample in a particular compound is plotted as a function of time. As the time, Δt, elapsing between administration of the tracer and the removal of the sample tends to zero, then the product first formed should have a higher percentage of the incorporated radioactivity, extra-polating to 100% at zero time (unless there is more than one port of entry of the tracer). Accordingly, only the first assimilation products of the tracer administered

will show negative slopes as a function of time, all the subsequent products will have positive slopes.

This technique was used to study the incorporation of sodium [^{14}C]formate and sodium [^{14}C]bicarbonate by cells of P. AM1 growing on formate.

Analysis of the pathway of incorporation of [^{14}C]formate into cell constituents.

A culture of P. AM1 in the logarithmic phase of growth on formate was centrifuged at room temperature (20°) and resuspended in 8 ml. of medium containing 20mM-sodium formate, 50 mM-tris hydrochloride pH7.65, 1 mM-sodium phosphate and essential salts (at a concentration one fifth of that used to grow the cells) to give a final cell concentration of 3 mg. dry wt./ml. The suspension was placed in a cylindrical vessel (10 cm. x 2.5 cm.) with a sintered-glass bottom through which moist air was vigorously blown to aerate the culture, the vessel being maintained at 30°. After 5 min. equilibration, 2 ml. of a solution of sodium [^{14}C]formate (32.7 µmoles; 200 µC of ^{14}C) was added. 1 ml. samples of cell suspension were then rapidly transferred with a hypodermic syringe into 3 ml. of boiling 95% v/v ethanol, at varying time intervals over 5 min. as shown in

TABLE III. 1.

DISTRIBUTION OF RADIOACTIVE CARBON AMONG THE NON-VOLATILE LABELLED COMPONENTS OF THE ETHANOL-SOLUBLE FRACTIONS OF SUSPENSIONS OF FORMATE-GROWN PSEUDOMONAS AM1 AFTER SHORT TERM INCUBATION WITH [^{14}C] FORMATE.

Sodium [^{14}C] formate (32.7 µmoles, 200 µC of ^{14}C) was added at zero time to 8 ml. of a bacterial suspension (31 mg. dry wt.) in 20mM-sodium formate, 50mM-tris hydrochloride pH 7.65, 1mM-sodium phosphate and essential salts. Samples (approx. 1 ml.) were transferred at the times shown by hypodermic syringe to 3 ml. hot ethanol, and analysed as described in chapter II.

Radioactivity (Counts/min.)

Time (sec.)	7	14	21	38	63	84	122	181	243
Malate	19	28	8	3	9	11	11	24	17
Glycine	5	16	11	16	12	16	20	15	31
Serine	39	84	67	45	80	104	74	50	26
Glutamate	11	38	8	36	95	142	144	215	178
Aspartate	0	0	30	26	73	51	186	132	104
Citrate	0	18	26	40	77	103	97	125	42
Trehalose	0	0	0	44	68	103	281	455	426
Phosphorylated compounds	0	52	31	139	392	576	549	646	477
Total	74	236	181	349	806	1106	1362	1662	1301

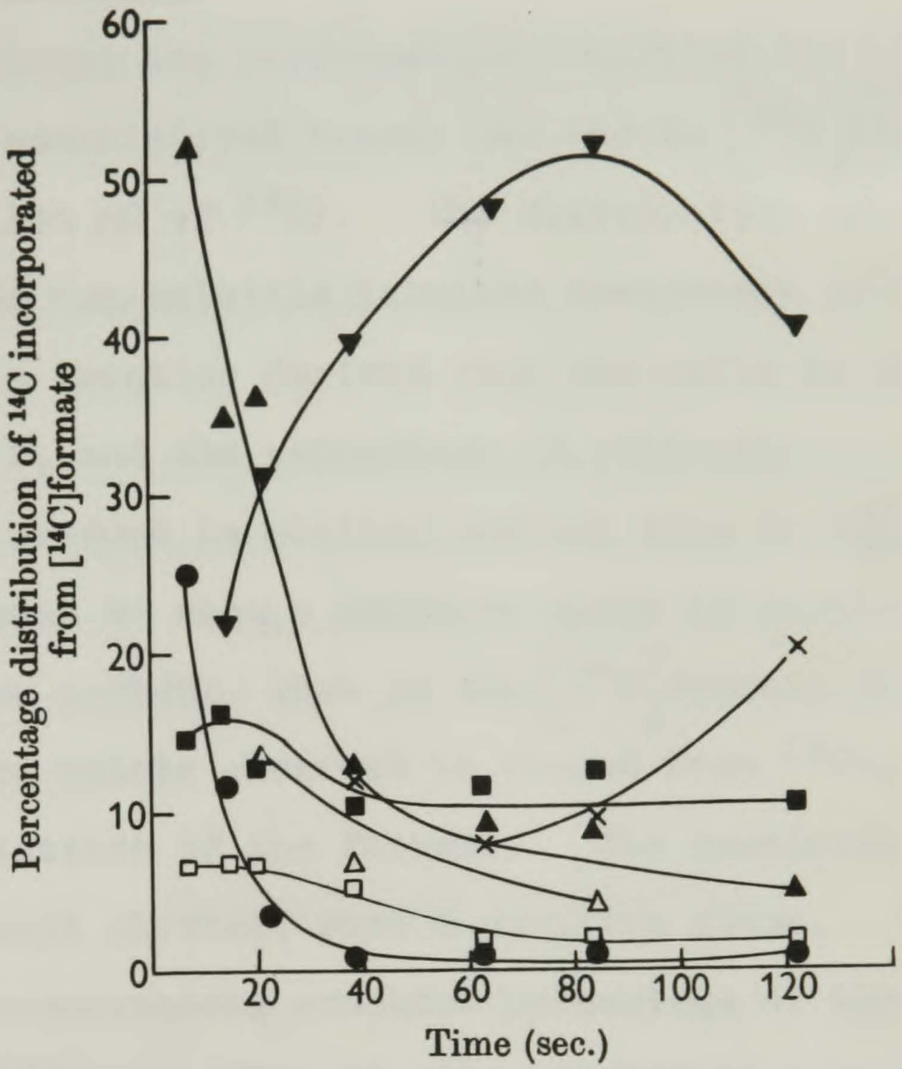
table III.1. These samples were analysed by two-dimensional paper chromatography and radioautography by the methods described in chapter II.

The distribution of radioactivity in the ethanol-soluble fraction of the cellular constituents is shown in table III.1, and the percentage of radioactivity in the different intermediates is plotted as a function of time in fig. 2. Of the curves shown in this fig., only serine and malate have negative slopes, indicating that these are both primary incorporation products. Trehalose and phosphorylated compounds have initially positive slopes, the former indeed containing 27% of the total radioactivity after 3 min. (table III. 1.). The remaining metabolites do not show any major increases or decreases.

In view of the known metabolic reactions involving carbon dioxide and reduced C_1 units, it appeared probable that the malate found in this experiment was derived not directly from formate but by fixation of carbon dioxide derived from formate by oxidation. In order to test this, a similar experiment was performed using sodium $[^{14}C]$bi-carbonate.

Fig. 2

Incorporation of [^{14}C] formate into components of the ethanol-soluble fraction of Pseudomonas AMI growing on formate.



● Malate; Δ aspartate; ▲ serine; □ glycine; ■ glutamate; ▼ phosphorylated compounds; X, trehalose.

Analysis of the pathway of incorporation of [^{14}C]bicarbonate into cell constituents.

The experiment was performed as described for [^{14}C]formate, save that the administered tracer was sodium [^{14}C]bicarbonate (5.9 μmoles; 140 μC of ^{14}C). The distribution of radioactivity in the non-volatile labelled components of the ethanol-soluble fraction derived from the cells is shown in table III, 2, and the percentage of radioactivity in each labelled product is plotted against time in fig. 3. The only compound to show a negative slope is malate. It thus appears probable that in the [^{14}C]formate experiment, the radioactive malate observed is formed from $^{14}CO_2$ arising by oxidation of the formate. The remaining compounds, except glycine, show a positive slope. Glycine contains an approximately constant percentage of the incorporated $^{14}CO_2$. After 1½ min., trehalose becomes labelled and increases in percentage radioactivity. No radioactive serine was found in any of the samples.

With both tracers, radioactive sugar phosphates were found. On dephosphorylation the distribution of radioactivity in these was found to be: glucose, 80%; fructose 10%; sedoheptulose, 10%.

TABLE III. 2.

DISTRIBUTION OF RADIOACTIVE CARBON AMONG THE NON-VOLATILE LABELLED COMPONENTS OF THE ETHANOL-SOLUBLE FRACTIONS OF SUSPENSIONS OF FORMATE-GROWN PSEUDOMONAS AM1 AFTER SHORT TERM INCUBATION WITH [^{14}C]BICARBONATE.

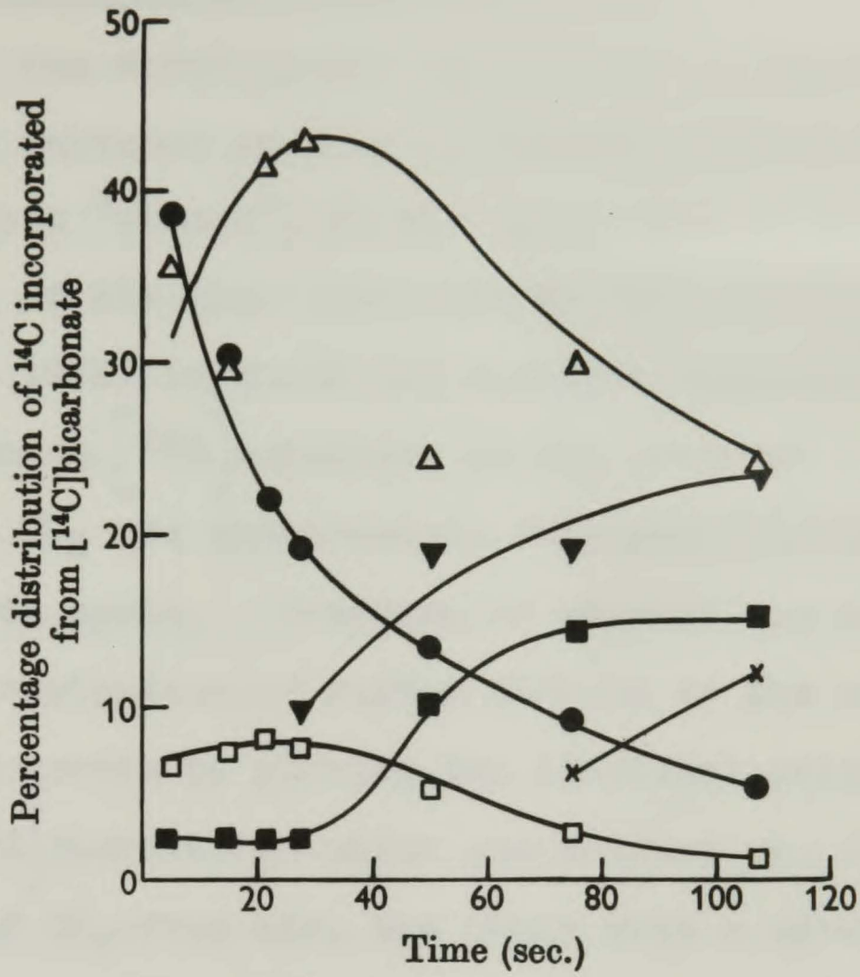
Sodium [^{14}C]bicarbonate (5.9 μmoles; 140 μc of ^{14}C) was added at zero time to 9 ml. of a bacterial suspension (28.3 mg. dry wt.) in 20mM-sodium formate, 50mM-tris hydrochloride pH 7.65, 1mM-sodium phosphate and essential salts. Samples (approx. 1 ml.) were transferred at the times shown by hypodermic syringe to 3 ml. hot ethanol, and analysed as described in chapter II.

Radioactivity (Counts/min.)

Time (sec.)	4	14	21	27	50	75	107	155	200
Malate	47	82	76	121	54	56	38	36	24
Glycine	8	20	29	46	21	15	13	8	1
Serine	0	0	0	0	0	0	0	0	0
Glutamate	3	7	8	16	40	86	105	113	82
Aspartate	43	80	145	266	96	186	170	209	159
Citrate	0	15	31	62	78	79	78	79	26
Trehalose	0	0	0	0	0	36	82	116	116
Phosphorylated compounds	0	0	0	58	72	114	165	143	116
Other compounds	20	65	57	50	34	30	47	66	0
Total	121	269	346	619	395	602	698	770	524

Fig. 3

Incorporation of [^{14}C] bicarbonate into components of the ethanol-soluble fraction of Pseudomonas AMI growing on formate.



● Malate; △ aspartate; □ glycine;

■ glutamate; ▼ phosphorylated compounds;

X trehalose.

Attempted assessment of the relative roles of formate and carbon dioxide in carbon assimilation.

Owing to the difficulties experienced in measuring the growth of cultures growing on formate, due to the tendency to form "clumps", it was impossible to perform an experiment of the type described by Peel (1961, p.24; Large et al., 1961) in which the specific radioactivity of cells grown on $[^{14}C]$ substrate in the presence and absence of 1% CO_2 was continuously followed through a complete growth cycle. However, an attempt was made to assess the contribution of carbon dioxide to the cellular carbon during growth by growing two identical cultures of P. AM1 on $[^{14}C]$ formate, in which one culture was aerated by a stream of CO_2-free air, the other with a stream of air containing 1% CO_2. After 48 hours growth, the cells were harvested. It was hoped to wash the cells and determine their specific radioactivity by direct combustion, but it proved impossible to obtain sufficient growth in the culture flushed with CO_2-free air for an accurate assessment of the specific radioactivity of the cellular material to be made.

Discussion.

The results obtained with formate-grown P. AM1 are very similar to those observed when the same organism is grown on methanol (Peel, 1961; Large et al., 1961). This indicates that despite their difference in reduction level, both C_1 substrates are assimilated by an essentially common pathway. It shows also that the growth of P. AM1 on formate does not involve an autotrophic pathway of the kind observed by Quayle & Keech (1958, 1959a) in the growth of P. oxalaticus on formate. The products obtained in short term kinetic experiments were quite different (see chapter I). This was confirmed by the demonstration that the key enzyme of the autotrophic RuDP cycle, carboxydismutase, is absent from formate-grown P. AM1 (chapter XII).

It is postulated that the common, non-autotrophic, pathway of formate and methanol incorporation involves the assimilation of a substantial fraction of cellular carbon at the level of carbon dioxide, although the experiment designed to demonstrate this in formate-grown cells by CO_2-flushing was found to be technically impracticable.

The extent of carbon dioxide assimilation during the heterotrophic growth of bacteria has been shown to vary

with the nature of the growth substrate (Wiame & Bourgeois, 1955). It was found in general that growth on C_3 substrates and glucose resulted in the incorporation of more $^{14}CO_2$ into cell material than growth on tricarboxylic acid cycle intermediates, and these authors suggested that the higher carbon dioxide incorporation observed with growth on C_3 substrates (and C_3 precursors) is due to the replenishment of the tricarboxylic acid cycle by carbon dioxide fixation. The experiments of Abelson, Bolton & Aldous (1952) with glucose-grown E. coli indicated that 7% of the cellular carbon had passed through a stage of carbon dioxide exchangeable with exogenous $^{14}CO_2$. The difficulty with this type of experiment lies in assessing how much of the $^{14}CO_2$ incorporated is due to a net fixation and how much to exchange reactions. Thus Kornberg & Quayle (1958) found in experiments in which air containing 5% CO_2 was used to flush cultures of Pseudomonas KB1 growing on uniformly-labelled acetate that there was a 15 - 17% drop in specific radioactivity of protein against a control flushed with CO_2-free air. Since in acetate-grown cells the mechanism for replenishment of tricarboxylic acid cycle intermediates is thought not to involve carbon dioxide fixation, but rather, operation of the glyoxylate cycle

(Kornberg, 1959a), this dilution of specific activity is probably due to exchange reactions (via the reversible $C_3 + CO_2 \rightleftharpoons C_4$ carboxylations).

Leadbetter & Foster (1958) concluded from their study of the contribution of carbon dioxide to cell material in P. methanica grown on methane (see chapter I) that during growth on this substrate, a significant proportion of the cellular carbon is derived from carbon dioxide and that this proportion is higher than in typical heterotrophic growth and lower than the 100% to be expected of autotrophic growth.

The evidence of Peel on the contribution of carbon dioxide to growth on methanol (chapter I), together with the substantial incorporation of $^{14}CO_2$ observed in formate-grown cells, indicates that P. AM1 when grown on either formate or methanol occupies, like P. methanica grown on methane, a position intermediate between the heterotrophs and the autotrophs. The former have less than 20% of their cellular carbon exchangeable with carbon dioxide whereas the latter, like formate-grown P. oxalaticus, have 90 - 100% of their cellular carbon exchangeable with carbon dioxide. Hence, any pathway proposed to explain the growth of P. AM1 on C_1 substrates must allow for about 50%

of the cellular carbon to be derived from carbon dioxide, and in view of the heavy labelling of malate observed above, it is probable that the carbon dioxide assimilation takes place via the carboxylation of a C_3 intermediate such as pyruvate or phosphoenolpyruvate.

The observation that serine is the first non-volatile ethanol-soluble assimilation product of $[^{14}C]$formate is similar to the observations of Peel (1961, p. 28) with methanol-grown P. AM1, Kaneda & Roxburgh (1959a) with methanol-grown P. PRL-W4, and Quayle (Large et al., 1961) with methanol-grown Hyphomicrobium vulgare (though in this last case, glycollate was also formed). Kaneda & Roxburgh suggested that the initial reactions of methanol assimilation in P. PRL-W4 involved the enzyme serine transhydroxymethylase. That such a mechanism might also be the case in methanol- and formate-grown P. AM1 and methanol-grown H. vulgare was suggested by Large et al. (1961). It was proposed that in these organisms a biosynthetic pathway for growth exists which must have the following characteristics: (a) it is different from the autotrophic type of pathway observed in formate-grown P. oxalaticus. (b) It involves mechanisms by which cell constituents are synthesised in about equal amounts from both reduced C_1 units and CO_2.

It was suggested that this pathway must involve (1) hydroxymethylation of glycine to form serine, by which C-3 of serine would arise from a reduced C_1 unit. (2) carboxylation of a C_3 unit arising from serine to form oxaloacetate or malate.

These two steps represent the successive synthesis of C_3 units from C_2 units and C_4 units from C_3 units. A crucial problem if this pathway is indeed operative, is the nature of the process by which glycine (or some other C_2 precursor) is synthesised from C_1 units. Radioactive glycine is present at the earliest times, whether [^{14}C]methanol, [^{14}C]formate or [^{14}C]bicarbonate is used, though only in small amounts (see figs. 2 and 3). Two possible pathways can be envisaged by which this glycine could be formed from C_1 units.

(a) A direct condensation of a reduced C_1 unit with CO_2 to give a C_2 precursor of glycine. This hypothesis is not well supported by the small amount of radioactivity found in glycine in the experiments described above.

(b) An indirect formation of glycine by some kind of cyclic mechanism. In this case, the precursors of glycine might be expected to be more heavily labelled at early times. The only compounds apart from serine, rapidly labelled in significant amounts at early times are the C_4 acids malate

and aspartate, and hence such C_4 compounds or their derivatives might well be implicated in such a cycle.

These two alternatives are summarised in fig. 1, Chapter I, (see Large et al., 1962, fig. 1) (Peel, 1961, fig. 9). There remains, of course, a third possibility, namely that the method of analysis is in fact missing key intermediates in the assimilatory pathway.

The work necessary to establish or reject this pathway falls naturally into two sections. The first is the establishment of the existence in P. AM1 of enzymes for the formation of serine from C_1 units, and the subsequent rearrangements and carboxylation reactions necessary for the synthesis of C_4 acids from this first stable product. The second is the elucidation of a mechanism by which glycine could be synthesised from C_1 units, either via a direct $C_1 + CO_2$ condensation or by some kind of cyclic mechanism possibly involving C_4 intermediates of the tricarboxylic acid cycle. This work is described in subsequent chapters. The first topic is dealt with in chapters IV and V, the second topic in chapters VIII and IX. In chapter VII are described whole cell studies on the labelling patterns of the individual atoms of various key intermediates, in order to determine whether they are in accord with the patterns predicted by the above hypotheses.

Chapter IV

FORMATE AND SERINE METABOLISM IN CELL-FREE EXTRACTS.

1. THE FORMATION OF SERINE

Since serine is the first radioactive product detected after administration of $[^{14}C]$formate to whole cells of Pseudomonas AM1 grown on formate, it appears likely, as suggested in chapter III, that one of the key reactions in the pathway of carbon assimilation is the hydroxymethylation of glycine to give serine:

$CH_2NH_2.COOH$ + "Active C_1" $\longrightarrow$ $CH_2OH.CHNH_2.COOH$ (i)

It has been shown (see Kisliuk, 1957; Blakley, 1958; Rabinowitz, 1960) that the product of the nonenzymic interaction of formaldehyde and tetrahydrofolic acid (H_4Pt G), characterised as $N^{5,10}$-methylenetetrahydrofolic acid, is an active donor of C_1 units in the serine transhydroxymethylase system. It also appears probable that the natural cofactors of this enzyme system are not in fact monoglutamates, but polyglutamyl derivatives of tetrahydrofolic acid. Thus Wright (1955, 1958) found that in the serine transhydroxymethylase system of Clostridium cylindrosporum, the natural cofactors were derivatives of reduced

pteroyl tri- and hepta-glutamates. Blakley (1957b) also found that polyglutamyl pterins were more active than H_4PtG in the glycine-serine interconversion catalysed by liver extracts.

For the formation of serine from formate by the enzyme systems at present known, the following sequence of reactions is necessary (Rabinowitz, 1960):

Formate + ATP + $H_4PtG \rightleftharpoons \underline{N}^{10}$-formyl$H_4PtG$ + ADP + P_i (ii)

catalysed by tetrahydrofolate formylase (formate-activating enzyme)(Rabinowitz & Pricer, 1957; Whiteley, Osborn, & Huennekens, 1959).

$\underline{N}^{10}$-formyl H_4PtG + $H^+ \rightleftharpoons \underline{N}^{5,10}$ methenylH_4PtG + H_2O (iii)

catalysed by methenyltetrahydrofolate cyclohydrolase (Rabinowitz, 1960).

$\underline{N}^{5,10}$ methenylH_4PtG + NADPH $\rightleftharpoons \underline{N}^{5,10}$methylene$H_4PtG$ + $NADP^+$ (iv)

catalysed by methylenetetrahydrofolate dehydrogenase (Hatefi, Osborn, Kay & Huennekens, 1957)

$\underline{N}^{5,10}$methyleneH_4PtG + Glycine $\rightleftharpoons$ Serine + H_4PtG (V)

catalysed by serine transhydroxymethylase (Blakley, 1955; Alexander & Greenberg, 1956; Huennekens, Hatefi & Kay, 1957).

Accordingly, it was decided to examine cell-free extracts of formate-grown Pseudomonas AM1 for the presence of these enzymes, and to see whether such extracts, when fortified

by suitable cofactors, would catalyse the synthesis of serine from labelled formate.

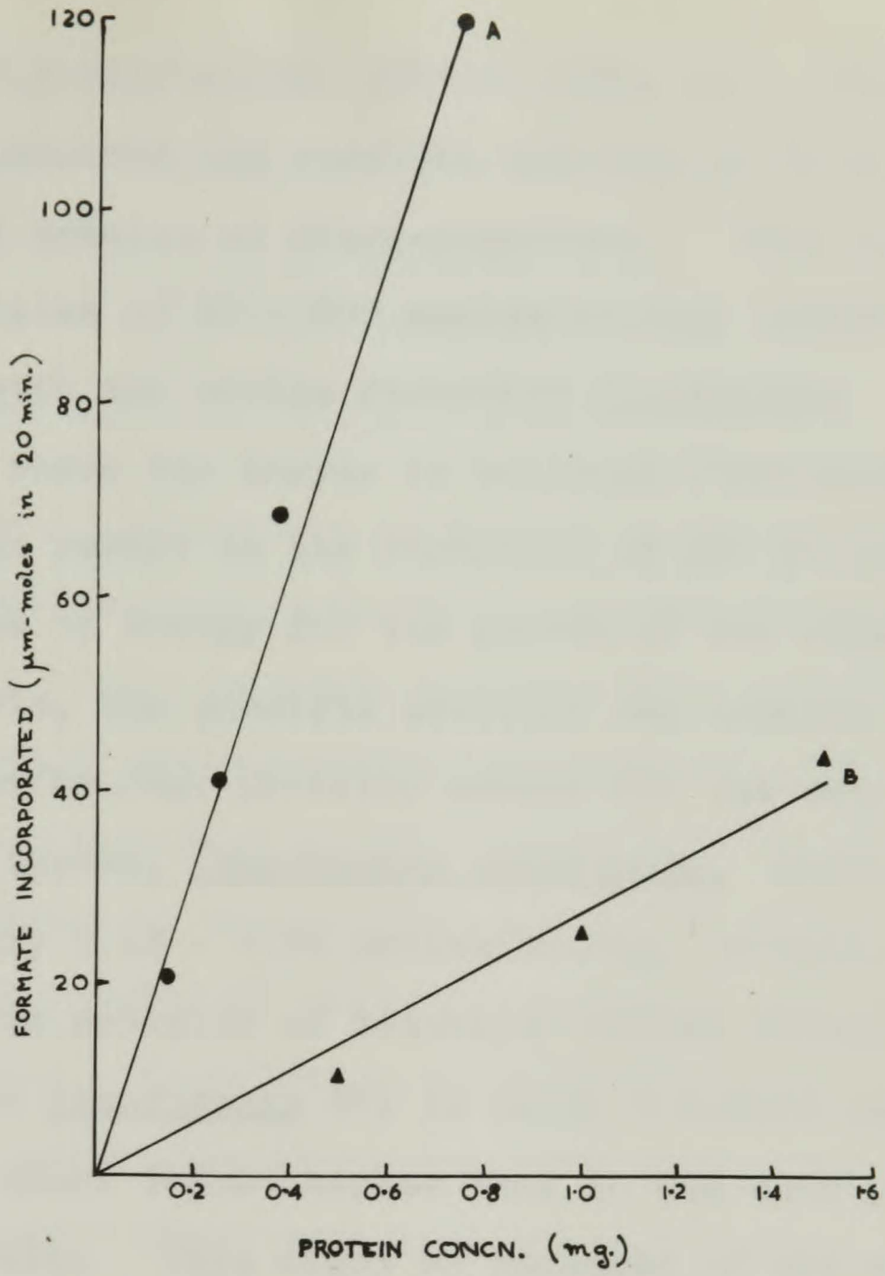
Preparation of tetrahydrofolic acid solution.

DL-Tetrahydrofolic acid was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. in the form of 500 mg. of solid dissolved in 2 ml. of water, pH7.0 - 7.4, stabilised by 0.1% v/v mercaptoethanol and sealed under N_2. The tube was opened, and the contents diluted by the addition of 2 ml. 0.01M-mercaptoethanol in 50mM-phosphate buffer pH 7.5. The diluted solution was distributed among 3 Thunberg tubes, each was filled with oxygen-free N_2, sealed and stored at -15^0. The concentration of this solution was estimated by adding 10 µl. to 60 ml. of 0.01 M-mercaptoethanol in 50mM-phosphate buffer pH7.5. The spectrum of this diluted solution was read against 0.01M-mercaptoethanol as blank on the Cary recording spectrophotometer. From the optical density at 297 mµ, the concentration of the original tetrahydrofolic acid solution was calculated from the molar extinction coefficient of H_4PtG at this wavelength (22 x 10^6 cm^2/mole, Hatefi, Talbert, Osborn & Huennekens, 1960).

Tetrahydrofolate formylase (formate activating enzyme).

This was assayed by a method which was basically a combination of the method of Greenberg, Jaenicke & Silverman (1955), which measures the incorporation of [^{14}C]formate into non-volatile material, after incubation in the mixture used by Whiteley et al. (1959). The reaction mixture contained 65 µmoles of maleate buffer pH7.5, 50 µmoles of KF, 2.5 µmoles of ATP, 0.77 µmole of DL-H_4PtG, 10 µmoles of mercaptoethanol, 40 µmoles of $MgCl_2$ and 10 µmoles (3 µC) of sodium [^{14}C]formate in a total volume of 1.2 ml. Varying amounts of extract were added, and the tubes incubated at 30° for 20 min. The reaction was stopped by the addition of 1 ml. of 3.5% w/v $HClO_4$ and 0.1 ml. aliquots were removed and assayed for radioactivity at infinite thinness on planchets as described in chapter II. There was a linear relationship between the incorporation of radioactivity and protein concentration (fig. 4), showing that the assay was performed under conditions of limiting enzyme concentration. A specific activity of 0.56 umole of formate fixed/hr./mg. protein was observed under these conditions with extracts of formate-grown cells. When extracts of cells grown on succinate as carbon source were tested, the specific activity of the formate activating

Fig. 4



The tetrahydrofolic acid-dependent fixation of formate by extracts of Pseudononas AMI.

The incubation mixture contained: 65 µmoles of maleate buffer pH 7.5, 50 µmoles of KF, 2.5 µmoles of ATP, 0.77 µmoles of DL-H_4PtG, 10 µmoles of mercapto-ethanol, 40 µmoles of $MgCl_2$, 10 µmoles (3µc) of sodium [^{14}C] formate, and extract in a total volume of 1.2 ml. Incubation was at 30° for 20 min. Reaction stopped by addition of 1ml. 3.5% W/V $HClO_4$ and 0.1 ml. aliquots assayed for acid-stable radio-activity. A, extract of formate-grown cells; B, extract of succinate-grown cells.

enzyme was 0.07 µmole/hr./mg. protein (fig. 4). Whiteley et al. (1959) measured the specific activity of this enzyme in a variety of species of micro-organisms. Very high specific activities of 60 - 600 µmoles/hr./mg. protein were observed with the purine fermenter Clostridium cylindrosporum where the enzyme is believed (Rabinowitz & Pricer, 1956) to result in the formation of ATP to provide the major source of energy for the growth of the organism. In other bacteria, the specific activity was usually about 0.6 - 1.2 µmoles/hr./mg. protein, except for the only species of Pseudomonas tested, Pseudomonas aeruginosa, where the activity was only 0.18 - 0.24 µmoles/hr./mg. protein. Thus the specific activity of tetrahydrofolate formylase in formate-grown Pseudomonas AM1 is about 2-3-fold higher than this, and about 8-fold higher than in the same organism grown on succinate. This might be expected if the enzyme plays a key role in carbon assimilation from formate.

When extracts of formate-grown P.AM1 were incubated with formate (9 µmoles), ATP (5 µmoles), DL-H_4PtG(0.5 µmole) and maleate buffer pH7.5 (100 µmoles) for 10 min., acidified with 0.05 ml. 6N-HCl, and the spectrum of the test system examined against a control containing all components except formate, an absorption maximum at 350-355 mµ was observed

This, under these conditions, is characteristic of $\underline{N}^{5},^{10}$methenylH_4PtG (Rabinowitz, 1960), which is formed under acid conditions from $\underline{N}^{10}$formylH_4PtG:-

$$\underline{N}^{10}\text{formyl}H_4\text{PtG} + H^+ \underset{\text{Base}}{\overset{\text{Acid}}{\rightleftharpoons}} \underline{N}^{5},^{10}\text{methenyl}H_4\text{PtG} + H_2O \quad \text{(iii)}$$

The nature of the products of H_4PtG - stimulated [^{14}C]formate incorporation was investigated by stopping the reaction anaerobically with $HClO_4$ or aerobically by heat. Aliquots of the reaction mixtures were chromatographed one-dimensionally by the ascending technique in 0.5M- or 1 M- formic acid (Greenberg et al., 1955), and the radioactive spots located by radioautography. The folic acid derivatives were located by their fluorescence beneath an ultra-violet lamp. There was a marked difference in the location of the radioactivity in the folic acid derivatives according to whether these were isolated aerobically or anaerobically. When the reaction was stopped aerobically, three derivatives were located by their UV fluorescence: A, R_f 0.4 in 0.5M - HCOOH, bright yellow fluorescence; B, R_f 0.55, faint blue fluorescence; and C, R_f 0.69, bright blue fluorescence. A contained no radioactivity, and on the basis of its fluorescence and the spectrum of the eluted spot, was probably xanthopterin (the principal oxidative

degradation product of H_4PtG, Blakley, 1957a). C contained only a little radioactivity and was probably, from its R_f, N^{10}-formylfolic acid (Greenberg et al., 1955). The identity of B, which contained 50-100% of the radioactivity, is not clear. It does not correspond in spectral, fluorescence, or R_f properties with N^{10} formyldihydrofolic acid, the expected product under these conditions. Under anaerobic conditions, three products were again obtained, as located by UV fluorescence: α, R_f 0.39 in 1M-HCOOH, bluish-white fluorescence, 50-75% of the incorporated radioactivity; β, R_f 0.6, had only a very faint fluorescence and was only slightly radioactive; γ, R_f 0.77, bright blue fluorescence, but not radioactive. The R_f of α is very close to that quoted (0.37) for $N^{5,10}$methenylH_4PtG (Greenberg, et al., 1955) and when eluted, its spectrum was similar to that of $N^{5,10}$methenylH_4PtG (Rabinowitz, 1960), having a peak between 320 and 360 mμ. The extinction of this peak agreed with that predicted for $N^{5,10}$methenylH_4PtG from the amount of [^{14}C]formate incorporated, on the basis of an extinction coefficient at 355 mμ of 22 x 10^6 cm.^{2}/mole (Whiteley et al., 1959).

Methylenetetrahydrofolate dehydrogenase.

Extracts of P. AM1 were found to catalyse the H_4PtG-dependent reduction of NADP by serine or formaldehyde. This is presumably due to the presence of the enzymes serine transhydroxymethylase, catalysing reaction (v) above, and methyleneH_4PtG dehydrogenase catalysing reaction (iv). These were assayed for by the method of Hatefi et al. (1957) by incubating 20 µmoles of DL-serine or 5 µmoles of formaldehyde with 1 µmole of NADP, 0.8 µmole of Dl-H_4PtG and 100 µmoles of maleate buffer pH 7.5 with extract in a total volume of 3 ml., and following the increase in extinction at 340 mµ consequent on the formation of NADPH, on the Cary recording spectrophotometer, against a blank containing all components except formaldehyde or serine. Formaldehyde reacts nonenzymically with H_4PtG under these conditions to give $N^5,^{10}$methylene H_4PtG (Kisliuk, 1957), so that in this case the methyleneH_4PtG dehydrogenase activity only is measured, whereas with serine both enzymes are involved. With extracts of formate-grown P.AM1, the specific activity of the system was 0.54 µmoles of NADPH formed/hr./mg. protein with serine as substrate and 22 umoles NADH formed/hr./mg. protein with formaldehyde. NAD would not replace NADP, and the reaction was dependent on extract,

H_4PtG, and formaldehyde or serine. At the conclusion of the reaction, the system was acidified by addition of 0.2 ml. N-HCl, and the effect on the test cuvette noted by scanning the spectrum between 340 and 370 mμ. The acidification destroys reduced nicotinamide nucleotides (Hatefi et al., 1957). Under these conditions, an absorption maximum at 355 mμ was observed. This corresponds to the presence of $N^{5,10}$methenylH_4PtG, which is the only tetrahydrofolic derivative to exhibit this particular absorption maximum. This compound is the enzymic product of the methyleneH_4PtG dehydrogenase system (Osborn & Huennekens, 1957), but all crude systems in which H_4PtG is active contain the enzyme methenylH_4PtG cyclohydrolase catalysing equilibrium (iii). Acidification pushes this reaction to the right. The much greater activity of formaldehyde over serine in this system is interesting. The contrary was observed in mammalian liver extracts by Hatefi et al., 1957. Probably it indicates that reaction (v) under these conditions is rate limiting. When these assays were repeated using extracts of succinate-grown cells, the specific activities were 13.1 and 0.28 μmoles NADPH formed/hr./mg. protein, with formaldehyde and serine respectively.

