STUDIES IN THE FORMATION

OF

SINGLE-CARBON COMPOUNDS FROM GLYCINE

John David Pitts, B.Sc.

Thesis presented for the degree of Doctor of Philosophy

University of Glasgow

April, 1963

I should like to express my gratitude to Professor J. N. Davidson for giving me the opportunity to pursue this research in his department. I am grateful to Dr G. W. Crosbie for his help, encouragement and friendship throughout the course of this investigation.

The results quoted in Tables 3.2.1.(iii). and 3.3.10.(i). were obtained in collaboration with Miss J. A. Stewart and Miss S. MacFarlane.

I should like to thank Miss A. Meikle for assistance in one of the isotopic experiments, and Miss R. Fevie and Miss J. Murdoch for help with bulk growth of bacteria. My thanks are also due to Mr R. Callander for preparing Fig. 3.1.3.C.

I acknowledge with pleasure the helpful discussions with Dr E. A. Dawes and Dr D. W. Ribbons.

I should like to thank the Medical Research Council for the award of a Scholarship for one year (1959-1960). The remainder of the work was carried out while holding the post of Assistant Lecturer in the University of Glasgow.

Finally, I should like to thank my wife for proof reading and for making many helpful suggestions.

CONTENTS

INTRODUCTION	page	2
METHODS	page	37
RESULTS	page	70
DISCUSSION	page	156
SUMMARY	page	174
REFERENCES	page	177

o o o O o o o

1. INTRODUCTION

1.1. GENERAL INTRODUCTION

1.2. GLYCINE METABOLISM

- 1.2.1. Serine hydroxymethyltransferase
- 1.2.2. Glycine - glyoxylate interconversions
  - 1.2.2.1. Glycine aminotransferase
  - 1.2.2.2. Glycine oxidase
  - 1.2.2.3. Glycine dehydrogenase
- 1.2.3. Biosynthetic reactions involving glycine
- 1.2.4. Glycine fermentation

1.3. GLYOXYLATE METABOLISM

- 1.3.1. Growth of microorganisms on C_2-compounds
- 1.3.2. The glyoxylate cycle
- 1.3.3. The glyoxylate carboligase reaction
- 1.3.4. The 3-hydroxyaspartate pathway
- 1.3.5. Growth of Escherichia coli on glycollate

1.4. TETRAHYDROPTEROYLGLUTAMATE AND THE C_1-POOL

- 1.4.1. C_1-H_4PtG compounds
- 1.4.2. The C_1-pool

1.5. INCORPORATION OF C_1-UNITS INTO CELLULAR COMPOUNDS

- 1.5.1. The purine ring
- 1.5.2. Thymine
- 1.5.3. Serine
- 1.5.4. Methionine

1.6. PRODUCTION OF C_1-UNITS IN ESCHERICHIA COLI

- 1.6.1. C_1-units for thymine methyl-group biosynthesis
- 1.6.2. Formation of C_1-units from glycine

1.1. GENERAL INTRODUCTION

Escherichia coli is a Gram negative eubacterium classified under the family of facultative anaerobes, the Enterobacteriaceae. Escherichia coli PA15 is an auxotrophic strain requiring either glycine or serine for growth.

Escherichia coli PA15 growing logarithmically in a simple glucose-ammonium salt medium supplemented with [2-^{14}C]glycine, incorporates the isotope into cellular DNA, RNA and protein. Isolation of these material followed by specific degradations, shows the principle sites of incorporation to be: (i) the C-2, C-5 and C-8 positions of the purine rings of the DNA and RNA, (ii) the thymine methyl-carbons of the DNA, and (iii) the C-2 of the protein glycine and the C-2 and C-3 of the protein serine (Crosbie, 1958).

This pattern is consistant with two pathways of incorporation. Firstly, direct utilisation of glycine for the synthesis of the purine C-4 and C-5, and for the serine C-1 and C-2. Secondly, the initial conversion of the glycine C-2 to a C_1-unit followed by incorporation of this unit into the C-2 and C-8 positions of the purines, the thymine methyl carbon and the C-3 of serine.

According to Nakada and Weinhouse (1953) the oxidative metabolism of glycine by rat liver can be represented by the equation:

$$NH_2.CH_2.COOH \longrightarrow CHO.COOH \longrightarrow H.COOH + CO_2$$

the C_1-unit formate being derived from the glycine C-2.

This pathway though, can only be a partial explanation of glycine incorporation into the C_1-positions in Escherichia coli, for cells grown in the presence of [^{14}C]formate extensively incorporate the isotope into the C-2 and C-8 positions of the purines, but not at all into the thymine methyl-carbon (Crosbie, 1958) whereas [2-^{14}C]glycine is incorporated into all these C_1-positions.

Hydroxymethyl-tetrahydropteroylglutamate (hydroxymethyl - H_4PtG), a C_1-unit at the oxidation level of formaldehyde, was suggested as the immediate precursor of the thymine methyl-carbon Friedkin and Kornberg, 1957; Crosbie, 1958). Oxidation of hydroxymethyl-H_4PtG to the oxidation level of formate would provide a suitable precursor for the C-2 and C-8 positions of the purines. This would necessitate a further step in any scheme of the type proposed by Nakada and Weinhouse, viz:

$$NH_2.CH_2.COOH \longrightarrow CHO.COOH \longrightarrow \text{'H.CHO'} + CO_2$$
$$\text{'H.CHO'} \downarrow$$
$$\text{'H.COOH'} \longrightarrow H.COOH$$

Otherwise an alternative pathway must be proposed.

The major purpose of this work was therefore to elucidate the enzymic reactions concerned in the conversion of glycine to C_1-units in Escherichia coli PA15, and to elucidate any possible role of glyoxylate as an intermediate in the conversion.

1.2. GLYCINE METABOLISM

1.2.1. SERINE HYDROXYMETHYLTRANSFERASE

Glycine can be omitted from the culture media of nearly all bacteria and it is readily synthesised by plants and by most animals. Metabolically it is closely related to serine. This was first shown by Roepke, Libby and Small (1944), who isolated an auxotrophic mutant of Escherichia coli which required either glycine or serine for growth.

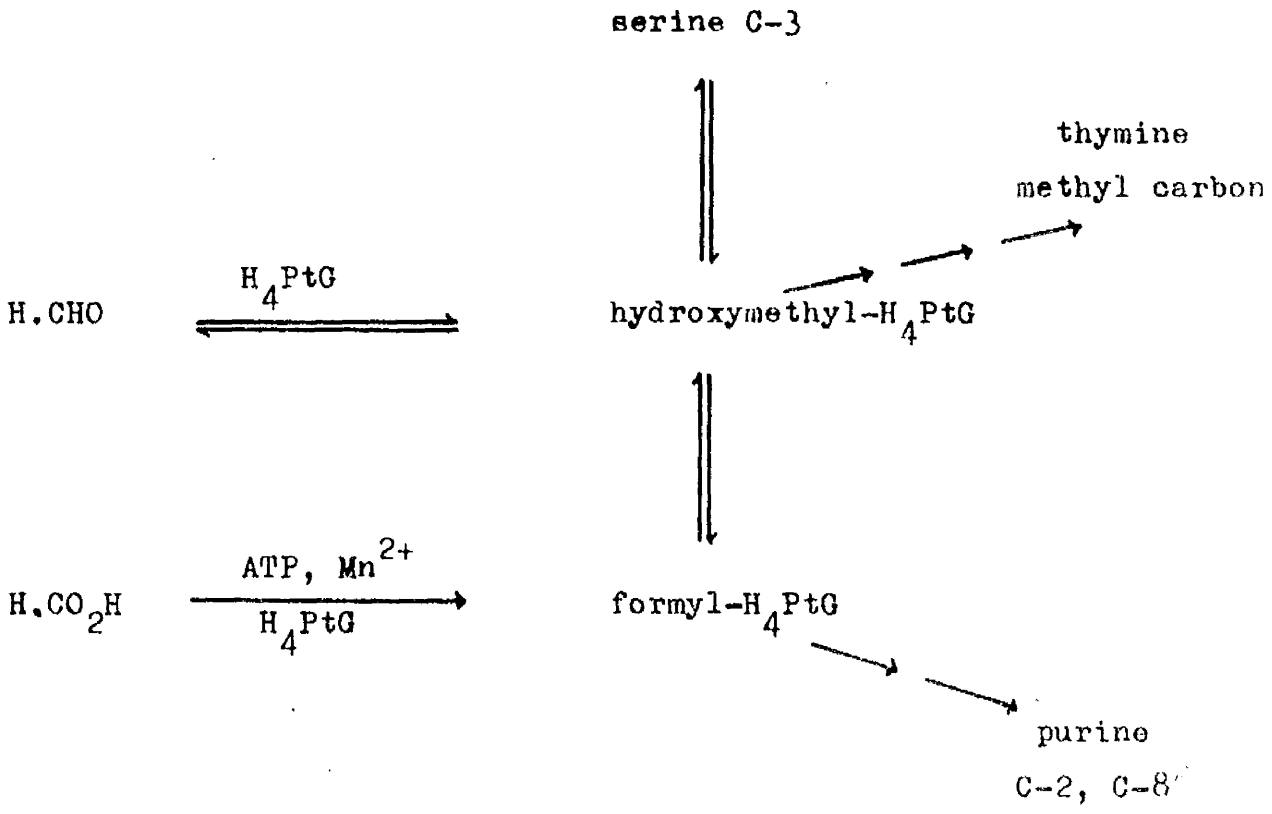
The reaction,

$$\begin{array}{l} CH_2OH \\ | \\ CH.NH_2.COOH \end{array} \rightleftharpoons \begin{array}{l} H.COOH \\ + \\ NH_2.CH_2.COOH \end{array}$$

was suggested (Shemin, 1946) and substantiated by Sakami (1948), who isolated [1-^{13}C, 3-^{14}C]serine from the liver protein of rats fed with [^{14}C]formate and [1-^{13}C]glycine. The *in vitro* incorporation of [^{14}C]formate into serine was shown by Siekevitz and Greenberg (1949), using rat liver homogenates.

The exact nature of the C_1-unit involved was questioned though, for suitable precursors of the serine C-3 were found at several oxidation levels. For example, the methyl-carbon of methionine (Siekevitz and Greenberg, 1950), the methyl-carbons of choline (Sakami, 1949), formaldehyde (Mitoma and Greenberg, 1952) and formate could all be incorporated into serine C-3. Formaldehyde appeared to be the most effective source.

FIG. 1.2.1.A.



Relationships between C_1-units and H_4PtG in serine biosynthesis

(Kisliuk and Sakami, 1955)

Folic acid was implicated in the reaction when it was shown (Elwyn and Sprinson, 1950) that folate deficient animals had a reduced ability to incorporate [^{15}N]serine into the glycine moiety of hippuric acid. The active form of folate was identified as H_4PtG (Blakely, 1954; Kisliuk and Sakami, 1954).

L-Serine and formate doubly labelled with deuterium and radioactive carbon ($^{14}CD_2OH.CHNH_2.COOH$ and D.COOH) were used as precursors of the thymine methyl-group in the pigeon. 1·5 and 0·9 atoms of deuterium accompanied the labelled carbon atoms repectively. This suggested that formate was not an intermediate in the production of C_1-units from serine suitable for thymine methyl-group formation (Elwyn and Sprinson, 1954). Using pigeon liver extracts, Blakely (1954) showed that [2-^{14}C]serine is formed from [2-^{14}C]glycine and unlabelled serine in the presence of H_4PtG. Formaldehyde and glycine are converted to serine by Dowex-1 treated pigeon liver extracts only on the addition of H_4PtG, but ATP, Mn^{2+}, H_4PtG and a source of reduced pyridine nucleotides (e.g. NAD^+, $NADP^+$ and glucose-6-phosphate) were required for the conversion of formate and glycine to serine (Kisliuk and Sakami, 1954).

The relationships between formaldehyde, formate, H_4PtG and the C_1-unit involved in serine biosynthesis were tentatively formulated by Kisliuk and Sakami (1955) and are shown in Fig. 1.2.1.A.

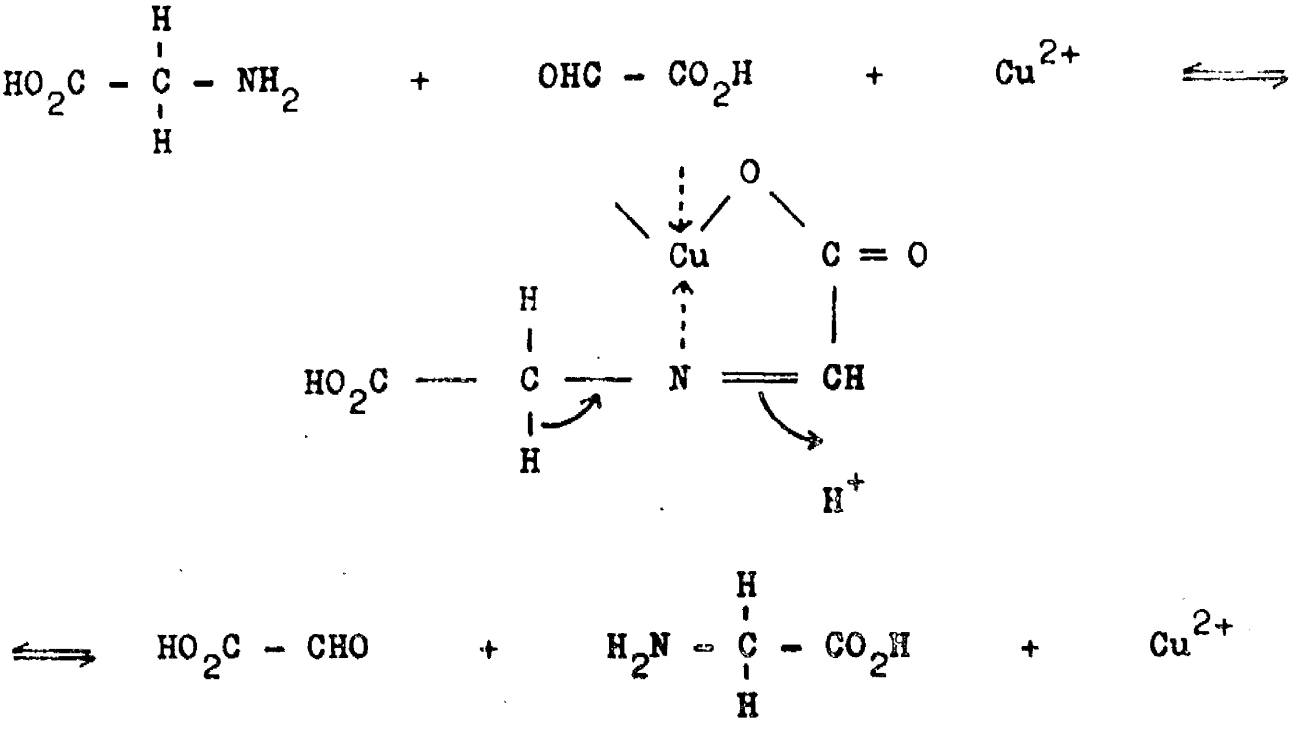
The enzyme serine hydroxymethyltransferase (L-serine: tetrahydrofolate 10-hydroxymethyltransferase, E.C. 2.1.2.1.) has been purified and characterised (Huennekens, Hatefi and Kay, 1957; Blakely, 1960). The C_1-donor is N-5,N-10-methylene-H_4PtG (see section 1.4.1.). Pyridoxal phosphate (PlP), which is firmly bound to the enzyme, and Mn^{2+} are necessary cofactors. The nature of the role of PlP in hydroxymethyl transfer is attributed to the characteristic Schiff base formed between the aldehyde-group of PlP and the amino-group of serine (Snell, 1960; Braunstein, 1960). This allows electron withdrawal from the C-2:C-3 bond, thus lowering the activation energy of the cleavage reaction.

$$\text{serine} + H_4\text{PtG} \xrightleftharpoons{\text{PlP, } Mn^{2+}} \text{N-5,N-10-methylene-}H_4\text{PtG} + \text{glycine} + H_2O$$

1.2.2. GLYCINE - GLYOXYLATE INTERCONVERSIONS

1.2.2.1. Glycine aminotransferase — Non-enzymic transamination between certain amino acids and glyoxylate, under physiological conditions of pH and temperature, was first reported by Nakada and Weinhouse (1953a), but the importance of non-enzymic, metal ion catalysed transamination in the special case of glycine and glyoxylate was not realised for some years. Fleming and Crosbie (1960) reported the conversion of [2-^{14}C] glycine and unlabelled glyoxylate to a mixture of [2-^{14}C]glycine and [2-^{14}C]glyoxylate in the presence of Cu^{2+}.

FIG. 1.2.2.1.A.



NON-ENZYMIC AMINO-TRANSFER BETWEEN GLYCINE AND GLYOXYLATE

$$NH_2.{}^{14}CH_2.COOH + CHO.COOH \xrightleftharpoons{Cu^{2+}} {}^{14}CHO.COOH + NH_2.CH_2.COOH$$

These authors proposed a mechanism (Fig. 1.2.2.1.A) similar to that described by Metzler, Ikawa and Snell (1954) for the non-enzymic transamination between alanine and pyruvate in the presence of PlP and metal ions.

The ease with which glycine and glyoxylate undergo transamination in the presence of trace amounts of certain metal ions means that reports of enzymic conversion (by either a glycine aminotransferase or a glycine oxidase type of reaction) of glycine to glyoxylate based on experiments involving [^{14}C]glycine and a pool of glyoxylate as an isotope trap must be treated with caution, unless the non-enzymic reaction has been efectively blocked with EDTA (Fleming and Crosbie, 1960).

Glycine:2-oxoacid aminotransferase activity has not been demonstrated conclusively in the glycine to glyoxylate direction, that is stoichiometric formation of a corresponding amino acid has not been shown when glycine is converted to glyoxylate in the presence of a keto acid. Bachrach (1957) though, presented evidence that the metabolism of glycine in Pseudomonas aeruginosa proceeds via glyoxylate and the isocitrate lyase (section 1.3.2.). Glyoxylate was metabolised by cell-free extracts in the presence of succinate, Mg^{2+} and glutathione. Glycine was not metabolised at all by these extracts, except in the presence of 2-oxoglutarate, ATP and PlP, when it was readily converted to glyoxylate. The author suggests the reaction

is brought about by a glycine aminotransferase.

The reverse reaction on the other hand, has been well documented. Meister, Sober, Tice and Fraser (1952) showed the conversion of glyoxylate to glycine in rat liver extracts in the presence of glutamate or glutamine. Campbell (1956) reported amino acid:glyoxylate aminotransferase activity in extracts of Pseudomonas 2RCC-1, and Stewart (1961) demonstrated the conversion of glyoxylate and glutamate to glycine and 2-oxoglutarate by extracts of Escherichia coli PA15, in the presence of EDTA. The enzyme glycine aminotransferase (glycine:2-oxoglutarate aminotransferase, E.C. 2.6.1.4.) has been partially purified from sonic extracts of Escherichia coli (Crosbie, 1962b).

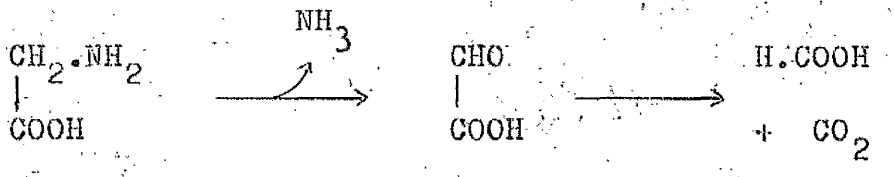
1.2.2.2. Glycine oxidase Glycine oxidase is a flavoprotein that occurs in the liver and kidney of animals and converts glycine to glyoxylate and ammonia (Ratner, Nocito and Green, 1944). The activity of the enzyme is low and Greenberg (1954) has disputed the biological significance of this pathway.

1.2.2.3. Glycine dehydrogenase Goldman and Wagner (1962) have shown the presence of an NAD^+ dependent glycine dehydrogenase in cell-free extracts of Mycobacterium tuberculosis. The equilibrium of the reaction favours the reductive amination of glyoxylate to give glycine. The authors suggest the reaction may represent a significant alternative pathway for the formation of glycine from C_2-compounds in the absence of trans-

amination.

Two reports concerning the oxidative degradation of glycine to formate and CO_2 suggest the initial conversion of glycine to glyoxylate, but neither identifies the mechanism (glycine aminotransferase or glycine oxidase) by which this occurs.

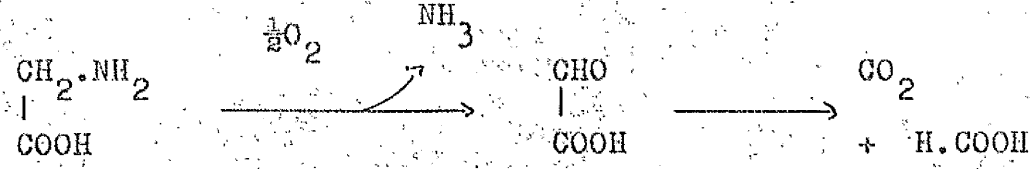
Nakada and Weinhouse (1953b) showed that glyoxylate was the initial product in the oxidative degradation of glycine to formate and CO_2 by rat liver extracts.



The evidence was based on a trapping technique. Unlabelled glyoxylate was added to a system oxidising [2-^{14}C]glycine to formate and CO_2, and at the end of the incubation the remaining glyoxylate was isolated as the 2,4-dinitrophenylhydrazone. Reduction of the hydrazone by the Wolff-Kischner procedure gave acetate which was further degraded by a modified Schmidt Reaction to show that the glyoxylate C-2 was active and the C-1 inactive. The authors believed transamination played the major role in this process, but without measures being taken to prevent the non-enzymic conversion of glycine to glyoxylate, it is difficult to assess the value of these observations (Fleming and Crosbie, 1960).

Extracts of Pseudomonas 2RCC-1 also convert glycine to

glyoxylate as the first step in the overall oxidation of glycine to formate and CO_2 (Campbell, 1955).

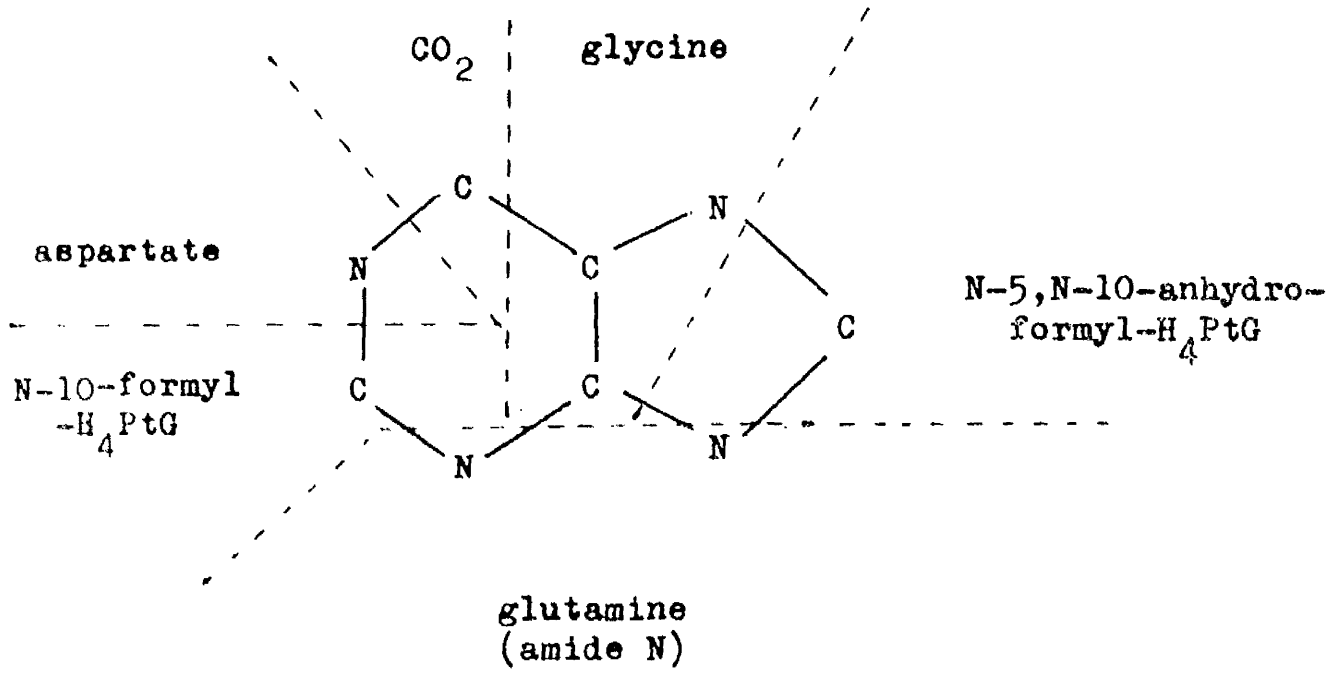


Campbell did not determine the mode of conversion of glycine to glyoxylate, but inferred that an oxidase was involved. In contrast to the Nakada and Weinhouse scheme the formate was derived from the glyoxylate C-1. This was shown by isolating [^{14}C]formate from a reaction mixture supplemented with [1-^{14}C]glycine. The formate was identified as the p-bromophenacyl ester (M.Pt. 140°) and counted as $Ba^{14}CO_3$ after oxidation to $^{14}CO_2$. When [2-^{14}C]glycine was used, the formate was not labelled.

These observations though are difficult to reconcile with the report of Neish and Lemieux (1952) that p-bromophenacyl formate has a M.Pt. of 92° (or 97·5 - 98°; section 2.6.6.) while p-bromophenacyl alcohol (formed by hydrolysis of p-bromophenacyl bromide) melts at 142°. Crosbie (1962a) suggests this puzzling degradation should be reinvestigated because of this discrepancy.

Callely and Dagley (1959) described the conversion of glycine to glyoxylate as the first step in the pathway of glycine utilization by a Pseudomonad grown on glycine as sole carbon source. The mechanism of conversion was thought to be

FIG. 1.2.3.A.



Biological origins of the purine ring atoms

the same as that reported by Campbell (1955, see above).

Wright (1957) isolated a mutant of the mould Neurospera crassa which would grown in a minimal medium supplemented with glycine, glyoxylate, glycollate or serine, which suggests a simple metabolic relationship between these compounds.

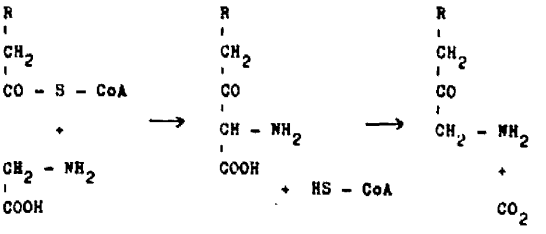
1.2.3. BIOSYNTHETIC REACTIONS INVOLVING GLYCINE

Glycine is incorporated as such into cellular protein and it is utilised for purine ring biosynthesis. It also condenses with acetyl-CoA and succinyl-CoA to give compounds which decarboxylate forming aminoacetone and 5-aminolaevulinate respectively.

The reactions involved in purine biosynthesis are well established. C-4, C-5 and N-7 are derived from glycine. Glycine reacts with 5-phosphoribosylamine in the presence of ATP to give glycinamide ribonucleotide which is further converted, in several stages, to inosinic acid. The biochemical origins of all the purine ring atoms are illustrated in Fig. 1.2.3.A.

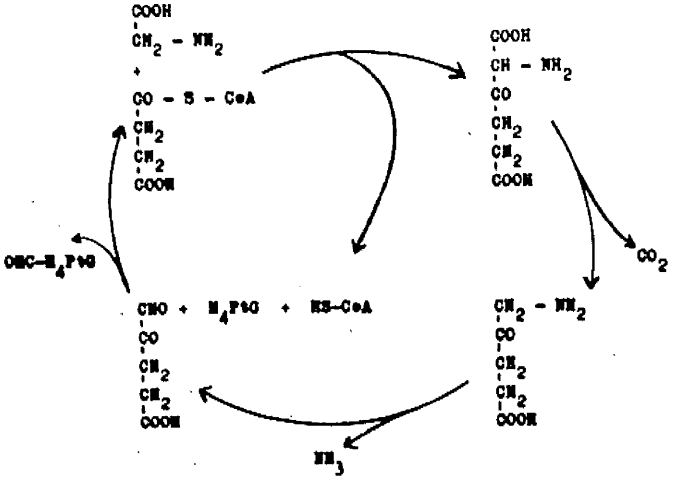
The condensation of glycine with acyl-CoA is shown in Fig. 1.2.3.B. The first reaction of this type to be recorded, involved succinyl-CoA (Shemin, Russell and Abramsky, 1954). The product of decarboxylation is 5-aminolaevulinate, two molecules of which can condense to form a pyrrole derivative, porphobilinogen.

FIG. 1.2.3.B.

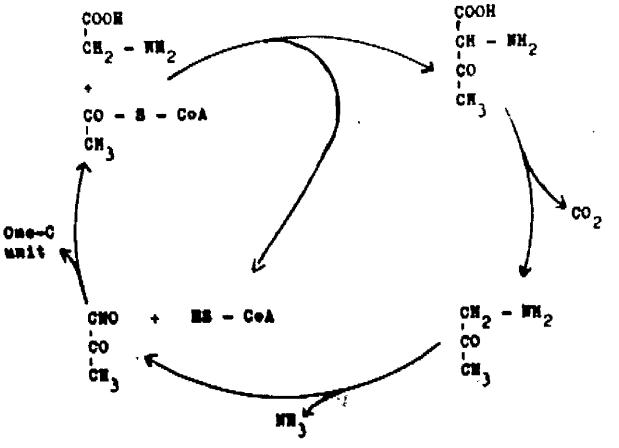


Condensation of acyl-CoA with glycine and subsequent decarboxylation.

Acetyl-CoA, R = H Succinyl-CoA, R = CH_2 - COOH



The "succinate - glycine" cycle, (Shemin, 1956)



Postulated "acetate - glycine" cycle, (Elliott, 1960)

Shemin (1956) showed that administration of [5-^{14}C]5-amino-laevulinate to animals leads to the labelling of formate, and of serine, methionine and purines in the C_1-positions, and has proposed that the "succinate-glycine cycle" (Shemin, Russell and Abramsky, 1954) could account for these observations. This alternative pathway of glycine metabolism which provides for the formation of a C_1-unit at the oxidation level of formate, from the 2-C of glycine, is shown in Fig. 1.2.3.B.

The condensation of glycine with acetyl-CoA, based on an analogy with the reaction just described, was postulated by Nemath, Russell and Shemin (1957). Elliott (1960) isolated an aminoketone which was synthesised from glycine by Staphylococcus aureus, with properties indistinguishable from aminoacetone. This confirmed the suggested condensation, and by further analogy Elliott proposed an "acetate-glycine" cycle which again allows the formation of a C_1-unit from glycine C-2. (Fig. 1.2.3.B.)

1.2.4. FERMENTATION OF GLYCINE

Glycine is reduced to acetate and ammonia in Clostridium stricklandii (Stadtman and Elliott, 1956) by a fermentation coupled to the oxidation of other amino acids (Rabinowitz, 1960). Extracts catalyse the reaction in the presence of dithiols such as 1,3-dithiolpropanol, ADP and orthophosphate.

$$NH_2.CH_2.COOH + P_i + ADP + R(SH)_2 \rightleftharpoons CH_3.COOH + NH_3 + ATP + R(SS)$$

It is interesting to note that this reaction is an apparent example of ATP formation being coupled to reduction (Stadtman and Elliott, 1956). Further purification of the extract showed the overall reaction to be stimulated by NAD^+, PlP and Mg^{2+}, and four moles of ATP to be formed per mole of glycine reduced. These results are explained in terms of a possible phosphorylated intermediate (Stadtman, Elliott and Tiemann, 1958).

The glycine C-1 and C-2 form the acetate C-1 and C-2 respectively in the Clostridium system, but this is not so in the fermentation of glycine to acetate by Diplococcus glycinophylus (Barker, Volcani and Cardon, 1948). Acetate formed from [2-^{14}C]glycine is heavily labelled in both carbons, while acetate formed from [1-^{14}C] glycine is predominately labelled in the C-1 position.

Sagers and Gunsalus (1958) partially explain these observations with the following sequence of reactions:

$$^{14}CH_2.NH_2.COOH + H_4PtG \longrightarrow {}^{14}C_1\text{-}H_4PtG + NH_3 + CO_2$$

$$^{14}C_1\text{-}H_4PtG + {}^{14}CH_2.NH_2.COOH \rightleftharpoons {}^{14}CH_2OH.{}^{14}CHNH_2.COOH$$

$$^{14}CH_2OH.{}^{14}CHNH_2.COOH \longrightarrow {}^{14}CH_3.{}^{14}CO.COOH$$

$$^{14}CH_3.{}^{14}CO.COOH \longrightarrow {}^{14}CH_3.{}^{14}COOH + CO_2$$

Further acetate formation by some form of CO_2 fixation, coupled to glycine oxidation, must account for the results with [1-^{14}C]glycine (Sagers and Gunsalus, 1961). This scheme will

be discussed in more detail in section 4.

1.3. GLYOXYLATE METABOLISM

1.3.1. GROWTH OF MICROORGANISMS ON C_2-COMPOUNDS

The tricarboxylic acid cycle performs a dual role in many bacterial cells - energy is provided by the oxidation of acetyl-CoA, and the various intermediates of the cycle form a primary source of carbon skeletons for the synthesis of cellular material.

If the cycle serves only as an energy source, oxaloacetate and the other C_4-dicarboxylic acids are regenerated, but if intermediates are drained away by anabolic processes they must necessarily be replaced by ancillary reactions, or the cycle will fail. Under conditions where CO_2 acceptor molecules (e.g. pyruvate, phosphopyruvate) are available, C_4-intermediates may be regenerated by CO_2 fixation reactions. Microorganisms though, grown on acetate, for example, do not have a ready supply of such CO_2 acceptor molecules, so the synthesis of oxaloacetate and other compounds containing more than two carbon atoms, must be explained differently.

Several mechanisms whereby a C_2-skeleton might be lengthened have been proposed:- (i) condensation of acetate and CO_2 to give a C_3-unit (e.g. in Escherichia coli, Roberts, Abelson, Cowie, Bolton and Britten, 1955), (ii) the oxidative condensation of two molecules of acetate to give succinate (Thunberg, 1920),

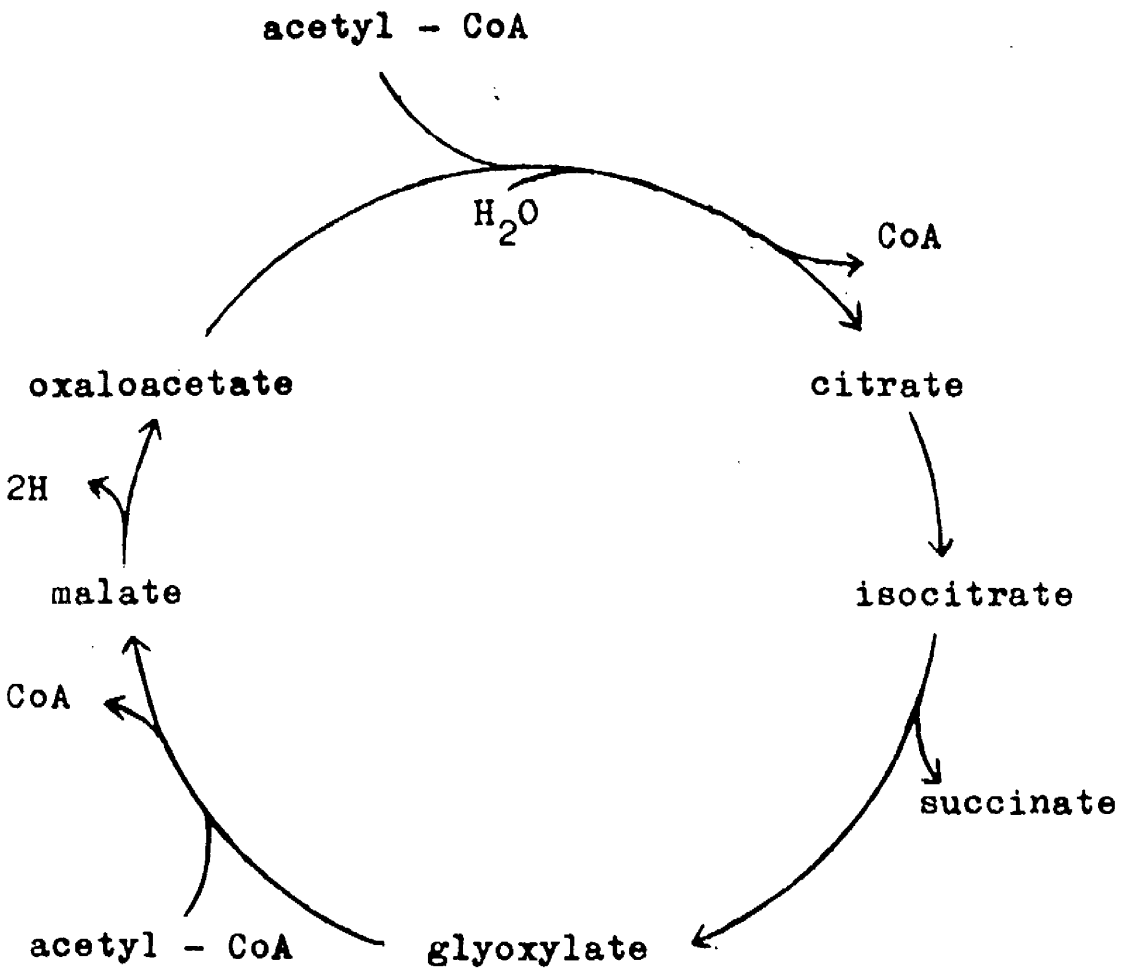
(iii) the "glyoxylate cycle" where two molecules of acetyl-CoA enter the cylce with the net production of one molecule of succinate (Kornberg and Madsen, 1957), (iv) condensation of two molecules of glyoxylate with the elimination of CO_2 to give a C_3-unit (Krakow and Barkulis, 1956) which is the precursor of pyruvate (Kornberg and Gotto, 1959), (v) the condensation of glycine and glyoxylate to give 3-hydroxyaspartate (Kornberg and Morris, 1963).

The importance of the first two reactions which are speculative, in microorganisms grown on C_2-compounds, has been questioned (Ajl, 1958; Kornberg, 1959), but the last three reactions which are well founded on in vitro enzyme studies satisfactorily explain the synthetic requirements of micro-organisms grown on acetate (Kornberg and Krebs, 1957), glycollate (Kornberg and Gotto, 1959; Kornberg and Morris, 1963), glycine (Callely and Dagley, 1959) and oxalate (Quayle and Keech, 1959). Acetate is utilised via scheme (iii), while glycollate, glycine and oxalate are first converted to glyoxylate and utilised via schemes (iv) and (v).

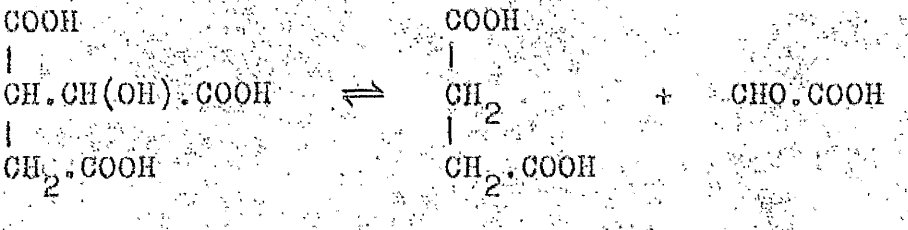
1.3.2. THE GLYOXYLATE CYCLE

The cleavage of isocitrate to succinate and glyoxylate by the enzyme isocitrate lyase (L_s-isocitrate glyoxylate-lyase, E.C. 4.1.3.1.) reported by several authors (e.g. Smith and Gunsalus, 1954; 1955),

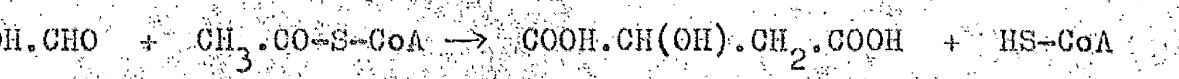
FIG. 1.3.2.A.



The glyoxylate cycle



and the formation of malate from acetyl-CoA and glyoxylate by malate synthetase (L-malate glyoxylate-lyase, E.C. 4.1.3.2.; Wong and Ajl, 1956)



can both be demonstrated in extracts of acetate grown Pseudomonas and Escherichia coli (Kornberg, 1959). Such extracts also contain malate dehydrogenase (L-malate:NAD oxidoreductase, E.C. 1.1.1.37.), citrate synthetase (citrate oxaloacetate-lyase, E.C. 4.1.3.6.) and aconitate hydratase (citrate (isocitrate) hydro-lyase, E.C. 4.2.1.3.), so a cyclic system was postulated (Kornberg and Madsen, 1958) as a by-pass to supplement the oxidative degradation of isocitrate to malate. This cycle, the glyoxylate cycle, allows the synthesis of one molecule of succinate for every two molecules of acetyl-CoA entering. The cycle is illustrated in Fig. 1.3.2.A.

