THIAMIN BIOSYNTHESIS

IN

SACCHAROMYCES CEREVISIAE

By

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ABSTRACT

The biosynthesis of thiamin is investigated by the administration of radioactively labelled compounds to yeast. Thiamin chloride hydrochloride is isolated from the yeast cells and degraded to determine the distribution of activity within its thiazole nucleus. Label from [2-^{14}C]-glycine, in two strains of S. cerevisiae (ATCC 24903 and 39916 H.J. Bunker), is located exclusively at C-2 of the thiazole moiety. Non-random incorporation of activity into the C_5 unit of the thiazole nucleus from [1,3-^{14}C]glycerol, D-[1-^{14}C]fructose, D-[U-^{14}C]-, D-[1-^{14}C]-, D-[2-^{14}C]-, D-[6-^{14}C]-, and D-[6-^{3}H,6-^{14}C]glucose is observed in S. cerevisiae (ATCC 24903). A new hypothesis, consistent with the positions of the label in the above experiments, is proposed for the biosynthesis of the thiazole unit. As the first step in this scheme, glycine and a 2-ketopentose are suggested to form a Schiff base which is converted to the thiazole moiety in a multistep sequence. The mode of incorporation of [1,3-^{14}C]-glycerol and the labelled hexoses indicates that the 2-ketopentose is formed from glycolytic intermediates by two pathways. D-Ribulose 5-phosphate, D-xylulose 5-phosphate, or a closely related compound is the most likely choice for the 2-ketopentose. Radioactivity from sodium [^{14}C]formate, [3-^{14}C]serine, [1,3-^{14}C]glycerol and the above hexoses is incorporated into the pyrimidine nucleus of thiamin. A new scheme is proposed to account for these results. Radioactivity from L-[Me-^{14}C]-methionine, DL-[2-^{14}C]tyrosine, sodium [1-^{14}C]acetate, sodium [3-^{14}C]-pyruvate, sodium L-[U-^{14}C]lactate, DL-[3-^{14}C]cysteine, D-[1-^{14}C]ribose, sodium 2-keto[5-^{14}C]glutarate, and [1-^{14}C]succinic acid is not incorporated into thiamin.

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Finally, I would like to dedicate this thesis to my parents, who have been a source of encouragement and support from the beginning.

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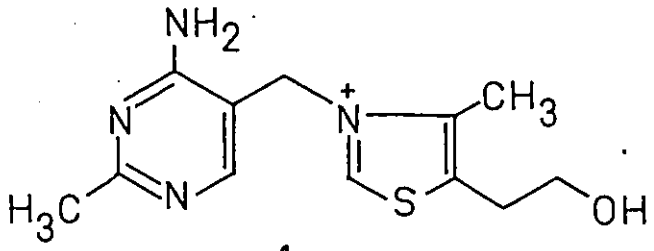
INTRODUCTORY REMARKS

An organic substance which is required in small amounts for the nutrition and normal growth of an organism, but which is not formed by that organism, has been given the term vitamin. Thirteen vitamins are required for the well-being of man.[1] Traditionally, these thirteen substances have been classified into two groups: the water-soluble (vitamin C and the B vitamins) and the fat-soluble (vitamins A, D, E and K) vitamins. Most water-soluble vitamins act as coenzymes while the fat-soluble vitamins have more varied functions.

Vitamin B_1, as it name suggests, was the first member to be identified in the B group of vitamins. The name thiamin chloride was proposed by R.R. Williams[2] for this substance and this, or the term thiamine, became accepted in North America, whereas the name aneurin(e) was used in Europe. These designations have been replaced by the name thiamin[3] which refers to the 3-(4-amino-2-methylpyrimidin-5-ylmethyl)-5-(2-hydroxyethyl)-4-methylthiazolium ion (1).



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Thiamin has been found to act as a coenzyme for some enzymes which catalyze carbon-carbon bond formations and cleavages. These reactions

are summarized in Chapter I and they will be referred to again in Chapter II, as it seems that thiamin is required for the formation of precursors involved in its own biosynthesis.

However, the biosynthesis of thiamin is not nearly as well established as its function. Evidence for the formation of thiamin pyrophosphate (the active form of the coenzyme) from two preformed heterocyclic units is presented in Chapter I to illustrate that the biosynthesis of thiamin comprises two completely separate problems: the biosynthesis of the pyrimidine and thiazole units. Many investigations have been undertaken to elucidate the basic precursors of these units and these are described in some detail. The technique most widely used in these experiments was the feeding of isotopically labelled compounds to micro-organisms. Table I summarizes the literature according to the labelled compound and micro-organism used in each investigation. The results of these numerous investigations have not led to a general agreement on the basic precursors of these units and are, in some cases, contradictory.

This thesis is an attempt to resolve the conflicting evidence concerning the biosynthesis of the thiazole moiety and to establish the basic precursors of this unit. Chapter II presents a new hypothesis for the biosynthesis of the thiazole moiety and the evidence which has been obtained in its support.

Some information concerning the origin of the pyrimidine moiety has also been collected and preliminary conclusions are given in Chapter III. However, the detailed solution of this aspect of thiamin biosynthesis will require a separate investigation.

CHAPTER I

INTRODUCTION

(i) Discovery and function of thiamin

The origin of the term vitamin has been closely associated with the isolation and characterization of thiamin. In 1884, the disease beriberi was eliminated from the Japanese navy by changing the diet.[4] Eijkman[6] observed that fowls, when fed polished rice, developed a disease which he called polyneuritis. Administration of rice polishings cured the disease. Eijkman suggested that a toxic principle, formed from starch in the intestine, caused the disease. Rice polishings would contain an antidote. Today's accepted explanation for this observation was proposed by Grijns in 1901.[7] He suggested that polyneuritis was partial starvation due to the absence of some essential substances. A few years later, the substance responsible for polyneuritis was given the name "vitamine"[8] as it was thought to be an amine which was vital to life. The present term, vitamin, has been generalized to refer to the group of organic compounds which are required in small amounts for the well being of man.

The search for this curative substance was thus focused on rice bran and a semicrystalline material was obtained in 1926.[9] Structural elucidation studies followed the determination of the molecular formula of the hydrochloride as $C_{12}H_{16}N_4OS \cdot 2HCl \cdot H_2O$.[10,11] Similar results were obtained for material extracted from yeast. The key degradative reaction used in the determination of the vitamin's structure was the quantitative

cleavage of thiamin chloride hydrochloride (2) by bisulfite [12] (Figure 1). The structures of the pyrimidine (4),[13] and thiazole (5) [14] components were determined separately and the full structure of thiamin was published in 1936 by Williams[15] and Grewe.[16] However, priority has been credited to R.R. Williams.

The chemical synthesis of thiamin, which confirmed the structural elucidation, was accomplished soon thereafter by three independent groups.[17-19] The chemically synthesized vitamin soon became the main source of thiamin because of the difficulties involved with the isolation of the vitamin from natural sources.

Within a year of the synthesis of thiamin, its pyrophosphate ester (3) was isolated from yeast[20] and shown to be the cofactor for the decarboxylation of pyruvic acid.[21]

In general, thiamin pyrophosphate (3) is the required cofactor for enzymes that cleave a carbon-carbon bond adjacent to a keto group.[22] These reactions may be classified into four main categories: non-oxidative decarboxylation of α-keto acids; oxidative decarboxylation of α-keto acids; formation of α-ketols; and, formation of acetyl phosphate. The substrates for these reactions are of two main structures, α-keto acids and 2-keto sugar phosphates. Figure 2 outlines the transformations of an α-keto acid, pyruvic acid (6), and Figure 3 presents the transformation of a 2-keto sugar phosphate, fructose 6-phosphate (14).

The non-oxidative decarboxylation of pyruvic acid (6) to acetaldehyde (9) is catalyzed by the enzyme pyruvic acid decarboxylase, whereas the oxidative decarboxylation yields S-acyl lipoic acid (13) which is converted to acetyl CoA. Acetyl CoA, among other functions, can provide

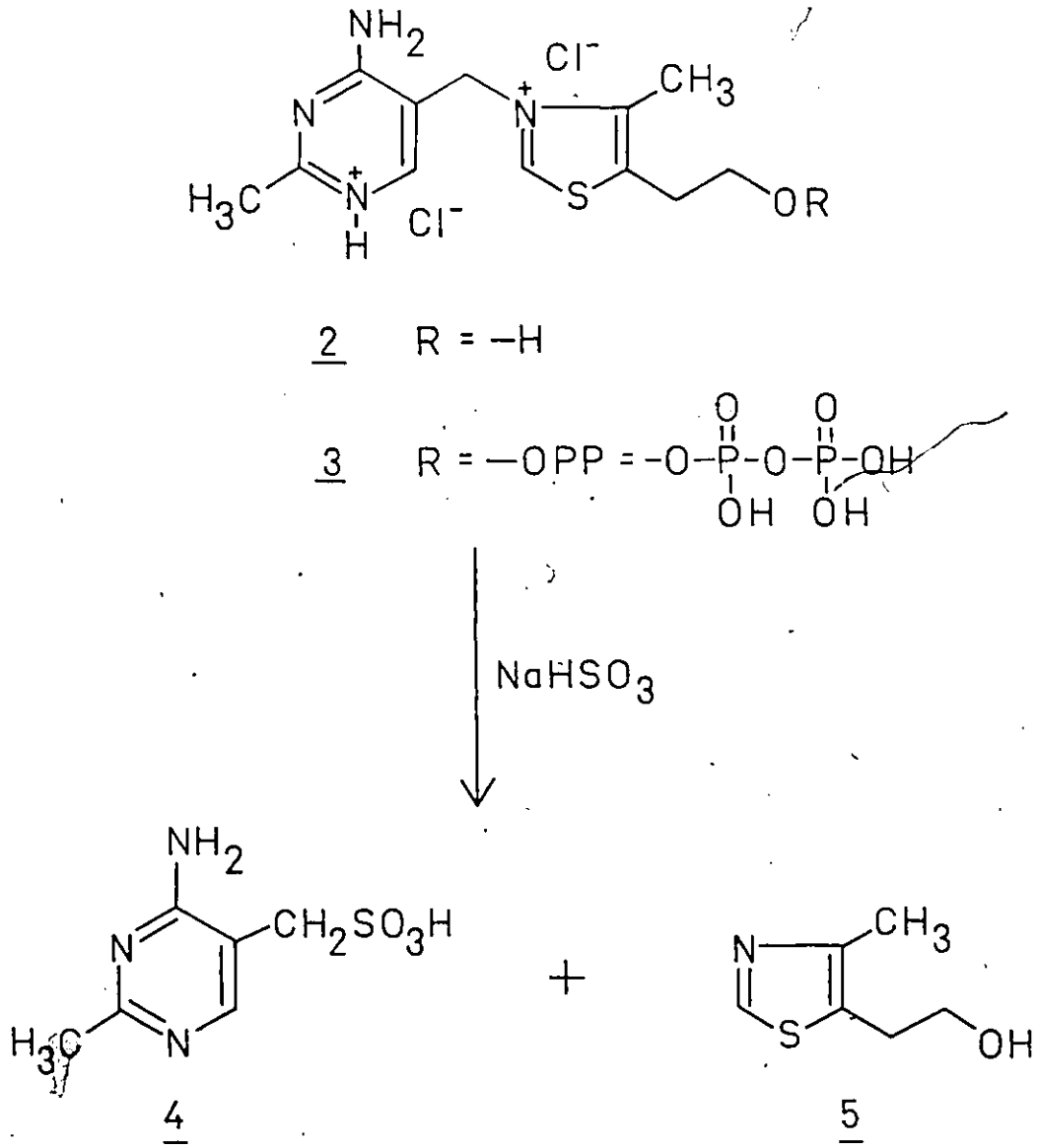


Figure 1: Bisulfite cleavage of thiamin chloride hydrochloride.

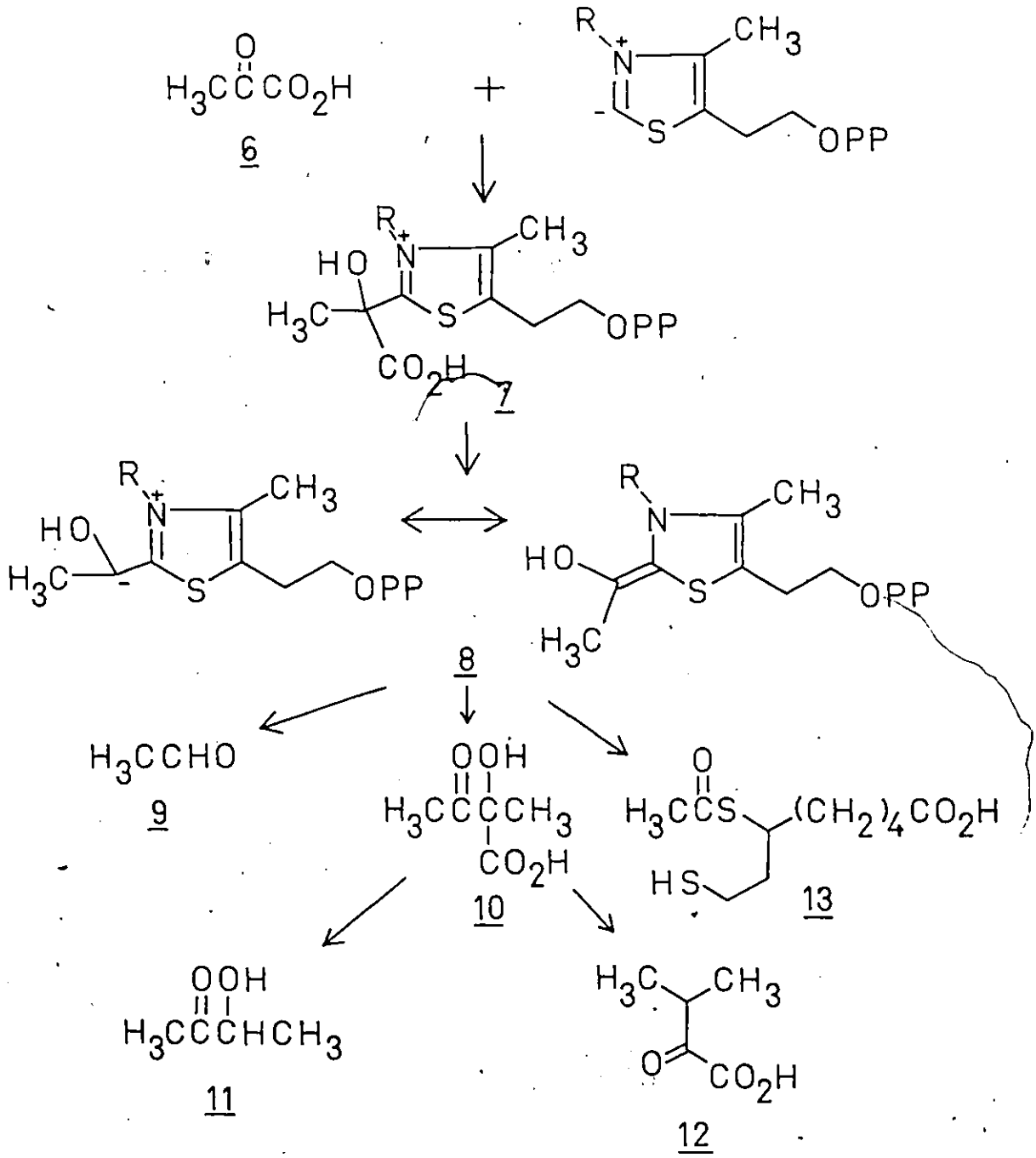


Figure 2: Products obtained from pyruvic acid via thiamin pyrophosphate catalyzed enzymic reactions. (R = the pyrimidine moiety of thiamin.)

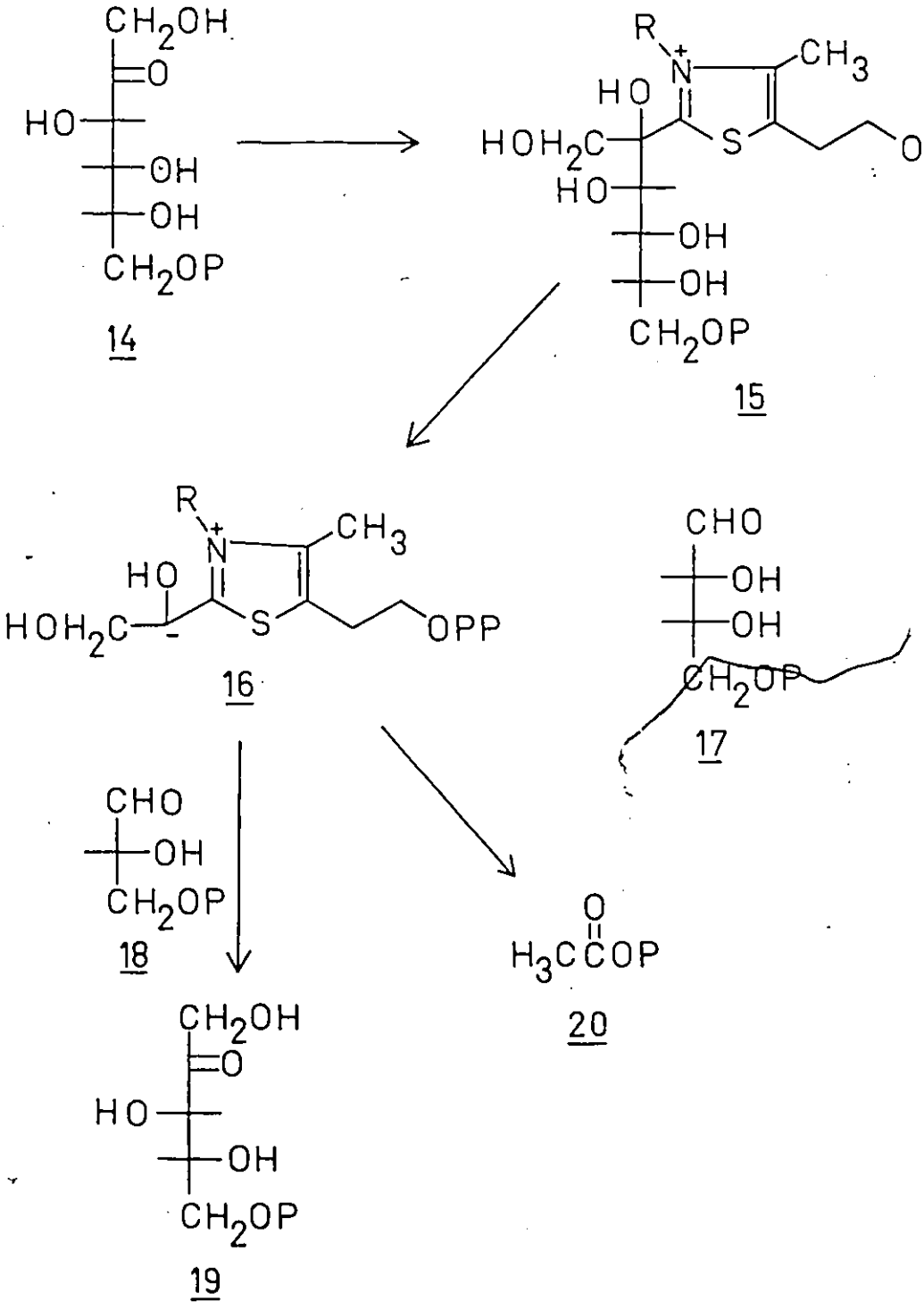


Figure 3. Products obtained from D-fructose 6-phosphate via thiamin pyrophosphate catalyzed enzymic reactions. (R = the pyrimidine moiety of thiamin.)

energy for the cell via the Krebs' Cycle.