Serine transhydroxymethylase.

This enzyme was assayed by manometric measurement of the serine formed from glycine and formaldehyde. The method of estimation was the oxidative decarboxylation of serine by sodium periodate at pH5.8 (Blakley, 1955).

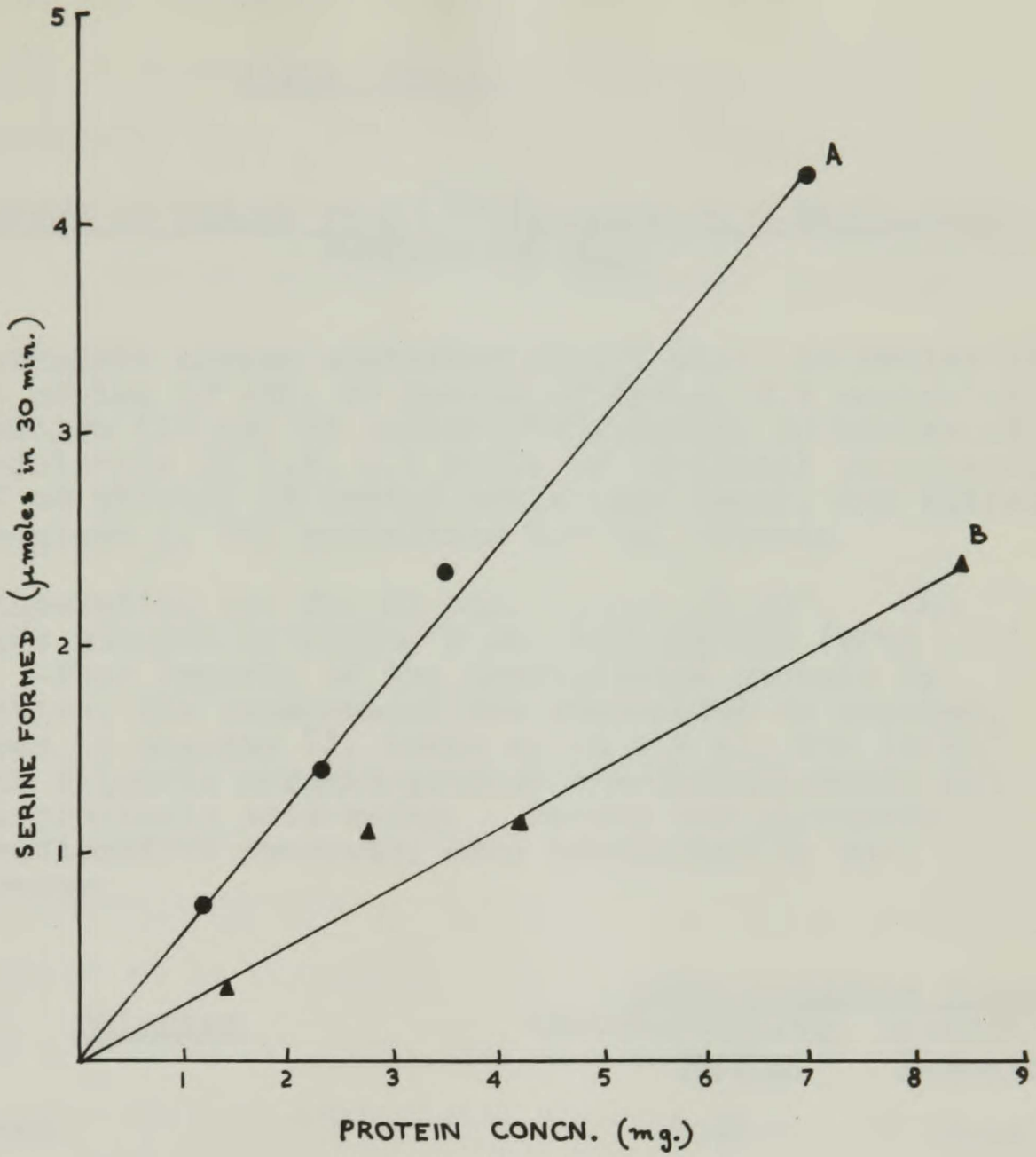
The reaction mixture for the enzyme assay contained 30 µmoles of glycine, 30 µmoles of formaldehyde, 0.3 µmole of H_4PtG or tetrahydropteroyl-γ-L-glutamyl-γ-L-glutamyl - γ-L-glutamate (tetrahydroteropterin H_4PtG3, a gift from Dr. J.R. Guest), 40 µmoles of phosphate buffer pH7.5 and 0.1 µmole of pyridoxal phosphate in a total volume of 1.5 ml. Varying amounts of extract were added to ensure that the enzyme was assayed under conditions of limiting enzyme concentration. The incubation was at 30° for varying time intervals from 15 to 60 min., and was carried out in Thunberg tubes under nitrogen to protect the tetrahydrofolic derivatives. The reaction was started by tipping in the extract from the side arm and was stopped by heating the opened tubes at 100° for 2 min. 1 ml. of each reaction mixture was transferred to micro-manometer flasks (K_{CO_2} about 0.5) containing, in the main compartment 500 µmoles of KH_2PO_4 pH5.8, and in the side arm 150 µmoles of sodium metaperiodate. The gas phase was air. After equilibration at 30°, the manometers were tipped

and the CO_2 evolution followed for 1 hr. This method has been used by Batt, Dickens & Williamson (1960). A linear relationship was observed between extract concentration and serine formation (fig. 5) and serine formation and time. Under the conditions used, the rate was not significantly faster with the tetrahydropteroyltriglutamate than with the monoglutamate, though both were some 17-fold higher than in the absence of reduced folic derivatives. It appears probable that substantial amounts of the natural folic acid cofactor were present in the untreated extract. The specific activity of the enzyme in formate-grown cells was 1.78 μmoles serine formed/hr./mg.protein. In parallel experiments with succinate-grown cells the specific activity was 0.82 μmoles serine formed/hr./mg. protein. This 2-fold difference in specific activity is evidence that serine transhydroxymethylase may be involved in the main biosynthetic pathway for growth of C_1 organisms.

The formation of serine from formate.

Having demonstrated that all the enzymes are present in extracts of Pseudomonas AM1 for the conversion of formate to serine, the next step was an attempt to see whether extracts, when supplemented by suitable cofactors, could

Fig. 5



The tetrahydrofolate-dependent formation of serine from glycine and formaldehyde by extracts of Pseudomonas AMI.

The incubation mixture contained: 30 umoles of glycine, 30 umoles of formaldehyde; 3 umoles of DL-H_4PtG, 40umoles of phosphate buffe pH 7.5, 0.1 umole of pyridoxal phosphate, and extract in a total volume of 1.5 ml. Incubation was at 30° for 30 min. Reaction steopped by heating and serine assayed manometrically as described in the text. A, extract of formate-grown cells; B, extract of succinate-grown cells.

TABLE IV. 1.

FORMATION OF SERINE FROM [^{14}C]FORMATE BY FORMATE-GROWN PSEUDOMONAS AM1.

The complete system contained in 1.7 ml.: 10 µmoles of glycine, 5 µmoles of ATP, 20 µmoles of $MgCl_2$, 2.5 µmoles of NADPH, 5 µmoles (10 µc) of sodium [^{14}C]formate, 50 µmoles of tris hydrochloride pH 7.5, 0.1 µmole of pyridoxal phosphate, 0.2 ml. of an extract of heated cells (see text), and extract of formate-grown P. AM1 containing 2.8 mg. protein.

The incubation was for 30 min. in air at 30°. The reaction was stopped by adding 3 ml. boiling 95% (v/v) ethanol. After removal of the precipitated protein by centrifugation, the supernatant was evaporated to dryness, as described in chapter II, taken up in 0.3 ml. 20% (v/v) ethanol and aliquots chromatographed one-dimensionally in butan-1-ol-propionic acid-water. Serine and glycerate, the only radioactive products, were identified by co-chromatography.

Tube	Omission	Total products formed. (Counts/min./mg. protein x 10^{-3}) Serine.	Glycerate.
1	None	6.45	0.44
2	ATP	1.42	0.17
3	Boiled cell extract	6.78	0.47
4	NADPH	0.15	0
5	Glycine	1.04	0.24
6	None, NADPH replaced by NADH	0.94	0.03
7	None, extract boiled	0	0

catalyse the formation of labelled serine from [^{14}C]formate. Table IV. 1 shows that extracts of formate-grown P. AM1 when incubated with [^{14}C]formate, pyridoxal phosphate, ATP, glycine and NADPH can indeed form serine. Addition of an extract of heated cells does not stimulate the reaction, which is dependent on ATP and glycine and in which NADH will not replace NADPH. Addition of tetrahydrofolic acid was not necessary for the reaction, indeed preliminary experiments had indicated that the addition of H_4PtG inhibits the formation of serine from formate (see also below). In addition to serine, one other radioactive product was detected which was identified by cochromatography as glycerate. Presumably this arises from serine (which contains 85-100% of the incorporated radioactivity) by pathways to be discussed later in this chapter.

This experiment shows that extracts of formate-grown P. AM1 must contain appreciable amounts of the natural folic acid cofactor. This is by no means unusual, thus Kisliuk & Woods (1960) found that dialysed extracts of Escherichia coli PA 15 catalysed the formation of the methyl group of methionine from serine in the absence of added cofactors. Treatment with Dowex 1 ion-exchange resin has been widely used (Rabinowitz & Pricer, 1956; Kisliuk & Woods, 1960) to remove folic acid cofactors from bacterial extracts. Accordingly, extracts of formate-grown P. AM1 were treated with Dowex 1 x 8 resin (chloride form; 100 - 200 mesh)

by the method of Kisliuk & Woods (1960). The extract was loaded at 4° on to a small column (1 x 3 cm.) which had previously been washed with water to constant pH. It was allowed to run through at 4° until an equal volume had been collected and the column was drained. When this Dowex-treated extract was incubated with [^{14}C]formate under the conditions shown in table IV. 2, there was a 94% inhibition of the serine-forming activity of untreated extracts. The ability of various folic acid coenzymes to restore this activity was then investigated. It was found that tetrahydrofolic acid was very poor in restoring serine-forming activity: there was a considerable incorporation of label from [^{14}C]formate into formylated folic derivatives but very little into serine. 0.55 mM-H_4PtG in fact only increased the activity of Dowex-treated extract 4-fold, whereas a 16-fold stimulation was required to bring the activity up to that of the untreated extract. In contrast, tetrahydropteroyl-γ-glutamyl-γ-glutamyl-γ-glutamate (H_4PtG_3) was much more effective in promoting serine synthesis at the considerably lower concentration of 0.15 mM. There was a 30-fold stimulation over the activity of the Dowex-treated extract alone. The effect of natural folic acid cofactors was also tested. A heated extract of P.AM1 was prepared by heating 2 g. wet wt. of methanol-grown P. AM1

TABLE IV. 2.

FORMATION OF SERINE FROM [^{14}C]FORMATE BY DOWEX-1-TREATED EXTRACTS.

The complete system contained in 2 ml.: 10 μmoles of glycine, 5 μmoles of ATP, 20 μmoles of $MgCl_2$, 2.5 μmoles of NADPH, 5 μmoles (10 μc) of sodium [^{14}C]formate, 50 μmoles of tris hydrochloride pH 7.5, 0.1 μmole of pyridoxal phosphate, 10 μmoles of mercaptoethanol, Dowex 1-treated extract (see text) containing 0.78 mg. protein or untreated extract containing 2.34 mg. protein, with the additions shown below.

The incubation was for 30 min. at 30° in Thunberg tubes under N_2. The reaction was stopped by the addition of 3 ml. boiling 95% (v/v) ethanol, and the reaction mixture treated as described in table IV. 1. Aliquots were chromatographed one-dimensionally in phenol-formic acid-water. The only products were serine (identified by cochromatography) and formylated folic derivatives.

Tube	Contents The above basic system with the additions or modifications shown below.	Total products formed. (counts/min/mg. protein x 10^{-3}).	
		Serine	Folic derivatives.
1	Untreated extract	4.32	0.94
2	Dowex 1-treated extract	0.26	0
	Dowex 1-treated extract with the following additions:		
3	H_4PtG (1.1 μmoles)	1.07	18.25
4	H_4PtG_3 (0.3 μmole)	7.83	13.42
5	H_4PtG_3 (0.1 μmole)	2.28	3.53
6	H_4PtG_3 (0.1 μmole) + H_4PtG (0.5 μmole)	1.33	8.67
7	Boiled cell extract of *P.* AM1 (see text)(0.2 ml.)	0.76	0
8	Boiled cell extract of *E. coli* PA 15 (see text)(0.2 ml.)	7.37	0
9	Extract boiled	0	0

with 3 ml. of water at 95° for 3 min. followed by cooling and centrifugation (Peel, 1961, p.64). 0.2 ml. of the supernatant were added to the Dowex-treated extract. A similar heated extract of Escherichia coli PA 15, prepared as described by Guest, Helleiner, Cross & Woods (1960) was a gift from Mr. F.G. Bull. This has been shown (Guest et al., 1960) to be a good source of the natural folic acid cofactor for the biosynthesis of methionine methyl groups from serine. Both of these preparations were tested for serine-forming ability with Dowex-treated extracts. Both had some effect. The boiled cell extract of P. AM1, however, was a very poor source of cofactors, less effective even than H_4PtG in restoring the endogenous level of serine formation. This may be because the preparation used was rather old, having been stored for 6 months at -15°. A similarly prepared extract of E.coli PA15 however, in amounts corresponding to 17 mg. dry wt. of organisms, was almost as effective as 0.15 mM - PtH_4G_3 in reactivating the Dowex-treated extract.

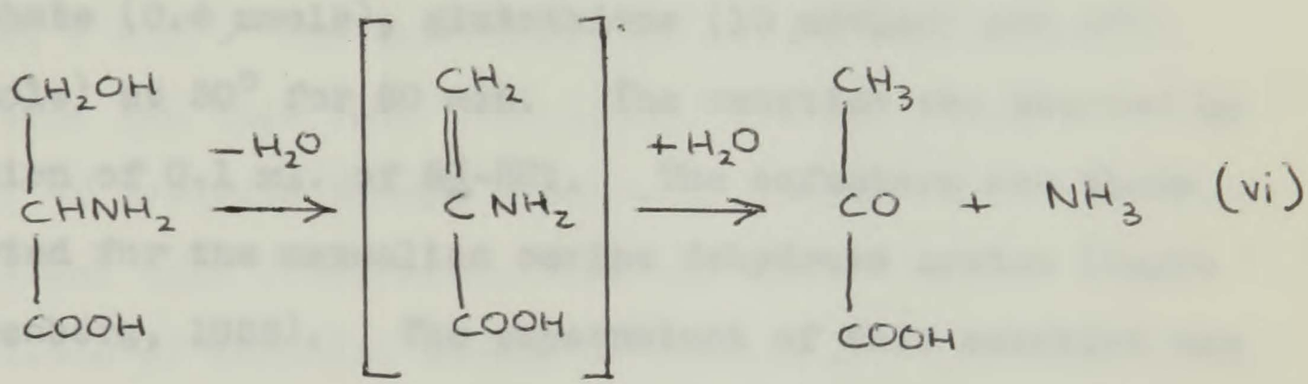
It is thus clear that H_4PtG is much less effective as a cofactor for serine formation from glycine and formate, than either the untreated extract, H_4PtG_3, or other natural sources of cofactor such as boiled extracts of E. coli PA15.

In fact, when tested together (table IV. 2, tubes 5 and 6) H_4PtG was found to inhibit the cofactor activity of H_4PtG_3. 0.25mM-H_4PtG produced a 42% inhibition of the serine-forming activity of 0.05 mM - H_4PtG_3. This may be compared with the observation (Kisliuk & Woods, 1960; Jones, Guest & Woods, 1961) that H_4PtG is a competitive inhibitor of the synthesis of the methionine methyl group when PtH_4G_3 or a boiled cell extract of E. coli PA 15 is the source of the cofactors.

2. METABOLIC TRANSFORMATIONS OF SERINE.

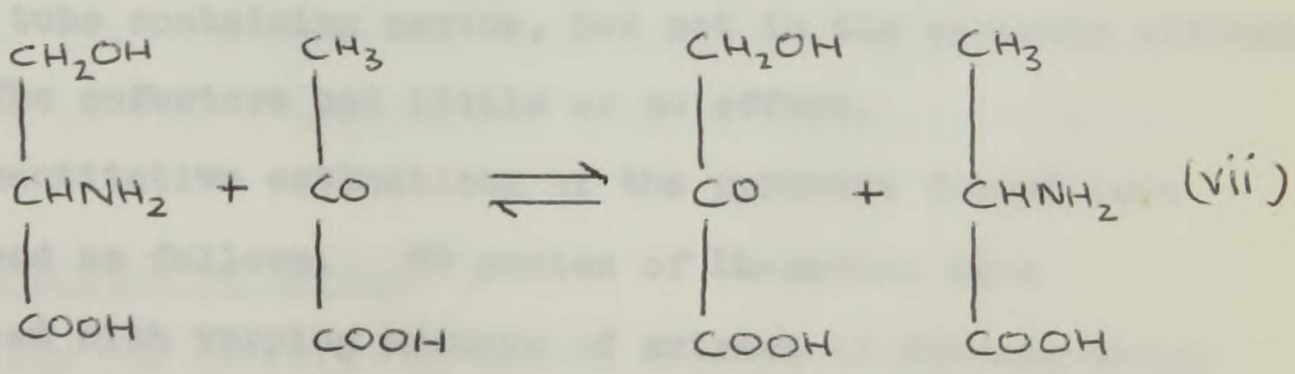
The studies on the incorporation of $[^{14}C]$formate by whole cells (chapter III) showed that, while serine is the principal product of formate assimilation at early times (52% of the total radioactivity is in serine after 7 sec.), at later times the percentage radioactivity in phosphorylated compounds and in trehalose increases rapidly. If, as this evidence seems to indicate, serine is the first stable assimilatory product of formate, then pathways must exist by which the radioactivity initially in serine can be transferred to phosphorylated derivatives, and thence to sugars. These pathways are presumably the routes for the synthesis of cellular material such as sugars, from formate.

If serine is indeed a general precursor of cellular carbon, then it is evident that before it can be transformed to other products, the amino group must be removed. Only two reactions are known in which the amino group of serine is removed: (a) deamination catalysed by the enzyme L-serine dehydrase (Yanofsky & Reissig, 1953; Sayre & Greenberg, 1955; Benziman, Sagers & Gunsalus, 1960):-



(vi)

leading to the formation of pyruvate, and (b) transamination, in which the amino group is transferred to a suitable keto acid acceptor (pyruvate or α-oxoglutarate) to give hydroxypyruvate:-



(vii)

This reaction has been demonstrated in dog liver (Sallach, 1956) and human liver (Hedrick & Sallach, 1960).

Formation of pyruvate from serine.

Extracts of formate-grown P. AM1 were incubated with 100 μmoles of tris hydrochloride pH7.5, 40 μmoles of DL-serine and in addition in some tubes, pyridoxal phosphate (0.4 μmole), glutathione (10 μmoles) and AMP (1 μmole) at 30° for 60 min. The reaction was stopped by addition of 0.1 ml. of 6N-HCl. The cofactors are those reported for the mammalian serine dehydrase system (Sayre & Greenberg, 1955). The supernatant of this solution was treated with 0.2 ml. of saturated 2, 4-dinitrophenylhydrazine in 2N-HCl, and the resultant 2, 4-DNP-derivatives extracted and chromatographed as described in chapter II with markers of authentic pyruvate and hydroxypyruvate 2,4-dinitrophenyl-hydrazones. The results showed that pyruvate was present in the tube containing serine, but not in the controls without it. The cofactors had little or no effect.

Quantitative estimations of the pyruvate formed were performed as follows. 20 μmoles of DL-serine were incubated with varying amounts of extract of formate-grown P. AM1 in the presence of GSH (10 μmoles), $MgCl_2$ (10 μmoles)

and 100 µmoles phosphate buffer pH 7.5 for 30 min. at pH 7.5 in a total volume of 1.3 ml. The reaction was stopped by the addition of 0.13 ml. of 50% w/v trichloracetic acid. After centrifugation, 0.7 ml. of the supernatant was taken, 0.3 ml. water added and 0.33 ml. saturated 2, 4-dinitrophenylhydrazine in 2N-HCl. After 10 min., 1.67 ml. of 2.5 N-NaOH were added, and incubated for a further 9 min. The extinction at 445 mu and 520 mu for each tube was then read (Freidemann & Haugen, 1943). The amount of pyruvate formed increased linearly with the amount of extract used (indicating that the assay is done under conditions of limiting enzyme concentration) and with time, but the rate was very slow - only 57 µm-moles pyruvate formed /hr./mg. protein. The pH optimum for pyruvate formation was 8.0. The reaction is complicated in crude extracts by the possibility of the removal of pyruvate by other reactions (e.g. transamination with the serine), but even so, the rate observed is probably too small for the reaction to play a significant role in carbon assimilation.

Transamination of serine.

Since the activity of serine dehydrase appeared to be so low, it is probable that removal of the serine amino

group takes place by transamination. In order to see whether transamination could take place under these conditions, extracts of formate-grown P. AM1 (containing 2.2 mg. protein) were incubated with 40 µmoles of sodium pyruvate or sodium α-oxoglutarate, 40 µmoles of DL-serine or glycine, 1 µmole of pyridoxal phosphate and 100 µmoles of phosphate buffer pH 7.5 for 60 min. at 30° under a layer of petroleum ether to exclude air. The reaction was stopped by heating at 100° for 4 min. The denatured protein was removed by centrifugation and aliquots chromatographed on paper in the following solvent systems: (a), methanol-butan-1-ol-water (10 : 10 : 5 by vol., Hardy, Holland & Nayler, 1955) which would separate serine and alanine, and (b), butan-1-ol-acetone-water-diethylamine, 10 : 10 : 5 : 2 by vol. (Hardy et al., 1955) to separate glycine and glutamate and serine and glutamate. Standards containing exactly 1.0 µmole of the amino acid to be detected were also chromatographed on the same paper. Solvent (a) was developed by the ascending method, solvent (b) by the descending method. After drying, the chromatograms were sprayed with 0.2% w/v ninhydrin in 95% v/v ethanol and heated 3 min. at 100°. The spots so detected (together with the standards) were cut out and eluted with 70% v/v ethanol. More ninhydrin was added,

TABLE IV. 3.

FORMATION OF ALANINE AND GLUTAMATE FROM SERINE AND GLYCINE BY TRANSAMINATION.

For details of the incubation, see text.

Tube.	Contents.	Chromatography solvent.	Total products formed (μmoles)	
			Glutamate.	Alanine.
1	α-Oxoglutarate	a, b	2.34	0.54
2	Pyruvate	a, b	2.34	0.10
3	α-Oxoglutarate + serine	b	4.64	
4	α-Oxoglutarate + glycine	b	5.40	
5	Pyruvate + serine	a		3.00

and the solutions kept at 100° for 15 min. The optical density of the eluted colour was then read at 575 mμ, together with the standard, against an eluted piece of blank paper. Approximate concentrations of amino acid were calculated from the standard, assuming that Beer's law is obeyed. The results are given in table IV. 3. When the blank glutamate and alanine are subtracted, there were 1.03 μmoles glutamate formed /hr./mg. protein from α-oxoglutarate in the presence of serine, 1.37 μmoles glutamate formed /hr./mg. protein from α-oxoglutarate in the presence of glycine, and 1.30 μmoles alanine formed/ hr./mg. protein from pyruvate in the presence of serine. These figures are only approximate, but even making allowances for the crudity of the method, there is nevertheless appreciable transaminase activity involving both glycine and serine in P. AM1.

Enzymic reduction of hydroxypyruvate and glyoxylate.

Hydroxypyruvate reductase (glycerate dehydrogenase) (Stafford, Magaldi & Vennesland, 1954) and glyoxylate reductase (Zelitch, 1955) were tested for spectrophotometrically as follows. Extract, 100 μmoles of phosphate buffer pH 7.5, 0.4 μmole of NADH and lithium hydroxypyruvate

(2 µmoles) or sodium glyoxylate (10 µmoles) were incubated in 3 ml. quartz cuvettes, and the decrease in optical density at 340 mµ measured against a blank containing all components except NADH and substrate, and the rate corrected for NADH oxidase activity in the absence of substrate.

Extracts of P. AM1 catalysed the reduction of both hydroxypyruvate and glyoxylate by NADH:-

Hydroxypyruvate + NADH + H^+ $\rightleftharpoons$ D-glycerate + NAD^+ (viii)

Glyoxylate + NADH + H^+ $\rightleftharpoons$ glycollate + NAD^+ (ix)

The specific activity with respect to these substrates was 41.6 µmoles NADH oxidised/hr./mg. protein for hydroxypyruvate and 4.6 µmoles /hr./mg. protein for glyoxylate at pH 7.5 in extracts of formate-grown cells. When extracts of succinate-grown cells were assayed under the same conditions the specific activities were 21.8 µmoles/hr./mg. protein for hydroxypyruvate and 1.97 µmoles /hr./mg. protein for glyoxylate.

Zelitch (1955) crystallised glyoxylate reductase from tobacco leaves, and found that it had hydroxypyruvate reductase activity. Stafford et al. (1954) purified glycerate dehydrogenase (hydroxypyruvate reductase) from parsley leaves and found that it had glyoxylate reductase activity. Similar observations were made with the same

enzyme purified from spinach leaves by Holzer & Holldorf (1957b). It appears probable that in plants these two activities are catalysed by the same enzyme.

It is possible that this may also be the case in P. AM1. Thus the ratio (specific activity with hydroxypyruvate)/(specific activity with glyoxylate) is closely similar in formate-grown cells (9.04) and succinate-grown cells (11.1) although the absolute values differ considerably. In order to explore this possibility further, an attempt was made to separate the two activities by ammonium sulphate fractionation of an extract of methanol-grown cells. The results of this are set out in table IV. 4. This shows that not only are the two activities recovered in the same ammonium sulphate fraction, but that the purification is the same for each (2.1-fold for hydroxypyruvate reductase, 2.16-fold for glyoxylate reductase) and that the ratio (specific activity with hydroxypyruvate)/(specific activity with glyoxylate) is the same for both crude (4.3) and the ammonium sulphate fraction (4.2)

As far as they go, the above data indicate that the two activities may well be due to the same enzyme. Recently, a hydroxypyruvate reductase has been purified from Aspergillus

TABLE IV. 4

ATTEMPT TO SEPARATE HYDROXYPYRUVATE AND GLYOXYLATE REDUCTASE ACTIVITIES.

A sonic extract of methanol-grown P. AM1 was prepared as described in chapter II, and centrifuged for 10 min. at 25000 x g to remove debris. To the supernatant (vol. 10 ml.) were added 3.44 ml. saturated ammonium sulphate (A.S.) solution to bring it to 30% saturation. The precipitate was removed by centrifugation, and the process repeated, taking a series of 10% fractions as indicated below. The precipitates were re-dissolved in 3 ml. 50mM-tris hydrochloride pH 7.5, dialysed overnight against 1.5 l. 10mM-tris hydrochloride pH 7.5, and then assayed as described in the text, save that phosphate buffer pH 6.5 was used.

A.S. Fraction	Protein concn. (mg./ml.)	Specific activity at pH 6.5 (μmoles/hr./mg. protein) with	
		Hydroxypyruvate	Glyoxylate
Crude extract before A.S. treatment.	4.6	62.5	1.46
0 - 30% saturation	0.86	Slight	None
30 - 40%	1.46	Slight	None
40 - 50%	1.06	Slight	None
50 - 60%	1.68	Slight	None
60 - 80%	1.98	131.2	3.15

niger which is free of glyoxylate reductase activity. (Behal & Hamilton, 1962).

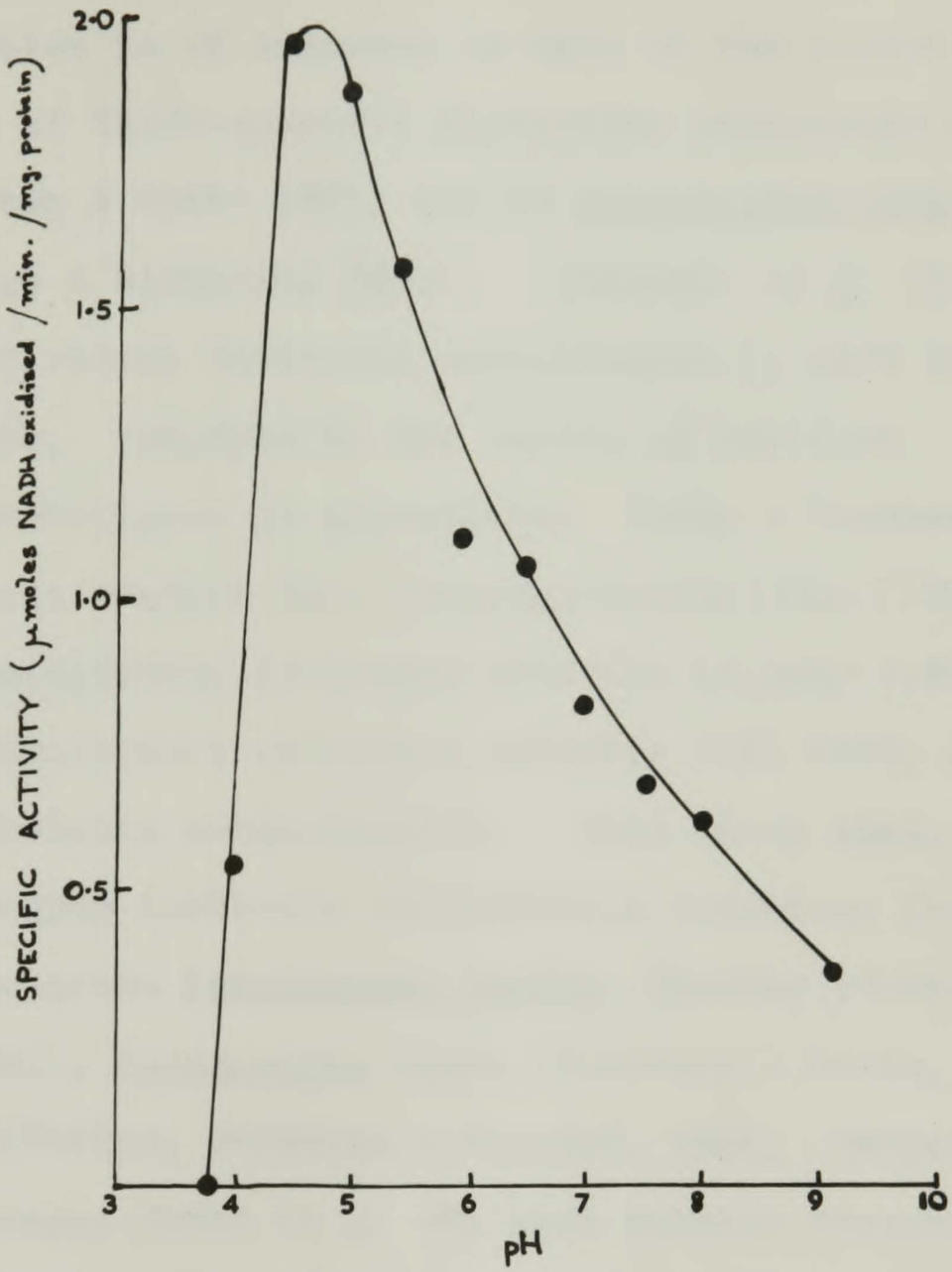
Further properties of glycerate dehydrogenase in crude extracts.

It appears probable that the natural substrate of the enzyme is hydroxypyruvate rather than glyoxylate, since the activity with the former substrate is much higher. The hydroxypyruvate- and glyoxylate-reducing activities closely resemble those observed by Gray, Gerhart, & Brooke (1959) in extracts of a soil micro-organism grown on glyoxylate. Some experiments were performed on the hydroxypyruvate reductase in crude extracts, in order to demonstrate its properties. For the NADH-linked reduction of hydroxypyruvate (reaction (viii) above), the pH optimum was found to be 4.5 (fig. 6), which is not unexpected in view of the proton requirement for reaction (viii).

The specificity of the reaction for substrate and pyridine nucleotide was also examined. NADPH was much less effective than NADH. The specific activity at pH6.5 for the reduction of hydroxypyruvate was respectively 63.9 μmoles /hr./mg. protein for NADH and 24 μmoles /hr./mg. protein for NADPH. With glyoxylate the figures were 3.45

Fig. 6

pH Optimum of hydroxypyruvate reduction by crude extracts of Pseudomonas AMI.