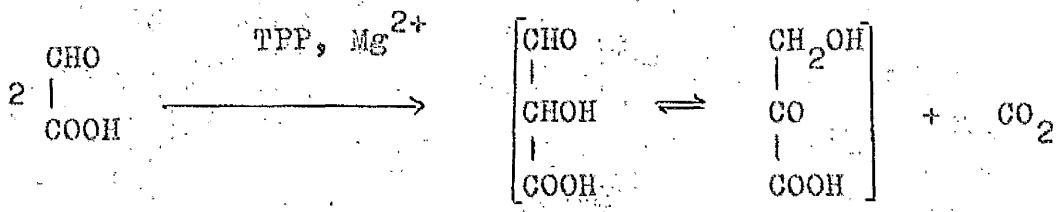
1.3.3. THE GLYOXYLATE CARBOLIGASE REACTION

The particular ability of Escherichia coli grown on glycollate to form CO_2 from glyoxylate both aerobically and anaerobically was reported by Krakow and Barkulis (1956).

Aerobically, oxygen uptake correlated with complete oxidation, by cell suspensions, of the glyoxylate to CO2 and water. Cell-free

extracts did not oxidise glyoxylate aerobically, but under anaerobic conditions approximately half a mole of CO_2 was liberated per mole of glyoxylate added. Mg^{2+} and thiamine pyrophosphate (TPP) added to extracts partially purified by ammonium sulphate fractionation, stimulated CO_2 production.

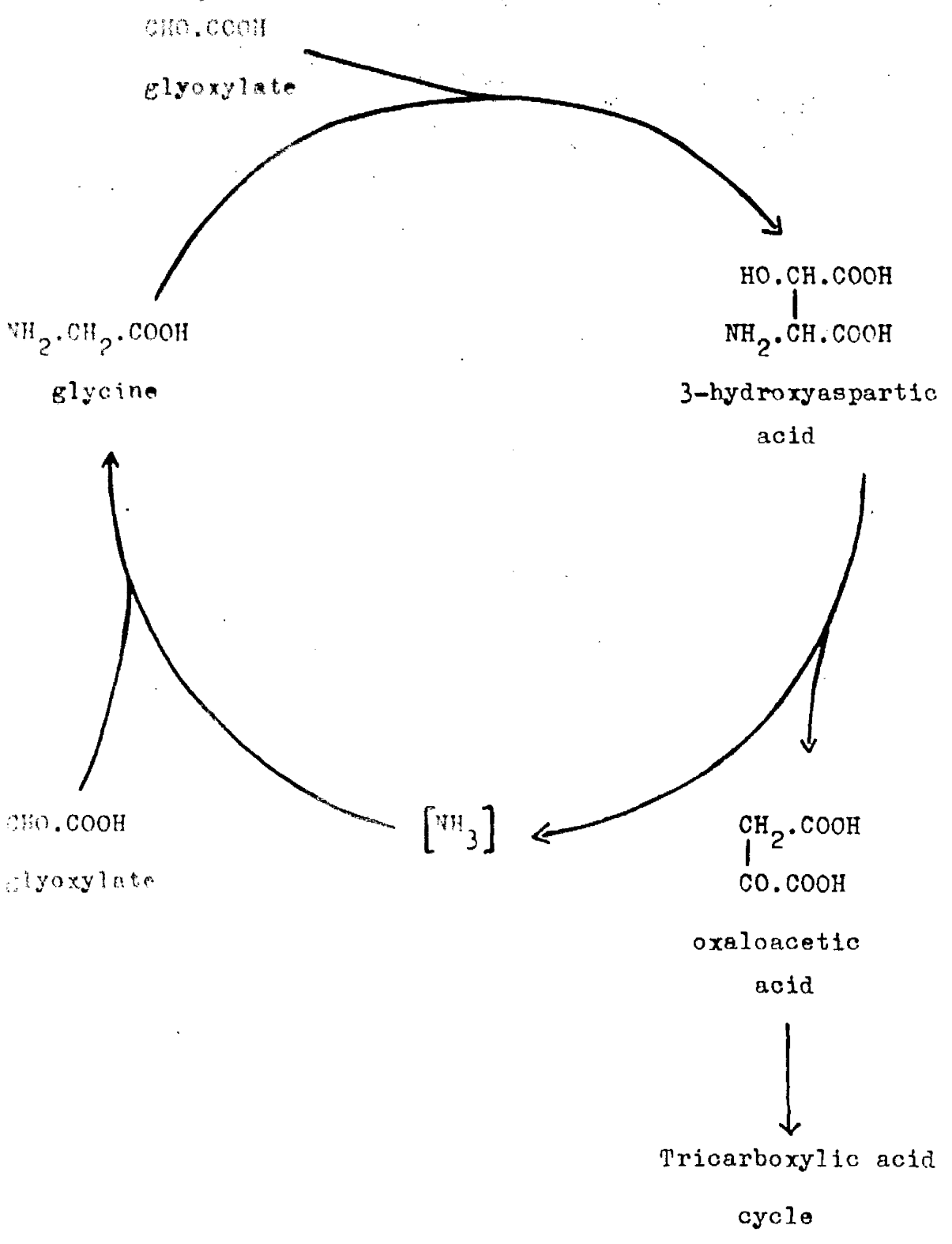
Krakow and Barkulis suggested the decarboxylation of glyoxylate produced a C_1-unit at the oxidation level of formaldehyde; condensation of this unit with a further molecule of glyoxylate to form a C_3-compound explained the observations.



A keto acid, identified as hydroxypyruvate, was isolated from the reaction mixture.

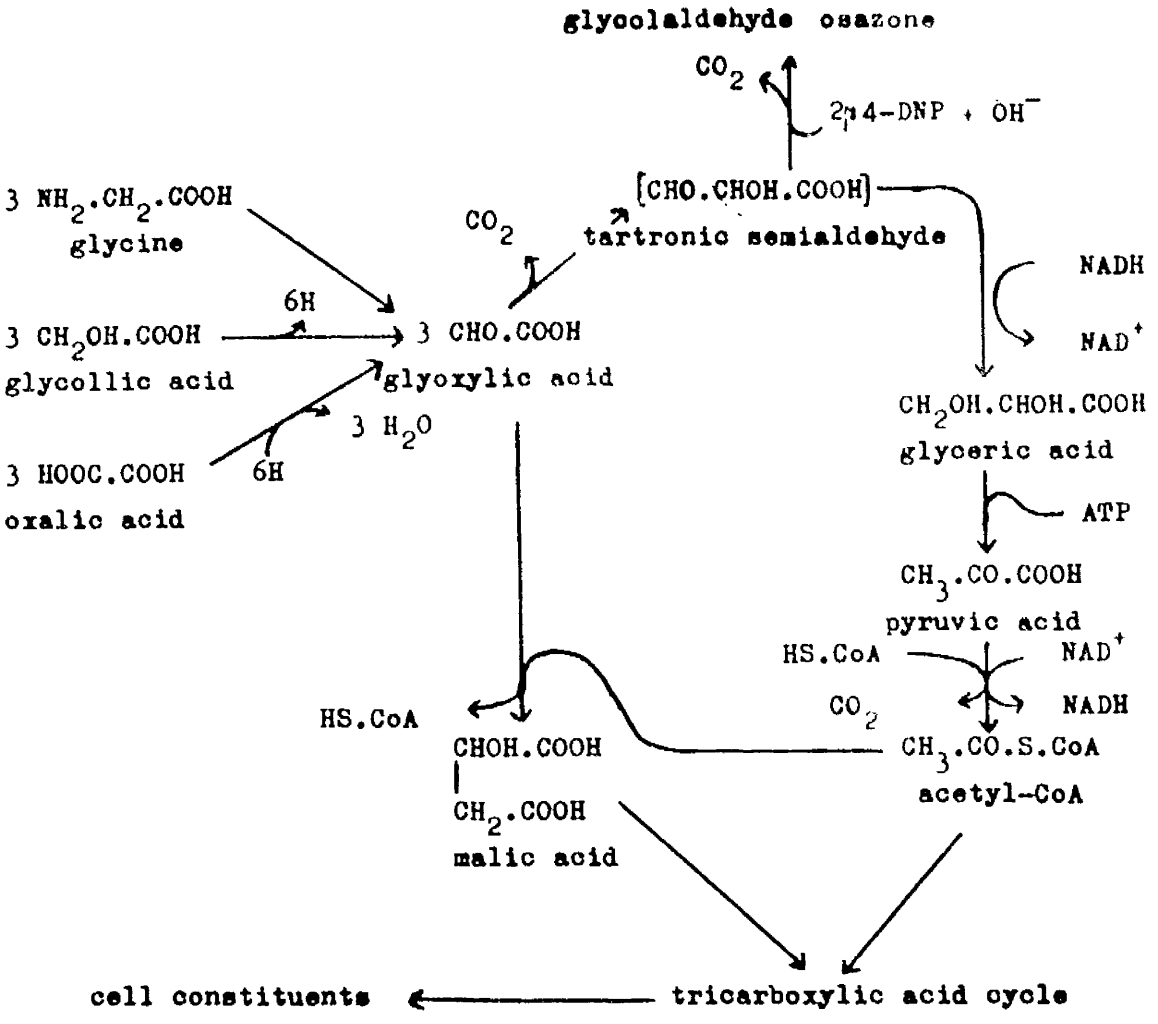
A partially purified extract of glycollate grown Pseudomonas ovalis Chester though, in the presence of NADH, TPP and Mg^{2+}, converts two moles of glyoxylate to one of glycerate by a pathway not involving hydroxypyruvate, or any compound in ready equilibrium with hydroxypyruvate. An unidentified intermediate forms a derivative with 2,4-dinitrophenylhydrazine which decarboxylates to the bis-2,4-dinitrophenylhydrazone of glyoxal on the addition of alkali (Kornberg and Gotto, 1959). The intermediate is probably tartronic semialdehyde (Krakow, Hayashi and Barkulis, 1959; Kornberg and Gotto, 1961).

FIG. 1.3.4.A.

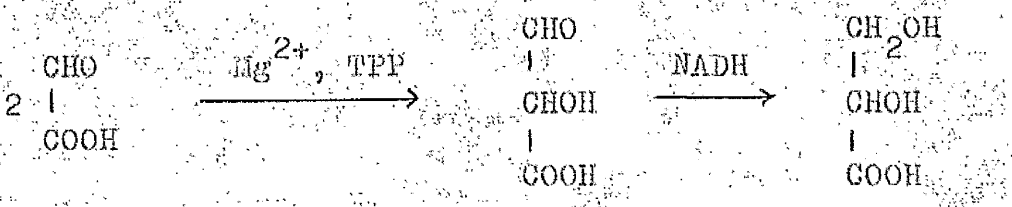


THE 3-HYDROXYASPARTATE PATHWAY

FIG. 1.3.5.A.



Pathways for the synthesis of cell constituents from C_2-compounds



1.3.4. THE 3-HYDROXYASPARTATE PATHWAY

Cell-free extracts of Micrococcus denitrificans grown on glycollate as sole carbon source, can form oxaloacetate by the 3-hydroxyaspartate pathway (Kornberg and Morris, 1963) to replace tricarboxylic acid cycle intermediates used for cell synthesis. The scheme is illustrated in Fig. 1.3.4.A. The cyclic conservation of nitrogen effects the net formation of one molecule of oxaloacetate per two molecules of glyoxylate.

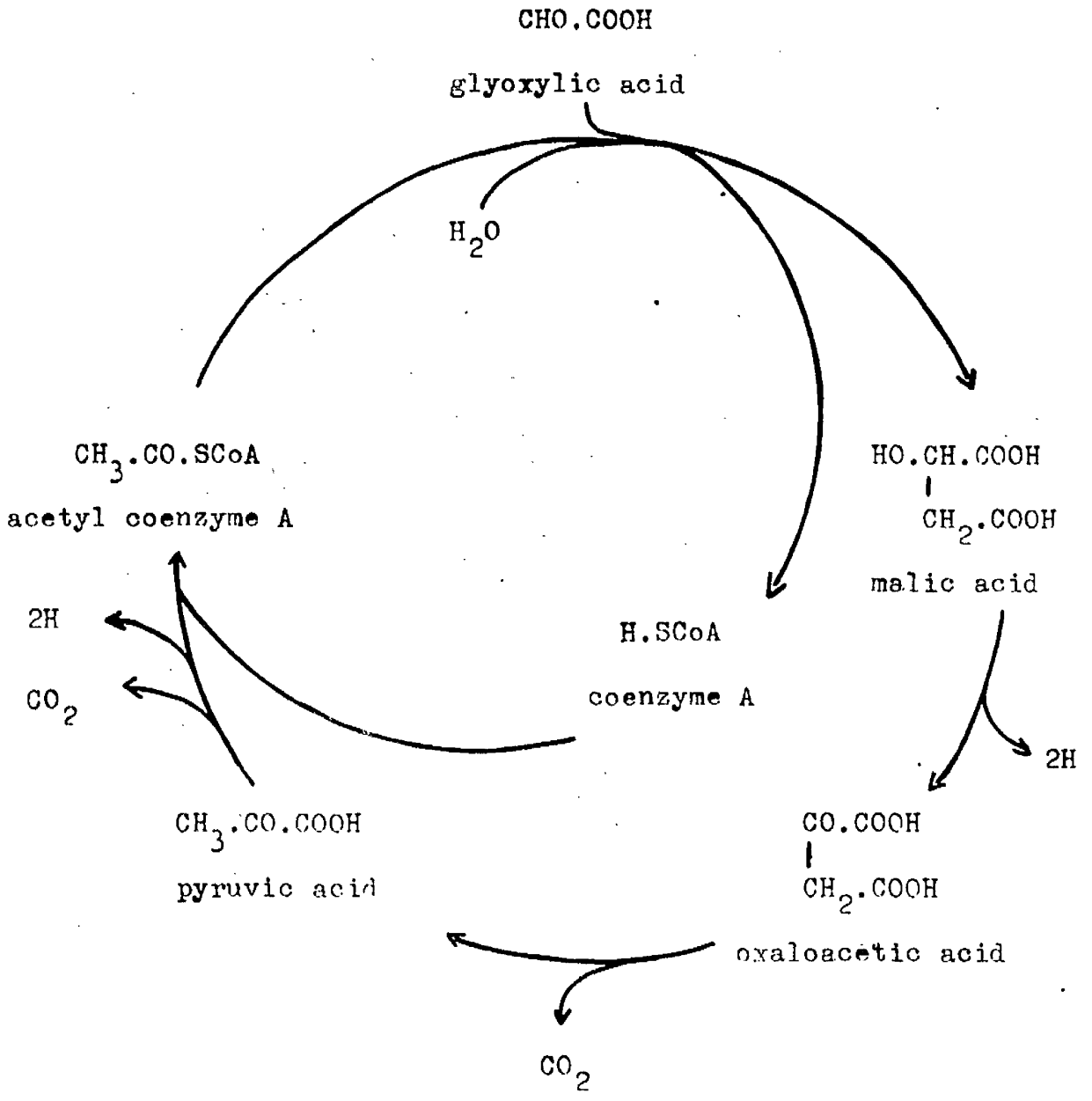
1.3.5. GROWTH OF ESCHERICHIA COLI ON GLYCOLLATE

When Escherichia coli is grown in a simple inorganic salt - glycollate medium, the energy required for growth, and the carbon skeletons of the cell constituents must necessarily be derived from glycollate. The pathways whereby the synthetic demands can be satisfied are outlined above and gathered together in Fig. 1.3.4.A.

To provide energy for cell growth, oxidation of glycollate must be coupled to reduction of pyridine nucleotides and the production of ATP. The tricarboxylic acid cycle is the major route for the terminal oxidation of acetate, but C_2-compounds at higher oxidation levels are oxidized differently.

A cyclic pathway, the dicarboxylic acid cycle, was

FIG. 1.3.5.B.



THE DICARBOXYLIC ACID CYCLE

proposed for the terminal oxidation of glyoxylate and of C_2-compounds which form glyoxylate (Kornberg and Sadler, 1960). The cycle is illustrated in Fig. 1.3.5.B. It differs from the tricarboxylic acid cycle in one important aspect -- a carbonyl compound condenses with acetyl-CoA in both, but acetyl-CoA is regenerated in the dicarboxylic acid cycle whereas the carbonyl compound is regenerated in the tricarboxylic acid cycle.

These pathways of glyoxylate metabolism can explain the synthetic and energy requirements of Escherichia coli grown on glycollate, for glycollate is readily transformed to glyoxylate by this organism (Kornberg and Sadler, 1961).

1.4. TETRAHYDROPTEROYLGLUTAMATE AND THE C_1-POOL

1.4.1. C_1-H_4PtG COMPOUNDS

5,6,7,8-Tetrahydropteroylglutamate (H_4PtG), tetrahydrofolate or coenzyme F is the active form of the vitamin, folic acid (Huennekens and Osborn, 1959; Rabinowitz, 1960; Jaenicke, 1961). In this form, or possibly as one of its polyglutamyl conjugates (Rabinowitz, 1959), its major function is concerned with the metabolism of C_1-units at various oxidation levels, viz: formate (formyl-H_4PtG), formaldehyde (hydroxymethyl-H_4PtG), and methanol (methyl-H_4PtG).

Four different types of reaction, each catalised by specific pteroproteins (Huennekens and Osborn, 1959), have

been recognised (Jaenicke, 1961):-

(i) formylation and transformylation,

$$H_4PtG + \text{"H.COOH"} \longrightarrow CHO\text{-}H_4PtG \xrightarrow{\text{acceptor}} CHO\text{-acceptor} + H_4PtG$$

(ii) hydroxymethylation and transhydroxymethylation,

$$H_4PtG + \text{"H.CHO"} \longrightarrow CH_2OH\text{-}H_4PtG \xrightarrow{\text{acceptor}} CH_2OH\text{-acceptor} + H_4PtG$$

(iii) interconversion of C_1-units,

$$CHO\text{-}H_4PtG \overset{2H}{\rightleftharpoons} CH_2OH\text{-}H_4PtG$$

(iv) methyl group formation,

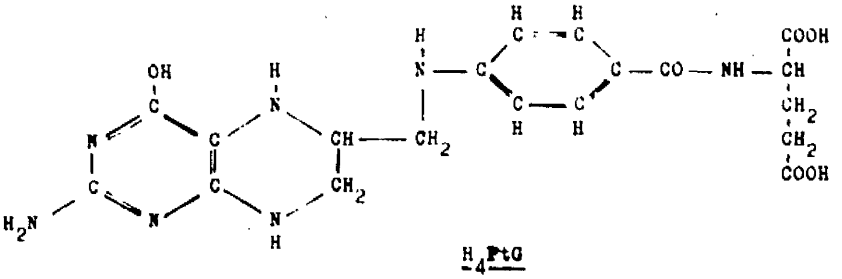
$$CH_2OH\text{-}H_4PtG \longrightarrow (CH_3\text{-}H_2PtG)^+ OH^- \xrightarrow{\text{acceptor}} CH_3\text{-acceptor} + H_2PtG$$

The enzyme, formyl-tetrahydrofolate synthetase (formate: tetrahydrofolate ligase (ADP), E.C. 6.3.4.3.) concerned in formate activation, requires the participation of ATP and Mg^{2+}. The protein has been isolated in a crystalline form from Clostridium cylindrosporum (Himes and Rabinowitz, 1962).

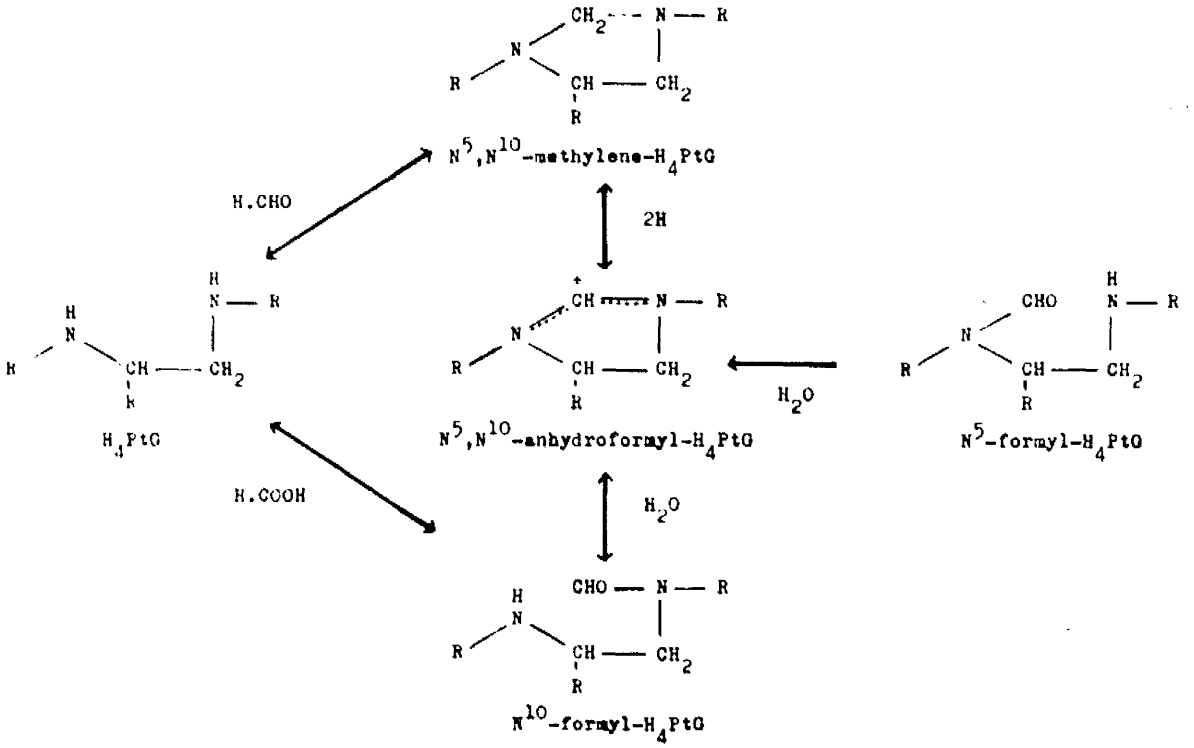
$$ATP + H.COOH + H_4PtG \overset{Mg^{2+}}{\rightleftharpoons} ADP + P_i + N\text{-}10,\text{formyl-}H_4PtG$$

Active formaldehyde can be formed non-enzymically (Jaenicke, 1956; Blakely, 1960) and enzymically (Huennekens, Osborn and Whiteley, 1958) from free formaldehyde, and also enzymically from the C-3 of serine (section 1.2.1.). The product is N-5,N-10,methylene-H_4PtG, the anhydro-, bridge form of hydroxymethyl-H_4PtG.

FIG. 1.4.1.A.



H_4PtG



Interconvertions of activated C_1 derivatives

N-5,N-10,methylene-H_4PtG can be oxidized by an NADP-dependent enzyme, N-5,N-10,methylene-tetrahydrofolate dehydrogenase, to N-5,N-10,methenyl-H_4PtG. N-5,N-10,methenyl-H_4PtG can be reversibly converted to N-10,formyl-H_4PtG by methenyl-tetrahydrofolatecyclohydrolase (5,10-methenyltetrahydrofolate 5-hydrolase (decyclizing), E.C. 3.5.4.9.) (Buchanan and Hartman, 1959), and it is formed from N-5,formyl-H_4PtG in the presence of ATP (Peters and Greenberg, 1958).

These relationships between the C_1-H_4PtG derivatives are represented in Fig. 1.4.1.A.

1.4.2. THE C_1-POOL

There appears to be a ready equilibrium between the many carbon atoms that can be metabolically liberated as single units (except perhaps in growing Escherichia coli; section 1.6.1.). Several examples involving this interchangeable pool of C_1-units are quoted in section 1.5.

1.5. INCORPORATION OF C_1-UNITS INTO CELLULAR COMPOUNDS

When the nature of the C_1-pool is realised, it is not surprising that many sources have been reported in the literature of C_1-units which are incorporated into purines, thymine, serine and methionine. However, the syntheses of these compounds do not depend on the origins of the C_1-units

but only on the nature of the C_1-H_4PtG derivatives directly involved. The primary sources of the carbon atoms will therefore not be discussed in this section which is concerned with the utilisation of C_1-units in anabolic processes.

1.5.1. PURINES

The pathway of biosynthesis of the purine ring includes three steps where carbon atoms are added singly. These reactions, in order, incorporate carbon atoms for the C-8, C-6 and C-2 positions, and the respective donors are methenyl-H_4PtG, CO_2 and formyl-H_4PtG, i.e. C-8 and C-2 are derived from the C_1-pool (Davidson, 1959).

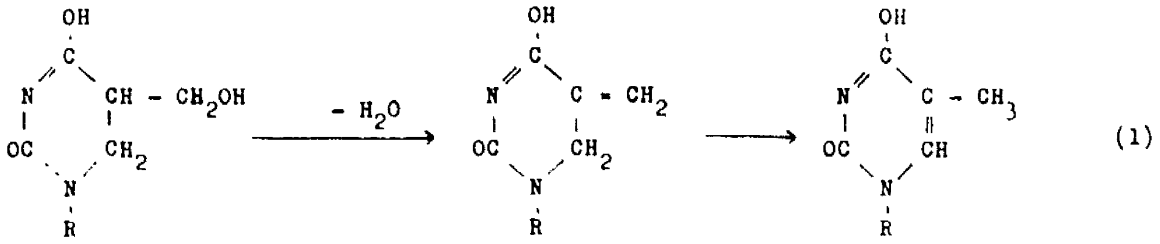
The exact nature of the C_1-donors and the properties of the enzymes concerned in the transfer reactions in chicken liver have been elucidated by Hartman, Buchanan and co-workers. The results have been reviewed by Buchanan and Hartman (1960).

The glycinamide ribonucleotide will accept formyl groups only from N-5,N-10,methenyl-H_4PtG, and the 5-amino-4-imidazole-carboxamide ribonucleotide only from N-10,formyl-H_4PtG. These reactions are very specific and were identified using enzyme preparations free from methenyl-tetrahydrofolate cyclohydrolase (Buchanan and Hartman, 1959; section 1.4.1.).

1.5.2. THYMINE

The introduction of a methyl-group into the pyrimidine

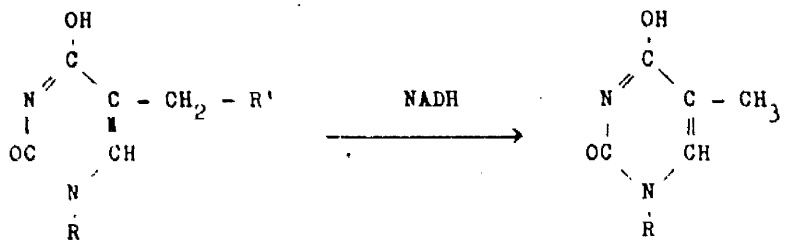
Synthesis of thymidine-5'-phosphate



(1)

hyrdoxymethyldihydro-deoxyuridine-5'-phosphate

thymidine-5'-phosphate

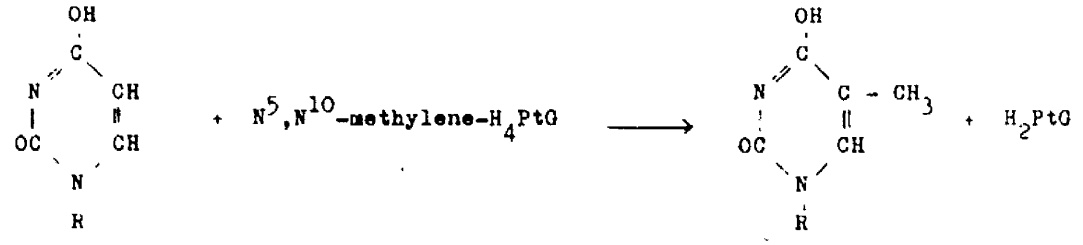


(2)

H_4PtG derivative of thymidine-5'-phosphate

($R' = H_4PtG$)

thymidine-5'-phosphate



(3)

uridine-5'-phosphate

thymidine-5'-phosphate

FIG. 1.5.2.A.

5-position is a step in thymine biosynthesis which has proved difficult to formulate.

H_4PtG was implicated (Friedkin and Roberts, 1956) and the reaction was shown to take place at the nucleotide level (Friedkin and Kornberg, 1957). The immediate C_1-donor is generally accepted to be N-5,N-10,methylene-H_4PtG (Rabinowitz, 1960).

$$\text{dUMP} + \text{N-5,N-10,methylene-}H_4\text{PtG} \xrightarrow{2H} \text{TMP} + H_4\text{PtG}$$

The production of thymidine-5'-phosphate from uridine-5'-phosphate and N-5,N-10,methylene-H_4PtG, according to this equation, represents a reduction. This addition of 2H could take place in one of several ways: (i) the initial formation of a dihydropyrimidine, addition of a hydroxymethyl-group, loss of water and rearrangement; Fig. 1.5.2.A., equation 1 (Green and Cohen, 1957); (ii) the condensation product of dUMP and methylene-H_4PtG cleaved reductively; Fig. 1.5.2.A., equation 2 (Friedkin and Kornberg, 1957); (iii) methylation of dUMP by methylene-H_4PtG to TMP with concomitant oxidation of the tetrahydropyrazine ring to give dihydropteroylglutamate (H_2PtG); Fig. 1.5.2.A., equation 3 (Humphreys and Greenberg, 1958).

Using a thymidylate synthetase from Escherichia coli, Wahba and Friedkin (1961) followed the formation of H_2PtG spectrophotometrically and showed the oxidation of H_4PtG to be equimolar with thymidylate synthesis.

$$dUMP + \text{N-5,N-10,methylene-}H_4PtG \xrightarrow{Mg^{2+}} TMP + H_2PtG$$

$$H_2PtG \xrightleftharpoons{NADPH} H_4PtG$$

These observations were essentially confirmed by the observations of McDougal and Blakely, 1961) working with an enzyme from Streptococcus fecalis. Dihydrofolate reductase (Osborn, Freeman and Huennekens, 1958) reduces H_2PtG to H_4PtG in the presence of NADPH.

1.5.3. SERINE

In mammalian and plant cells serine can be formed from glucose via 3-phosphoglycerate, 3-phosphohydroxypyruvate and phosphoserine. Enzyme studies have shown that this route may operate in Escherichia coli, but the presence of isocitrate lyase and glycine aminotransferase in glucose grown Escherichia coli K 12 (and other glycine - serine auxotrophs) suggested that an alternative pathway via isocitrate, glyoxylate and glycine may be of some importance (Umbarger, 1961).

The formation of serine from glycine is discussed in section 1.2.1.

1.5.4. METHIONINE

The formation of methionine in extracts of Escherichia coli from homocysteine and serine requires the presence of H_4PtG and a protein bound cobalamin factor. This factor is possibly concerned in the synthesis of an active pteroylglutamate derivative,

probably tetrahydropteroyltriglutamate (H_4PtG_3) (Foster, Tejerina and Woods, 1961).

Wilmanns, Rucker and Jaenicke (1960) proposed a mechanism for the methylation of homocysteine in extracts of pig liver which involved N-5,methyl-H_2PtG as the active donor. A methylated form of reduced pteroylglutamate has also been islolated by Larrabee and Buchanan (1961) from an Escherichia coli system synthesising methionine from homocysteine and methylene-H_4PtG. Guest and Woods (1962) suggest the immediate donor is N-5,N-10, methylene-H_4PtG, in Escherichia coli, so methylation requires either concomitant formation of H_2PtG (cf. the Friedkin scheme for the methylation of dUMP; section 1.5.2.) or the coupled oxidation of some unspecified hydrogen carrier, and the liberation of unchanged H_4PtG. Aminopterin fails to inhibit the methylation, in the presence of catalytic amounts of H_4PtG, but inhibits completely the regeneration of H_4PtG from H_2PtG. Accordingly, Guest and Woods postulate the second mechanism.

1.6. PRODUCTION OF C_1-UNITS IN ESCHERICHIA COLI

1.6.1. C_1-UNITS FOR THYMINE METHYL-GROUP BIOSYNTHESIS

Several compounds, including formate, glycine and serine, can supply the C_1-pool, which in turn is used extensively for cellular synthesis in Escherichia coli growing in simple media.

Crosbie (1958) observed: (i) serine C-3 is utilised quantitatively for thymine methyl-group formation by a route that does not involve glycine; (ii) glycine C-2 is utilized to an extent that depends on its extracellular concentration (cf. Koch, 1955) and by a route not involving serine, and (iii) formate is not utilized at all, but is incorporated into the purine C-2 and C-8 positions.

The first observation can be explained (section 1.2.1.), but the route whereby glycine C-2 is converted to C_1-units is unknown. It is difficult to reconcile the third observation with the picture of a mobile C_1-pool. The nature of the block in growing Escherichia coli, preventing the production of methylene-H_4PtG from formyl derivative, remains unexplained, except in terms of compartmentation.

1.6.2. FORMATION OF C_1-UNITS FROM GLYCINE

Three ways of converting glycine C-2 to a C_1-unit can be considered: (i) via glyoxylate to formaldehyde or formate; (ii) via the acetate-glycine cycle; (iii) via the succinate-glycine cycle to formate. Because formate is not utilized for thymine methyl-group biosynthesis while glycine C-2 is, any pathway postulated must allow for the formation of "active formaldehyde" (Crosbie, 1958). None of these three have been shown, experimentally, to do this. Finally, the conversion may be via a new pathway not yet formulated.

2. METHODS AND MATERIALS

2.1. ORGANISM : ESCHERICHIA COLI PA15

- 2.1.1. Maintenance of organism
- 2.1.2. Growth of organism
- 2.1.3. Nutritional requirements

2.2. PREPARATION OF CELL EXTRACTS

- 2.2.1. Alumina grinding
- 2.2.2. Sonic oscillation

2.3. FRACTIONATION OF CELL EXTRACTS

- 2.3.1. Protamine sulphate treatment
- 2.3.2. Ammonium sulphate fractionation
- 2.3.3. Acetone fractionation
- 2.3.4. Dialysis and Sephadex treatment

2.4. ENZYME ASSAYS

- 2.4.1. Glycine aminotransferase
- 2.4.2. Glycine-splitting enzyme
  - 2.4.2.1. Carbon dioxide production
  - 2.4.2.2. Formaldehyde production
  - 2.4.2.3. Ammonia production
  - 2.4.2.4. Benzyl viologen reduction

2.5. ISOLATION METHODS

- 2.5.1. Fractionation of cell DNA, RNA and protein
- 2.5.2. Purine and pyrimidine bases
- 2.5.3. Amino acids
- 2.5.4. Keto acids
- 2.5.5. Formaldehyde
- 2.5.6. Formate
- 2.5.7. Degradation of adenine to glycine

2.6. ASSAY METHODS

- 2.6.1. Aminoacetone
- 2.6.2. Ammonia
- 2.6.3. p-Bromophenacyl formate
- 2.6.4. 2,4-Dinitrophenyl-amino acids
- 2.6.5. Formaldmethone
- 2.6.6. Glycine
- 2.6.7. Glycollate
- 2.6.8. Glyoxylate
- 2.6.9. Glyoxylate-2,4-dinitrophenylhydrazone
- 2.6.10. Protein
- 2.6.11. Purine and pyrimidine bases
- 2.6.12. Tetrahydropteroylglutamate

2.7. ISOTOPE ASSAY TECHNIQUES

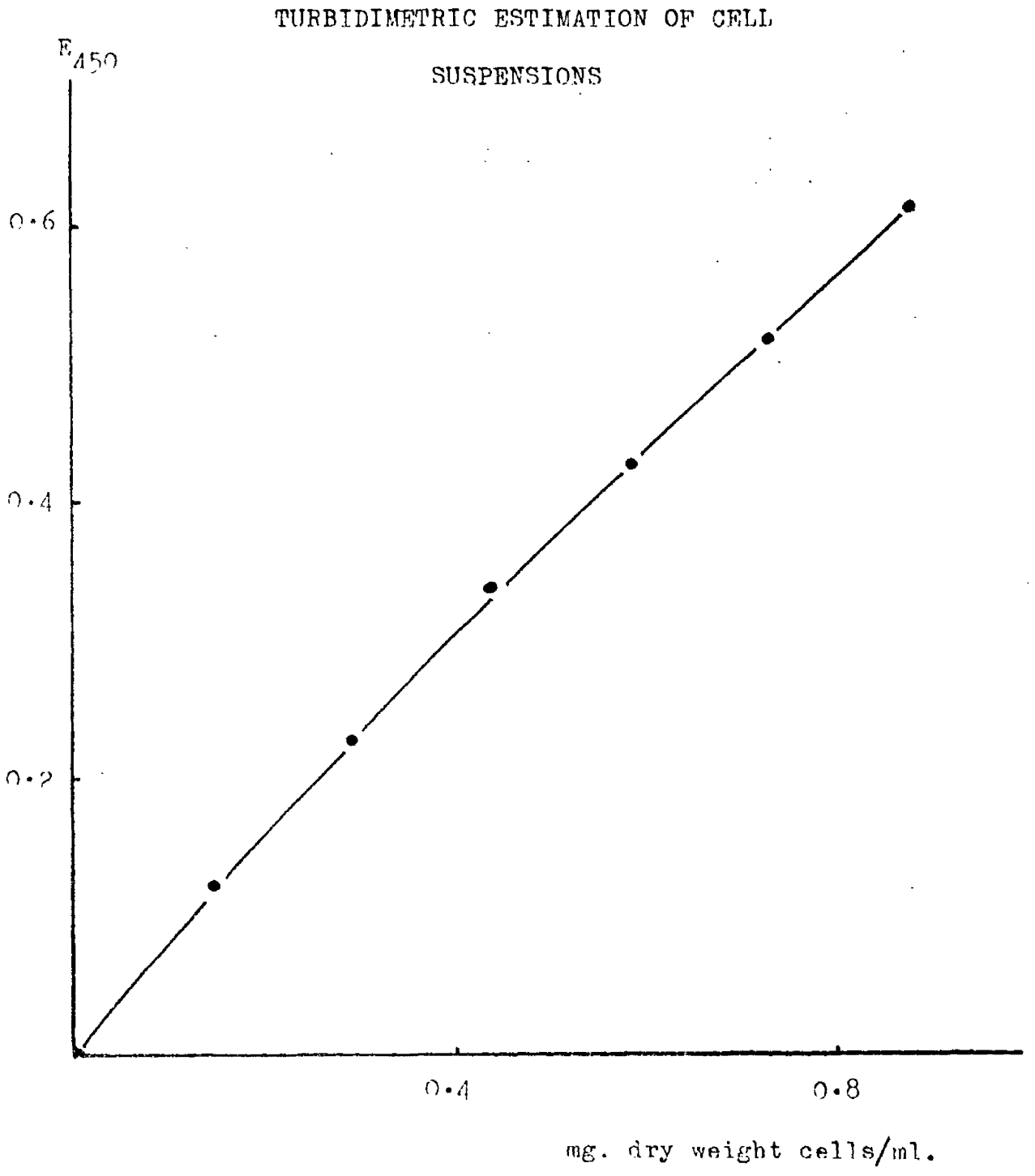
- 2.7.1. Assays at infinite thinness
- 2.7.2. Carbon dioxide
- 2.7.3. Formate

2.8. RADIOAUTOGRAPHY AND PHOTOGRAPHY

2.9. MATERIALS

- 2.9.1. General materials
- 2.9.2. H_4PtG
- 2.9.3. Aminoacetone
- 2.9.4. [2-^{14}C]glycollate

FIG. 2.1.2.A.



Homogeneous cell suspensions of E. coli PA15 were divided into two parts. One part was diluted 1:100 and the extinction of the diluted suspension measured at 450 mμ. 1·0 ml. of the second part was heated at 120° to constant weight. 1/100th of this weight is plotted against the corresponding extinctions at 450 mμ for various suspensions.

2.1. ORGANISM : ESCHERICHIA COLI PA15

2.1.1. MAINTENANCE OF ORGANISM

Escherichia coli PA15 was obtained from Professor D. D. Woods. It is an auxotrophic strain requiring either glycine or serine for growth. Stock cultures were maintained on slopes prepared from Oxoid CM4 Tablets (Oxo Ltd, England), subcultured monthly, grown for 24 hr. at 37° and stored at 4°.