The α-ketol, acetoin (11), is formed by micro-organisms and plants by two known mechanisms.[23] In bacteria, the formation of α-acetolactic acid (10) can be pictured as the condensation of a C_2 unit derived from pyruvate (6) with a second molecule of pyruvate (6). A specific decarboxylase catalyzes the formation of acetoin (11) from α-acetolactic acid (10). Under anaerobic conditions, acetoin is reduced to 2,3-butanediol. In plants and yeasts, the enzyme carboligase catalyzes the formation of acetoin from a C_2 unit derived from pyruvate (6) and free acetaldehyde (9) formed by the action of pyruvate decarboxylase on pyruvate (6). The chemical analogies for the formation of acetoin (11) are the Acyloin Condensation[24] and the Benzoin Condensation.[25]

Thiamin pyrophosphate (3) is also important as a coenzyme in amino acid biosynthesis.[26] α-Oxoisovaleric acid (12), the precursor of valine and leucine, is formed from α-acetolactic acid (10) by rearrangement and reduction. The first step in the biosynthetic route to isoleucine is an analogous condensation of a C_2 unit derived from pyruvate with α-oxobutyrate.

The enzyme transketolase catalyzes the formation of the α-ketol D-xylulose 5-phosphate (19) from D-fructose 6-phosphate (14) and D-glyceraldehyde 3-phosphate (18). Acetyl phosphate (20) is formed from D-fructose 6-phosphate (14) in the presence of the enzyme phosphoketolase. The second product in both of these reactions is D-erythrose 4-phosphate (17).

A clue to the mechanism of these transformations was given by the observation of the catalysis of the acyloin condensation by a number

of thiazolium compounds, including thiamin.[27] Thiamin was also found to catalyze both the decarboxylation of pyruvate and the formation of acetoin from pyruvic acid and acetaldehyde without the aid of enzymes.[28] These reactions were thought to involve the acyl anion, RCO^-, or some stabilized equivalent, and a number of proposals were made to account for the stabilization of this species by thiamin.[29,30] However, Breslow's nuclear magnetic resonance observation of rapid deuterium exchange in thiazolium salts[31] led him to propose the mechanism outlined in Figure 2 for the thiamin catalyzed transformation of pyruvate to acetaldehyde[32] and for the thiamin catalyzed Benzoin Condensation.[33] Breslow's proposal included the intermediates (7) and (8).

α-Lactyl thiamin pyrophosphate (7) was isolated by paper chromatography from the enzymic reaction of pyruvate with pyruvate decarboxylase from brewer's yeast.[34]

However, the evidence for the α-hydroxyethylthiamin pyrophosphate zwitterion (8) as an intermediate in these reactions is much stronger. α-Hydroxyethylthiamin was chemically synthesized and, in the presence of thiamin pyrophosphokinase and pyruvate decarboxylase, was converted to acetaldehyde.[35] Its isolation from enzymic incubations has been described.[36] α-Hydroxyethylthiamin was also detected in higher plants and it was thought to be present as its pyrophosphate ester.[37] The pyrophosphate ester (protonated 8) was later synthesized[38] and identified in extracts of Escherichia coli,[39] in the enzymic reactions of pyruvate with wheat germ decarboxylase,[39] and in a brewer's yeast decarboxylase.[34] The same compound was also found in the reaction mixture of the pyruvate dehydrogenase catalyzed conversion of pyruvic acid (6) to acetyl CoA.[40,41] Evidence for the formation of the zwitterion (8) from α-hydroxyethylthiamin

pyrophosphate was provided by the observation of the rate of exchange of the α-proton by nuclear magnetic resonance spectroscopy.[42]

The term "active aldehyde" was in general use[43] to describe the stabilized acyl anion involved in these transformations. This term derives from the statement by Neuberg[44] that "this condensation ... can be brought about without any difficulty if the aldehyde which furnishes the acetyl group is in statu nascendi ...". The intermediate anion (8) can be regarded as this "active aldehyde".

To explain the ketol condensation that is carried out by the enzyme transketolase, Racker et al.[45] postulated the formation of an "active glycolaldehyde" which condenses with the "acceptor aldehyde" to form a ketosugar. This "active glycolaldehyde" was assumed to be tightly bound to the enzyme as no free glycolaldehyde was detected. Denaturation of the protein liberated glycolaldehyde and when ^{14}C labelled glycolaldehyde was placed in solution along with a 2-ketosugar phosphate and transketolase, the 2-ketosugar phosphate became labelled;[46] this indicated exchange between free glycolaldehyde and "active glycolaldehyde". By analogy with "active acetaldehyde", "active glycolaldehyde" is considered as the zwitterion (16). The protonated form has been chemically synthesized.[38] α,β-Dihydroxyethyl thiamin pyrophosphate (protonated 16) has been isolated from an incubation of [U-^{14}C]fructose and transketolase by ion-exchange chromatography.[47] When incubated with transketolase and inactive glycolaldehyde, this derivative yielded radioactive erythrulose. An improved method for the preparation of this derivative[48,49] led to the isolation of sufficient quantities for chemical degradation.[50] An incubation was carried out with [3-^{14}C]hydroxypyruvate. α,β-Dihydroxyethylthiamin pyro-

phosphate was isolated, dephosphorylated with phosphatase and separated by electrophoresis from thiamin. The derivative was cleaved with bisulfite and the α-hydroxyethylthiazole was isolated by electrophoresis and paper chromatography. This substituted thiazole was treated with hydroxylamine and an active spot with the same R_f value as glycerohydroxamic acid was obtained on paper chromatography. The glycerohydroxamic acid was derived from C-2 of the thiazole ring and the covalently bound glycolaldehyde. Other than R_f value, no effort was made to characterize this compound.

The formation of sedoheptulose 7-phosphate from this isolated compound and ribose 5-phosphate by transketolase was reported as additional evidence for the intermediacy of "active glycolaldehyde" (16) in the transketolase reaction.[51]

The enzyme phosphoketolase has been isolated from the bacteria Leuconostoc mesenteroides,[52] Acetobacter xylinum[53] and Lactobacillus plantarum.[54] In each case, thiamin pyrophosphate is the required cofactor and D-xylulose 5-phosphate is a substrate for all three enzymes. Only the enzymes isolated from the first two bacterial species, however, could utilize D-fructose 6-phosphate. Breslow[55] suggested a mechanism in which the "active glycolaldehyde" intermediate (16) rearranged to give a 2-acetyl thiamin derivative which would react with inorganic phosphate to generate acetyl phosphate (20). Model studies had shown the kinetic lability of such an intermediate.[56] A second possible mechanism is the addition of phosphoric acid across the double bond of the intermediate enol. There is no experimental evidence either to support or to contradict these mechanisms.[57] However, the zwitterion (16) is a likely intermediate as α,β-dihydroxyethylthiamin pyrophosphate, obtained from the

action of pyruvic oxidase on [2-^{14}C]hydroxypyruvate, was converted to acetyl phosphate (20) by phosphoketolase.[58]

(ii) Formation of thiamin pyrophosphate from preformed heterocyclic units

The ultimate goal of a biosynthetic investigation is the detailed description of the biosynthetic sequence leading to the desired product from the well-known primary constituents of the cell. The biosynthesis of thiamin from the preformed pyrimidine (4-amino-5-hydroxymethyl-2-methylpyrimidine (21)) and thiazole (5-(2-hydroxyethyl)-4-methylthiazole (5)) moieties has been well investigated, and reviewed;[5,29,59-67] this is presented in Figure 4. Some of the enzymes involved in this sequence of reactions have been purified. However, the pyrimidine (21) and thiazole (5) precursors have structures which are unique to thiamin; these units are not primary constituents of the cell and their origins from simpler substances is not at all well established.

The ready availability of chemically synthesized thiamin and pyrimidine and thiazole derivatives led to nutritional studies which provided the first information concerning thiamin biosynthesis. Experiments revealed that the thiamin requirements of the fungus Phycomyces blakesleeanus[68-70] and excised pea roots[71] could be met with a mixture of the pyrimidine and thiazole moieties. The pyrimidine moiety was supplied as either the 5-bromomethyl, 5-ethoxymethyl or the 5-aminomethyl derivatives of (21). Neither the pyrimidine nor the thiazole derivative alone could replace the thiamin requirement, and the amount of growth was limited by the component present in the smallest amount. This suggested that these compounds were converted to thiamin before they had any effect on the organism.

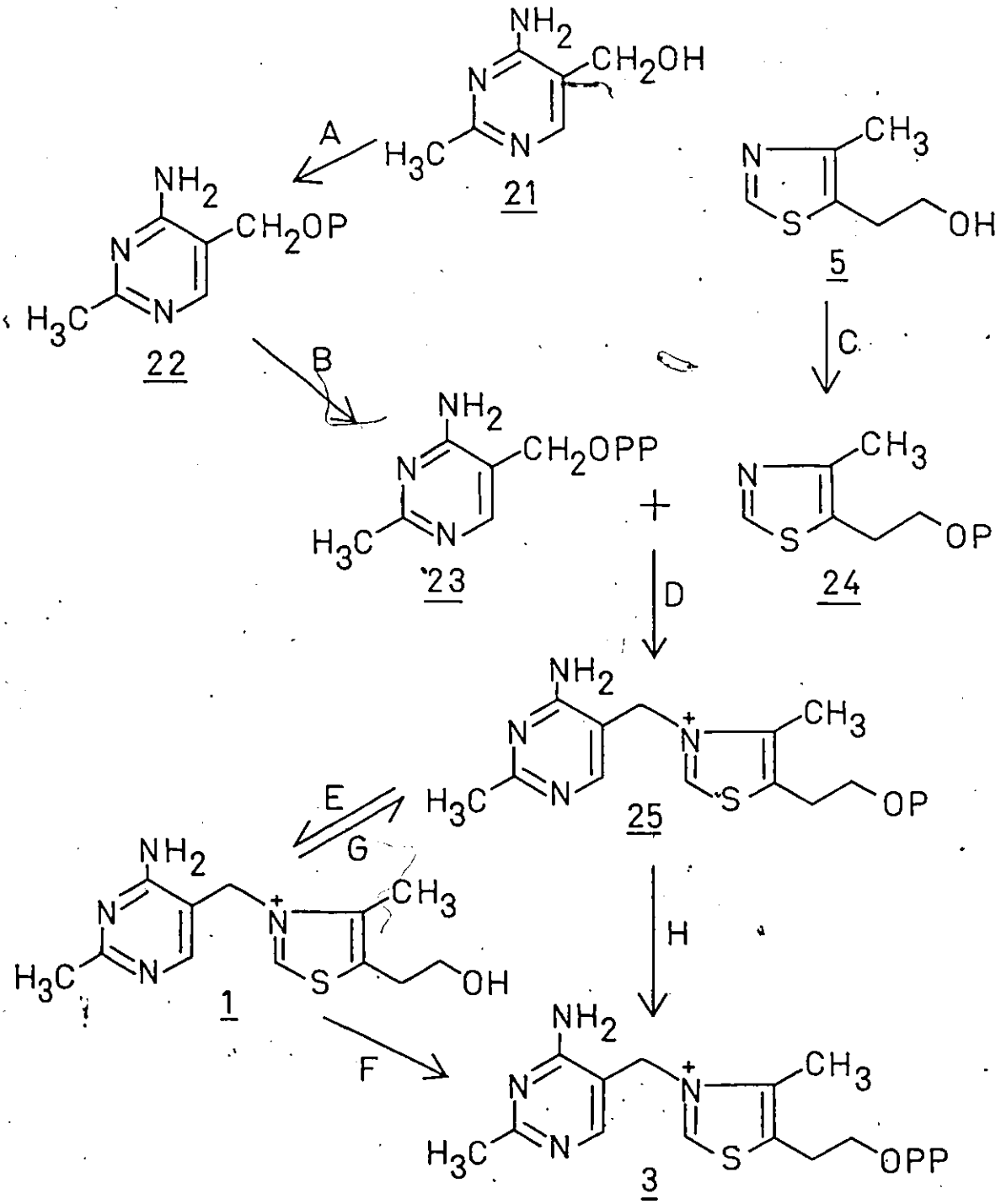


Figure 4: Biosynthesis of thiamin from preformed heterocyclic compounds.

The synthesis of thiamin from the pyrimidine and thiazole units supplied to pea roots was shown with the fungus Phytophthora cinnamomi,[72] since the thiamin requirement of this fungus could only be met by the intact vitamin and not by a mixture of the pyrimidine and thiazole moieties.

In Staphylococcus aureus[73] and excised tomato roots[74] the thiazole (5) satisfied the thiamin requirement; this suggested that these organisms have the ability to synthesize the pyrimidine portion of thiamin.

The thiamin requirements of many fungi were investigated and the results summarized.[65,75] The fungi were classified into five categories according to whether they require preformed thiamin, pyrimidine and thiazole moieties, pyrimidine moiety, thiazole moiety, or have no requirement.

Similar observations have been made on examination of bacteria. Lactobacillus fermenti [76] and Flavobacterium aquatile[77] require preformed thiamin and these organisms, among others, have been used for the quantitative assay of thiamin.[78] The thiamin requirements of other bacteria and yeasts have been summarized.[79]

These early nutritional studies produced the currently accepted idea that thiamin was derived from preformed pyrimidine and thiazole units. These investigations were taken one step further with Neurospora crassa mutants,[80] which differed from the wild type in only a single gene. One mutant required intact thiamin and another required the thiazole (5). The first mutant accumulated pyrimidine and thiazole moieties and the second one accumulated only the pyrimidine moiety. These results suggested that the first mutant lacked the enzyme necessary for the synthesis of thiamin from its pyrimidine and thiazole precursors and that the second mutant lacked the ability to manufacture the thiazole precursor. When the thia-

zole (5) was supplied to the latter mutant, the organism could synthesize thiamin. From his work with the same series of N. crassa mutants, Harris[81] suggested two alternative pathways of thiamin biosynthesis. In addition to the coupling of the pyrimidine and thiazole units to form thiamin directly, he postulated a second route which involved an intermediate containing a partially formed thiazole already attached to the pyrimidine moiety. A mutant which accumulated a substance that appeared to have the properties of this intermediate was also described. Eberhart and Tatum[82] re-investigated this mutant and they found no evidence for the accumulation of such an intermediate. Instead, they suggested that the substance accumulated was a phosphorylated form of thiamin.

Harris[81] also made a distinction between the thiazole (5) supplied in the medium, and an endogenous precursor of the thiazole moiety which is used preferentially to couple with the pyrimidine unit. Perhaps the endogenous precursor was the thiazole phosphate (24) which would be used preferentially for the formation of thiamin.

Feeding experiments, using isotopically labelled compounds, gave the first direct evidence that thiamin is synthesized from a pyrimidine and a thiazole precursor. [^{14}C]4-Amino-5-aminomethyl-2-methylpyrimidine[83] and [2-^{14}C]-5-(2-hydroxyethyl)-4-methylthiazole[84] were chemically synthesized and were found to be incorporated into thiamin by a number of thiamin requiring fungi. The organisms used required either the pyrimidine or the thiazole moiety or both for growth, but all showed incorporation of the labelled compounds. A tracer experiment of this type does not indicate that a compound is on the direct pathway to thiamin from simple cellular constituents but only that it can be converted to a substance which is on

the direct pathway.[85] From current knowledge,[59] 4-amino-5-aminomethyl-2-methylpyrimidine falls into the latter category.

The first indication of the coupling process between the thiazole and pyrimidine moieties came from studies of the enzyme, thiaminase, which catalyzes the cleavage of thiamin. Woolley[86] suggested that the quaternary nitrogen of thiamin could be formed only with the expenditure of energy and Fujita[87] used thiaminase to catalyze the synthesis of thiamin from the thiazole (5), and the pyrimidine moiety, substituted at C-7, with pyridine. The enzyme was simply catalyzing a nitrogen exchange reaction.

Using a cell-free extract from baker's yeast, Harris and Yavit[88] found thiamin to be synthesized from the pyrimidine (21) and the thiazole (5). In addition, they observed that the lag period of this synthesis was eliminated if the pyrimidine phosphate (22) was used as a substrate, and that thiamin synthesis was inhibited by the addition of phosphatases to the extract. These observations were confirmed by Leder[89] who found that the synthesis of thiamin from the pyrimidine phosphate (22) and the thiazole (5) required the participation of two enzymic compounds, one of which was stable at 55°C and the other not. Also, the presence of an ATP generating system was required.

Nose et al.,[90,91] using an enzymic extract from Oriental baker's yeast, concluded that two steps were required for the synthesis of thiamin: the activation of the pyrimidine (21) and the thiazole (5), and the condensation of these activated units to form thiamin. Chemically synthesized phosphate esters of the pyrimidine and thiazole moieties were incubated with the enzymic extract. The pyrimidine pyrophosphate (23) and the thiazole phosphate (24) were the most active of the compounds tried and thus

they were suggested as the most likely activated thiamin precursors.[92] Also, the enzymic synthesis of the pyrimidine pyrophosphate was suggested as a two-step reaction.

The initial product of this coupling reaction was found to be thiamin monophosphate (25),[93,94] and not free thiamin (1) as had been suggested by Harris and Yavit.[88] Thiamin monophosphate (25) was detected because the cell-free extract had been heated to inactivate the phosphatases. Synthetic samples of the pyrimidine pyrophosphate (23) and the thiazole phosphate (24) eliminated the ATP requirement for thiamin synthesis.

Camiener and Brown[94,95] isolated the pyrimidine phosphate (22) and presented chromatographic evidence which indicated the presence of the pyrimidine pyrophosphate (23), the thiazole phosphate (24), thiamin monophosphate (25), thiamin (1) and thiamin pyrophosphate (3), in cell-free extracts of baker's yeast. They proposed a scheme identical with that given in Figure 4, with the route from thiamin monophosphate (25) to thiamin pyrophosphate (3) via free thiamin (1).