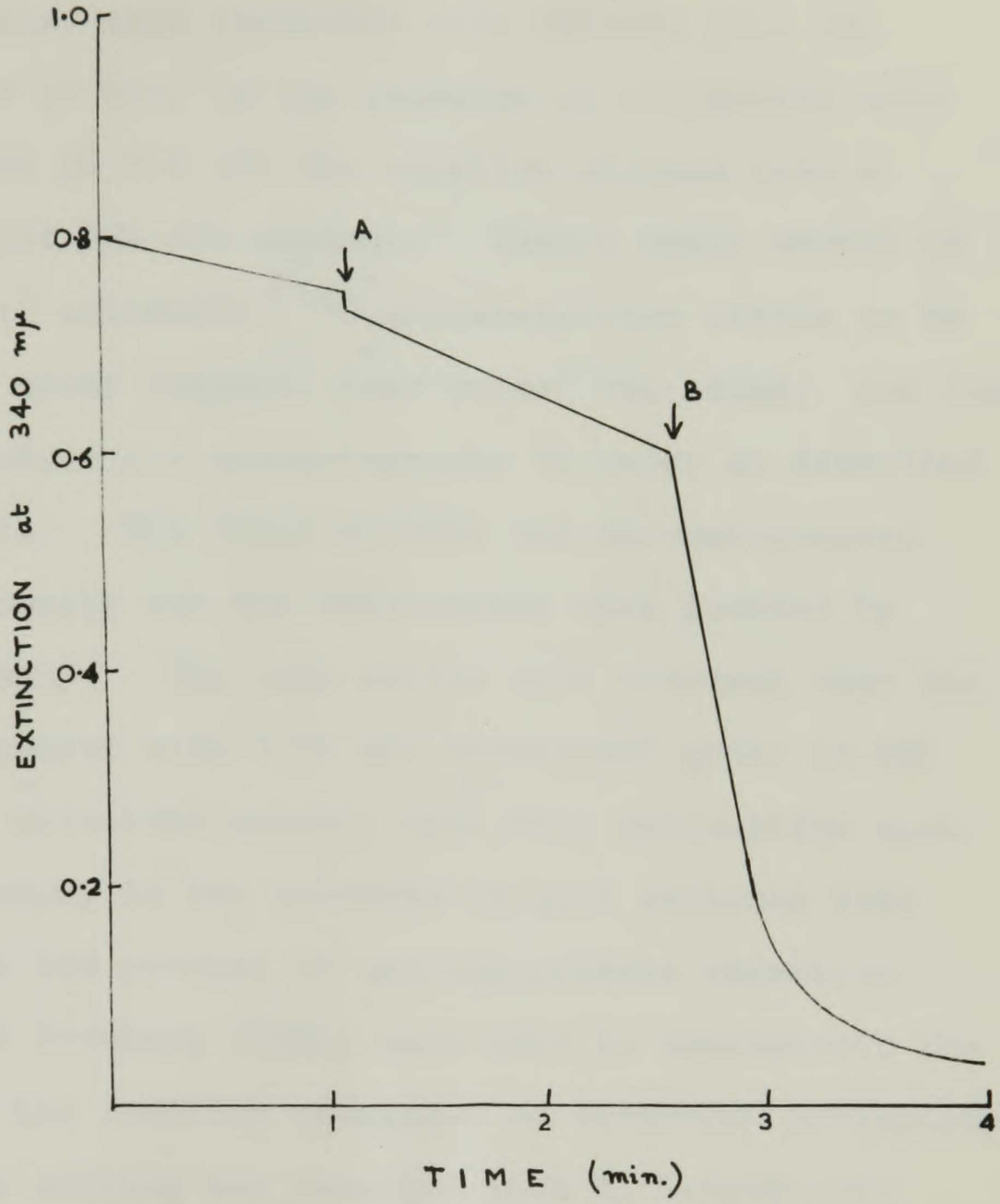
3 ml. silica cuvettes (light path, 1 cm.) contained: 100 μmoles of buffer, 0.4 μmoles of NADH and extract. Rate of decrease in extinction at 340 mμ on addition of 2 μmoles of lithium hydroxypyruvate followed on the Cary recording spetrophotometer. Acetate buffer below pH 5.5, phosphate above pH 5.5.

with NADH, and with NADPH no detectable activity. This last observation is of interest in view of the recent observations of NADPH-specific glyoxylate reductases in plants (Zelitch & Gotto 1961) and in Aspergillus niger (Franke, Jilge & Eichhorn, 1961). Extracts of P. AM1 were found to reduce tartronic semialdehyde (a gift from Dr. A.M. Gotto; prepared by the action of purified glyoxylate carboligase on glyoxylate; Gotto & Kornberg, 1961) much less readily than hydroxpyruvate (fig.7).The tartronic semialdehyde reductase activity is only 4.6% of the hydroxypyruvate reductase activity with NADH, at equimolar substrate concentration. This shows that, unlike the enzyme tartronic semialdehyde reductase found in glycollate-grown Pseudomonas Ovalis Chester (Gotto & Kornberg, 1961), Pseudomonas B_2aba (Kornberg & Gotto, 1961) and E. coli (Krakow, Barkulis & Hayashi, 1961; Sadler, 1961), the enzyme found in P. AM1 more closely resembles the plant enzyme. The absence of lactate dehydrogenase in extracts of P. AM1 was evident from the fact that pyruvate was inactive in the above assay whereas hydroxypyruvate and glyoxylate were active.

In an attempt to characterise glycerate as the product of the reaction, lithium hydroxypyruvate (6 μmoles) and

Fig. 7

Substrate specificity of hydroxypyruvate reduction by crude extracts.



1.5 ml. silica cuvettes (light path, 1 cm.) contained: 100 μmoles of phosphate buffer pH 6.5, 0.2 μmole of NADH and extract. At A, 2 μmoles of tartronic semialdehyde were added; at B, 2 μmoles of lithium hydroxypyruvate. (Taken from a trace by a Cary recording spectrophotometer.)

NADH (8 µmoles) were incubated with extract (3.1 mg. protein) for 10 min. in the presence of 100 µmoles tris hydrochloride pH 7.5 and the reaction stopped with 3 volumes of hot 95% v/v ethanol. Then a small amount of a solution of authentic [^{14}C]glycerate (too little to be detected by spray reagent) (see below) was added; and the mixture prepared for chromatography on paper as described in chapter II. The whole mixture was chromatographed two-dimensionally and the radioactive spot located by radioautography. The only yellow spot produced when the paper was sprayed with 0.1% w/v bromcresol green in 95% v/v ethanol coincided exactly with this radioactive spot. Cochromatography in two solvents is good evidence that glycerate is the product of hydroxypyruvate reduction.

Gotto & Kornberg (1961) were able to demonstrate the reversal of the reaction catalysed by tartronic semialdehyde reductase by pulling the reaction with 2, 6-dichlorophenolindophenol (DCPIP) and NADH dehydrogenase:-

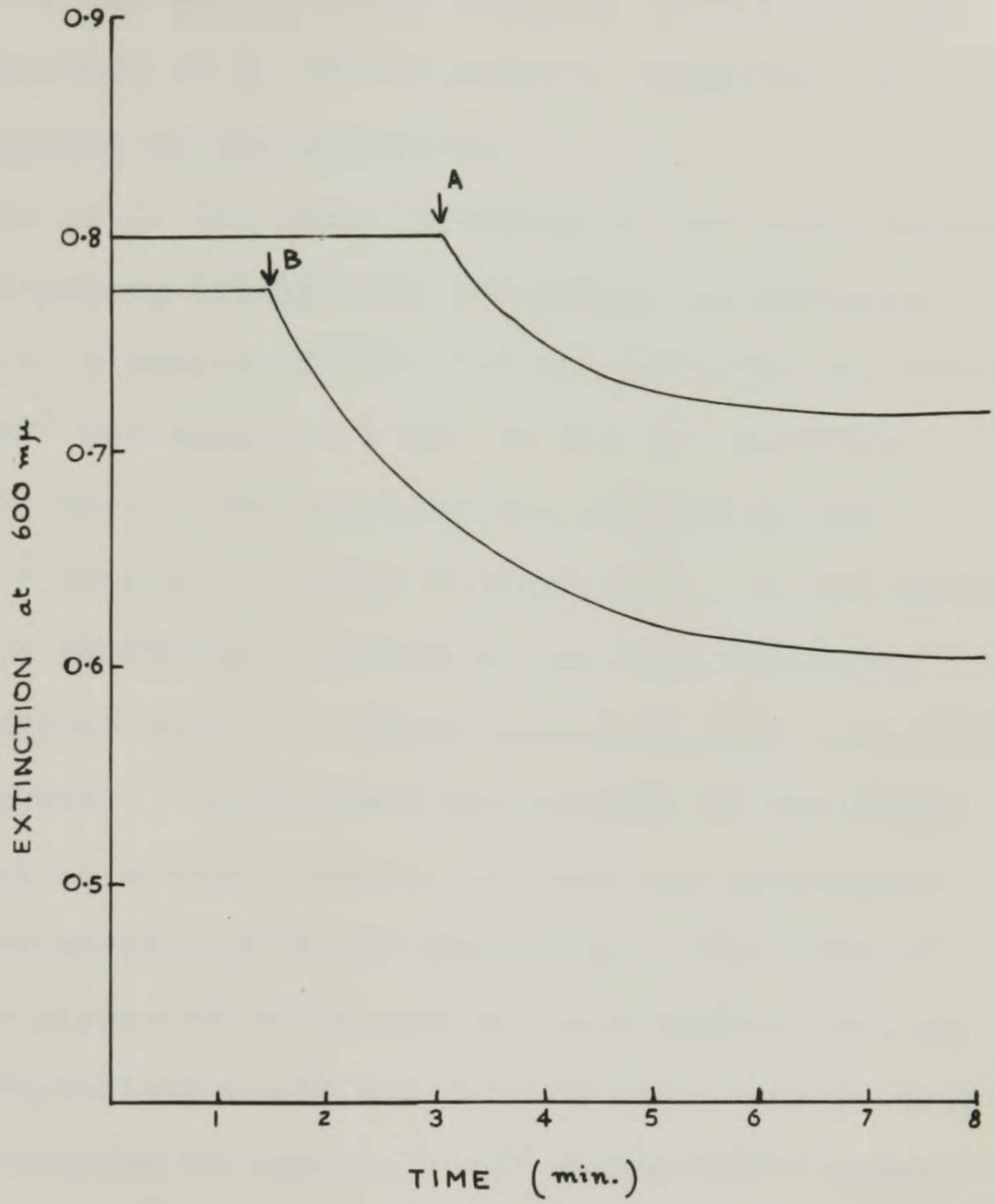
$$\text{D-Glycerate} + NAD^+ \rightleftharpoons \text{tartronic semialdehyde} + NADH + H^+ \quad (x)$$

$$NADH + H^+ + \text{DCPIP} \rightleftharpoons NAD^+ + \text{leucoDCPIP} \quad (xi)$$

In this system NAD^+ acted catalytically and the reduction of DCPIP (measured as decrease in extinction at 600 mµ) at pH 8.6 was used as an assay for the TSA reductase. This

Fig. 8

Oxidation of glycerate by crude extracts.



1.5 ml. silica cuvettes (light path, 1cm.) contained: 100 μmoles of phosphate buffer pH 8.6, 5 μmoles of KCN, 0.4 ml. of 0.01% W/V DCPIP, 0.6 μmole of NAD and extract. At A, 0.250 μmole of sodium DL-glycerate added; at B, 0.250 μmole of potassium D-glycerate. (Taken from a trace by a Cary recording spectrophotometer.)

method was adopted to study the reversal of the hydroxypyruvate reductase of P. AM1 in order to determine whether D- or L-glycerate is the substrate.

Extracts of P. AM1 were incubated in the assay system of Gotto & Kornberg (1961) with 100 µmoles of phosphate buffer pH 8.6, 5 µmoles of KCN, 0.4 ml. of 0.01% w/v DCPIP, 0.6 µmole NAD^+ and water to 1 ml. in 1.5 ml. cuvettes (light path 1 cm). The reaction was started by the addition of 4 µmoles of sodium DL-glycerate, and the decrease in extinction at 600 mµ followed on the Cary recording spectrophotometer against a blank containing all components except glycerate. Under these conditions, it was found that a change in optical density of 0.68 was equivalent to the oxidation of 1 µmole of glycerate. The rate of oxidation of glycerate at pH 8.6 was 10.4 µmoles /hr./mg. protein. Glycollate would not replace glycerate in this system. Potassium DL-lactate would reduce DCPIP under these conditions, but at a 3-fold higher concentration the rate was only 37% of that with glycerate. In order to determine which enantiomorph is active in the system, limiting amounts of sodium DL-glycerate and potassium D-glycerate (a gift from Dr. A.M. Gotto) (0.250 µmoles) were taken, and the reaction allowed to go to completion.

Fig. 8 shows that under these conditions D-glycerate is twice as effective as DL-glycerate, and hence the D enantiomorph is the substrate for the reaction.

The formation of 3-phosphoglycerate from glycerate.

Glycerate kinase, catalysing reaction (xii)

D-Glycerate + ATP $\longrightarrow$ 3-phosphoglycerate + ADP (xii)

has been demonstrated in rat liver (Holzer & Holldorf, 1957a), rapeseed (Ozaki & Wetter, 1960), yeast (Black & Wright, 1956) and bacteria (Kornberg & Gotto, 1961). Attempts to demonstrate reaction (xii) in P. AM1 by incubation of glycerate and ATP with extract, stopping the reaction by heating, and estimating the PGA formed with purified phoshoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (C.F. Boehringer und Söhne, Mannhein, Germany) were not successful. Accordingly, a qualitative approach was attempted. Sodium [^{14}C]glycerate was obtained from 3-phospho[^{14}C]glycerate (prepared by incubation of extracts of formate-grown P. oxalaticus with sodium[^{14}C]-bicarbonate and ribulose-1, 5-diphosphate; Quayle & Keech, 1959b) by dephosphorylation with a preparation of Polidase-S followed by paper chromatography and radioautography. The [^{14}C]glyceric acid was incubated with extract of formate-

TABLE IV. 5.

FORMATION OF 3-PGA FROM GLYCERATE.

The system contained in 1 ml. : 50 μmoles of tris hydrochloride pH 7.5, 4 μmoles of $MgCl_2$, 5 μmoles of glutathione, 10 μmoles of KF, sodium D-[^{14}C]glycerate (1000 counts/min.), extract (containing 4 mg. protein) and either 5 μmoles of ATP or no ATP.

The incubation was for 60 min. at 30°, and was stopped by the addition of 2 ml. boiling 95% v/v ethanol. The deproteinised solution was chromatographed two-dimensionally, and the products located by radioautography as described in chapter II. The 3-PGA was identified as described in the text.

Tube.	Contents	Radioactivity (counts/min.) in:	
		Glycerate	3-Phosphoglycerate
1	No ATP	935	0
2	ATP (5 μmoles)	15.4	541

grown P. AM1 and ATP as shown in table IV. 5. In the tube which contained ATP, 95% of the radioactivity was found in the phosphate area; the tube without ATP gave rise only to unchanged [^{14}C]glycerate. The radioactive phosphate area was eluted, 2 µmoles authentic sodium 3-phosphoglycerate added, the mixture shaken with about 10 mg. Dowex 50 (H^+ form) and cochromatographed in methanol-formic acid (90% w/w)- water (80: 15: 5 by vol., Bandurski & Axelrod, 1951). The radioactive spot located by radio-autography agreed exactly with the blue spot obtained by the phosphate spray described in Chapter II. Thus extracts of P. AM1 can phosphorylate glyceric acid to 3-PGA. Presumably the PGA so formed will give rise to the sugar phosphates and trehalose which are formed rapidly in whole cells (chapter III) by recognised metabolic pathways.

Conversion of L-serine to glycerate.

Experiments on the enzymic incorporation of [^{14}C]formate by extracts of formate-grown P. AM1 (see section 1 of this chapter) showed that in addition to serine, small amounts of glyceric acid were formed. In view of the presence of a transaminase, catalysing reaction (vii) and glycerate dehydrogenase, catalysing reaction (viii), it is clear

TABLE IV. 6.

CONVERSION OF [^{14}C] SERINE TO GLYCERIC ACID BY FORMATE-GROWN P. AM1.

The system contained, in 1.3 ml.: extract of formate-grown P. AM1 (containing 1.2 mg. protein), 60 μmoles of phosphate buffer pH 7.5, 0.1 μmole of pyridoxal phosphate, and 0.34 umole (1 μC) of L-[^{14}C] serine, with the additions shown below.

The incubation was for 30 min. at 30° under N_2, and the reaction stopped by the addition of 3 ml. boiling 95% v/v ethanol. The deproteinised solutions were processed as described in chapter II and aliquots chromatographed two-dimensionally. The products were identified by cochromatography.

Tube	Additions	Products formed Counts/min./mg. protein x 10^{-}		
		Serine	Glycerate	Total
1	None	53.9	0.23	54.13
2	Sodium pyruvate (4 μmoles)	40.4	0.55	40.95
3	Sodium pyruvate (4 μmoles) NADH (3 μmoles)	40.16	7.02	47.18
4	None; boiled extract	50.9	0	50.90

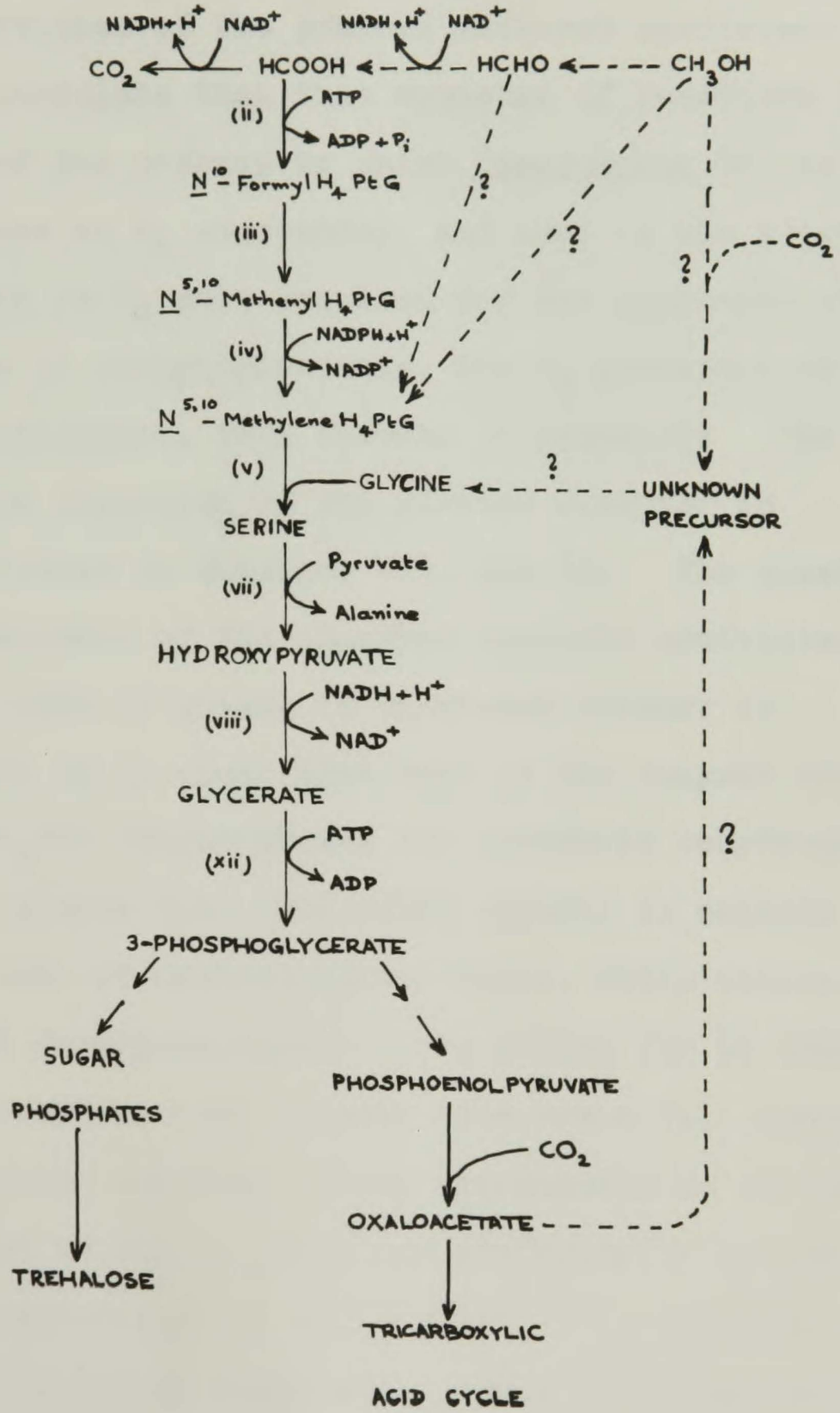
that glyceric acid could be formed from serine under these conditions. The ability of extracts of formate-grown P. AM1 to catalyse the formation of labelled glycerate from L - [^{14}C]serine was then tested. The results of this experiment are shown in table IV. 6. Glyceric acid was identified by cochromatography in 3 solvents. In the complete system (tube 3) there was a 15% conversion of serine to glycerate. In tube 2, where pyruvate but not NADH was supplied there is a 20% drop in the total recovered radioactivity. This presumably is due to the formation of [^{14}C]hydroxypyruvate by transamination (reaction vii) which would be destroyed by the techniques used in this experiment.

Discussion.

The experiments described in this chapter demonstrate the presence of all the enzymes required to catalyse reactions (ii) - (v); (vii), (viii) and (xii), which would result in the complete conversion of formate and glycine to 3-phosphoglyceric acid. This sequence of reactions is set out in fig. 9. In addition, the tracer experiments show that in fact these enzymes, present in extracts, can indeed act in the concerted manner postulated to bring about the synthesis of phosphoglycerate from formate.

Fig. 9

The formation of 3-phosphoglycerate from formate and glycine.



Conjectual reactions in dotted lines. The Roman numerals in brackets refer to the equations in Ch. IV.

Two questions, however, immediately arise. Are the specific activities of the enzymes measured sufficient to warrant the postulate that this sequence of reactions is indeed part of the pathway by which Pseudomonas AM1 is enabled to grow on C_1 compounds; and what is the source of the glycine or C_2 unit required for the synthesis of each molecule of phosphoglycerate, the C_3 precursor of cellular constituents, from formate or methanol? The problem of the formation of the glycine acceptor is considered further in chapters VIII and IX. The question of the relationship of the observed specific activities to the observed rate of growth is discussed further in chapter V, but it is clear that some of the enzymes such as methyleneH_4PtG dehydrogenase and glycerate dehydrogenase are present in more than sufficient amounts to explain the growth rate even of methanol-grown cells, while others, notably H_4PtG formylase appear to be rather low in activity. It is of interest to note however (see table V.1., chapter V), that the specific activity of all the enzymes in the pathway postulated in fig. 9 which can conveniently be measured, are consistently higher in cells grown on C_1 substrates than in cells grown on succinate. This is strong evidence that they may play a more important role in cells grown on

C_1 substrates than merely the synthesis of glycine and serine for protein constituents.

In view, however, of the observation that H_4PtG is not as active as other folic acid derivatives in the synthesis of serine from formate, it may well be that some of the enzymic activities measured with H_4PtG as cofactor, would in fact have been much higher had the natural derivative (or some closer relative to it e.g. H_4PtG_3) been used instead of H_4PtG in these assays.

Chapter V

ENZYMIC ACTIVITIES OF EXTRACTS OF FORMATE-GROWN *PSEUDOMONAS* AM1.

In chapter III, it was suggested that during growth on C_1 compounds, *Pseudomonas* AM1 assimilates carbon in approximately equal proportions from both carbon dioxide and a "reduced C_1 unit." The presence of enzymes in formate-grown *P.* AM1 which could bring about the formation of serine (the first labelled product of $[^{14}C]$formate assimilation) and its conversion to 3-phosphoglyceric acid by cell free extracts was demonstrated in chapter IV. The major port of entry of carbon dioxide into cellular material is thought to be the carboxylation of a C_3 unit to malate or aspartate (chapter III), and chapter X describes the search for enzymes bringing about such a reaction.

This chapter describes the demonstration in formate-grown *P.* AM1 of various enzymes concerned in the metabolic inter-relationships of C_2 compounds, C_3 compounds, and some of the intermediates of the tricarboxylic acid cycle.

Metabolic interrelationships involving C_2 compounds.

Experiments on postulated reactions for the formation of C_2 units which could be precursors of glycine (one molecule of which is necessary for the assimilation of every molecule of "reduced C_1 unit" by the serine transhydroxymethylase reaction, see chapter IV) are described in chapter IX. Only enzymes for which C_2 compounds are substrates will be described here.

Glyoxylate carboligase. This enzyme, like serine transhydroxymethylase, results in the net formation of a C_3 compound (tartronic semialdehyde) from a C_2 compound (glyoxylate)

$$2\ CHO.COOH \xrightarrow{TPP} CHO.CH(OH).COOH + CO_2 \qquad (i)$$

It plays a key role in the synthesis of cell constituents by organisms growing on C_2 compounds such as glycollate (Krakow & Barkulis, 1956; Kornberg & Gotto, 1959, 1961), glycine (Callely & Dagley, 1959) and oxalate (Quayle & Keech, 1959a, Quayle, Keech & Taylor, 1961).

The enzyme was assayed manometrically in Warburg vessels containing in the main compartment, extract, 10 µmoles of $MgCl_2$, 1 µmole of TPP, and 100 µmoles of phosphate buffer pH 7.5. The two side-arms contained respectively

20 µmoles of sodium glyoxylate and 0.2 ml. 10N-H_2SO_4. The gas phase was N_2. No CO_2 evolution could be detected, indicating that this enzyme is not present in P. AM1. Thus serine transhydroxymethylase is the only reaction which has been detected by which C_3 compounds can be synthesised from C_2 compounds in this organism.

Glyoxylate reductase. This has been discussed in connection with hydroxypyruvate reductase (glycerate dehydrogenase) in chapter IV.

Malate synthetase. This enzyme (Wong & Ajl, 1956; Dixon, Kornberg & Lund, 1960) catalyses the aldol condensation of acetyl coenzyme A with glyoxylate to form malate.

$$CH_3.CO.SCoA + CHO.COOH \longrightarrow HOOC.CH(OH).CH_2.COOH + CoASH \quad (ii)$$

It has been shown to play an essential role in the growth of micro-organisms on acetate (see Kornberg, 1959a, b) and glycollate (Kornberg & Gotto, 1961; Kornberg & Sadler, 1961). The enzyme was assayed by the spectrophotometric method of Dixon & Kornberg (1959), in which acetyl CoA (0.025 µmole), $MgCl_2$ (2 µmoles), tris hydrochloride pH 7.1 (18 µmoles) are incubated in 0.5 ml. silica cuvettes (light path 1 cm.), and the decrease in optical density at

232 mμ, consequent on the cleavage of the thiolester bond on the addition of glyoxylate, is measured. The enzyme was found to be present with a specific activity of 1.74 μmoles acetyl CoA cleaved /hr./mg. protein. With extracts of cells grown on succinate, the corresponding figure was 3.03 μmoles /hr./mg. protein. In view of the fact that malate synthetase has been detected at similar levels of activity in all species of micro-organisms so far tested (Kornberg & Elsden, 1961), it appears unlikely that at this level of activity it plays any unique role in growth on C_1 substrates. It is interesting to note that in Pseudomonas AM1 grown on ethanol the specific activity of malate synthetase is much higher, being 4.32 μmoles / hr./mg. protein (see chapter IX, and below).

Glyoxylate dehydrogenase (Oxalyl coenzyme A reductase). This enzyme (Quayle, Keech & Taylor 1961; Quayle & Taylor, 1961; Quayle, 1962) plays a key role in the biosynthesis of cell constituents by P. oxalaticus when it grows on oxalate as sole source of carbon and energy:

$$HOOC.CO.SCoA + NADPH + H^+ \rightleftharpoons HOOC.CHO + NADP^+ + CoASH \quad (iii)$$

It was assayed as described by Quayle & Taylor (1961) by measurement of the coenzyme A-dependent reduction of

$NADP^+$ in the presence of glyoxylate. The assay system contained, in 1.5 ml. silica cuvettes (light path, 1 cm.): 27 μmoles of pyrophosphate buffer pH 8.6, 2.5 μmoles of cysteine, 5 μmoles of sodium glyoxylate, 0.2 μmole of CoA, 0.2 μmole of NADP and water to 1 ml. The specific activities of extracts of cells grown on formate and on succinate were respectively 0.49 and 0.64 μmoles of NADPH formed /hr./mg. protein. An examination of most of the known organisms which can grow on C_1 substrates or oxalate as carbon source for the presence of this enzyme is reported in chapter XII. It appears probable from this comparison that the glyoxylate dehydrogenase does not play a significant role in the growth of P. AM1 on formate.

The enzymes of the tricarboxylic acid cycle.

Spectrophotometric assays for the following enzymes of the tricarboxylic acid cycle were performed as described below: condensing enzyme, isocitrate dehydrogenase, and malate dehydrogenase. Malic enzyme and isocitratase were also assayed. The assays were performed on extracts of formate-grown cells, and for comparison (see below) also on extracts of cells grown on succinate.

Condensing enzyme was assayed by measuring the

oxaloacetate-dependent decrease in optical density at 232 mμ consequent on the cleavage of the thiolester group of acetyl-CoA under the conditions described by Dixon & Kornberg (1959):

Oxaloacetate + Acetyl-CoA $\rightleftharpoons$ Citrate + CoASH (iv)

The specific activities for formate- and succinate-grown cells were respectively 2.25 and 7.01 μmoles/hr./mg. protein.

Isocitrate dehydrogenase was assayed by measuring the rate of increase in extinction at 340 mμ on the addition of NADP to threo-D_s-isocitrate at pH 7.5 in the presence of $MgCl_2$ (Ochoa, 1948):-

threo-D_s-(+)-Isocitrate + $NADP^+$ $\rightleftharpoons$ α-Oxoglutarate + CO_2 + NADPH + H^+ (v)

The specific activities for formate- and succinate-grown cells were respectively 10.4 and 20.0 μmoles /hr./mg. protein.

Malate dehydrogenase was assayed as described by Ochoa (1955) in which the rate of decrease in extinction at 340 mμ consequent upon the oxidation of NADH by oxaloacetate at pH 7.0 is measured:-

Oxaloacetate + NADH + H^+ $\rightleftharpoons$ L-Malate + NAD^+ (vi)

The specific activities were 44.2 μmoles/hr./mg. protein for formate-grown cells and 81.3 for succinate-grown cells.

Malic enzyme was assayed as described by Ochoa, Mehler & Kornberg (1948) in which the reduction of NAD or NADP is followed at 340 mμ in the presence of extract, Mn^{++} and an excess of L-malate at pH 7.4:-

L-Malate + $NADP^+$ $\rightleftharpoons$ Pyruvate + CO_2 + NADPH + H^+ (vii)

(Malate dehydrogenase will not catalyse the oxidation of malate under these conditions). No malic enzyme could be detected in formate-grown cells. Succinate-grown cells were not tested.

Isocitratase (Olson, 1954; Smith & Gunsalus, 1954) was assayed by the method of Dixon & Kornberg (1959) which follows the formation of glyoxylate phenylhydrazone from threo-D_s-isocitrate spectrophotometrically at 324 mμ:

threo-D_s-(+)-Isocitrate $\rightleftharpoons$ Succinate + Glyoxylate (viii)

Glyoxylate + Phenylhydrazine $\longrightarrow$ Glyoxylate phenylhydrazone (ix)

No activity was found in formate - or succinate-grown cells. Ethanol-grown cells however were found (see chapter IX) to contain the enzyme. This observation may indicate (Kornberg, 1960; Kornberg & Elsden, 1961) that the catabolism of ethanol in P. AM1 proceeds via acetate. The increased level of malate synthetase activity (see above) in ethanol-grown cells affords additional support for this hypothesis.

β-Hydroxybutyrate dehydrogenase.

In view of the finding (Peel & Quayle, 1961) that P. AM1 contains appreciable amounts of poly-β-hydroxybutyrate, it was of interest to see whether a key enzyme of β-hydroxybutyrate metabolism, β-hydroxybutyrate dehydrogenase, was present in P. AM1, as it is present in high activity in the photosynthetic bacteria which also contain the polymer (Doudoroff, Merrick & Contopoulou, 1961; Carr & Lascelles, 1961). The enzyme was assayed by measuring the decrease in extinction at 340 mμ when acetoacetate is added to extract and NADH at pH 7.5 as described by Williamson, Mellanby & Krebs (1962):-

$$\text{Acetoacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{D(-)-}\beta\text{-Hydroxybutyrate} + \text{NAD}^+ \qquad \text{(x)}$$

The specific activities measured for formate-and succinate-grown cells were respectively 4.97 and 11.0 μmoles /hr./mg. protein.

Discussion.

A complete summary of the specific activities of the enzymes whose assay is described in this chapter and chapter IV is given in table V.1.

One of the important criteria (see below)

in the demonstration of the metabolism of a particular compound is to show that enzymes are present in cell-free extracts of the organism which could bring about the postulated transformations. The specific activity of these enzymes should be of the order required by their contribution to the growth or energy requirements of the organism. Furthermore, when the organism is grown on other substrates which do not require the involvement of these particular enzymes in either anabolism or catabolism, the specific activity of such enzymes is often markedly lower. In such cases it is often possible to follow these changes in enzymic activity during adaptation of an organism to growth on a new substrate. This is well exemplified by the enormous differences in specific activity of the enzymes of the biosynthetic pathway postulated for growth on glycollate in Pseudomonas B_2aba observed when the organism is grown on glycollate compared with when it is grown on succinate (Kornberg & Gotto, 1961).

Accordingly, the level of specific activities of various enzymes detected in formate-grown cells was also measured in cells grown on succinate as sole carbon and energy source. Succinate was chosen as a substrate since

P. AM1 grows as well on succinate as it does on methanol (i.e. considerably faster than on formate), and because there is no reason to suspect that the metabolic transformations undergone by succinate during growth on this substrate are any other than the reactions of the tricarboxylic acid cycle and its ancillary processes. The results of the two comparative sets of assays are set out in table V.1. The conditions for the assays were identical, and all the spectrophotometric assays were performed at 22°.

Of the enzymes in table V.1., only the first five had specific activities greater in formate-than in succinate-grown cells. But these five enzymes are precisely the ones which have been suggested in chapter IV (see fig. 9), as playing a key role in the formation of C_3 compounds from methanol or formate.

The suggestion (chapter IV) that serine transhydroxymethylase plays a key role in the formation of C_3 compounds is not new. Kaneda & Roxburgh (1959a) suggested that the enzyme is the principal mechanism for the assimilation of carbon by Pseudomonas PRL-W4 grown on methanol, and the same enzyme has been shown to play a key role in both the formation of C_3 compounds and in energy-generation in the

TABLE V. 1.

COMPARISON OF SPECIFIC ACTIVITIES OF ENZYMES IN EXTRACTS OF P. AM1 GROWN ON FORMATE OR SUCCINATE.

Enzyme	pH of Assay	Specific Activity (μmoles of product formed /hr./mg. protein). Growth substrate		Ratio of Specific Activities: Formate-grown
		Formate	Succinate	succinate-grown
H_4PtG Formylase	7.5*	0.56	0.07	8.0
Methylene H_4PtG dehydrogenase				
Serine substrate	7.5*	0.54	0.28	1.93
HCHO substrate		22.0	13.1	1.68
Serine trans-hydroxymethylase	7.5*	1.78	0.82	2.17
Glycerate dehydrogenase	7.5*	41.6	20.6	2.02
Glyoxylate reductase	7.5*	4.6	1.97	2.33
Glyoxylate carboligase	7.5	0	0	-
Malate synthetase	7.1	1.74	3.03	0.58
Glyoxylate dehydrogenase	8.6	0.49	0.64	0.77
Condensing enzyme	7.1	2.25	7.01	0.32
Isocitrate dehydrogenase	7.5	10.4	20.0	0.52

TABLE V. 1 (continued)

Enzyme	pH of Assay	Specific Activity (μmoles of product formed /hr./mg. protein). Growth substrate: Formate	Succinate	Ratio of Specific Activities: Formate-grown succinate-grown
Malate dehydrogenase	7.0	44.2	81.3	0.54
Malic enzyme	7.4	0	-	-
Isocitratase	6.85	0	0	-
β-Hydroxybutyrate dehydrogenase	7.5	4.97	11.0	0.45

* Assay conditions described in chapter IV.

Assay conditions for the remaining enzymes are described in this chapter.

anaerobe Diplococcus glycinophilus grown on glycine (Sagers & Gunsalus, 1961; Klein & Sagers, 1962). The absence of the enzyme glyoxylate carboligase would indicate that other mechanisms for the conversion of C_2 to C_3 units observed in organisms grown on glycollate, glycine and oxalate are not operative in this organism.