2.1.2. GROWTH OF ORGANISM

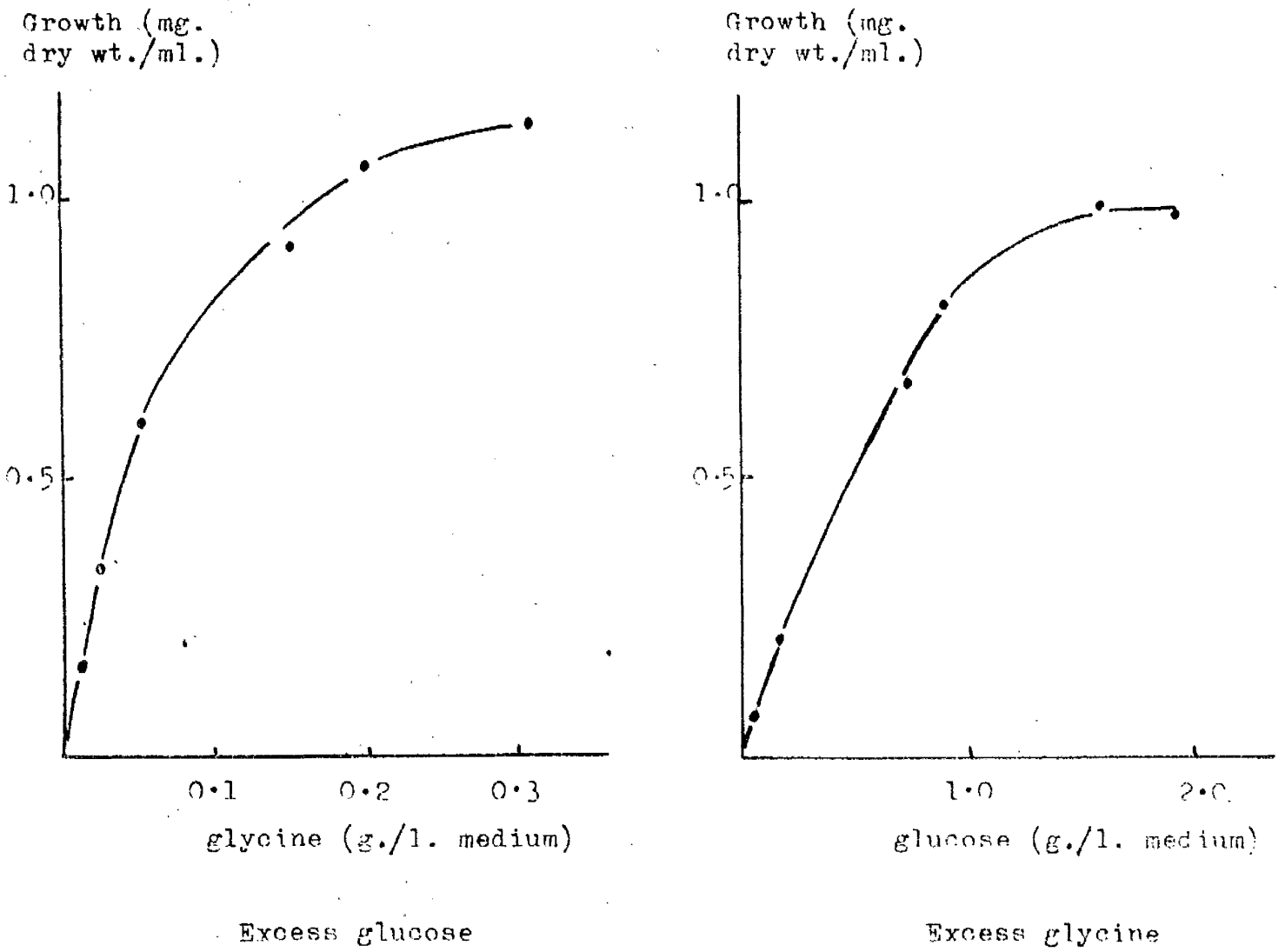
Basal medium contained (g./l.): KH_2PO_4, 5·4; $(NH_4)_2SO_4$, 1·2; $MgSO_4.7H_2O$, 0·4; glucose, 5·0; glycine, 1·0; brought to pH 7·0 with 5N-NaOH (cf. Dagley and Dawes, 1949).

For bulk growth the cells were trained by inoculating 200 ml. basal medium from a stock culture, and incubating at 37° with aeration. Cells growing logarithmically (20 ml. suspension) were subcultured into 1·0 l. basal medium. This in turn, after incubation, was used to inoculate (200 ml. suspension per flask) five 10 l. flasks of basal medium with logarithmically growing cells. Growth was followed turbidimetrically (extinctions of cell-suspensions were measured at 450 mu, Fig. 2.1.2.A.) during incubation at 37° with aeration. The cells were harvested in late log. phase.

2.1.3. NUTRITIONAL REQUIREMENTS OF ESCHERICHIA COLI PA15

Escherichia coli PA15 will grow in basal medium under

FIG. 2.1.3.A.



ESCERICHIA COLI PA15: GROWTH RESPONSE CURVES

Curves were obtained by incubating 25 ml. basal medium containing excess glycine (0·5 g./l.) or excess glucose (3·25 g./l.) and other concentrations of glucose or glycine (respectively) as shown, at 37° with aeration, until full growth. Growth was followed by measuring the extinctions of suspensions at 450 mμ. Extinctions were converted to mg. dry wt. bacteria/ml. with a previously constructed calibration curve.

aerobic and anaerobic conditions. The yields (stationary phase) are 1·2 and 0·7 g. dry cells / l., respectively.

The growth response curves for glycine in the presence of excess glucose, and glucose in the presence of excess glycine are shown in Fig. 2.1.3.A.

The identity of the strain (as a Gram-negative organism requiring glycine for growth) was checked regularly.

2.2. PREPARATION OF CELL EXTRACTS

Cell cultures in late log. phase were cooled to 0° and harvested by centrifuging, either batchwise, for volumes under 2 l., or by continuous flow using a Sharples air-turbine centrifuge. The cells were washed twice with 0·067M-phosphate buffer pH 7·0 at 0°.

2.2.1. ALUMINA GRINDING

Packed cells were ground at 0° in a chilled glass mortar with enough alumina (grade 3/50, slow cutting Polishing Alumina, Griffin and George Ltd., Glasgow.) to give a dry paste (McIlwain, 1948). More alumina was added to maintain this consistency as cells were ruptured. The mixture was stirred with ice-cold 0·067M-phosphate buffer pH 7·0, and centrifuged at 20,000 g. for 30 min. The supernatant was termed the 'alumina crude extract'.

2.2.2. SONIC OSCILLATION

Packed cells were suspended at 0° in 0·067M-phosphate buffer pH 7·0 and subjected to high frequency vibration in a 50 watt, 9 Kc Ratheon Magnetostrictor Oscillator, cooled by a rapid flow of cold water. 10 min. was found to give extracts of maximum activity (Fig. 2.2.2.A.). The supernatant, after centrifuging at 20,000 g. for 30 min., was termed the 'sonic crude extract'.

2.3. FRACTIONATION OF CELL EXTRACTS

2.3.1. PROTAMINE SULPHATE TREATMENT

Crude extracts were treated with 1% protamine sulphate solution in 0.067M-phosphate buffer pH 7·0 at 0° till no further precipitation occured. After centrifuging at 20,000 g. for 20 min at 0°, the supernatants were removed and termed the 'protamine sulphate extracts'. The ratio of extinctions $\frac{E_{280}}{E_{260}}$ were: crude extracts, 0·55 to 0·59; protamine sulphate extracts, 0·92 to 0·99.

2.3.2. AMMONIUM SULPHATE FRACTIONATION

Protamine sulphate extracts were fractionally precipitated with saturated ammonium sulphate at pH 7·0. Protein fractions at 0-25%, 25-50% and 50-75% saturation of the salt were collected, dissolved in 0·067M-phosphate buffer pH 7·0 and dialysed at 0° (section 2.3.4.). The fractions were termed

respectively, the '0-25%, 25-50% and 50-75% ammonium sulphate extracts'.

2.3.3. ACETONE FRACTIONATION

25-50% ammonium sulphate extracts were further fractionally precipitated with acetone at pH 7·0. Protein fractions at 0-20%, 20-40% and 40-60% acetone (v/v) were collected, dissolved in 0·067M-phosphate buffer pH 7·0, and the acetone removed by a second ammonium sulphate precipitation at 50% salt saturation. The protein fractions were redissolved in buffer and dialysed (section 2.3.4.). The fractions were termed respectively, the '0-20%, 20-40% and 40-60% acetone extracts'.

2.3.4. DIALYSIS AND SEPHADEX TREATMENT

Extracts were dialysed in 18/32" Visking Tubing (H.M.C., London, W.1.) at 0° with stirring for 18 hr., against 200 volumes 0·067M-phosphate buffer pH 7·0.

1·5 × 20 cm. columns of Sephadex G.75 (Pharmacia, Sweden) were used to separate small molecular weight material (M.Wt. < 40,000) from the larger proteins of the extract. The columns were eluted at 0° with 0·067M-phosphate buffer pH 7·0, and 2 ml. fractions were collected. The distribution of the single protein peak was determined by measuring extinctions at 280 mu.

2.4. ENZYME ASSAYS

2.4.1. GLYCINE AMINOTRANSFERASE

Glycine aminotransferase was assayed by following [1-^{14}C]glycine formation from glutamate and [1-^{14}C]glyoxylate.

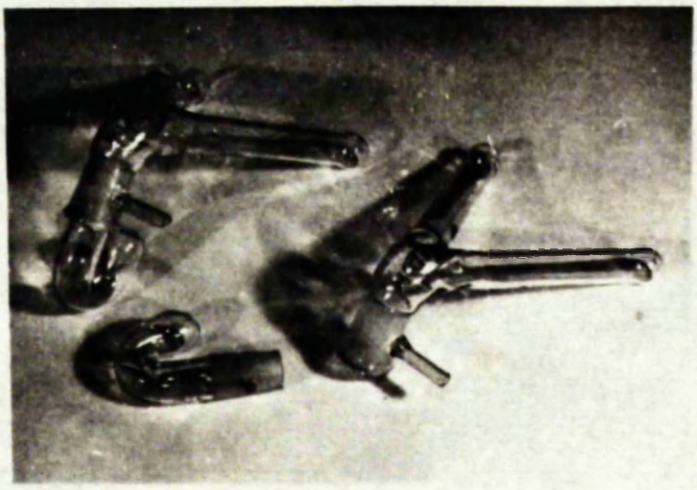
Assay mixtures contained: extract; [1-^{14}C]glyoxylate, 2·0 µmoles (33 µC/m-mole); glutamate, 2·0 µmoles; PlP, 0·1 µmole; EDTA, 2·0 µmoles, in a total volume of 1·0 ml. 0·067M-phosphate buffer pH 7·0. After incubating for 45 min. at 37°, the reaction was stopped by adding 0·2 ml. formic acid - acetic acid mixture (10% formic acid, 31% acetic acid in water).

Acidified reaction mixture (50 µl.) was dispensed for electrophoresis (40 v/cm.) in a formic acid - acetic acid solvent (2·5% formic acid, 7·8% acetic acid in water) on Whatman No. 1 paper, for 1 hr. The glycine was located with a Geiger-Muller end-window tube and rate-meter. The active areas were eluted in 5·0 ml. water overnight, and the glycine plated at infinite thinness for counting (section 2.7.1.). Aminotransferase activity was expressed as glycine formed (total counts/min.)/hr./mg. protein.

2.4.2. GLYCINE-SPLITTING ENZYME

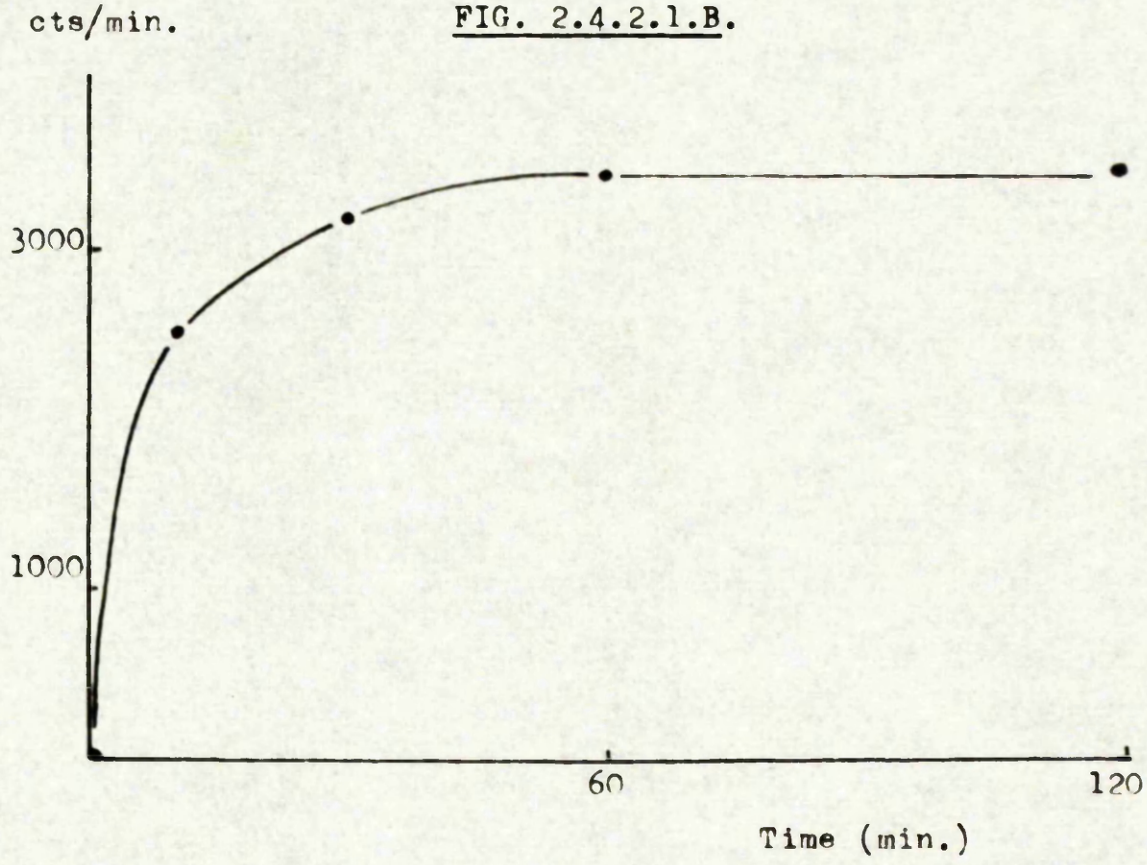
2.4.2.1. Carbon dioxide production $^{14}CO_2$ liberated enzymically from [1-^{14}C]glycine was collected in NaOH and counted as $Ba^{14}CO_3$.

FIG. 2.4.2.1.A.



Modified Thunberg tube

FIG. 2.4.2.1.B.



Variation of $^{14}CO_2$ recovery with time after acidification of $Na^{14}CO_3$ in modified Thunberg tubes

2 ml. $Na_2{}^{14}CO_3$ (5 μ-moles) acidified at time 0 with 2 ml. 2N-H_2SO_4. Liberated $^{14}CO_2$ absorbed in 2ml. 2N-NaOH (section 2.4.2.1.). Activity in alkaline trap at indicated time intervals determined as $Ba^{14}CO_3$ (section 2.7.2.).

Assay mixtures contained: extract; [1-^{14}C] glycine, 5·0 μ-moles (750 μC / m-mole); EDTA, 2·0 μ-moles; and additions of PlP, 0·2 μ-mole; H_4PtG, 1·0 μ-mole; NAD^+, 1·0 μ-mole, in a total volume of 3·0 ml. 0·067M-phosphate buffer pH 7·0.

The mixture, in one leg of a two-legged Thunberg tube (Fig. 2.4.2.1.A.), was incubated for 45 min. at 37°, then acidified by tipping 2 ml. 2N-H_2SO_4 from the second leg. $^{14}CO_2$ was absorbed in 2·0 ml. 2N-NaOH in the cap. After two hours, when all the liberated $^{14}CO_2$ had been absorbed (Fig. 2.4.2.1.B), the caps were removed and 2·0 ml 0·2M-Na_2CO_3 were added. The [^{14}C] carbonate was precipitated as $Ba^{14}CO_3$ for counting (section 2.7.2.).

Anaerobic assay conditions were obtained by setting up the tubes with no extract or H_4PtG, and with M-sodium dithionite in the 2N-NaOH. After repeated evacuation and refilling with nitrogen, the tubes were left overnight. The extract, deoxygenated by similar means, and H_4PtG were added and immediately the tubes were evacuated and refilled with nitrogen pretreated with alkaline dithionite. 2·0 ml. 0·2M-Na_2CO_3 were added to the alkaline dithionite solution from the caps (after incubation and absorption), followed by 5 ml. sat.-$KMnO_4$ to oxidize the dithionite to sulphate. (Barium dithionite is insoluble and interferes with $Ba^{14}CO_3$ activity assays.)

The oxidized solution, in one leg of another modified

Thunberg tube, was acidified with 5 ml. 2N-H_2SO_4 by tipping, and the $^{14}CO_2$ was collected in 4·0 ml. 2N-NaOH in the cap for plating as $Ba^{14}CO_3$.

Enzyme activities were expressed either as μ-moles $^{14}CO_2$ liberated / hr. / mg. protein, or as $Ba^{14}CO_3$ activity (at inf. thickness, counts / min.)/ hr. / mg. protein.

Note

Activity $Ba^{14}CO_3$ at infinite thickness (A counts/min.) is proportional to the specific activity (S counts/μ-mole $Ba^{14}CO_3$/ min.), and the specific activity of a carbonate sample can be determined by plating as Na_2CO_3 at infinite thinness. Hence the proportionality constant (K) can be calculated: $K = \frac{S}{A}$.

In each assay total $^{14}CO_2$ counts liberated from [1-^{14}C] glycine are eventually distributed between 400 μ-moles carrier carbonate and 0-5 μ-moles formed enzymically, i.e. 402·5 ± 0·75% μ-moles. Accordingly, 402·5.S = total $^{14}CO_2$ (counts/min.) liberated by the glycine-splitting enzyme (to within ± 0·75%).

∴ total $^{14}CO_2$ liberated enzymically = 402·5.S (±0·75%)

= 402·5.KA (±0·75%)

The activity of $Ba^{14}CO_3$ at infinite thickness (A) is therefore proportional to the total $^{14}CO_2$ liberated enzymically, and can be used as an index of enzymic activity.

If the specific activity of the original [1-^{14}C] glycine is S_g (counts/μ-mole/min.) then the specific activity of the $^{14}CO_2$

derived from the glycine C-1 must be S_g counts/μmole/min. Hence, the amount of CO_2 liberated enzymically = $(\frac{402 \cdot 5 \cdot KA}{S_g}) \pm 0 \cdot 75\%$ μmoles.

2.4.2.2. Formaldehyde production [^{14}C]Formaldehyde can be isolated from reaction mixtures containing: extract; [2-^{14}C] glycine, 4·0 μmoles (1,500 μC/m-mole); EDTA, 2·0 μmoles; and additions of PlP, 0·2 μmole; H_4PtG, 1·0 μmole; NAD^+, 1·0 μmole, in a total volume of 3·0 ml. 0·067M-phosphate buffer pH 7·0.

After incubation at 37° for 45 min. the mixture was acidified with 2·0 ml. 2N-H_2SO_4 and 1·0 ml. 0·033M-formaldehyde was added. The formaldmethone was prepared after steam distillation of the formaldehyde from the mixture (section 2.5.5.).

Note

35·5 × (specific activity [^{14}C] formaldmethone) ±7% is equivalent to the total [^{14}C] formaldehyde (counts/min.) formed enzymically and present in the reaction mixture at the time of acidification. (cf. section 2.4.2.1.)

2.4.2.3. Ammonia production Free ammonia present in the supernatants of reation mixtures specified in sections 2.4.2.1. and 2.4.2.2., acidified with ammonia-free acid, was determined by the method of Paul (1958; section 2.6.2.). Enzyme activity was expressed as μmoles ammonia formed/hr./mg. protein.

2.4.2.4. Benzyl viologen reduction The oxidation of

EXTRACTION OF DNA, RNA AND PROTEIN FROM ESCHERICHIA COLI

(Each washing: 20 ml./1 g.)

At 0°

1. Wash cells twice with water
2. with 5%-TCA (containing 10%-acetone)
3. twice with 5%-TCA
4. with Et_2O (+ sufficient EtOH to give friable ppt.)
5. Repeat 1 to 4.

At room temp.

1. Wash with EtOH
2. with EtOH/$CHCl_3$ (3:1)
3. with EtOH/Et_2O (3:1)
4. with EtOH
5. with Et_2O

Digest remaining acid-insoluble, non-lipid fraction for 18 hr. at 37° with N-KOH (1 ml./100 mg.). Adjust to pH 2 with PCA at 0°.

Supernatant contains RNA. Adjust to pH 7 with KOH at 0°. Remove ppt. at 0°. Reduce volume.

RNA

Residue contains DNA & protein.

1. Wash twice with 5%-TCA
2. twice with Et_2O (+ sufficient EtOH to give friable ppt.)
3. with EtOH
4. with Et_2O

Extract twice for 10 min. at 70° with N-PCA (1·5 ml./100 mg.).

Combined extracts contain DNA. Evaporate to dryness.

DNA

Residue contains protein.

1. Wash twice with 5%-TCA
2. with Et_2O (+ sufficient EtOH to give friable ppt.)
3. with EtOH
4. with Et_2O

Protein

FIG. 2.5.1.A.

glycine by the glycine-splitting enzyme can be coupled to the reduction of benzyl viologen under strictly anaerobic conditions.

Assay mixtures contained: glycine, 5·0 µmoles; PlP, 0·2 µmole; NAD^+, 1·0 µmole; benzyl viologen, 5·0 µmoles, in a total volume of 3·0 ml. 0·067M-phosphate buffer pH 7·0. Assays were performed in 10 x 120 mm. tubes designed for the Unicam SP. 600 Spectrophotometer, sealed with rubber tubing and a screw clip, and repeatedly evacuated and filled with nitrogen. Oxygen-free extract, H_4PtG (1·0 µmole) and other additions as indicated in the text, were made with hypodermic syringes through the rubber.

The reduction of benzyl viologen was followed at 37° by measuring the increase in extinction at 555 mu.

2.5. ISOLATION METHODS

2.5.1. FRACTIONATION OF CELL DNA, RNA AND PROTEIN

The procedure for the fractionation of cell DNA, RNA and protein is outlined in Fig. 2.5.1.A. It was based on the methods of Schmidt and Tannhauser (1945) and Ogur and Rosen (1950). Techniques for the separation of nucleic acids from biological materials have been reviewed recently by Hutchison and Munro (1961).

2.5.2. PURINE AND PYRIMIDINE BASES

The purine and pyrimidine nucleotides from the RNA hydrolysate

(Fig. 2.5.1.A) were further hydrolysed to purine bases and pyrimidine nucleotides with N-HCl at 100° for 1 hr. (Smith and Markham, 1950) and the DNA (Fig. 2.5.1.A.) hydrolysed to purine and pyrimidine bases with 72% perchloric acid at 100° for 1 hr. (Wyatt, 1951). The RNA hydrolysate was evaporated to dryness over $CaCl_2$ and KOH and the residue dissolved in a small volume of water. The DNA hydrolysate was neutralised with KOH, the precipitate of potassium perchlorate removed at 0° and the supernatant evaporated to small volume.

The bases and nucleotides were separated by two-dimensional chromatography on Whatman No. 1 paper.

Solvent 1 (ascending): 65 ml. isopropanol, 16.5 ml. conc. HCl (sp. gr. 1.19) and water to 100 ml. (Wyatt, 1951)

Solvent 2 (descending): 86% n-butanol (v/v) in water, with 5% ammonia (sp. gr. 0.880) by vol. added (Markham and Smith, 1949)

R_f values:	Solvent 1	Solvent 2
adenine	0.38	0.25
guanine	0.23	0.11
cytosine	0.47	0.24
thymine	0.76	0.35

The bases were located by inspection in u.v. light, cut out and eluted in 5.0 ml. 0.1N-HCl overnight.

2.5.3. AMINO ACIDS

(1) Cell protein was hydrolysed with 6N-HCl in a sealed tube at 120° overnight, and the hydrolysate evaporated to dryness over $CaCl_2$ and KOH. The amino acids were converted to the 2,4-dinitrophenyl-derivatives (DNP-derivatives) by the method described by Sanger (1945).

200 mg. amino acids and 400 mg. $NaHCO_3$ in 5 ml. water were added to 400 mg. 2,4-dinitrofluorobenzene in 10 ml. methanol and the mixture left in the dark for 24 hr. with occasional shaking. Methanol was removed by evaporation under reduced pressure and the unreacted 2,4-dinitrofluorobenzene removed from the aqueous bicarbonate solution with ethyl acetate. The DNP-amino acids were extracted, after acidification, with ethyl acetate and separated by two-dimentional chromatography on Whatman No. 1 paper (Koch and Weidel, 1956).

Solvent 1 (ascending): n-butanol saturated with 0·1% NH_3

Solvent 2 (descending): 1·5M-phosphate buffer pH 6·0 (M-NaH_2PO_4 + 0·5M-Na_2HPO_4)

R_f values:	Solvent 1	Solvent 2
DNP-glycine	0·30	0·27
DNP-serine	0·27	0·40

The DNP-amino acid spots were cut out, eluted in 2 ml. 1% $NaHCO_3$ overnight and extracted with ethyl acetate after acidification. The ethyl acetate solution was washed with water

and evaporated to small volume *in vacuo*. After plating and counting (section 2.7.1.) the DNP-amino acids were eluted from the planchettes in 3·0 ml. 1% $NaHCO_3$ for assay (section 2.6.4.).

(2) Amino acids were also separated by ascending chroatography on Whatman No. 1 paper in phenol/water (80:20, w/w). The phenol was removed by washing the chromatogram in ether before dipping in acetone containing 1% ninhydrin. The amino acid spots were developed with gentle heating and preserved by spraying with saturated $ZnSO_4$.

R_f values:	glycine	0·39
	glutamic acid	0·27

2.5.4. KETO ACIDS

Keto acids were isolated as the 2,4-dinitrophenylhydrazones (keto acid-DNPs). 0·1% 2,4-Dinitrophenylhydrazine hydrochloride in 2N-HCl was added to the keto acid solution. After 2 to 3 hr. the keto acid-DNPs were extracted with ethyl acetate, and from this were taken into 1% $NaHCO_3$ which was washed twice with ethyl acetate. After acidification with 2N-H_2SO_4 the keto acid-DNPs were re-extracted into ethyl acetate and washed with water. the solution was evaporated to small volume at room temperature.

The keto acid-DNPs were separated by ascending chromatography on Whatman No. 1 paper in n-butanol saturated with 0·3% NH_3.

R_f values:

glyoxylate-DNP (trans-isomer)	0·18
glyoxylate-DNP (cis-isomer)	0·35
pyruvate-DNP (trans-isomer)	0·26
pyruvate-DNP (cis-isomer)	0·45
2-oxoglutarate (both isomers)	0·03

2.5.5. FORMALDEHYDE

Formaldehyde was isolated from acidified reaction mixtures by steam distillation. The first 50 ml. distillate were collected (containing more than 95% formaldehyde from the reaction mixture) and 50 ml. 0·4% dimedon solution were added. Crystals of formaldmethone separated overnight and were recrystallised three times from ethanol-water (M.Pt. 188·5° - 189·5°).

2.5.6. FORMATE

Formate was isolated by steam distillation from acidified reaction mixtures, purified by column chromatography, assayed by titration and characterised as the p-bromophenacyl ester.

0·5% H_2SO_4 was added to a vigorously stirred suspension of celite 545 in ether (8·0 ml. acid/10 g. celite). A water-cooled column (45 x 1 cm.) was packed with acidified celite. A solution of the sodium salts of the steam volatile acids was evaporated to 0·25 ml, mixed with acidified celite (to form a dry paste) and acidified with 0·2 ml. 10N-H_2SO_4. After application to the column, the organic acids were eluted with ether saturated

TABLE 2.5.6.A.

THE PREPARATION OF p-BROMOPHENACYL FORMATE

Method of preparation	M.Pt. of product	Activity of product (cts/10 min./μg.)
Vogel (1956) or Judefind & Reid (1920)	141°	0(1)
Neish & Lemieux (1952)	97·5°-98°	93·4

(1) count indistinguishable from background

p-Bromophenacyl formate was prepared from sodium formate (0·013 μC/μg.) and p-bromophenacyl bromide according to the methods indicated. The product obtained by the methods of Vogel or Judefind and Reid was identified as p-bromophenacyl alcohol: M.Pt. p-bromophenacyl alcohol (prepared by the method of Judefind and Reid (1920)) - 142°; mixed M.Pt. with product - 141°.

with 0·5% H_2SO_4 (Phares *et al.*, 1952).

Formic acid is ether volatile, so 5 ml. fractions with 5 ml water added, were immediately titrated with 0·01N-NaOH (indicator: phenol red). 95-98% formic acid was collected in fractions 8 to 12.

The purified formate was concentrated and the p-bromophenacyl ester prepared according to the method of Neish and Lemieux (1952). The formate solution was made just acid to phenol red with 0·1N-HCl and an exactly equimolar quantity of p-bromophenacyl bromide in ethanol added. After refluxing for 30 min. the p-bromophenacyl formate was recrystallised from aqueous ethanol; M.Pt., 97·5°-98°.

When the derivative was prepared according to the method of Judefind and Reid (1920) or Vogel (1956) the product was indistinguishable from p-bromophenacyl alcohol; M.Pt., 141°. p-bromophenacyl bromide appears to be hydrolysed to the alcohol by extended refluxing (1 hr.) in the presence of excess formate (Table 2.5.6.A.). The colourless needles of the alcohol are easily distinguished from the colourless plates of p-bromophenacyl formate.

2.5.7. DEGRADATION OF ADENINE TO GLYCINE

The adenine spots from six chromatograms (section 2.5.2.) of nucleic acid hydrolysates were eluted in water and the combined eluates concentrated. The bridge carbon atoms were liberated as

glycine by heating with conc. HCl in a sealed tube at 180° overnight. The contents of the tube were evaporated to dryness over KOH and $CaCl_2$ and DNP-glycine prepared (section 2.5.3.).

2.6. ASSAY METHODS

2.6.1. AMINOACETONE

Aminoacetone was estimated by the method of Elliott (1960).

2.6.2. AMMONIA

Ammonia was determined using a modified Nessler's reagent (Paul, 1958).

Reagents:- Modified Nessler's Reagent; 3·5 g. gum acacia in 750 ml. water, added to 4·0 g. KI and 4·0 g. HgI_2 in 25 ml. water and the total volume adjusted to 1·0 l.

Alkaline Nessler's Reagent; modified Nessler's reagent and 2N-NaOH freshly mixed in the ratio 2:3.

5·0 ml. alkaline Nessler's reagent were added to the ammonia (5-50 µg. N) in 2·0 ml. 0·4N-H_2SO_4. After 15 min. the extinction at 490 mµ was measured. A standard ammonium sulphate solution in 0·4N-H_2SO_4 (10·0 µg. N/ml.) was treated similarly.

2.6.3. p-BROMOPHENACYL FORMATE

p-Bromophenacyl formate was estimated spectrophotometrically at 256 mu in n-propanol. The extinction coefficient at the absorption maximum (256 mµ) is $1·79 \times 10^7$ $cm^2 . mol^{-1}$.

2.6.4. 2,4-DINITROPHENYL-AMINO ACIDS

DNP-amino acids were estimated spectrophotometrically at 360 mμ in 1% $NaHCO_3$. The extinction coefficients at 360 mμ are: DNP-glycine, 1.70×10^7 $cm^2 . mol^{-1}$.; DNP-serine, 1.68 $cm^2 . mol^{-1}$. (cf. Krol, 1952).

2.6.5. FORMALDMETHONE

Formaldmethone was estimated spectrophotometrically at 256.5 mμ in ethanol. The extinction coefficient at the absorption maximum (256.5 mμ) is 2.57×10^7 $cm^2 . mol^{-1}$.

2.6.6. GLYCINE

The formaldehyde liberated when excess ninhydrin reacts quantitatively with glycine can be estimated using chromotropic acid. The method is a modification of the procedure of Alexander, Landwehr and Seligman (1945), who isolated the formaldehyde by steam distillation before chromotropic acid treatment.

Reagents:- Phosphate buffer, pH 5.5; add 3.5 g. K_3PO_4 to 100 ml. 20% KH_2PO_4.

Ninhydrin reagent: 1% solution in water.

Chromotropic acid reagent: dissolve 0.2 g. chromotropic acid in 4 ml. water and make up to 100 ml. with 15N-H_2SO_4.

10 ml. tubes with C14 glass stoppers were used. Glycine

CALIBRATION CURVES

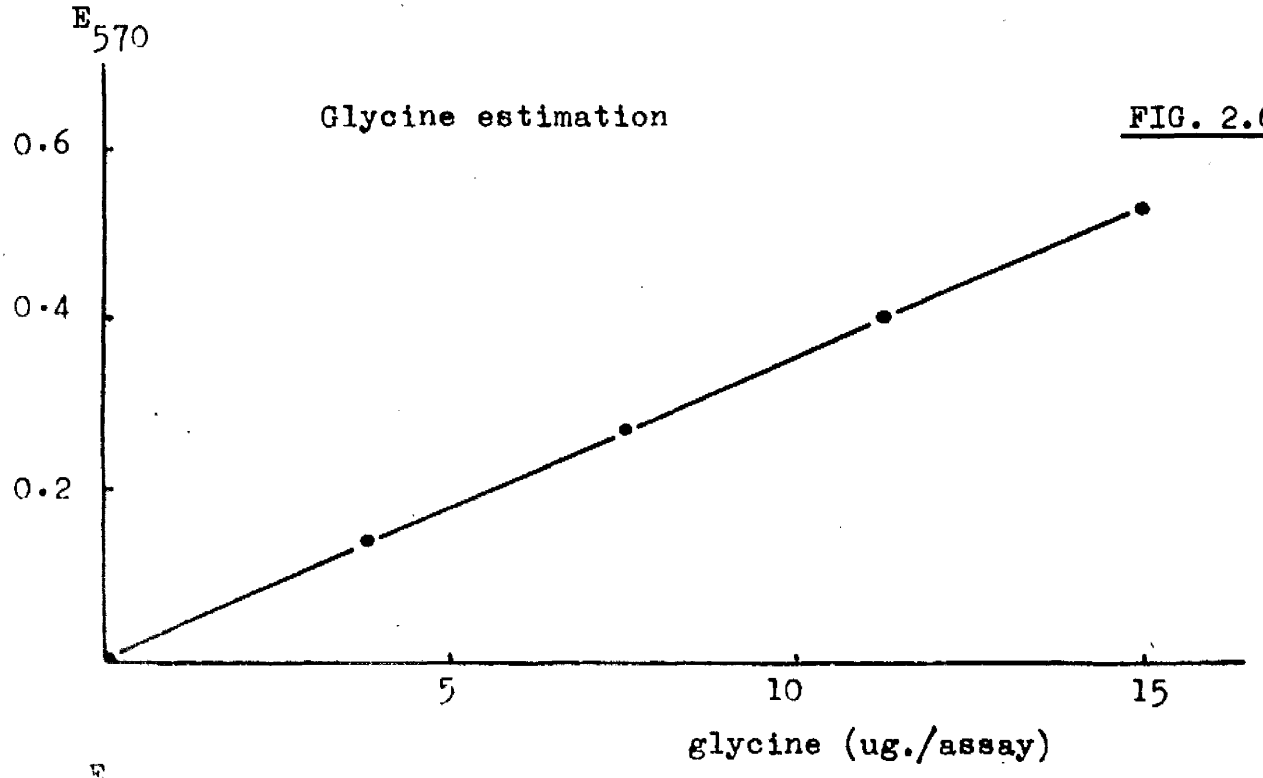


FIG. 2.6.6.A.

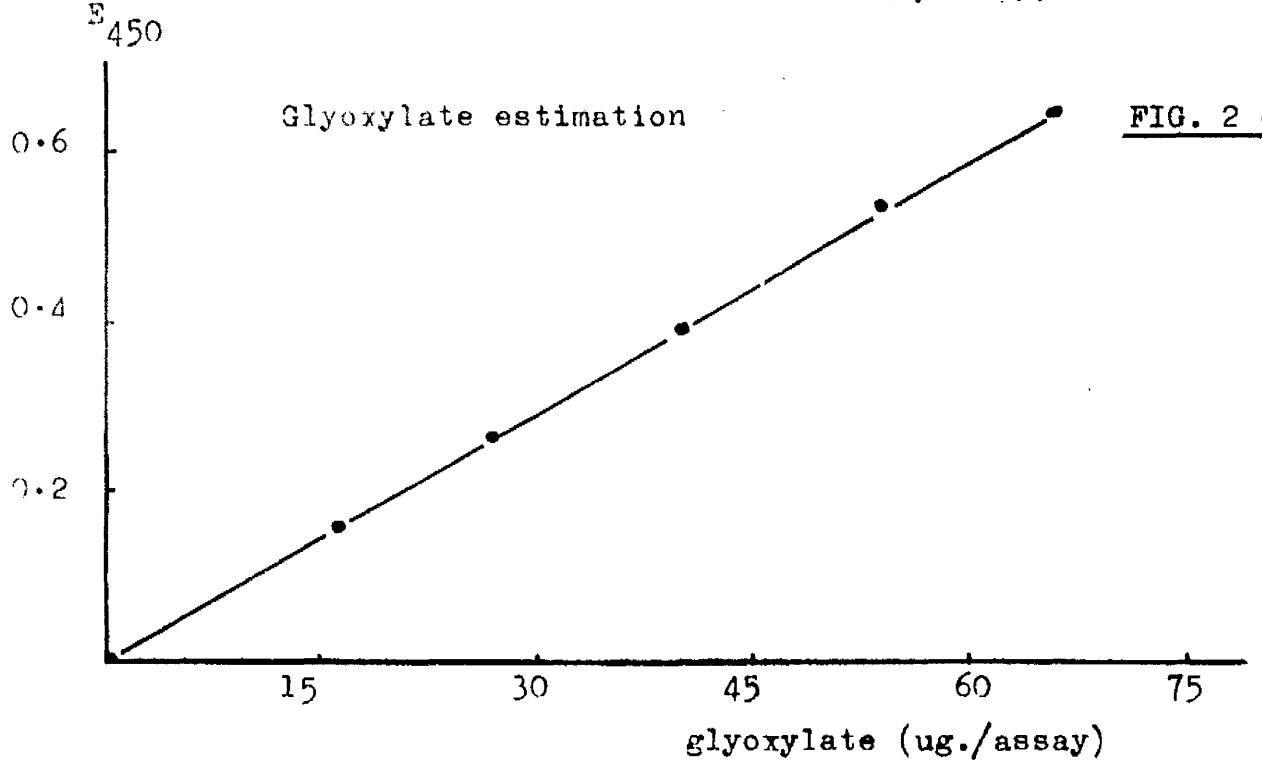
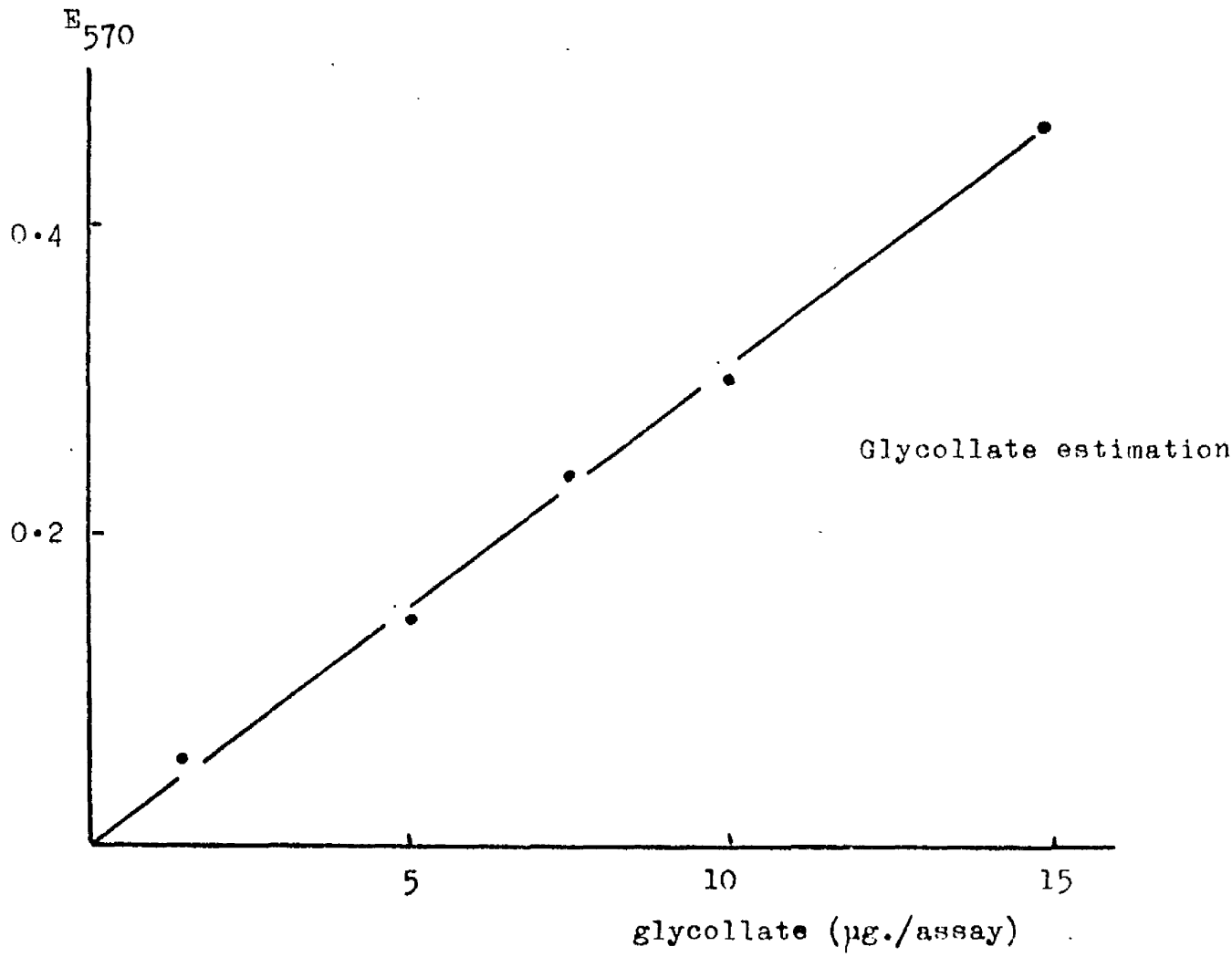


FIG. 2 6.8.A.