Evidence in the support of this route was obtained when the thiazole phosphate (24) was isolated from an enzymic extract of baker's yeast and identified by ultraviolet spectroscopy and bioautographic methods[96] and enzymic fractions were found which converted thiamin (1), but not thiamin monophosphate (25), to thiamin pyrophosphate (3). A phosphatase was assumed to be present since a purified extract gave thiamin monophosphate (25) as the only product of coupling, whereas the crude extract produced all three forms of thiamin.

The formation of the thiazole phosphate (24) was demonstrated using

^{35}S labelled thiazole (5) and paper chromatography.[97] The incubation was followed with time and it was concluded that thiamin monophosphate (25) was formed and then converted to thiamin (1).

Evidence for the stepwise formation of the pyrimidine pyrophosphate (23) with the pyrimidine phosphate (22) as an intermediate was obtained using enzymic fractions from baker's[98] and brewer's[99] yeast. The first phosphorylation was catalyzed by an enzyme which was stable at 55°C and able to use the nucleoside phosphates CTP, GTP and UTP in addition to ATP as the phosphorylating agent. The conversion of the pyrimidine phosphate (22) to the pyrimidine pyrophosphate (23) was catalyzed by a heat-labile and ATP specific enzyme. No free pyrimidine (21) was detected in the mixture. Unfortunately, the two enzymes were found in the same purified fraction so the evidence given above only suggests the presence of two separate enzymes.

The enzyme, thiamin monophosphate pyrophosphorylase, which catalyzes the synthesis of thiamin monophosphate (25) from the pyrimidine (21) and thiazole (5) moieties was purified 500-fold from baker's yeast.[100] The enzyme requires Mg^{++} for optimal activity and is specific for the pyrimidine pyrophosphate (23) and the thiazole phosphate (24). This enzyme has been obtained in a crystalline form.[101]

The synthesis of thiamin monophosphate (25) from the pyrimidine (21) and thiazole (5) units has been described not only in yeast, but also in the fungus Phycomyces blakesleeanus,[102] in plants[103] and in Escherichia coli.[104] Thiamin monophosphate pyrophosphorylase has been purified 175-fold from E. coli,[105] and it differs from the yeast enzyme in some respects. The former is much smaller in molecular weight

(17000 vs 340000)[101] and is inactivated at 45°C, whereas the yeast enzyme is stable at 50°C. Both, however, are inhibited by ATP and other high-energy phosphate compounds.[105,106]

Thiamin pyrophosphate (3) is the active form of the coenzyme and two routes from thiamin monophosphate (25) are shown in Figure 4. Thiamin monophosphate (25) can be converted to thiamin (1) and this pyrophosphorylated to thiamin pyrophosphate (3), or thiamin monophosphate (25) can be phosphorylated to give thiamin pyrophosphate (3) directly. The available evidence suggests that the first route (steps E and F) occurs in yeast and the latter one (step H) in E. coli.

Weil-Malherbe[107] observed the synthesis of thiamin pyrophosphate (3) in a cell-free enzyme preparation from yeast. A longer induction period was noted for thiamin monophosphate (25) than for thiamin (1). He concluded that thiamin monophosphate (25) was not an intermediate in the formation of thiamin pyrophosphate (3) from thiamin (1).

The transfer of a pyrophosphate group from ATP to thiamin was shown in a ^{32}P labelling experiment.[108,109] A sample of ATP labelled with ^{32}P in the γ and β phosphates in the ratio of 11.5 was incubated with a 100-fold purified thiamin pyrophosphokinase from baker's yeast.[110] This enzyme was inactive to thiamin monophosphate (25) and had a broad nucleotide specificity.[111] Thiamin pyrophosphate (3) was isolated and degraded to thiamin monophosphate (25) by acid hydrolysis. The distribution of ^{32}P in the α and β phosphates of thiamin pyrophosphate (3) was equal to the distribution in ATP.

The existence of thiamin pyrophosphokinase and phosphatase in yeast has led to the view that thiamin monophosphate (25) must be dephos-

phorylated before it can be converted to thiamin pyrophosphate (3), but there is no evidence for a specific phosphatase.[62] There is also some evidence for a thiamin monophosphate kinase in yeast.[59]

In E. coli, thiamin pyrophosphate (3) appears to be formed directly (step H) from thiamin monophosphate (25). However, thiamin pyrophosphokinase activity has been found in the membrane fraction of E. coli cells,[112] but it has been suggested that its function is to participate in the transport of thiamin across the membrane.[112] Separate enzymes which catalyze the synthesis of thiamin monophosphate (25) from thiamin (1)[113] (step G) and the synthesis of thiamin pyrophosphate (3) from thiamin monophosphate (25)[114] have been demonstrated in the soluble fraction of E. coli extracts. Thiamin monophosphate kinase has been purified 60-155 fold from crude extracts of E. coli.[115]

The above information, obtained from experiments with cell-free extracts or purified extracts, was confirmed with E. coli mutants which required the phosphorylated forms of thiamin.[116,117] One mutant required thiamin pyrophosphate (3) and accumulated thiamin monophosphate (25) when incubated with thiamin, which suggested a lack of thiamin monophosphate kinase. A second mutant required thiamin monophosphate (25) or thiamin pyrophosphate (3) and the cells accumulated free thiamin from the medium, which suggested a lack of thiamin kinase. A cell-free extract of the latter mutant was able to phosphorylate thiamin monophosphate (25) but not thiamin (1), whereas a cell-free extract of the first mutant was able to phosphorylate thiamin (1) but not thiamin monophosphate (25).

The route(s) to thiamin pyrophosphate (3) given in Figure 4

are quite well established. The steps indicated (A to H) have been found in cell-free extracts and the enzymes responsible for steps D, F and H have been purified. The origins of the pyrimidine (21) and the thiazole (5) moieties remain as the largest unsolved problems.

(iii) Origin of the thiazole moiety

Although the biosynthesis of the thiazole moiety has been investigated since shortly after the structural elucidation of thiamin, there is no general agreement on the basic precursors of this portion of the molecule. This is reflected in recent reviews of thiamin biosynthesis.[59-62]

Feeding of radioactively labelled compounds to whole organisms has been the technique most widely used in these investigations. An experiment of this type involves the isolation and purification to constant specific activity of the desired compound from the organism. Derivatives can also be prepared to check the radiochemical purity of the isolated compound. The location of the label is determined by chemical degradation. Ideally, the molecule should be broken apart so that each atom is obtained on its own. The sum of the specific activities of all the carbon atoms in the molecule should be the same as the specific activity of the compound degraded. If this is not so, then either one or more of the compounds in the degradation is impure, or the mechanism of reactions used in the degradations is not as expected.

The results of experiments of this type, undertaken to elucidate the biosynthesis of the thiazole moiety, are in some cases, contradictory and in all cases incomplete. The term "incomplete" is used deliberately,

since only one of the six carbon atoms was isolated by chemical degradation, and the suggested hypotheses are based solely on incorporation results. Incorporation measurements were used to judge the relative importance of labelled compounds as precursors to the thiazole moiety, but this type of comparison assumes that the compounds are taken up by the organism and transported to the site of biosynthesis at the same rate. Such an assumption is unwarranted even for a given organism, and when results that have been obtained from different organisms are compared, this assumption is clearly invalid. In fact, negative results might be due solely to permeability problems. An additional difficulty with this type of investigation concerns the radiochemical purity of the compound isolated. If great care is not taken in checking the purity of the isolated substance, there is always the chance of contamination by minute amounts of very active compounds.

The general approach to thiazole biosynthesis has been the isolation of thiamin and use of the bisulfite cleavage reaction (Figure 1) to separate the pyrimidine and thiazole components. The pyrimidine sulfonic acid (4) is a solid and may be recrystallized to constant radioactivity, but the thiazole (5) is an oil. Generally, the thiazole (5) has not been converted to a solid derivative which could be purified by crystallization and sublimation. A solution of the thiazole (5) was usually obtained and the concentration was measured by ultraviolet spectroscopy. A portion of this solution was then used for radioactivity measurements. Since a procedure of this type would neither detect nor remove a small amount of very active contaminant, it had to be assumed that the measured radioactivity was associated entirely with the thiazole (5).

The six biogenetic schemes in Figure 5 reflect the varied and contradictory results which have originated during the past forty years from investigations of the type described above. As an introduction to the biosynthesis of the thiazole moiety and the problems associated with its investigation, the evidence for each hypothesis will be critically examined.

From nutritional studies with isolated pea roots, Bonner and Buchman[72] suggested that the thiazole moiety was biosynthesized from thioformamide (26) and 5-hydroxypentan-2-one (27) (Route A, Figure 5) or from thioformamide (26) and 3-chloro-5-hydroxypentan-2-one. This hypothesis was not confirmed in the micro-organisms Phycomyces and Neurospora.[80] In relation to this, [^{35}S]-γ-mercapto-γ-acetopropylacetate was fed to a number of fungi[118] but no incorporation into thiamin was obtained. Through the use of mutants, Nakayama[119] concluded that γ-aceto-γ-mercaptopropanol was not involved in the normal synthesis of the thiazole moiety in E. coli.

Nakayama[119-122] using thiamin auxotrophs of E. coli and N. crassa, found that cystine and thiazolidine-4-carboxylic acid (29) could maintain the growth of these strains and that thiamin and its thiazole moiety were detected in the culture medium of Neurospora.[122] 4-Methylthiazole (30) supported growth, but 4,5-dimethylthiazole did not. These observations led to the suggestion that cysteine (28), along with presumably a C_1 unit, would form thiazolidine-4-carboxylic acid (29), which would be converted to 4-methylthiazole (30). An unspecified C_2 unit would then be added to give the β-hydroxyethyl substituent of the thiazole moiety (Route B, Figure 5).

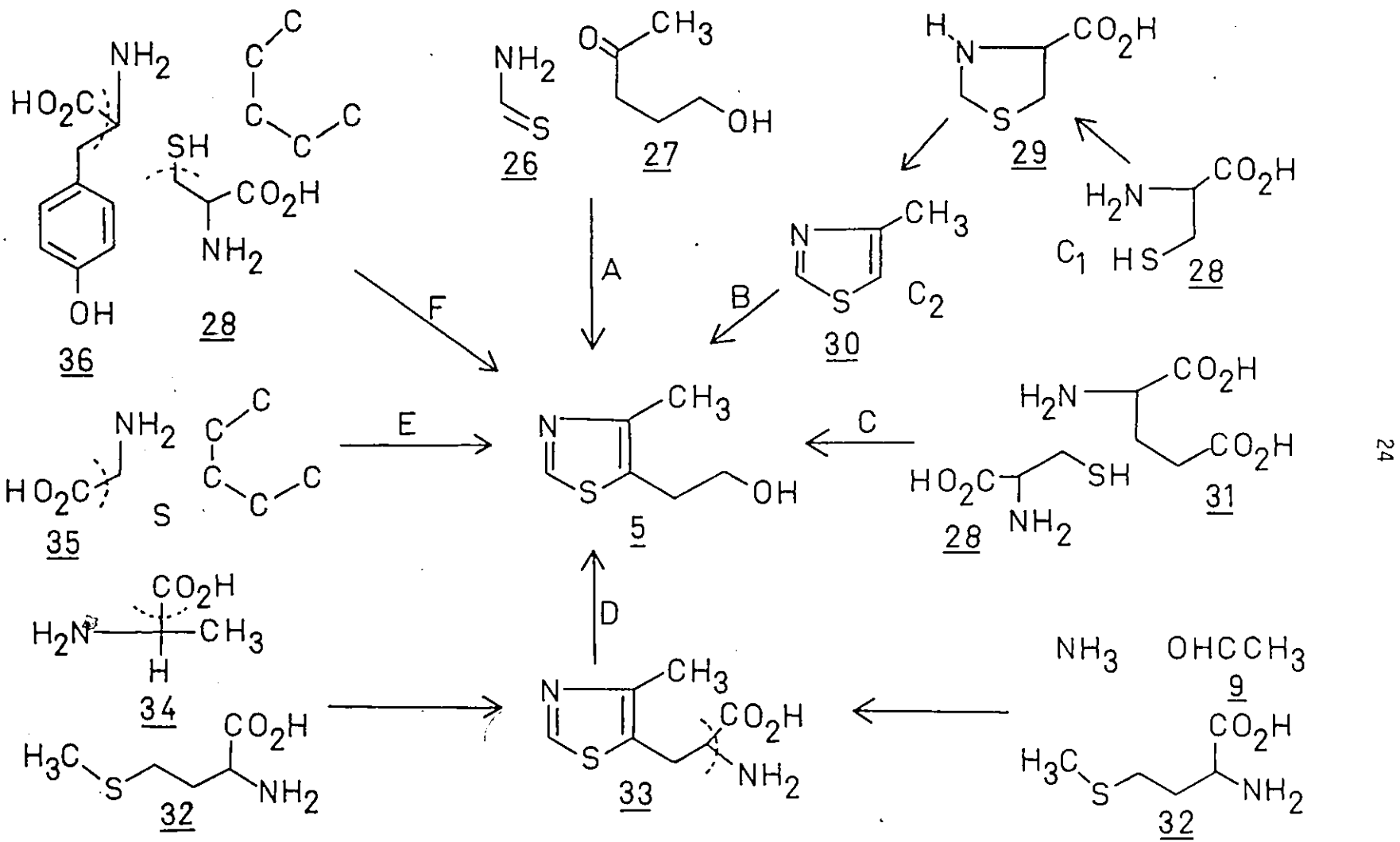


Figure 5: Hypothetical schemes accounting for the biogenetic origin of the thiazole moiety.

The intermediacy of 4-methylthiazole (30) was tested by Korte et al.[84] [2-^{14}C]-4-Methylthiazole was chemically synthesized and fed to a number of fungi but no incorporation into thiamin was obtained. Also, in a competition experiment with [^{35}S]sulfate, 4-methylthiazole (30) diluted the incorporation of label from [^{35}S]sulfate into thiamin by only a small extent.[123] In a similar experiment, thiazolidine-4-carboxylic acid (29) was more effective in diluting the label from [^{35}S]sulfate.[123] However, the interpretation of such an experiment has to take into account the relative rates of membrane transport for the two suggested precursors and the possible hydrolysis of the thiazolidine ring to cysteine (28) and formaldehyde. These observations, along with the necessity for an unlikely reduction of a carboxylic acid to a methyl group, and the unknown origin of the β-hydroxyethyl substituent, make this hypothesis seem improbable.

If, as Nakayama suggests, a one-carbon unit were to combine with cysteine to form a thiazolidine ring as a first step to the thiazole moiety, then formate might be expected as a precursor. One report[124] has suggested that formate is incorporated into the thiazole moiety, but when a degradation was carried out to isolate C-2, the expected site of activity, no activity was found. This degradation is shown in Figure 6. Treatment of the thiazole (5), obtained by bisulfite cleavage of thiamin (Figure 1), with methyl iodide yielded the methiodide (37) which was reduced to the thiazolidine derivative (38) by sodium borohydride. Hydrolysis of the thiazolidine ring in the presence of mercuric chloride yielded formaldehyde (39) which was trapped as its dimethone derivative (40). This experiment may be interpreted in one of two ways. Either the activity was associated with the remainder of the molecule (C-4,-4',-5,-6,-7)

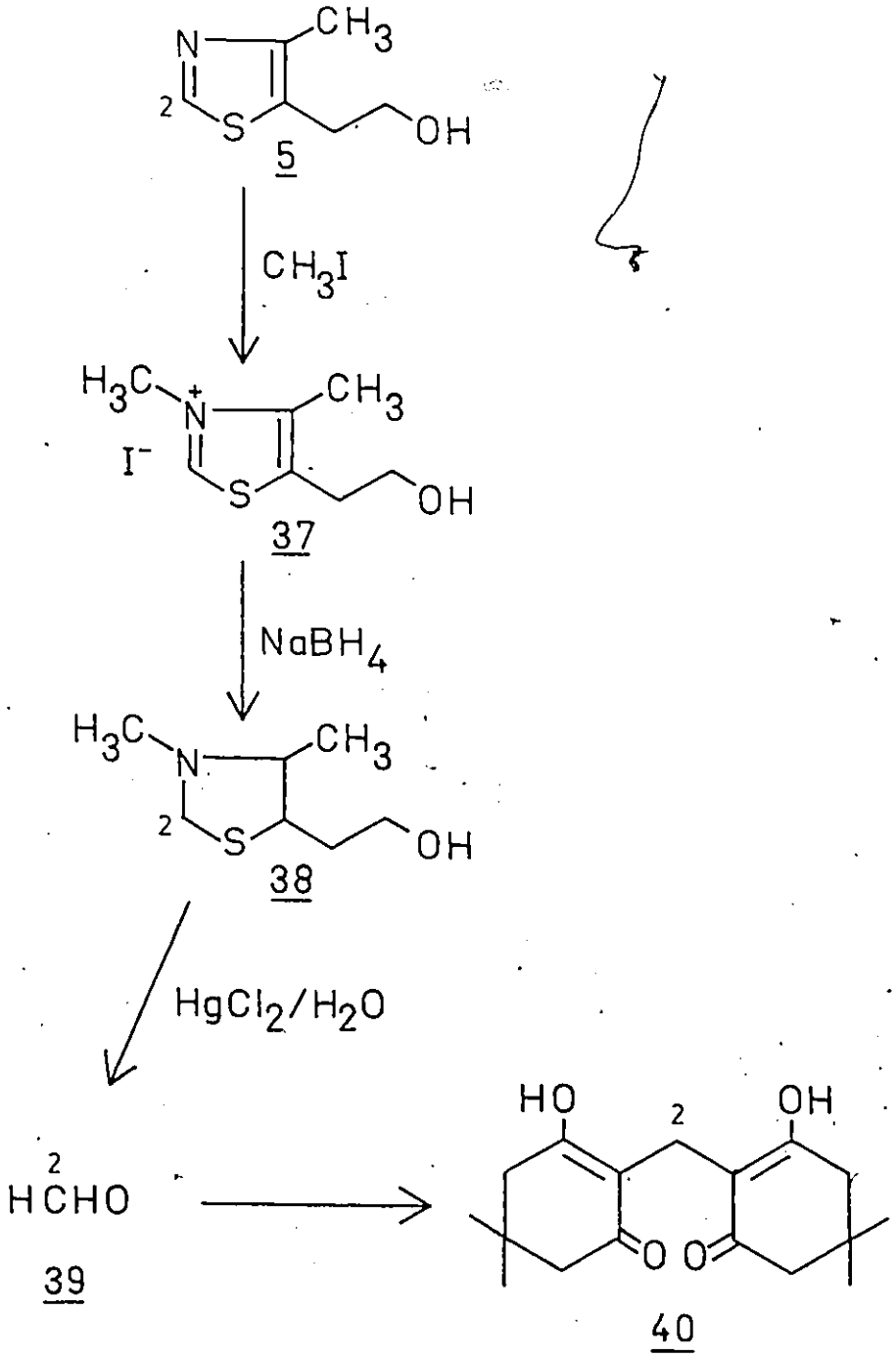


Figure 6: Degradation of the thiazole (5) to obtain C-2 as formaldehyde dimethone (40).