No other instance can be found in the literature of the concept that tetrahydrofolate formylase plays a key role in the assimilation of carbon by an organism grown on formate. The only other instance of growth on formate which has been investigated is the growth of P. oxalaticus on formate, which was found to involve its oxidation to CO_2 and the assimilation of the latter by the RuDP cycle (Quayle & Keech 1959 a,b). A key role for the formate-activating enzyme, however, has been proposed in the generation of ATP in the purine fermenter Clostridium cylindrosporum (Rabinowitz & Pricer, 1956).

Formate is at a reduction level of -2 (if the reduction level of cell material $\left[CH_2O\right]$ is taken as 0) and a reductive step is accordingly necessary to bring it to the reduction level of cellular carbon. It is suggested that the enzyme methylenetetrahydrofolate dehydrogenase plays a key role in this reduction. The specific activity of this enzyme

is very high in formate-grown cells, as might be expected, but it is also high in methanol-grown cells (Peel, 1961, p. 84, obtained the figure of 24.2 µmoles /hr./mg. protein) where its role is more obscure (since methanol needs oxidation rather than reduction to bring it to the level of cellular material). However, its specific activity is so high in C_1-grown P. AM1 that it clearly plays some important role in the metabolism of C_1 substrates in this organism. (Peel, 1961, p. 83 suggested that its role is in the oxidation of C_1 compounds; experiments described in chapter VI render this suggestion much less likely). The activity of this enzyme was then investigated in a variety of organisms (see chapter XII) in order to determine whether its occurrence in high activity could be correlated with the ability to grow on C_1 substrates.

Kornberg & Elsden (1961) have listed the criteria which must at least be satisfied before a postulated sequence of metabolic reactions may be considered as established. These are "(1) The intermediates of the sequence must acquire label in the prescribed order. (2) The position of the isotope within a labelled intermediate must conform with the postulated sequence of events. (3) The enzymes catalysing component reactions of the

sequence must be shown to be present in the organism. (4) The rate of such component reactions must be shown to be adequate to account for the rate of the over-all reaction."

Criterion (1) is clearly established for the proposed pathway, both in whole cells (chapter III) and extracts (chapter IV). Criterion (2) is investigated and discussed in chapter VII. Criteria (3) and (4) have been investigated in chapters IV and V. Clearly criterion (3) is fulfilled in that all the enzymes for the postulated sequence have been demonstrated. This leaves the problem of the relation of the specific activities of the enzymes of the proposed biosynthetic pathway to the over-all rate of the reaction, i.e. the growth rate of the cells.

Since the observed mean generation time for growth of P. AM1 on formate is 20 hr., then 2 g. dry wt. of cells become 4 in 20 hr. By plotting this growth rate graphically, it can be shown that in 30 min., 2 g. cells become 2.04 g., i.e. 2 g. cells synthesise 0.04 g. cell material in 30 min. Hence 1 g. of cells synthesises $\frac{0.04}{60}$ g. cell material /min. If we assume that 50% of the dry weight of the cell is carbon (a figure which was obtained for E. coli by Roberts et al., 1955) then 1 g. of cells synthesises $\frac{0.04}{60 \times 2} = 3.3 \times 10^{-4}$ g. carbon/min. = 27.8 μmoles carbon/min. Since the

total cellular protein contains 300 mg. of carbon per g. dry wt. of cells (Roberts et al , 1955), this corresponds to a synthesis rate of 27.8 µmoles carbon/min./300 mg. protein carbon. Now protein contains 50% of its dry weight as carbon (Roberts et al., 1955), so the above figure can be expressed as 27.8 µmoles carbon /min./600 mg. protein. Hence the rate of carbon assimilation is $\frac{27.8}{600} \times 60$ µmoles carbon /hr./mg. protein = 2.78 µmoles carbon /hr./mg. protein. If 50% of the cellular carbon is derived from CO_2 (Large et al., 1961) then 1.39 µmoles carbon /hr./mg. protein must arise from reduced C_1 units.

In view of the many assumptions involved in this calculation, it can be regarded as no more than an indication of the order of magnitude of the activities demanded of the enzymes of carbon assimilation. It is clear, however, that of the first four enzymes in table V. 1, only tetrahydrofolate formylase falls below this figure, and even there the activity is 40% of the above calculation. Bearing in mind the above reservation, it appears that this discrepancy may not be significant. It is clear, however, that if the pathway postulated in chapter IV is indeed operative, formate activation is probably the rate-limiting step in growth.

Chapter VI

OXIDATIVE METABOLISM OF PSEUDOMONAS AM1.

Pseudomonas AM1, like all other aerobic micro-organisms, obtains the energy it requires for growth by the oxidative catabolism of its growth substrate. In micro-organisms grown on compounds containing three or more carbon atoms, the processes associated with the oxidation of their growth substrates are designed to modify the molecule in such a way as to enable it to enter the tricarboxylic acid cycle, which is the universal principal pathway of the oxidative catabolism of carbon compounds. In addition to its role as a pathway of terminal oxidation, the concept has developed in recent years (Krebs, Gurin & Eggleston, 1952) of the important role of the tricarboxylic acid cycle as a pathway for the generation of the precursors of cellular protein (oxaloacetate, α-oxoglutarate and pyruvate, which together are the precursors of 11 of the 20 amino acids found in proteins ; Roberts et al., 1955).

It appears probable that in P. AM1, energy is not generated by the tricarboxylic acid cycle, but by the direct oxidation of the C_1 growth substrates to carbon

dioxide:-

$$CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2 \qquad (i)$$

This is supported by the observation that during the short term growth of P. AM1 on [^{14}C]methanol, large quantities of [^{14}C]formate accumulate (Peel, 1961, p. 31).

Since most of the enzymes of the tricarboxylic acid cycle are present in formate-grown cells (see chapter V) it appears likely that their role is purely biosynthetic. This is supported by the observation that in succinate-grown cells, where the cycle has presumably a catabolic in addition to a biosynthetic function, the specific activities of the enzymes of the tricarboxylic acid cycle are much higher (table V.1.).

Oxidative properties of whole cells.

Washed cells of formate-grown P. AM1 readily oxidised methanol, formaldehyde and formate with Qo_2 values which were usually similar to those in table VI. 1. Methylamine was only oxidised after a lag of 3 hr. Intermediates of the tricarboxylic acid cycle were much less readily oxidised, which may indicate the presence of permeability barriers, or possibly this may indicate that they are not intermediates in the oxidation of C_1 substrates. Succinate-grown cells

TABLE VI. 1.

OXIDATION OF VARIOUS SUBSTRATES BY FORMATE- and SUCCINATE-GROWN P. AM1.

Manometer vessels contained: main compartment, 1 ml. of cell suspension (2 - 4 mg. dry wt.), 100 μmoles of sodium-potassium phosphate buffer pH 7.5; side arm, substrate (10 μmoles); centre well, 0.2 ml. 2N-KOH. Total volume of contents was 2.7 ml. Incubation was at 30° for 3 - 4 hrs.

Substrate	Q_{O_2} (μl./hr./mg. dry wt.) Formate-grown cells	Succinate-grown cells
None	-8	-29
Methanol	-146	-32
Formaldehyde	-121	-65
Formate	-53	-39
Methylamine	-57 *	
Succinate	-22	-73
Malate	-23	-81
Fumarate	-16	
Citrate	-9	
Glycine	-17	
Serine	-14	

* After a lag of 3 hr.

were found not to oxidise methanol or formate, but formaldehyde was oxidised quite readily.

5mM-sodium hypophosphite was found to produce complete inhibition of formate oxidation (see Takamiya, 1953) and significantly reduced the rate of methanol and formaldehyde oxidation. 1mM-2, 4-dinitrophenol did not affect the rate of oxidation but raised the total oxygen consumption from 64% of the theoretical for methanol to 70%, and from 80% of the theoretical for formate to 98%. As observed by Harrington & Kallio (1960) in P. methanica, the oxidation of methanol by whole cells was completely inhibited by 5mM-EDTA. In the case of formaldehyde, however, the inhibition was only 50% (Harrington & Kallio did not observe any inhibition), and the oxidation of formate was unaffected.

Oxidative properties of extracts.

Only very slight oxygen uptakes could be detected manometrically on incubating Hughes press extracts of formate-grown P.AM1 with methanol, formaldehyde or formate or with the addition of NAD or NAD and phenazine metho-sulphate.

Oxidation of methanol.

When cell-free extracts of formate-grown cells were incubated with 100 µmoles of phosphate buffer pH 7.5, 10 µmoles of glutathione or cysteine, and NAD or NADP (0.5 µmole) in 3 ml. silica cuvettes, and the reaction initiated by addition of 10 µmoles of methanol, no increase in extinction at 340 mµ could be detected. This seems to indicate the absence of a nicotinamide nucleotide-linked methanol dehydrogenase. This observation is in agreement with that of Harrington & Kallio (1960). These authors were unable to detect methanol dehydrogenase activity in methanol-grown P. methanica (Iowa strain). Kaneda & Roxburgh (1959c), on the contrary, found evidence for an NAD-linked methanol dehydrogenase in methanol-grown P. PRL-W4, though they did not determine its specific activity.

Having failed to demonstrate any nicotinamide nucleotide-dependent methanol dehydrogenase, Harrington & Kallio (1960) tested for the peroxidative oxidation of methanol by the procedure of Keilin & Hartree (1936, 1945), who found that a catalase-hydrogen peroxide complex would oxidise a number of different substrates by the reaction

$$AH_2 + H_2O_2 \xrightarrow{\text{Catalase}} A + 2\,H_2O \qquad \text{(ii)}$$

(see Mason, 1957). In the normal activity of catalase AH_2 is H_2O_2, but AH_2 can be ethanol, methanol, propan-1-ol, nitrite, formaldehyde, azide, iodide, or formate. Harrington & Kallio generated hydrogen peroxide by means of glucose oxidase and glucose. When extract and methanol were added there was an increase in oxygen uptake over the net observed for equations (iii) and (iv)

$$\text{Glucose} + O_2 \longrightarrow \text{Gluconate} + H_2O_2 \qquad \text{(iii)}$$

$$H_2O_2 \longrightarrow H_2O + 1/2\,O_2 \qquad \text{(iv)}$$

By reducing the amount of H_2O_2 decomposing by reaction (iv) by the operation of reaction (v)

$$CH_3OH + H_2O_2 \longrightarrow HCHO + 2H_2O \qquad \text{(v)}$$

an increase in oxygen uptake was observed. Harrington & Kallio showed that the oxygen uptake was dependent on glucose concentration and hence on the hydrogen peroxide concentration.

The experiments of Peel (1961, p. 82) had previously shown that sonic and Hughes press extracts of methanol-grown P. AM1 were inactive in this system. Harrington & Kallio, however, prepared their extract by grinding with alumina. It was found that alumina-ground cell-free extracts of both freshly harvested and lyophilised methanol-

grown P. AM1 would produce similar results to the observations of Harrington & Kallio.

Extracts were prepared as follows. Finely powdered calcined aluminium oxide (A.R.) was repeatedly washed with distilled water until the pH of the suspension had fallen from 10 to 6. The washed alumina was thoroughly dried at 120° and cooled. Alumina (160 mg.) was mixed with 100 mg. lyophilised methanol-grown cells or 500 mg. wet wt. freshly harvested cells in a mortar at 4° and ground with a pestle for 5 min. The material was then extracted with 2.5 ml. phosphate buffer (0.05 M, pH 7.5), the mixture transferred to a centrifuge tube and the debris removed. The extract contained 6 - 8 mg. protein/ml. When such extracts were incubated in manometer vessels as described in table VI. 2, there was a greater oxygen uptake in the complete system than in the controls, exactly as described by Harrington & Kallio (1960). The significance of these results is discussed in detail below, but it is probable that the mere demonstration of a peroxidative oxidation is not entirely sufficient to account for the oxidation of methanol: the significant oxygen uptake observed in this experiment with methanol and extract alone (vessel 3, table VI, 2) (an observation confirmed by Johnson, 1962)

TABLE VI. 2.

PEROXIDATIVE OXIDATION OF METHANOL BY EXTRACTS OF METHANOL-GROWN P. AM1.

The complete system, in manometer vessels, contained: main compartment, 100 μmoles phosphate buffer pH 7.5, 3 μmoles of glucose, 6 μmoles of methanol and water; side arm, extract (prepared as described in the text, containing 4 mg. protein) and glucose oxidase suspension (5 mg.); centre well, 0.2 ml. 2N-KOH. Total volume 2.7 ml. Oxygen uptake followed for 150 min. at 30°.

Vessel	Omission	Oxygen uptake (μl.)
1	None	51
2	Glucose, methanol	3
3	Glucose, glucose oxidase	18
4	Methanol, glucose oxidase	3
5	Methanol, extract	31
6	Methanol	31
7	Extract	26

Theoretical oxygen uptake for reactions (iii) and (iv) together (see text) is 33 μl.

could not be explained on this basis.

In view of the low activity of enzymes catalysing the oxidation of methanol and formaldehyde in P. AM1, Peel (1961) suggested a scheme by which methanol oxidation could be achieved via tetrahydrofolic derivatives. The postulated reactions were:-

$$CH_3OH + H_2PtG \rightleftharpoons \underline{N}^{5,10}\text{methylene}H_4PtG \quad \text{(vi)}$$

$$\underline{N}^{5,10}\text{methylene}H_4PtG + NADP^+ \rightleftharpoons N^{5,10}\text{methenyl}H_4PtG + NADPH \quad \text{(vii)}$$

$$N^{5,10}\text{methenyl}H_4PtG + H_2O \rightleftharpoons N^{10}\text{formyl}H_4PtG + H^+ \quad \text{(viii)}$$

$$N^{10}\text{-formyl}H_4PtG + ADP + P_i \rightleftharpoons ATP + \text{Formate} + H_4PtG \quad \text{(ix)}$$

$$\text{Formate} + NAD^+ \longrightarrow CO_2 + NADH + H^+ \quad \text{(x)}$$

$$H_4PtG + NADP^+ \rightleftharpoons H_2PtG + NADPH + H^+ \quad \text{(xi)}$$

Sum:

$$CH_3OH + 2NADP^+ + NAD^+ + ADP + P_i + H_2O \longrightarrow$$

$$CO_2 + 2NADPH + NADH + 3H^+ + ATP \quad \text{(xii)}$$

This scheme would provide a metabolic role for the enzymes catalysing reactions (vii), (ix) and (x) whose presence in high specific activity has already been shown in chapter IV, and would at the same time explain the low activity of enzymes catalysing the non-H_4PtG-linked oxidation of C_1 substrates. Peel demonstrated in methanol-grown cells the enzyme dihydrofolate reductase catalysing reaction (xi)

(though it was of low specific activity and was very little less active in succinate-grown cells). He was, however, unable to obtain evidence for the key reaction of the sequence, reaction (vi), which has not previously been postulated.

If a sequence of reactions such as that above is in operation, then it should be possible to demonstrate this by the use of inhibitors. Aminopterin has been shown quite conclusively (Futterman, 1957; Osborn, Freeman & Huennekens, 1958) to be a non-competitive inhibitor of dihydrofolate reductase (reaction (xi)). If the latter enzyme does indeed play a role in the oxidation of methanol, then both the growth of P. AM1 and the oxidation of methanol by resting cell suspensions should be inhibited by suitable concentrations of aminopterin.

The effect of aminopterin on the oxidation of methanol, formaldehyde and formate by methanol-grown P. AM1 was examined manometrically. 5mM-aminopterin had no effect on the oxidation of any of these substrates, and methanol oxidation was unaffected by 10mM-aminopterin. It would seem from this experiment that either aminopterin cannot enter the cells, or else that methanol-oxidation does not involve an aminopterin-sensitive step. In order to determine

whether it could enter the cells, the effect of aminopterin on growth was examined.

A culture of methanol-grown P. AM1 was divided between two sterile Fernbach flasks and growth followed nephelometrically. After 4 hr., when both were found to be growing at the same rate, aminopterin was added to one flask to a final concentration of 3mM. Growth was followed for a further 5 hr., but no difference between the two cultures could be detected. This experiment thus does not enable us to distinguish whether aminopterin cannot enter the cells or whether the reaction is aminopterin-insensitive. If the latter is the case then clearly the scheme shown above cannot be operative. Recent experiments by Johnson (1962) in cell-free extracts have shown that the cell-free oxidation of methanol is not inhibited by 1mM-aminopterin.

Controversy also at present surrounds the problem of methanol oxidation in mammalian tissues. Two opposing schools of thought exist. One, based on the observations of Keilin & Hartree (1936, 1945) prefers the peroxidative mechanism outlined above, mediated by catalase. This idea was supported by the fact that liver alcohol dehydrogenase, crystallised by Theorell & Bonnichsen (1951),

would not catalyse the NAD-dependent oxidation of methanol (though it would catalyse the reduction of formaldehyde by NADH). However, Kini & Cooper (1961) purified a methanol-oxidising system from rhesus monkey liver and showed that it was identical with alcohol dehydrogenase. The peroxidative mechanism has been reasserted more recently by Tephly, Parks & Mannering (1962). Both mechanisms are acknowledged to exist, the dispute relates to which predominates *in vivo*.

Oxidation of formaldehyde.

When Hughes press extracts of formate-grown *P.* AM1 were tested spectrophotometrically for NAD- and NADP-dependent oxidation of formaldehyde, only a slight amount of NAD-dependent formaldehyde dehydrogenase activity was observed (less than 0.1 μmole NAD reduced /hr./mg. protein). The rate was not affected by omission of glutathione. The low rate may be due to partial inactivation of the formaldehyde oxidising system by the methods of cell-rupture used, or may reflect a true low level of activity. This observation contrasts with those of Kaneda & Roxburgh (1959c) and Harrington & Kallio (1960). The former authors found no NAD-linked formaldehyde dehydrogenase activity in methanol-grown *P.* PRL-W4. The latter authors

demonstrated in methanol-grown P. methanica (Iowa strain) the presence of a glutathione-dependent NAD-linked formaldehyde dehydrogenase similar to that observed in mammalian liver by Strittmatter & Ball (1955). NADP would not replace NAD, and other thiol compounds would not replace glutathione. The enzyme was specific for formaldehyde, and had a pH optimum of 9.

An alternative mechanism for formaldehyde oxidation would involve its conversion (enzymically or non-enzymically) to methylenetetrahydrofolate and the oxidation of the latter to formate by reactions (vii) - (ix) and the oxidation of formate to CO_2 (see below) by reaction (x). All the enzymes for this reaction sequence are present in high activity in P- AM1. The problem of methanol oxidation however, cannot be solved by this sequence, as shown above. The question as to how formaldehyde is actually oxidised in vivo remains open.

Oxidation of formate.

Formate dehydrogenase activity was quite clearly detectable in the spectrophometric assay. The presence in extracts of formate-grown cells of a formate dehydrogenase of specific activity of 3 μmoles NADH formed /hr./mg. protein

was demonstrated. The enzyme was inactive with NADP, and 50% inhibited by addition of 1mM-sodium hypophosphite. Inhibition was 100% if the hypophosphite were preincubated with extract for 60 min., before adding formate.

Discussion.

If we assume that 50% of the cell dry weight is protein, then the Q_{O_2} values in table VI. 1 may be correlated with enzyme activity to give figures of 13, 11 and 4.6 µmoles of O_2 utilised /hr./mg. protein respectively for methanol, formaldehyde and formate. Hence any enzyme involved in energy-liberation in P. AM1 ought to have a specific activity of this order of magnitude. The only nicotinamide nucleotide-linked enzyme detected in extracts of formate-grown cells, formate dehydrogenase, had a specific activity of 3 µmoles of NAD reduced /hr./mt. protein i.e. 1.5 µmoles of O_2 consumes /hr./mg. protein. This figure is 40% of the rate observed in whole cells. Only very low dehydrogenase activity for methanol and formaldehyde could be detected. In particular, the methanol dehydrogenase found by Kaneda & Roxburgh (1959b) in Pseudomonas PRL-W4 and the glutathione-dependent formaldehyde dehydrogenase found by Harrington & Kallio (1960) in P. methanica (Iowa

strain) could not be found. As demonstrated by the latter authors, methanol could be oxidised peroxidatively by extracts of P. AM1 in the presence of a H_2O_2-generating system. The objection to the latter pathway of methanol oxidation in whole cells of P. AM1 is that whereas in animal tissue there are many conceivable sources of H_2O_2 (e.g. uricase, xanthine oxidase), no natural generating system for peroxide has been demonstrated in P. methanica or P. AM1, and since a stoicheiometric quantity of H_2O_2 is required for each mole of methanol oxidised, the suggested peroxidative pathway raises as many problems as it solves. It is clear that much more work is necessary in both mammalian and bacterial systems before the exact role, if any, of the peroxidative oxidation of methanol in vivo is elucidated.

The suggestion of Peel that the oxidation of methanol and formaldehyde proceeds via tetrahydrofolate derivatives appears improbable in view of the fact that aminopterin does not inhibit growth or the oxidation of methanol by methanol-grown cells, nor the cell-free methanol-oxidising system of Johnson (1962).

The problem of formaldehyde oxidation is not quite as obscure. Recent experiments by Johnson (1962) have

clearly established the presence of an NAD-linked formaldehyde dehydrogenase in P.AM1 but its investigation is rendered difficult by strong NADH oxidase activity.

Only in the case of formate oxidation has it been possible to demonstrate a system with activity approaching that observed in whole cells. The pathway of oxidation of the other C_1 substrates still needs further investigation.

Effect of glycine and serine on the oxidation of methanol by methanol-grown cells.

The experiments of Peel (1961, p. 79) showed that in the presence of glycine or serine there was an increase in total oxygen uptake during the oxidation of methanol of about 20%. Since the oxidation of glycine or serine alone could not be demonstrated manometrically, the effect observed in the presence of methanol was investigated further, using ^{14}C-labelled amino acids.

Double side arm manometer vessels contained in the main compartment, 1 ml. of cell suspension (containing about 2 mg. dry wt. of washed cells of methanol-grown P. AM1), 100 µmoles of phosphate buffer pH 7.5; in the centre well, 0.2 ml. of 2.5 N-NaOH (CO_2-free); in one side arm, 5 µmoles (1 µc.) of L-$[^{14}C]$serine or $[1\text{-}^{14}C]$ or

[2-^{14}C]glycine, and in some cases 5 µmoles of methanol; and in side arm 2, 0.2 ml. of 5N - H_2SO_4. The reaction was followed for about 2½ hr. until the substrates had been completely oxidised. The acid was then tipped and the manometers shaken without reading for a further 30 min. Then the manometers were removed from the bath, dried, and the vessels detached. To each centre well was added 0.2 ml. of 0.20M - Na_2CO_3. The contents of each were transferred quantitatively to graduated tubes and made up to 2.2 ml. with 0.20M-Na_2CO_3. 0.6 ml. samples were removed from each, converted to $BaCO_3$ and assayed for radioactivity as described in chapter II. The results were expressed as total counts/min. at infinite thinness in each centre well. The tracers were diluted 10-fold and aliquots plated on planchets at infinite thinness and assayed for radioactivity. Hence the radioactivity in the centre wells could be expressed as a percentage of that in the substrate added. A duplicate series of incubations was also performed in which the acid was not tipped. After incubation, the contents of each manometer were removed quantitatively, the cells centrifuged down, washed once with 0.9% KCl containing 20mM-glycine or serine, drained and resuspended in 0.5 ml. distilled water. 1.5 ml. of

boiling 95% v/v ethanol was added to each suspension, and the precipitated protein removed by centrifugation. The precipitate was washed with 0.2 ml. 20% v/v ethanol and the washings and supernatant combined. These were then evaporated to dryness under N_2 at 60°, taken up in 0.2 ml. 20% v/v aqueous ethanol and 0.1 ml. samples analysed by two-dimensional paper chromatography and radio-autography. The glycine, serine and glutamate were separated as described in chapter II, and the products identified by CO-chromatography. The ethanol-insoluble fraction from the cells was hydrolysed in 0.5 ml. 6N-HCl for 15 hr. at 110° in hard glass tubes sealed at the ends. The tubes on cooling were opened, their contents removed quantitatively, and evaporated to dryness under N_2 at 60°. They were then taken up in 0.5 ml. water and again evaporated to remove all traces of HCl. The residue was then taken up in 0.2 ml. 20% v/v aqueous ethanol and 0.1 ml. analysed by paper chromatography in the same way as the ethanol-soluble fraction. The results are set out in table VI. 3.

It is clear that glycine is slightly oxidised, but that the oxygen uptake is too small to measure manometrically. Glycine carbon has also been incorporated into a number of materials in the ethanol-soluble fraction, and into protein

Table VI.3

FATE OF (^{14}C) GLYCINE AND (^{14}C) SERINE INCUBATED WITH RESTING CELLS OF METHANOL-GROWN PSEUDOMONAS AM 1 IN THE PRESENCE OF UNLABELLED METHANOL.

A washed cell suspension (2mg. dry wt./ml.) of methanol-grown P. AM 1 was incubated with phosphate buffer pH 7.5 and in addition (1-^{14}C) or (2-^{14}C) glycine or (^{14}C) serine (5 μmoles; 1 μc of ^{14}C) and unlabelled methanol (5 μmoles) as shown below, in manometer flasks as described in the text. The CO_2 evolved was trapped in 2.5N CO_2-free NaOH after an acid tip. Non-acidified cells were removed, washed and analysed for aqueous ethanol-soluble and -insoluble radioactive compounds as described in the text.

Vessel No.	Contents	Total radioactivity of starting traces (counts/min. at infinite thinness x 10^{-3})	Total O_2 uptake (μl) (corrected for endogenous respiration)	Radioactivity (Counts/min at infinite thinness) in Respiratory CO_2	Ethanol-soluble fraction	Ethanol-insoluble fraction
1	Methanol	-	65	-	-	-
2	(1-^{14}C)Glycine	73.63	0	4,790	Gly1656 Glu144 Tre582 Phosph540 Unid.60 TOTAL 1326	Gly1460 Ser441 Glu276 Ala459 Unid189 TOTAL 1365
3	(1-^{14}C)Glycine + Methanol	73.63	82	11,880	Gly972 Glu120 Tre1018 Phosph793 TOTAL 1931	Gly1872 Ser1035 Glu1180 Ala957 Unid1065 TOTAL 4237
4	(2-^{14}C)Glycine + Methanol	60.87	70	4,130	Gly732Glu513 Tre988 Phosph1152 Unid132 TOTAL 2785	Gly2460 Ses798 Glu501 Ala627 Unid375 TOTAL 2301
5	(^{14}C) Serine	70.76	0	2,450	Ser51 Gly174 Glu36 Tre213 Phosph81 TOTAL 504	Gly279 Ser 399 Glu90 Ala78 Unid15 TOTAL 462
6	(^{14}C)Serine + Methanol	70.76	82	1,518	Ser66 Gly261 Glu171 Tre453 Phosph 255 TOTAL 1140	Ser 477Gly 396 Glu 210 Ala132 TOTAL 807 Unid 69

TOTALS do not include the starting tracer.

and possibly other ethanol-insoluble materials. This incorporation is enhanced in all cases in the presence of methanol. The effect of methanol on the oxidation of glycine as measured by $^{14}CO_2$ formation is to increase it, but to decrease the oxidation of serine. [1-^{14}C]Glycine is more readily converted to $^{14}CO_2$ in the presence of methanol than is [2-^{14}C]glycine (16.1% as against 6.8%). In the ethanol-soluble fraction most of the radioactivity from labelled glycine was found in trehalose and phosphorylated compounds (which are presumably the precursors of trehalose). A small amount is found in glutamate, but surprisingly, none in serine, not even when methanol is present. In the ethanol-insoluble fraction the radioactivity from glycine is distributed almost equally between serine, alanine and glutamate.

With [^{14}C]serine, the major products in the ethanol-soluble fraction are glycine, trehalose and phosphorylated compounds in roughly equivalent amounts, and a smaller amount of glutamate. In the protein hydrolysate glycine is the most important product, glutamate rather less and alanine also rather less important.

Discussion.

It is not possible to conclude from this data whether the increased oxygen uptake observed when methanol is oxidised by resting cells in the presence of glycine and serine is due to a methanol-dependent oxidation of the amino acids, or a decreased assimilation of methanol leading to an increase in its oxidation. It is clear, however, that lack of oxygen uptake in manometric experiments is not to be taken as indicating that a particular substrate cannot enter the cell and be metabolised. Similar conclusions were reached by Leadbetter & Foster (1958) after studying the incorporation of [^{14}C]amino acids by cultures of P. methanica growing on methane. They observed an extensive incorporation of the radioactive amino acids into other amino acids and into protein and extensive oxidation to $^{14}CO_2$. They concluded that "...numerous substances, by themselves incapable of supporting growth of the methane bacteria could be oxidised and used extensively for cellular biosynthesis provided the organisms were utilising methane concomitantly." The experiments reported above extend this observation to non-growing cells both in the presence and in the absence of the growth substrate and energy source (methanol).

SECTION III

METHANOL AND CARBON DIOXIDE METABOLISM IN CELLS GROWN ON METHANOL

Chapter VII

DISTRIBUTION OF RADIOACTIVITY IN METABOLITES OF ^{14}C METHANOL IN METHANOL-GROWN *PSEUDOMONAS* AM1

A study was made, in collaboration with Dr. J.R. Quayle, of the distribution of radioactivity in certain key intermediates of the postulated reaction scheme, shown in fig. 1. The intermediates studied were glycine, serine and phosphoglyceric acid obtained from the short term growth of *P.* AM1 on [^{14}C]methanol in the presence and absence of non-radioactive carbon dioxide. In addition, Dr. Quayle studied the distribution of radioactivity in the malic acid derived from this source, and also the labelling patterns in glycine, serine and malic acid obtained after short exposure of methanol-grown *P.* AM1 to sodium [^{14}C]bicarbonate.

Brief incubation of cells with [^{14}C]methanol.

Cultures of methanol-grown *P.* AM1 were harvested by centrifugation at room temperature while in the exponential phase of growth. The cells were resuspended in growth medium containing 10mM-KH_2PO_4 adjusted to pH7.0 with

2N-NaOH, 10mM-ammonium chloride and 10mM-methanol to a cell density of 6 mg. dry wt. of bacteria/ml. The bacterial suspensions (50 ml. or 25 ml.) were placed in a sintered glass funnel (8 cm. diameter x 5 cm. deep) through which was blown either air or air-carbon dioxide (99 : 1, by vol., prepared by mixing 4 volumes of nitrogen with 1 volume of oxygen-carbon dioxide [95 : 5v/v]). After 5 min. incubation at 30° [^{14}C]methanol (500 µc. or 250 µc, see tables) was added to the suspensions by means of a hypodermic syringe. After 1 minute's growth on the labelled substrate the entire contents of the funnel were tipped into 4 vol. of boiling 95% v/v ethanol. Each resulting extract was cooled, centrifuged, and the insoluble residue was further extracted with two 5 ml. portions of aqueous 20% v/v ethanol. These extracts were combined with the supernatant and evaporated under reduced pressure (at about 30°) to a volume of approx. one-fiftieth of the original. Samples (0.1 ml.) of the concentrated aqueous-ethanol-soluble cell extract were applied to the origin positions of paper chromatograms and analysed by two-dimensional chromatography as described in chapter II. The radioactive compounds were located by radioautography, and eluted from the paper with water.

Mixtures of radioactive glycine and serine thus obtained were separated by further paper chromatography. Phosphate esters were dephosphorylated, and the glyceric acid was separated from the mixture of sugars so formed by two-dimensional paper chromatography in the phenol-formic acid-water and butanol-propionic acid-water solvent systems.

Preparation of samples for degradation.

Each purified radioactive compound eluted from the final chromatograms was mixed with about 300 mg. of the authentic unlabelled compound and then crystallised to constant specific activity. In most cases three recrystallisations were necessary. Glyceric acid was crystallised as the calcium salt. The solvent used in all cases was aqueous ethanol. The samples were finally dried in high vacuum at 80°, to remove all traces of ethanol.

Combustion of labelled compounds.

The specific activity of the labelled compounds was determined by total oxidation of a 20 mg. sample of crystals from each recrystallisation step to carbon dioxide using the chromic anhydride-iodate combustion reagent of van

Slyke & Folch (1940). The apparatus and methods used are described in detail by Sakami (1955, p.1.). The material was oxidised in the boiling combustion reagent through which a stream of CO_2-free air was passing. The radioactive CO_2 formed was trapped in CO_2-free 2.5 N-NaOH (see chapter II). The ^{14}C was precipitated as $BaCO_3$, assayed for radioactivity and the specific activity calculated as described in chapter II.

Degradation of glycine.

Glycine was degraded by ninhydrin decarboxylation:

$$\underset{2}{CH_2}\underset{}{NH_2}.\underset{1}{COOH} \xrightarrow{\text{Ninhydrin}} \underset{2}{HCHO} + \underset{1}{CO_2} + NH_3 \quad (i)$$

About 75 mg. (1 m-mole) of recrystallised glycine was dissolved in saturated KH_2PO_4, and decarboxylated with 2 m-moles of ninhydrin at 100° in a stream of CO_2-free air in the apparatus described by Sakami (1955, p. 70), the CO_2 from the carboxyl group being trapped in 2.5 N-NaOH. The formaldehyde from carbon atom 2 was steam distilled from the ninhydrin reaction mixture, cooled to 0°, and oxidised to formic acid by sodium hypoiodite

$$HCHO + NaOI \longrightarrow HCOOH + NaI \quad (ii)$$

The yield of formaldyhyde (determined by back-titration of the excess iodine used in the hypoiodite oxidation)

was rather poor (30 - 40%), but was quite sufficient for an accurate determination of its specific activity. The formate formed in reaction (ii) was oxidised to CO_2 by mercuric ions in acetate buffer pH3 (Piria, 1946):

$$HCOOH + 2Hg^{++} \longrightarrow CO_2 + Hg_2^{++} + 2H^+ \qquad \text{(iii)}$$

as described in detail by Sakami (1955, p. 55). The CO_2 formed, derived from carbon atom 2 of glycine, was trapped in CO_2-free 2.5 N - NaOH and converted to $BaCO_3$ for radioassay as described in chapter II.

Degradation of serine and glyceric acid.

Serine and calcium glycerate were degraded by periodate oxidation as described by Sakami (1955, p.72):

$$\underset{3}{CH_2OH}.\underset{2}{CHNH_2}.\underset{1}{COOH} + 2IO_4^- \longrightarrow$$

$$\underset{3}{HCHO} + \underset{2}{HCOOH} + \underset{1}{CO_2} + NH_3 + 2IO_3^- \qquad \text{(iv)}$$

the CO_2 from the carboxyl group being trapped in 2.5 N-NaOH. About 100 mg. of serine or calcium glycerate (i.e. 1m-mole of serine or 0.8 m-mole of glyceric acid) in sodium phosphate buffer pH 5.8 was oxidised at room temperature by sodium metaperiodate (3 m-moles) in a stream of CO_2-free air. The yield of CO_2 was nearly quantitative. The iodate, periodate and phosphate in the reaction mixture were then removed by precipitation with 1M-$SrCl_2$ and left

to stand overnight. After removal of the strontium salts by centrifugation, the formic acid from C-2 was oxidised to CO_2 by the Piria method as described above (equation (iii)). The CO_2 was trapped in 2.5N-NaOH. This procedure does not oxidise the formaldehyde from carbon atom 3. This was then distilled off, oxidised to formic acid by hypoiodite (reaction (ii)) as described for glycine above, and the formate oxidised to CO_2 by a second Piria oxidation. Yields of CO_2 were about 80% from C-2 and about 70% from C-3.