Assay procedures are described in sections 2.6.6. and 2.6.8.

FIG. 2.6.7.A.

CALIBRATION CURVE



Assay procedure is described in section 2.6.7.

(4-20 µg.) in 1·0 ml. water was added to 0·5 ml. phosphate buffer and 0·5 ml. ninhydrin reagent, stoppered firmly and placed in a boiling water bath for 20 min. After cooling and adding 1·0 ml. chromotropic acid reagent and 2·0 ml. conc. H_2SO_4, the tubes were quickly restoppered and replaced in the boiling water bath for 45 min. The extinctions of the stable purple solutions were measured at 570 mµ. The calibration curve is shown in Fig. 2.6.6.A.

2.6.7. GLYCOLLATE

Glycollic acid is oxidized by hot conc. H_2SO_4 to formaldehyde which can be determined with chromotropic acid. This assay is based on the procedure of Dagley and Rodgers (1953).

Reagents:- chromotropic acid reagent; see section 2.6.6.

10 ml. test-tubes with C14 glass stoppers were used. Glycollate (4-20 µg.) in 0·2 ml. water was added to 0·5 ml. chromotropic acid reagent and 5·0 ml. conc. H_2SO_4. The firmly stoppered tubes were placed in a boiling water bath for 1 hr. When cool, the extinctions were measured at 570 mµ. The calibration curve is shown in Fig. 2.6.7.A.

2.6.8. GLYOXYLATE

Glyoxylate was estimated by a method based on that of Friedman and Haugen (1943) for keto acid determinations.

Reagents:- 2,4-dinitrophenylhydrazine reagent; 0·1% in

2N-HCl.

All operations were carried out at 25°. 1·0 ml. 2,4-dinitrophenylhydrazine reagent was added to 3·0 ml. gloxylate solution (1-20 µg./ml.). After 20 min., 5·0 ml. 1·5N-NaOH were added. The orange colour produced is unstable and the extinctions at 450 mµ were measured between 10 and 16 min. after the addition of the alkali, when the intensity was within 2% of the maximum. The calibration curve is shown in Fig. 2.6.8.A.

2.6.9. GLYOXYLATE-2,4-DINITROPHENYLHYDRAZONE

Glyoxylate-DNP was estimated spectrophotometrically at 367 mµ in 0·5% $NaHCO_3$. The extinction coefficient at the absorption maximum (367 mµ) is $2{\cdot}02 \times 10^7$ $cm^2 . mol^{-1}$.

2.6.10. PROTEIN

The protein concentrations of cell extracts were measured using the Folin phenol reagent and the procedure of Lowry, Rosebrough, Farr and Randall (1951).

2.6.11. PURINE AND PYRIMIDINE BASES

Solutions of purine and pyrimidine bases were assayed by measuring the extinctions at the absorption maxima and also at another chosen wavelength (Crosbie, Smellie and Davidson, 1953). Standard values (Δ_{st}) of the differences in extinctions for the bases (10.0 µg./ml.) in 0·1N-HCl are:-

	Absorption maximum (mμ)	Chosen wavelength (mμ)	Δ_{st}
adenine	262·5	290	0·935
cytosine	275	290	0·546
thymine	264	290	0·533
guanine (in 1·6N-HCl)	249	290	0·539

2.6.12. 5,6,7,8-TETRAHYDROPTEROYLGLUTAMATE

H_4PtG was estimated spectrophotometrically at 297 mμ in 0·067M-phosphate buffer pH 7·0 containing 0·01M-2-mercaptoethanol. The extinction coefficient at the absorption maximum (297 mμ) is $2·71 \times 10^7$ $cm^2 . mol^{-1}$. Measurements were made in stoppered cuvettes in the absence of oxygen.

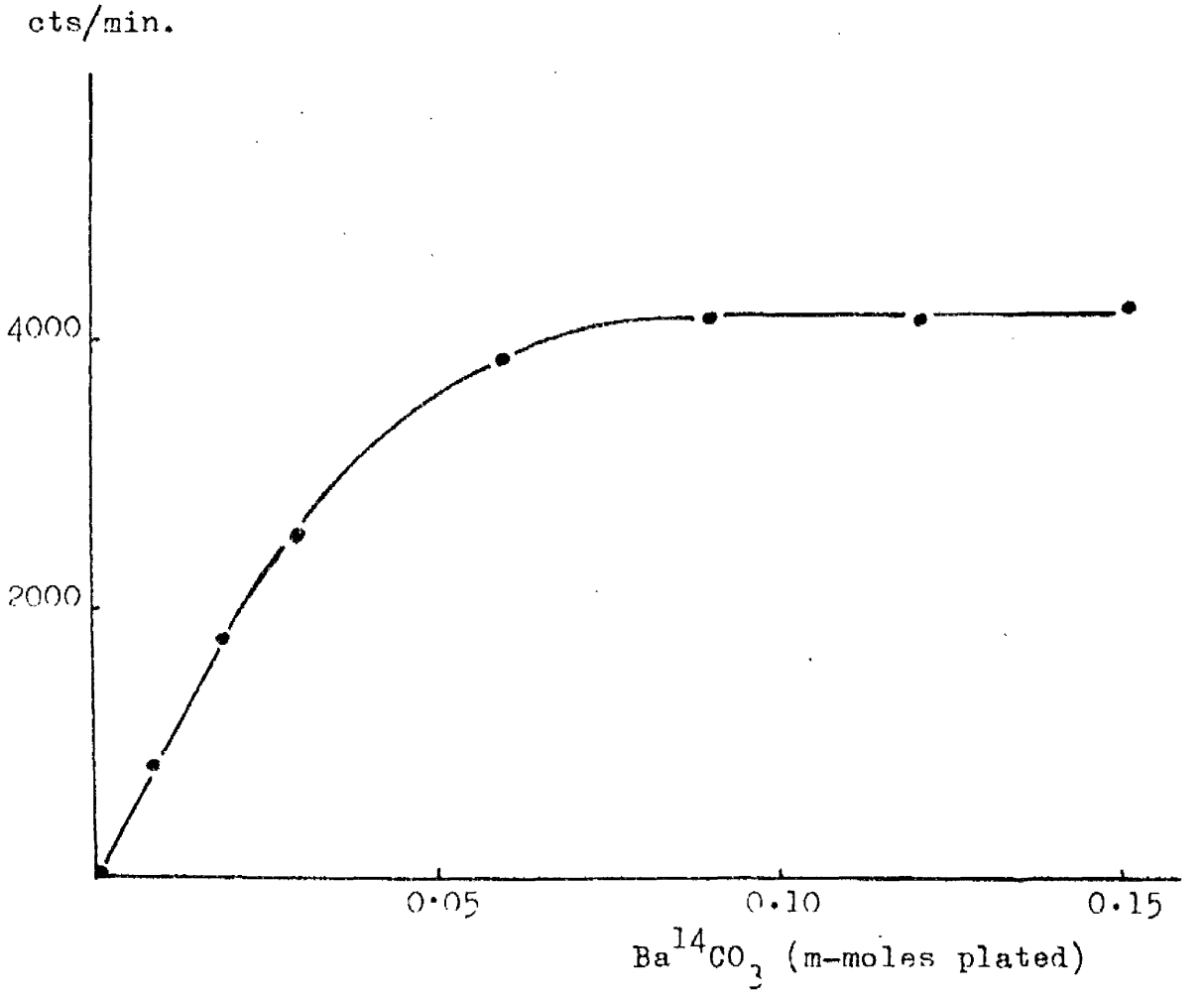
2.7. ISOTOPE ASSAY TECHNIQUES

2.7.1. ASSAYS AT INFINITE THINNESS

Radioactive determinations were carried out using a mica end-window counter or a window-less gas-flow counter (Nuclear Chicago Automatic Counting Unit). Known quantities of material were plated on nickel planchettes (effective area 1·8 cm^2.) at infinite thinness. Different amounts of the same compound were plated to check the absence of self-absorption.

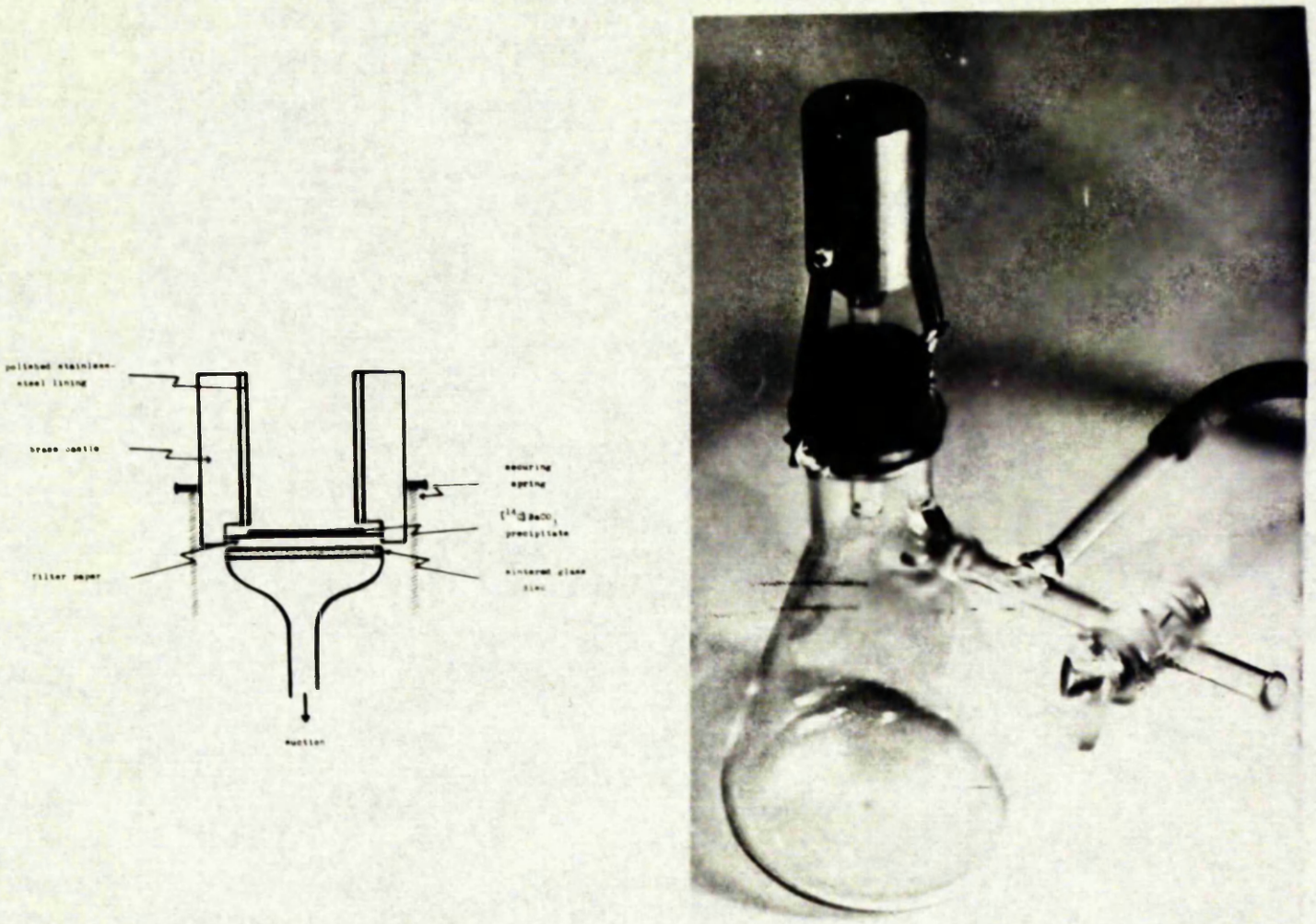
Samples were counted for a time such that the "95/100" error was less than 5%, i.e. 95% of the measurements for a given sample had an error of less than 5% (see Calvin,

FIG. 2.7.2.A.



SELF-ABSORPTION CURVE FOR $Ba^{14}CO_3$

FIG. 2.7.2.B.



PLATING APPARATUS FOR $Ba^{14}CO_3$

Heidelberger, Reid, Tolbert and Yankwich, 1949).

$$\text{"95/100" error} = \left(\frac{1.96\sqrt{[Ct + Bgd]} \times 100}{Ct - Bgd}\right)\%$$

2.7.2. CARBON DIOXIDE

$^{14}CO_2$ collected from acidified reaction mixtures in 2N-NaOH was precipitated as $Ba^{14}CO_3$ and counted at infinite thickness.

The plating apparatus is shown in Fig. 2.7.2.B. A filter paper (2·0 cm. diameter), trapped between the scintered disc and the castle, collected the precipitate on a circular area (1·5 cm^2.) accurately defined by the polished stainless-steel lining of the castle.

0·5 ml. alkaline 0·1M-$Na_2{}^{14}CO_3$ was added to 3 ml. $BaCl_2$ (5% solution) in the castle, and the precipitated $Ba^{14}CO_3$ filtered by suction. A total of 1·5 ml. 0·1M-$Na_2{}^{14}CO_3$ (0·15 m-mole) was plated by repeating the operation twice. Precipitating all the $Ba^{14}CO_3$ in one operation caused the plate to crack when dry; precipitation in three layers prevented this. After washing with acetone the filter paper and precipitate were removed as a unit, dried, and the activity determined with a mica end-window counter to a "95/100" error level of less than 5%.

Fig. 2.7.2.A. illustrates the self-absorption curve for increasing amounts of $Ba^{14}CO_3$ plated this way.

2.7.3. FORMATE

[^{14}C]Formate was oxidized to $^{14}CO_2$ with Pirie's Solution (Pirie, 1946), 8% $HgCl_2$, 2% sodium acetate and 2% acetic acid, using the techinique described by Sakami (1955). The $^{14}CO_2$ was collected in 2N-NaOH and counted as $Ba^{14}CO_3$ (section 2.7.2.).

2.8. RADIOAUTOGRAPHY AND PHOTOGRAPHY

Radioautographs were prepared by leaving chromatograms of [^{14}C]compounds in contact with Kodak "Industrex D" X-ray film for three weeks.

Contact prints of chromatograms were made on Ilford "Contact Document 50M" paper, exposed with ultra-violet or visible light.

2.9. MATERIALS

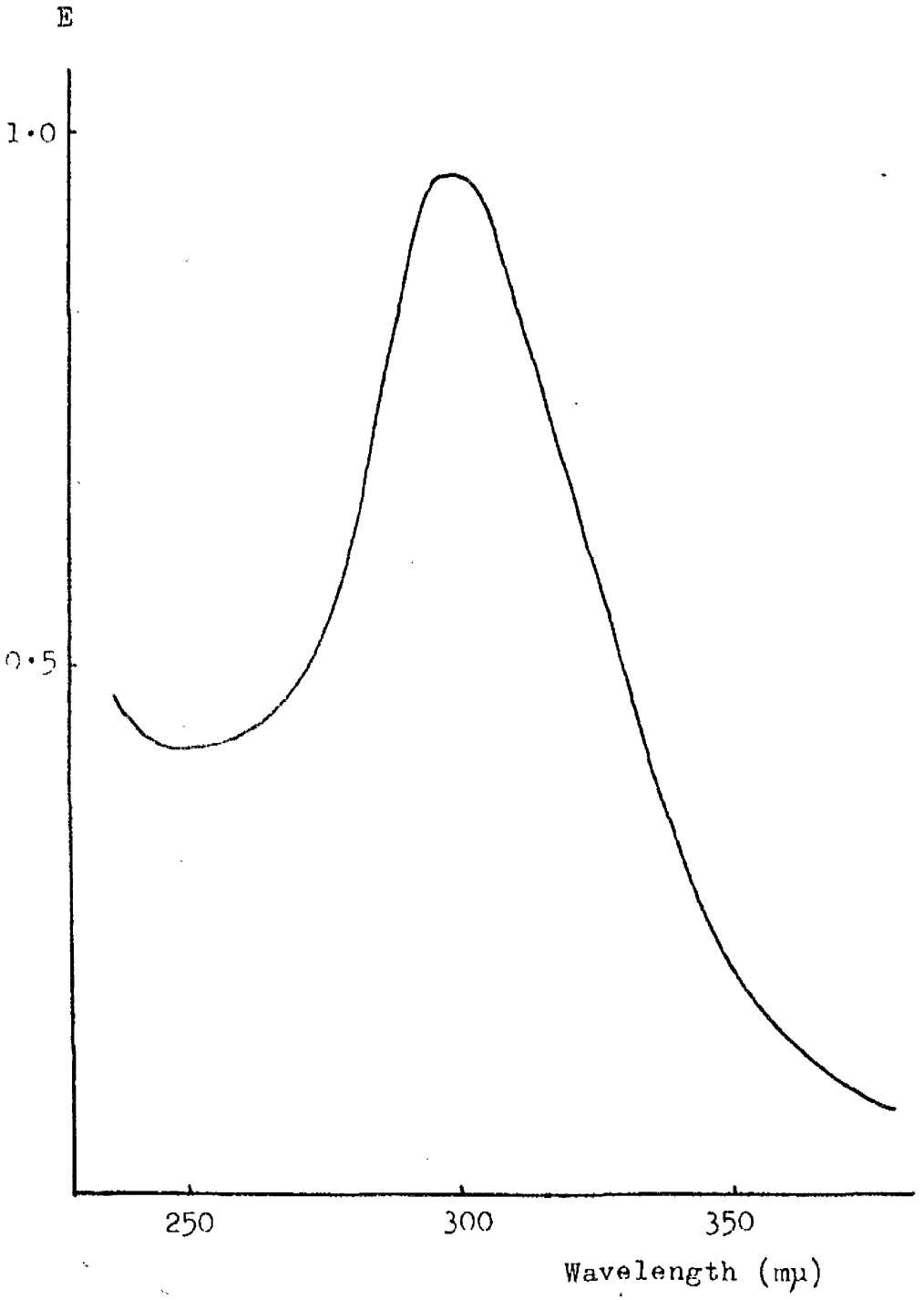
2.9.1. GENERAL MATERIALS

NAD^+, NADH, $NADP^+$, NADPH, acriflavine, benzyl viologen, p-bromophenacyl bromide, folic acid, platinum oxide, protamine sulphate and pyridoxal-5'-phosphate were obtained from British Drug Houses Ltd.

Glycollic acid, glyoxylic acid and EDTA were obtained from L. Light and Co. Ltd.

Ninhydrin (indanetrione hydrate) was obtained from Hopkin and Williams Ltd.

FIG. 2.9.2.A.



ABSORPTION SPECTRUM OF H_4PtG AT pH 7·0

Compounds labelled with radioactive carbon (^{14}C) were obtained from the Radiochemical Centre, Amersham, England.

2.9.2. 5,6,7,8-TETRAHYDROPTEROYLGLUTAMIC ACID

A modification of the method suggested by O'Dell, Vandenbelt, Bloom and Pfiffner (1947) was used.

Folic acid (200 mg.) in glacial acetic acid (120 ml.) was hydrogenated in the presence of Adam's catalyst (hydrated platinum oxide, 150 mg.). 95% of the theoretical hydrogen uptake (21.5 ml. at 760 mm. Hg and 15°) was recorded in 20 min. After 3 hr. hydrogenation the acetic acid solution was separated from the catalyst by syphoning through a scintered glass filter under nitrogen, and lyopholysed. The pale buff powder was stored in vacuum sealed ampoules at -75°.

The absorption spectrum of H_4PtG in phosphate buffer pH 7.0 (containing 0.01M-2-mercaptoethanol) was determined in the absence of oxygen (section 2.6.12) and is illustrated in Fig. 2.9.2.A. The absorption maximum is at 297 mμ; Rabinowitz (1960) quoted 298 mu.

2.9.3. AMINOACETONE

Aminoacetone was prepared by the phthalidide method (Gabriel and Colman, 1902).

Potassium phthalimide (10 g.) and chloroacetone (10 ml.) were heated at 125° in xylene (10 ml.) for 1 hr. The solvent

and excess chloroacetone were removed by steam distillation. The acetonyl phthalimide was purified with charcoal, recrystallised from water and hydrolysed by boiling with 50 ml. 2N-HCl for 4 hr. After cooling, the phthalic acid was removed by filtration and the aminoacetone solution evaporated to dryness at 37° over $CaCl_2$ and KOH. The residue was dissolved in warm ethanol, the NH_4Cl removed by centrifugation, and the crude product recrystallised from ethanol-ether. The colourless product had no definite melting point and was very deliquescent. Aminoacetone toluene-p-sulphonate was prepared (Openshaw, 1955) to characterise the product; M.Pt. 126-129°; Elliott (1960) quoted 130·5°.

2.9.4. [2-^{14}C] GLYCOLLATE

[2-^{14}C]sodium glycollate was prepared from [2-^{14}C] sodium bromacetate and purified by the method described by Jayasuriya (1956).

3. RESULTS

3.1. GLYCINE AMINOTRANSFERASE

3.1.1. Trapping experiments with formate and glyoxylate

3.1.2. Glycine and glyoxylate dissimilation by crude extracts

3.1.3. Glycine aminotransferase

3.1.4. Trapping experiments with active extract

3.1.5. Alternative methods of extract preparation

3.1.6. Conversion of [2-^{14}C] glycollate to [2-^{14}C] glycine

3.1.7. Radioactive assay of glycine aminotransferase

3.1.8. Transamination coupled to glyoxylate removal reactions

3.2. INCORPORATION OF [2-^{14}C] GLYCINE INTO GROWING *E. COLI* PA15

3.2.1. Dilution of incorporation with glyoxylate

3.2.2. Dilution of incorporation with aminoacetone

3.3. GLYCINE-SPLITTING ENZYME

3.3.1. Conversion of glycine to formaldehyde and CO_2 by whole cells

3.3.2. Conversion of glycine to formaldehyde and CO_2 by cell-free extracts

3.3.3. Quantitative assay of $^{14}CO_2$ production from [1-^{14}C] glycine

3.3.4. Glycine catabolism by crude extracts: effect of PLP and NAD^+

3.3.5. Purification of the glycine-splitting enzyme system

3.3.6. Conversion of glycine to formaldehyde and CO_2 by partially purified extracts: effect of glyoxylate

3.3.7. The stoichiometry of the reaction

3.3.8. Sephadex treatment

3.3.9. Glycine oxidation coupled to benzyl viologen reduction

3.3.10. Inhibition of NADH oxidase: anaerobic conditions of assay

3.3.11. Flavoprotein nature of the glycine--splitting enzyme

3.3.12. Inhibition of the reaction by NADH

When E. coli is grown in a glucose-inorganic salt medium (Medium A; Dagley and Dawes, 1949) supplemented with [2-^{14}C]glycine or [3-^{14}C]serine, the C_1-positions of the purine rings, thymine and serine are extensively labelled (Crosbie, 1959), but when the medium is supplemented with [^{14}C]formate the purine C_1-positions are labelled while the thymine methyl-carbon and the serine C-3 are not (Delluva, 1953; Koch and Levy, 1955; Crosbie, 1959). The labelling patterns are shown in Table 3.(i).

The immediate C_1-donors of the purine C-2 and C-8 positions are N-10,formyl-H_4PtG and N-5,N-10,methenyl-H_4-PtG respectively, and the donor of the thymine methyl and the serine C-3 is N-5,N-10,methylene-H_4PtG (section 1.5.), i.e. C_1-units at the oxidation level of formate are required for purine synthesis, but at the oxidation level of formaldehyde for thymine and serine synthesis.

Apparently then, glycine C-2 and serine C-3 can provide methylene-, methenyl- and N-10,formyl-H_4PtG, while formate can only supply methenyl- and N-10,formyl-H_4PtG in growing E. coli.

Koch and Levy analysed the distribution of activity in the purine ring when E. coli was grown in the presence of [^{14}C]formate and found 79% in the C-8 position and only 3.5% in the C-2. The block preventing incorporation of formate into the thymine

TABLE 3.(i).

INCORPORATION OF [2-^{14}C] GLYCINE, [3-^{14}C] SERINE AND [^{14}C] FORMATE INTO GROWING *E. COLI*

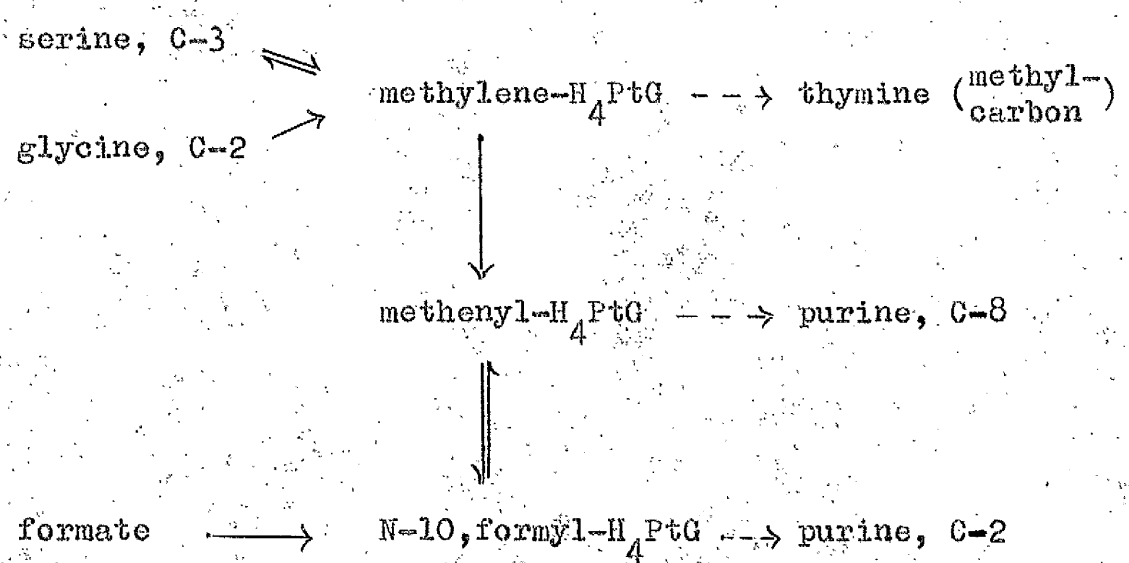
	[2-^{14}C] glycine incorporation	[3-^{14}C] serine incorporation	[^{14}C] formate incorporation
	specific activities (cts/μ-mole/min.)		
Glycine supplement	2,700	-	-
Serine supplement	-	8,500	-
RNA adenine	6,900	17,900	5,100
DNA adenine	6,300	17,900	-
Adenine C-4, C-5 [1]	2,600	-	-
Guanine C-4, C-5 [1]	-	0	0
Guanine C-2, C-4 C-5 and C-8 [2]	-	-	1,500
Thymine	2,500	8,500	0
Cytosine	0	500	0
Protein glycine	2,600	0	0 [3]
Protein serine	5,100	8,500	0 [3]
Protein alanine	0	-	0 [3]

1 isolated as glycine (section 2.5.7.)
2 isolated as 4-guanidinoglyoxaline (Hunter, 1936)
3 autoradiographic evidence

E. coli grown for 24 hr. at 37° in 1 l. glucose- inorganic salt medium (Medium A, Dagley and Dawes, 1949) supplemented with [2-^{14}C] glycine (250 mg.), [3-^{14}C] serine (250 mg.) or [^{14}C] formate (250 mg., added in log. phase). Bases and amino acids were isolated and assayed as described in section 2. Specific activities expressed as 0 were insignificantly different from background.

E. coli PA15 was used for the glycine incorporation experiment. *E. coli* 6386 was used for the serine and formate incorporation experiments; this is a uracil-less mutant and a uracil supplement was added to the medium. The analyses for the serine and formate incorporation experiments were described by Crosbie (1959).

methyl-carbon must occur therefore at the N-5,N-10,methylene-tetrahydrofolate dehydrogenase step.



The reason for the irreversibility of this reaction is not understood but may be explained by postulating rapid utilization of methenyl-H_4PtG with slow reduction to methylene-H_4PtG and rapid formation of the latter compound from alternative sources, or by compartmentation (a veiled term referring to unknown kinetic phenomena).

E. coli PA15 is an auxotrophic strain requiring either glycine or serine for growth and when grown in basal medium (section 2.1.2.) containing glycine, the glycine C-2 is used quantitatively for thymine methyl-group synthesis (Table 3.(i).) For this reason the organism was chosen to study the mechanism by which glycine is converted to C_1-units at the oxidation level of formaldehyde.

Nakada and Weinhouse (1953) adduced evidence for the enzymic oxidation of glycine to formate via glyoxylate in rat liver, viz:

$$NH_2.CH_2.COOH \rightleftharpoons CHO.COOH \longrightarrow H.COOH + CO_2$$

The conclusions of these authors, based on isotope trapping techniques, may have been vitiated however by the demonstration of a facile non-enzymic transamination between glycine and glyoxylate in the presence of trace amounts of certain metal ions (Fleming and Crosbie, 1960).

This scheme though, cannot explain the incorporation pattern of [2-^{14}C]glycine into growing E. coli without alteration to allow the formation of a C_1-unit at the oxidation level of formaldehyde, viz:

$$NH_2.CH_2.COOH \rightleftharpoons CHO.COOH \longrightarrow "H.CHO" \longrightarrow H.COOH$$

The formaldehyde need not occur free, but only as an H_4PtG derivative.

Janke and Tayenthal (1937) demonstrated the formation of glyoxylate from glycine by washed cell suspensions of E. coli, and this conversion has been reported in other organisms (section 1.2.2.). Paretsky and Werkman (1950), using extracts of Achromobacter, showed the production of formaldehyde from glycine, and assumed that glyoxylate was an intermediate, decarboxylating to give formaldehyde. Hydrogen peroxide was

produced also, and this oxidyzed the formaldehyde to formate.

Krakow and Barkulis (1956), while studying the glyoxylate carboligase reaction (section 1.3.3.) in E. coli, suggested a C_1-unit at the oxidation level of formaldehyde might be formed by the decarboxylation of glyoxylate, and this could then condense with a further molecule of glyoxylate to form tartronic semialdehyde or hydroxypyruvate.

For these reasons it was decided to evaluate the role of glyoxylate in C_1-unit production from glycine in E. coli PA15, using an experimental procedure similar to that described by Nakada and Weinhouse (1953) but with suitable precautions to avoid the ambiguity caused by non-enzymic transamination.

3.1.1. TRAPPING EXPERIMENTS WITH FORMATE AND GLYOXYLATE

If a cell-free system converts [2-^{14}C]glycine to [^{14}C]formate an added pool of unlabelled formate should mix freely with the labelled product, and the activity of the formate when isolated at the end of the experiment should be indicative of the extent of the enzymic reaction. Similarly, if glyoxylate is an intermediate in this reaction, added unlabelled glyoxylate should trap activity and dilute the activity recovered in the formate pool.

[2-^{14}C]glycine was incubated with an alumina crude extract, EDTA and pools of formate, or glyoxylate and formate. At the

TABLE 3.1.1.(i).

CONVERSION OF [2-^{14}C] GLYCINE TO GLYOXYLATE AND FORMATE BY EXTRACTS OF E. COLI PA15

Additions	Glyoxylate-specific activities (cts/μ-mole/10 min.)	Formate-specific activities (cts/μ-mole/10 min.)	$BaCO_3$ activity (cts/10 min.)
Formate, EDTA	-	3	70
Glyoxylate, formate, EDTA	61	19	85
Glyoxylate, formate, EDTA(x)	46	24	71
Glyoxylate, formate	1295	12	91
Glyoxylate, formate(x)	1123	8	78

(x) extract heated to 100° for 15 min.

Reaction mixtures in 10.0 ml. 0.067M-phosphate buffer pH 7.0, contained:- alumina crude extract (9.2 mg. protein); [2-^{14}C] glycine, 0.5 m-mole (10 μC/m-mole); and (as indicated) formate, 1.0 m-mole; glyoxylate, 0.25 m-mole; EDTA, 0.5 m-mole. After incubation at 37° for 2 hr. the reactions were stopped by adding 2 ml. 5N-H_2SO_4. Glyoxylate was isolated as glyoxylate-DNP and formate as p-bromophenacyl formate and assayed by the methods described in section 2. Formate was also oxidized to CO_2 with mercuric chloride, precipitated as $BaCO_3$ and counted at infinite thickness (section 2.7.3.).

end of the incubation the pools were isolated and the activities determined.

Glyoxylate was added to the reaction mixture as the sodium salt and isolated as the 2,4-dinitrophenylhydrazone (section 2.5.4.).

Formaldehyde interacts with proteins and inhibits enzyme reactions, so formate, added as sodium formate, was used as the C_1-unit trap. However, formate is difficult to recover in a pure form and a somewhat complicated isolation procedure had to be adopted.

The steam distillates from the incubation mixtures, acidified with H_2SO_4, were evaporated to small bulk and the steam-volatile acids separated on celite columns (Phares et al, 1952; section 2.5.6.).

Formic acid was eluted as a discrete peak, estimated by titration with 0·01N-NaOH, and the neutralized solution concentrated. Part was converted to the p-bromophenacyl derivative (section 2.5.6.) and the remainder was oxidized to CO_2 by boiling with $HgCl_2$ in acetic acid (Pirie, 1946). The oxidation is specific for formate. The CO_2 was collected in 2N-NaOH and counted as $BaCO_3$ (section 2.7.3.).

The activity of the isolated pools showed no evidence for the enzymic conversion of glycine to formate or glyoxylate (Table 3.1.1.(i).). The small background activities of the

glyoxylate and formate, even in the presence of EDTA and a heated extract, were later accounted for as [^{14}C] impurities in the [2-^{14}C] glycine. Non-enzymic transamination was evident in the reaction mixtures without EDTA, but there was not any indication of enzymic formate production from the [2-^{14}C] glyoxylate so produced.

The experiment was repeated with a freshly prepared alumina crude extract and exactly similar results were obtained. Fleming (1960) was unable to demonstrate formate or glyoxylate production from glycine by rat-liver extracts, in the presence of EDTA.

3.1.2. GLYCINE AND GLYOXYLATE DISSIMILATION BY CRUDE EXTRACTS

The results quoted above leave open two interpretations; (i) *E. coli* PA15 does not possess enzyme systems for converting glycine to glyoxylate and formate, or (ii) the enzymes are not extracted in an active form by the alumina grinding process of McIlwain.

To obtain some index of enzymic activity, the rates of glycine and glyoxylate dissimilation by alumina crude extracts were measured.

Glycine estimation Alexander, Landwehr and Seligman (1945) described a specific micromethod for the spectrophotometric determination of glycine. The procedure involved the quantitative reaction of glycine with excess ninhydrin at

pH 5·5, followed by steam distillation of the liberated formaldehyde and subsequent estimation with chromotropic acid.

The method was found to be tedious and the results difficult to reproduce because the steam distillation step was rarely quantitative.

The procedure was modified by omitting the distillation. Ninhydrin withstood heating in 15N-H_2SO_4 at 100° without interferring with the formaldehyde assay, so it was possible to perform both stages of the Alexander et al method, the ninhydrin treatment to liberate formaldehyde and estimation of the latter with chromotropic acid in 15N-H_2SO_4, in a single glass-stoppered tube. The optimum times for the ninhydrin and chromotropic acid treatments were determined by trial. The method is described in section 2.6.6. and the calibration curve (Fig 2.6.6.A.) indicates the accuracy of the determination.

It was necessary to use high initial concentrations of glycine in the dissimilation experiment so that the acidified samples could be diluted 1:100 for estimation. This ensured that the extinction of the solutions was not unduly high because of charring of extract material in 15N-H_2SO_4 at 100°.

Glyoxylate was assayed by a modification of the Friedman and Haugen procedure (section 2.6.8.).

Glycine, in the presence and absence of 2-oxoglutarate, and glyoxylate, in the presence and absence of glutamate, were

TABLES 3.1.2.(i) and (ii).

(i) GLYCINE DISSIMILATION BY EXTRACTS OF E. COLI PA15

Additions	Glycine concn. (μ-moles/reaction mixture)			
	0 min.	15 min.	30 min.	60 min.
Extract, glycine, EDTA(x)	28.2	28.5	28.3	28.2
Extract, glycine, EDTA	28.7	29.0	29.0	28.9
Extract, glycine, EDTA, 2-oxoglutarate	28.1	28.0	28.1	28.0

(ii) GLYOXYLATE DISSIMILATION BY EXTRACTS OF E. COLI PA15

Additions	Glyoxylate concn. (μ-moles/reaction mixture)			
	0 min.	15 min.	30 min.	60 min.
Extract, glyox., EDTA(x)	27.5	29.0	29.8	30.0
Extract, glyox., EDTA	28.2	27.7	28.8	28.9
Extract, glyox., EDTA, glutamate	28.0	28.9	29.9	28.0

(x) extract heated to 100° for 15 min.

Reaction mixtures in 3.5 ml. 0.067M-phosphate buffer pH 7.0, contained (as indicated):- alumina crude extract (10.1 mg. protein); glycine, 100 μ-moles; glyoxylate, 100 μ-moles; EDTA, 100 μ-moles; 2-oxoglutarate, 50 μ-moles; glutamate, 50 μ-moles. 0.1 ml. samples were withdrawn at the specified times, diluted to 10.0 ml. (after the addition of 1 drop 2N-H_2SO_4), and glycine or glyoxylate estimated by the methods described in section 2.

incubated with EDTA and alumina crude extracts at 37°. Samples were withdrawn for glycine and glyoxylate estimations at intervals (Tables 3.1.2.(i) and (ii).).

Neither glycine nor glyoxylate were found to be dissimilated to any significant extent under these conditions.

3.1.3. GLYCINE AMINOTRANSFERASE

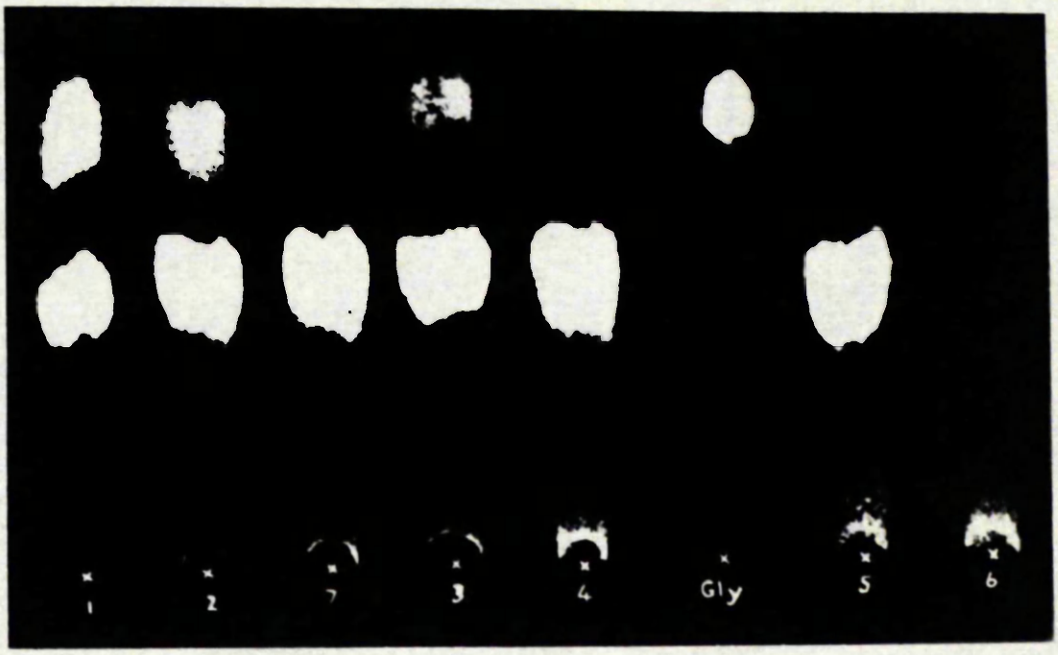
The keto acid estimation of Friedman and Haugen is not specific for glyoxylate and in order to check the validity of the glyoxylate dissimilation experiment, 2,4-dinitrophenylhydrazones were prepared of the keto acids present in the reaction mixtures at the end of the incubation, and these were separated chromatographically.

Glyoxylate-DNP was the only 2,4-dinitrophenylhydrazone present except in the mixture including extract, glyoxylate, glutamate and EDTA, which contained a trace of material with the same R_f as 2-oxoglutarate-DNP. A chromatogram of the amino acids indicated the presence in the same mixture of small amounts of glycine. There was no similar chromatographic evidence for the enzymic formation of glyoxylate from glycine and 2-oxoglutarate in the corresponding tube from the glycine dissimilation experiment.