which was not isolated, or the original activity in the thiazole (5) was due to an active impurity which was removed during the degradation. Had the radioactivity of the intermediates (37) or (38) been determined and been found to be negligible, then the latter explanation would hold. This seems to be the case as a later report by the same authors,[125] in which an improved purification method was used, stated that inactive thiazole (5) was obtained from a [^{14}C]formate feeding. It is generally accepted that formate is not a precursor of the thiazole moiety.[126-129]

Plaut,[67] in a review on the biosynthesis of thiamin, proposed that the thiazole moiety could be formed in a manner analogous to the thiazolidine portion of penicillin. Cysteine (28) and a five-carbon amino acid, glutamic acid (31) were suggested as the basic building blocks (Route C, Figure 5). Cysteine (28) has been tested as a precursor by feeding [^{35}S]cystine,[128,130,131] [^{35}S]cysteine,[132] and [3-^{14}C]cysteine.[132] A small amount of incorporation of activity into the thiazole moiety was obtained when radioactively labelled sulfur was used but no incorporation was obtained from [3-^{14}C]cysteine. These results suggest that cysteine (28) acts only as a sulfur donor and not as a source of a C-S unit, as required by Route C in Figure 5. Cysteine (28) gave only a small dilution of label in a competition experiment with [^{35}S]sulfate in yeast;[123] this again suggests that cysteine is not directly involved in the biosynthesis of the thiazole moiety. Cysteine (28), in a similar experiment carried out in E. coli, was effective in diluting the incorporation of label from [^{35}S]sulfate, and the authors concluded that cysteine (28) is the likely direct donor of sulfur.[133]

The question of whether or not the carbon skeleton of cysteine (28)

was incorporated into the thiazole (5) was further probed by the feeding of labelled serine, the biosynthetic precursor of cysteine. In yeast, incorporation into the thiazole moiety was reported from [U-^{14}C]serine and [3-^{14}C]serine.[134] The thiazole was degraded as shown in Figure 6, but the formaldehyde dimethone (40) derived from C-2 of the thiazole (5) was inactive. The remainder of the molecule was not isolated. The activity obtained in the thiazole (5), as in the [^{14}C]formate feeding, appears to be due to the presence of an inactive impurity and is not located in the unisolated portion of the thiazole (5). The [3-^{14}C]serine experiment was repeated by the same group and inactive thiazole (5) was isolated by the revised purification procedure.[125] The nonincorporation of serine was confirmed in E. coli.[133]

Feeding experiments with [U-^{14}C]glutamic acid[129,132,134] have shown very little incorporation into thiamin. This implies that glutamic acid (31) is not a precursor. Also, the reduction of a carboxylic acid group to a methyl group seems to be an unlikely transformation.

Forty years ago, Harington and Moggridge[135] suggested that the thiazole moiety could arise from ammonia, acetaldehyde (9) and methionine (32) via the intermediate α-amino-β-(4-methylthiazol-5-yl)propionic acid (33) (Route D, Figure 5). They found that this compound was converted into the thiazole moiety by fermenting yeast.[136] Independently, Buchman and Richardson[137] suggested the same intermediate and found that pea roots were able to convert this substance to the thiazole moiety. This transformation has also been shown in excised tomato roots[138] but does not seem to take place in Phycomyces blakesleeanus[139] and Staphylococcus aureus.[140]

Methionine has been tested as a source of sulfur. It was found to dilute the incorporation of activity from [^{35}S]sulfate in yeast[123] but not in E. coli.[133] Also, label from [^{35}S]methionine[128,130,131] has been incorporated into thiamin. In support of this scheme, it was reported that label from L-[Me-^{14}C]methionine was incorporated into the thiazole moiety in yeast and that the ^{35}S/^{14}C ratio of L-[Me-^{14}C,^{35}S]-methionine was maintained on incorporation into the thiazole moiety.[128,130] From this result, it was concluded that the CH_3-S- portion of the methionine (32) molecule was incorporated into the thiazole (5) as an intact unit. The possibility that the labelled atoms could have been broken apart and incorporated by different routes into the thiazole (5) cannot be completely ruled out. However, the efficiencies of incorporation over the two routes would have to be the same and this would make this possibility seem rather unlikely.

In Bacillus subtilis, [Me-^{14}C]methionine was incorporated into thiamin[129] and the position of the label, C-2 of the thiazole (5) as predicted by Route D, Figure 5, was confirmed by the degradation shown in Figure 7.[141,142] Thiamin chloride hydrochloride (2) was reduced to the thiazolidine derivative (41) by sodium borohydride. The thiazolidine derivative (41) was split by sodium bisulfite into the pyrimidinesulfonic acid (4) and the thiazolidine derivative (42) which was hydrolyzed to formaldehyde (39). Again, the formaldehyde (39) was trapped as its dimethone derivative (40). The specific activity of the thiazole moiety was measured either by difference or directly on a sample obtained from the bisulfite cleavage of thiamin. This degradation was reported in a preliminary communication[141] in which no experimental details are given.

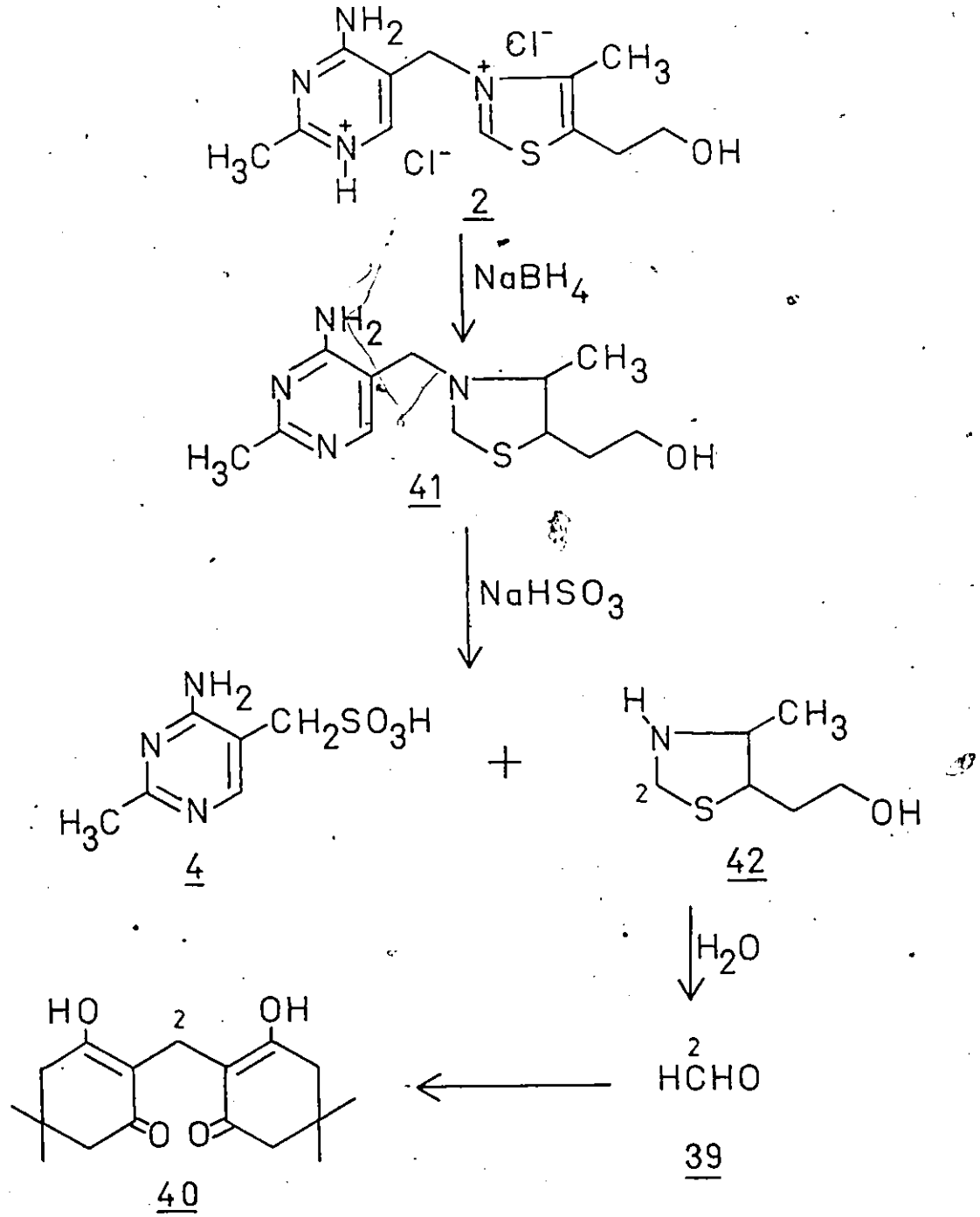


Figure 7: Degradation of thiamin chloride hydrochloride (2) to isolate C-2 of the thiazole moiety as formaldehyde dimethone (40).

Thus, the purity and identity of the compounds involved cannot be judged.

The incorporation of the methyl group of methionine into the thiazole moiety could not be confirmed in Salmonella typhimurium,[143] E. coli,[133] or in a second strain of yeast.[124,134] Nor could the inference that the carbon atoms of methionine (other than the carboxy group) accounted for C-5 and the β-hydroxyethyl side chain be confirmed in feedings of [2-^{14}C]methionine,[143] [3,4-^{14}C]methionine,[134] and [G-^{14}C]methionine.[133] It must be mentioned, however, that these experiments were carried out by the groups which could not reproduce the [Me-^{14}C]methionine incorporations. The workers which obtained incorporation from labelled methionines did not try any experiments with methionine labelled with ^{14}C in positions other than the methyl group. Incorporation was reported[129] from [^{3}H]methionine, and [^{3}H,Me-^{14}C]methionine, with a ^{3}H/^{14}C ratio of 1.3, yielded the thiazole moiety with a ^{3}H/^{14}C ratio of 0.4. The distribution of tritium in the doubly labelled methionine was not reported. In the absence of this information, the ^{3}H/^{14}C ratio of the thiazole moiety derived from [^{3}H,Me-^{14}C]-methionine cannot be interpreted on the basis of Route D in Figure 5. Without knowledge of the method of preparation of the tritiated methionine, it cannot even be assumed that the tritium was equally distributed over the eight non-exchangeable protons of methionine. No degradations were carried out on this sample. It is, therefore, not known whether this incorporation was specific or random.

Johnson et al.[128] modified the Harington proposal by replacing ammonia and acetaldehyde (9) with alanine (34). Both alanine (34) and acetate were incorporated, but alanine (34) was considered to be the most likely

precursor. This route requires the loss of C-1 of alanine (34) and [1-^{14}C]alanine was not incorporated into thiamin in yeast.[134] The incorporation of [U-^{14}C]alanine was reported in Bacillus subtilis[129] but [U-^{14}C]-alanine was not incorporated into thiamin by S. typhimurium,[143] or yeast.[134] [^{3}H]Alanine was also incorporated into the thiazole moiety and [^{3}H,^{14}C]-alanine was incorporated with the ratio of ^{3}H/^{14}C maintained.[129] Again, such a result cannot be interpreted unequivocally because the distribution of both ^{3}H and ^{14}C in [^{3}H,^{14}C]alanine was not reported. If alanine was incorporated as in Route D in Figure 5, and if the ^{3}H was distributed equally over the four non-exchangeable positions and the ^{14}C was present in the three carbons in equal amounts, the ^{3}H/^{14}C ratio of the isolated thiazole should have been higher than that of the [^{3}H/^{14}C]alanine that was fed.

A number of incorporation results have been reported[129] for [1,2-^{14}C]-acetate, [1-^{14}C]acetate and ^{3}H/^{14}C doubly labelled acetates. Incorporation of acetate into the thiazole moiety in yeast was found to be low.[134] No degradations to determine the location of label in the thiazole moiety were carried out in the above experiments and no checks on the purity of the samples were made. It is difficult to interpret such experiments.

Evidence that contradicted the methionine-alanine hypothesis emerged from radioactive-tracer experiments with glycine. It was found by unequivocal degradation (Figure 6) of the radioactively labelled thiamin obtained from a feeding experiment in yeast with [2-^{14}C]glycine that most of the activity of the molecule was localized at C-2 of the thiazole nucleus.[124] Administration of [2-^{14}C]glycine in the presence of unlabelled methionine gave an identical result[125] and, in an experiment with [^{15}N]glycine, thiamin was isolated whose thiazole moiety (but not

the pyrimidine unit) was enriched with ^{15}N.[134] A scheme (Route E, Figure 5) that implicated glycine (35) in the biosynthesis of the thiazole unit was proposed.[125] This scheme requires the loss of C-1 of glycine (35) and experiments[125,134] with [1-^{14}C]glycine indicated, after an improved purification procedure, that label from this compound is not incorporated into the thiazole unit.

Iwashima and Nose[144] showed that the requirement for thiazole in an E. coli mutant could be partially met by glycine (35), and that thiamin synthesis, in washed cell suspensions provided with the pyrimidine moiety, was stimulated by glycine (35) as well as by the thiazole moiety (5). They concluded that glycine may serve as a precursor of the thiazole unit. A feeding experiment with E. coli[145] suggested that [U-^{14}C]glycine was incorporated into the thiazole unit, but the radioactivity of this molecule was obtained by difference instead of by a direct measurement. Radioactivity measured in this way has an increased chance of error and such results should be considered as less reliable. [2-^{14}C]Glycine was not incorporated into the thiazole moiety by S. typhimurium.[143]

The published data concerning the biosynthesis of the thiazole unit were further complicated when phenylalanine was found to inhibit thiazole biosynthesis in an E. coli mutant.[146] This inhibition was overcome by tyrosine and the authors suggested that tyrosine (36) was a precursor of the thiazole moiety. This was confirmed by experiments with radioactively labelled tyrosines.[147] When either [U-^{14}C]tyrosine or [2-^{14}C]tyrosine was administered to E. coli, most of the activity was located at C-2 of the thiazole moiety, as determined by the degradation developed by Linnett and Walker[124] (Figure 6). [2-^{14}C]Tyrosine was also

fed to S. typhimurium[143] and again most of the activity was found at C-2 of the thiazole unit. In addition, a competition experiment between [2-^{14}C]tyrosine and unlabelled glycine and unlabelled methionine was carried out, but neither the incorporation nor distribution of label in the thiazole unit was changed. A scheme showing the involvement of tyrosine was then proposed (Route F, Figure 5). Tyrosine (36), in analogy with glycine (35), might supply a C-N unit to the thiazole moiety and an experiment with [^{15}N]tyrosine[148] showed incorporation of ^{15}N into the thiazole unit.

The results obtained for glycine and tyrosine are similar not only in that each amino acid seems to contribute a C-N unit to the thiazole moiety but that each requires a C_5 unit to complete the thiazole molecule. No suggestions were made in any of these investigations as to the origin of the C_5 unit. Experiments with DL-[2-^{14}C]mevalolactone,[134] potassium DL-[2-^{14}C]mevalonate[134] and D-[1-^{14}C]ribose[125] did not yield radioactively labelled thiazole. This implies that these compounds were not involved in the origin of this C_5 unit.

Other schemes have been proposed for the biosynthesis of the thiazole moiety, but there is little evidence in their support. In a footnote, Buchman and Richardson[137] mention a hypothesis for the formation of the thiazole moiety from a thiomethylpentose. Parada and Ortega,[149] in a study of the growth inhibition of a temperature-sensitive thiazoleless mutant of S. typhimurium, suggested that the C_5 unit is derived from a pentose-like compound which is formed from acetaldehyde and a C_3 compound, by means of an aldolase or transaldolase-type reaction. Calcium DL-[1-^{14}C]-glycerate did not serve as a source of this three-carbon unit.[125] This

idea was based on the methionine-alanine results by Johnson et al.[128] and presumably methionine was considered as a source of the C-2-S unit.

The experiments described above involve many labelled compounds and also many different types of micro-organisms. Table 1, found at the end of this chapter, lists organisms and the compounds fed and serves as a summary and guide to the literature published to-date on this seemingly complex problem.

The above review shows that the biosynthetic origins of the thiazole moiety of thiamin are far from clear even though a great deal of time and effort has been devoted to this problem. The obvious criticism of these investigations is the lack of a degradation for the C_5 unit of the thiazole (5). Without such a degradation, no evaluation can be made of the various sets of incorporation results in terms of a valid biogenetic hypothesis.