Labelling patterns obtained after growth on [^{14}C]methanol in the absence of exogenous CO_2.

The distribution of radioactivity in the individual carbon atoms of glycine, serine and phosphoglyceric acid isolated after a 1 min. exposure of cells of P. AM1 growing on methanol to [^{14}C]methanol is shown in table VII.1. In addition the percentages of total radioactivity in the malic acid from the same experiment (degraded by Dr. J.R. Quayle) were:

HOOC .	CH(OH).	CH_2.	COOH
1	2	3	4
23.1	20.1	30.2	26.6

The most striking feature of these results is the uniform

Table VII.1

DISTRIBUTION OF RADIOACTIVE CARBON IN GLYCINE, SERINE AND PHOSPHOGLYCERIC ACID DERIVED FROM PSEUDOMONAS AM 1 GROWING ON (^{14}C) METHANOL IN AIR

(^{14}C) Methanol (92.5 μmoles; 500 μC of ^{14}C) was added at zero time to an aerated suspension of bacteria (300 mg. dry wt.) in 50 ml. of 10 mM-phosphate buffer (pH 7.0), 10mM-NH_4Cl and 10mM-methanol. After 1 min. incubation, the suspension was poured into 200 ml. of boiling ethanol. The isolation of radioactive compounds from the resulting solution is described in the text.

Compound	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from each C atom (counts/min. at infinite thinness/mg. $BaCO_3$)	Sum of specific activities of individual C atoms	Specific activity of $Ba^{14}CO_3$ obtained from combustion X no. of C atoms in the molecule	Percentage of total radioactivity in molecule contributed by each C atom
GLYCINE					
COOH	(1)	13.3	27.0	28.7	49.1
$CHNH_2$	(2)	13.7			50.9
SERINE					
COOH	(1)	5.46			24.1
$CHNH_2$	(2)	6.03	22.7	22.7	26.6
CH_2OH	(3)	11.2			49.4
PHOSPHOGLYCERIC ACID					
COOH	(1)	8.66			29.7
CHOH	(2)	8.32	29.2	34.0	28.5
$CH_2OPO_3H_2$	(3)	12.2			41.8

distribution of radioactivity in the glycine. This is reflected in the serine. The β-carbon atom (C-3) of serine, however, contains 50% of the radioactivity in the molecule, suggesting that it arises from methanol more directly than the carbon atoms of glycine. This would indeed be the case if serine were formed by the addition of a hydroxymethyl group derived from methanol to a molecule of glycine. The pattern of labelling in serine is reflected in phosphoglycerate, but in the latter, the difference in radioactivity between C-3 and C-1,2 is less, indicating a partial randomisation of the radioactivity via some structurally-symmetrical intermediate. Since randomisation always results in the elimination of differences between individual atoms, and can never lead to an enhancement of differences, it is clear that phosphoglycerate has been formed from serine and not vice versa. Moreover this difference means that the two compounds cannot be in equilibrium, i.e. there must be an essentially irreversible enzymic step between serine and phosphoglycerate.

An interesting point is that the labelling patterns in phosphoglycerate and carbon atoms 1-3 of malic acid are closely similar. If we decarboxylate the malic acid

molecule above at C-4, the distribution of radioactivity in the remaining carbon atoms becomes: C-1 31.4; C-2 27.4; C-3 41.2 which is almost identical with the labelling pattern in phosphoglycerate (table VII.1.). It is clear from this that malate and phosphoglycerate are in equilibrium, indicating that all the enzymic steps separating them (including the carboxylation) are reversible.

It thus appears that while malate and phosphoglycerate are in equilibrium with one another, yet serine could not be produced from either of these two intermediates. The partial randomisation observed above could take place by the reversible conversion of malate to fumarate. Hence then phosphoglycerate and malate must have been formed from serine and not versa.

The similarity of the labelling in the two carbon atoms of glycine has been mentioned above. When Dr. J.R. Quayle degraded glycine, serine and malate formed from $[^{14}C]$bicarbonate in cells of P. AM1 growing on non-radioactive methanol, he found the following labelling patterns:

Glycine:			Serine:		
CH_2NH_2.	COOH		CH_2OH.	$CHNH_2$.	COOH
2	1		3	2	1
15.3	84.7		2.3	15.4	82.3

Malic acid:

HOOC.	CH_2.	CH(OH).	COOH
4	3	2	1
49.7	5.8	6.4	38.1

Since methanol is readily oxidised to CO_2 by the cells, it would seem possible that the fixation of radioactivity from $[^{14}C]$methanol into the carboxyl group of glycine is in fact due to fixation of $^{14}CO_2$ formed from the $[^{14}C]$-methanol. In order to test this possibility a culture of methanol-grown cells was incubated with $[^{14}C]$methanol in the presence of non-radioactive carbon dioxide (see below).

With the labelling patterns from $[^{14}C]$bicarbonate it is clear that the conclusion above that the C-3 atom of serine is derived more directly from a reduced C_1 unit than from CO_2 is correct. The close similarity of the labelling patterns in glycine and C-1, 2 of serine with $[^{14}C]$methanol is also observed with $[^{14}C]$bicarbonate, indicating that these two are interconvertible by the

reversible addition of a reduced C_1 unit, but it is not possible to tell from either set of data whether glycine normally arises from serine or vice versa.

Labelling patterns obtained after growth on [^{14}C]methanol in the presence of exogenous CO_2.

When the above experiment was repeated, save that the cells growing on methanol were exposed to [^{14}C]methanol for 1 min. during which they were aerated with air containing 1% carbon dioxide, it was found that the labelling patterns of glycine and serine were markedly changed from the patterns observed when the cells were aerated with air only. The new patterns observed are shown in table VII.2. The uniform labelling pattern observed with glycine was changed to one in which the carboxyl group contained only 24% of the total radioactivity. This changed pattern is repeated in C-1, 2 of serine, so that the net effect with serine is to increase even more markedly the percentage of radioactivity in C-3. This confirms the observations made directly with [^{14}C]bicarbonate, that the carboxyl group of glycine arises from carbon dioxide (which in turn in the growing cell is presumably derived from methanol by oxidation), while the methylene group is derived from

Table VII.2

DISTRIBUTION OF RADIOACTIVE CARBON IN GLYCINE AND SERINE DERIVED FROM PSEUDOMONAS AM 1 GROWING ON (^{14}C) METHANOL IN THE PRESENCE OF AIR-CARBON DIOXIDE (99:1)

(^{14}C) Methanol (46.9 μmoles; 250 μC of ^{14}C) was added at zero time to a suspension of bacteria (140 mg. dry wt.) in 25 ml. of 10 m*M*-sodium potassium phosphate (pH 7.0), 10 m*M*-NH_4Cl and 10 m*M*-methanol. The suspension was flushed with air-carbon dioxide (99:1, v/v). After 1 min. incubation, the suspension was poured into 100 ml. of hot ethanol. The isolation of radioactive compounds from the resulting solution is described in the text.

Compound	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from each C atom (counts/min. at infinite thinness/mg. $BaCO_3$)	Sum of specific activities of individual C atoms	Specific activity of $Ba^{14}CO_3$ obtained from combustion X no. of C atoms in the molecule	Percentage of total radioactivity in molecule contributed by each C atom
GLYCINE					
COOH	(1)	3.85	16.1	17.7	23.8
CH_2NH_2	(2)	12.3			76.2
SERINE					
COOH	(1)	4.10			8.3
$CHNH_2$	(2)	14.9	49.3	48.7	30.3
CH_2OH	(3)	30.3			61.4

some C_1 unit formed from methanol but not in rapid equilibrium with carbon dioxide.

Discussion.

The labelling patterns observed in serine and glycine under all conditions tested, are consistent with the suggestion that serine is synthesised from glycine by addition of a C_1 unit, providing that there exists a mechanism for deriving methylene-tetrahydrofolate from methanol. Such a mechanism has been demonstrated for formate in chapter IV. It has been known for some years that methanol can act as a precursor of the hydroxymethyl group of serine in mammalian tissue (Siegel & Lafaye, 1950).

The fact that the labelling patterns of serine, phosphoglycerate and malate are more in accord with formation of malate by carboxylation of a C_3 fragment (probably in equilibrium with PGA) rather than the reverse is an important one. It supports the conclusion (chapter III, Large et al. 1961) that the serine observed together with malate at the earliest times of incubation of P. AM1 with $[^{14}C]$ substrate is itself a primary incorporation product of the reduced C_1 substrates, and is not merely

derived from malate. It has already been shown (chapter IV) that enzymic pathways for the conversion of serine to PGA and hence presumably to phosphoenolpyruvate are present in P. AM1, and it will be shown in chapter X that phosphoenolpyruvate can be carboxylated to malate by extracts of methanol-grown cells. Since glycerate kinase catalyses an essentially irreversible reaction, reversal of the reaction sequence

Serine ⇌ Hydroxypyruvate ⇌ Glycerate → PGA ⇌ PEP ⇌ Malate (v)

would not be expected to occur. Hence the labelling pattern of carbon atoms 1-3 in malate and PGA would not appear in serine.

If serine synthesis from glycine plays a major role in the formation of C_3 compounds from C_2 compounds, then a mechanism must exist for the prior synthesis of glycine, or its C_2 precursors, from C_1 units. The labelling data show quite clearly that such a mechanism must involve the derivation of C-2 of glycine from methanol and the carboxyl group from carbon dioxide. Direct condensation of CO_2 and a reduced C_1 unit is a possibility, although the quantitatively low incorporation of tracer into glycine in whole cells of P. AM1 does not support this idea (chapter III). Cleavage of a C_4 compound to give two

C_2 compounds one or both of which could form glycine is an alternative mechanism (fig. 1, chapter I). Evidence for this is the rapidity with which malate is labelled on incubation of whole cells with [^{14}C]methanol, [^{14}C]-formate or [^{14}C]bicarbonate, and the fact that if cleavage of a molecule of malate of the above labelling patterns took place, it would lead to the following patterns: with malate from [^{14}C]methanol:
C-1 53.4; C-2 46.6 (from C-1 and C-2 respectively) and C-1 53.1; C-2 46.9 (from C-4 and C-3 respectively, cf. the figures for glycine in table VII.1.). With malate from [^{14}C]bicarbonate the pattern would be: C-1 85.6; C-2 14.4 (from C-1 and C-2 respectively) and C-1 89.6; C-2 10.4 (from C-4 and C-3 respectively, cf. the figures for glycine quoted above).

Though the data above indicate that the carboxyl group of glycine arises from carbon dioxide, the labelling patterns do not enable us to decide between the two alternative mechanisms for glycine synthesis suggested in chapter III. The incorporation of radioactive carbon dioxide into the carboxyl group of glycine has been observed by many workers: in Clostridium kluyveri grown on ethanol, acetate and carbon dioxide (Tomlinson &

Barker, 1954; Tomlinson, 1954); in acetate-grown Torulopsis utilis and E. coli (Ehrensvärd et al, 1951; Cutinelli et al., 1951a); and in the photometabolism of acetate by Rhodospirillum rubrum (Cutinelli et al.,1951b). Many of these observations could be explained in terms of $C_3 + CO_2$ reactions, but in Brucella abortus (Marr & Wilson, 1951; Tepper & Wilson, 1958), 40% of the $^{14}CO_2$ fixed during growth on a complex medium appeared in glycine. No convincing metabolic evidence has yet been produced of the enzymic mechanism of this last fixation, and it may be that the pathway of glycine formation in P. AM1 is the same as in one or more of these unknown processes. An explanation of the observation of CO_2-fixation into C-1 of glycine by C. cylindrosporum by Barker & Elsden (1947) may well lie in recent observations on glycine metabolism in Diplococcus glycinophilus (Sagers & Gunsalus, 1961).

Chapter VIII

THE GLYCINE-FORMATION PROBLEM: WHOLE CELL EXPERIMENTS

In the postulated scheme for growth on C_1 compounds (fig. 1), glycine occupies a key position. Its mode of synthesis from C_1 units is not known. Two possible schemes have been proposed: (a) the indirect formation of glycine by cleavage of a C_4 derivative, and (b) the direct formation of glycine by a $C_1 + CO_2$ condensation.

This chapter describes an attempt to investigate the indirect formation of $[^{14}C]$glycine from possible C_4 precursors. The C_4 acid chosen was sodium $[2,3 - {}^{14}C_2]$-succinate, and its metabolism was investigated in cells of Pseudomonas AM1 grown on methanol and also on sodium succinate by the short term kinetic incorporation techniques described in chapter III. If succinate is a precursor of glycine, we should expect to see glycine as an early labelled product, with serine appearing later.

Incorporation of $[^{14}C]$succinate by methanol- and succinate-grown cells.

A culture of methanol-grown P. AM1 was harvested by

centrifugation at room temperature. 32 mg. dry wt. were suspended in 5.5 ml. of growth medium containing 10mM-phosphate buffer pH 7.0, 10mM-NH_4Cl, and 100 mM-methanol. This suspension was aerated vigorously in the vessel described in chapter III. After 5 min. equilibration at 30°, 1 ml. of sodium [2, 3-$^{14}C_2$]succinate (73 μmoles; 100 μC of ^{14}C) was added and samples rapidly removed and transferred to boiling 95% v/v ethanol. After cooling, the killed cell suspensions were prepared for two dimensional chromatography as described in chapter II. The radioactive products were located by radioautography and identified by co-chromatography. The distribution of ^{14}C in the non-volatile metabolic products is given in table VIII.1. Fig. 10 shows this radioactivity as a percentage of the total, expressed as a function of time. The following compounds have negative slopes: malate, fumarate, aspartate and citrate, as expected, and also glycine. Glutamate, phosphates, trehalose and serine have initially positive slopes.

The unexpected finding of a negative slope to the glycine curve, together with its high percentage of radioactivity (9%) in the 6 sec. sample, are indications that there is possibly a direct pathway for the formation of glycine from succinate other than via C_3 compounds and

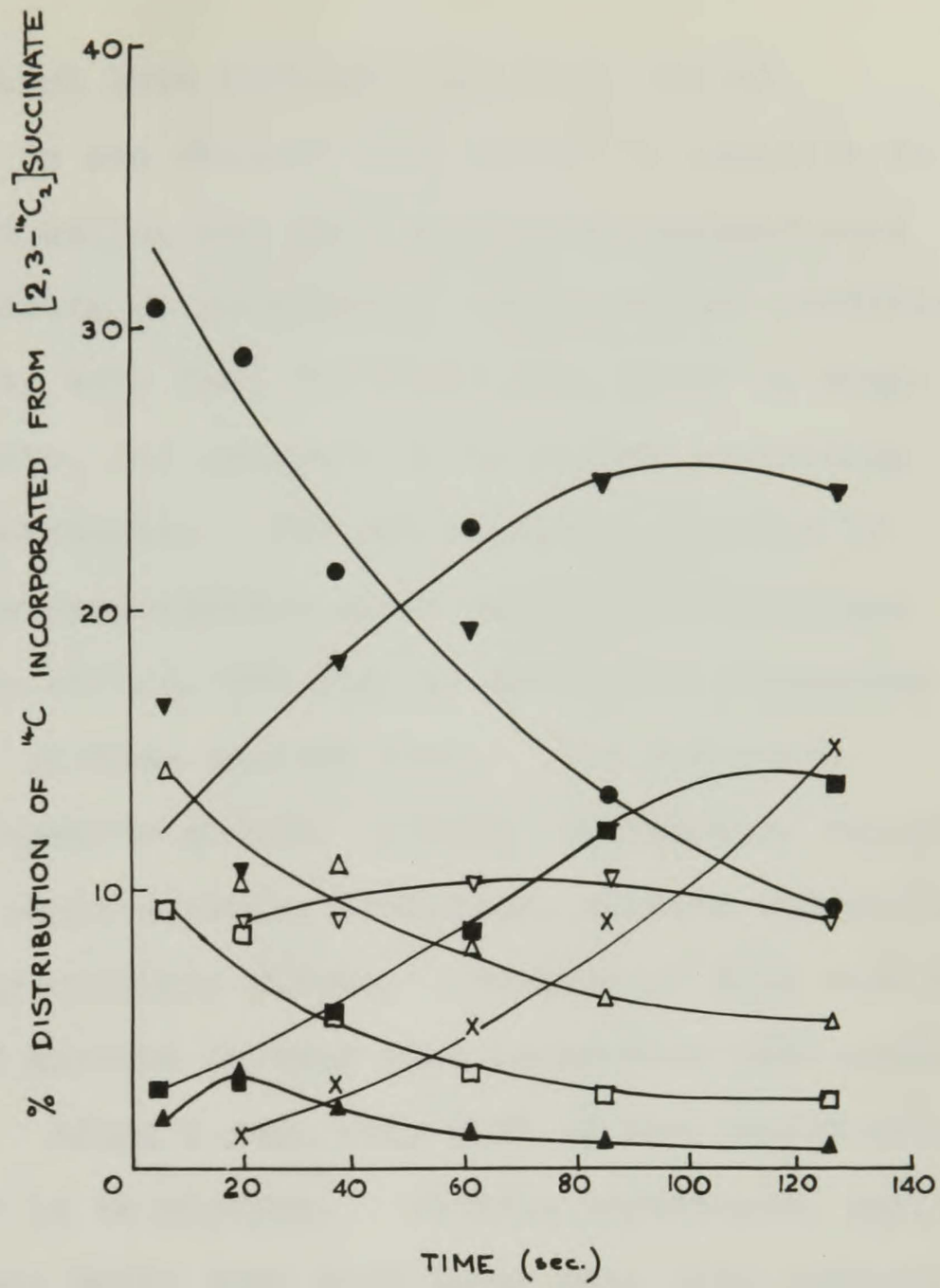
TABLE VIII. 1.

DISTRIBUTION OF RADIOACTIVE CARBON AMONG THE NON-VOLATILE LABELLED COMPONENTS OF THE ETHANOL-SOLUBLE FRACTIONS OF SUSPENSIONS OF METHANOL-GROWN PSEUDOMONAS AM1 INCUBATED WITH [^{14}C]SUCCINATE.

Sodium [2,3-$^{14}C_2$]succinate (73 μmoles; 100 μc of ^{14}C) was added at-zero time to 5.5 ml. of a bacterial suspension (32 mg. dry wt.) in 100mM-methanol, 10mM-phosphate buffer pH 7.0 and 10mM-NH_4Cl. Samples (approx. 1 ml.) were removed by hypodermic syringe at the times indicated, and treated as described in the text.

	Radioactivity (Counts/min.)						
Time (sec.)	6	20	37	61	85	126	207
Malate	1635	3843	4732	7174	5009	5095	4219
Fumarate	632	128	0	0	0	0	0
Glycine	493	1162	1251	1060	1038	1352	1180
Serine	107	481	514	468	419	497	743
Glutamate	156	460	1232	2608	4560	7320	15930
Aspartate	768	1443	2459	2495	2377	2821	2296
Citrate	567	706	1583	1879	1900	2992	3587
Trehalose	0	180	690	1588	3389	7910	16231
Alanine	0	1220	2012	3192	3975	4993	8655
Phosphorylated compounds	928	1431	4001	6030	9349	12807	20010
Other compounds	0	2355	3582	4526	5077	7101	9623
Total	5286	13409	22056	31020	37093	52888	82274

Fig. 10



Incorporation of [2, 3 - $^{14}C_2$] succinate into compounds of the ethanol-soluble fraction of Pseudomonas AMI growing on methanol.

● Malate; △ aspartate; ▲ serine; □ glycine; ■ glutamate; ▼ phosphorylated compounds; X trehalose; ▽ alanine.

phosphates, which have initially positive slopes.

In order to see whether this effect is peculiar to methanol-grown cells, the same experiment was performed with cells growing on succinate. Experimental conditions were as before, save that the cells were grown on 50mM-sodium succinate, and resuspended in medium containing 10mM-sodium succinate. The non-volatile products of [^{14}C]succinate assimilation under these conditions are given in table VIII.2, and fig. 11 shows the percentage radioactivity plotted against time. The following curves have negative slopes: malate, phosphates, aspartate and glycine, while citrate, trehalose, alanine and glutamate have initially positive slopes. However in this case the percentage of glycine is very much lower than with methanol-grown cells. After 5 sec., only 2.6% of the incorporated radioactivity is in glycine. In this experiment, serine does not appear until very much later than with methanol-grown cells, and when it does, only negligible amounts are present.

These experiments provide good preliminary evidence (a) that glycine can arise from succinate by a route not involving C_3 compounds in equilibrium with alanine or phosphates, and (b) that this route is of greater quantitative importance in cells grown on methanol than

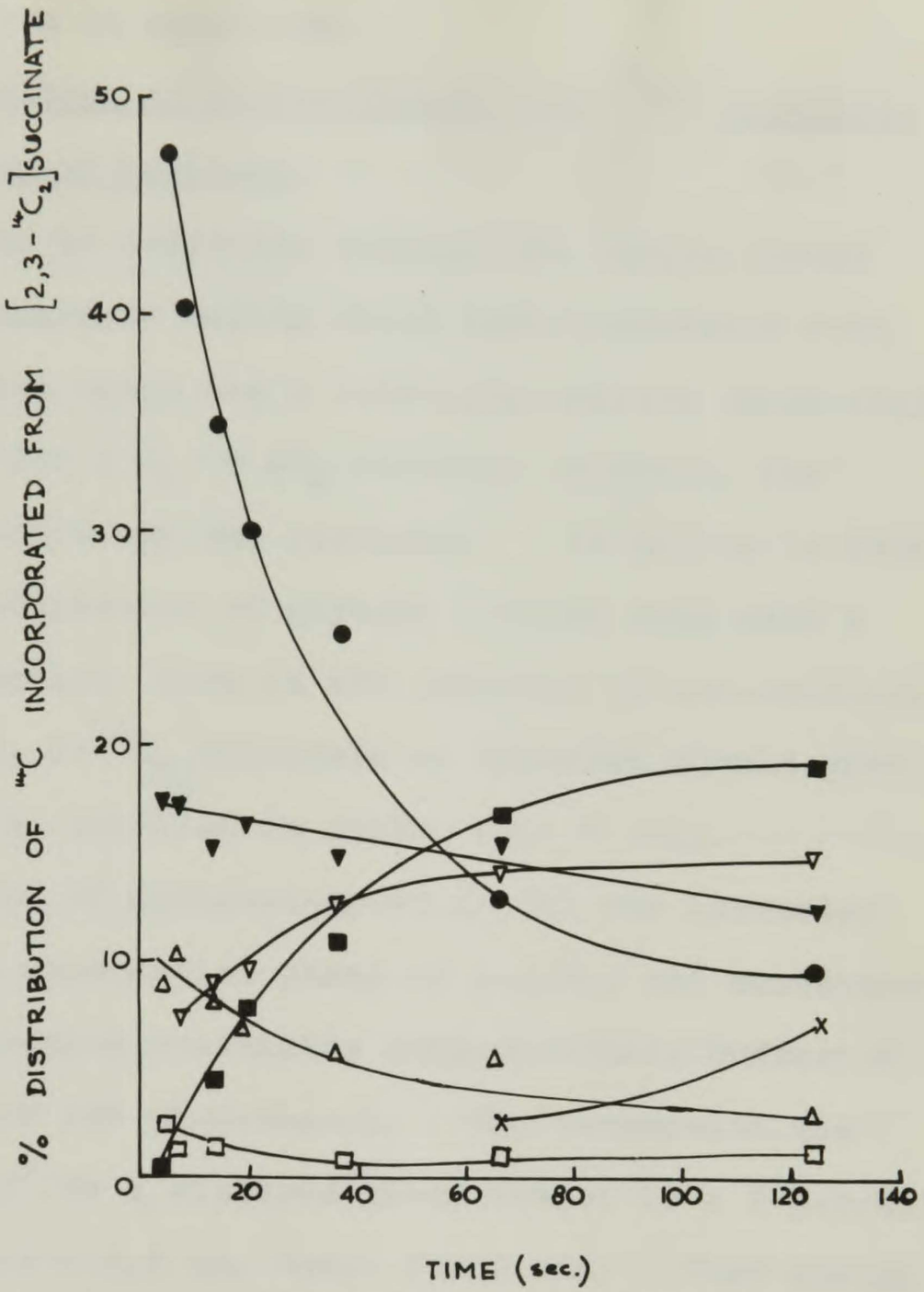
TABLE VIII. 2.

DISTRIBUTION OF RADIOACTIVE CARBON AMONG THE NON-VOLATILE LABELLED COMPONENTS OF THE ETHANOL-SOLUBLE FRACTIONS OF SUSPENSIONS OF SUCCINATE-GROWN PSEUDOMONAS AM1 INCUBATED WITH [^{14}C]SUCCINATE.

Sodium[2,3-$^{14}C_2$]succinate (73 μmoles; 100 μc. of ^{14}C) was added at zero time to 5.5 ml. of a bacterial suspension (33 mg. dry wt.) in 10mM-sodium succinate, 10mM-phosphate buffer pH 7.0 and 10mM-NH_4Cl. Samples (approx. 1 ml.) were removed by hypodermic syringe at the times indicated, and treated as described in the text.

	Radioactivity (counts/min.)						
Time (sec.)	5	8	14	20	37	67	125
Malate	3975	2847	2873	3588	1224	1060	1678
Glycine	217	79	135	38	38	97	190
Serine	0	0	0	11	0	18	40
Glutamate	77	114	363	674	522	1400	3365
Aspartate	766	735	667	604	280	457	512
Citrate	451	630	606	879	330	501	880
Trehalose	0	0	0	0	0	228	1271
Alanine	0	532	735	851	612	1153	2630
Phosphorylated compounds	1463	1203	1237	1398	718	1269	2146
Other compounds	1470	873	1584	1532	1113	1937	4856
Total	8419	7003	8200	8575	4837	8120	17568

Fig. 11



Incorporation of [2, 3 - $^{14}C_2$] succinate into components of the ethanol-soluble fraction of Pseudomonas AMI growing on succinate.

● Malate; Δ aspartate; □ glycine;
■ glutamate; ▼ phosphorylated compounds;
X trehalose; ∇ alanine.

in cells grown on succinate.

Labelling pattern of serine formed from [^{14}C]succinate during growth on methanol.

In order to determine whether the serine formed from [^{14}C]succinate during short term incubation with methanol-grown cells has a labelling pattern which would be expected for a $C_4 \longrightarrow 2C_2$ cleavage reaction, the following experiment was performed. If serine is formed by hydroxymethylation of glycine derived from such a cleavage reaction, then in the presence of non-radioactive methanol, [2, 3-$^{14}C_2$]succinate or fumarate should give rise to serine labelled in carbon atom 2 only.

A culture of methanol-grown P. AM1 was harvested while in the exponential phase of growth, and resuspended in 9 ml. of medium containing 10mM-phosphate buffer pH 7.0, 10mM-NH_4Cl and 100 mM-methanol. The suspension was aerated at 30° in a sintered glass funnel (3 x 2 porosity, 4 cm. diameter x 4.5 cm. deep) for 5 min. Then sodium [2, 3-$^{14}C_2$]succinate (73 µmoles, 100 µc of ^{14}C) was added and incubated with the growing cells for 90 sec. The whole culture was then tipped into 40 ml. of boiling 95% v/v ethanol. After concentration as described in chapter VII, the non-volatile radioactive products were isolated

by two-dimensional paper chromatography. The glycine-serine-glutamate areas from 12 chromatograms, after location by radioautography were eluted and combined. They were separated into their component amino acids as described in chapter II. The mixture was found to consist of glutamate, glycine and serine in a ratio 32 : 7 : 1. The radioactive serine was eluted from the chromatogram with water, 230 mg. of non-radioactive crystalline DL-serine was added and the mixture re-crystallised to constant specific radioactivity. This was determined by wet combustion with the van Slyke-Folch reagent as described in chapter VII.

The recrystallised radioactive serine (53 mg.) was then degraded by periodate oxidation as described in chapter VII. The yield of CO_2 from C-1 was 94%, that from C-2 was 64%, and that from C-3 was 69%. The distribution of radioactivity in these carbon atoms is shown in table VIII.3.

75.6% of the total radioactivity in the molecule is found in carbon atom 2, while carbon atom 3 contains only 6.8%. This is in accordance with a cleavage of the labelled succinate giving rise to $\left[2\text{-}\ {}^{14}C\right]$glycine and hydroxymethylation of the latter by an "active C_1

TABLE VIII. 3.

DISTRIBUTION OF RADIOACTIVE CARBON IN SERINE DERIVED FROM METHANOL-GROWN PSEUDOMONAS AM1 INCUBATED WITH [^{14}C] SUCCINATE

Sodium [2,3-$^{14}C_2$] succinate (73 μmoles; 100 μc. of ^{14}C) was added at zero time to an aerated suspension of bacteria (60 mg. dry wt.) in 9 ml. of 10mM-phosphate buffer pH 7.0, 10mM-NH_4Cl and 100mM-methanol. After incubation for 90 sec., the suspension was poured into 40 ml. of boiling 95% v/v ethanol. The isolation of radioactive compounds from the resulting solution is described in chapter II and the degradation of the labelled serine is described in chapter VII.

(1)	Formula	CH_2OH.	$CHNH_2$.	COOH
(2)	Carbon atom	3	2	1
(3)	Specific radioactivity of $Ba^{14}CO_3$ derived from each C atom (counts/min. at infinite thinness/mg. $BaCO_3$)	0.94	10.41	2.42
(4)	Sum of specific activities of individual C atoms		13.77	
(5)	Specific activity of $Ba^{14}CO_3$ obtained from combustion X no. of C atoms in molecule		15.09	
(6)	Percentage of total radioactivity in molecule contributed by each C atom.	6.8	75.6	17.6

unit" derived from unlabelled methanol to form [2- ^{14}C]-serine. An alternative explanation of the data would be the formation of [2, 3 - $^{14}C_2$]serine from the succinate via phosphorylated C_3 derivatives, and an exchange reaction (not involving a net synthesis) of the C-3 of serine with an "active C_1 unit" derived from unlabelled methanol. This alternative is rendered unlikely by the results in chapter VII, where it was shown that while C_4 derivatives could be formed from serine during growth on methanol, the labelling pattern ruled out the possible formation of serine from C_4 derivatives.

Discussion.

The data presented above constitute evidence for the indirect formation of glycine (required for the synthesis of C_3 compounds) by some pathway from C_4 dicarboxylic acids not involving the intermediate formation of C_3 compounds and phosphorylated derivatives. Possible enzymic mechanisms by which such a transformation could come about are not known. In the hope of elucidating this possible pathway further, and also to investigate alternative possible mechanisms of glycine synthesis via a direct $C_1 + CO_2$ condensation, further experiments were

performed using cell-free extracts. These are described in chapter IX.

Chapter IX

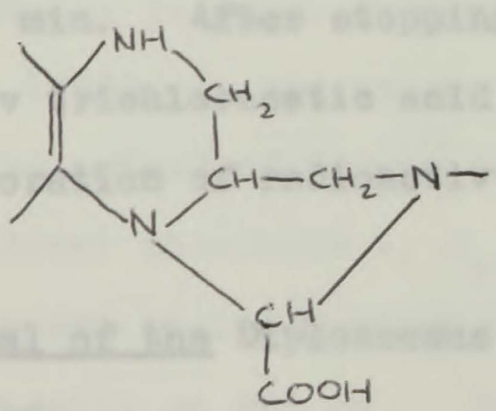
THE GLYCINE-FORMATION PROBLEM: EXTRACT EXPERIMENTS

In this chapter are described some of the experiments which were performed in cell-free extracts in a further attempt to shed light on the pathways by which C_2 compounds are formed during growth on methanol and formate.

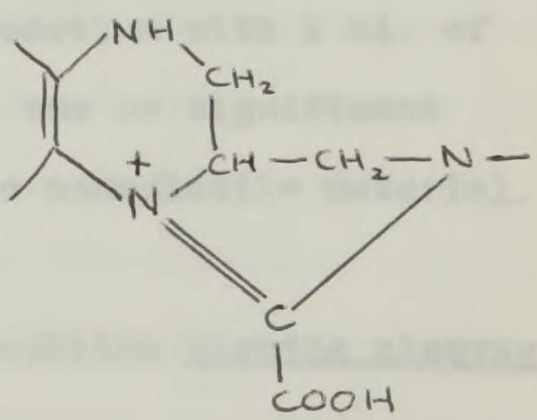
1. DIRECT SYNTHESIS OF C_2 COMPOUNDS.

Carboxylation of a tetrahydrofolic derivative.

A possible mechanism for the formation of a C_2 compound from a C_1 compound and CO_2 would be the direct carboxylation of a hydroxymethyl or formyl tetrahydrofolic acid derivative to give products such as I or II:



I



II

The chemical formation of compound I, the glyoxalyl analogue of $N^{5,10}$-methylene H_4PtG, and its oxidation to II, the oxalyl analogue of $N^{5,10}$-methenylH_4PtG, have been described by Scrimgeour & Huennekens (1960). Compound I could give rise to glyoxylate and thence to glycine, while II could give rise to oxalate and thence to glyoxyate via glyoxylate dehydrogenase present in low specific activity in Pseudomonas AM1 (chapters V and XII).

In order to investigate this possibility, extracts of formate-grown P. AM1 were incubated with DL-tetrahydrofolic acid (1 μmole), 20 μmoles of formate or formaldehyde, 2.5 μmoles of ATP, 65 μmoles of maleate buffer pH7.5, 40 μmoles of $MgCl_2$, 10 μmoles of cysteine and 6.3 μmoles (10 μc) of sodium $[^{14}C]$bicarbonate at 30° for 15 min. After stopping the reaction with 1 ml. of 10% w/v trichloracetic acid, there was no significant incorporation of radioactivity into nonvolatile material.

Reversal of the Diplococcus glycinophilus glycine cleavage reaction.

Another possible mechanism would be a reversal of the mechanism for glycine cleavage shown to play a key

role in the anaerobic growth of Diplococcus glycinophilus on glycine (Sagers & Gunsalus, 1961). These authors demonstrated that in the presence of NAD, pyridoxal phosphate and an electron acceptor such as benzyl viologen, there is a release of CO_2 from the carboxyl group of glycine with the concomitant transfer of the α carbon atom to tetrahydrofolic acid to give methylene H_4PtG:

$$CH_2NH.\ COOH + H_4PtG + NAD^+ \longrightarrow$$
$$\underline{N}^{5,10}\text{methylene } H_4PtG + CO_2 + NH_3 + NADH + H^+ \quad (i)$$

The same reaction has recently been demonstrated also in E. coli PA 15 (Pitts & Crosbie, 1962).

In order to explain earlier observations (Barker, Volcani & Cardon, 1948) that $^{14}CO_2$ can give rise to doubly labelled acetate, Sagers & Gunsalus postulated that the above reaction (and other reactions in their proposed metabolic pathway) were reversible. The experiments of Peel (1961, p. 55) attempted to demonstrate reaction (i) by the method of Sagers & Gunsalus (1961) in which the pyridoxal phosphate -, H_4PtG- and NAD-dependent reduction of benzyl viologen by glycine is followed spectrophotometrically at 555 mμ. Sonic extracts of methanol-grown cells did not show any activity. Peel also failed to demonstrate a reversal of the system by measuring the

TABLE IX. 1.