A fuller investigation using chromatographic analysis, confirmed the presence, in alumina crude extracts, of an aminotransferase which converted glyoxylate and glutamate to glycine

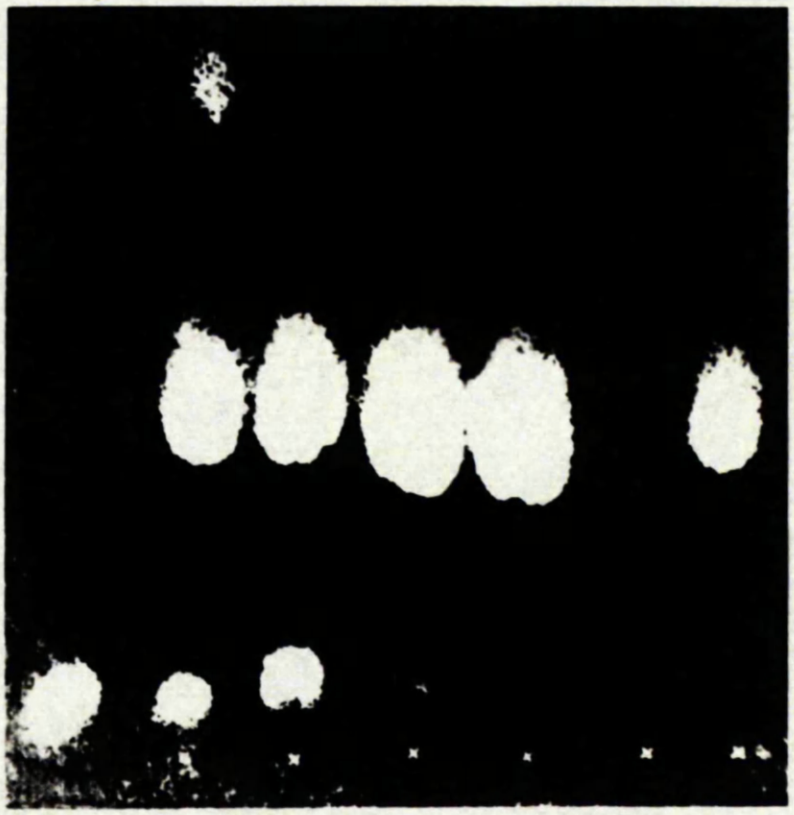
FIG. 3.1.3.A.

CONVERSION OF GLYOXYLATE AND GLUTAMATE TO GLYCINE AND 2-OXO-GLUTARATE BY ALUMINA CRUDE EXTRACTS



1 2 7 3 4 Gly 5 6

Amino acid chromatogram



2-oxo glut. 2 1 3 4 5 Glyox.

Keto acid chromatogram

FIG. 3.1.3.A.

(legend)

CONVERSION OF GLYOXYLATE AND GLUTAMATE TO GLYCINE AND 2-OXOGLUTARATE BY ALUMINA CRUDE EXTRACTS

The components of the systems shown in the chromatograms are:-

1. glyoxylate, glutamate, extract.
2. glyoxylate, glutamate, extract, EDTA.
3. glyoxylate, glutamate.
4. glyoxylate, glutamate, EDTA.
5. glutamate, extract, EDTA.
6. glyoxylate, extract, EDTA.
7. glyoxylate, glutamate, boiled extract, EDTA.

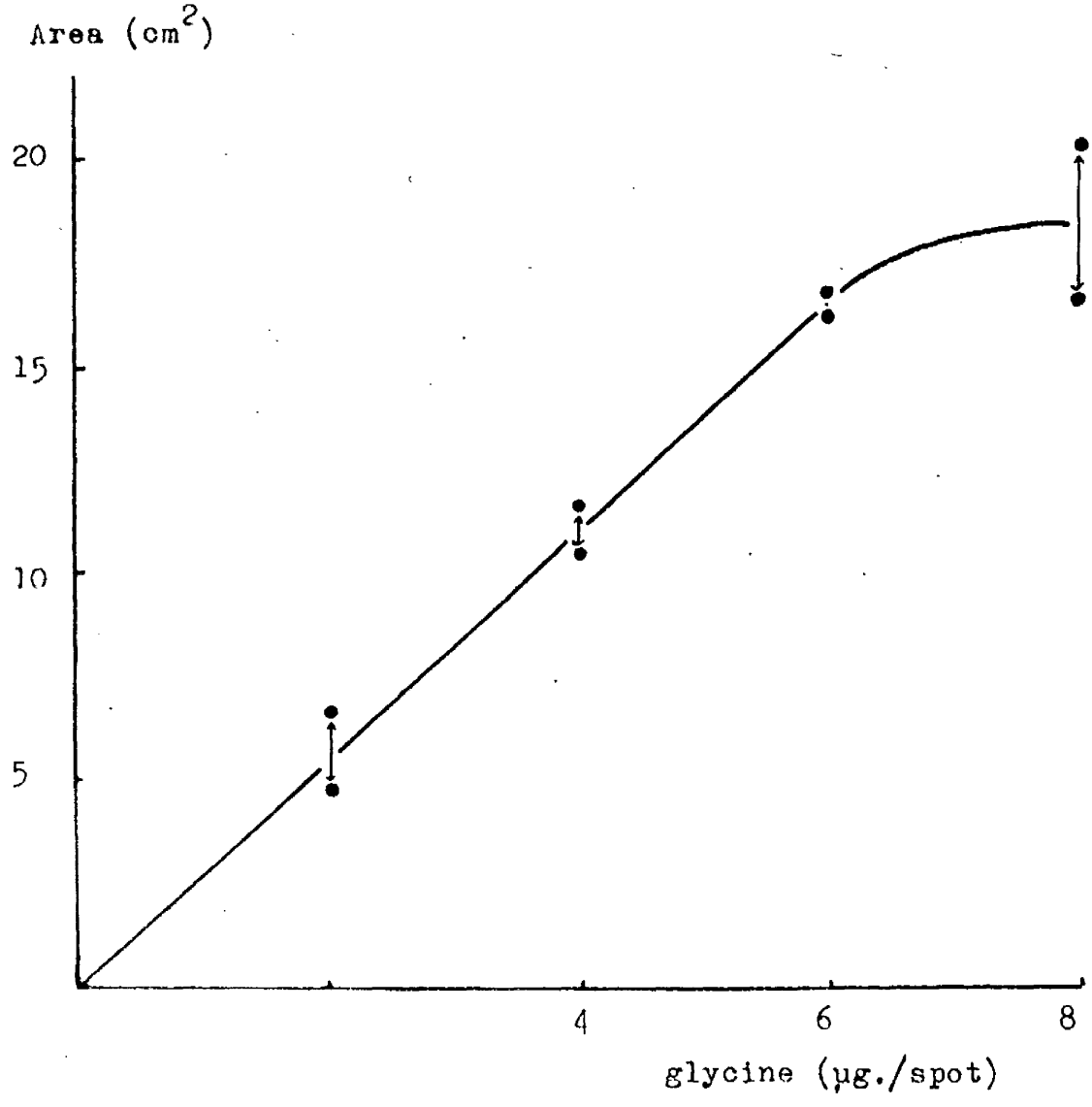
The reaction mixtures in 3·5 ml. 0·067M-phosphate buffer pH 7·0, contained (as indicated):- sodium glyoxylate (70 µ-moles); sodium glutamate (40 µ-moles); EDTA (70 µ-moles); extract (alumina crude extract, 3·8 mg. protein/mixture). After incubation at 37° for 1 hr. the reaction was stopped by adding 0·5 ml samples to 0·5 ml. ethanol. After centrifugation 50 µl. were dispensed for chromatography as described in section 2.

and 2-oxoglutarate in the presence of EDTA (Fig. 3.1.3.A.), but the same techniques were unable to demonstrate the reverse reaction.

The non-enzymic transamination between glyoxylate and glutamate (Nakada and Weinhouse, 1953a) was completely suppressed at pH 7·0 and 37° by the presence of EDTA (Fig. 3.1.3.A.), but if a mixture of glyoxylate and glutamate were dried on the base-line of a chromatogram with hot air, non-enzymic transamination took place even in the presence of EDTA. The solutions applied to chromatograms were therefore dried with cold air. General non-enzymic transamination between 2-keto acid and 2-amino acid mixtures dried on filter papers had previously been observed at temperatures in excess of 80°, but not at all at 28° (Giri and Kalyanker, 1953).

Several methods were tried to convert the chromatographic analysis into a quantitative assay for transaminase activity; (i) the glycine spots were eluted in 3 ml. phosphate buffer pH 5·5, and the glycine conventration of the eluate determined with ninhydrin and chromotropic acid; (ii) after dipping the chromatograms in acetone containing 1% ninhydrin, the amino acid spots were developed at 37° for 2 hr. (optimum time and temperature for maximum colour development, as determined by trial) and eluted in 3 ml. 5% $ZnSO_4$. The extinctions of the eluates were measured at 485 mμ (the absorption maximum); (iii) the glycine spots were developed to maximum intensity

FIG. 3.1.3.B.

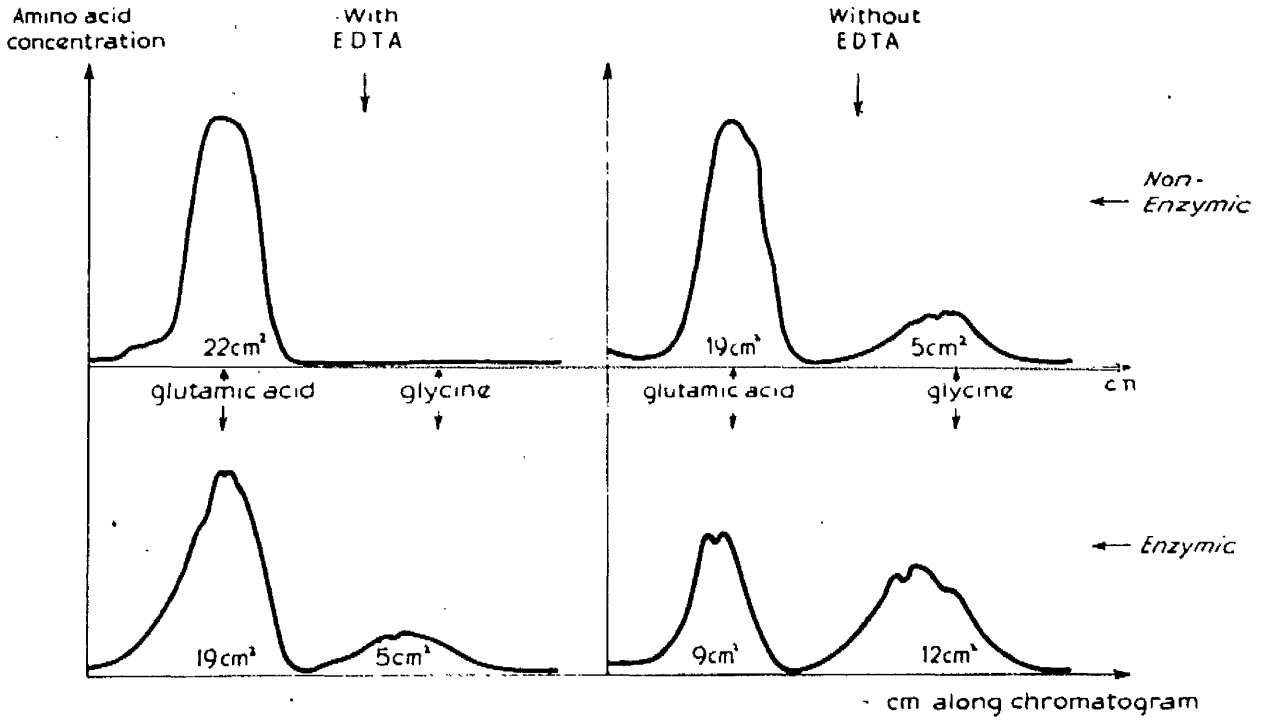


CHROMATOGRAPHIC ESTIMATION OF GLYCINE

Glycine solutions (20·0 µl. containing 0–8 ug. glycine) were applied to Whatman No. 1 paper and the chromatogram run with phenol:water (80:20 w/w). After washing in ether, the chromatogram was dipped in acetone containing 1% ninhydrin and dried at 37° for 2 hr. then sprayed with sat. $ZnSO_4$. After drying the chromatogram lanes were analyzed in a recording densitometer with an automatic integrator (Beckman/ Spinco Model RB Analytrol) which gave directly the areas under the absorption peaks representing the glycine spots.

FIG. 3.1.3.C.

GLYCINE AMINOTRANSFERASE ACTIVITY: CHROMATOGRAPHIC ASSAY



Reaction mixtures in 5·0 ml. 0·067M-phosphate buffer pH 7·0, contained: sodium glyoxylate, 100 µ-moles; sodium glutamate, 100 µ-moles; and (as indicated) EDTA, 100 µ-moles; alumina crude extract (1·9 mg. protein). After incubation at 37° for 1 hr., the reaction was stopped by adding 0·5 ml. ethanol to 0·5 ml. reaction mixture. After centrifugation, 30 µl. supernatant was chromatographed in phenol:water (section 2.5.3.) After treatment with ninhydrin the spots were developed by heating (see text) and treated with sat. $ZnSO_4$. Cromatogram strips were analysed with a recording densitometer (Beckman/ Spinco Model RB Analytrol).

with ninhydrin and heating (as in ii), then sprayed with saturated $ZnSO_4$. After drying, chromatogram lanes were analysed in a recording densitometer with an automatic integrator (Beckman/Spinco Model RB Analytrol) which gave directly the areas under the absorption peaks representing the amino acid spots.

Methods (i) and (ii) did not give concordant results for repeated determinations, but method (iii) gave a reproduceable straight-line calibration curve between the limits 0 and 6 ug. glycine per spot (Fig. 3.1.3.B.). This method was used to estimate the aminotransferase activity (Fig. 3.1.3.C.). In the presence of EDTA, the rate of glycine formation from glyoxylate and glutamate by the aminotransferase was 0·23 µ-mole/hr./mg. protein (alumina crude extract).

The enzyme catalyzing this transamination was designated glycine:2-oxoglutarate aminotransferase even though the forward reaction had not been demonstrated, for an enzyme of this name, operating in both directions, had previously been identified in crude extracts of barley, oat, mung bean and white lupine seedlings (Wilson, King and Burris, 1954).

3.1.4. TRAPPING EXPERIMENTS WITH AN ACTIVE EXTRACT

The chromatographic analysis technique provided a measure of the aminotransferase activity in the direction glyoxylate to glycine, but failed to show the reverse reaction. The radioactive

TABLE 3.1.4.(i).

GLYOXYLATE FORMATION FROM [2-^{14}C]GLYCINE BY EXTRACTS OF *E. COLI* PA15

Additions	Glyoxylate-specific activities (cts/μ-mole/10 min.)	
	0 hr.	3 hr.
-	1900	3790
EDTA	1900	1917
Extract	1900	3662
Extract, EDTA	1900	2025
Extract, EDTA, 2-oxoglutarate	1900	1972

Reaction mixtures in 5·5 ml. 0·067M-phosphate buffer pH 7·0, contained:- [2-^{14}C]glycine, 25 μ-moles (1 μC/μ-mole); glyoxylate, 25 μ-moles; and (as indicated) crude alumina extract (4·2 mg. protein); EDTA, 25 μ-moles; 2-oxoglutarate, 25 μ-moles. After incubation at 37° for 3 hr. the glyoxylate was isolated and assayed by the methods described in section 2. The glyoxylate-specific activity at zero time (see section 3.1.1.) was measured in a reaction mixture containing glycine and glyoxylate.

trapping procedure (section 3.1.1.) was more sensitive, but [^{14}C] glyoxylate was not available and the reverse reaction, namely glyoxylate to glycine, could not be studied.

The two methods were therefore combined. An extract shown to have glycine aminotransferase activity (glyoxylate to glycine) by chromatographic analysis, was incubated with [2-^{14}C] glycine, 2-oxoglutarate and EDTA to study the glycine to glyoxylate reaction in a trapping experiment. As the results show (Table 3.1.4.(i).), there is no evidence for enzymic [2-^{14}C] glyoxylate formation from [2-^{14}C] glycine despite the fact that the extract showed activity in the reverse direction.

3.1.5. ALTERNATIVE METHODS OF EXTRACT PREPARATION

The activity of the aminotransferase was small in one direction and not apparent in the other, so alternative methods of preparing cell extracts were tried to increase the reaction rate.

(i) glass-grinding. Packed cells were ground at 0° with glass beads (200 mesh) in an exactly similar way to the alumina-grinding method of McIlwain (1948, section 2.2.1.).

(ii) acetone-powder. An acetone-powder was prepared by the procedure of Szulmajster and Woods (1960). The dry powder was mixed in a Potter-Elvehjem homogenizer with 0·067M-phosphate buffer pH 7·0 at 0° (20 mg./ml.) and centrifuged at 20,000 g. for 20 min.

(iii) sonic disruption. Cells were suspended in 0·067M-phosphate buffer pH 7·0 (0·5 g. wet weight/ml.) and disrupted by sonic oscillation in a Ratheon Magnetostrictor Oscillator (section 2.2.2.).

Extracts prepared these ways, and an alumina crude extract, were assayed qualitatively by chromatographic analysis. Glass-ground and acetone-powder extracts showed little activity. Alumina ground and sonic extracts were equally active.

3.1.6. CONVERSION OF [2-^{14}C] GLYCOLLATE TO [^{14}C] GLYCINE

Chromatographic evidence suggested that glyoxylate was converted to glycine by an aminotransferase: unequivocal evidence was sought using [2-^{14}C] glyoxylate prepared enzymically from [2-^{14}C] glycollate.

E. coli PA15 will grow in a sodium glycollate-inorganic salt medium supplemented with glycine (Stewart, 1961). Glyoxylate plays a central role in the metabolism of such cells (section 1.3.5.) and glycollate is rapidly converted to glyoxylate by sonic crude extracts.

[2-^{14}C] Glycollate was prepared (4 μ-moles/ml.; 5 μC/μ-mole) by the procedure of Jayasurya (1956) from [2-^{14}C] 2-bromacetate (section 2.9.3.).

Sonic crude extracts of glucose and glycollate grown cells were incubated, separately and together, with [2-^{14}C] glycollate, glutamate and EDTA. The incubation products

TABLE 3.1.6.(i).

CONVERSION OF GLYCOLLATE TO GLYCINE BY EXTRACTS OF GLUCOSE AND GLYCOLLATE GROWN *E. COLI* PA15

Additions	% total counts	
	Base line	Glycine
Glycollate extract	97·3	2·7
Glycollate extract + glucose extract	93·9	6·1
Glucose extract	99·7	0·3

Reaction mixtures contained, in 1·0 ml. 0·067M-phosphate buffer pH 7·0:- glycollate, 2·0 μ-moles (5 μC/μ-mole); glutamate, 2·0 μ-moles; EDTA, 2·0 μ-moles; and (as indicated), sonic crude extract glycollate grown cells (6·2 mg. protein); sonic crude extract glucose grown cells (5·9 mg. protein). After incubation at 37° for 1 hr., the reaction products were separated by high-voltage electrophoesis in a formic acid-acetic acid solvent (section 2.4.1.). The glycine spots, located with a Geiger-Müller end window tube and ratemeter, and the active spots remaining on the base line were eluted in 5·0 ml. water, and plated at infinite thinness for counting.

TABLE 3.1.7.(i).

GLYCINE AMINOTRANSFERASE ACTIVITIES IN EXTRACTS OF GLUCOSE AND GLYCOLLATE GROWN E. COLI PA15

Additions	Glycine formed (total counts/10 min.)
-	40
Glucose extract	9,500
Glycollate extract	908

Reaction mixtures in 1·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- [1-^{14}C]glyoxylate, 2·0 µ-moles (33 µC/m-mole); glutamate, 2·0 µ-moles; EDTA, 2·0 µ-moles; PlP, 0·1 µ-moles; and (as indicated) sonic crude extract glucose grown cells (8·1 mg. protein); sonic crude extract glycollate grown cells (6·7 mg. protein). After incubation for 45 min. at 37°, the reaction products were separated by high-voltage paper electrophoresis in a formic acid-acetic acid solvent. The glycine spots were eluted in 5·0 ml. water and the glycine plated at infinite thinness for counting (the method is described in section 2.4.1.).

were separated by high voltage electrophoresis and [2-^{14}C]glycine formation assayed as total counts isolated per tube (section 2.4.1.)

The glycollate extract showed slight aminotransferase activity, but the addition of the glucose extract increased [2-^{14}C]glycine production by a factor of three (Table 3.1.6.(i).).

3.1.7. RADIOACTIVE ASSAY OF GLYCINE AMINOTRANSFERASE

Later, when [1-^{14}C]glyoxylate became available, and using more precise assay conditions (section 2.4.1.), aminotransferase activities were assayed in the two extracts. [^{14}C]glycine production from [1-^{14}C]glyoxylate in the presence of glutamate and EDTA was much less with extracts of glycollate-grown cells than with extracts of glucose-grown cells (Table 3.1.7.(i).).

3.1.8. TRANSAMINATION COUPLED TO GLYOXYLATE REMOVAL REACTIONS

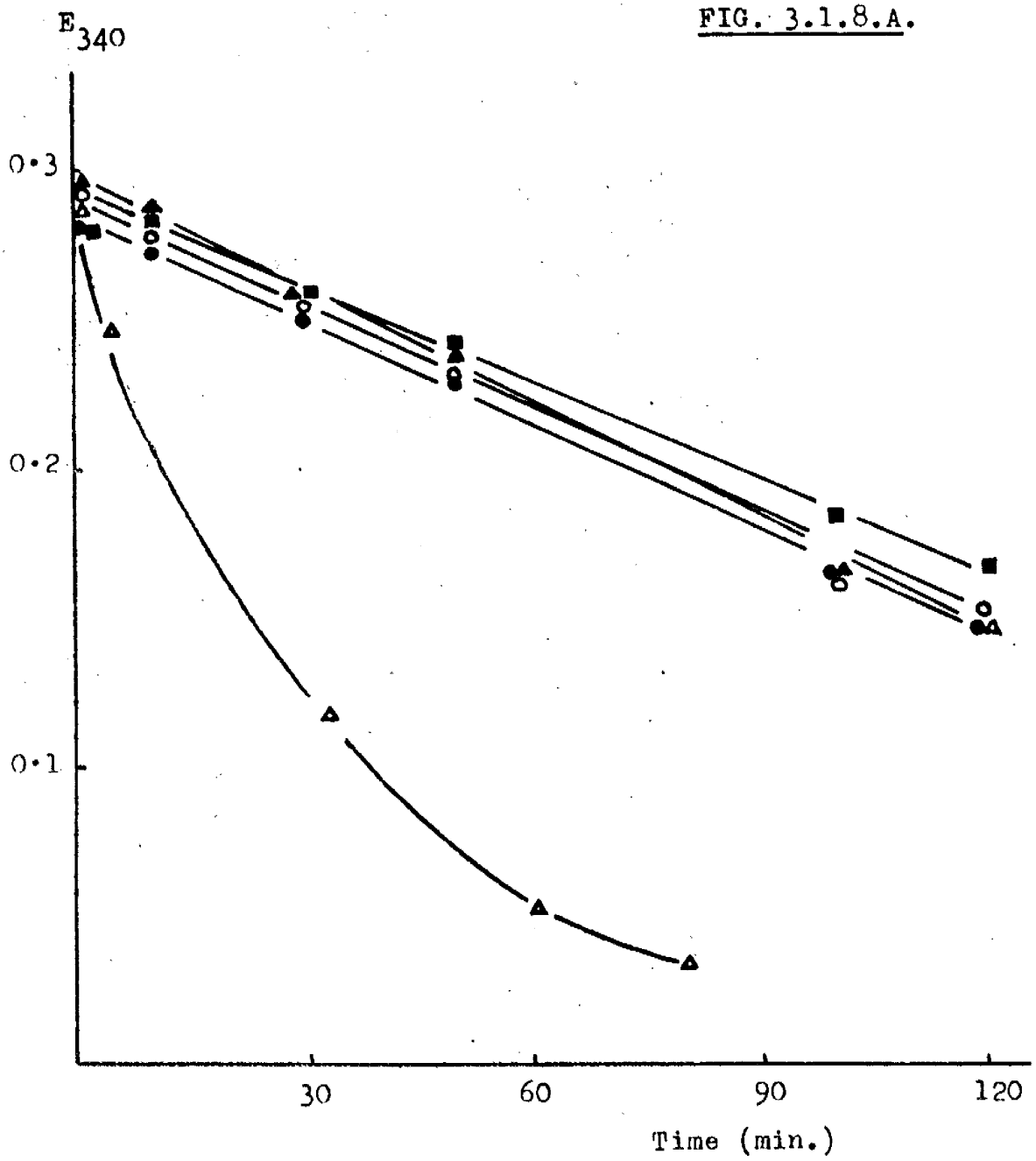
When *E. coli* is grown aerobically on glucose the cells contain malate synthetase but virtually no isocitrate lyase (Reeves and Ajl, 1960). Dixon, Kornberg and Lund (1960) failed to show reversal of malate synthetase (i.e. the formation of glyoxylate from malate): the standard free energy change of the reaction is about -12,000 cal. (Kornberg and Elsden, 1961). Glucose grown *E. coli* PA15 possess a glycine:2-oxoglutarate aminotransferase and the equilibrium of the reaction is greatly in favour of glycine production, but the cells have no source of glyoxylate.

Despite the equilibrium, if glyoxylate is not available in the growing cell, one possible function of the aminotransferase could be the formation of glyoxylate from glycine. Demonstration of the reaction in this direction would support a scheme of C_1-unit production from glycine involving glyoxylate.

Attempts were therefore made to force the reaction in the forward direction in the in vitro system, by coupling possible glyoxylate formation to rapid and irreversible glyoxylate removal reactions, e.g. the glyoxylate caboligase reaction and the glyoxylate reductase reaction.

Glyoxylate carboligase Mixed extracts of glucose and glycollate grown cells were incubated with [2-^{14}C]glycine, 2-oxoglutarate and EDTA. A pool of unlabelled tartronic semialdehyde (prepared enzymically from glyoxylate using an extract of glycollate grown E. coli as source of glyoxylate carboligase) was added at the end of the incubation. 2,4-Dinitrophenylhydrazine hydrochloride was added and the acidic 2,4-dinitrophenylhydrazones (isolated by a method similar to that described in section 2.5.4.) were separated on an alumina column eluted with chloroform. The orange-yellow product, when added to 5N-NaOH, gave the purple coloration typical of tartronic semialdehyde bis-2,4-dinitrophenylhydrazone (Callely and Dagley, 1959). The activity of the product was indistinguishable from background.

FIG. 3.1.8.A.



GLYOXYLATE REDUCTASE

FIG. 3.1.8.A.

(legend)

GLYOXYLATE REDUCTASE

The components of the systems illustrated on the graph are:-

- △ - NADPH, glyoxylate, extract.
- ● - NADPH, glycine, extract.
- ▲ - NADPH, 2-oxoglutarate, extract.
- ■ - NADPH, 2-oxoglutarate, glycine, extract
- o - NADPH, extract.

The reaction mixtures in 3·0 ml. 0·1M-tris buffer, pH 7·2, contained:- $MgCl_2$, 4·0 µ-moles; EDTA, 10 µ-moles; and (as indicated) NADPH, 0·2 µ-mole; glyoxylate; 10 µ-moles; glycine, 10 µ-moles; 2-oxoglutarate, 10 u-moles; 60-80% ammonium sulphate extract. The mixtures were incubated in quartz cuvettes at 37°, and the reactions followed by measuring the changes in extinction at 340 mµ.

Glyoxylate reductase Glyoxylate is reduced to glycollate by cell-free extracts of E. coli, in the presence of NADPH, and Mg^{2+}. Glyoxylate production from glycine and 2-oxoglutarate (in the presence of EDTA) by transamination can be followed in such a system by measuring the change in extinction at 340 mμ.

Glyoxylate, and a mixture of glycine, 2-oxoglutarate and EDTA were incubated with NADPH, $MgCl_2$ and extract, with suitable controls, and the reaction followed at 37°. Only the mixture with glyoxylate present showed any significant decrease in extinction at 340 mμ.; there was no evidence for glyoxylate formation by transamination.

3.2. INCORPORATION OF [2-^{14}C]GLYCINE INTO GROWING E. COLI PA15

The conversion, by a cell-free system, of glycine to C_1-compounds which can be isolated and assayed is an ideal arrangement for studying the role of possible intermediates and for determining the mechanism of the reaction. The only C_1-compound suitable for this type of experiment is formate, but studies have failed to show the production of formate from glycine by cell-free extracts.

The utilization of glycine C-2 for the synthesis of TMP from dUMP, or methionine from homocysteine by *in vitro* systems would provide an equally valuable arrangement for the investigation.

However, Birnie (1959) using alumina-ground extracts of *E. coli* was able to show with difficulty only very low levels of thymidylate synthesis from dUMP and serine, an efficient C_1-donor. Kisliuk and Woods (1960) demonstrated the synthesis of methionine from homocysteine by extracts of *E. coli* PA15 with serine or glycine as C_1-donors. The procedure was repeated with a sonic crude extract of *E. coli* PA15, an extract of heated organisms, H_4PtG and glycine or serine. The methionine formed was assayed microbiologically with *Leuconostoc mesenteroides* (N.C.I.B. catalogue No. 8018).

Formation of methionine from homocysteine and serine was easily detected, but with glycine as C_1-donor the yield

was too small and variable for the method to be suitable as an assay of C_1-unit production.

The one system available where glycine C-2 was readily and quantitatively converted to C_1-units appeared to be E. coli PA15 growing in basal medium, and this system was reverted to in an attempt to elucidate the intermediates of the conversion.

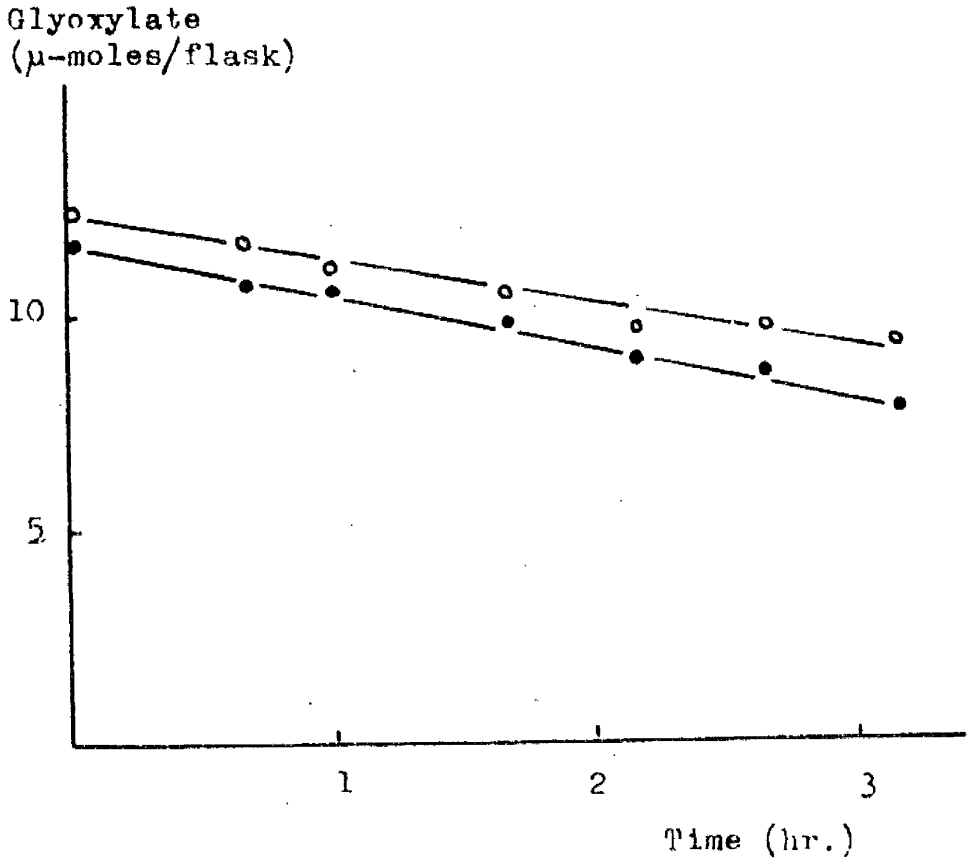
Intermediates in a synthetic scheme are maintained by the cell at suitable concentrations for further reactions, and these intermediates (extractable with short exposure to cold 5% TCA - Britten and McClure, 1962) are collectively named the pool of metabolic intermediates, or more simply, the 'pool'.

Exogenous compounds which permeate bacterial cell walls are often concentrated within the cell, but the ultimate chemical fates of these compounds may be different from those of identical compounds synthesized by the cell and constituting the 'pool'.

McClure and Britten (1962) demonstrated exchange between the 'pool' amino acids of E. coli and amino acids in the medium, even in the absence of an energy source, but the rate of exchange was limited and dependent, not on the external concentration, but on the 'pool' size.

If the incorporation of activity from [2-^{14}C]glycine

FIG. 3.2.1.A.



GLYOXYLATE DISSIMILATION BY WASHED CELL SUSPENSIONS

Reaction mixtures in 20 ml. 0·067M-phosphate buffer pH 7·0, contained:- washed cells (250 mg. dry wt.); glyoxylate, 1 mg.

Glyoxylate removal from the medium (●):- 1 ml. samples were removed, at the time intervals indicated, and the cells removed by centrifuging. Glyoxylate was estimated in the supernatants (section 2.6.8.).

Total glyoxylate dissimilation by the cells (○):- 1·0 ml. samples were removed, at the time intervals indicated, and added to 1·0 ml. 5% TCA. After centrifuging, glyoxylate was estimated in the supernatants (section 2.6.8.).

into the C_1-positions of thymine, serine and the purine ring is diluted in the presence of an unlabelled supplement added to the medium, the supplement is probably chemically identical with, or an immediate precursor of, an intermediate in the scheme of C_1-unit formation.

An experiment of this type depends on four premises: (i) the supplement does not inhibit cell growth, (ii) the supplement enters the cells, (iii) the unlabelled supplement within the cells is in equilibrium with the 'pool', (iv) the supplement is not converted to glycine which is then converted to C_1-units by a different pathway, and (v) the supplement is not converted to C_1-units by an independent pathway, thus diluting incorporation at the C_1-unit stage. Glyoxylate, aminoacetone and 5-aminolaevulinate are possible intermediates suitable for this type of investigation.

3.2.1. DILUTION OF INCORPORATION WITH GLYOXYLATE

Glyoxylate added to the basal medium does not inhibit the growth of *E. coli* PA15. Washed-cell suspensions remove glyoxylate from a buffered solution; part of this glyoxylate is recovered from the cells by extraction with cold 5% TCA (Fig. 3.2.1.A.), but the remainder is metabolized.

E. coli PA15 was grown in basal medium supplemented with [2-^{14}C] glycine and glyoxylate. After 24 hr. the cells were

TABLE 3.2.1.(i).

DISSIMILATION OF GLYOXYLATE BY GROWING *E. COLI* PA15

	Total keto acid concentration	% glyoxylate	% pyruvate
Before growth	0·24 mg./ml.	100	0
After growth	0·23 mg./ml.	57	43

The keto acid concentration of the medium, before and after growth, of the incorporation experiment (with added glyoxylate) described in Table 3.2.1.(ii), was measured by the Friedman and Haugen procedure (section 2.6.8.).

The keto acids present in the medium at the finish were separated by paper chromatography (section 2.5.4) and the concentration of each component measured by eluting the spots in 3·0 ml. 0·5% $NaHCO_3$. The solutions were estimated spectrophotometrically (section 2.6.9.), and the proportion of each keto acid present, calculated.

TABLE 3.2.1.(ii).

INCORPORATION OF [2-^{14}C]GLYCINE INTO GROWING E. COLI PA15

I. MEDIUM SUPPLEMENTED WITH GLYOXYLATE

	Control cells	Cells with glyoxylate supplement
	Specific activities (cts/μ-mole/min.)	
Glycine supplement	13,700	13,700
DNA adenine	22,500	26,100
RNA adenine	26,200	24,700
Adenine C-4,C-5 [1]	14,100	16,200
Thymine	12,200	11,300
Cytosine	0	0

1 adenine C-4,C-5 isolated as glycine (section 2.5.7.)

E. coli PA15 grown for 24 hr. at 37° in 1 l. basal medium supplemented with [2-^{14}C]glycine (200 mg.). Bases and amino acids were isolated as described in section 2. Specific activities expressed as 0 were insignificantly different from background.

Glyoxylate (250 mg.) was added to the medium in a duplicate experiment.

TABLE 3.2.1.(iii).

INCORPORATION OF [2-^{14}C]GLYCINE INTO GROWING *E. COLI* PA15

II. GROWTH ON GLYCOLLATE

	Glucose-grown cells	Glycollate-grown cells
	Specific activities (cts/μ-mole/min.)	
Glycine supplement	74,000	74,000
RNA adenine	150,000	149,000
DNA adenine	118,000	144,000
Thymine	57,000	67,000
Cytosine	0	0

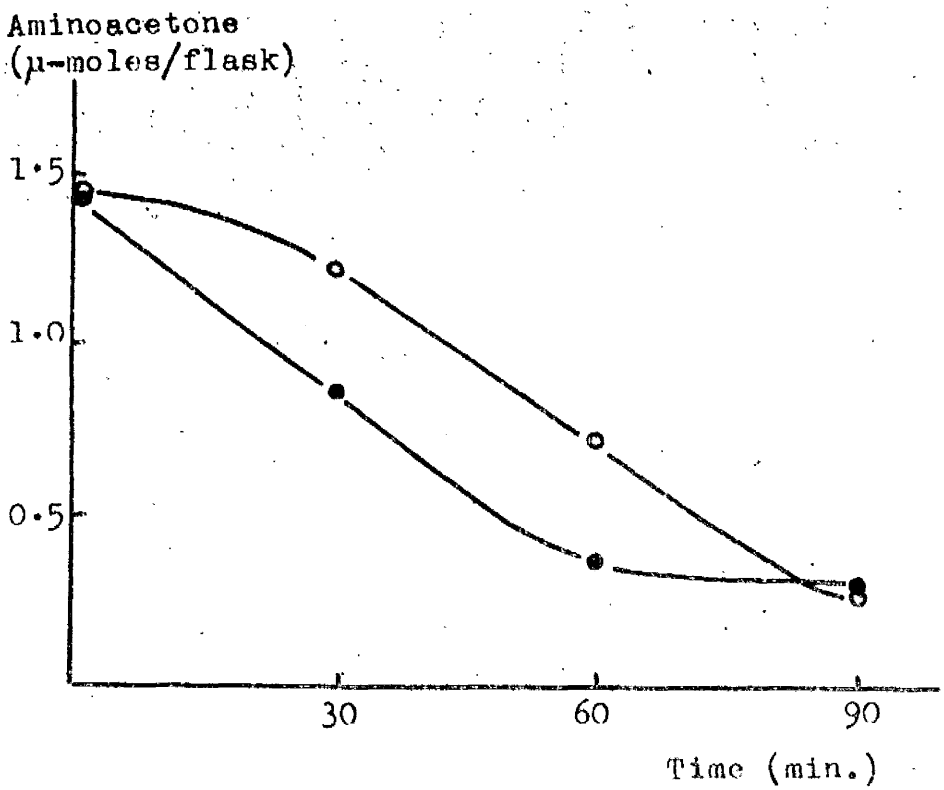
E. coli PA15 grown for 24 hr. at 37° in basal medium (1 l.) and a sodium glycollate (3·75 g./l.) -inorganic salt medium (1 l.; Stewart, 1961) supplemented with [2-^{14}C]glycine (200 mg.). Bases were isolated and assayed as described in section 2. Specific activities expressed as 0 were insignificantly different from background.

harvested and the DNA, RNA and protein isolated and degraded by the methods described in section 2. Total keto acids present in the medium at the beginning and end of growth were estimated the procedure of Friedman and Haugen (section 2.7.8.). Chromatograms of the keto acid-2,4-dinitrophenylhydrazones were prepared and the proportions of the different keto acids present were determined by a method similar to that described in section 2.7.9. Glyoxylate was present at the beginning and equal amounts of glyoxylate and pyruvate were present at the end (Table 3.2.1.(i).).

The incorporation of the [2-^{14}C]glycine into the C_1-positions was found to be unaffected by the presence of extracellular glyoxylate (Table 3.2.1.(ii).).

The value of this negative result depends on the validity of premise (iii) above, i.e. unlabelled glyoxylate in the cell mixes with the 'pool' glyoxylate, and there are no criteria that can be used to judge this. However, further evidence can be adduced in favour of a pathway not involving glyoxylate by altering the properties of the glyoxylate 'pool' in the growing cell. This will probably change the conditions governing the equilibrium between glyoxylate derived from exogenous sources and 'pool' glyoxylate. E. coli PA15 will grow in a glycollate-inorganic salt medium supplemented with glycine, and glyoxylate plays a central role in the metabolism of such cells. A large flux of glyoxylate will accordingly pass through

FIG. 3.2.2.A.



AMINOACETONE DISSIMILATION BY WASHED CELL SUSPENSIONS E. COLI PA15

Reaction mixtures contained in 5·0 ml. 0·067M-phosphate buffer pH 7·0 :- washed cells (10 mg. dry wt.); aminoacetone, 100 μg.

Aminoacetone removal from the medium (●):- cells were removed by centrifuging, at the time intervals indicated, and aminoacetone estimated in the supernatant (section 2.6.1.)

Total aminoacetone dissimilation by the cells (○):- 2·0 ml. 5% TCA was added to the suspensions, at the time intervals indicated, and aminoacetone estimated (section 2.6.1.) in the supernatants after centrifuging.

TABLE 3.2.2.(i).