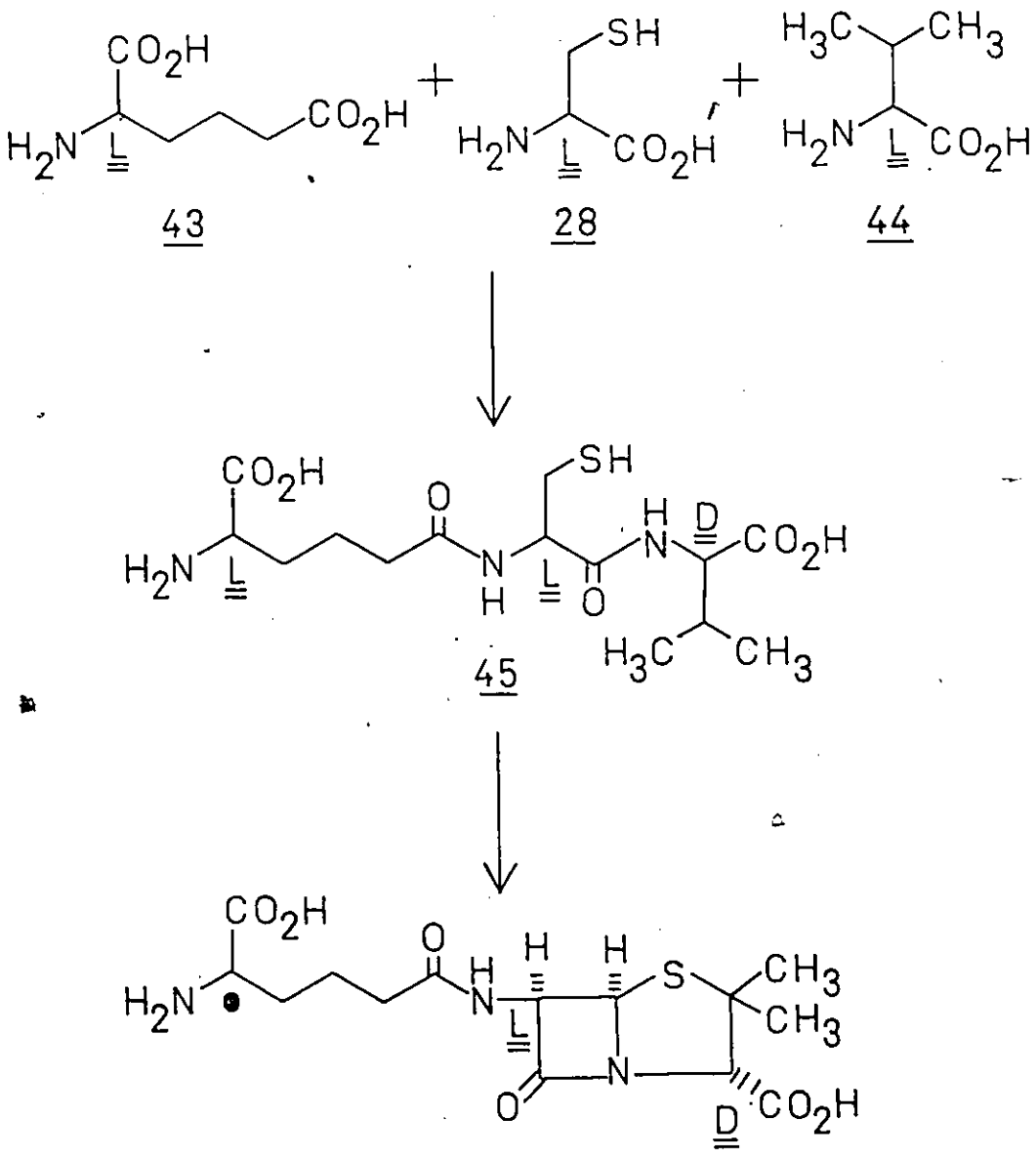
(iv) Naturally occurring substances with structures similar to the thiazole moiety

Very often, a biogenetic hypothesis can be generated through consideration of compounds of similar structure which have been found in nature. This approach was used very successfully by Sir Robert Robinson[150] in the days before the availability of tracers. Compounds with nitrogen and sulfur in the 1 and 3 positions of a five-membered ring form a small class of natural products. The ring may be a thiazole, thiazoline or a thiazolidine. These compounds, excluding thiamin, fall into one of the following categories: penicillin, luciferin; or, a peptide antibiotic. Their biosynthetic origins in general are not well

understood.

The biosynthesis of penicillin has been well-investigated[151,152] and α-aminoadipic acid (43), cysteine (28) and valine (44) have been identified as the basic precursors. A tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (45) has been identified as an intermediate but the mechanistic details of the transformation of this tripeptide into penicillin are not known. As shown in Figure 8, the L-enantiomers of the amino acids are used to form the tripeptide (45) but the valine unit is inverted to the D-configuration in this process. The tripeptide is cyclized to penicillin with retention of the configuration at the cysteine asymmetric centre. If the configuration of the α-aminoadipyl asymmetric centre is inverted to D, then the product is penicillin N (46), but if no inversion occurs, then the penicillin with the L-configuration at this centre, or isopenicillin N (47), is obtained.

Luciferin (49) provides an example of a benzthiazole ring and a thiazoline ring in the same molecule. Both rings are, however, thought to be derived from cysteine. The scheme outlined in Figure 9 was proposed by McCapra[153] and is based on two feeding experiments. Labelled p-benzoquinone (48) was found to be incorporated into luciferin[154] but no degradation was carried out to show that this incorporation was non-random. The second feeding experiment was carried out with DL-[1-^{14}C]-cystine[153] and the radioactively labelled luciferin was degraded. The label was found to be localized in the carboxy group of the thiazoline ring. The derivation of this thiazoline ring from cysteine (28) is easily imagined to be as shown in Figure 9. A complex rearrangement was



46 ● = D Penicillin N

47 ● = L Isopenicillin N

Figure 8: Biosynthesis of penicillins.

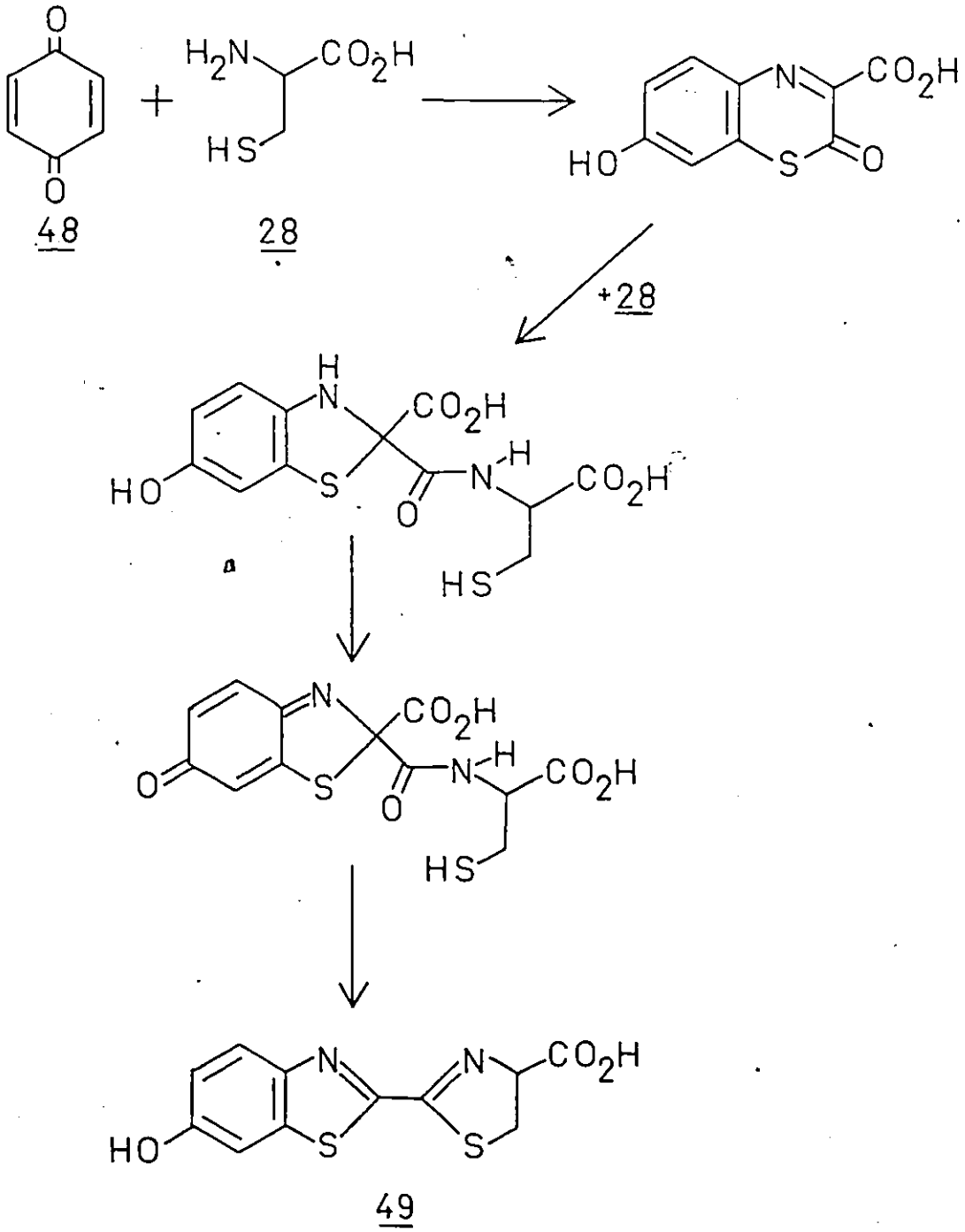


Figure 9: Proposed biosynthesis of luciferin (49).

proposed, however, to account for the lack of incorporation of [1-^{14}C]-cysteine into the benzthiazole portion of the molecule. No evidence has been presented which incorporates cysteine into the benzthiazole ring system.

The third category, i.e., peptide antibiotics, contains the largest number of members, yet the biosynthesis of these compounds is the least understood. They all bear a common structural resemblance and their structures may be described by the partial structures (50)-(54) in Figure 10. Often more than one of the units (50)-(54) is present in the structure of these substances. The members of this group and their partial structures are actithiazic acid (54),[155] althiomycin (50)(52),[156] bacitracin (50),[157] berninamycin (53),[158] bleomycin (52),[159] bottromycin (51),[160] dysidenin (51),[161] isodysidenin (51),[162] micrococcin P (52),[163] nosiheptide (52),[164] siomycin A (52),[165] tallysomycin (52),[166] thiostrepton (50)(52),[167] and zorbamycin (50)(52).[168] A possible biogenetic scheme for the origin of these partial structures is also outlined in Figure 10. The first step would be the condensation of cysteine with an acid or an aldehyde to form the five-membered ring. The reaction of cysteine with formaldehyde proceeds readily and the equilibrium favors the thiazolidine ring over the starting materials.[169] The condensation of cysteine with acids has been suggested to occur first by amide formation, i.e., the formation of a peptide chain, and then cyclization to thiazolines and subsequent conversion to thiazoles.[170] The thiazole structure (50) can be dehydrogenated to give the thiazole (52). This type of dehydrogenation has been observed in chemical systems[171] and does not require vigorous conditions. The thiazole (51) can be derived directly from (50)

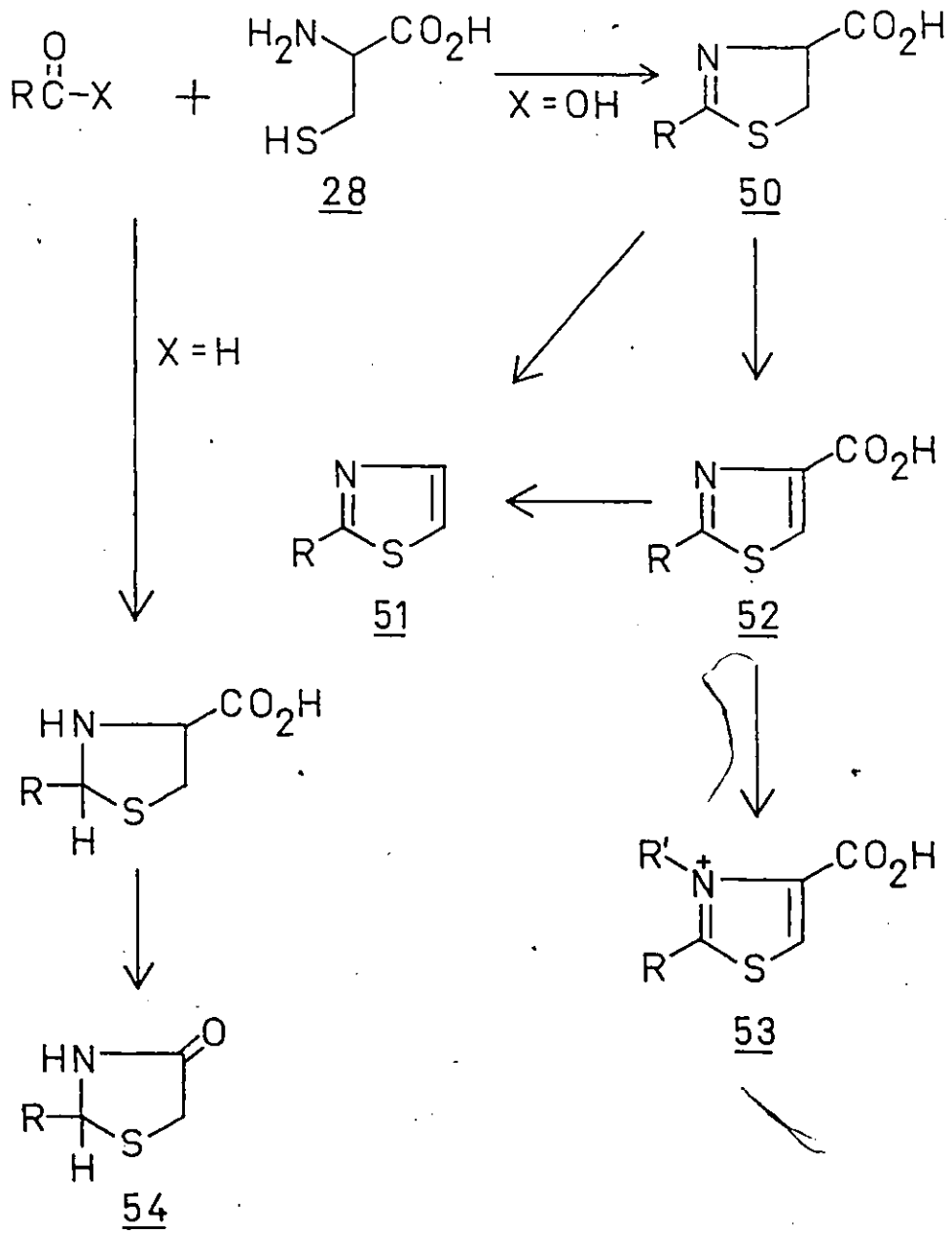


Figure 10: Biogenetic scheme of the origin of thiazolidine, thiazoline and thiazole ring systems in peptide-derived natural products.

or from (52) depending on whether decarboxylation occurs as a first or second step. Alkylation of the nitrogen of (52) would generate (53). The formation of a thiazolidine ring followed by oxidative decarboxylation would give (54). The derivation of these structures is still speculative but the scheme presented in Figure 10 is chemically reasonable.

The thiazole structure contained in thiamin is unique, even when compared with other natural thiazole structures. The unique feature is the β-hydroxyethyl substituent at position 5 of the thiazole ring. Only penicillin and the benzthiazole ring system of luciferin have substituents at this position and these are quite different from a β-hydroxyethyl group. The methyl group at C-4 of the thiazole moiety of thiamin is also unique. These differences make it very likely that the thiazole moiety of thiamin is derived from precursors other than those implicated in the origin of penicillin, luciferin or the peptide antibiotics.

(v) Origin of the pyrimidine moiety

The biosynthesis of the pyrimidine moiety in thiamin has been more extensively investigated than that of the thiazole moiety. A number of degradations have been developed and used to locate the site of the label in the pyrimidine unit derived from feeding experiments with radioactively labelled compounds. In spite of these investigations, the basic precursors are still not known with any degree of certainty. Again, no one group of investigators has managed to carry out a complete study and to present evidence for the origins of all six carbon atoms in the pyrimidine moiety.

On the basis of these investigations, six hypotheses have been

proposed. The known biosynthetic pathways for the biosynthesis of nucleic acid pyrimidines and purines have each provided a basis for three hypotheses. The available evidence concerning the origin of the pyrimidine moiety will be described in terms of these hypotheses.

Unlike the thiazole ring, the pyrimidine ring is a very common naturally occurring structure, as it is the basic framework for the nucleic acid bases uracil (58), cytosine (60) and thymine (59). The methyl group located at C-2, however, is a structural feature which is unique to the pyrimidine moiety of thiamin. The first hypotheses for the biosynthesis of the pyrimidine moiety were modelled on the biosynthetic route known for the nucleic acid pyrimidines.[172-174] The origin of the pyrimidine unit of thiamin by this type of pathway has been well tested by feeding experiments with radioactively labelled compounds. Both the basic precursors, aspartic acid (56) and formic acid (61), and the postulated intermediates, orotic acid (57) and uracil (58), have been fed.

David and Estramareix[127] modelled their hypothesis, shown as Route A in Figure 11, on the known biosynthesis of nucleic acid pyrimidines. Orotic acid (57), the known precursor of the nucleic acid pyrimidines, is derived from carbamoyl phosphate (55) and aspartic acid (56). Decarboxylation yields uracil (58), which was known to be converted to 5-hydroxymethylcytosine (62), a nucleic acid base found in bacteriophages, by a C_1 transfer. Formaldehyde was known as the precursor of this C_1 unit.[175] They also postulated that the methyl group at C-2 was derived from acetyl CoA via the intermediate (64) formed by a condensation of acetyl CoA and 5-hydroxymethylcytosine (62). Decarboxylation of the intermediate (64) would yield the pyrimidine (21).

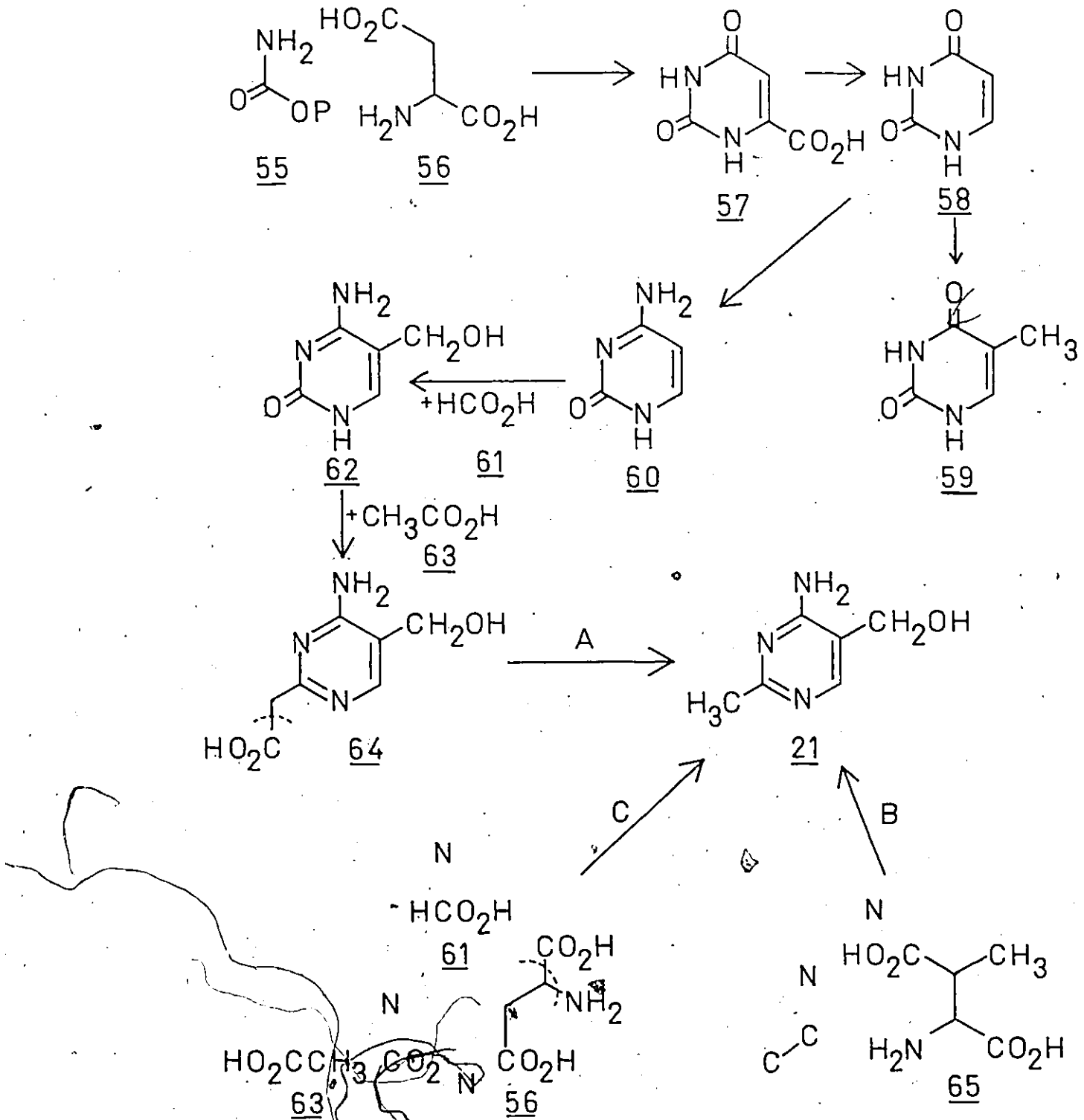


Figure 11: Proposed biogenetic schemes for the pyrimidine moiety.