ASSAY FOR THE GLYCINE-CLEAVAGE REACTION OF D. GLYCINOPHILUS IN METHANOL-GROWN PSEUDOMONAS AM1.

Manometer vessels contained: main compartment 1.3 μmoles (5 μc.) of [1-^{14}C] glycine, 1.5 μmoles of DL-H_4PtG, 0.5 μmole of NAD, 0.5 μmole of NADP, 100 μmoles of phosphate buffer pH 7.5, 10 μmoles of mercaptoethanol, 2.9 μmoles of benzyl viologen, and water to a total volume of 1.7 ml. Centre well: 0.2 ml. CO_2-free 2.5N-NaOH. First side arm: extract (about 3 mg. protein); second side arm: 0.2 ml. 10 N-H_2SO_4. The gas phase was N_2. After tipping the extract, the flasks were incubated for 60 min. at 30°.

The acid was then tipped, and the incubation continued for 30 min. The manometers were then removed from the bath, dried, and the flasks detached. To each centre well was added 0.2 ml. of 0.20 M-Na_2CO_3. The contents were transferred quantitatively to a graduated tube, and made up to 2.2 ml. with 0.20 M-Na_2CO_3. The carbonate was converted to $BaCO_3$ and assayed for radioactivity as described in chapter II, using a shielded GM tube.

Vessel	Contents	Total ^{14}C found in respiratory CO_2. (Counts/min. at infinite thinness)
1	Complete.	756
2	No H_4PtG, NAD, NADP, pyridoxal phosphate or benzyl viologen.	308
3	Complete; but extract boiled.	223

anaerobic fixation of [^{14}C]bicarbonate into non-volatile material in an incubation mixture containing formaldehyde, H_4PtG, NADH, pyridoxal phosphate and NH_4^+ ions.

A further confirmation of the non-operation of t is pathway in P. AM1 was obtained by repeating another experiment of Sagers & Gunsalus in which the formation of radioactive CO_2 from [1-^{14}C]glycine is followed in the presence of extract, NAD, NADP, H_4PtG, pyridoxal phosphate and benzyl viologen. Details of this experiment are set out with the results in table IX.1. Only a very small amount of radioactivity was found (formed from 5 μC of [^{14}C]glycine).

Glycine-carbon dioxide exchange reaction.

In order to ascertain whether glycine is a product of $^{14}CO_2$ fixation, a test was made for the exchange of radioactivity from [^{14}C]bicarbonate into glycine. Such exchange reactions have been observed in Brucella abortus (Marr & Wilson, 1951; Tepper & Wilson, 1958) on a massive scale, and are also known other organisms (see chapter VII for further details). Extract of methanol-grown cells was incubated with glycine (10 μmoles), an extract of boiled cells (see chapter IV), various possible

TABLE IX. 2

TEST FOR CARBON DIOXIDE-GLYCINE EXCHANGE REACTION.

The complete system contained: 10 µmoles of glycine, 100 µmoles of tris hydrochloride pH 7.5, boiled cell extract (see chapter IV), sodium [^{14}C] bicarbonate (8 µmoles; 10 µc. of ^{14}C), 2.5 µmoles of ATP, 0.1 µmole of CoA, 1 µmole of NADH, 1 µmole of NADPH, 0.8 µmole of DL-H_4PtG, sonic extract of methanol-grown P. AM1, and water to 1.7 ml.

The incubation was for 30 min. at 30°, and the reaction was terminated by adding 3.5 ml. boiling 95% v/v ethanol. Aliquots (0.1 ml.) were dried at infinite thinness, irrigated with 0.1 ml. 90% w/v formic acid, and assayed for radioactivity as described in chapter II, using a shielded GM tube.

Tube.	Omission.	Total radioactivity incorporated. (Counts/min.)
1	None	146
2	Glycine, cofactors	126
3	Cofactors	189
4	Boiled cell extract	80
5	Boiled cell extract, cofactors.	66
6	Complete, but extract boiled	63

cofactors, sodium [^{14}C]bicarbonate (8 μmoles; 10 μC of ^{14}C) and tris hydrochloride pH 7.5. After incubation, aliquots of the reaction mixture were dried on planchets and assayed for radioactivity. None was detected (see table IX. 2 for details).

Synthesis and carboxylation of formyl coenzyme A.

A fourth possible mechanism for the direct enzymic synthesis of C_2 units would be the activation of formate to formyl coenzyme A, carboxylation (reaction iii) or transcarboxylation (reaction iv) of the latter to oxalyl coenzyme A, and reduction of the latter to glyoxylate by the glyoxylate dehydrogenase known to be present in the cell (chapter V):

$$HCOOH + ATP + CoASH \longrightarrow HCOSCoA + AMP + PP_i \quad (ii)$$

$$HCOSCoA + CO_2 \longrightarrow HOOC.COSCoA \quad (iii)$$

$$HCOSCoA + E \overset{C_1}{\rightleftharpoons} HOOC.COSCoA + E \quad (iv)$$

$$HOOC.COSCoA + NADPH + H^+ \rightleftharpoons HOOC.CHO + NADP^+ + CoASH \quad (v)$$

$$HOOC.CHO + Glutamate \rightleftharpoons HOOC.CH_2NH_2 + \alpha\text{-Oxoglutarate} \quad (vi)$$

The first step in investigating the above series of reactions is to determine whether any activation of formate to formyl hydroxamic acid can be detected. Two recent reports have indicated the existence in micro-organisms

of an enzyme, formokinase, catalysing the reaction:

$$HCOOH + ATP \rightleftharpoons HCOOPO_3H_2 + ADP \qquad \text{(vii)}$$

assayed by the formation of formyl hydroxamate:-

$$HCOOPO_3H_2 + NH_2OH \longrightarrow HCONHOH + P_i \qquad \text{(viii)}$$

It has been found in Escherichia coli B, Aerobacter aerogenes, P. fluorescens, Clostridium welchii (Nakagawa, Suzuki & Takeda, 1961) and C. cylindrosporum (Sly & Stadtman, 1962). In addition the latter authors have presented evidence for the existence of specific phosphotransformylase and deacylase enzymes catalysing the reactions:

$$HCOOPO_3H_2 + CoASH \rightleftharpoons HCOSCoA + P_i \qquad \text{(ix)}$$

$$HCOSCoA + H_2O \longrightarrow HCOOH + CoASH \qquad \text{(x)}$$

In order to test for a formokinase reaction, or for an analogous reaction such as reaction (ii) above, extracts of formate-grown P. AM1 were incubated with hydroxylamine, CoA, ATP, glutathione and formate, acetate or succinate as described by Jones & Lipmann (1955). The results are shown in table IX.3. While acetate and succinate give a considerable amount of hydroxamic acid under these conditions, there was no detectable formation of hydroxamate with formate.

The apparent lack of a direct activating system

TABLE IX. 3.

HYDROXAMATE ASSAY FOR ACID ACTIVATION IN FORMATE-GROWN PSEUDOMONAS AM1.

The complete system contained, in 1 ml.: 0.1 μmoles of CoA, 10 μmoles of ATP, 20 μmoles of potassium acetate or succinate or sodium formate, 100 μmoles of tris hydrochloride pH 7.5, 50 μmoles of KF, 10 μmoles of $MgCl_2$, 10 μmoles of glutathione, extract of formate-grown cells (containing 1.5 mg. protein), and 200 μmoles of neutral hydroxylamine.

The incubation was for 20 min. at 30°, and was terminated by adding 1.5 ml. of reagent containing 10% w/v $FeCl_3.6H_2O$ and 3.3% w/v trichloracetic acid in 0.66 N-HCl. After centrifugation, the extinction of the supernatant was read at 540 mμ. against a blank containing all components except acetate, succinate or formate.

Substrate	Hydroxamate formed in 20 min. (μmoles/mg.protein)
Potassium acetate	0.73
Potassium succinate	0.62
Sodium formate	0

for the formation of formyl CoA may not rule out the presence of an indirect activating system involving e.g. acetyl or succinyl CoA and a thiophorase enzyme:

$$RCOSCoA + HCOOH \rightleftharpoons HCOSCoA + RCOOH \qquad (xi)$$

Such a mechanism operates in the formation of oxalyl-coenzyme A by soil micro-organisms (Jakoby, Ohmura & Hayaishi, 1956; Quayle, Keech & Taylor, 1961).

Extract of formate-grown P. AM1 containing about 2 mg. protein was incubated with 40 μmoles of phosphate buffer pH 7.5, 10 μmoles of cysteine, 0.1 μmole of coenzyme A, 10 μmoles of ATP, 10 μmoles of acetate or succinate, 10 μmoles (5 μC) of sodium [^{14}C]formate and water to 1 ml. The mixture was incubated for 15 min. at 30°. Then there was added 1 ml. of 2M neutralised hydroxylamine hydrochloride. After a further 10 min, at 30°, the reaction was stopped by addition of 25 ml. of 95% v/v ethanol to precipitate the excess hydroxylamine. This was removed by centrifugation and the supernatant concentrated by distillation of the ethanol under reduced pressure. The products were then chromatographed on Whatman no. 4 paper in phenol-water (72 : 28 w/v, Jakoby et al., 1956) with formyl, succinyl and acetyl hydroxamate markers. The spots were located by spraying with 5% w/v

$FeCl_3.6H_2O$ in ethanol 0.1 N in HCl (Stadtman & Barker, 1950), and radioactivity located by radioautography. Succinyl and acetyl hydroxamates were found in the tubes in which succinate and acetate were present, and very small amounts of a compound having the same R_f as formyl hydroxamate were detected by radioautography, but which did not give a colour with $FeCl_3$. The compound co-chromatographed with authentic formyl hydroxamate.

When the experiment was repeated using acetyl CoA instead of acetate, CoA and ATP, there was less radioactivity in formyl hydroxamate in the presence of acetyl CoA and extract than in a control containing only extract and $[^{14}C]$formate. Since the latter did not contain any active acetate or acetate-activating system the experiment was repeated using a boiled enzyme control. There was as much $[^{14}C]$formyl hydroxamate in the control as in the other tubes indicating that the reaction is non-enzymic.

The use of acetone instead of ethanol as an hydroxamate-extracting agent showed conclusively that the $[^{14}C]$formyl hydroxamate had arisen from ethyl formate, formed by non-enzymic esterification of $[^{14}C]$formate by the ethanol extracting agent.

It thus seems that formyl CoA cannot be directly or indirectly implicated in the formation of C_2 compounds.

Peel (1961, p.56) has shown that no formation of glyoxylate could be detected when the "formyl CoA formation system" was incubated with $NaHCO_3$ or oxaloacetate, thus ruling out carboxylation and transcarboxylation reactions of the types shown in equations (iii) and (iv) above.

2. INDIRECT FORMATION OF C_2 COMPOUNDS.

The indirect formation of C_2 compounds from C_4 dicarboxylic acids, possibly by a cyclic mechanism as shown in fig. 1 (b), was then investigated.

Cleavage of malate.

Peel (1961, p. 59) was unable to detect the formation of glyoxylate from malate by an aldol cleavage reaction (Gunsalus, 1959), perhaps of the types shown below:-

$$\text{Malate} \longrightarrow \text{Glyoxylate} + \text{Acetate} \qquad \text{(xii)}$$

$$\text{Malate} + \text{ATP} + \text{CoA} \longrightarrow \text{Glyoxylate} + \text{AcetylCoA} + \text{ADP} + P_i \qquad \text{(xiii)}$$

These are analogous to known reactions for the cleavage of citrate (Gunsalus, 1959).

Cleavage of β-hydroxyaspartate.

A likely mechanism for a C_4 cleavage reaction might

be the aldol cleavage of β-hydroxyaspartate to glyoxylate and glycine:

HOOC. CH(OH). $CHNH_2$. COOH → HOOC. CHO + CH_2NH_2. COOH (xiv)

analogous to the enzymic cleavage of threonine to acetaldehyde and glycine by threonine aldolase. β-hydroxyaspartate has been synthesised and its metabolism studied by Kornguth & Sallach (1960).

The metabolism of DL-β-hydroxyaspartate (probably a mixture of diastereoisomers, a gift from Prof. Sir Hans Krebs) was studied in extracts of methanol-grown P. AM1. β-Hydroxyaspartate (10 μmoles) was incubated at pH 7.5 with extract (3.5 mg. protein) both live and boiled, and in the presence of semicarbazide (12 μmoles) at pH 6.0, and also at pH 7.5 with 8 μmoles of α-oxoglutarate. The incubation was for 1 hr. at 30°, after which the reaction was stopped by addition of hot ethanol, except in the presence of semicarbazide, when saturated 2,4-dinitrophenylhydrazine in 2N-HCl was used to convert the semicarbazones to 2,4-dinitrophenylhydrazones. After extraction and chromatography, no evidence for the 2,4-DNP-derivatives of glyoxylate was found. The remaining tubes were centrifuged, concentrated and aliquots chromatographed two-dimensionally as described in chapter II.

After spraying with ninhydrin, the only product not present in the controls was an intense colour in the glycine-serine-glutamate area of the tube containing live extract, β-hydroxyaspartate and α-oxoglutarate. This was probably glutamate. To another aliquot was added 2000 counts/min. of authentic [^{14}C]glutamate and it was chromatographed in the butan-1-ol-acetone-water-diethylamine solvent (chapter II). The ninhydrin positive spot agreed exactly with the radioactive area, thus showing that β-hydroxyaspartate can transaminate enzymically with α-oxoglutarate. However no evidence of any glycine or glyoxylate was obtained in this experiment.

Cleavage of [^{14}C]dicarboxylic acids.

For a final test for the formation of glycine from C_4 dicarboxylic acids, a ^{14}C tracer technique was used. This is sufficiently sensitive for the formation of only slight amounts of glycine, such as might be observed even in the absence of added cofactors, to be detected. Accordingly, extracts (both live and boiled) of methanol-grown P. AM1 were incubated with the sodium salts of [2, 3-$^{14}C_2$]succinate, [2, 3-$^{14}C_2$]fumarate and DL-[2-^{14}C]malate (this last a gift from Dr. J.A. Bassham).

Table IX.4

NON-VOLATILE PRODUCTS OF METABOLISM OF (^{14}C) DICARBOXYLIC ACIDS BY CRUDE EXTRACTS OF METHANOL-GROWN PSEUDOMONAS AM 1

The system contained: sodium salts of (^{14}C) dicarboxylic acids as indicated (each tube containing 0.5μc of ^{14}C), 100 μmoles of phosphate buffer pH 7.5, live or boiled extract (containing 4.5 mg. protein) and water to 1 ml.

Incubation was for 60 min. at 30°. Reaction stopped by addition of 3 ml. of boiling 95% v/v ethanol, and the deproteinised supernatant concentrated and aliquots chromatographed two-dimensionally on paper as described in chapter II. Products were located by radioautography and identified by co-chromatography.

Tube No.	1	2	3	4	5	6
Contents	Live extract (^{14}C) fumarate (0.31 μmole)	Boiled extract (^{14}C) fumarate (0.31 μmole)	Live extract (^{14}C) succinate (0.36 μmole)	Boiled extract (^{14}C) succinate (0.36 μmole)	Live extract DL-(^{14}C)malate (0.56 μmole)	Boiled extract DL-(^{14}C)malate (0.56 μmole)
Total radioactivity (counts/min. x 10^{-3}) in:						
Aspartate	2.80	1.10	5.66	0	1.31	0
Citrate	0.28	0	0.33	0	0.10	0
Glutamate	1.84	0	6.25	0	0.42	0
Malate	13.80	0	11.72	0	12.58	13.63
Fumarate	3.01	17.40	2.25	0	0.94	0
Succinate	0	0	0.66	25.40	0	0.43
Total	21.73	18.50	26.67	25.40	15.35	14.06

The incubation was in air at 30° for 60 min., started by addition of extract and stopped by the addition of 3 ml. boiling 95% v/v ethanol. The deproteinised mixture was treated as described in chapter II, and the products analysed by two-dimensional paper chromatography, located by radioautography and identified by co-chromatography. Details of the experiment and the products are shown in table IX. 4. No glycine or serine was detected in the separated amino acid area from any of the tubes: the radioactivity was solely in tricarboxylic acid cycle intermediates and the amino acids derived therefrom. The experiment thus only serves to demonstrate that the reactions of the tricarboxylic acid cycle can take place in extracts. The large amount of glycine formed from [^{14}C]succinate in whole cells (amounting to 40% of the glutamate found, after a 1 min. incubation, see chapter VIII), might have led one to suppose that even in extracts which had not been supplemented by addition of cofactors, small amounts of glycine might be detected, but this appears not to be the case.

3. ETHANOL IN RELATION TO C_2 METABOLISM.

Whole cells of methanol-grown P. AM1 readily oxidised

ethanol with a Q_{O_2} about 85% of that observed for methanol. It was also found that P. AM1 would readily grow on ethanol as sole carbon source (Peel & Quayle, 1961; Peel, 1961, p. 21). This is a property which P. AM1 does not share with any other C_1-growing organism except P. extorquens (Peel & Quayle, 1961). It seemed likely that growth on ethanol would involve formation of acetate and synthesis of cell constituents via the glyoxylate cycle. However, if this were not the case, the metabolism of the C_2 compound ethanol might be relevant to the pathway of formation of glycine in methanol-grown P. AM1. With this in mind, the possible occurrence of the glyoxylate cycle during growth was examined by testing for the presence of the key enzymes malate synthetase and isocitratase.

Two cultures of P. AM1 were given equal inocula. Both contained the Jayasuriya salt medium (chapter II), and in addition one had 0.5% v/v methanol and the other 0.1% v/v ethanol as sole sources of carbon.

These were grown for 24 hr. at 30°. The cells were then harvested by centrifugation, and sonic extracts prepared as described in chapter II. These extracts were tested for the activity of isocitratase and malate

TABLE IX. 5.

ACTIVITY OF THE KEY ENZYMES OF THE GLYOXYLATE CYCLE IN METHANOL- AND ETHANOL-GROWN PSEUDOMONAS AM1.

Parallel cultures of cells were grown respectively on 0.5% v/v methanol and 0.1% v/v ethanol. After harvesting by centrifugation, cell-free extracts were prepared by ultrasonic disruption as described in chapter II. After centrifugation to remove whole cells and debris, the supernatants were tested for isocitratase and malate synthetase activity by the spectrophotometric methods of Dixon & Kornberg (1959), at 22°.

Carbon source for growth	Methanol	Ethanol
Specific activity (μmoles/hr./mg.protein) of:		
Isocitratase	< 0.08	0.69
Malate synthetase	1.74	4.22
Ratio $\frac{\text{Isocitr. sp. activity}}{\text{M.S. sp. activity}}$	< 0.046	0.164

synthetase, the key enzymes of the glyoxylate cycle, by the method of Dixon & Kornberg (1959). The results are shown in table IX. 5. The ratio (isocitratase specific activity) / (malate synthetase specific activity) is usually < 0.05 for compounds which do not give rise to acetate and about 0.4 - 0.9 for cells which do (Kornberg & Elsden, 1961). The presence of isocitratase in high specific activity in ethanol-grown cells thus indicates that growth on this compound involves acetate and the glyoxylate cycle, and hence is unlikely to give any information on the mechanism of glycine or C_2 unit formation in cells grown on C_1 compounds.

Discussion.

Despite evidence obtained from whole cell experiments (see chapter VIII) in favour of the indirect pathway of glycine formation via C_4 intermediates (alternative (b) in fig. 1), the extract experiments have failed to give further information of this point.

Chapter X

THE FORMATION OF C_4 ACIDS BY CARBOXYLATION REACTIONS

Two alternative reaction mechanisms for the formation of glycine from C_1 units were proposed in chapter III (see fig. 1). Any mechanism for the pathway of carbon assimilation must account for the following observations:

(1) 50% of the cellular carbon of methanol-grown Pseudomonas AM1 is derived from a compound in equilibrium with exogenous carbon dioxide (Large, Peel & Quayle, 1961).

(2) the carboxyl group of glycine and hence of serine is derived from carbon dioxide (chapter VII).

(3) the major products formed on administration of [^{14}C]bicarbonate to methanol- or formate-grown P. AM1 are malate and aspartate (chapter III).

Most of the evidence obtained in whole cells (chapter VIII) and extracts (chapter IX) favours the indirect mechanism (mechanism (b) in fig. 1) for glycine formation. This mechanism embodies two features: the carboxylation of a

C_3 compound (derived from serine) to give a C_4 acid such as oxaloacetate or malate, and the cleavage of the latter to two C_2 units, one or both of which could give rise to glycine. This could, from the observed labelling patterns in malate (Large et al., 1962) give rise to glycine with a labelling pattern similar to that observed (chapter VII).

Six carboxylation reactions involving the conversion of C_3 acids to oxaloacetate or malate are known. These are:

A. The reversible, nicotinamide nucleotide-dependent carboxylation of pyruvate to form malate, catalysed by malic enzyme (Ochoa, Mehler & Kornberg, 1948).

$$\text{Pyruvate} + \text{NADPH (or NADH)} + H^+ + CO_2 \rightleftharpoons \text{Malate} + \text{NADP}^+ \text{ (or NAD}^+\text{)} \qquad \text{(i)}$$

B. The reversible, nucleoside diphosphate-dependent carboxylation of phosphoenolpyruvate to oxaloacetate, catalysed by phosphoenolpyruvate carboxykinase (Utter & Kurahashi, 1954):

$$\text{Phosphoenolpyruvate} + \text{NuDP} + CO_2 \rightleftharpoons \text{Oxaloacetate} + \text{NuTP} \qquad \text{(ii)}$$

where Nu = adenosine, inosine or guanosine.

C. The irreversible carboxylation of phosphoenolpyruvate to oxaloacetate and orthophosphate, catalysed by phosphoenolpyruvate carboxylase (Bandurski & Greiner, 1954):

Phosphoenolpyruvate + CO_2 ⟶ Oxaloacetate + P_i (iii)

D. The reversible, orthophosphate-dependent carboxylation of phosphoenolpyruvate to oxaloacetate with the formation of inorganic pyrophosphate, catalysed by phosphoenolpyruvate carboxytransphosphorylase (Siu, Wood & Stjernholm, 1961):

Phosphoenolpyruvate + P_i + CO_2 ⇌ Oxaloacetate + PP_i (iv)

E. The ATP-dependent carboxylation of pyruvate to oxaloacetate with the cleavage of the triphosphate to ADP and orthophosphate catalysed by pyruvate carboxykinase (Utter & Keech, 1960). The reaction, which is not known to be reversible, requires catalytic amounts of acetyl coenzyme A, and is inhibited by avidin.

Pyruvate + CO_2 + ATP $\xrightarrow{\text{Acetyl CoA}}$ Oxaloacetate + ADP + P_i (v)

F. The reversible, ATP dependent carboxylation of pyruvate to oxaloacetate similar to the above but not requiring acetyl CoA. The enzyme has been purified from P. citronellolis and shown to contain a biotin prosthetic

group (Seubert & Remberger, 1961).

$$\text{Pyruvate} + CO_2 + ATP \rightleftharpoons \text{Oxaloacetate} + ADP + P_i \qquad \text{(vi)}$$

Since the fixation of $^{14}CO_2$ into C_4 acids is of such importance in growth on methanol, an examination of the pathways of fixation of [^{14}C]bicarbonate in extracts of methanol-grown P. AM1 was undertaken by Peel (1961, p.61) in an attempt to discover whether one or more of the above reactions, or some other reaction, was involved. He found no evidence for the presence of malic enzyme or pyruvate carboxykinase. There was a small incorporation of ^{14}C into non-volatile fixation products when extracts were incubated with [^{14}C]bicarbonate in the presence of phosphoenolpyruvate (PEP). This incorporation was decreased when IDP, ADP or GDP was added. The incorporation was stimulated considerably by addition of a boiled cell extract, prepared as described in chapter IV.

The fixation stimulated by boiled extract was inhibited by IDP and phosphate. Boiled extract had no effect on incorporation of ^{14}C when PEP was replaced by pyruvate. When the fixation products were analysed by two-dimensional paper chromatography, the products were: with PEP and [^{14}C]bicarbonate alone: 66 µm-moles fixed, of which 46% was malate, 46% aspartate and 10% fumarate.

When boiled cell extract was added, the PEP dependent fixation of ^{14}C was increased to 533 µm-moles, of which 53% were malate, 24% aspartate, and 10% fumarate. The appearance of both malate and aspartate as products indicates that the primary fixation product is oxaloacetate (which is labile under these conditions). It seemed probable that the increase in fixation in the presence of boiled cell extract might really be due to stabilisation of the oxaloacetate formed by carboxylation by its conversion to non-labile malate and aspartate by co-factors such as reduced nicotinamide nucleotides and glutamate present in the boiled cell extract. Accordingly, an attempt was made to reproduce the stimulatory effect of the boiled cell extract with NADH. Extracts of P. AM1 had very strong NADH oxidase activity, but when large substrate amounts of NADH were used, it was found that the stimulatory effect of 0.5 ml. of boiled extract could entirely be replaced by 2 µmoles of NADH. Under these conditions, however, no aspartate was found in the fixation products, which were 21% fumarate and 79% malate. In order to determine whether the reductive carboxylation of the PEP was catalysed by one enzyme or by one of the above PEP carboxylation reactions coupled with malate

dehydrogenase

$$\text{Oxaloacetate} + \text{NADH} + H^+ \rightleftharpoons \text{Malate} + \text{NAD}^+ \qquad \text{(vii)}$$

purification of the crude system was necessary.

Preparation of sodium phosphoenolpyruvate.

The silver-barium salt of PEP (500 mg.) was dissolved in 0.2N-HNO_3 (11.2 ml., the theoretical amount for the conversion of Ag^+ and Ba^{2+} to their nitrates). AgCl and $BaSO_4$ were precipitated by the addition of a slight excess of sodium chloride and sodium sulphate. The precipitate was removed by centrifugation and the pH of the supernatant adjusted to 7 with 1N-NaOH.

Radioactive assay used for the enzyme purification.

0.3 ml. of enzyme solution (at any stage of purification) was incubated for 30 min. at 30° with 100 μmoles of tris hydrochloride pH 7.5, 1 μmole of $MgCl_2$, 2 μmoles of glutathione, 20 μmoles of $NaH^{14}CO_3$ (containing 2 μC of ^{14}C), 2.5 μmoles of sodium PEP, 3 μmoles of NADH and water to 2 ml. After stage 3 of the enzyme purification (see below) it was necessary to add in addition 0.1 ml. of a solution of malate dehydrogenase (containing 375 units by the assay of Ochoa, 1955). This was prepared by diluting the stock suspension of malate dehydrogenase (Boehringer) 10-fold with 10mM-tris hydrochloride pH 7.5

and dialysing for 2 hr. against 1.5 l. of the same buffer to remove ammonium sulphate. The latter was found to inhibit the carboxylation reaction. The reaction was stopped by addition of 3 ml. of boiling aq. 95% v/v ethanol, and 0.1 ml. samples were removed and assayed for fixed non-volatile radioactivity at infinite thinness on planchets as described in chapter II. Samples of the tracer solution were diluted 100-fold with 30 mM-NaOH and assayed for radioactivity under the same conditions as the test samples (omitting the formic acid treatment). 1 unit of enzyme activity is defined as the amount of enzyme required to fix 1 μm-mole of $^{14}CO_2$ into non-voltaile products in 30 min. The measured activity was found to be proportional to the amount of enzyme used.

Preliminary experiments on the crude extract.

The assay used above was developed in the study of crude extracts which had considerable NADH oxidase activity, and hence the incubation mixture for the assay contains excess NADH. NADH was prepared immediately prior to use by dissolving the solid in 20 mM-tris hydrochloride pH 8. A protracted series of preliminary experiments was necessary before the procedure outlined below was developed. These

involved the use of solid and saturated ammonium sulphate solution and alumina C γ gel. During these experiments, it was found that the carboxylation reaction was inhibited by ammonium sulphate at quite low concentrations (of the order of 5mM). The crude extract was not stimulated by the addition of malate dehydrogenase (dialysed as described above), nor was the ammonium sulphate precipitate after step 3, indicating that malic dehydrogenase is still present in excess. NADPH would not replace NADH in the assay.

PURIFICATION OF THE CARBOXYLATING ENZYME.

Step 1: Preparation of bacterial extract. Frozen cells of methanol-grown P. AM1 (21 g., wet wt.) were crushed in a Hughes press (Hughes, 1951) at -25°. The crushed cells were extracted with 100 ml. of ice-cold 40mM - tris hydrochloride pH 7.5 containing 10 mM-mercaptoethanol and about 1 mg. each of crystalline ribonuclease and deoxyribonuclease. The resulting extract was centrifuged at 25000 x g for 10 min. at 2° and the solid material discarded. All subsequent operations were performed at 2°.

Step 2: Treatment with protamine sulphate. To the supernatant (vol. 99 ml., containing 1130 mg. of protein) was added, with stirring, a solution containing 113 mg. of protamine sulphate in 2mM sodium acetate buffer pH 5, and the resulting pink curdy precipitate removed by centrifugation and discarded.

Step 3: Ammonium sulphate precipitation and dialysis. To the supernatant (vol. 128 ml.) was added 31.3 g. of ammonium sulphate with stirring, to give 40% saturation. The resulting precipitate was centrifuged down. To the supernatant (136 ml.) was added a further 8.56 g. of ammonium sulphate to bring the percentage saturation to 50. The resulting precipitate was removed by centrifugation. The supernatant (134 ml.) was brought to 60% saturation by the addition of 8.85 g. of ammonium sulphate. The precipitate was centrifuged down, and the supernatant discarded. The three precipitates were each dissolved in 10 ml. of 50mM-tris hydrochloride pH 7.5 and dialysed overnight against 1.5 l. of 10mM-tris hydrochloride pH 7.5. The activity was found to be almost entirely in the protein fraction precipitating between 40 and 50% saturation. This was subjected to ion-exchange chromatography.

Step 4: Ion-exchange chromatography. 7 g. of diethylaminoethylcellulose (DEAE-cellulose, Whatman DE 50) was slurried in 5mM-tris hydrochloride pH 7.5, and poured into a chromatographic column (2.5 cm. x 15 cm.). The column was then washed with 1 l. of 5mM-tris hydrochloride pH 7.5 and the enzyme preparation from step 3 applied to the top of the column. The column was then eluted with an increasing gradient of potassium chloride in 5mM-tris hydrochloride pH 7.5. This was formed by connecting together the bottoms of two 500 ml. polythene bottles, the first containing 500 ml. of 1 M-KCl, the other 500 ml. of 5mM-tris hydrochloride pH 7.5. The second bottle was stirred mechanically, and the outflow was allowed to drip on to the top of the column. The levels of the solutions in both bottles dropped at the same rate throughout. Fractions (4 ml.) were collected automatically at a flow rate of 40 ml./hr. Under these conditions, the carboxylating enzyme was eluted at concentrations of chloride between 0.14 M and 0.19 M (see fig. 12). This corresponded to fractions 52 - 58, which were combined and the resulting solution (25 ml.) was stored at 2°.

The purification procedure is summarised in table X.1.

Table X.1

PURIFICATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE FROM METHANOL-GROWN PSEUDOMONAS AM 1

For experimental details, see text.

Step	Fraction	Vol. (ml)	Activity (units/ml)	Protein (mg/ml)	Total units	Total protein (mg)	Specific Activity (units/mg of protein	Purification	Yield %
1	Supernatant obtained from crude extract after centrifugation	99	2479	11.4	245,000	1,130	217	1	100
2	Supernatant after removal of protamine sulphate precipitate by centrifugation	129	1318	5.9	168,700	755	224	1.02	68.9
3	Fraction precipitated by ammonium sulphate (40-50%), after dialysis	11.4	13800	7.9	157,500	90.1	1,750	8.06	64.3
4	Selected combined fractions after column chromatography	24.5	3750	0.62	92,000	15.2	6,050	27.9	37.6

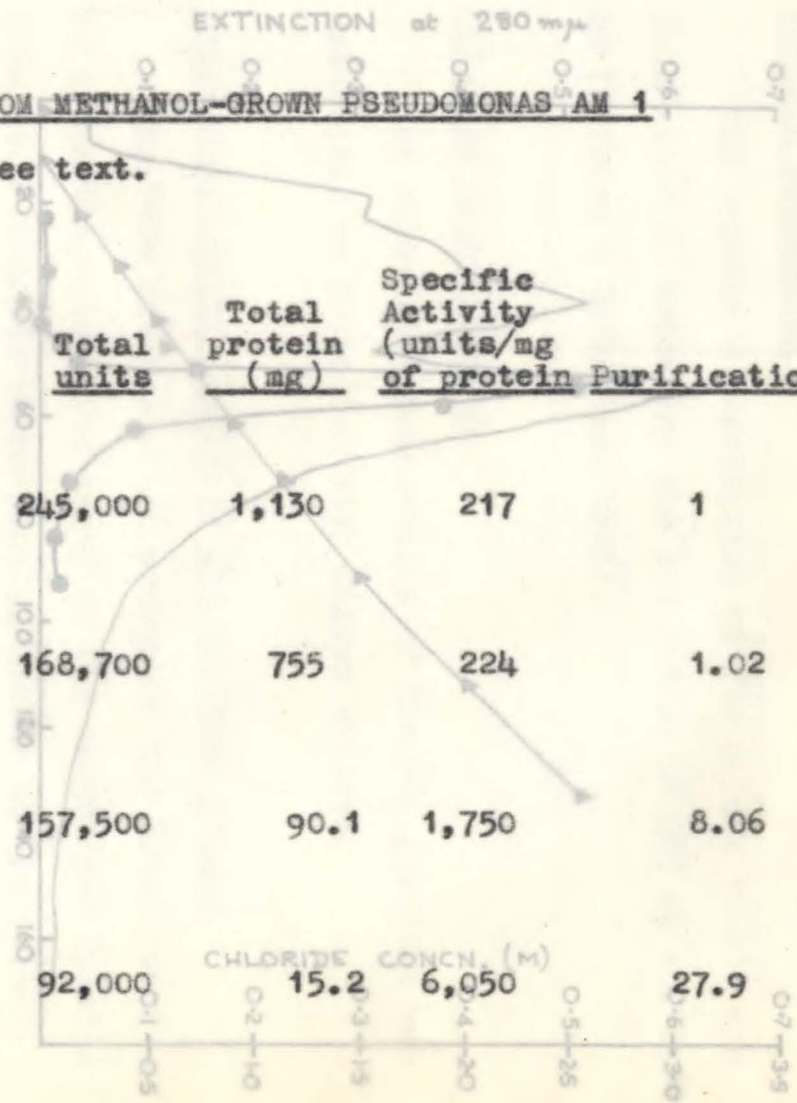
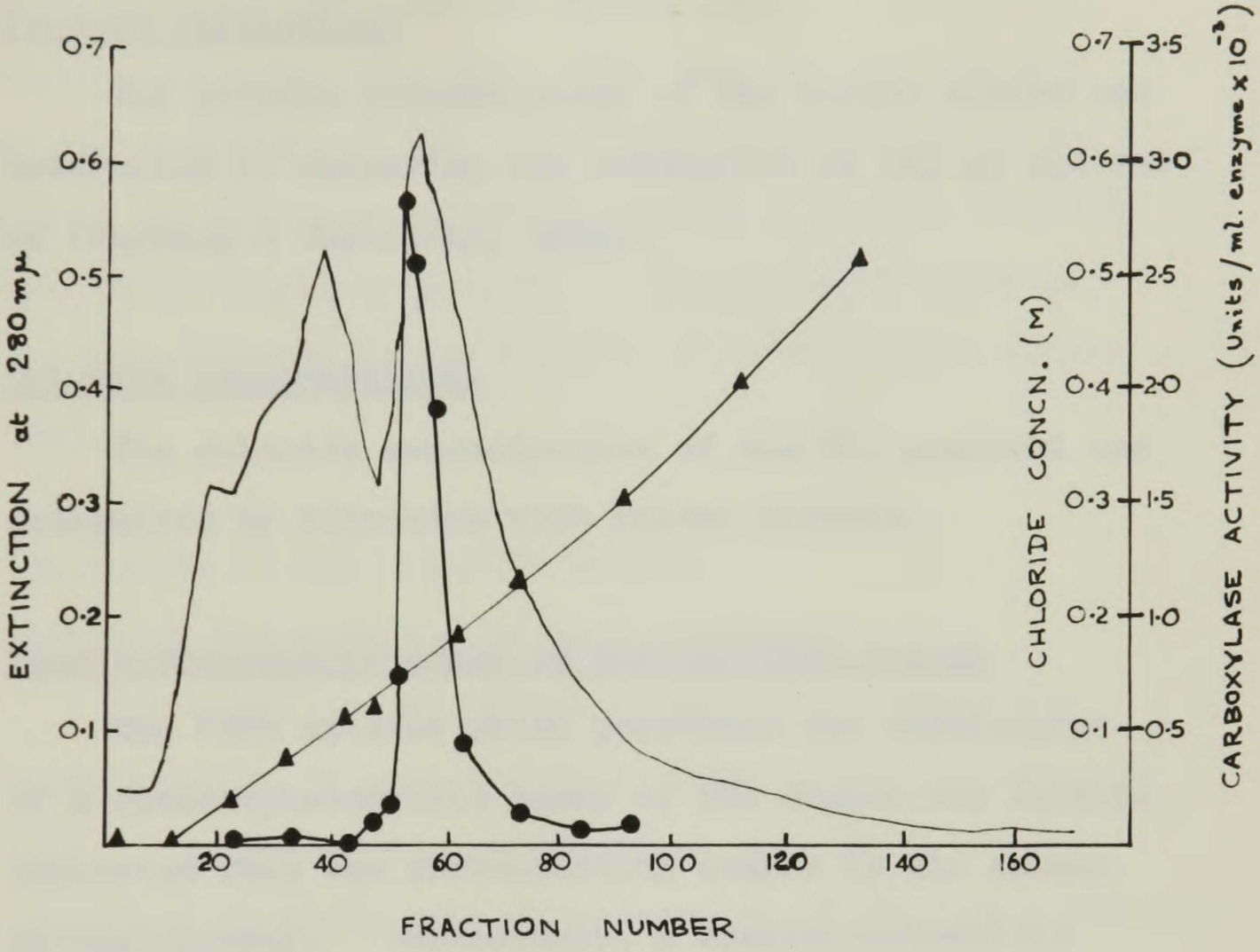


Fig. 12

Elution of PEP carboxylase from DEAE-cellulose column.