INCORPORATION OF [2-^{14}C] GLYCINE INTO GROWING E. COLI PA15

III. MEDIUM SUPPLEMENTED WITH AMINOACETONE

	Control cells	Cells with aminoacetone supplement
	Specific activities (cts/μ-mole/min.)	
Glycine supplement	27,000	27,000
RNA adenine	68,000	68,900
Thymine	25,400	24,900
Cytosine	0	100
Protein glycine	25,600	26,100
Protein serine	51,000	52,900

E. coli PA15 grown for 24 hr. at 37° in 1 l. basal medium supplemented with [2-^{14}C] glycine (200 mg.). Bases and amino acids were isolated as described in section 2.

Aminoacetone (500 mg.) was added to the medium in a duplicate experiment.

the 'pool'.

E. coli PA15 were grown in a glycollate-inorganic salt medium (Stewart, 1961) supplemented with [2-^{14}C]glycine, and the cells treated as in the previous experiment. [2-^{14}C]Glycine was incorporated without dilution (with respect to cells grown in parallel on glucose) into the C_1-positions of thymine, serine and the purine ring (Table 3.2.1.(iii).). This suggests that glyoxylate is unlikely to lie on the pathway of C_1-unit production.

3.2.2. DILUTION OF INCORPORATION WITH AMINOACETONE

Similar experiments were performed with aminoacetone, which does not inhibit cell growth when added to the basal medium, and is rapidly metabolized by washed-cell suspensions of E. coli PA15 (Fig. 3.2.2.A.).

E. coli PA15 was grown in basal medium supplemented with [2-^{14}C]glycine and aminoacetone. After 24 hours the cells were harvested and treated as before. 73% of the aminoacetone disappeared from the medium during cell growth.

The presence of aminoacetone did not dilute the incorporation of [2-^{14}C]glycine into the C_1-positions (Table 3.2.2.(i).).

Note. Washed cell suspensions were shown to convert [1-^{14}C]glycine to $^{14}CO_2$, and [2-^{14}C]glycine to formaldehyde, so further investigations were directed to the study of this

simpler system, and incorporation studies in the presence of extracellular 5-aminolaevulinate were not carried out.

COLLECTION OF ENZYMICALLY LIBERATED $^{14}CO_2$

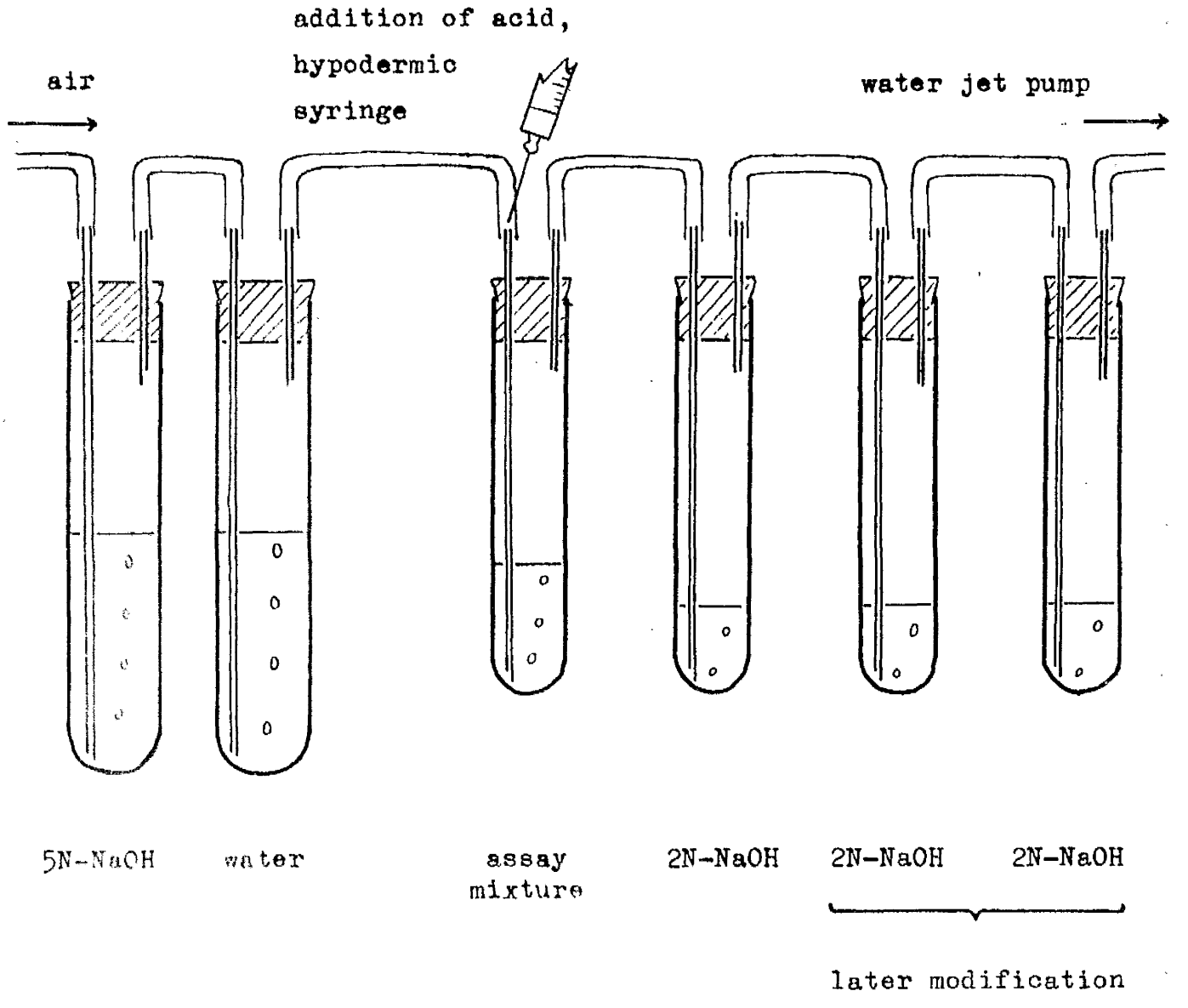


FIG. 3.3.1.A.

3.3. THE GLYCINE-SPLITTING ENZYME

3.3.1. CONVERSION OF GLYCINE TO FORMALDEHYDE AND CO_2 BY WHOLE CELLS

The production of $^{14}CO_2$ from [1-^{14}C]glycine provided an index of glycine catabolism by washed-cell suspension of *E. coli* PA15, while concurrent experiments with [2-^{14}C]glycine were designed in an attempt to show [^{14}C]formaldehyde production.

Whole cells maintain a 'pool' of metabolic intermediates, and it was hoped that cells metabolizing [2-^{14}C]glycine would maintain a small but finite concentration of [^{14}C]formaldehyde (or some compound which could readily be hydrolyzed to [^{14}C]formaldehyde, e.g. hydroxymethyl-H_4PtG) which could be isolated by acid treatment of the cells.

E. coli PA15, freshly grown, harvested and washed, were suspended in phosphate buffer with [1-^{14}C]glycine or [2-^{14}C]glycine, and slow streams of CO_2-free air were drawn through the incubation mixtures. The liberated $^{14}CO_2$ was collected by bubbling the outgoing air through 2N-NaOH. The experimental arrangement is illustrated in Fig. 3.3.1.A. After the addition of carrier carbonate, the $^{14}CO_2$ was counted as $Ba^{14}CO_3$ at infinite thickness (section 2.7.2.).

At the end of the incubation the cell suspensions were

TABLE 3.3.1.(i).

CONVERSION OF GLYCINE TO FORMALDEHYDE AND CO_2 BY WHOLE CELLS

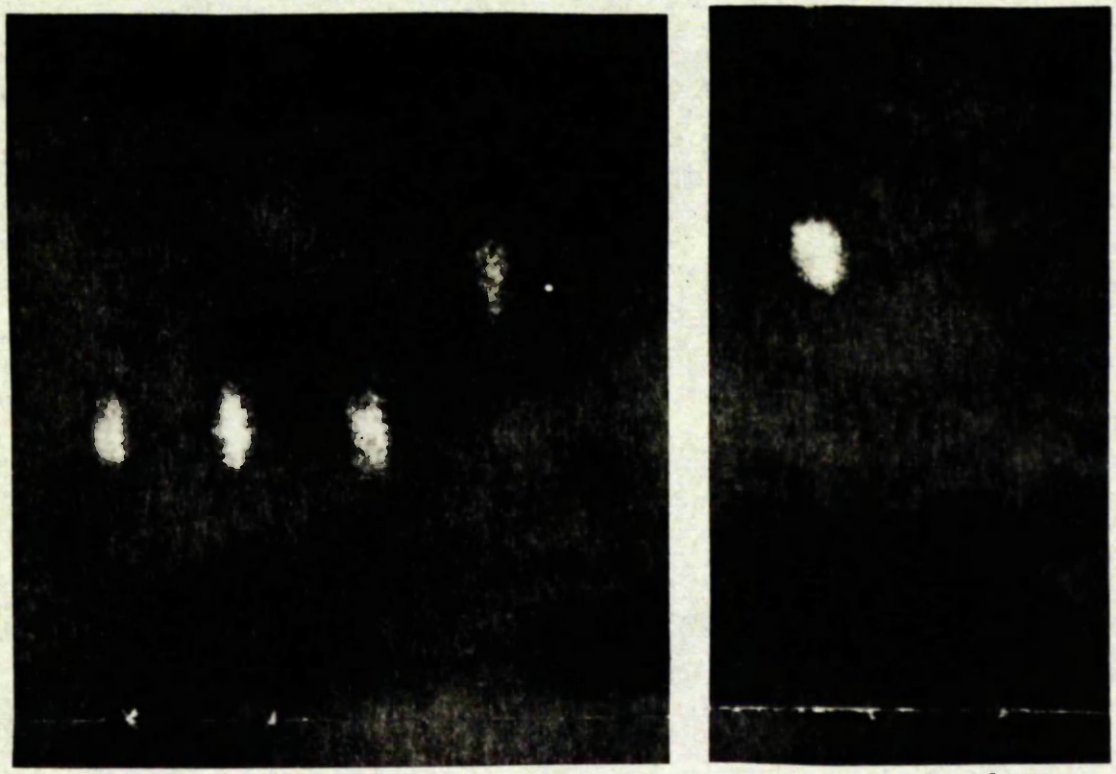
Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/10 min.)	Formaldehyde (cts/μ-mole/ 10 min.)
[1-^{14}C] glycine(i)	110	0
[1-^{14}C] glycine	56,400	0
[2-^{14}C] glycine(i)	160	0
[2-^{14}C] glycine	19,900	530

(i) cells heated to 100° for 15 min.

Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- washed cell suspension E. coli PA15 (41 mg. dry weight); and (as indicated) [1-^{14}C]glycine, 5·0 μ-moles (750 μC/m-mole); [2-^{14}C]glycine, 4·0 μ-moles (1,500 μC/m-mole). After incubation for 37° for 3 hr. ($^{14}CO_2$ collected as described in the text), the reaction was stopped by the addition of 2 ml. 2N-H_2SO_4. The liberated $^{14}CO_2$, trapped in 2·0 ml. 2N-NaOH, was counted as $Ba^{14}CO_3$ (section 2.7.2.). The formaldehyde was isolated and assayed by the methods described in section 2.4.2.2.

FIG. 3.3.1.B.

CONVERSION OF GLYCINE TO PYRUVATE BY WHOLE CELLS



glyox. assay boiled control pyruv. assay boiled control

Keto acid chromatogram Radioautograph

The reaction mixture contained in 3·0 ml. 0·067M-phosphate buffer pH 7·0:- washed cell suspension E. coli PA15 (41 mg. dry weight); [2-^{14}C]glycine, 4·0 µ-moles (1,500 µC/m-mole). After incubation at 37° for 3 hr. the reaction was stopped by the addition of 2·0 ml. 2N-H_2SO_4. Carrier sodium glyoxylate was added (1·0 µ-mole). Keto acid 2,4-dinitrophenylhydrazones were prepared and separated by paper chromatography (section 2.5.4.). [^{14}C]keto acids were identified by radioautography (section 2.8.).

acidified, the cells removed by centrifuging and the supernatants divided into two parts. One part was steam distilled after the addition of carrier formaldehyde, and treated with dimedon. The formaldmethones formed were purified and assayed for activity. The other part was treated with 2,4-dinitrophenylhydrazine hydrochloride after the addition of carrier glyoxylate, and the keto acid-2,4-dinitrophenylhydrazones separated by paper chromatography. Active keto acids were identified radioautographically.

Both glycine C-1 and C-2 were oxidized to CO_2 by whole cells. The cells metabolizing [2-^{14}C]glycine contained a small amount of active material which could be isolated as [^{14}C]formaldehyde (Table 3.3.1.(i).). The radioautograph (Fig. 3.3.1.B.) showed that no detectable quantities of glyoxylate were formed in the course of the oxidation, but active spots with the same R_f values as cis- and trans-pyruvate-DNP were apparent, from both the [1-^{14}C]glycine and [2-^{14}C]glycine incubations.

The following pattern of metabolism was suggested from these observations: (i) glycine C-2 is converted to formaldehyde (or some compound which is hydrolyzed to formaldehyde under the acid conditions of the isolation procedure), (ii) glycine C-1 is converted to CO_2, (iii) formaldehyde is oxidized to CO_2 (probably via formate), (iv) formaldehyde (or a C_1-derivative of formaldehyde) reacts

with glycine to form a C_3-unit (probably serine) which is converted to pyruvate, and (v) glyoxylate does not appear to be involved in the reaction scheme.

3.3.2. CONVERSION OF GLYCINE TO FORMALDEHYDE AND CO_2 BY CELL-FREE EXTRACTS

These pathways could be further elucidated by two approaches: (i) isotope trapping and isotope dilution techniques with possible intermediates in the whole-cell system, or (ii) isolation and purification of a cell-free extract which converts glycine to formaldehyde and CO_2.

The former approach suffered from similar limitations to the growing-cell system (section 3.2.), while the latter was the first step towards precise enzyme studies, and was accordingly tried first.

A major difficulty in the cell-free system was to find a suitable trap to prevent further metabolism of C_1-compounds formed at the oxidation level of formaldehyde. The organisation of the whole cell maintains a small formaldehyde 'pool', probably in the form of an H_4PtG derivative. In the cell-free system, C_1-units formed from glycine will be enzymically accepted by H_4PtG, and in an extract containing only trace amounts of H_4PtG there must necessarily be rapid oxidation, transfer or liberation of the C_1-units to regenerate the H_4PtG. If, however, a large excess of synthetic H_4PtG is

TABLE 3.3.2.(i).

CONVERSION OF GLYCINE TO FORMALDEHYDE AND CO_2 BY CELL-FREE EXTRACTS

Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/10 min.)	Formaldehyde (cts/μ-mole/ 10 min.)
[1-^{14}C] glycine, H_4PtG (i)	140	0
[1-^{14}C] glycine	2,100	74
[1-^{14}C] glycine, H_4PtG	16,000	68
[2-^{14}C] glycine, H_4PtG (i)	74	10
[2-^{14}C] glycine	441	35
[2-^{14}C] glycine, H_4PtG	2,501	95,000

(i) extract heated to 100° for 15 min.

Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- alumina crude extract (12·5 mg. protein); and (as indicated) [1-^{14}C] glycine, 5·0 μ-moles (750μC/m-mole); [2-^{14}C] glycine, 4·0 μ-moles (1,500 μC/m-mole); H_4PtG, 1·0 μ-mole. After incubation at 37° for 3 hr. ($^{14}CO_2$ collected as described in the text), the reaction was stopped by the addition of 2 ml. 2N-H_2SO_4. The liberated $^{14}CO_2$, trapped in 2·0 ml. 2N-NaOH, was counted as $Ba^{14}CO_3$; section 2.7.2. The formaldehyde was isolated and assayed by the methods described in section 2.4.2.2.

TABLE 3.3.2.(ii).

CONVERSION OF FORMATE TO CARBON DIOXIDE BY CELL-FREE EXTRACTS

Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/10 min.)	Formaldehyde (cts/μ-mole/ 10 min.)
None (i)	126	7
None	58,900	12
H_4PtG	59,400	0

(i) extract heated to 100° for 15 min.

Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- [^{14}C]formate, 3·5 μ-moles (3 μC/μ-mole); alumina crude extract (12·5 mg. protein); and (as indicated) H_4PtG, 1·0 μ-mole. After incubation at 37° for 3 hr. ($^{14}CO_2$ collected as described in the text), the reaction was stopped by the addition of 2 ml. 2N-H_2SO_4. The liberated $^{14}CO_2$, trapped in 2·0 ml. 2N-NaOH, was counted as $Ba^{14}CO_3$ (section 2.7.2.). The formaldehyde was isolated and assayed as described in section 2.4.2.2.

added to the system, C_1-H_4PtG compounds may accumulate.

Sonic crude extracts were incubated with [1-^{14}C]glycine and [2-^{14}C]glycine in the presence and absence of H_4PtG. $^{14}CO_2$ and [^{14}C]formaldehyde were collected and assayed for activity as before; (Table 3.3.2.(i).).

Highly active formaldehyde was isolated from the reaction mixture containing [2-^{14}C]glycine and H_4PtG, which confirmed the trapping capacity of this compound. H_4PtG also stimulated the formation of $^{14}CO_2$ from [1-^{14}C]glycine and from [2-^{14}C]glycine (derived from the oxidation of formaldehyde, via formate which is rapidly converted to CO_2 by these extracts; Table 3.3.2.(ii).) which suggested that H_4PtG may be intimately connected with the liberation of both formaldehyde and CO_2 from glycine.

Much evidence had been accumulated against the involvement of glyoxylate in the C_1-unit production scheme, and it is unlikely that CO_2 liberation is H_4PtG dependent in the 'acetate-glycine' and 'succinate-glycine' cycles. It was therefore tentatively suggested that glycine is split directly to ammonia, formaldehyde and CO_2.

$$NH_2.CH_2.COOH \xrightarrow[H_4PtG]{+H_2O,\ -2H} NH_3 + H.CHO + CO_2$$

3.3.3. QUANTITATIVE ASSAY OF $^{14}CO_2$ PRODUCTION FROM [1-^{14}C]GLYCINE

In order to determine the cofactor requirements of the overall reaction it was necessary to develop a quantitative

TABLE 3.3.3.(i).

QUANTITATIVE ASSAY OF $^{14}CO_2$ PRODUCTION

Method	$Ba^{14}CO_3$ (cts/min.)		
	Expt. 1	Expt. 2	Expt.3
Aeration train	1279	6302	3792
Modified aeration train	3291	5986	4802
Warburg flasks	3926	3883	3803
Modified Thunberg tubes	13,630	14,670	13,921

Reaction mixtures contained, in 3·0 ml. 0·067M-phosphate buffer pH 7·0:- sonic crude extract (3·6 mg. protein; 10·7 mg. protein in modified Thunberg tube experiments); [1-^{14}C]glycine, 5·0 µ-moles (750 µC/m-mole); H_4PtG, 1·0 µ-mole; PlP, 1·0 µ-mole; NAD^+, 1·0 µ-mole. The experiments were performed as described in the text. $^{14}CO_2$ was collected in 2N-NaOH and counted as $Ba^{14}CO_3$ (section 2.7.2.).

assay. The amount of formaldehyde isolated varies with the efficiency of the trap and a large proportion is oxidized to CO_2. Glycine disappearance in crude extracts may indicate the effect of several reactions. CO_2 production from glycine C-1 appears to be associated with C_1-unit formation, and at least gives a quantitative record of the reactions which split the C-C bond of glycine.

Any quantitative assay depending on CO_2 production must be conducted in such a way that total collection of CO_2 is ensured. To test this, a reaction mixture containing [1-^{14}C]glycine, H_4PtG and a sonic crude extract was incubated in triplicate and the absorbed CO_2, after the addition of carrier carbonate, was counted as $BaCO_3$. The three results were widely scattered (Table 3.3.3.(i).).

The experimental arrangement, shown in Fig. 3.3.1.A., was modified by the addition of two further tubes of 2N-NaOH in series, for the collection of the CO_2, and the addition of 2 ml. 2N-H_2SO_4 (by hypodermic syringe through the rubber tubing, Fig. 3.3.1.A.) to each reaction mixture at the end of the incubation period. Aeration was continued for three hours to transfer any CO_2 liberated by acidification of the buffered reaction mixture, to the alkaline traps. Carrier carbonate was added to the combined CO_2 traps and the activity estimated as $BaCO_3$. The results of an experiment performed in triplicate, showed a reduced scatter (Table 3.3.3.(i).), but the variations

were still unsatisfactory.

A similar experiment was carried out in three Warburg flasks with Cl4 glass stoppers. The centre-well contained 0·2 ml. 5N-NaOH, the main compartment, [1-^{14}C]glycine, H_4PtG and extract, and the side-arm, 0·5 ml. 5N-H_2SO_4. The flasks were filled with nitrogen to prevent atmospheric oxidation of H_4PtG during the course of the incubation. After 3 hr., the acid was tipped and the flasks left overnight. After removing the alkali from the centre-well and adding carrier carbonate, the activity was assayed as $Ba^{14}CO_3$. The duplication of the three results was good (Table 3.3.3.(i).).

The closed system of the Warburg flask had obvious advantages over the previous methods, but the design was not ideal. Thunberg tubes, modified by the addition of a second leg (Fig.2.4.2.1.A.) had the same advantages and were simpler to manipulate. An experiment in triplicate (details in section 2.4.2.1.) showed good duplication (Table 3.3.3.(i).) and the tubes were adopted for further experiments concerned with $^{14}CO_2$ production.

3.3.4. GLYCINE CATABOLISM BY CRUDE EXTRACTS: EFFECT OF PlP & NAD^+

Rat liver homogenates convert glycine to formate (from C-2) and CO_2 (from C-1) and oxidize formate to CO_2 (Nakada and Weinhouse, 1953; Weinhouse and Friedman, 1954). The oxidation

of glycine to CO_2 is decreased in liver homogenates from animals fed with a purified diet unsupplemented with folic acid, and CO_2 formation from glycine, and from formate, is impaired in liver homogenates from vitamin B_6-deficient rats (Schulman and Richert, 1959). It would appear therefore that folic acid and vitamin B_6 are involved in glycine catabolism to C_1-units and CO_2.

During the course of the present studies, Sagers and Gunsalus (1961) showed the requirement for PlP and NAD^+ when glycine and H_4PtG are converted to hydroxymethyl-H_4PtG, CO_2 and ammonia by extracts of the strict anaerobe, Diplococcus glycinophilus. These authors showed that glyoxylate was inert in the system, and suggested that the frequent observations of glyoxylate mediation in aerobic glycine metabolism may reflect a difference in pathways of C_1-unit production by aerobic, as opposed to anaerobic organisms.

The effects of PlP and NAD^+ were accordingly investigated with dialyzed crude extracts of E.coli PA15. The reaction was assayed quantitatively by CO_2 production from [1-^{14}C]glycine (section 2.4.2.1.) while concurrent experiments with [2-^{14}C]glycine showed any formaldehyde production (section 2.4.2.2.). Though the latter results had no quantitative significance, they demonstrated concomitant formaldehyde production from glycine C-2 when CO_2 was liberated from glycine C-1.

TABLE 3.3.4.(i).

CONVERSION OF GLYCINE TO FORMALDEHYDE AND CARBON DIOXIDE:

EFFECT OF PYRIDOXAL PHOSPHATE

Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/10 min.)	Formaldehyde (cts/μ-mole/ 10 min.)
[1-^{14}C] glycine, H_4PtG, PlP(i)	700	-
[1-^{14}C] glycine, H_4PtG	13,000	-
[1-^{14}C] glycine, H_4PtG, PlP	105,000	-
[2-^{14}C] glycine, H_4PtG, PlP(i)	-	1410
[2-^{14}C] glycine, H_4PtG,	-	108,200
[2-^{14}C] glycine, H_4PtG, PlP	-	108,800

(i) extract heated to 100° for 15 min.

Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- sonic crude extract (11·4 mg. protein); and (as indicated) [1-^{14}C]glycine, 5·0 μ-moles (750 μC/m-mole); [2-^{14}C]glycine, 4·0 μ-moles (1,500 μC/m-mole); H_4PtG, 1·0 μ-mole; PlP, 1·0 μ-mole. The tubes were incubated at 37° for 3 hr. Collection, isolation and assay of CO_2 and formaldehyde are described in section 2.4.2.

TABLE 3.3.4.(ii).

CONVERSION OF GLYCINE TO FORMALDEHYDE AND CARBON DIOXIDE: EFFECT OF NAD^+

Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/ 10 min.)	Formaldehyde (cts/μ-mole/ 10 min.)
[1-^{14}C] glycine, NAD^+ (i)	510	-
[1-^{14}C] glycine	105,000	-
[1-^{14}C] glycine, NAD^+	107,200	-
[2-^{14}C] glycine, NAD^+ (i)	-	530
[2-^{14}C] glycine	-	108,800
[2-^{14}C] glycine, NAD^+	-	107,500

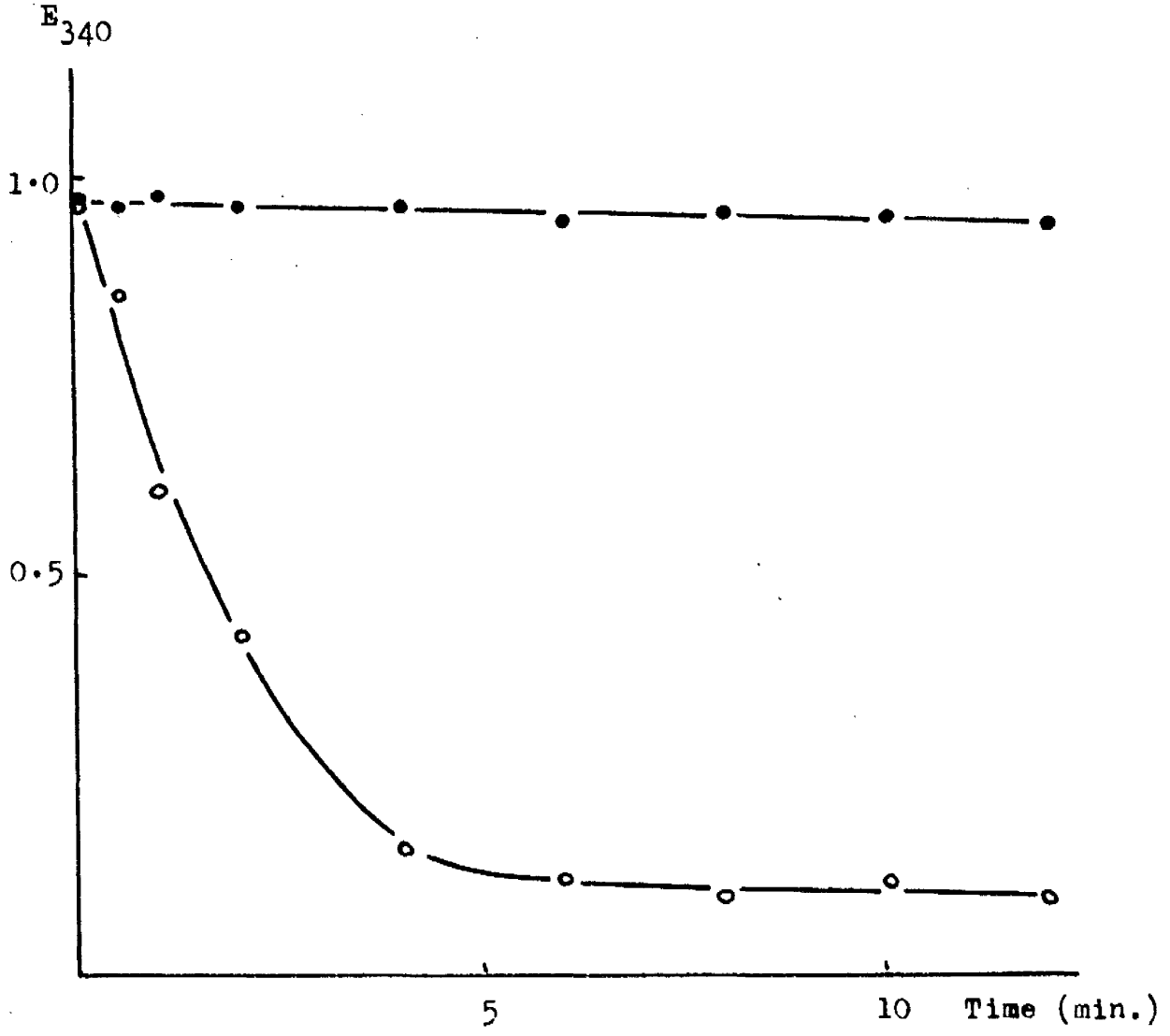
(i) extract heated to 100° for 15 min.

The reaction mixtures and assay procedures were the same as those described for Table 3.3.4.(i). Mixtures contained:- extract, H_4PtG, PlP, and (as indicated) [1-^{14}C]glycine, [2-^{14}C]glycine and NAD^+ (1.0 μ-mole).

FIG. 3.3.4.A.

NADH OXIDASE ACTIVITY

(Sonic crude extract)



Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- NADH, 0·5 μ-mole; and, o - sonic crude extract (1·1 mg. protein); ● - sonic crude extract (heated to 100° for 15 min., 1·1 mg. protein). The mixtures, in quartz cuvettes, were incubated at 18° and the reactions followed by measuring the extinctions at 340 mμ.

PlP produced a dramatic stimulation of $^{14}CO_2$ production (Table 3.3.4.(i).) while NAD^+ showed no effect (Table 3.3.4.(ii).), but in crude extracts where NAD^+, present only in trace amounts, might be regenerated, the latter result is not very significant. These extracts, in fact, contained a very active NADH oxidase (Fig. 3.3.4.A.).

The reactions which convert glycine to formaldehyde and CO_2 appear to involve H_4PtG and PlP. Requirement for these cofactors does not clarify the mechanism of conversion (both could be involved in any scheme previously suggested). Purification of the enzyme system and a thorough quantitative characterization of the reaction are required.

3.3.5. PURIFICATION OF THE GLYCINE-SPLITTIN ENZYME SYSTEM

Using $^{14}CO_2$ formation from [1-^{14}C]glycine as a quantitative criterion for the activity of the enzyme system, several purification procedures were attempted. The protein concentration at each stage was measured with the Folin phenol reagent by the method of Lowry *et al* (1951). All operations were carried out at 0°, unless otherwise stated.

Sonic crude extracts were treated with protamine sulphate (1% solution in 0·067M-phosphate buffer pH 7·0) till no further precipitation was detected. The precipitate was removed by centrifuging. The ratio of extinctions E_{280}/E_{260} changed from about 0·57 for the crude, to about 0·95 for the protamine

TABLE 3.3.5.(i)

PARTIAL PURIFICATION OF THE GLYCINE-SPLITTING ENZYME SYSTEM

Extract	Vol. (ml.)	Activity (units/ ml.)	Total units ($\times 10^3$)	Protein (mg./ml.)	Purity (units /mg.)	Yield (%)	Purification
Sonic crude	45	6,830	308	10·6	608	100	1·0
Protamine sulphate	55	4,900	270	6·4	776	88	1·3
0-25% $(NH_4)_2SO_4$	12	410	5	1·8	227	2	-
25-50% $(NH_4)_2SO_4$	12	12,190	146	10·1	1,205	47	2·0
50-75% $(NH_4)_2SO_4$	12	308	4	14·3	22	1	-

The experimental details for the different operations are described in section 2.3.

The activity was measured at each stage by measuring $^{14}CO_2$ production from [1-^{14}C]glycine by the method described in section 2.4.2.1.

treated extracts. About 25% of the protein was also lost in this step, but there was little reduction of the total activity (e.g. 10%).

Protamine sulphate extracts were fractionally precipitated with saturated ammonium sulphate. Protein fractions at 0-25%, 25-50% and 50-75% saturation of the salt were collected (the details of this, and the other purification methods, are described in section 2.3.). About half the original activity was found in the 25-50% ammonium sulphate fraction, and the purification achieved was about 2-fold.

The quantitative details of a typical partial purification of this nature are given in Table 3.3.5.(i)., presented according to the format recommended by Dixon and Webb (1958).

Protamine sulphate extracts were 'titrated' with calcium phosphate gel. 0, 9, 18, 27, 36 and 45 mg. gel were added to 3 ml. lots of extract in 0·067M-phosphate buffer pH 7·0; after 10 min. the mixtures were centrifuged and the supernatants assayed for protein concentration and glycine-splitting activity. Only the first fraction, with no gel added, showed activity. This could be due to: (i) total absorption of activity on 9 mg. gel, (ii) separation of the enzyme system into two parts, or (iii) inactivation of the enzyme system by calcium phosphate.

Calcium phospahte gel (18 mg.) was added to a protamine sulphate extract (4·5 mg. protein/ml.), and after 10 min.

TABLE 3.3.5.(ii).

FURTHER PURIFICATION OF THE GLYCINE-SPLITTING ENZYME SYSTEM

Extract	Vol. (ml.)	Activity (units/ml.)	Total units ($\times 10^3$)	Protein (mg./ml.)	Purity (units/mg.)	Yield (%)	Purification
Sonic crude	10	1,242	12·4	8·1	153	100	1·0
Protamine sulphate	12	962	11·5	5·0	192	93	1·25
25–50% $(NH_4)_2SO_4$	5	1,342	6·7	4·6	291	54	1·9
0–20% acetone + 25–50% $(NH_4)_2SO_4$	1·5	2,962	4·5	0·5	5,940	36	39

The experimental details for the different operations are described in section 2.3.

The activity was measured at each stage by estimating $^{14}CO_2$ production from [1-^{14}C]glycine by the method described in section 2.4.2.1.

TABLE 3.3.5.(iii).

TABLE 3.3.5.(iv).

STABILITY OF THE GLYCINE-SPLITTING ENZYME SYSTEM

(a)

Extract	Carbon dioxide ($Ba^{14}CO_3$; cts/min.)
Extract	7,802
Extract + 0·1M-cysteine	7,783
Extract(i)	916
Extract(i) + 0·1M-cysteine	3,222

(i) extract kept at 37° for 3 hr. before assay

(b)

Number of freezing-thawing operations	Carbon dioxide ($Ba^{14}CO_3$; cts/min.)
Once	7,944
Twice	7,005
5 times	4,557

The assay conditions and experimental procedure are described in section 2.4.2.1. 25-50% ammonium sulphate extract contained 10·0 mg. protein/ml.

(a) Stability of enzyme system at 37°.

(b) Stability of enzyme to freezing and thawing.

the mixture was centrifuged. The precipitated gel was eluted with 25% ammonium sulphate and mixture centrifuged. The 25-50% ammonium sulphate fraction was isolated from both supernatants. These fractions were assayed for protein concentration and glycine-splitting activity, separately and combined. No activity was detected and alternative methods of purification were investigated.

A 25-50% ammonium sulphate fraction, with added cysteine (0·1 molar) was fractionally precipitated with acetone. Protein fractions were collected at 0-20%, 20-40% and 40-60% acetone concentration, at -5°, -10° and -15° respectively. The precipitates were dissolved in 0·067M-phosphate buffer pH 7·0, and further fractionated with ammonium sulphate precipitation at 25-50% salt saturation. The three fractions were assayed for protein concentration and glycine-splitting activity. Only the 0-20% acetone fraction was active. The quantitative details of the fractionation are shown in Table 3.3.5.(ii): a 40-fold purification was achieved.

The activity of 25-50% ammonium sulphate fractions, stored at -15°, remained constant indefinitely. Extracts stored at 0° were inactive after two weeks; extracts kept at 37° lost 90% of glycine splitting activity after 3 hr. (Table 3.3.5.(iii).). Extracts containing 0·1M-cysteine lost only 60% of the activity in this time at 37°. Freezing and thawing an extract several times reduced the activity (Table 3.3.5.(iv).). Accordingly,

bulk preparations of extracts were stored in 10 ml. lots at -15°.

3.3.6. CONVERSION OF GLYCINE TO FORMALDEHYDE AND CO_2 BY PARTIALLY PURIFIED EXTRACTS: EFFECT OF GLYOXYLATE

A 25-50% ammonium sulphate extract which converts glycine to formaldehyde and CO_2 can be used to study unambiguously, the possible role of glyoxylate in C_1-unit production.

[1-^{14}C]Glycine was incubated with glyoxylate and a 25-50% ammonium sulphate extract in the presence of H_4PtG, EDTA and PlP. The glyoxylate was isolated as the 2,4-dinitrophenylhydrazone at the end of the incubation, and assayed for activity. The $^{14}CO_2$ produced was estimated by the method described in section 2.4.2.1.

This estimation procedure involved the addition of 200 µ-moles carrier sodium carbonate, so it was not possinle to ostimate the specific activity of the enzymically produced $^{14}CO_2$, but only the total counts.

The specific activity of the glyoxylate, if it gained activity from the glycine in the course of the reaction, and if no glyoxylate was produced from unlabelled sources, would rise to a maximum at the end of the incubation. The number of counts that could have been transferred from the glyoxylate to the CO_2 could therefore be calculated. It was the product of the maximum specific activity of the glyoxylate

TABLE 3.3.6.(i). &
TABLE 3.3.6.(ii).

CONVERSION OF GLYCINE TO FORMALDEHYDE AND CARBON DIOXIDE BY PARTIALLY PURIFIED EXTRACTS: EFFECT OF A GLYOXYLATE POOL

	Specific activities (cts/μ-mole/sec.)	Total counts (per sec.)
[1-^{14}C]glycine		
Initial glycine	41,300	78,600
Initial glyoxylate	25	
Final glyoxylate	148	
Max. theoretical CO_2 counts from glyoxylate pool		742
Carbon dioxide		36,900
[2-^{14}C]glycine		
Initial glycine	82,300	329,200
Initial glyoxylate	10	
Final glyoxylate	31	
Max. theoretical HCHO counts from glyoxylate pool		157
Formaldehyde		22,850

Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- protamine sulphate extract (10·2 mg. protein); EDTA, PlP, 2·0 μ-moles; H_4PtG, 1·0 μ-moles; glyoxylate, 5·0 μ-moles; and (as indicated) [1-^{14}C]glycine, 1·9 μ-moles (750 μC/μ-mole); [2-^{14}C]glycine, 4·0 μ-moles (1,500 μC/μ-mole). After incubation (under N_2) at 37° for 3 hr. the reactions were stopped by tipping 2 ml. 2N-H_2SO_4. Carbon dioxide and formaldehyde (total counts) were estimated as described in section 2.4.2. Glyoxylate was isolated and assayed as glyoxylate-DNP (sections 2.5.4 and 2.6.9.). Calculations to obtain max. theoretical counts from glyoxylate pool are explained in the text.

(i.e the final specific activity), and the maximum amount of glyoxylate that could have been present (i.e. the initial amount added). This number of counts was far short of the total counts actually isolated as $^{14}CO_2$.(Table 3.3.6.(i).).

An exactly similar situation was found in the case of [^{14}C]formaldehyde production from [2-^{14}C]glycine. In this experiment, excess H_4PtG was used to trap the formaldehyde produced, but even so some was further oxidized, so the figure shown for the [^{14}C]formaldehyde total counts would have been even greater if this oxidation had not taken place. (Table 3.3.6.(ii).).

These results, in conjunction with the earlier work, invalidate any scheme for the conversion of glycine to formaldehyde and CO_2 via the intermediate formation of free glyoxylate.