At that time, this scheme was consistent with the available data. Nakayama[119] had reported that the thiamin requirements of E. coli mutants could be satisfied by a mixture of thymine (59) and uracil (58). Formate was also known to be incorporated into the pyrimidine moiety of thiamin,[126,127,176] but the site of labelling was not known.

The observation by Guthrie et al.,[177] that the pyrimidine (21) reversed the inhibition of the growth of Bacillus subtilis by amethopterin, a folic acid inhibitor, was the first indication that a C_1 transfer was involved in the biosynthesis of the pyrimidine moiety. This observation was followed by a [^{14}C]formate feeding,[126,176] and the 2-methyl and 5-hydroxymethyl substituents were suggested as the most likely sites of labelling.[126]

For the hypothesis suggested by David and Estramareix[127] (Route A, Figure 11) to be correct, the activity derived from [^{14}C]formate would have to be located in the 5-hydroxymethyl substituent (C-7). The degradation[178] outlined in Figure 12 was used to show that [^{14}C]formate labelled a site other than C-7 of the pyrimidine moiety. Thiamin chloride hydrochloride (2) via thiamindisulfide, was converted to the 5-aminomethylpyrimidine (66) which, on treatment with sodium nitrite, was converted to the 5-hydroxymethylpyrimidine (21). Potassium permanganate oxidation converted the hydroxymethyl derivative (21) into the carboxylic acid (67). On heating, this acid was decarboxylated to 4-amino-2-methylpyrimidine (68). The carbon dioxide liberated was trapped as barium carbonate. A labelled sample of thiamin obtained by the incubation of yeast with [^{14}C]-formate was degraded and almost all the activity of the pyrimidine (67) was recovered in 4-amino-2-methylpyrimidine (68).[179] The carbon dioxide,

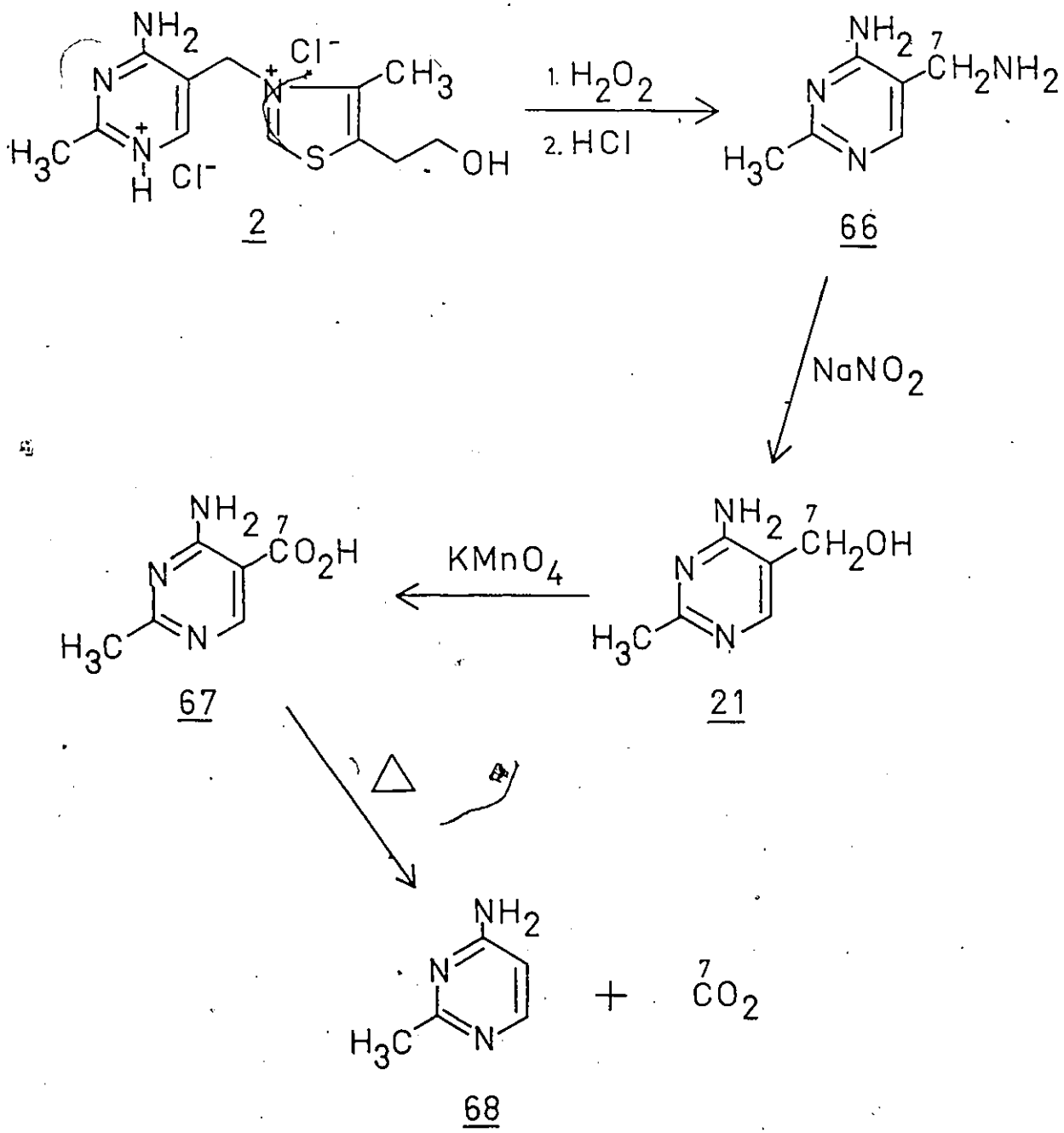


Figure 12: Degradation to isolate C-7 of the pyrimidine moiety.

derived from C-7, was inactive. Thus, the 5-hydroxymethyl substituent did not appear to arise by a C_1 transfer as is the route for 5-hydroxymethylcytosine.

The nucleic acid pyrimidine intermediate orotic acid (57) was tested by feeding [2-^{14}C]orotic acid to E. coli[180] and by the feeding of [6-^{14}C]orotic acid to both E. coli[180] and to yeast.[128] In each case, no incorporation into thiamin was observed. Similarly, no incorporation from [U-^{14}C]uracil was found in E. coli.[180]

The above experiments indicated that a common route to both the nucleic acid pyrimidines and the pyrimidine moiety of thiamin was unlikely. Even so, a number of feeding experiments were carried out with radioactively labelled aspartic acids (56), but the results obtained are not conclusive. [U-^{14}C]Aspartic acid was fed to yeast[134] and to E. coli[133] and each time no incorporation into thiamin was observed. In an experiment carried out with [U-^{14}C]aspartate in B. subtilis,[129] however, incorporation into the pyrimidine moiety was reported. [4-^{14}C]-Aspartate was incorporated one-tenth as much as [U-^{14}C]aspartate by E. coli.[180] The incorporation of [2-^{14}C]aspartate into thiamin by E. coli was comparable to the incorporation obtained from [^{14}C]formate.[181] The incorporation was measured by the oxidation of thiamin to thiochrome which was subjected to paper chromatography. The chromatogram was then scanned to determine the amount of activity associated with each spot.

A suggestion[65] that β-methylaspartic acid (65), instead of aspartic acid (56), was a precursor to the pyrimidine moiety (Route B, Figure 11), was based on the incorporation of [^{3}H]β-methylaspartate into thymine (59).[182]

This hypothesis would generate a pyrimidine with a methyl group at C-5 which did not originate from a C_1 transfer. The methyl group would have to be oxidized to a hydroxymethyl group at a later stage. β-Methylaspartate (65) is derived from glutamic acid (31) via the glutamate mutase reaction,[183] but feedings with [U-^{14}C]glutamic acid[129,134] did not show any incorporation into thiamin.

The most immediate question which had to be answered at this point, was the site labelled by [^{14}C]formate. It was not C-7, but could it have been the methyl group at C-2 as suggested by Pine and Guthrie,[126] or was it another position in the pyrimidine molecule?

The pyrimidine moiety, obtained by feeding [^{14}C]formate to yeast, was subjected to a series of degradations developed by Estramareix and his coworkers.[184] A preliminary degradation (Figure 13) isolated C-2 and the methyl group (C-2'). The pyrimidinesulfonic acid (4) obtained by bisulfite cleavage (Figure 1), was hydrolyzed to the 4-hydroxypyrimidinesulfonic acid (69) by reflux in hydrochloric acid. Kuhn-Roth oxidation yielded acetic acid (63) from C-2,-2'. The acetic acid was inactive[184] and thus formate was not incorporated into C-2,-2' of the pyrimidine moiety.

A degradation (Figure 14) was developed to isolate C-4,-5,-6,-7 of the pyrimidine moiety.[185] Thiamin chloride hydrochloride (2) was converted to hydroxythiamin (70) by reflux in hydrochloric acid. Cleavage of the hydroxythiamin with thioglycolic acid[186] yielded the pyrimidine thioether (71) and reduction by sodium in ethanol gave 1,3-diamino-2-methylpropane (72) which was trapped as its dipicrate. This compound included C-4,-5,-6,-7 yet contained only 73% of the activity of the pyrimidine

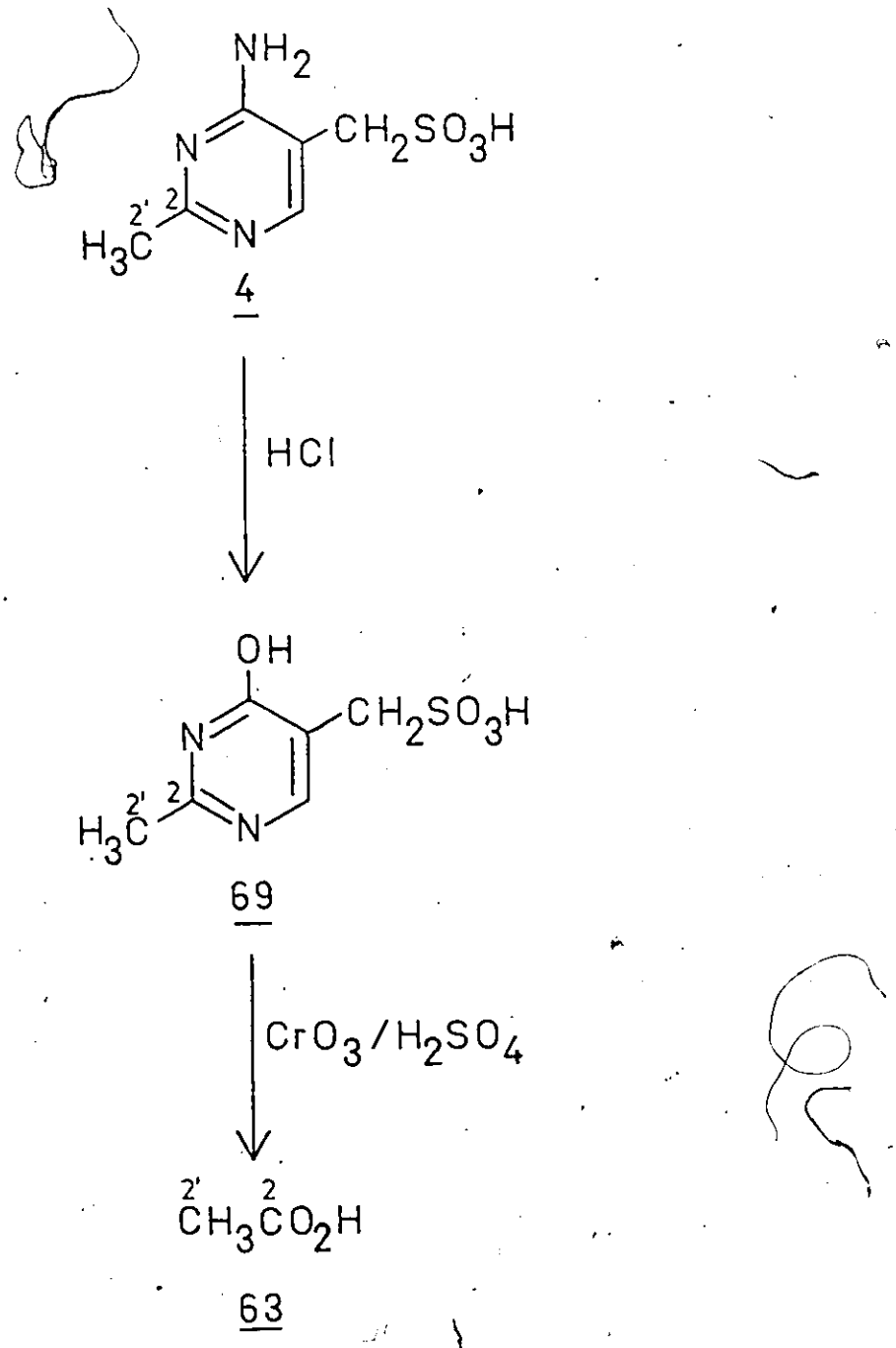


Figure 13: Degradation to obtain C-2,-2' of the pyrimidine moiety.

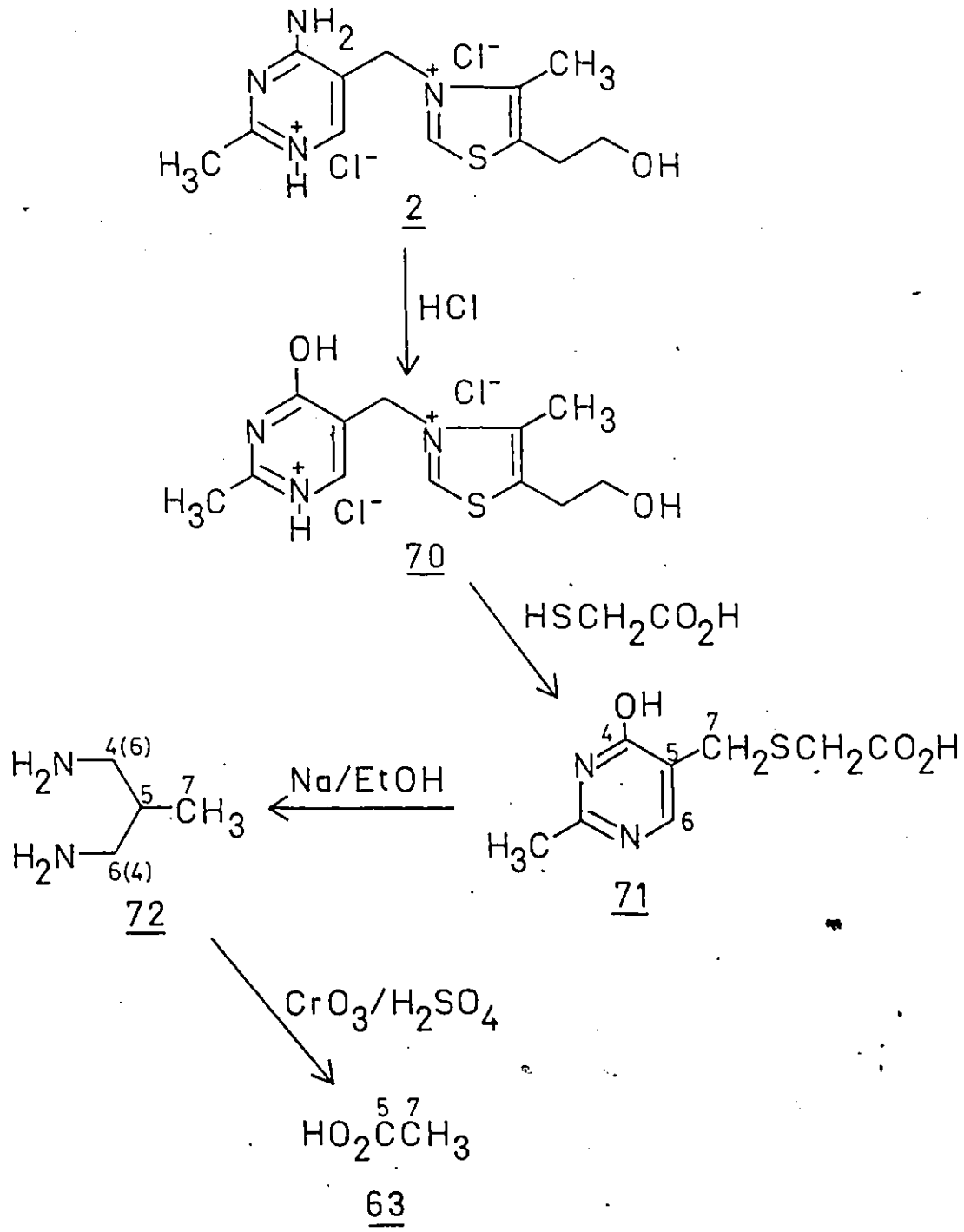


Figure 14: Degradation which isolated C-4,-5,-6,-7 of the pyrimidine moiety and also C-5,-7 of the pyrimidine moiety.

thioether (71).[187] A result of 100% had been expected but the authors claimed that the thiomethylpyrimidine (71) was not extensively purified because of the small amount of material available for degradation. Kuhn-Roth degradation of the 1,3-diamino-2-methylpropane (72) yielded inactive acetic acid:[184,188] this implies that the radioactivity from [^{14}C]formate is contained in either C-4 or C-6 of the pyrimidine moiety.