For conditions see text. The continuous line represents the extinctin of fraction (vol. = 4 ml.) at 280 mμ (1 cm. light path); ● carboxylase activity (μmoles CO_2 fixed in 30 min. /ml. enzyme; ▲ gradient of chloride concentration (M).

Protein estimation.

The protein concentration of the column eluate was determined by measuring the extinction at 260 mμ and 280 mμ (Warburg & Christian, 1941).

Chloride determination.

The chloride concentration of the KCl gradient was determined by titration with silver nitrate.

Spectrophotometric assay of the purified enzyme.

The NADH oxidase which prevented the development of a spectrophotometric assay of the enzyme was finally separated from the carboxylating enzyme by the column chromatography. Accordingly, a spectrophotometric assay was devised for study of the purified enzyme. The activity was assayed in a Cary recording spectrophotometer by measuring the rate of NADH oxidation (decrease in extinction at 340 mμ) in the following reaction mixture at 22°. The complete system, contained in 1.5 ml. silica cuvettes (light path, 1 cm.) consisted of 40 μmoles of tris hydrochloride pH 8.5, 20 μmoles of $NaHCO_3$, 0.2 μmole of NADH, 2 μmoles of $MgCl_2$, 1 μmole of sodium PEP, 0.03 ml. of the dialysed malate dehydrogenase

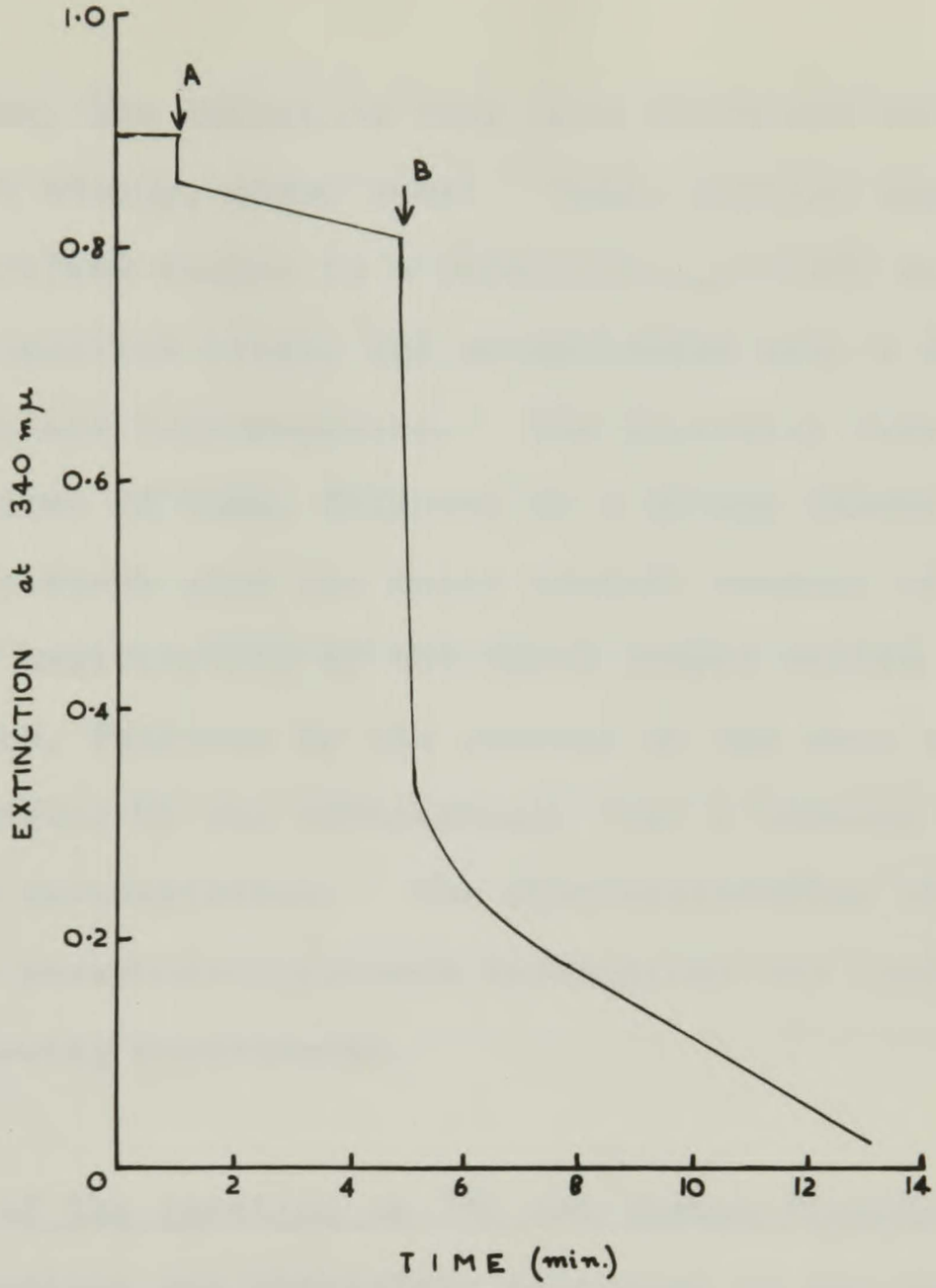
(113 units by the assay of Ochoa, 1955), 0.05 ml. of enzyme and water to 1 ml. The reaction was started by the addition of PEP. One unit was defined as the amount of enzyme required to oxidise 1 µm-mole of NADH/ min. under these conditions. The measured activity was proportional to the amount of carboxylating enzyme used.

PROPERTIES OF THE PURIFIED ENZYME.

Nature of the enzyme-catalysed reaction.

The crude enzyme extract catalysed a reductive carboxylation of phosphoenolpyruvate in the presence of NADH. As the enzyme purification proceeded, it was necessary to add malate dehydrogenase to the assay mixture. With the purified enzyme, when malate dehydrogenase was omitted from the spectrophotometric assay mixture, there was only a slow oxidation of NADH on addition of PEP. When malate dehydrogenase was added to this system, there was a stimulation in the rate of NADH oxidation. The extent of this stimulation varied with the pH. At pH 7.0, the stimulation of rate was 1.7-fold, at pH 7.5 2.4-fold, and at pH 8.5 it was 4.8-fold (fig. 13). A very rapid oxidation of NADH was observed immediately on adding malate

Fig. 13



Effect of the addition of malate dehydrogenase to the purified PEP carboxylase.

The reaction mixture contained: PEP carboxylase (31 µg.), 40 µmoles of tris hydrochloride pH 8.5, 20 µmoles of $NaHCO_3$, 0.2 µmoles of NADH, 2 µmoles of $MgCl_2$, total volume 1 ml. in 1.5 ml. silica cuvettes (light path 1 cm.). At A, was added 1 µmole of PEP; at B, 113 units of malate dehydrogenase. (Taken from a trace by a Cary recording spectrophotometer.).

dehydrogenase, the oxidation rate then decreased and settled to a steady, lower rate. These results suggest that the purified enzyme is a phosphoenolpyruvate carboxylase, catalysing reaction (iii), and contaminated with a small amount of malate dehydrogenase. The initially very rapid oxidation of NADH, followed by a slower steady rate is consistent with the rapid initial removal of accumulated oxaloacetate by the added excess malate dehydrogenase, followed by its removal at the same rate as its formation by the carboxylase, i.e. a measure of the rate of carboxylation. The characterisation of the enzyme as a phosphoenolpyruvate carboxylase was confirmed by the following experiments.

Dependence of the reaction on PEP and carbon dioxide.

The reaction was absolutely dependent on the presence of PEP. None of the following compounds could serve as substrate when tested at 1 mM concentration in the spectrophotometric assay; sodium 3-phosphoglycerate, sodium pyruvate, sodium pyruvate + ATP (1 mM), L-α-glycerophosphate, lithium hydroxypyruvate, sodium DL-glycerate, Dl-serine, and potassium Dl-lactate.

It was difficult to demonstrate a complete dependence

on carbon dioxide. At pH 8.6, in the spectrophotometric assay, the reaction proceeded at maximal velocity even in the absence of added sodium bicarbonate. However, if the buffer, magnesium chloride and PEP solutions were prepared from CO_2-free water, the reaction proceeded at only 60% of the previous rate. The original rate was completely restored on addition of sodium bicarbonate. Radioactivity from $[^{14}C]$bicarbonate was incorporated into the reaction products.

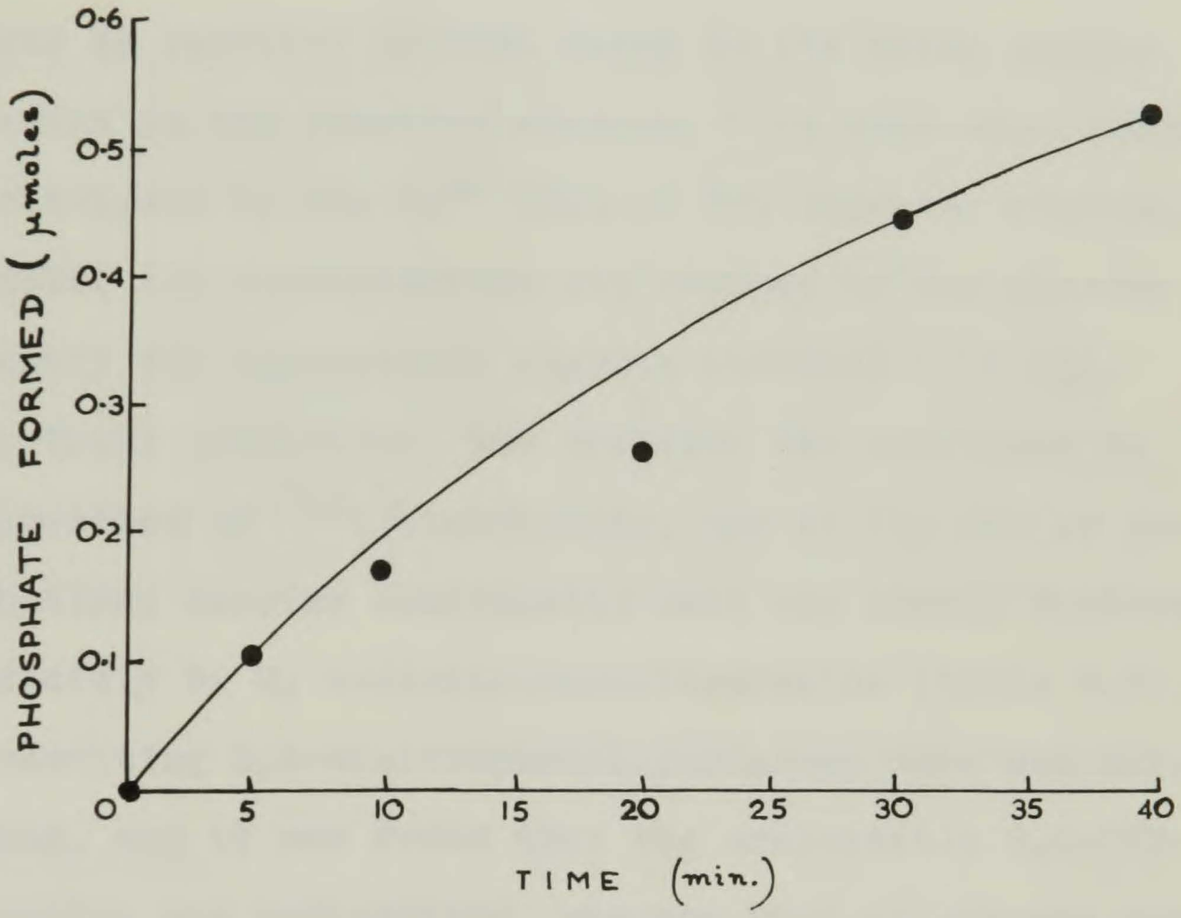
The enzyme must clearly be a phosphoenolpyruvate carboxylase, as the reaction proceeds at maximal velocity in the absence of added nucleotides and phosphate (see below), thus eliminating the possibility of reactions (ii) and (iv).

Identification of reaction products.

When PEP was carboxylated in the presence of the enzyme, the liberation of orthophosphate could be followed by estimation by the method of Allen (1940). This is shown in fig. 14. From the initial slope of this curve the specific activity of the enzyme at pH 8.5 and 30° was calculated to be 0.338 μmoles P_i liberated/min/mg. protein. This can be compared with a corresponding

Fig. 14

Formation of inorganic orthophosphate by PEP carboxylase.



The system contained in 1 ml.: 50 μmoles of tris hydrochloride pH 8.5, 2.5 μmoles of sodium PEP, 50 μmoles of $NaHCO_3$, 2 μmoles of $MgCl_2$, and 62 μg. of enzyme. Incubated at 30°. Reaction stopped by heat, and phosphate estimated as described in the text.

spectrophotometric assay of 0.386 μmoles NADH oxidised/ min./mg. protein at pH 8.5 and 22°.

Difficulty was encountered in establishing oxaloacetate as reaction product owing to its rapid decarboxylation in the reaction mixture. As this decarboxylation was catalysed by the Mg^{2+} ions in the reaction mixture, the metal ion concentration was reduced to the minimum necessary for appreciable enzymic reaction (0.5 mM). Under these conditions, the reaction was performed in the presence of $[^{14}C]$bicarbonate, and at the end of the incubation, carrier oxaloacetic acid was added, followed immediately by 2, 4-dinitrophenylhydrazine (table X.2). The resulting 2,4-dinitrophenylhydrazones were chromatographed, and it was found that the oxaloacetic 2,4-DNP-derivative was radioactive, whereas that of pyruvic acid (derived from oxaloacetic acid by decarboxylation) was not. This shows that the PEP was being carboxylated at the β-position to give oxaloacetate. No evidence was found for an enzyme-catalysed exchange of $^{14}CO_2$ with the carboxyl groups of oxaloacetic acid in the absence of PEP.

TABLE X. 2

IDENTIFICATION OF OXALOACETATE AS THE PRODUCT OF CARBOXYLATION OF PHOSPHOENOLPYRUVATE.

The complete reaction mixture contained: 100 μmoles of tris hydrochloride pH 8.5, 2.5 μmoles of PEP, 0.5 μmole of $MgCl_2$, 8 μmoles of sodium [^{14}C]bicarbonate (10 μc. of ^{14}C), 62 μg. of enzyme, and water to 1 ml.

Incubation was for 30 min. at 30°. At the end of this time, 10 μmoles of carrier oxaloacetic acid were added to each tube, followed by 2 ml. of 0.1% w/v 2,4-dinitrophenylhydrazine in 2N-HCl. After standing 15 min. at 30°, the 2,4-DNP derivatives were extracted into 2 ml. of ethyl acetate. Aliquots were chromatographed in butan-1-ol-ethanol- aq. NH_3 (sp. gr. 0.880)-water (140 : 20 : 1 : 39 by vol.) and the radioactivity in the spots of oxaloacetic and pyruvic 2,4-DNP-derivatives assayed directly.

Reaction mixture	Radioactivity (Counts/min.) in 2,4-DNP-derivatives of: Oxaloacetic acid	Pyruvic acid
Complete	1142	0
PEP replaced by oxaloacetate (10 μmoles)	0	0
Complete system in which enzyme had previously been boiled.	0	0

Stoicheiometry of the reaction.

The changes in concentration of PEP, orthophosphate pyruvate and NADH observed when the carboxylase was coupled with malate dehydrogenase are shown in table X.3. Phosphate was estimated by the method of Allen (1940). PEP was estimated by measuring the decrease in extinction at 340 mμ in the presence of NADH, ADP and lactate dehydrogenase when pyruvate kinase was added (Bücher & Pfleiderer, 1955). The complete assay system contained, in 3 ml. silica cuvettes: 0.5 μmole of ADP, 25 μmoles of $MgCl_2$, 250 μmoles of KCl, 150 μmoles of triethanolamine hydrochloride buffer pH 7.5, 0.005 ml. of lactate dehydrogenase suspension, the sample for assay (which contained 0.05 - 0.15 μmole of PEP) and NADH to give an optical density of 0.55 - 0.75. The reaction was started by adding 0.005 ml. of pyruvate kinase suspension and the decrease in extinction at 340 mμ was measured in a Beckman model DU spectrophotometer. The reaction was complete in 1½ min. under these conditions. Pyruvate was estimated by measuring the decrease in extinction at 340 mμ consequent on the addition of NADH and 0.005 ml. of lactate dehydrogenase suspension to a sample of the incubation mixture.

TABLE X. 3.

STOICHEIOMETRY OF THE PHOSPHOENOLPYRUVATE CARBOXYLASE REACTION.

The complete reaction mixture contained: 40 μmoles of tris hydrochloride pH 8.5, 20 μmoles of sodium bicarbonate, 3 μmoles of NADH, 2 μmoles of $MgCl_2$, 1 μmole of PEP, 0.03 ml. of malate dehydrogenase, 31 μg. of PEP carboxylase, water to 1 ml.

Incubation was for 90 min. at 30°, and the reaction was terminated by heating for 3 min. at 70°. NADH was assayed by change of extinction of 340 mμ. Other reactants assayed as described in the text.

Omission	Change in reactant concentration (μmoles)			
	NADH	PEP	Orthophosphate	Pyruvate
None	-0.674	-0.655	+0.646	+0.019
PEP	-0.172	0	0	-
Malate dehydrogenase	-0.487	-0.662	+0.621	+0.072
None; boiled enzyme used	-0.122	-0.030	0	-

The results of table X.3 show that the disappearance of one mole of phosphopyruvate is accompanied by the appearance of one mole of orthophosphate and one mole of NAD^+. Thus the coupled reaction can be represented as the sum of reactions (iii) and (vii).

Metal ion activation.

The reaction was absolutely dependent on the presence of divalent metal ions. Mg^{2+} was the most effective activator; of the other divalent cations tested, only Mn^{2+} showed significant activity (table X.4).

Inhibition of the reaction.

The following compounds at a concentration of 1mM did not inhibit the carboxylase: sodium iodoacetate, sodium 3-phosphoglycerate, L-α-glycerophosphate, lithium hydroxypyruvate, sodium DL-glycerate, DL-serine, potassium Dl-lactate, sodium pyruvate, sodium pyruvate + 1mM-ATP, potassium fluoride (10mM). The only substances which were found to inhibit the reaction were phosphate and ammonium ions. Both these effects had been noted in crude extracts, and for this reason tris hydrochloride buffers were always used, also dialysis of the ammonium

TABLE X. 4.

EFFECT OF METAL IONS ON THE RATE OF THE CARBOXYLATION REACTION

The reaction mixture contained, in 1.5 ml. silica cuvettes (light path, 1 cm.) : 1 μmole of sodium PEP, 31 μg. of purified enzyme, 40 μmoles of tris hydrochloride pH 8.5, 20 μmoles of $NaHCO_3$, 0.2 μmole of NADH, 0.03 ml. (113 units) of malate dehydrogenase, and water to 1 ml.

The reaction was started by the addition of 3 μmoles of the metal salt shown below.

Addition	Relative velocity
None	0
$MgCl_2$	100 *
$MnCl_2$	34
$CaCl_2$	0
$SrCl_2$	1.75
$BaCl_2$	1.75
$CoCl_2$	0
$ZnCl_2$	0
$MgCl_2$ + EDTA (2.5 μmoles)	18.6

* This corresponded to 11.8 μm-moles NADH oxidised/min.

sulphate precipitate at stage 3 of the purification, and dialysis of the malate dehydrogenase preparation used in the assay of the purified enzyme were necessary. The effect of the presence of these inhibitors on the rate of the reaction at different PEP concentrations was studied. As seen in fig. 15, the inhibition by 40mM-phosphate is competitive, while the inhibition by 5mM-ammonium chloride is non-competitive. The effect with phosphate may be due to competition with the enzyme for Mg^{2+} ions.

Effect of nucleoside diphosphates.

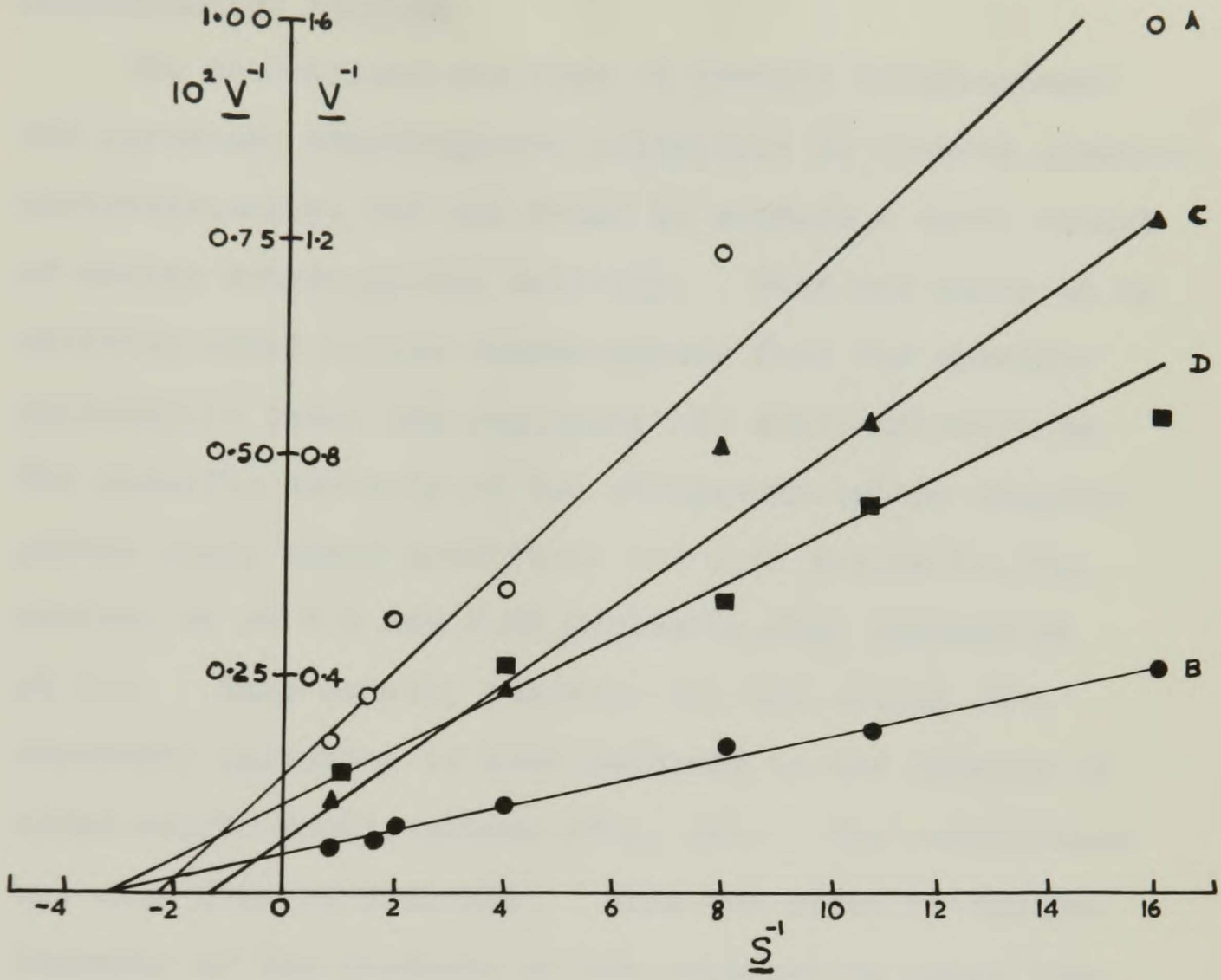
The presence of GDP (1mM) was without effect on the activity of the carboxylase, and the presence of ADP (1mM) caused a slight inhibition.

Stability and pH optimum.

The purified enzyme was stable in 5mM-tris hydrochloride pH 7.5 for at least a month at 2°, but was completely inactivated after storage at -15° for 14 days. The activity was completely destroyed in 2 min. at 50°. The engyme showed a sharp maximum in activity at pH 8.5 (fig. 16) with a 10-fold difference in activity between pH 7.5 and pH 8.5.

Fig. 15

Effect of PEP concentration on the PEP carboxylase reaction in the presence and absence of inhibitors.



The left-hand ordinate (V = µm-moles CO_2 fixed in 30 min. by 124 µg. of enzyme) refers to the radioactive assay, the right hand ordinate (V = µm-moles NADH oxidised/min. by 31 µg. of enzyme) refers to the spectrophotometric assay. A, radioactive assay at pH 7.5 and 30°. B, spectrophotometric assay at pH 8.5 and 22°. C, spectrophotometric assay pH 8.5, 22° in the presence of 40 mM-phosphate (pH 8.5). D, spectrophotometric assay, pH 8.5, 22°, in the presence of 5 mM-ammonium chloride. S = concentration of PEP (mM).

Contaminating enzymes.

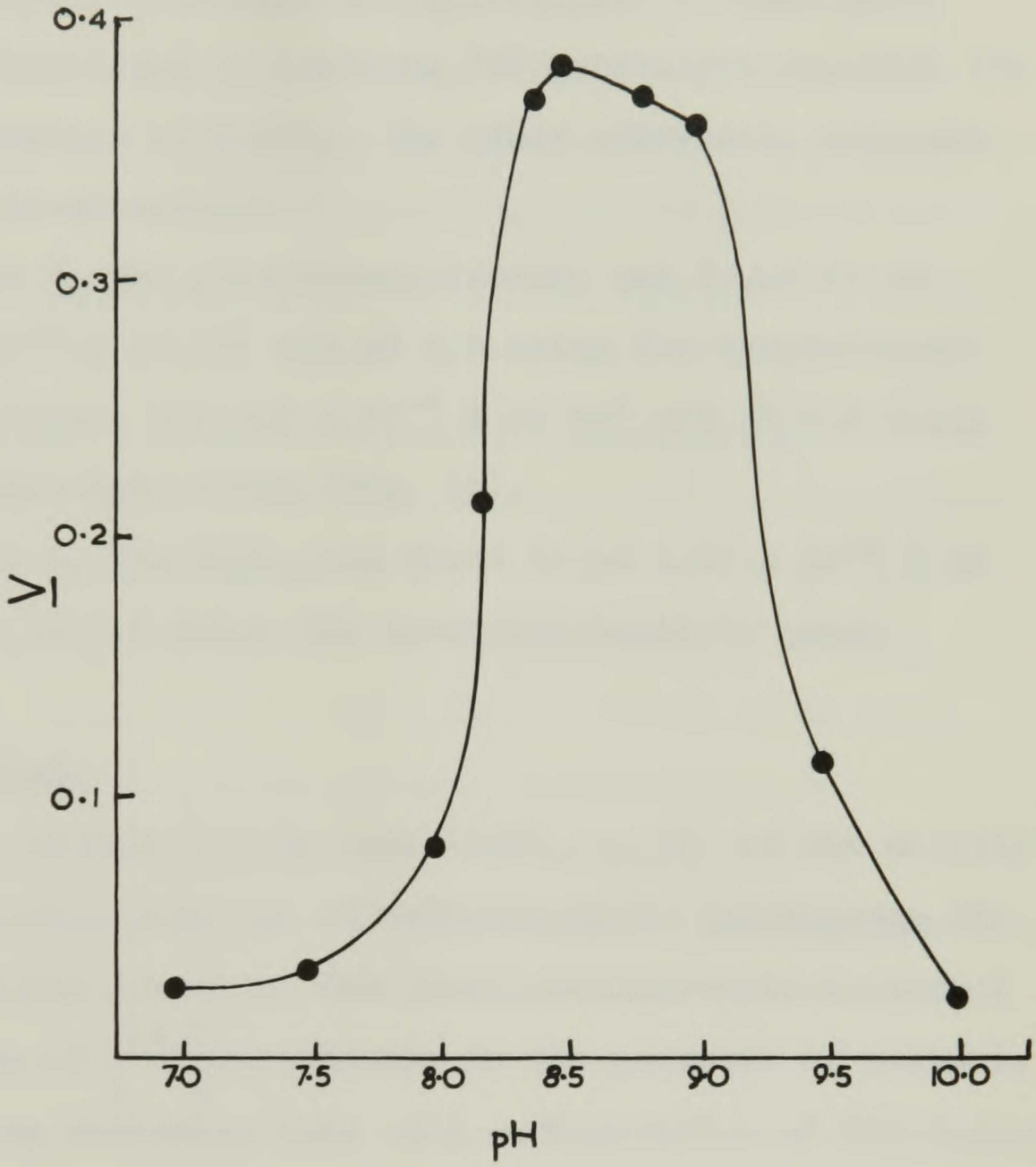
The carboxylase was free of lactate dehydrogenase and glycerate dehydrogenase activities as assayed spectrophotometrically, but was found to contain a small amount of malate dehydrogenase activity. This was measured by omitting added malate dehydrogenase from the spectrophotometric assay and replacing PEP with oxaloacetate. The specific activity of the endogenous malate dehydrogenase under these conditions was 1.93 µmoles/hr./mg. protein at pH 7.5 and 2.86 µmoles/hr./mg. protein at pH 8.5. This exactly accounts for the slight PEP-dependent oxidation of NADH observed in the absence of added malate dehydrogenase (fig. 13). The carboxylase was also free of fumarase. This was shown by chromatography of the products of the radioactive assay two-dimensionally on paper. In stages 1 - 3 of the purification, there were two radioactive products, malate 79% and fumarate 21%, but the product of $[^{14}C]$bicarbonate fixation at stage 4 was entirely malate.

Effect of reactant concentration on reaction rate.

The K_m value for PEP was determined by plotting the reciprocal of reaction velocity against reciprocal

Fig. 16

pH Optimum of PEP carboxylase



Standard spectrophotometric assay. Tris hydrochloride buffer throughout.

V = umoles NADH oxidised /min./mg. protein.

of substrate concentration (Lineweaver & Burk, 1934) under conditions of limiting PEP concentration, with the concentration of $NaHCO_3$, the other substrate, constant and in large excess.

The K_m for phosphoenolpyruvate was found to be 3.3×10^{-4} M at 22° and pH 8.5 using the spectrophotometric assay, and 4.4×10^{-4} M at 30° and pH 7.5 using the radioactive assay (fig. 15).

The K_m for $MgCl_2$ was found to be 1.96×10^{-4} M at 22° and pH 8.5 using the spectrophotometric assay.

Discussion.

An examination by Peel (1961, p. 61) of the ability of cell-free extracts of methanol-grown Pseudomonas AM1 to catalyse pyruvate- and phosphoenolpyruvate-dependent fixation of $[^{14}C]$bicarbonate in the presence of suitable cofactors indicated that only carboxylation of PEP occurred to an appreciable extent. Fractionation of the enzyme system responsible shows it to be a phosphoenolpyruvate carboxylase as shown in (iii). The absence of malic enzyme, indicated by the tracer assay (Peel 1961) was also indicated by spectrophotometric assay involving nicotinamide nucleotide reduction (Ochoa et al., 1948)

(see chapter V). The absence of PEP carboxykinase, indicated by the tracer assay (Peel, 1961) was also indicated by an assay of the crude extract involving incorporation of $[^{14}C]$bicarbonate into a pool of oxaloacetate in an exchange reaction, as described by Utter & Kurahashi (1955). Hence, as has been observed in the autotrophic bacterium Thiobacillus thiooxidans (Suzuki & Werkman, 1957, 1958), phosphoenolpyruvate carboxylase appears to play the major role in formation of C_4 dicarboxylic acids, such as oxaloacetate or malate, from C_3 substrates. The presence of the reversible PEP carboxytransphosphorylase, demonstrated in Propionibacterium shermanii by Siu et al. (1961) is rendered unlikely by the observation that $^{14}CO_2$ fixation is inhibited by phosphate both in crude extracts (Peel, 1961) and also with the purified enzyme.