3.3.7. THE STOICHIOMETRY OF THE REACTION

Before designing an experiment to determine the stoichiometry of the reaction, some of the fundamental properties of the system were investigated, viz: (i) the effects of various combinations of cofactors on $^{14}CO_2$ production from [1-^{14}C]glycine by a partially purified extract, (ii) the variation of $^{14}CO_2$ production with time, (iii) the variation of $^{14}CO_2$ production with enzyme concentration, (iv) the variation of $^{14}CO_2$ production with [1-^{14}C]glycine concentration,

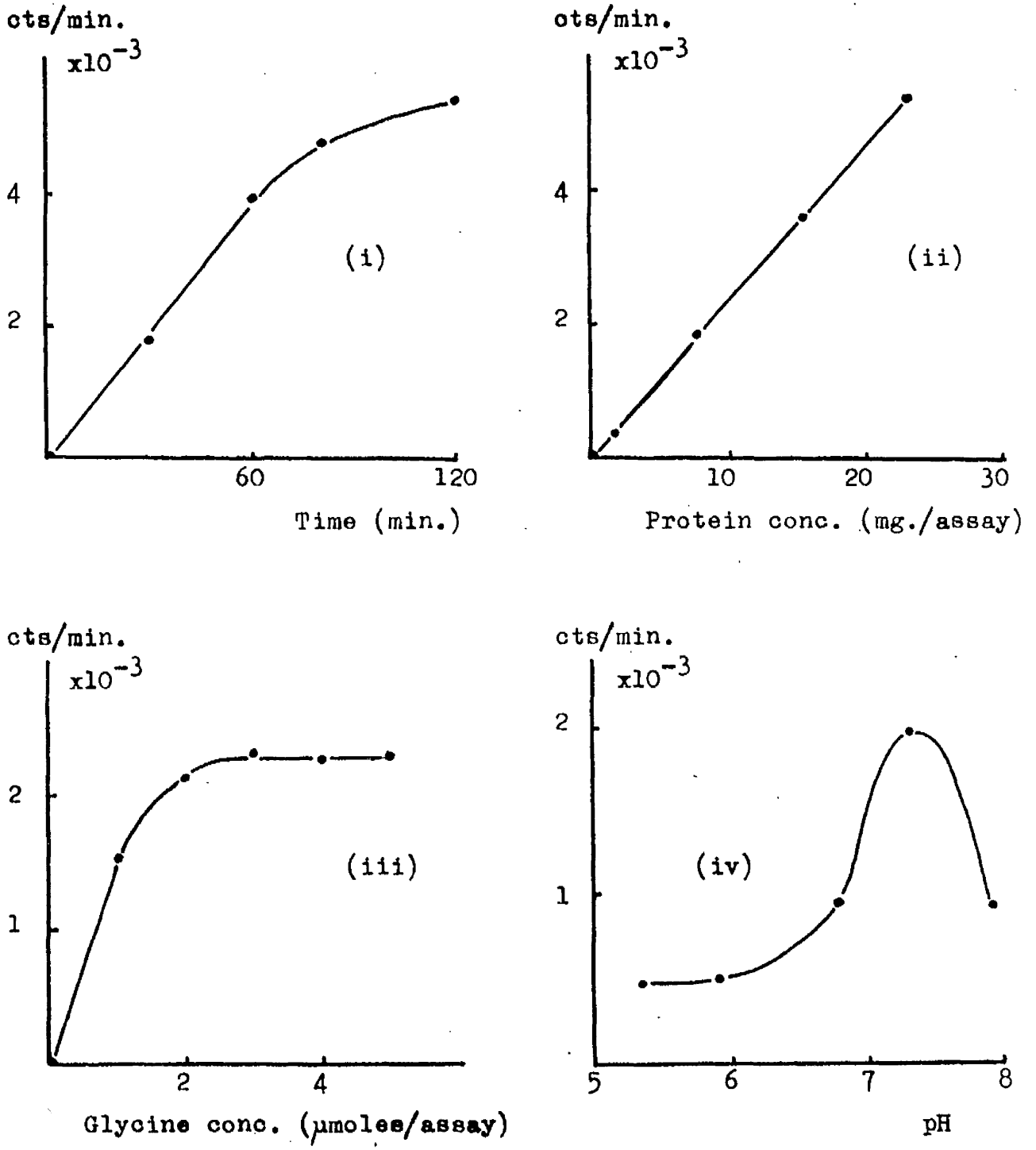
TABLE 3.3.7.(i)

CONVERSION OF GLYCINE TO CO_2 BY A PARTIALLY PURIFIED EXTRACT: COFACTOR REQUIREMENTS

Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/10 min.)
–	200
PlP	8,290
H_4PtG	2,410
NAD^+	120
PlP, NAD^+	8,530
H_4PtG, NAD^+	2,400
PlP, H_4PtG	11,140
PlP, H_4PtG, NAD^+	13,530

The reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- protamine sulphate-treated extract (7·9 mg. protein); [1-^{14}C]glycine, 5·0 µ-moles (750 µC/m-mole); and (as indicated) PlP, 1·0 µ-mole; H_4PtG, 1·0 µ-mole; NAD^+, 1·0 µ-mole. The tubes were incubated at 37° for 45 min. Collection and assay of $^{14}CO_2$ are described in section 2.4.2.1.

$^{14}CO_2$ PRODUCTION FROM 1-^{14}C GLYCINE BY 25-50% AMMONIUM SULPHATE EXTRACTS



Variation of $^{14}CO_2$ production with (i) time, (ii) protein concentration, (iii) glycine concentration and (iv) pH. Assay conditions and $^{14}CO_2$ determination procedure are described in section 2.4.2.1. (protein concns.: (i) & (iii), 6·8 mg./assay; (iv), 8·1 mg./assay.)

FIG. 3.3.7.A.

and (v) the pH optimum for $^{14}CO_2$ production.

The cofactor requirements for the reaction, using a 25-50% ammonium sulphate extract, were similar to those previously observed using crude extracts (Table 3.3.7.(i).).

The reaction proceeds at a constant rate for more than an hour (Fig. 3.3.7.A.) and varies directly with the protein concentration of the 25-50% ammonium sulphate extract (between the limits 0 and 24 mg. protein). The enzyme is saturated with respect to substrate at a glycine concentration of 0·25 µ-mole/mg. 25-50% ammonium sulphate extract protein (Fig. 3.3.7.A.)

M-Phosphate buffers of different pH were prepared. [1-^{14}C]glycine, extract, H_4PtG, PlP and NAD^+ in 1·5 ml. 0·067M-phosphate buffer pH 7·0 were added to 1·5 ml. lots of M-phosphate buffer of known pH, and the CO_2 production assayed in the usual way (section 2.4.2.1.). The pH of 10 ml. 0·067M-phosphate buffer pH 7·0 added to 10 ml. M-phosphate buffer pH 5·35, was 5·37.

The optimum pH of the reaction is 7·3. 75% of the maximum activity is recorded at pH 7·0 (Fig. 3.3.7.A.).

Further investigations could now be carried out using conditions where product formation was proportional to reaction rate, and where the reaction rate was limited only by the absence of necessary cofactors.

TABLE 3.3.7.(ii).

CONVERSION OF GLYCINE TO FORMALDEHYDE, CO_2 AND AMMONIA BY A PARTIALLY PURIFIED EXTRACT: STOICHIOMETRY AND COFACTOR REQUIREMENTS.

Additions	Glycine removed (μ-moles)	Carbon dioxide formed (μ-moles)	Formaldehyde formed (μ-moles)	Ammonia formed (μ-moles)
[1-^{14}C]glycine				
H_4PtG, PlP, NAD$^+$ (i)	0	0	-	0
-	0	0·1	-	0·2
PlP	1·8	1·8	-	1·9
H_4PtG	0·2	0·1	-	0·5
PlP, NAD$^+$	1·9	1·5	-	1·8
H_4PtG, NAD$^+$	0·4	0·2	-	0·3
PlP, H_4PtG	2·0	1·9	-	1·7
PlP, H_4PtG, NAD$^+$	1·8	1·8	-	1·8
[2-^{14}C]glycine				
H_4PtG, PlP, NAD$^+$ (i)	0	0	0	0
-	0	0	0	0
PlP	1·8	1·2	0·1	1·8
H_4PtG	0·1	0·1	0·1	0·3
PlP, H_4PtG	1·8	0·2	0·3	1·8
PlP, H_4PtG, NAD$^+$	1·9	0·4	0·3	1·9

(i) extract heated to 100° for 15 min.

Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- 25-50% ammonium sulphate extract (6·8 mg. protein); and (as indicated) [1-^{14}C]glycine, 5·0 μ-moles (750 μC/m-mole); [2-^{14}C]glycine, 4·0 μ-moles (1·5 mC/m-mole); PlP, 1·0 μ-mole; H_4PtG, 1·0 μ-mole; NAD$^+$, 1·0 μ-mole. After incubation under N_2 at 37° for 45 min. reaction stopped with 2N-H_2SO_4. Assay procedures are described in section 2.4.2. and in the text.

To determine the stoichiometry of the reaction, $^{14}CO_2$ formation was measured as described in section 2.4.2.1. and [^{14}C]formaldehyde as described in section 2.4.2.2. [^{14}C]glycine disappearance was estimated as the activity remaining in the acidified reaction mixtures after steam distillation, measured by plating known fractions of the total at infinite thinness and noting the proportion of total counts which disappeared. Ammonia was estimated in the acidified reaction mixtures as described in section 2.4.2.3. The results are compiled in Table 3.3.7.(ii).

For each mole of glycine that is metabolized, one mole of carbon dioxide and one mole of ammonia are liberated. Formaldehyde is produced concurrently, probably mole for mole with the CO_2. H_4PtG and PlP stimulate the reaction, but NAD^+ has no effect.

It appears that the liberation of CO_2, formaldehyde and ammonia from glycine by the glycine splitting enzyme system depends on the presence of both H_4PtG and PlP. If the overall reaction represents two or more steps, then the first step, liberation of CO_2 or liberation of ammonia, would most likely, by analogy with other reactions, be PlP dependent (cf. Snell, 1961). The second step, liberation of a C_1- unit would be H_4PtG dependent (cf. Jaenicke, 1961). If both ammonia formation and CO_2 formation are dependent on H_4PtG and PlP, then the overall conversion of glycine to ammonia, CO_2 and a C_1-unit

TABLE 3.3.8.(i). &

TABLE 3.3.8.(ii).

CONVERSION OF GLYCINE TO CO_2 AND AMMONIA BY A SEPHADEX-TREATED EXTRACT: EFFECT OF PlP

PlP supplement (μ-moles)	Carbon dioxide ($Ba^{14}CO_3$; cts/min.)	Ammonia (μ-moles)
0	873	0·27
0·0001	1,407	1·04
0·01	3,923	1·34
0·5	3,917	1·36

CONVERSION OF GLYCINE TO CO_2 AND AMMONIA BY A SEPHADEX-TREATED EXTRACT: EFFECT OF H_4PtG

H_4PtG supplement (μ-moles)	Carbon dioxide ($Ba^{14}CO_3$; cts/min.)	Ammonia (μ-moles)
0	1,180	0·61
0·0001	3,394	1·21
0·01	4,010	1·11
1·0	3,919	1·31

Reaction mixtures contained in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- sephadex-treated, 25-50% ammonium sulphate extract (10·0 mg. protein); [1-^{14}C]glycine, 5·0 μ-moles (750 μC/m-mole); NAD^+, 1·0 μ-mole; and PlP, 1·0 μ-mole; H_4PtG, 1·0 μ-mole (or as indicated). After incubation at 37° for 20 min., under nitrogen, the reaction was stopped by tipping 2 ml. 2N-H_2SO_4. Carbon dioxide and ammonia were assayed by the methods described in section 2.4.2.

might be envisaged as a single complex reaction. It was therefore important to determine the exact nature of the involvement of these cofactors in glycine catabolism, by purification of the enzyme system from trace amounts of PlP and H_4PtG, and showing which parts of the reaction have an absolute requirement for both cofactors.

3.3.8. SEPHADEX TREATMENT

25-50% Ammonium sulphate extract (10 ml.) was passed through a 20 x 1·5 cm. column packed with Sephadex G75, at 0°. The protein was eluted with 0·067M-phosphate buffer pH 7·0, and the single peak collected in 12 ml.

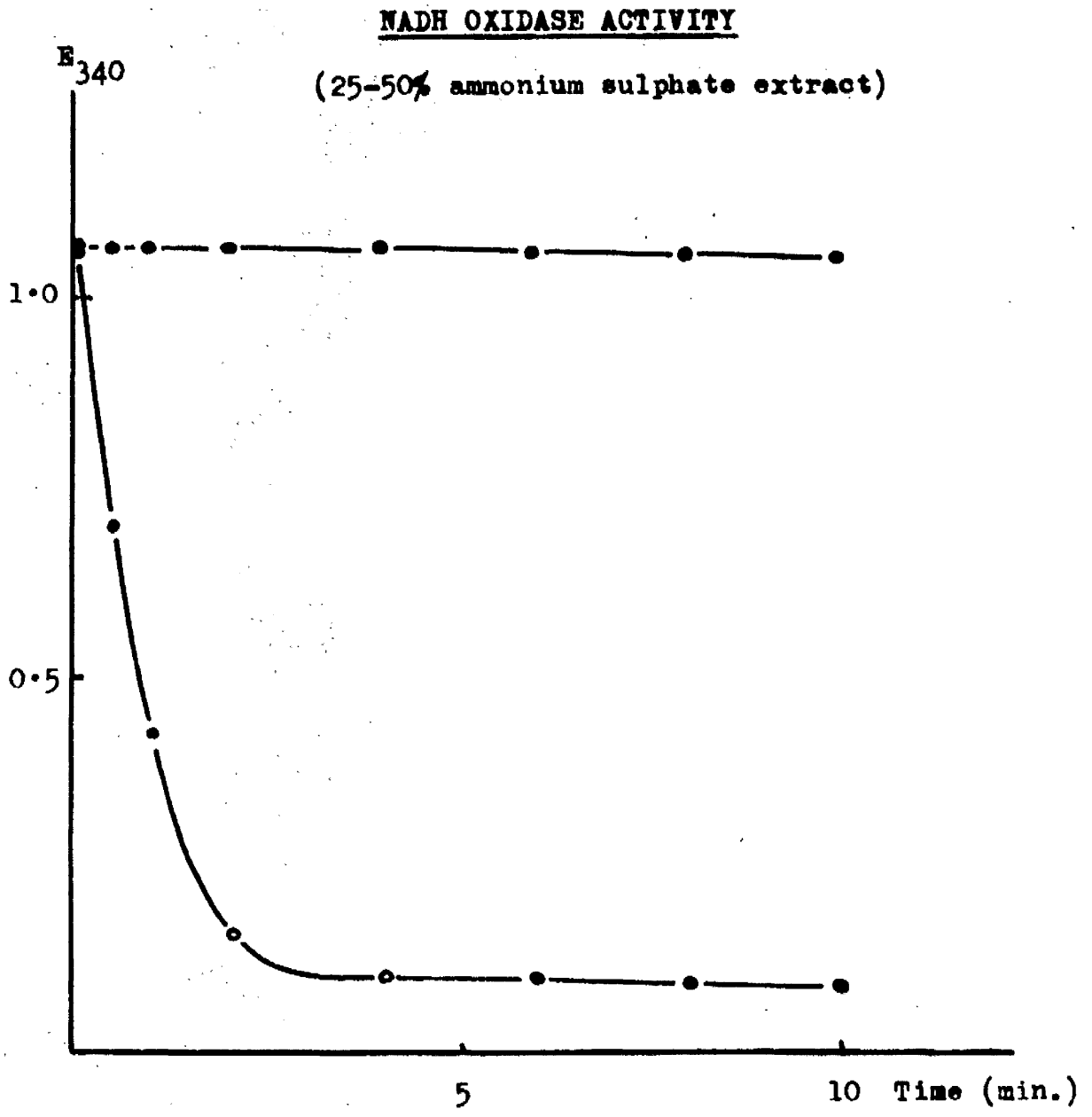
The activities of this extract were determined with respect to CO_2 and ammonia formation in the presence of excess H_4PtG and increasing amounts of PlP, and also in the presence of excess PlP and increasing amounts of H_4PtG. The experimental details are described in section 2.4.2.1. and 2.4.2.3.

Both CO_2 and ammonia production depend on the concurrent presence of trace amounts of H_4PtG and PlP (Tables 3.3.8.(i). and 3.3.8.(ii).). The requirement for only catalytic amount of these cofactors probably explains the background activity of an extract purified only to this extent, in the absence of added either H_4PtG or PlP.

3.3.9. GLYCINE OXIDATION COUPLED TO BENZYL VIOLOGEN REDUCTION

The overall conversion of glycine to formaldehyde, ammonia

FIG. 3.3.9.A.



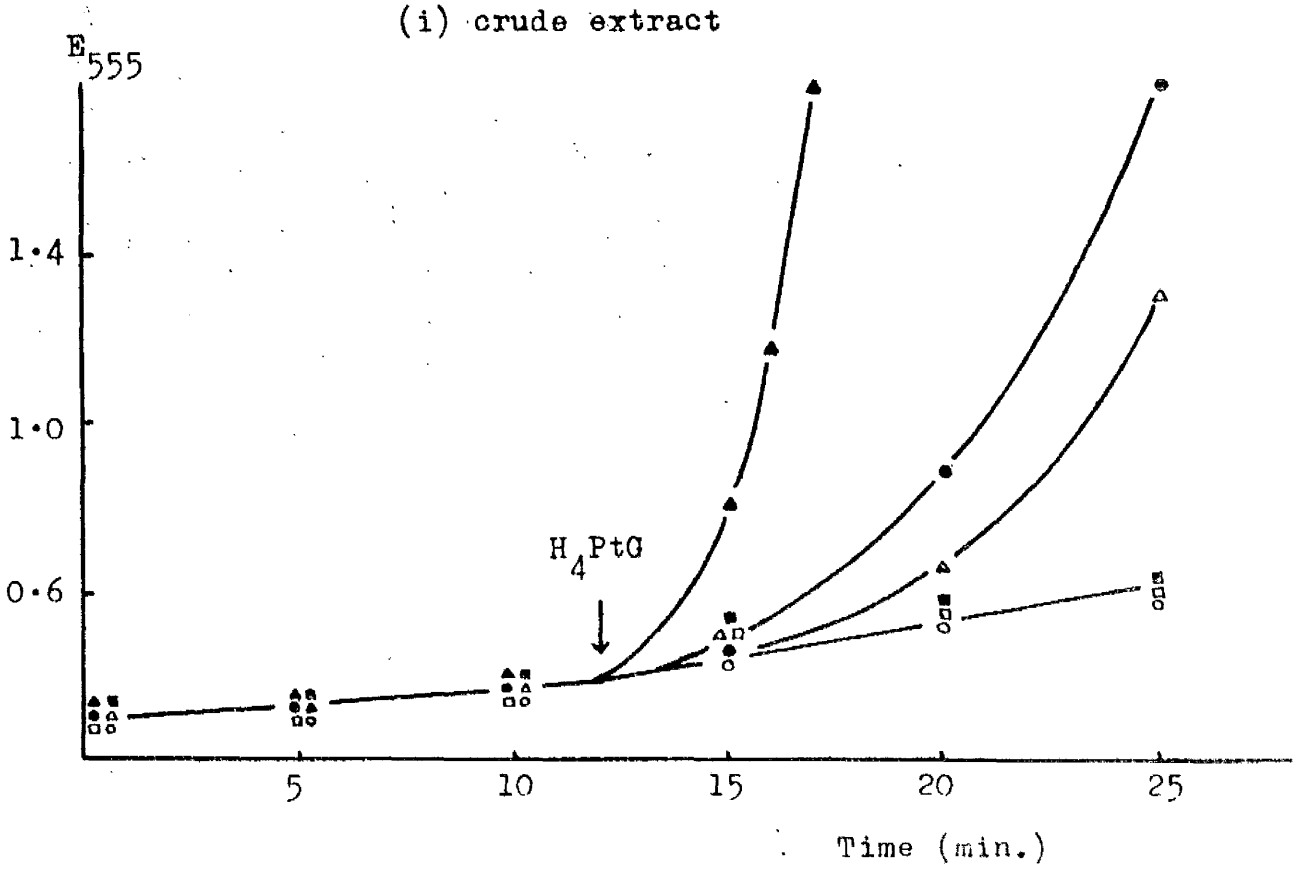
Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer, pH 7·0, contained:- NADH, 0·5 μ-mole; and ○ - 25-50% ammonium sulphate extract (1·6 mg. protein); ● - 25-50% ammonium sulphate extract (heated to 100° for 15 min., 1·6 mg. protein). The mixtures, in quartz cuvettes, were incubated at 18°, and the reactions followed by measuring the extinctions at 340 mμ.

and CO_2 represents an oxidation. Extracts of Diplococcus glycinophilus (Sagers and Gunsalus, 1961; Klein and Sagers, 1962) utilize glycine as an electron donor in the presence of H_4PtG, PlP and NAD^+, and a low potential dye such as benzyl viologen. The benzyl viologen is reduced while the glycine is converted to hydroxymethyl-H_4PtG and CO_2.

Unlike the Diplococcus glycinophilus system, partially purified extracts of E. coli PA15 are not dependent on the presence of added NAD^+ for the production of CO_2 and formaldehyde from glycine (but these extracts contain an active NADH oxidase, Fig. 3.3.9.A.). To gain more information about the nature of the oxidation accompanying glycine splitting, crude and 25-50% ammonium sulphate extracts were incubated with glycine, benzyl viologen and various combinations of H_4PtG, PlP and NAD^+.

The purple coloured reduced form of benzyl viologen is rapidly oxidized to the colourless form by atmospheric oxygen. Accordingly, all parts of the reaction mixtures were deoxygenated under reduced pressure, and the incubations were performed under vacuum in 10 x 120 mm. tubes designed for the Unicam SP600 spectrophotometer, sealed with rubber tubing and a screw-clip. Additions were made with a hypodermic syringe through the rubber. The reduction of benzyl viologen was followed at 37° by measuring the increase in extinction at 555 mμ (section 2.4.2.4.).

REDUCTION OF BENZYL VIOLOGEN



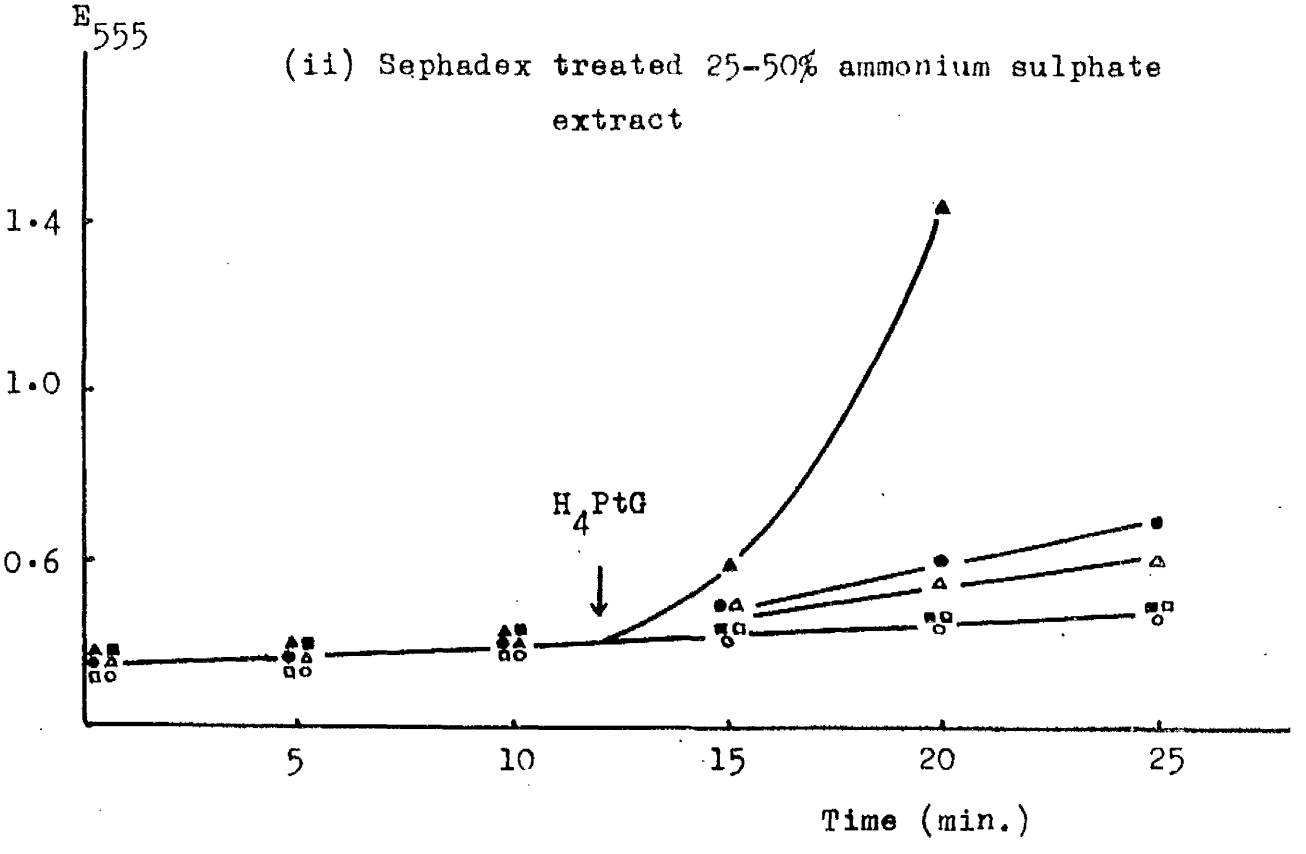


FIG. 3.3.9.B.

FIG. 3.3.9.B.

(legend)

REDUCTION OF BENZYL VIOLOGEN

The components of the systems illustrated in the graphs are:-

▲ - extract, glycine, NAD^+, PlP.

● - extract, glycine, NAD^+.

△ - extract, glycine, PlP.

■ - extract, NAD^+, PlP.

o - extract, NAD^+.

□ - extract, glycine.

The reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- benzyl viologen, 5·0 μ-moles; and (as indicated) extract (sonic crude, 12·5 mg. protein; 25-50% ammonium sulphate, 6·8 mg. protein); glycine, 5·0 μ-moles; H_4PtCl_6, 1·0 μ-mole; PlP, 2·0 μ-moles; NAD^+, 2·0 μ-moles. The mixtures were incubated at 37° under anaerobic conditions and the reactions followed by measuring the changes in extinctions at 555 mμ. The details are described in section 2.4.2.4.

H_4PtG, PlP and NAD^+ are all required to couple to oxidation of glycine to benzyl viologen reduction at the maximum rate (Fig. 3.3.9.B.). When PlP is omitted (25-50% ammonium sulphate extract) the rate decreases about 10-fold. When NAD^+ is omitted the rate decreases about 20-fold, and any further omission causes the rate to fall almost to zero.

This presents a confusing situation. NAD^+ is required for the transfer of electrons from glycine to benzyl viologen, but apparently, is not required for the complete catabolism of glycine to ammonia, CO_2 and formaldehyde.

3.3.10 INHIBITION OF NADH OXIDASE: ANAEROBIC CONDITIONS OF ASSAY

One immediate difference between the assay of glycine oxidation by ammonia, CO_2 and formaldehyde production, and the assay by coupling to benzyl viologen reduction, is the strict anaerobic conditions necessarily employed in the latter.

In a partially purified, well dialyzed extract, the only quantitatively significant electron acceptor available is molecular oxygen. In the presence of atmospheric oxygen NADH is rapidly oxidized to NAD^+ (Figs. 3.3.4.A and 3.3.9.A.), by crude and partially purified extracts.

The preceeding assays of enzyme activity involving H_4PtG had been carried out under an atmosphere of nitrogen to protect the H_4PtG from oxidation. The assay mixtures though, had not been freed from dissolved oxygen, and this oxygen

(about 1 μ-mole/ml.), regenerating catalytic amounts of NAD^+, could have accounted for the glycine oxidation.

A procedure was therefore adopted to exclude all oxygen from the reaction mixtures. The modified Thunberg tubes were set up with assay mixtures (apart from enzyme and H_4PtC) in one leg, 2N-H_2SO_4 in the other leg and 2N-NaOH containing M-sodium dithionite in the cap. The tubes were repeatedly evacuated and filled with oxygen-free nitrogen, then left overnight to allow any oxygen remaining to be absorbed by the alkaline dithionite.

The extract, deoxygenated under reduced pressure, and H_4PtC were added, in a rapid operation, to the reaction mixtures in the tubes. The tubes were immediately evacuated and filled with oxygen-free nitrogen.

At the end of the incubation the reaction mixtures were acidified by tipping. After a further two hours, carrier carbonate was added to alkaline dithionite, $^{14}CO_2$ traps, which were then transferred to one of the leg compartments of further modified Thunberg tubes.

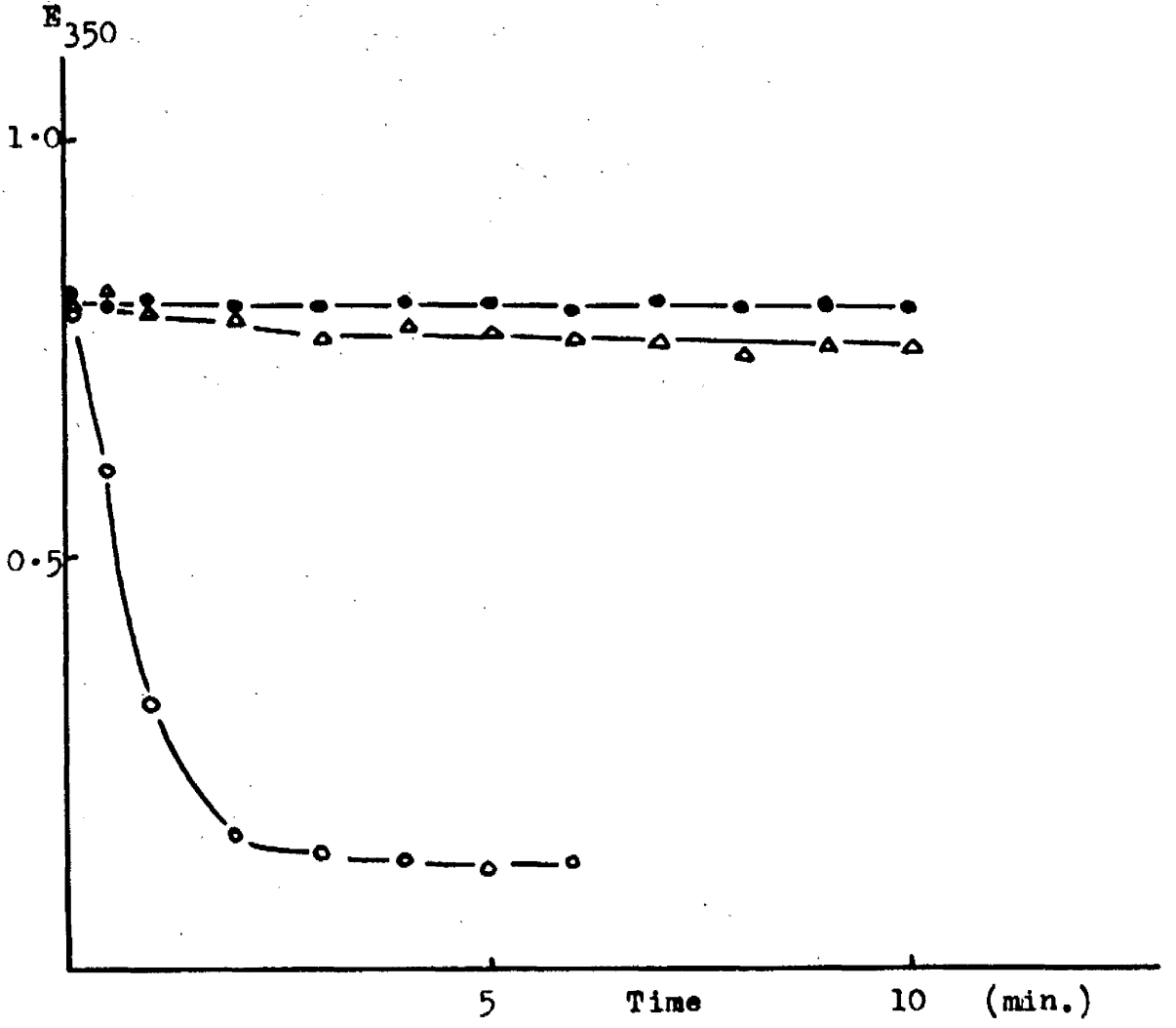
Barium dithionite is precipitated when $BaCl_2$ is added to an alkaline solution of sodium dithionite, and it was therefore necessary to separate the dithionite and the carbonate before precipitating the carbonate as the barium salt for counting.

Excess saturated $KMnO_4$ was added to the mixed alkaline

FIG. 3.3.10.A.

NADH OXIDASE ACTIVITY

(in the presence & absence of O_2)



Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer, pH 7·0, contained:- NADH, 0·5 μ-mole; 25-50% ammonium sulphate extract, (0·8 mg.). ○ - aerobic incubation; △ - anaerobic incubation; ● - extract heated to 100° for 15 min. The mixtures, contained in 10 x 120 mm. glass tubes (designed for the Unicam SP600 Spectrophotometer), were incubated at 16°, and the reactions followed by measuring the extinctions at 350 mμ.

TABLE 3.3.10.(i).

CONVERSION OF GLYCINE TO CARBONDIOXIDE BY PARTIALLY PURIFIED EXTRACTS, UNDER AEROBIC AND ANAEROBIC CONDITIONS; EFFECT OF NAD^+

Assay conditions	Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/min.)		
		Expt. 1	Expt. 2	Expt. 3
Aerobic	-	18,723	3,617	8,570
	NAD^+	20,220	5,520	21,703
Anaerobic	-	7,742	849	2,063
	NAD^+	12,500	2,879	6,861

Reaction mixtures in 3·0 ml. 0·067M-phosphate buffer pH 7·0, contained:- 25-50% ammonium sulphate extract (8·1mgprotein, Expt. 1); Sephadex-treated protamine sulphate extract (3·2 mg. protein; Expt. 2); Sephadex-treated protamine sulphate extract (10·9 mg. protein; Expt 3); [1-^{14}C]glycine, 5·0 μ-moles (750 μC/m-mole); PIP, 0·2 μ-mole; H_4PtG, 1·0 μ-mole; and (as indicated) NAD^+, 5·0 μ-moles. The assay procedures for aerobic and anaerobic conditions are described in section 2.4.2.1. Mixtures were incubated at 37° for 45 min.

dithionite-carbonate solutions to oxidize the dithionite to sulphate. The sulphate-carbonate solution was acidified by tipping 5N-H_2SO_4, and the liberated CO_2 was absorbed in 2N-NaOH in the caps. The sodium carbonate, so trapped, was counted as $BaCO_3$ as described in section 2.7.2.

The rate of NADH oxidation by a 25-50% ammonium sulphate extract in a deoxygenated reaction mixture under oxygen-free nitrogen was measured by following the decrease in extinction at 350 mμ in a 10 x 120 mm. spectrophotometer tube (cf. section 3.3.9; these tubes were designed for the Unicam SP600 spectrophotometer which cannot be used at wavelengths shorter than 350 mμ. The reaction should have been followed at 340 mμ.). The activity of the extract under these conditions, with respect to NADH oxidation, was small (Fig. 3.3.10.A.).

The glycine-splitting enzyme system was assayed under anaerobic conditions (as explained above) by measuring $^{14}CO_2$ production from [1-^{14}C]glycine. The rate of $^{14}CO_2$ formation by a 25-50% ammonium sulphate extract in the presence of H_4PtG and PlP and absence of molecular oxygen, was stimulated by the addition of NAD^+ (Table 3.3.10.(i).). The reaction rate, however, in both anaerobic assays (with and without NAD^+) was less than in comparable aerobic assays (Table 3.3.10.(i).).

The experiment was repeated with two different preparations of Sephadex-treated, protamine sulphate extracts and a similar

pattern was obtained (table 3.3.10.(i).). In addition, in these experiments, the addition of NAD^+ stimulated CO_2 production under aerobic conditions. This effect had previously been looked for, but not found.

It appears that NAD^+ is involved in glycine oxidation by partially purified extracts of E. coli PA15, but the exact nature of the involvement is not clear. If the reaction were simply NAD^+-dependent, the addition of NAD^+ in amount equimolar with the [1-^{14}C]glycine concentration, should allow maximum rate of reaction whether or not the system is anaerobic. Anaerobic conditions, however, inhibit the reaction, even in the presence of excess NAD^+.

3.3.11. FLAVOPROTEIN NATURE OF THE GLYCINE-SPLITTING ENZYME

If the enzyme, or enzyme system, is flavoprotein in nature and can couple the oxidation of glycine directly to the reduction of molecular oxygen, with out the mediation of NAD^+ and NADH oxidase, then anaerobic inhibition of the reaction in the presence of excess NAD^+, could be understood.

Friedmann and Vennesland (1958) purified the enzyme, dihydroorotic dehydrogenase, from Zymobacterium oroticum, and characterized some of its properties. The enzyme is a flavoprotein containing equal amounts of FMN and FAD, and it catalyzes the following reactions: (i) reduction of orotate by NADH, (ii) oxidation of dihydroorotate by NAD^+, (iii) oxidation

TABLE 3.3.11.(i).

CONVERSION OF GLYCINE TO CO_2: EFFECT OF ATABRINE

Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/min.)
-	7,947
Atabrine	7,630

Reaction mixtures, in 3·0 ml. 0·067M-phosphate buffer pH 7·0 contained:- sonic crude extract (10·6 mg. protein); [1-^{14}C]glycine, 5·0 µ-moles (750 µC/m-mole); PLP, 1·0 µ-mole; NAD^+, 1·0 µ-mole; and (as indicated) atabrine, 5·0 µ-moles. The mixtures were incubated at 37° for 2 hr. under aerobic conditions. CO_2 formation was assayed by the method described in section 2.4.2.1.

of dihydroorotate by molecular oxygen, and (iv) oxidation of NADH by molecular oxygen. The authors suggested that all these reaction were catalyzed by one flavoprotein, or two flavoproteins in very close association.

If the enzyme associated with glycine catabolism in E. coli PA15 has similar properties to dihydroorotic dehydrogenase of Zymobacterium oroticum, then the relative effects of NAD^+ and oxygen will depend entirely on the particular rates of the reactions involved.

Atabrine (mepacrine) is, along with other antimalarials, a flavoprotein antagonist (Madinaveitia, 1946; Beinert, 1960), but when added to assays of $^{14}CO_2$ production from [1-^{14}C]glycine, no inhibition was observed (Table 3.3.11.(i).).

Further purification of the enzyme (especially from flavoprotein dependent oxidation of NADH) would be required before flavoprotein propertied could be established.

3.3.12. INHIBITION OF THE REACTION BY NADH

A further explanation, which could account for the inhibition of glycine catabolism under anaerobic conditions in the presence of NAD^+, can be proposed if NADH reduces the reaction rate by some form of product inhibition. Under anaerobic conditions NADH will accumulate, but not in the presence of molecular oxygen.

[1-^{14}C]Glycine was incubated with a partially purified

TABLE 3.3.12.(i).

CONVERSION OF GLYCINE TO CO_2 BY PROTAMINE SULPHATE EXTRACTS: NADH INHIBITION

Additions	Carbon dioxide ($Ba^{14}CO_3$; cts/min.)
NAD^+	6,832
NADH, NAD^+	797
NADH(i)	6,771

(i) preincubated at 37° with aeration (before the addition of [1-^{14}C]glycine and H_4PtG) till the extinction at 340 mμ. showed complete oxidation of NADH.

Reaction mixtures contained, in 3·0 ml. 0·067M-phosphate buffer pH 7·0:- protamine sulphate extract (8·1 mg. protein); [1-^{14}C]glycine, 5·0 μ-moles (750 μC/m-mole); PlP, 0·2 μ-mole; H_4PtG, 1·0 μ-mole; and (as indicated) NAD^+, 1·0 μ-mole; NADH, 1·0 μ-mole. The tubes were incubated (aerobic conditions) at 37° for 45 min., and CO_2 production estimated by the procedure described in section 2.4.2.1.

The mixture containing: extract, glycine, PlP, H_4PtG, NADH and NAD^+ was prepared in duplicate. One was assayed for CO_2 production (see above); the extinction of the second (prepared with unlabelled glycine, and similarly incubated at 37°) was followed at 340 mμ. The NADH in this mixture was completely oxidized after 56 min.

extract, H_4PtG, PlP and NAD^+, in the presence and absence of NADH. $^{14}CO_2$ production was reduced by 90% when NADH was added (Table 3.3.12.(i).).

Fawcett, Ciotti and Kaplan (1961) reported the presence of inhibiting material in commercial preparations of NADH, especially after prolonged storage.

To overcome the possibility of inhibition of this nature, extract, PlP and NADH were aerated at 37° till the extinction of the solution at 340 mμ. showed the complete oxidation of NADH. [1-^{14}C]glycine and H_4PtG were then added to the reaction mixture in a modified Thunberg tube, and the rate of $^{14}CO_2$ production measured. The reaction was not inhibited by any compounds remaining in the reaction mixture after aeration (Table 3.3.12.(i).).

DISCUSSION

In growing E. coli glycine C-2 is converted to C_1-units at the oxidation levels of formaldehyde and formate, whereas under similar conditions formate is utilized for the synthesis of C_1-units only at the oxidation level of formate (Crosbie, 1959; Pitts, Stewart and Crosbie, 1961). In the rat, on the other hand, formaldehyde (Hamill, Hermann, Byerrum and Fairley, 1956) and formate (Totter, Volkin and Carter, 1951; Mannell and Rossiter, 1955) will serve as precursors of C_1-units at both oxidation levels.

N-5,N-10,methenyl-H_4PtG, apparently, is not reduced to N-5,N-10,methylene-H_4PtG in growing E. coli (section 3.1.), so any pathway of C_1-unit formation from glycine must provide for the formation of methylene-H_4PtG by a route not involving the methenyl derivative, i.e.

$$NH_2.CH_2.COOH \longrightarrow \text{"H.CHO"} \longrightarrow \text{"H.COOH"}$$

A scheme which fulfils this requirement was suggested by Paretsky and Werkman (1950) to account for the catabolism of glycine in Achromobacter.

$$NH_2.CH_2.COOH \longrightarrow CHO.COOH \longrightarrow HCHO \longrightarrow H.COOH$$

The intermediate participation of glyoxylate was suggested without the support of experimental evidence.

The conversion of glycine to glyoxylate, however, occurs widely in nature, and under the influence of several different enzymes. In the liver and kidney of animals glycine is oxidized

to glyoxylate by the flavoprotein, glycine oxidase (Ratner, Nocito and Green, 1944) and a similar pathway probably operates in Pseudomonas (Campbell, 1955; Callely and Dagley, 1959). In Mycobacterium tuberculosis the oxidation is brought about by the NAD^+ dependent glycine dehydrogenase (Goldman and Wagner, 1962).

Wilson, King and Burris (1954) reported the presence of a glycine:2-oxoglutarate aminotransferase in crude extracts of plant seedlings. The extracts, which also contained glycollic oxidase (Zelitch and Ochoa, 1953), effected the overall conversion of [1-^{14}C]glycollate (via [1-^{14}C]glyoxylate) to [1-^{14}C, ^{15}N]glycine, in the presence of [^{15}N]glutamate, and catalized the formation of [^{14}C]glutamate from [^{14}C]2-oxo-glutarate in the presence of glycine.