Carbons 4 and 6 of the pyrimidine moiety were distinguished by the following degradation[184,189] (Figure 15) in which all the carbons in the pyrimidine moiety were isolated. The pyrimidine thioether (71) was converted to 2,5-dimethyl-4-hydroxypyrimidine (73) by treatment with Raney nickel. After quaternization of the nitrogens with methyl iodide, the pyrimidine (74) was hydrolyzed in sodium hydroxide to yield a mixture of propionic (76), acetic (63) and formic (61) acid. The formic acid derived from C-6 was oxidized to carbon dioxide and the propionic and acetic acids were converted to their p-bromophenacyl derivatives which were separated by silica gel chromatography. The propionic acid was further degraded by a Schmidt reaction to ethylamine which was oxidized to acetic acid to separate C-4 from C-5 and C-7.

Almost all (99%) of the activity contained in the pyrimidine (71 or 73) moiety was recovered in the propionic acid (76) or in C-4,-5,-7. The acetic acid (C-2,-2') and the formic acid (C-6) were inactive. The acetic acid obtained from the propionic acid was also inactive, whereas the carbon dioxide derived from C-4 which was trapped as barium carbonate, contained 82% of the pyrimidine activity. A result of 100% would have been expected but the low result can be explained: either the carbon dioxide from the atmosphere or some other inactive acid may have been present in

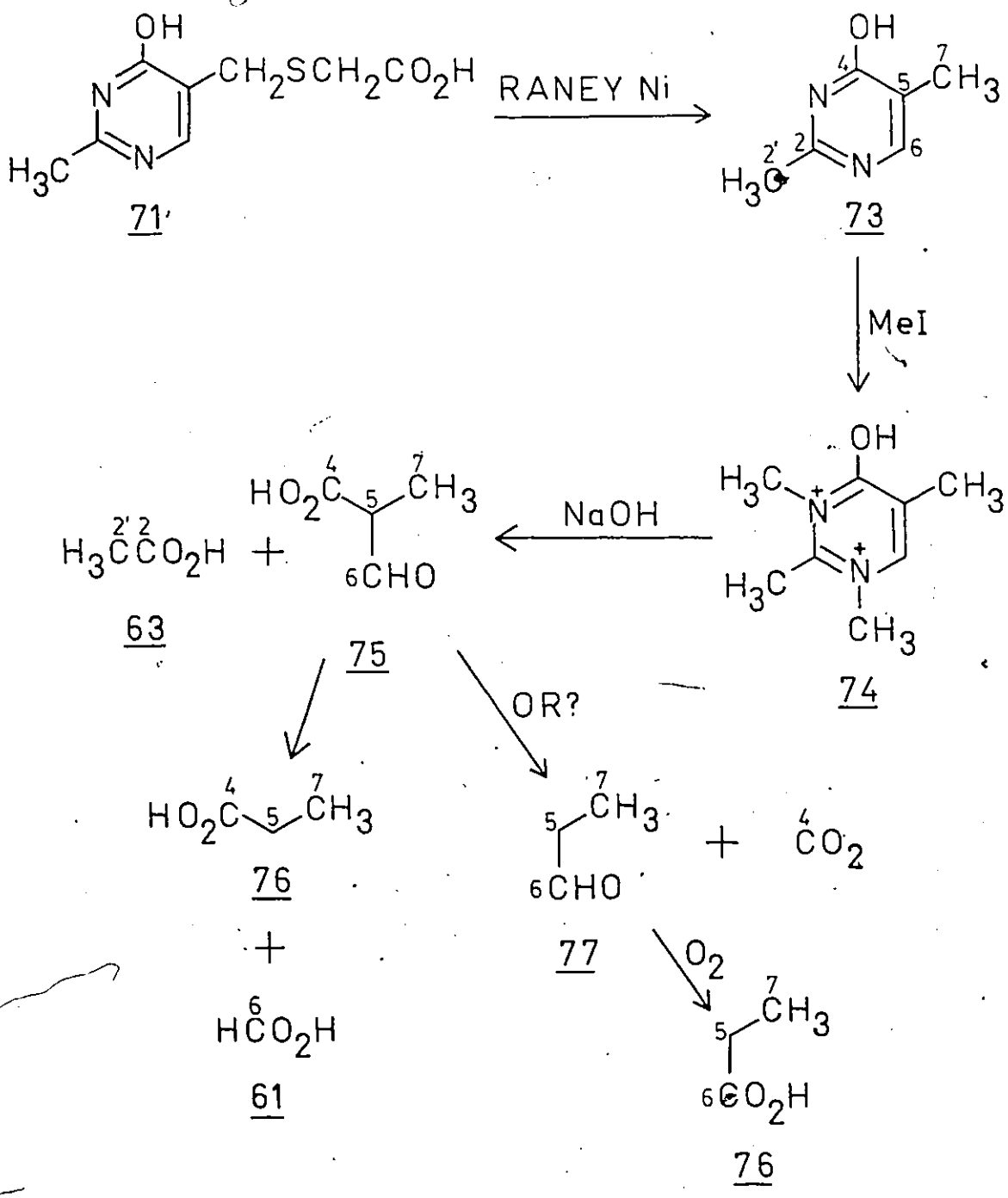


Figure 15: Degradation of the pyrimidine moiety to propionic, acetic and formic acid.

the reaction mixture.

Carbons 4, 5 and 7 are the most likely source of the propionic acid in the above degradation, but if the hydrolysis proceeds via 2-formylpropionic acid (75), a decarboxylation instead of a retro-aldol is likely. Propanal (77) would thus derive from C-5,-6,-7. Air oxidation could form propionic acid (76) with the origin of its carboxyl group C-6 and not C-4 of the pyrimidine. [4-^{14}C]-2,5-Dimethyl-4-hydroxypyrimidine (73) was chemically synthesized and subjected to the above reactions to check the validity of the degradation in locating activity at C-4. Approximately 94% of the activity of this material was located in the propionic acid (C-4,-5,-7) and no activity was recovered in either the acetic acid (C-2,-2') or the formic acid (C-6).[184]

The origin of the C_2 unit (C-2,2') has also been investigated in order to distinguish between the two possibilities, namely that either an acetate unit is incorporated intact, or that only the methyl group of acetate is incorporated, as proposed by David and Estramareix[127] (Route A, Figure 11). Goldstein and Brown[180] fed [2-^{14}C]acetate to *E. coli* and reported some degree of incorporation, but no degradations were carried out. Thus, this experiment does not distinguish between the two alternatives. A more extensive investigation, with the organism *B. subtilis* was carried out by Tomlinson and his coworkers.[129,190] They degraded the pyrimidine obtained from a [2-^{14}C]acetate experiment by the method of David *et al.*[185] (Figure 14) and found only 6% of the activity in the 1,3-diamino-2-methylpropane (72). This suggested that the activity was located in the unrecovered two-carbon unit. Since this result was again consistent with both proposals, a series of experiments with

$^{3}H/^{14}C$ doubly labelled acetates was carried out. A preliminary experiment with [^{3}H]acetate gave labelled pyrimidine. [2-^{3}H,2-^{14}C]Acetate was incorporated into the pyrimidine moiety, but the $^{3}H/^{14}C$ ratio dropped from 1.0 to 0.48. Without confirmation of the radiochemical purity of this feeding solution, it cannot be assumed that all of the tritium was associated with acetic acid.[191] If tritiated water were present in the feeding solution, the $^{3}H/^{14}C$ ratio of acetate would be lower than the reported value. Thus, it cannot be assumed that [2-^{3}H,2-^{14}C]acetate is incorporated into the pyrimidine moiety with a loss of tritium relative to ^{14}C. Even if the [2-^{3}H,2-^{14}C]acetate was pure, the observed $^{3}H/^{14}C$ ratio of the pyrimidine moiety was lower than predicted by either of the hypotheses. Such a result could be explained by exchange processes, but without knowledge of the amount of exchange, it cannot distinguish between the two possibilities. In an effort to resolve this problem, two experiments with [2-^{3}H,1-^{14}C]acetate were undertaken. Starting with a $^{3}H/^{14}C$ ratio of 1 in each case, pyrimidine moieties with $^{3}H/^{14}C$ ratios of 10.1 and 11.1, respectively, were isolated. These experiments showed a much greater incorporation of ^{3}H relative to ^{14}C; this implies that C-2 of acetate was incorporated much more extensively than C-1 of acetate. The obvious conclusion from these results is that the methyl group of the pyrimidine moiety is generated by the decarboxylation pathway.

These results, however, do not agree with those of Kumaoka and Brown[192] who found that [1-^{14}C]acetate was incorporated to a greater extent than [2-^{14}C]acetate into the pyrimidine moiety. Moreover, the incorporation of label from [2-^{14}C]acetate was diluted by the presence of inactive formate. It was concluded that acetate was not a direct precursor

of the pyrmidine moiety.

A third explanation exists for this incorporation of acetate. If the acetate was utilized by the cell via the glyoxylate and Krebs' cycles, then the oxaloacetate and the phosphoenol pyruvate generated would be labelled mainly by C-2 of acetate. A C_2 unit derived from phosphoenol pyruvate would be labelled in both positions by [2-^{14}C]-acetate but would not contain any label from [1-^{14}C]acetate. This route to the pyrimidine C-methyl unit can easily be distinguished from the decarboxylation route by a [2-^{14}C]acetate feeding. The pyrimidine would have to be degraded to determine the activity in these two positions. If all of the activity were found in the methyl group, then the decarboxylation route would be favoured, but if the activity was distributed over the two carbons, C-2,-2', then the incorporation of acetate via the glyoxylate and Krebs' cycles would be the favoured route. On the basis of published data, a distinction cannot be made between these alternatives.

Tomlinson et al.[129] suggested Route C in Figure 11 which includes the generation of the methyl group via the decarboxylation of acetate. Carbonate was proposed as the source of C-2 of the pyrimidine ring and the authors carried out a feeding experiment with [^{14}C]carbonate. However, no incorporation of activity into the pyrimidine ring was observed. The pyrimidine moiety was labelled in a [^{14}C]formate experiment, but no degradations were carried out. By analogy to the results of David et al.[184,188] C-4 of the pyrmidine moiety was suggested as the location of label derived from [^{14}C]formate.

Aspartic acid (56) was suggested as the precursor of the remainder of the pyrimidine molecule. Presumably, because it was accepted that the

nucleic acid pyrimidines and the thiamin pyrimidine did not share a common origin, the manner of the suggested incorporation of aspartic acid (56) into thiamin differed from that of the nucleic acid pyrimidines. [^{14}C]-Aspartate was incorporated into the pyrimidine moiety and this was degraded by the method of David et al.[185] (Figure 14) to 1,3-diamino-2-methylpropane (72). Since all of the expected sites of label (C-5,-6,-7) were recovered in the degradation product (72), this compound should have had the same specific activity as the pyrimidine which had been degraded. Only 73% of the activity was recovered. 1,3-Diamino-2-methylpropane (72) contains four of the six carbons of the pyrimidine moiety. A random incorporation of [^{14}C]aspartic acid into the pyrimidine would lead to 67% of the activity being recovered in the product (72). The pyrimidine could have been contaminated with an active impurity and this impurity could have been lost in the degradation. This seemed to have been the case when this degradation had been used previously.[187] From these results, it cannot be stated with certainty that aspartic acid (56) is a precursor to the pyrimidine moiety. A better method for the investigation of this problem would have been to use specifically labelled aspartic acids and degradations of the pyrimidine moiety to determine whether the incorporation was non-random.

The first indication of a relationship between the biosynthesis of the pyrimidine moiety of thiamin and nucleic acid purine biosynthesis was the observation of the inhibition of the biosynthesis of the pyrimidine moiety by adenosine in Aerobacter aerogenes.[193] Through the use of Salmonella typhimurium mutants, Newell and Tucker[194] found that adenosine not only caused an inhibition of thiamin biosynthesis, but

also a de-repression. The technique involved the growth of the organism in the presence of adenosine and then the resuspension of the cells in fresh minimal medium. The cells, whose synthesis of thiamin had been inhibited in the growing culture, synthesized large quantities of thiamin when placed in the fresh adenosine-free medium. A similarly large synthesis of the pyrimidine moiety (21) was found in a mutant blocked along the pathway of thiazole biosynthesis.

Newell and Tucker also found that the pyrimidine (21) was synthesized in large amounts from intermediates on the purine pathway by the above mutant.[195-197] Single site mutants of S. typhimurium LT2 were used to determine the intermediates common to both the pyrimidine and purine pathways. Some of these mutants required for growth both purines and the pyrimidine moiety of thiamin, while others had only a purine requirement. The nutritional needs of the mutants with the dual requirement could be satisfied by 5-aminoimidazole ribonucleotide (AIR, 78) and compounds prior to this on the purine pathway. Intermediates located beyond AIR (78) on the purine pathway could not satisfy these requirements. Thus, it was suggested that the early part of the biosynthetic pathway of the pyrimidine moiety might be common to the early part of the purine biosynthetic pathway and that AIR (78) was the last common intermediate.

Confirmation of this hypothesis was obtained by the feeding of ^{14}C-labelled glycines, a known purine precursor, to the S. typhimurium mutant, which synthesized the pyrimidine (21) in large quantities and excreted it into the medium.[195,196] Radioactivity from both carbons of glycine (35) was incorporated into the pyrimidine moiety. In mutants which were also auxotrophic for glycine, pyrimidine moiety biosynthesis was

strictly dependent on the presence of added glycine and label from the [^{14}C]glycines was incorporated from both carbon atoms with little or no dilution of specific activity.

A mutant which was able to use the ribonucleoside form of AIR (78) and which also excreted the pyrimidine moiety into the medium was found to convert chromatographically pure [5-^{14}C]AIR ribonucleoside into the pyrimidine moiety with no dilution of specific activity.[195,197] The radioactive [5-^{14}C]AIR ribonucleoside was obtained from the incubation of a mutant, which excretes AIR ribonucleoside into the medium, with [1-^{14}C]glycine. The AIR ribonucleoside was purified by chromatography but it is possible that all of the [1-^{14}C]glycine was not removed. This provides evidence that AIR (78) is also an intermediate in the biosynthesis of the pyrimidine moiety (21), but no degradations were carried out. Thus, it cannot be assumed that the label was introduced without randomization by degradation and resynthesis.

Newell and Tucker[197] proposed a scheme for the conversion of AIR (78) to the pyrimidine (21) (Route D, Figure 16) which involved the insertion of a carbon atom into the imidazole ring to generate the pyrimidine ring. Two other C_1 transfers were proposed for the origin of the methyl and the hydroxymethyl substituents. However, the authors rejected this proposal for two reasons. They considered that the methyl group should be derived from methionine or the one-carbon pool. They did not find any incorporation from [Me-^{14}C]methionine or [G-^{14}C]-methionine into the pyrimidine moiety.[196] Secondly, formate was known as the precursor of C-4 of the pyrimidine moiety in yeast[184,188] and not the methyl group. Formate is known as the precursor of C-2 of AIR (78) and Route D in Figure 16 predicts that C-2 of AIR (78) becomes C-2, not C-4, of the

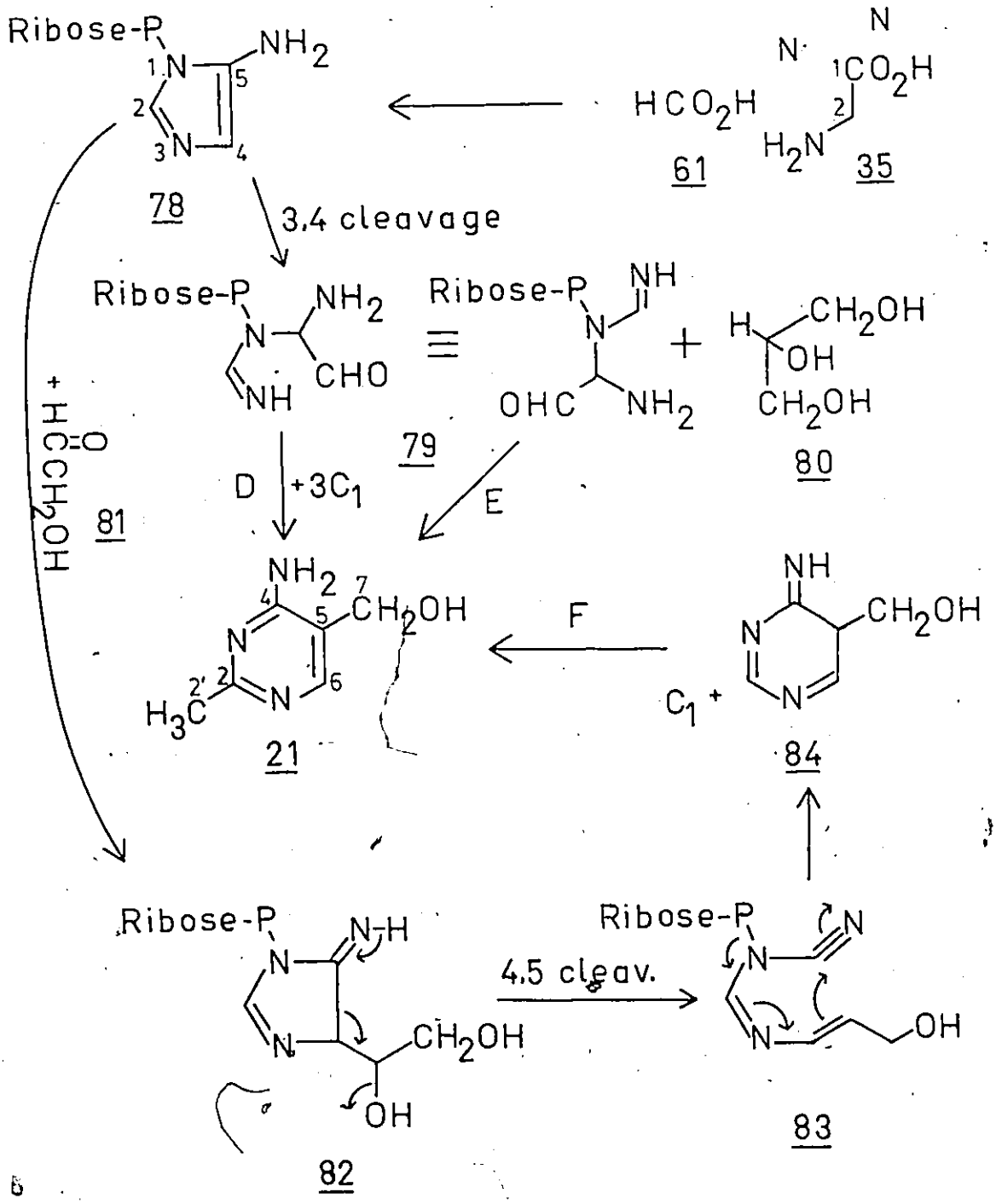


Figure 16: Hypotheses for the biogenesis of the pyrimidine moiety from the purine intermediate AIR.

pyrimidine (21).