Phosphoenolpyruvate carboxylase has been studied extensively in plants. Bandurski (1955) purified the enzyme 10-fold from spinach leaf acetone powder. Tchen & Vennesland (1955) and Walker (1957) studies the engyme in relatively crude preparations from wheat germ and succulent plants respectively. Other studies in plants have demonstrated similar systems: in succulent plants

(Saltman, Kunitake, Spolter & Stitts, 1956), barley roots (Young & Graham, 1958), spinach chloroplasts (Rosenberg, Capindale & Whatley, 1958), and other sources. The role of carboxylation of C_3 compounds in plant metabolism has recently been reviewed in detail by Walker (1962).

In bacteria, the only detailed report of the occurrence of the enzyme is in the autotroph Thiobacillus thiooxidans (Suzuki & Werkman, 1957), although the enzyme may well play a role in the incorporation of carbon dioxide into aspartate and oxaloacetate by Staphylococcus aureus (Hancock & McManus, 1960) and the minute streptococci (Martin & Niven, 1960). A report that the enzyme occurs in Propionibacterium shermanii (Pomerantz, 1958) may need reinvestigation in view of the subsequent demonstration of PEP carboxytransphosphorylase (Siu et al., 1961) in the same organism.

The present work reveals many similarities in the properties of the enzyme purified from P. AM1 with those of the plant enzyme, e.g. K_m values for PEP : 3.3×10^{-4} M for the Pseudomonas enzyme, 5×10^{-4} M for the spinach enzyme (Bandurski, 1955), 1.9×10^{-4} M for the preparation from succulent plants (Walker, 1957). As

with the enzyme from spinach, there is a requirement for divalent cations, of which Mg^{2+} is the most active. The other enzyme preparations from plants and T. thiooxidans were equally activated by either Mg^{2+} or Mn^{2+}. The enzyme from Pseudomonas AM1 differs from the enzymes obtained from wheat germ and succulent plants in being inhibited, instead of stimulated, by low concentrations of phosphate. The sharp pH optimum of 8.5 for the reaction observed in the present work may be compared with the broad optimum over pH 7 - 9 (Bandurski, 1955) or that at pH 7 (Walker, 1957). A detailed comparison of the enzymes from 4 sources with the Pseudomonas AM1 enzyme is given in table X.5.

Siu (1962) has recently raised the possibility that the PEP carboxylase activity of plants may be the result of the coupled reaction of PEP carboxytransphosphorylase (iv) with inorganic pyrophosphatase (viii):

$$PP_i + H_2O \longrightarrow 2\ P_i \qquad \text{(viii)}$$

Such a hypothesis would explain the irreversibility of the PEP carboxylase reaction and the stimulation of OAA formation by orthophosphate with the wheat germ enzyme and the succulent plant preparation (table X.5). Siu

Table X.5

COMPARATIVE PROPERTIES OF PHOSPHOENOLPYRUVATE CARBOXYLASE FROM DIFFERENT SOURCES.

	1	2	3	4	5
Reference	Bandurski & Greiner (1953) Bandurski (1955)	Tchen & Vennesland (1955) Tchen, Loewus & Vennesland (1955)	Walker (1957) Walker & Brown (1957)	Suzuki & Werkman (1957, 1958)	Large, Peel & Quayle Biochem. J. in press
Source of enzyme	Spinach leaf	Wheat germ	Kalanchoe crenata Sedum spectabile (succulent plants)	Thiobacillus thiooxidans	Pseudomonas AM 1
Preparation	Acetone powder. Extracted with water. Protamine sulphate added. A.S. ppt. dialysed (Stage I) Further A.S. precipitation dialysed gave Stage II. 10-fold purification. 20% yield	Wheat germ extracted with water, centrifuged and dialysed. Some preparations treated with $MnCl_2$.	Macerated leaf blended in buffer. homogenate strained through muslin and centifuged. Protein precipitated by A.S. to 60% saturation, redissolved and dialysed.	Cells treated with ion exchange resin. Then sonicated. After centrifugation, supernatant dialysed.	Cells crushed in Hughes press Treated with protamine sulphate. Fraction pptd. by A.S. between 40-50% saturation dialysed and chromotographed on DEAE-cellulose column.
Assay Method	Estimation of oxaloacetate manometrically and colorimetrically. Spectrophotometric, coupling with NADH and malate dehydragenase.	Spectrophotometric, using NADH and the malate dehydrogenase present in the preparation.	Spectrophotometric using NADH and the malate dehydrogenase present in the preparation	Fixation of CO_2 into oxaloacetate 2,4-DNP-derivative	Fixation of CO_2 into non-volatile product in the presence of malate dehydrogenase and NADH Spectrophotometric coupling with NADH and malate dehydrogenase.
Cofactor requirements	Absolute ion dependence. Only Mg^{2+} effective: Mn^{2+} would not replace. Stage II had absolute requt. for -SH compounds. ADP inhibits.	Mg^{2+} stimulates, but an absolute ion dependence was not established. EDTA inhibits completely in the absence of added Mg^{2+}. This inhibition reversed by excess Mg^{2+}. Mn^{2+} equally effective. No stimulation by -SH compounds. Not inhibited by iodoacetate. ATP, ADP, AMP were without effect. Phosphate stimulated, but this effect was variable. Stimulation at 0.03 mM and at 2.7 mM rate was doubled.	Absolute ion dependence. Mg^{2+} or Mn^{2+} equally effective. Co^{2+} and Cd^{2+} had some effect. Phosphate stimulated up to 10mM but inhibited above this concentration. No -SH requirement.	Omission of Mg^{2+} causes 84% decrease in activity. Mn^{2+} equally effective. Nucleotides had slight inhibition. -SH compounds did not stimulate.	Absolute ion dependence. Mn^{2+} only 34% as effective as Mg^{2+}. Phosphate inhibits above 10 mM and does not stimulate below this concentration. Nucleotides and -SH compounds do not stimulate.
K m values	PEP 5×10^{-4} M	Not recorded	PEP 1.9×10^{-4} M (Spectrophotometric) 1.5×10^{-4} M (by phosphate formation) HCO_3 2.2×10^{-4} M	HCO_3 1.2×10^{-3} M	PEP 3.3×10^{-4} M $MgCl_2$ 1.9×10^{-4} M
pH Optimum	Broad over pH7-9 Falls off below 7 rapidly.	Not recorded	pH 7.0	Not recorded	pH 8.5
Contaminating activities	Enolase	PG-mutase, enolase, malate dehydrogenase, alcohol dehydrogenase.	Malate dehydrogenase	Presumably many enzymes	Small amount of malate dehydrogenase.

showed that the PEP carboxylase of spinach is also stimulated by orthophosphate. Using the spinach enzyme, which contained no inorganic pyrophosphatase activity, $^{32}P_i$ was not incorporated into PP_i and non-radioactive PP_i was not formed in the reaction. Thus it is clear that the PEP carboxylase of plants differs from the PEP carboxytransphosphorylase of propionibacteria. The role of P_i in the stimulation of the plant enzyme remains unknown. The present work supports the evidence that PEP carboxylase cannot be the same enzyme as the carboxytransphosphorylase, since in this case there is no stimulation of the reaction by orthophosphate.

Two problems are raised by the discovery that the CO_2-fixing enzyme is PEP carboxylase. Both stem from the fact that this carboxylation reaction is irreversible (ΔG^o = -7.2 to - 7.9 kcal. at 25° and unit activities; Tchen, Loewus & Vennesland, 1955). The labelling patterns reported in chapter VII showed that the pattern in 3-phosphoglyceric acid was the same as that in C-1 to C-3 of malic acid, indicating that the two acids are reversibly interconvertible. This would imply, since the PEP carboxylase is irreversible, that in addition there is some reversible C_3 carboxylation enzyme in P. AM1.

But the cell-free studies indicate that PEP carboxylase is the only enzyme present. The same problem arises in connection with growth on succinate, where the formation of C_3 compounds demands a decarboxylation of C_4 dicarboxylic acids. The possibility thus seems to remain that some undetected carboxylation reaction may exist in P. AM1.

SECTION IV

PRELIMINARY EXPERIMENTS ON OTHER C_1 ORGANISMS

Chapter XI

GROWTH OF OTHER BACTERIAL SPECIES ON C_1 SUBSTRATES

At the commencement of work in this laboratory on organisms which can grow on C_1 compounds as sole source of carbon and energy, a survey of the literature revealed that only a very few species had been recorded as being able to grow on C_1 substrates (table I. 1, chapter I).

More recently, however, reports have appeared which indicated that in the genus Pseudomonas, the ability to grow on formate as sole carbon source may be more widespread. Thus while Seleen & Stark (1943) found that none of the fluorescent Pseudomonads tested by them was capable of growth on 0.3% formate in a mineral medium containing phosphate, NH_4^+ and Mg^{2+}; Rhodes (1959) reported that out of 169 different isolated strains of Pseudomonas tested on 0.1% w/v formate in a mineral medium containing NaCl, $MgSO_4$, NH_4^+ and phosphate (Koser, 1923), only 6 strains failed to grow. Lysenko (1961) examined all available strains of Pseudomonas for ability to grow on a range of substrates including 0.1% formate in the medium of Koser (1923) and 0.3% formate in the medium of Seleen & Stark (1943) and

found that of 30 species tested, only 7 failed to grow on formate in either medium.

The discrepancy between the above observations could be explained if the 0.3% formate concentration used by Seleen & Stark were inhibiting growth. Other possible explanations might lie in the different mineral salt media used and in the different criteria of growth.

In order to investigate these conflicting observations, a study was made of the growth of 6 species of Pseudomonas from the National collection of Industrial Bacteria on methanol, methylamine and sodium formate (0.1% w/v or v/v) in the mineral salt medium of Jayasuriya (1955) (chapter II). In addition, some species were tested in the medium of Seleen & Stark (1943).

The strains tested were Pseudomonas aeruginosa, NCIB 8626 (ATCC 9027); P. fluorescens 112, NCIB 8027 (ATCC 9721); P. chlororaphis, NCIB 8730; P. synxantha, NCIB 8179 (ATCC 9890); P. fragi, NCIB 8542 (ATCC 4973); and P. aureofaciens, NCIB 9265 (NRRL B 1453 P). All these organisms are reported as either positive or variable in their growth on formate by Lysenko (1961). The cultures were obtained from the National Collection

in a lyophilised form. The cultures were recovered from the desiccates in the recommended manner (National Collection Catalogue, 1954) and inoculated into 10 ml. of liquid medium containing mineral salts in the concentrations used by Jayasuriya (1955) (chapter II) and 50 mM-sodium succinate. Most strains grew thickly overnight on succinate. This culture was then used as an inoculum for the C_1 substrate cultures. A loopful of the liquid culture was transferred to each of four 50 ml. growth flasks (a 50 ml. conical flask with a thin-walled test-tube sealed into the side near the base to act as a side arm for nephelometric measurements). These flasks contained respectively 10 ml. of Jayasuriya salt medium with in addition 0.1% v/v methanol or 0.1 w/v methylamine hydrochloride, or 0.1% w/v sodium formate, or no carbon source. After receiving equal inocula, these 4 flasks were incubated in stationary culture at 30° for two weeks. Growth was estimated by comparing the turbidities in the flasks with substrate with the turbidity of the flask containing no substrate. In no case, with either Jayasuriya salt medium or Seleen-Stark salt medium, was growth detected with any of the strains on any C_1 substrate.

With regard to formate, these observations agree with those of Seleen & Stark rather than Rhodes & Lysenko. The other substrates have not been tested before.

In contrast to these observations, some tests were made with organisms which have been shown to grow on C_1 compounds. When cultures of Protaminobacter alboflavus NCIB 8167 and Pr. ruber NCIB 2879 were tested under similar conditions, the latter grew readily on all three substrates (methanol and formate had not been tested before; den Dooren de Jong, 1926, 1927) while the former grew only slightly on formate and not at all on methanol, though it grew on methylamine. When Pseudomonas extorquens (Bacillus e., Vibrio e., Flavobacterium e., see chapters I and XII, a gift from Dr. L. Janota-Bassalik) was also tested under these conditions, it grew on all three substrates including methylamine, which had not previously been tested (Bassalik, 1913).

Discussion.

The conflicting results observed with regard to growth on formate may be due to the different criteria used to estimate growth. Thus in particular, Lysenko

used a pH indicator as the principal criterion of growth.

It is clear however, that despite the above reports of growth of Pseudomonas species on formate, growth on formate or on any other of the C_1 substrates tested, on the scale observed with, e.g. P. AM1 or P. oxalaticus, is not a widespread property of the genus Pseudomonas, or of any other group of micro-organisms.

Chapter XII

DISTRIBUTION OF THE KEY ENZYMES OF ONE - CARBON AND OXALATE METABOLISM IN BACTERIA

Two pathways are at present known by which micro-organisms are enabled to synthesise their cellular constituents from C_1 compounds. One of these is the autotrophic pathway involving the ribulose diphosphate cycle, demonstrated by Quayle & Keech (1959 a, b) in Pseudomonas oxalaticus growing on formate. The other is the present pathway, which has so far been shown to operate in P. AM1 growing on methanol and formate and in Hyphomicrobium vulgare growing on methanol (Large, Peel & Quayle, 1961). The autotrophic pathway is characterised by the presence of the enzyme carboxy-dismutase:

$$\text{Ru DP} + CO_2 + H_2O \longrightarrow 2 \text{ 3-Phosphoglycerate} \quad (i)$$

This enzyme (Quayle, Fuller, Benson & Calvin, 1954; Weissbach, Smyrniotis & Horecker, 1954) is the key enzyme of the photosynthetic carbon fixation cycle (Bassham et al., 1954; Bassham & Calvin, 1960). The evidence that this cycle is the major, if not the only, pathway for the net synthesis of polycarbon compounds

from carbon dioxide in green plants is very strong (Bassham & Kirk, 1960). The enzyme occurs universally in green plant tissue, and has been shown to be involved in photosynthesis by the photosynthetic bacteria. There is also some evidence for its presence in the chemosynthetic autotrophs Hydrogenomonas spp., Micrococcus denitrificans, Thiobacillus spp., and Nitrobacter agilis (for details see Quayle, 1961). In addition to its role in the growth of green plants and chemosynthetic autotrophs on CO_2 and in the growth of photosynthetic bacteria on organic substrates anaerobically in the light, it has also been found, as noted above, in formate-grown P. oxalaticus, but not in P. oxalaticus grown on any other substrate.

The occurrence of carboxydismutase in P. AM1.

In view of the fact that 3-phosphoglycerate was not a major early product of formate - or bicarbonate-assimilation by whole cells of formate-grown P. AM1, and since the enzyme was absent from methanol-grown P. AM1 (Peel, 1961, p. 51) and H. vulgare (Large et al.,1961) it appeared unlikely that carboxydismutase would be present in P. AM1 grown on formate. When extracts of formate-grown cells were incubated at 30° with sodium $[^{14}C]$-

bicarbonate (5 µmoles, 10 µc), 2.5 µmoles of $MgCl_2$, 2 µmoles of glutathione, 5 µmoles of tris hydrochloride pH 7.8 and water to 0.4 ml. (Quayle & Keech, 1959b) for 15 min., there was no significant stimulation of fixation of ^{14}C into non-volatile material in the presence of Ru DP (0.15 µmole) (see table XII.1.). It is thus unlikely that the reductive pentose phosphate cycle (Ru DP) cycle is a pathway of carbon assimilation in formate-grown P. AM1, despite its proved role in growth on the same substrate by P. oxalaticus.

Distribution of the enzymes of one-carbon metabolism.

The enzymic details of the non-autotrophic pathway of growth on C_1 compounds have been only partially elucidated (chapters IV and V), but of those which have been investigated, the enzymes which catalyse the inter-conversions of C_1-derivatives of tetrahydrofolic acid, and the enzymes involved in the formation and transformation of serine are probably characteristic of this pathway.

In order to investigate whether either of the above pathways is operative in organisms which can grow on C_1 compounds, a detailed examination was made of the occurrence of two enzymes, each taken as characteristic of its own

particular pathway: carboxydismutase (for the autotrophic pathway) and methylene-tetrahydrofolate dehydrogenase (for the non-autotrophic pathway). The latter enzyme catalyses the reaction

$$\underline{N}^{5,10}\text{methylene}H_4\text{PtG} + NADP^+ \rightleftharpoons \underline{N}^{5,10}\text{methenyl}H_4\text{PtG} + NADPH \qquad \text{(ii)}$$

In addition, an investigation into the distribution of the key enzyme for the growth of bacteria on oxalate, glyoxylate dehydrogenase (Quayle, Keech & Taylor, 1961; Quayle & Taylor, 1961; Quayle, 1962) was made. This enzyme (see also chapter V) catalyses the reaction:

$$CHO.COOH + NADP^+ + CoASH \rightleftharpoons COSCoA.COOH + NADPH + H^+ \qquad \text{(iii)}$$

Bacterial strains studied.

The following organisms were examined: Pseudomonas oxalaticus OX1, National Collection of Industrial Bacteria Catalogue no. 8642; P. AM1; P. extorquens (syn. Bacillus e., Vibrio e., Flavobacterium e.), a culture of which was a gift from Dr. L. Janota-Bassalik, Institute of Plant Physiology, University of Warsaw, Poland; Protaminobacter ruber, NCIB 2879; Ps. ODI, NCIB 8735; Hyphomicrobium vulgare, a culture of which was a gift from Dr. R. Näveke, Staatsinstitut für Allgemeine Botanik, Hamburg 36, Germany; and P. methanica, a culture of which

was a gift from Dr. J.J. Perry, Department of Bacteriology, University of Texas, Austin, Texas, U.S.A. These were maintained as follows: P. oxalaticus on 5mM-oxalate slopes as described by Quayle & Keech (1959a); P. extorquens, Pr. ruber and H. vulgare on 0.1 M-methylamine hydrochloride slopes as described for P. AM1 in chapter II; and P. methanica on mineral-agar plates under methane-air (1 : 1 by vol.) as described by Leadbetter & Foster (1958).

Assay methods used.

Carboxydismutase was assayed as described above, methylene H_4PtG dehydrogenase as described in chapter IV, and glyoxylate dehydrogenase as described in chapter V.

Growth conditions.

The mineral medium of Jayasuriya (1955) was used for all liquid cultures. In addition the concentrations of carbon sources used were: formate 100mM, oxalate 50mM, methanol 0.5% v/v, methylamine 0.5% w/v, succinate 50mM. Inocula from slopes were grown for 24 hr. in 10 ml. medium at 30°, and these were then used to inoculate 400 ml. of medium in a Carrel culture flask (chapter II). The 400 ml. was shaken at 30° for 48 - 60 hr. and harvested

by centrifugation. H. vulgare was grown in stationary culture in Fernbach flasks for two weeks as described by Large et al. (1961). Cell-free extracts were prepared either by ultra-sonic disruption or by crushing in the Hughes press as described in chapter II. The extracts were centrifuged at 25000 x g. for 10 min., and the clear supernatant used for assay.

Enzyme specific activities.

The results of these enzyme assays are set out in table XII. 1. Carboxydismutase was absent from all the organisms tested except for formate-grown P. oxalaticus. This indicates that the autotrophic pathway of growth on C_1 compounds is not common. Despite a wide variation in specific activity (1.6 to 24.7) the enzyme methylene-H_4PtG dehydrogenase is found in all the organisms tested. However, it is much lower in formate-grown P. oxalaticus than in any of the other organisms tested. All the pink group and H. vulgare had specific activities for this enzyme at least 6-fold higher. This is a strong indication that all the pink group of C_1 organisms may assimilate carbon by the same pathway as P. AM1. Should this indeed prove to be the case, then the suggestion of Peel (1961, p.22) that these organisms, though at present

TABLE XII. 1.

SPECIFIC ACTIVITIES OF CARBOXYDISMUTASE, METHYLENETETRAHYDROFOLATE DEHYDROGENASE AND GLYOXYLATE DEHYDROGENASE IN VARIOUS SPECIES OF BACTERIA.

For maintenance and growth of the cultures, and preparation of extracts, see text. Carboxydismutase was assayed by measuring the RuDP-dependent fixation of $[^{14}C]$-bicarbonate as described in the text, methylene H_4PtG dehydrogenase by measuring the H_4PtG-dependent reduction of NADP in the presence of formaldehyde as described in chapter IV, and glyoxylate dehydrogenase by measuring the CoA-dependent reduction of NADP in the presence of glyoxylate as described in chapter V.

Species	Growth substrate	Enzyme specific activity: Carboxydismutase ++=present -=absent	Methylene H_4PtG dehydrogenase μmoles/hr./mg. protein	Glyoxylate dehydrogenase μmoles/hr./mg. protein
Pseudomonas oxalaticus	Formate	++ *	1.6	0
	Oxalate	-		17.4
Pseudomonas AM1	Methanol	-	24.2	0.52
	Formate	-	22.0	0.49
	Succinate	-	13.0	0.64
Pseudomonas extorquens	Methanol	-	5.9	2.93
	Formate	-	16.4	0.425
	Oxalate	-		9.04
Protaminobacter ruber	Methylamine	-	18.3	0.735
	Methanol	-	11.9	0.332
	Formate	-	24.7	0.592
Pseudomonas OD1	Oxalate	-	0	54.6
	Malate			9.6

TABLE XII. 1.(continued)

Hyphomicrobium vulgare	Methanol	-	6.95	0
Pseudomonas methanica	Methanol	-	7.55	0.112

* ++ corresponds to 1.8 μmoles CO_2 fixed/hr./mg. protein (J.R. Quayle, personal communication).

in different genera are all strains of a parent species of pink pseudomonad, will be greatly strengthened. With regard to H. vulgare, the whole cell evidence (Large et al., 1961) already points to the operation of the same pathway as P. AM1, so that the occurrence of methylene-H_4PtG dehydrogenase in this organism also may be regarded as further evidence relating this enzyme to the non-autotrophic pathway.

The enzyme glyoxylate dehydrogenase is present in all organisms tested except formate-grown P. oxalaticus and methanol-grown H. vulgare. However, in most of the organisms of the pink group, it is very low in specific activity when the cells are grown on C_1 substrates. With oxalate-grown organisms on the other hand, the enzyme is very much higher in specific activity, as would be expected from its role in growth on oxalate.

The observations of this chapter provide confirmatory evidence that at least in H. vulgare and the pink organisms tested, the pathway of growth on C_1 compounds is similar to that in P. AM1. It would be interesting to test the remaining colourless strains of C_1 organisms shown in table I.1. for these enzymes, but P. aminovorans is by now probably lost, and Vibrio oxaliticus is also unobtainable.

SECTION V

GENERAL DISCUSSION

Chapter XIII

GENERAL DISCUSSION

The aim of the work described above was to elucidate the metabolic pathways by which micro-organisms are able to grow on C_1 compounds as sole carbon and energy source. It has been found that during growth of Pseudomonas AM1 on formate, the first stable detectable product of formate assimilation is serine, and the initial product of carbon dioxide assimilation is malate. These observations resembled those of colleagues, who made similar findings on the growth of P. AM1 and Hyphomicrobium vulgare on methanol (Large, Peel & Quayle, 1961). The scheme for the pathway of methanol assimilation proposed by Peel (1961, p. 36) has also been adopted to explain growth on formate. In this pathway the C_1 unit gives rise to serine by the action of serine transhydroxymethylase, serine is converted to some other C_3 derivative which is then converted to malate by carbon dioxide fixation. The synthesis of glycine, the essential acceptor for C_1 unit fixation might occur by the direct condensation of carbon dioxide and a C_1 unit, or indirectly

by a cyclic process.

The presence of all the enzymes necessary for the conversion of glycine and formate to 3-phosphoglycerate (regarded as the key precursor of cellular material) has been demonstrated in extracts of formate-grown cells. The specific activities of these enzymes are adequate to account for the observed growth rate. These specific activities are lower in cells grown on succinate, thus lending support to the view that they play a particular role in growth on C_1 compounds. An enzyme catalysing the formation of C_4 acids from phosphoenolpyruvate and CO_2, detected in crude extracts by Peel (1961, p. 63), has been purified and found to be a phosphoenolpyruvate carboxylase, catalysing the irreversible conversion of PEP to oxaloacetate. Studies in cell-free extracts shed very little light on the pathway by which glycine could be formed, but studies on the metabolism of succinate in cells growing on methanol provided some evidence that this compound could act as a precursor of glycine.

The patterns of radioactive labelling in molecules of glycine, serine and 3-phosphoglycerate isolated and degraded after short term incubations with $[^{14}C]$methanol and $[^{14}C]$succinate confirm the above hypothesis by showing

that: (a) glycine and serine are readily interconvertible; (b) serine may be a precursor of malate but that serine cannot be formed from malate; (c) glycine might be produced by cleavage of malate and (d) succinate could act as a precursor of glycine.

The distribution of the hetero-trophic pathway of carbon assimilation from C_1 units demonstrated in P. AM1 has been found to be widespread. Out of 6 C_1 organisms tested, a key enzyme of this pathway was found in high specific activity in all species except P. oxalaticus.

A number of significant problems remain yet to be solved. The most important is the question of how glycine, the acceptor molecule for C_1 unit fixation, is synthesised. It appears probable that it arises by some indirect cyclic mechanism, perhaps involving C_4 dicarboxylic acids. In view of the negative results obtained on the transformation of radioactive C_4 derivatives in extracts, the most profitable approach to this problem would seem to be isolation of a glycine- or serine- requiring auxotroph of P. AM1 by classical irradiation techniques. Such a mutant might accumulate precursors of glycine in the culture medium. The other major problem is the mechanism of methanol oxidation, which is important

because it is the source of the energy required by the cell for growth. These two problems are at present under study by colleagues. Progress on this latter problem will be more rapid now that cell-free preparations that can oxidise methanol and formaldehyde have been obtained (Johnson, 1962). The solution of these two well defined problems should present the key to one of the outstanding unsolved questions in microbial intermediary metabolism, growth on C_1 compounds.

SECTION VI

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APPENDICES

Appendix I.

NOMENCLATURE AND ABBREVIATIONS.

The following abbreviations are used in this thesis:

AMP, ADP, ATP	Adenosine - 5' - mono -, di- and tri-phosphates.
A.S.	Ammonium sulphate.
CoA, CoASH	Coenzyme A.
DCPIP	2, 6-Dichlorophenolindophenol
2, 4-DNP-derivative	2, 4-Dinitrophenylhydrazone.
EDTA	Ethylenediaminetetraacetic acid.
GDP	Guanosine - 5' - diphosphate.
GM tube	Geiger-Müller tube.
H_2PtG	Dihydrofolic acid.
H_4PtG	Tetrahydrofolic acid.
H_4PtG_3	Tetrahydropteroyl - γ -glutamyl - γ - glutamyl - γ - glutamate.
IDP	Inosine - 5' - diphosphate.
NAD, NADH	Oxidised and reduced forms of nicotinamide - adenine dinucleotide (formerly DPN).
NADP, NADPH	Oxidised and reduced forms of nicotinamide - adenine dinucleotide phosphate (formerly TPN).
P.	Pseudomonas.
P_i	Inorganic orthophosphate.
PEP	Phosphoenolpyruvate.
PGA	3 - Phosphoglyceric acid.
PP_i	Inorganic pyrophosphate.
RuDP	Ribulose - 1, 5 - diphosphate.
TPP	Thiamine pyrophosphate.
TSA	Tartronic semialdehyde.

Appendix II

Commercially available special chemicals were obtained from the following manufacturers:

C.F. Boehringer und Söhne, Mannheim, Germany:
NAD, NADH, NADP, NADPH, PEP (silver-barium salt), coenzyme A (70% pure), oxaloacetic acid (80% pure), PGA (barium salt), crystalline lactate dehydrogenase, pyruvate kinase, phosphoglycerate kinase, triose phosphate dehydrogenase, purified malate dehydrogenase.

Sigma Chemical Co., St. Louis, Mo., U.S.A.:
ATP, ADP, IDP, GDP, sodium glyoxylate, glucose oxidase, TPP.

Schwarz Laboratories Inc., New York, N.Y., U.S.A.:
Polidase-S

Nutritional Biochemicals Corp. Cleveland, Ohio, U.S.A.:
Tetrahydrofolic acid, phenazine methosulphate.

L. Light & Co. Ltd., Colnbrook, Bucks.:
Calcium DL-glycerate, crystalline ribonuclease and deoxyribonuclease.

Roche Laboratories Ltd., Welwyn Garden City, Herts.:

Pyridoxal phosphate.

California Corporation for Biochemical Research,

Los Angeles, Ca., U.S.A.:

Aminopterin.

Appendix III

Generous gifts of special chemicals not commercially available were:

Potassium D-glycerate, Tartronic semialdehyde	Dr. A.M. Gotto.
Lithium hydroxypyruate	Mr. D.H. Williamson.
Ribulose-1, 5-diphosphate	Dr. J.R. Quayle.
DL-β-Hydroxyaspartic acid	Prof. Sir Hans Krebs, F.R.S.
DL-Tetrahydrofolic acid, DL-Tetrahydroteropterin	Dr. J.R. Guest.
DL-[2 - ^{14}C]Malic acid	Dr. J.A. Bassham.

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Hyphomicrobium vulgare	Dr. R. Näveke, Hamburg.
Pseudomonas extorquens	Dr. L. Janota-Bassalik, Warsaw.
Pseudomonas methanica	Dr. J.J. Perry, Austin, Texas.

Appendix IV

Some of the work in this thesis has already been published.

1. Large, P.J., Peel, D. & Quayle, J.R. (1961). Microbial Growth on C_1 Compounds. 2. Synthesis of Cell Constituents by Methanol- and Formate-grown Pseudomonas AM1, and Methanol-grown Hyphomicrobium vulgare. Biochem. J. 81, 470.
   reports the work in chapter III.

2. Large, P.J., Peel, D. & Quayle, J.R. (1962). Microbial Growth on C_1 Compounds. 3. Distribution of Radioactivity in Metabolites of Methanol-grown Pseudomonas AM1 after incubation with $[^{14}C]$Methanol and $[^{14}C]$Bicarbonate. Biochem. J. 82, 483.
   reports the work in chapter VII.

3. Large, P.J., Peel, D. & Quayle, J.R. (1962). Microbial Growth on C_1 Compounds. 4. Carboxylation of Phosphoenolpyruvate in Methanol-grown Pseudomonas AM1. Biochem. J. 85, 243.
   reports the work in chapter X.

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ABSTRACT

ABSTRACT

Pseudomonas AM1, a pink bacterium which can grow on the one-carbon compounds methanol, formate and methylamine as sole carbon and energy sources has been the principal object of study in the work described. The pathway of carbon incorporation during growth on formate has been studied by chromatographic examination of the non-volatile ethanol-soluble radioactive cell constituents in samples taken over a period of 2 minutes after administration of [^{14}C]formate or [^{14}C]bicarbonate to cells growing on non-radioactive formate. It was found that the primary product of [^{14}C]formate fixation was serine, and that of [^{14}C]bicarbonate fixation was malate. At later times phosphorylated compounds, other amino acids, and trehalose acquired radioactivity. It is concluded that the assimilation of formate in P. AM1 does not follow an autotrophic pathway, in which 3-phosphoglyceric acid would be the principal early product.

A pathway for synthesis of cell constituents operating during growth on formate has been proposed. This involves the conversion of the reduced C_1 compound to methylenetetrahydrofolate, the fixation of this into

serine by serine transhydroxymethylase, the conversion of the serine to phosphoenolpyruvate and carboxylation of the latter to C_4-dicarboxylic acids. The operation of such a biosynthetic route necessitates the synthesis of glycine from C_1 units. It is suggested that this process could come about by a direct condensation of two C_1 compounds, or by a cyclic series of reactions involving the formation of glycine from the C_4 end-product.

In an attempt to obtain further evidence for this hypothesis an investigation into the enzymes present in cell-free extracts of formate-grown P. AM1 has been made. It was found that all the enzymes are present for the conversion of formate and glycine to 3-phosphoglycerate. Extracts supplemented by the appropriate cofactors could bring about the conversion of $[^{14}C]$formate to $[^{14}C]$-glycerate and $[^{14}C]$glycerate to 3-phospho$[^{14}C]$glycerate. The specific activities of all the enzymes involved in these transformations were higher in cells grown on formate than in cells grown on succinate. This observation is taken as evidence that these enzymes do indeed play a special role in the growth of P. AM1 on C_1 compounds. The specific activities of all the enzymes are of the correct order of magnitude to explain the overall observed

rate of growth on formate.

Glycine, serine and phosphoglyceric acid have been isolated from the ethanol-soluble fraction of cells grown for 1 min. on [^{14}C]methanol, and degraded. Similar degradations have also been performed on glycine and serine derived from cells grown for 1 min. on [^{14}C]-methanol but aerated with air containing 1% v/v carbon dioxide. The distribution of radioactivity indicates that the carboxyl groups of glycine and serine are derived from carbon dioxide, that C-3 of serine is derived from a reduced C_1 unit, and that C-2 of glycine and C-2 of serine are also derived from a reduced C_1 unit, but less directly than C-3. Comparison of these patterns with those of malate derived from the same source indicated that serine could not have been formed from malate, but that malate could have been formed from serine.

Cell-free extracts of methanol-grown P. AM1 contain an enzyme which on purification has been shown to be a phosphoenolpyruvate carboxylase, catalysing the irreversible formation of oxaloacetate and orthophosphate from phosphoenolpyruvate and bicarbonate. The properties of the enzyme have been investigated. The enzyme is specific for phosphoenolpyruvate (for which K_m is 3.3×10^{-4} M),

inhibited by phosphate and ammonium ions, not stimulated by ADP or GDP, and activated specifically by Mg^{2+} ions. The pH optimum is 8.5. The enzyme has been assigned a key role in the formation of C_4 compounds from C_3 compounds.

Attempts have been made, both in whole cells and in extracts to determine the origin of the glycine necessary for the above biosynthetic scheme. Studies on the metabolites formed from [2, 3-$^{14}C_2$]succinate after short-term incubation with whole cells of methanol-grown P. AM1 indicated that glycine could possibly be formed from C_4 dicarboxylic acids by a pathway not involving C_3 compounds or phosphates. It was not found possible, however, to demonstrate this in extracts. Experiments on the carboxylation of reduced C_1 units in extracts were also unsuccessful.

Experiments on the oxidation of methanol, formaldehyde and formate by washed suspensions and extracts of formate- and methanol-grown P. AM1 have been performed. Whole cells rapidly oxidised all three substrates. The only enzyme demonstrable in extracts with a specific activity of the correct order to explain the oxidation rate of whole cells was an NAD-linked formate dehydrogenase. Evidence against the participation of

reduced folic acid derivatives in the oxidation of methanol has been obtained. The peroxidative oxidation of methanol by a catalase-hydrogen peroxide complex in extracts of P. AM1 has been demonstrated, but arguments are advanced against this as the mechanism of methanol oxidation by whole cells.

The distribution of an enzyme of the postulated heterotrophic pathway of growth on C_1 compounds, methylenetetrahydrofolate dehydrogenase, and of carboxydismutase, the key enzyme of the autotrophic pathway of growth on formate has been examined in 6 species of organisms which can grow on C_1 substrates. Formate-grown P. oxalaticus was the only organism in which carboxydismutase could be detected. Methylenetetrahydrofolate dehydrogenase was present in high specific activity in all the organisms tested except for formate-grown P. oxalaticus where the activity was low.

The significance of the experimental results is discussed in relation to the problem of microbial growth on C_1 substrates as sole carbon and energy sources.