Enzymic transamination of glyoxylate and glutamate has also been reported in rat liver extracts (Meister, Sober, Tice and Fraser, 1952) and in Pseudomonas (Campbell, 1956), and it was suggested that the conversion of glycine to glyoxylate by a transamination process, is the first step in the oxidation of glycine to formate and CO_2 by rat liver (Nakada and Weinhouse, 1953).

C_1-units are formed from glycine by the scheme of Nakada and Weinhouse, but this pathway cannot explain the observations in E. coli, unless a further step is introduced, at the

oxidation level of formaldehyde. The pathway is then similar to that suggested by Paretsky and Werkman.

The conversion of glyoxylate to formaldehyde and CO_2 was postulated by Krakow and Barkulis (1956) to explain the glyoxylate carboligase reaction. The reaction is dependent on the presence of thiamine diphosphate (TPP) and is analogous to 2-acetolactate formation from pyruvate by the pyruvate carboligase of Aerobacter aerogenes :-

$$2\ H.CO.COOH \xrightarrow{TPP} CO_2 + H.CO.CH(OH).COOH$$

glyoxylate — tartronic semialdehyde

$$2\ CH_3.CO.COOH \xrightarrow{TPP} CO_2 + CH_3.CO.C(OH).CH_3.COOH$$

pyruvate — 2-acetolactate

Some TPP-derivatives remain enzyme bound throughout the course of the reaction, e.g. "active glycolaldehyde" participating in the transketolase reaction, is not in equilibrium with free glycolaldehyde. Likewise, possible "active fomaldehyde" formed from glyoxylate, may not be in equilibrium with free formaldehyde or hydroxymethyl-H_4PtG.

Crude extracts of E. coli PA15 convert glyoxylate and glutamate to glycine and 2-oxoglutarate (in the presence of

EDTA which blocks non-enzymic transamination reactions). The enzyme, glycine:2oxoglutarate aminotransferase, has been partially purified by protamine sulphate treatment and ammonium sulphate fractionation (the activity is precipitated between 60-80% saturation of the salt): the reaction is PlP-dependent (Crosbie, 1962b) :-

$$\text{glyoxylate} + \text{glutamate} \xrightleftharpoons{\text{PlP}} \text{glycine} + \text{2-oxoglutarate}$$

The equilibrium of the reaction *in vitro*, favours glycine formation from glyoxylate. Some reaction (less than 1%) has been detected in the reverse direction with partially purified extracts (e.g. increase in glyoxylate-pool specific activity when [1-^{14}C]glycine or [2-^{14}C]glycine is incubated with a protamine sulphate extract, EDTA and glyoxylate; Tables 3.3.6.(i). and 3.3.6.(ii).). The free energy change at 79° for the non-enzymic, metal catalyzed reaction (in the glycine to glyoxylate direction) has been calculated by Metzler *et al* (1954) as + 2 kcal.: at 37° the equilibrium will undoubtedly favour glycine formation.

A second, less active aminotransferase, glycine: phenylpyruvate aminotransferase, is also present in extracts of *E. coli* PA15, and can be resolved from the 2-oxoglutarate dependent enzyme by fractional heat denaturation (Crosbie, 1962b). This reaction has only been studied in the glyoxylate to glycine direction.

Studies with cell-free extracts do not appear to support a scheme of C_1-unit formation from glycine via glyoxylate - glycine is not converted significantly to gloxylate, and glyoxylate is not oxidized to formate.

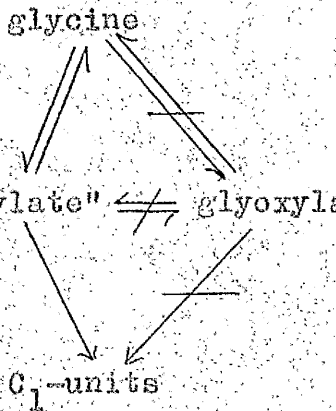
However, the properties of an enzymic reaction studied *in vitro* do not always reflect the role of the enzyme *in vivo*. The equilibrium of the isocitrate lyase reaction favours the formation of isocitrate from succinate and glyoxylate (ΔF = -2 kcal.), but the reaction functions in the reverse direction in the cell.

E. coli PA15, grown in basal medium supplemented with [2-^{14}C]glycine, incorporates isotope into the C_1-positions of thymine and the purines: if glyoxylate is added to the medium, the incorporation pattern is unchanged.

The interpretation of results of this nature suffers from several limitations which are outlined in section 3.2., but further evidence was adduced in favour of a route not involving glyoxylate by growing the organism in a glycollate-inorganic salt medium supplemented with [2-^{14}C]glycine. The central position of glyoxylate in the metabolism of such cells necessarily requires a large flux of glyoxylate molecules through the glyoxylate pool. Even under these conditions, though, the pattern of isotope incorporation remains unaltered.

This last experiment suffers from similar limitations to

the previous one, but the two results taken together appear to indicate that glyoxylate does not lie on the pathway of C_1-unit formation from glycine. Some form of enzyme-bound "glyoxylate", on the other hand, not in equilibrium with free glyoxylate, may be involved.



The results also suggest that glyoxylate is not converted to glycine by transamination, in the growing cell.

Extracts of glycollate-grown E. coli PA15 showed only slight glycine aminotransferase activity when assayed with [1-^{14}C]glyoxylate (section 3.1.8.). Stewart (1961) suggested that the reduced activity (with respect to the glucose-grown cells) may be caused by (i) the enzyme being present in only small amounts in glycollate-grown cells, (ii) the glyoxylate being removed from the assay by faster alternative reactions; (iii) dissimilation of the glycine formed by transamination. Further investigation showed that the poor assay result was due to a combination of glyoxylate catabolism by other reactions, and glycine dissimilation.

If the glycine aminotransferase is present in glycollate-grown E. coli, which have a ready supply of glyoxylate for glycine production, the organism should no longer be a glycine-less mutant. The glycine requirements of glycollate-grown E. coli PA15, however, are identical to those of the glucose-grown cells (Stewart, 1961).

The enzyme is present in extracts of cells grown either on glucose or on glycollate, but it is not utilized for the conversion of glycine to glyoxylate, or glyoxylate to glycine. It is difficult to envisage the purpose of such an enzyme, unless it is concerned with other transamination reactions within the cell, and is able to accept glyoxylate as substrate in the cell-free system.

In the incorporation experiments with E. coli PA15 grown in the presence of [2-^{14}C]glycine, the ratio of the purine ring specific activity to the original glycine specific activity, should be 3, because glycine C-2 is incorporated into the C-2, C-5 and C-8 positions of the ring. In fact, the ratio varies between 2 and 3, and depends on the extracellular glycine concentration (Koch, 1955). On the other hand, the thymine methyl group and the the serine C-3 are always derived quantitatively from glycine C-2.

This suggests that formate, or formyl-H_4PtG is available from sources other than glycine in growing E. coli. Formate produced from pyruvate, for example, would dilute the

incorporation of activity from glycine C-2 into the purine C_1-positions (mainly at C-8; Koch and Levy, 1955), but not into the thymine methyl or serine C-3 because of the block preventing the reduction of methenyl-H_4PtG to methylene-H_4PtG (section 3.1.), in growing E. coli.

Three possible pathways of glycine catabolism were suggested by Nakada, Friedmann and Weinhouse (1955) :- (i) via glyoxylate to formate and CO_2 (shown by these authors to operate in rat liver), (ii) via serine to pyruvate (which can be converted to acetate and formate in E. coli; Strecker, 1951), (iii) via the 'succinate-glycine' cycle (Shemin, 1956) to formate and CO_2 (Huennekens and Osborn, 1959).

The second pathway does not allow the net production of C_1-units, and the first pathway does not appear to function in E. coli. The 'succinate-glycine' cycle (or the 'acetate-glycine' cycle) can only explain glycine catabolism in E. coli if C_1-units are formed at the oxidation level of formaldehyde. The C_1-units derived from these cycles have not been characterized: [^{14}C]formate is derived from [5-^{14}C]5-aminolaevulinate in the rat (Shemin, 1956), but formate may not be the initial product.

Aminoacetone, added to E. coli PA15 growing in a basal medium supplemented with [2-^{14}C]glycine, does not dilute the specific activities of the C_1- units. The value of this

observation, like the previous similar one with glyoxylate, depends on the intimate mixing of aminoacetone derived from extracellular sources and possible intermediate aminoacetone. If there are no barriers of this nature, the 'acetate-glycine' cycle is unlikely to be a major pathway of C_1-unit formation from glycine in E. coli. If the hypothetical pathway, aminoacetone, methylglyoxal, lactate, pyruvate to acetate and formate is the real explanation of Elliott's observations (1960b), then the 'acetate-glycine' cycle cannot explain glycine catabolism in E. coli, because there is no provision for formaldehyde production.

Those investigations were abandoned when washed-cell suspensions of E. coli PA15 were shown to convert glycine C-2 to formaldehyde.

Cell-free extracts also convert glycine to formaldehyde, which can be trapped as an H_4PtG derivative, CO_2 and ammonia:-

$$NH_2{\cdot}CH_2{\cdot}COOH + H_4PtG + NAD^+ \xrightarrow{PlP} HOCH_2{-}H_4PtG + CO_2 + NH_3 + NADH + H^+$$

The enzyme system has been partially purified and characterized. Glycine is oxidized to CO_2, ammonia and a compound isolated as formaldehyde on acidification, in the presence of H_4PtG, PlP and NAD^+.

The overall reaction could be the result of two or more enzymic steps or represent the conversion catalyzed by a single

enzyme. The observations presented in the previous section favour a single enzyme (or two enzymes in very close association) for three reasons.

(i) The enzyme system has been partially purified without resolution into parts. The maximum purification achieved, however, was only 40-fold.

(ii) Possible intermediates added to the active system do not participate in the reaction. If the conversion occurs in more than one step, an intermediate formed must be transferred from one enzyme to the next. Transfers of this nature, unless the two enzymes are very specifically orientated, involve the liberation of the intermediate from the first enzyme, followed reaction with the second. A finite, steady-state concentration of the intermediate will be present in the medium to react with the second enzyme.

Possible intermediates which could be involved in a stepwise, PLP-dependent degradation of glycine are glyoxylate and methylamine.

Deamination of glycine to give glyoxylate and pyridoxamine phosphate could be followed by regeneration of PLP by (a) transamination with a suitable keto acid, or (b) oxidation by pyridoxamine phosphate oxidase (Turner and Happold, 1961). This latter enzyme could not be detected in crude extracts of *E. coli* FA15.

Decarboxylation of glycine to give methylamine and CO_2

could be followed by oxidation of the methylamine-PlP Schiff base to formaldehyde, ammonia and PlP, or liberation of methylamine and PlP followed by oxidation of methylamine, or liberation of formaldehyde and pyridoxamine phosphate followed by oxidation of pyridoxamine phosphate.

Unlabelled glyoxylate is not in ready equilibrium with any intermediate of $^{14}CO_2$ or [^{14}C]formaldehyde production from [1-^{14}C]glycine or [2-^{14}C]glycine, and [^{14}C]methylamine is not readily converted to ammonia and [^{14}C]formaldehyde by the glycine-splitting enzyme system.

(iii) The oxidation of glycine (coupled to benzyl viologen reduction), the production of CO_2 and the production of ammonia are all dependent on the concurrent presence of H_4PtG, PlP and NAD^+. If the reaction occured in several steps, the first could involve the condensation of glycine with enzyme-bound PlP, followed by decarboxylation or deamination. In either case, H_4PtG would be the expected cofactor responsible for the liberation of a C_1-unit and ammonia from methylamine, or a C_1-unit and CO_2 from glyoxylate, by a second enzyme.

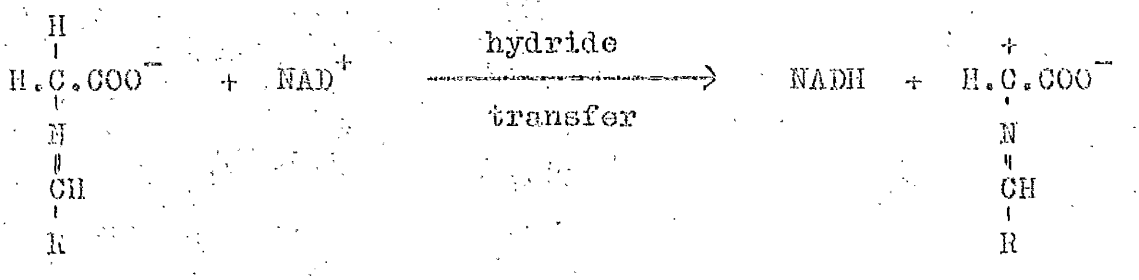
The requirement of both cofactors, plus NAD^+, for glycine catabolism suggests, as do the other observations, a concerted reaction on a single enzyme, or a closely bound enzyme complex.

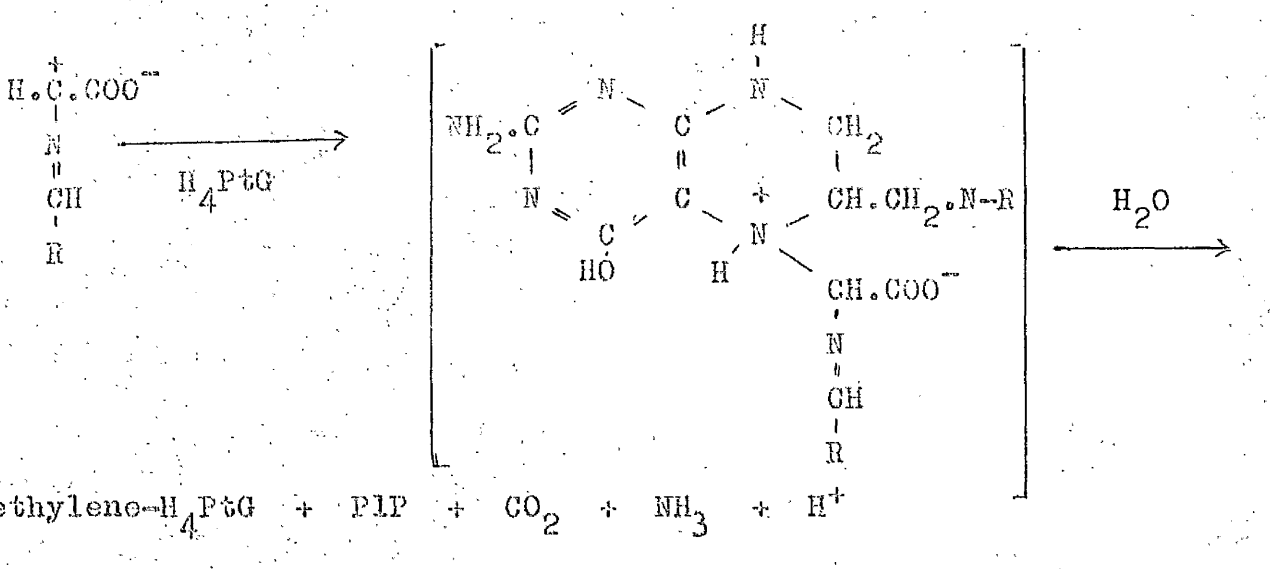
Sagers and Gunsalus (1961), using extracts of Diplococcus glycinophilus, reported the conversion of glycine and H_4PtG

to hydroxymethyl-H_4PtG, CO_2 and ammonia in the presence of PlP and NAD^+. These authors did not suggest a mechanism, but glyoxylate was inert in the system, and presumed therefore, not to be an intermediate.

Richert, Amberg and Wilson (1962) demonstrated an exactly similar reaction in extracts of avian liver. Again, glycine catabolism was dependent on the presence of H_4PtG, PlP and NAD^+. Glyoxylate was dismissed as an intermediate of quantitative significance on grounds similar to those cited in the present work with *E. coli* PA15.

A hypothetical formulation of the reaction sequence was proposed by Richert *et al* :-





The condensation of glycine with PlP to form a Schiff base will create an electron defficient centre at the glycine C-2, suitable for nucleophilic attack by the N-8 of the H_4PtG. Whether this attack is of the S_N1 type involving the formation of a carbonium ion by loss of a hydride ion (as suggested by Richert et al), or whether it is of the S_N2 type involving the concerted participation of H_4PtG, the Schiff base and NAD^+ at the reaction centre, it is impossible to define. The mechanism awaits further characterization by studies on a purified enzyme system.

The pathway of glycine catabolism via formaldehyde, CO_2 and ammonia, occurs in microorganisms and in animals.

The glycine-splitting enzyme is present in extracts of E. coli PA15 grown aerobically and anaerobically. The strict anaerobe, Diplococcus glycinophilus (Sagers and Gunsalus, 1961), and chick, duck and pigeon liver (Richert, Amberg and Wilson, 1962) contain a similar enzyme.

The oxidation of glycine to formaldehyde and ammonia by Achromobacter sp. (Paretsky and Werkman, 1950) may proceed by a related scheme (glyoxylate was only inferred to be an intermediate by these authors). Jenny and Leuthardt (1961) showed that glycine C-2 was converted to a C_1-unit in guinea-pig liver, which condensed with a further molecule of glycine to give serine. Serine was rapidly converted to pyruvate by

liver slices, and this scheme explained the observed labelling of isolated glutamate derived from 2-oxoglutarate and the TCA cycle. These authors suggested glyoxylate as an intermediate in C_1-unit production, but only by analogy with the earlier scheme suggested by Nakada and Weinhouse (1953).

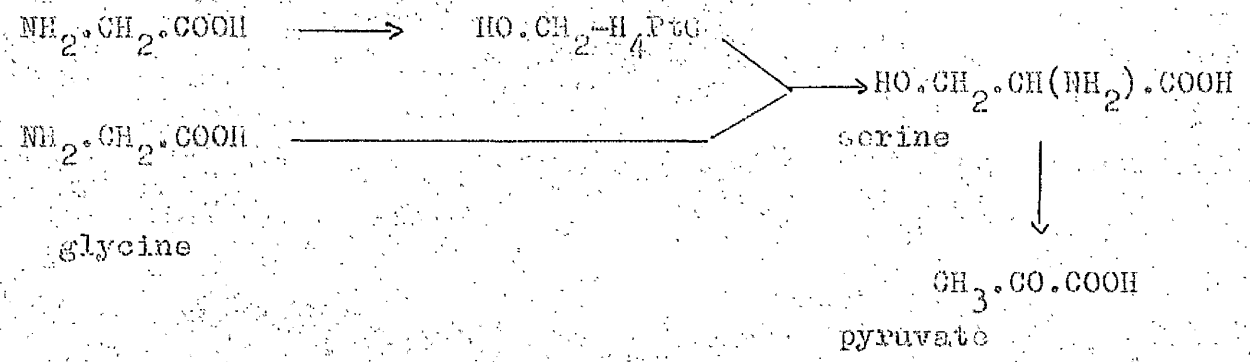
A similar metabolic route has been elucidated by observations made with cell-free extracts of Clostridium acidi-urici (Radin and Barker, 1953; Sagers and Beck, 1956) to account for the production of doubly labelled acetate from [2-^{14}C]glycine. This pattern of labelling has also been observed with extracts of Diplococcus glycinophilus, and the enzymic steps have been characterized (Sagers and Klein, 1961; Klein and Sagers, 1962). A $^{14}C_1$-unit is formed from glycine C-2, which condenses with [2-^{14}C]glycine to give [2,3-^{14}C]serine. [2,3-^{14}C]serine is deaminated to give [2,3-^{14}C]pyruvate, which is converted in a number of steps, probably involving acetyl-CoA and acetyl phosphate, to acetate, doubly labelled and CO_2.

By this scheme, 2 moles of glycine are fermented with the production of 1 mole of acetate and 2 moles of CO_2, and a ballance of 2 pairs of electrons are liberated (Sagers and Gunsalus, 1961). The measured fermentation balance, however, shows the production of 3 moles of acetate and 2 moles of CO_2 from 4 moles of glycine, with no hydrogen gas formation. Sagers and Gunsalus suggested that the 8 electrons liberated

by the oxidation of 4 moles of glycine are utilized for the reduction of 2 moles of CO_2, or the equivalent thereof, to 1 mole of acetate. This suggestion is supported by the observation of Barker, Volcani and Carter (1948) that $^{14}CO_2$ is incorporated into both carbons of acetate by cells of Diplococcus glycinophilus.

Dagley, Trudgill and Callely (1961) suggested that the cell-constituents of Pseudomonas (A), grown with glycine as sole carbon source, were synthesized from glycine via glyoxylate, tartronic semialdehyde, glycerate and pyruvate. The cells also contained malate synthetase, and the synthetic scheme is similar to that of Pseudomonas grown on glycollate (Fig. 1.3.5.A.).

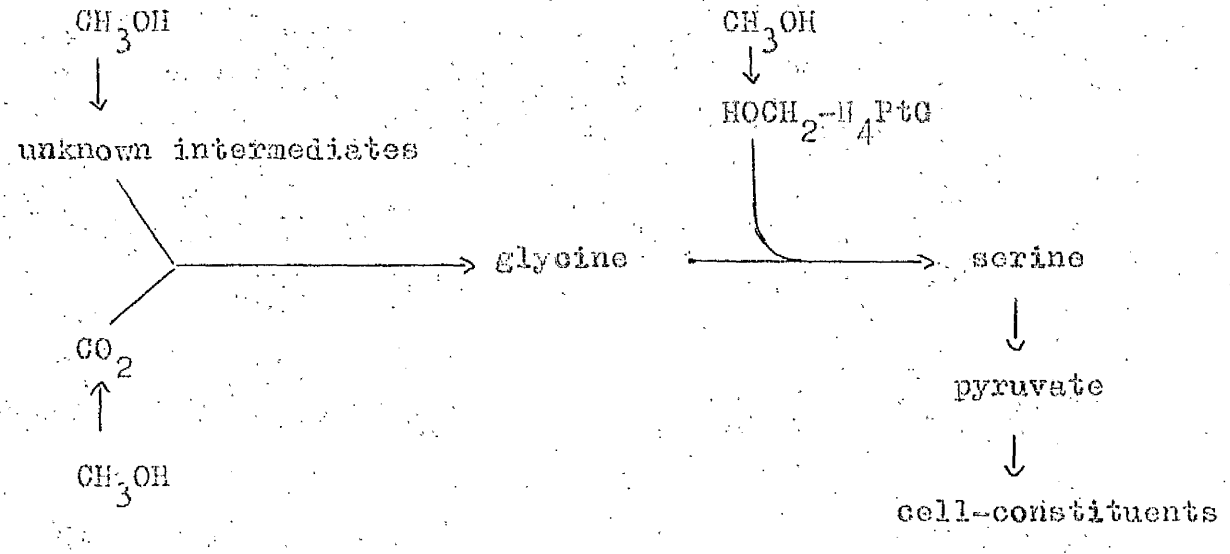
The presence of the glycine-splitting enzyme allows growth on glycine as sole carbon source, by a different scheme :-



A reaction scheme of this type probably operates in E. coli PA15. Chromatograms of keto acid-2,4-dinitrophenyl-

hydrazones show the formation of [^{14}C]pyruvate from [1-^{14}C]glycine and [2-^{14}C]glycine, by cell-free extracts (e.g. in the experiments described in Tables 3.3.6.(i) and (ii).). The operation of this pathway should allow the organism to grow on glycine as sole carbon source. This prediction has been confirmed, but growth is very slow. The rate limiting step has not been elucidated.

Large, Peel and Quayle (1962) suggested the following scheme for the synthesis of cell-constituents from methanol by Pseudomonas AM1, grown on methanol as sole carbon source.



Large and Quayle (1963) have confirmed parts of the scheme by studies of enzyme activities in cell-free extracts, but have not identified the exact nature of the precursors of glycine. If the glycine-splitting enzyme operated in the anabolic direction, an explanation could be offered for this part of the pathway. Attempts to show glycine formation from

[^{14}C]carbonate, formaldehyde and ammonia in the presence of H_4PtO, PLP and NADH, in extracts of E. coli PA15, have so far been unsuccessful.

SUMMARY

1. A review of glycine and glyoxylate metabolism is presented.

2. Growing Escherichia coli PA15 incorporate glycine C-2 into the methyl group of DNA thymine, the C-2 and C-8 positions of the RNA and DNA purine rings and the C-3 position of protein serine. The pathway whereby glycine C-2 is converted to suitable C_1-units at the oxidation levels of formaldehyde and formate, has been investigated.

3. Cell-free extracts convert glyoxylate and glutamate to glycine and 2-oxoglutarate by transamination. The reaction is not inhibited by EDTA which completely inhibits non-enzymic transamination under the conditions used.

4. The data indicate that the reverse reaction, the conversion of glycine to glyoxylate in the presence of 2-oxoglutarate and EDTA, does not play a significant role in glycine catabolism by cell-free extracts.

5. The incorporation of [2-^{14}C]glycine into the thymine, serine and purines is unaffected by the addition of glyoxylate or aminoacetone to the medium, and the incorporation pattern is not changed when the cells are grown on glycollate instead of glucose.

6. Washed-cell suspensions convert glycine C-1 to carbon dioxide and glycine C-2 to formaldehyde (or a compound readily hydrolysed to formaldehyde) and carbon dioxide.

7. An enzyme system, partially purified from crude extracts,

converts 1 mole of glycine to 1 mole of carbon dioxide (derived from C-1), 1 mole of ammonia and a C_1-compound (derived from C-2) which can be isolated as formaldehyde. H_4PtG and PlP are necessary cofactors; the reaction is not dependent on metal ions which are chelated by EDTA. The overall reaction represents the oxidation of glycine: NAD^+ is the primary electron acceptor, but catalytic amounts can be regenerated in the system by oxygen or benzyl viologen.

8. Free glyoxylate is not in equilibrium with any intermediate of the glycine-splitting reaction.

9. Cell-free extracts readily oxidize formate to carbon dioxide, but do not convert glyoxylate to formate in significant amounts.

10. A scheme for the formation of C_1-units from glycine is suggested, which involves the concerted participation of H_4PtG, PlP and NAD^+. The initial products of the reaction are carbon dioxide, ammonia, NADH and hydroxmethyl-H_4PtG (or methylene-H_4PtG).

11. The metabolic significance of the glycine-splitting reaction is discussed.

REFERENCES

REFERENCES

Ajl, S. J. (1958). Physiol. Rev. 38, 196.

Alexander, B., Landwehr, G. & Seligman, A. M. (1945). J. biol. Chem. 160, 51.

Bachrach, U. (1957). Biochem. J. 66, 559.

Barker, H. A., Volcani, B. E. & Cardon, B. P. (1948). J. biol. Chem. 173, 803.

Beinert, H. (1960). The Enzymes, vol. 2, part A, p. 339. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.

Birnie, G. D. (1959). Ph.D. Thesis, Glasgow.

Blakely, R. L. (1954). Biochem. J. 58, 448.

Blakely, R. L. (1960a). Biochem. J. 74, 71.

Blakely, R. L. (1960b). Biochem. J. 77, 459.

Braunstein, A. E. (1960). The Enzymes, vol. 2, part A, p. 113. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.

Britten, R. J. & McClure, F. T. (1962). Bact. Rev. 26, 292.

Buchanan, J. M. & Hartman, S. C. (1959). Advanc. Enzymol. 21, 199.

Callely, A. G. & Dagley, S. (1959). Nature, Lond. 183, 1793.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. E. (1949). Isotopic Carbon. New York: John Wiley & Sons, Inc.

Campbell, L. L. (1955). J. biol. Chem. 217, 669.

Campbell, L. L. (1956). J. Bact. 71, 81.

Cardon, B. P. & Barker, H. A. (1946). J. Bact. 52, 629.

Cavalieri, L. F., Tinker, J. & Brown, G. B. (1949). J. Amer. chem. Soc. 71, 3973.

Crosbie, G. W. (1958). Biochem. J. 69, 1P.

Crosbie, G. W. (1959). Unpublished

Crosbie, G. W. (1962a). Nature, Lond. 193, 597.

Crosbie, G. W. (1962b). Unpublished

Crosbie, G. W., Smellie, R. M. S. & Davidson, J. N. (1953). Biochem. J. 54, 287.

Dagley, S. & Dawes, E. A. (1949). Biochem. J. 45, 331.

Dagley, S.& Rodgers, A. (1953). Biochim. biophys. Acta. 12, 591.

Dagley, S., Trudgill, P. W. & Callely, A. G. (1961). Biochem. J. 81, 623.

Davidson, J. N. (1959). The Biochemistry of the Nucleic Acids. London: Methuen & Co. Ltd.

Delluva, A. M. (1953). Arch. Biochem. Biophys. 45, 443.

Dixon, G. H., Kornberg, H. L. & Lund, P. (1960). Biochim. biophys. Acta. 41, 217.

Dixon, M. & Webb, E. C. (1958). Enzymes. London: Longmans, Green & Co, Ltd.

Elliott, W. H. (1960a). Biochem. J. 74, 90.

Elliott, W. H. (1960b). Biochem. J. 74, 478.

Elwyn, D. & Sprinson, D. B. (1950). J. biol. Chem. 184, 475.

Elwyn, D. & Sprinson, D. B. (1954). J. biol. Chem. 207, 467.

Fawcett, C. P., Ciotti, M. M. & Kaplan, N. O. (1961). Biochim. biophys. Acta. 54, 210.

Fleming, L. W. (1960). B.Sc. Thesis, Glasgow.

Fleming, L. W. & Crosbie, G. W. (1960). Biochim. biophys. Acta. 43, 139.

Foster, M. A., Tejerina, G. & Woods, D. D. (1961). Biochem. J. 81, 1P.

Friedkin, M. & Kornberg, A. (1957). The Chemical Basis of Heredity. p. 609. Baltimore: John Hopkins Press. Ed. McElroy, W. D. & Glass, B.

Friedkin, M. & Roberts, D. (1956). J. biol. Chem. 220, 653.

Friedman, T. E. & Haugen, G. E. (1943). J. biol. Chem., 147, 415.

Friedmann, H. C. & Vennesland, B. (1958). J. biol. Chem. 233, 1398.

Gabriel, S. & Colman, J. (1902). Ber. dtsch. chem. Ges. 35, 3805.

Giri, K. V. & Kalyanker, G. D. (1953). Naturwiss. 40, 224.

Goldman, D. S. & Wagner, M. J. (1962). Biochim. biophys. Acta. 65, 297.

Green, M. & Cohen, S. S. (1957). J. biol. Chem. 225, 387.

Greenberg, D. M. (1954). Chemical Pathways of Metabolism. vol. 2, p. 53. Ed. Greenberg, D. M. New York: Academic Press Inc.

Guest, J. R. & Woods, D. D. (1962). Biochem. J. 82, 26.

Hamill, R. L., Hermann, R. L., Myrrum, R. U. & Fairley, J. L. (1956). Biochim. biophys. Acta. 21, 394.

Himes, R. H. & Rabinowitz, J. C. (1962). J. biol. Chem. 237, 2903.

Huennekens, F. M., Hatefi, Y. & Kay, L. D. (1957). J. biol. Chem. 224, 435.

Huennekens, F. M. & Osborn, M. T. (1959). Advanc. Enzymol. 21, 369.

Huennekens, F. M., Osborn, M. J. & Whiteley, H. R. (1958). Science, 128, 120.

Humphreys, G. K. & Greenberg, D. M. (1958). Arch. Biochem. Biophys. 78, 275.

Hutchison, W. C. & Munro, H. N. (1961). Analyst, 86, 768.

Jaenicke, L. (1956). Fed. Proc. 15, 281.

Jaenicke, L. (1961). The Mechanism of Action of Water-Soluble Vitamins, p. 38. Ed. by deReuck, A. V. S & O'Connor, M. London: Churchill Ltd.

Janke, A. & Tayenthal, W. (1937). Biochem. Z. 289, 76.

Jayasuria, G. C. N. (1956). Biochem. J. 64, 469.

Jonny, E. & Leuthardt, F. (1961). Helv. chim. acta. 44, 78.

Judefind, W. L. & Reid, E. E. (1920). J. Amer. chem. Soc. 42, 1043.

Kisliuk, R. L. & Sakami, W. (1954). J. Amer. chem. Soc. 76, 1456.

Kisliuk, R. L. & Sakami, W. (1955). J. biol. Chem. 214, 47.

Kisliuk, R. L. & Woods, D. D. (1960). Biochem. J. 75, 467.

Klein, S. M. & Sagers, R. D. (1962). J. Bact. 83, 121.

Koch, A. L. (1955). J. biol. Chem. 217, 931

Koch, A. L. & Levy, H. R. (1955). J. biol. Chem. 217, 947.

Koch, G. & Weidel, W. (1956) Z. Physiol. Chem. 303, 213.

Kornberg, H. L. (1959). Annu. Rev. Microbiol. 13, 49.

Kornberg, H. L. & Elsden, S. R. (1961). Advanc. Enzymol. 23, 401.

Kornberg, H. L. & Gotto, A. M. (1959). Nature, Lond. 183, 1791.
Kornberg, H. L. & Gotto, A. M. (1961). Biochem. J. 78, 69.
Kornberg, H. L. & Krebs, H. A. (1957). Nature, Lond. 179, 988.
Kornberg, H. L. & Madsen, N. B. (1957). Biochim. biophys. Acta. 24, 651.
Kornberg, H. L. & Madsen, N. B. (1958). Biochem. J. 68, 549.
Kornberg, H. L. & Morris, J. G. (1963). Nature, Lond. 197, 456.
Kornberg, H. L. & Sadler, J. R. (1960). Nature, Lond. 185, 153.
Kornberg, H. L. & Sadler, J. R. (1961). Biochem. J. 81, 503.
Krakow, G. & Barkulis, S. S. (1956). Biochim. biophys. Acta. 21, 593.
Krakow, G., Barkulis, S. S. & Hayashi, J. A. (1961). J. Bact. 81, 509.
Krakow, G., Hayashi, J. A. & Barkulis, S. S. (1959). Fed. Proc. 18, 265.
Krebs, H. A. (1954). Chemical Pathways of Metabolism, vol. 1, p. 109. Ed. by Greenberg, D. M. New York: Academic Press Inc.
Krol, S. (1952). Biochem. J. 52, 227.
Large, P. J., Peel, D & Quayle, J.R. (1962). Biochem J. 82, 483.
Large, P. J. & Quayle, J. R. (1963). Unpublished.
Larrabee, A. R. & Buchanan, J. M. (1961). Fed. Proc. 20, 9.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). J. biol. Chem. 193, 265.
McDougall, B. M. & Blakely, R. L. (1961). J. biol. Chem. 236, 832.
McIlwain, H. (1948). J. gen. Microbiol. 2, 288.
Madinaveitia, J. (1946). Biochem. J. 40, 373.
Mannell, W. A. & Rossiter, R. J. (1955). Biochem. J. 61, 418.
Markham, R. & Smith, J. D. (1949). Biochem. J. 45, 294.
Meister, A., Sober, H. A., Tice, S. V. & Fraser, P. E. (1952). J. biol. Chem. 197, 319.
Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). J. Amer. chem. Soc. 76, 648.
Mitoma, C. & Greenberg, D. M. (1952). J. biol Chem. 196, 599.

Nakada, H. I., Friedman, B. & Weinhouse, S. (1955). J. biol. Chem. 216, 583.

Nakada, H. I. & Sund, L. P. (1958). J. biol. Chem. 233, 8.

Nakada, H. I. & Weinhouse, S. (1953). J. biol. Chem. 204, 831.

Nakada, H. I. & Weinhouse, S. (1953a). Arch. Biochem. Biophys. 42, 257.

Neish, A. C. & Lemieux, R. U. (1952). Canad. J. Chem. 30, 454.

Nemath, A. M., Russell, C. S. & Shemin, D. (1957). J. biol. Chem. 229, 415.

Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). J. biol. Chem. 193, 691.

O'Dell, B. L., Vandenbelt, J. M., Bloom, E. S. & Pfiffner, J. J. (1947). J. Amer. chem. Soc. 69, 250.

Ogur, M. & Rosen, G. (1950). Arch. Biochem. 25, 262.

Openshaw, H. T. (1955). Laboratory Manual of Qualitative Organic Analysis, p. 73. Cambridge, England: University Press.

Osborn, M. J., Freeman, M. & Huennekens, F. M. (1958). Proc. Soc. expt. biol. Med. 97, 429.

Paretsky, D. & Werkman, C. H. (1950). Arch. Biochem. 25, 288.

Paul, J. (1958). Analyst. 83, 37.

Peters, J. M. & Greenberg, D. M. (1958). J. Amer. chem. Soc. 80, 2719.

Phares, E. F., Mossbach, E. H. & Denison, F. W. (1952). Analyt. Chem. 24, 660.

Pirie, N. W. (1946). Biochem. J. 40, 100.

Pitts, J. D. & Crosbie, G. W. (1962). Biochem. J. 83, 35P.

Pitts, J. D., Stewart, J. A & Crosbie, G. W. (1961). Biochim. biophys. Acta. 50, 361.

Quayle, J. R. & Keech, D. B. (1959). Nature, Lond. 183, 1794.

Rabinowitz, J. C. (1959). Fed. Proc. 18, 306

Rabinowitz, J. C. (1960a). The Enzymes, vol. 2, part A, p. 113. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.

Rabinowitz, J. C. (1960b). Annu. Rev. Microbiol. 13, 441.

Racker, E. (1952). Biochem. biophys. Acta. 9, 577.

Radin, N. S. & Barker, H. A. (1953). Proc. nat. Acad. Sci., Wash. 39, 1196.

Ratner, S., Nocito, V. & Green, D. E. (1944). J. biol. Chem. 152, 119.

Reeves, H. C. & Ajl, S. (1960). J. Bact. 79, 341.

Richert, D. A., Amberg, R. & Wilson, M. (1962). J. biol. Chem. 237, 99.

Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). Carnegie Institute of Washington Publication 607, Washington, D.C.

Roepke, R. R., Libby, R. L. & Small, M. H. (1944). J. Bact. 48, 401.

Sagers, R. D. & Beck, J. V. (1956). J. Bact. 72, 199.

Sagers, R. D., Benziman, M. & Gunsalus, I. C. (1961). J. Bact. 82, 233.

Sagers, R. D. & Klein, S. M. (1961). Bact. Proc. p. 187.

Sagers, R. D. & Gunsalus, I. C. (1958). Bact. Proc. p. 119.

Sagers, R. D. & Gunsalus, I. C. (1961). J. Bact. 81, 541.

Sakami, W. (1948). J. biol. Chem. 176, 995.

Sakami, W. (1949). J. biol. Chem. 178, 519

Sakami, W. (1955). Handbook of Isotope Tracer Methods. p. 55. Ed. by Sakami, W. Cleveland, Ohio: Western Reserve University School of Medicine.

Sanger, F. (1945). Biochem. J. 39, 507.

Schmidt, G. & Thannhauser, S. J. (1945). J. biol. Chem. 161, 83.

Schulman, H. P. & Richert, D. A. (1959). J. biol. Chem. 234, 1781.

Shemin, D. (1946). J. biol. Chem. 162, 297.

Shemin, D. (1956). Harvey Lectures, 50, 258.

Shemin, D., Russell, C. S. & Abramsky, T. (1954). J. biol. Chem. 215, 613.

Siekevitz, P. & Greenberg, D. M. (1949). J. biol. Chem. 180, 845.

Siekevitz, P. & Greenberg, D. M. (1950). J. biol. Chem. 186, 275.

Smith, R. A. & Gunsalus, I. C. (1954). J. Amer. chem. Soc. 76, 5002.

Smith, R. A. & Gunsalus, I. C. (1955). Nature, Lond. 175, 774.

Smith, J. D. & Markham, R. (1950). Biochem. J. 46, 509.

Snell, E. E. (1961). Mechanism of Action of Water-Soluble Vitamins, p18. Ed. by deReuck, A. V. S. & O'Connor, M. London: Churchill Ltd.

Stadtman, T. C. & Elliott, P. (1956). J. Amer. chem. Soc. 78, 2020.

Stadtman, T. C., Elliott, P. & Tiemann, L. (1958). J. biol. Chem. 231, 961.

Stewart, J. A. (1961). B.Sc. Thesis, Glasgow.

Strecker, H. J. (1951). J. biol. Chem. 189, 815.

Szulmajster, J. & Woods, D. D. (1960). Biochem. J. 75, 3.

Thunberg, T. (1920). Skand. Arch. Physiol. 40, 1.

Totter, J. R., Volkin, E. & Carter, C. E. (1951). J. Amer. chem. Soc. 73, 1521.

Turner, J. M. & Happold, F. C. (1961). Biochem. J. 78, 364.

Umbarger, H. E. (1961). Bact. Proc. p. 187.

Vogel, A. I. (1956). Textbook of Practical Organic Chemistry, 3rd edn. London: Longmans, Green & Co.

Wahba, A. J. & Friedkin, M. (1961). J. biol. Chem. 236, PC11.

Weinhouse, S & Friedmann, B. (1954). J. biol. Chem. 210, 423.

Wilmanns, W., Rucker, B. & Jaenicke, L. (1960). Z. Physiol. Chem. 322, 283.

Wilson, D. J., King, K. W. & Burris, R. H. (1954). J. biol. Chem. 208, 863.

Wong, D. T. O., & Ajl, S. J. (1956). J. Amer. chem. Soc. 78, 3230.

Wright, B. E. (1957). Arch. Biochem. Biophys. 31, 332.

Wyatt, G. R. (1951). Biochem. J. 48, 584.

Zelitch, I. & Ochoa, S. (1953). J. biol. Chem. 201, 707.