As an explanation for the incorporation of formate into C-4 of the pyrimidine (21), Newell and Tucker[197] proposed a second scheme (Route E, Figure 16) which involved the cleavage of the imidazole ring of AIR (78) at the same position and the combination of the intermediate (79) with a three-carbon compound rather than three C_1 units. Glycerol (80) or α-glycerophosphate were proposed for the three-carbon compound. The interesting features of this scheme are that the carbon atoms of glycine become C-2,-2' of the pyrimidine (21) and that no C_1 transfers are required for the formation of the methyl or the hydroxymethyl substituents.

A third possible scheme (Route F, Figure 16) has been proposed for the conversion of AIR (78) to the pyrimidine (21).[198,199] This route differs from Route E in that C-4 and C-6 of the pyrimidine are derived from glycine and also in that C-2 rather than C-4 is suggested as the carbon derived from formate. The difference between Route F and Route D is that in Route F, C-6 of the pyrimidine is derived from C-2 of glycine, whereas C-5 of the pyrimidine is derived from C-2 of glycine via Route D. These three routes are easily distinguished by tracer experiments with specifically labelled compounds and degradation of the resulting pyrimidine to determine the location of the activity.

A [^{14}C]formate experiment was carried out in yeast[128] and the pyrimidinesulfonic acid (4) obtained by bisulfite cleavage (Figure 1) was degraded by permanganate oxidation to acetic (63) and propionic (76) acids (Route A, Figure 17). The products were identified on the basis of their retention times in gas-liquid chromatography. The acetic acid (63)

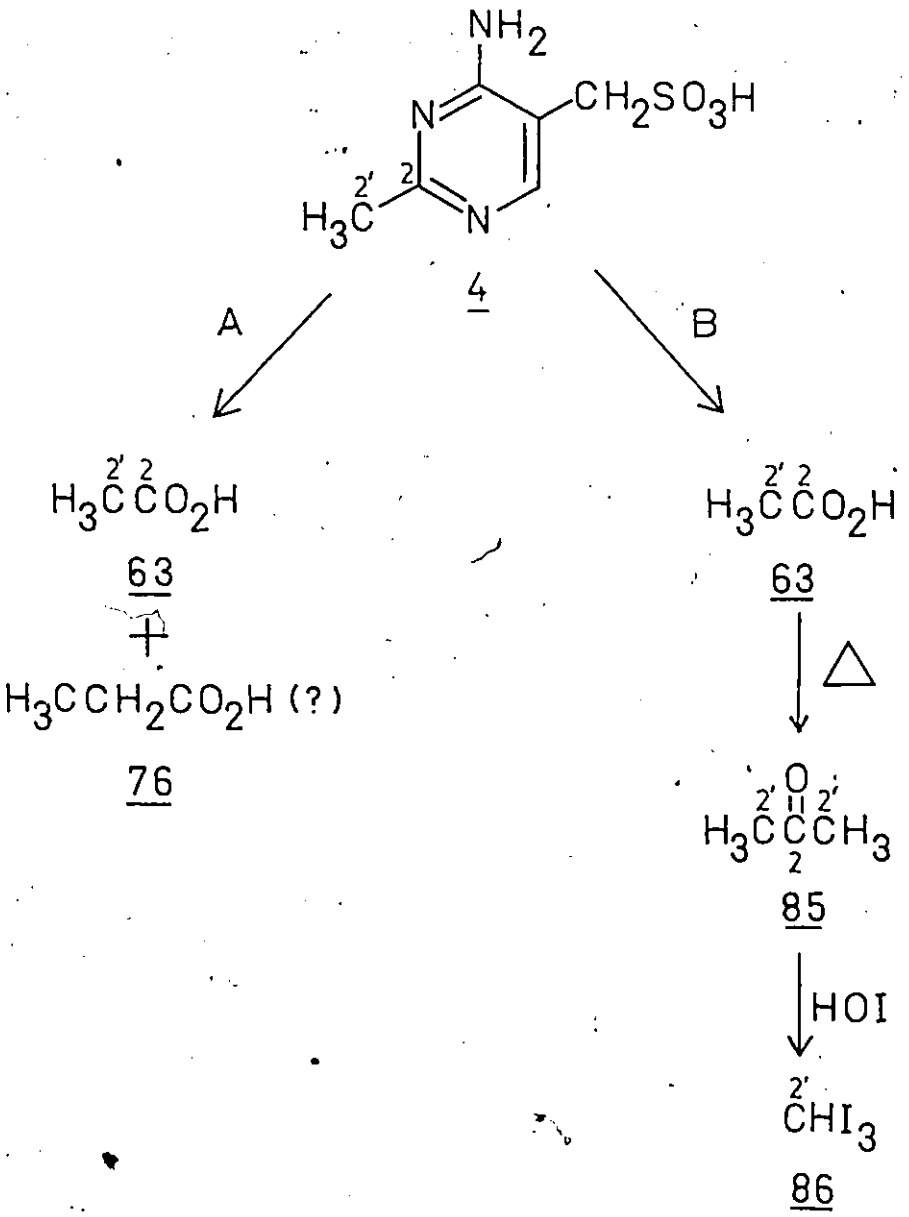


Figure 17: Degradation of the pyrimidinesulfonic acid (4) to isolate C-2,-2'.

was thought to derive from C-2,-2' of the pyrimidine and activity was found to be associated with the gc peak assigned to this compound. The origin of the propionic acid from the pyrimidine was not clear but no activity was associated with the gc peak attributed to this compound. In contrast to the results obtained previously in yeast,[184,188] the activity associated with formate was found not at C-4 of the pyrimidine moiety but at either C-2 or C-2'.

A much more extensive degradation was carried out to determine the position of the pyrimidine moiety derived from formate in E. coli.[192] Carbons 2 and 2' were obtained by Kuhn-Roth degradation of the pyrimidine-sulfonic acid (4) (Route B, Figure 17). Only 67% of the activity associated with the pyrimidine (4) was recovered in the acetic acid (63). The acetic acid (63) was converted to its barium salt and pyrolysis of this compound yielded acetone (85) and carbon dioxide; the latter was trapped as barium carbonate. Iodoform (86) was obtained by the hypoiodite oxidation of acetone (85). The inactive iodoform suggested that the activity was located in the carboxyl carbon of acetate or in C-2 of the pyrimidine moiety. This was confirmed as the barium carbonate obtained from the pyrolysis of barium acetate was active. Since only 67% of the activity was obtained in C-2,-2', the degradation outlined in Figure 18 was used to isolate the other four carbons of the pyrimidine moiety. The pyrimidine (71) was obtained from thiamin as in Figure 14, and then converted to the dimethylpyrimidine (73) as outlined in Figure 15. The dimethyl derivative (73) was partially hydrogenated over Adams catalyst to 87 which yielded β-aminoisobutyric acid (88) on acid hydrolysis. When this degradation was carried out on the pyrimidine moiety derived from [^{14}C]formate, no

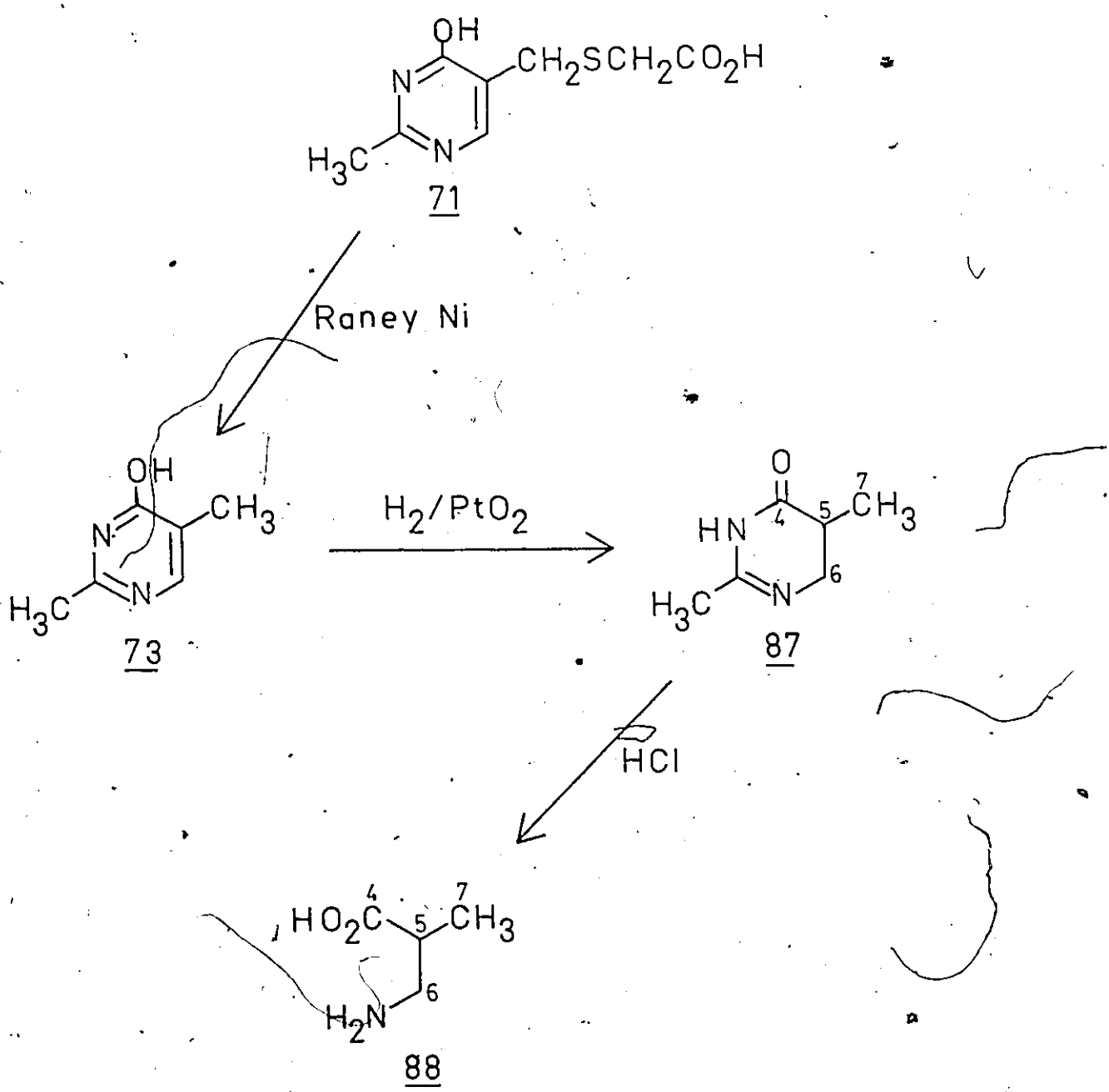


Figure 18: Degradation of the pyrimidine moiety to obtain C-4,-5,-6,-7 as β-aminoisobutyric acid.

activity was recovered in the β-aminoisobutyric acid (88).

Estramareix and Lesieur[198] used S. typhimurium supplied by Newell and Tucker, to confirm the incorporation of formate into C-2,-2' of the pyrimidine moiety via the degradation outlined in Figure 15. They also found that C-4 of the pyrimidine moiety was derived from C-1 of glycine. These results are not consistent with Route E in Figure 16, but still fit Routes D and F. A [2-^{14}C]glycine experiment showed that C-6 of the pyrimidine moiety, as suggested in Route F, and not C-5 as suggested by Route E, was derived from C-2 of glycine.[199]

The incorporation of [2-^{14}C]glycine into the pyrimidine moiety by S. typhimurium was confirmed by Bellion et al.,[143] but the position of the label was not determined.

Recently, the incorporation of glycine into the pyrimidine moiety by E. coli has been confirmed using ^{13}C, ^{2}H and ^{15}N labelled glycines.[200] The position of the label in the pyrimidine moiety was determined from the mass spectral fragmentation patterns which were assigned from pyrimidines that were synthesized with labels in known positions. In complete agreement with the results obtained from ^{14}C-labelled glycines and chemical degradation, C-4 and C-6 of the pyrimidine moiety were derived from [1-^{13}C]- and [2-^{13}C]glycine, respectively. [^{15}N]Glycine was incorporated only into N-1 of the pyrimidine moiety. Again, these results are consistent with Route F in Figure 16. Considerable loss of deuterium was observed for the incorporation of [2-$^{2}H_2$]glycine into the pyrimidine moiety. The reversible carboxylation of AIR (78) at C-4 of the imidazole ring was offered as an explanation for this observation. This carboxylation is the next step in the purine pathway and if it was reversible,

any deuterium at C-4 of AIR (78) would be exchanged with the protons from the medium.

Route F in Figure 16 fits all of the known results for the biosynthesis of the pyrimidine moiety except the incorporation of formate into C-4 by yeast. However, the problem is still not solved as the precursors to the methyl group and C-5 and C-7 have not been identified. Glycolaldehyde (81) has been suggested as the C_2 unit which combines with AIR (78) and Route F outlines a possible mechanism for the ring expansion. A second possibility exists for the incorporation of glycine. Threonine, which is synthesized in some micro-organisms from glycine and acetaldehyde, could be incorporated into the imidazole precursor. Rearrangement and oxidation of the methyl to a hydroxymethyl would lead to the pyrimidine moiety. Alternatively, glycolaldehyde instead of acetaldehyde, could combine with glycine to form 4-hydroxythreonine which would yield the intermediate (82) directly.

Four compounds which have been proposed as intermediates in the biosynthesis of the pyrimidine portion of thiamin have been isolated by chromatographic methods from Neurospora crassa cultures.[201-203] They were identified as the 5-aminomethyl-(89), 5-formyl-(90), 5-hydroxymethyl-(21) and 5-methoxymethyl-(91) derivatives of 4-amino-2-methylpyrimidine by chemical, chromatographic and spectroscopic techniques.[203] They were assumed to be related as shown in Figure 19. The 5-aminomethyl derivative (89), postulated to be formed first, was assumed to yield the 5-formyl derivative (90) which could be reduced to the 5-hydroxymethylpyrimidine (21). The 5-methoxymethyl derivative (91) was derived from the 5-hydroxymethylpyrimidine (21). [^{14}C]Formate was incorporated into

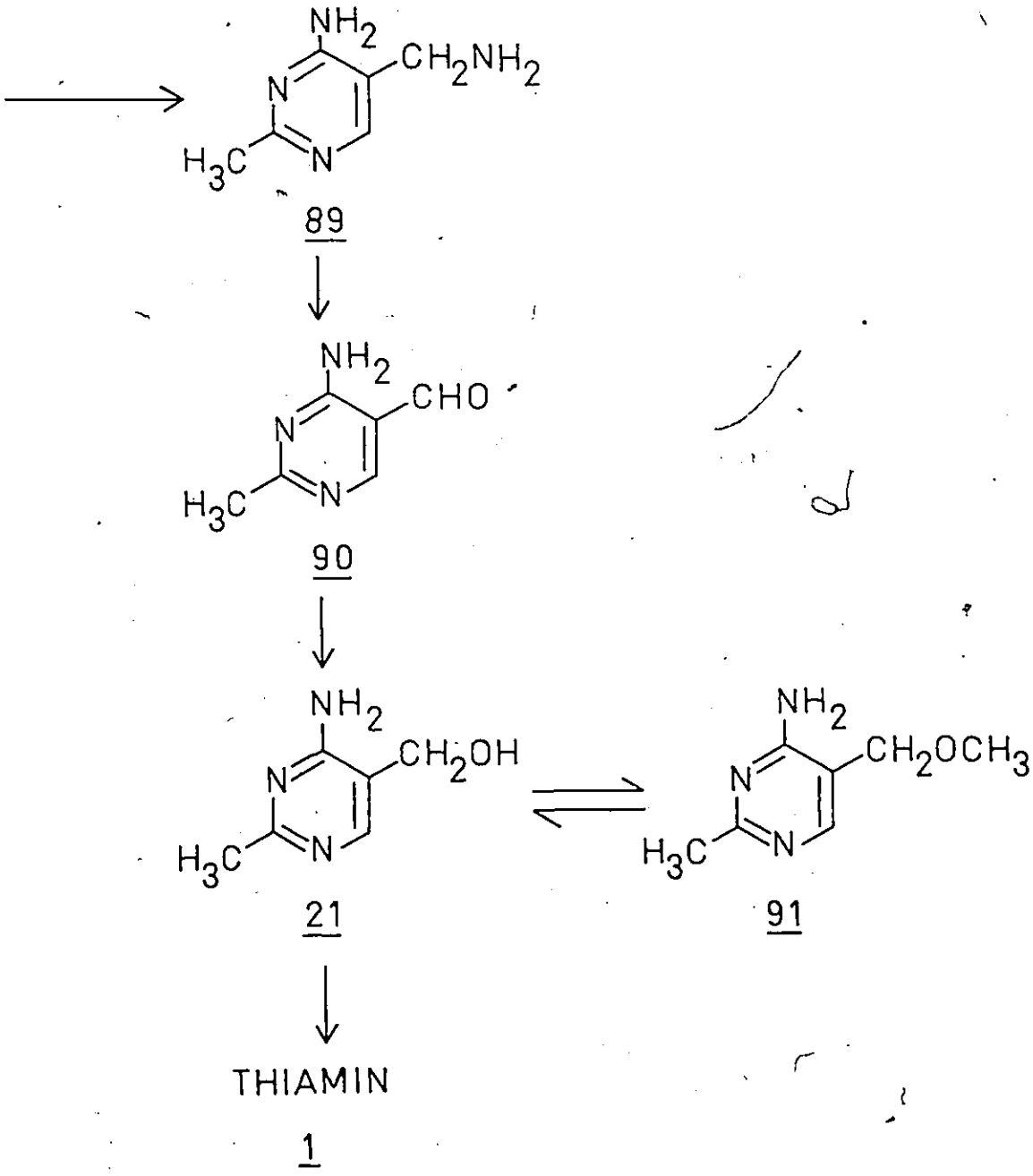


Figure 19: Hypothetical pathway for the biosynthesis of the pyrimidine moiety based on compounds isolated from N. crassa.

the 5-aminomethyl-(89) and the 5-hydroxymethyl-(21) derivatives, but no evidence was given in the paper to support the proposed sequence. It is possible, since long incubation times were used, that the 5-hydroxymethylpyrimidine (21) was formed first and that the other compounds are derived from it. It has been shown that, in cell-free preparations from yeast, these compounds are converted to the 5-hydroxymethylpyrimidine (21),[95,204,205] and some of the properties of the enzymes involved in these transformations have been measured.

The investigations described above have been summarized in Table 1 according to the organism and the position of the label in the compound used in the experiment. This table is intended as a guide to the literature of this problem.

Although many experiments have been described in the preceding pages, the pathway for the biosynthesis of the pyrimidine moiety has not been elucidated. In fact, there is no complete scheme that is supported by unequivocal evidence. Thus, the biosynthesis of the pyrimidine moiety is also an area which requires further investigation.

Table 1 Summary of literature feeding experiments
(Numbers in Table refer to references)

	Organism	Labelled Substrates: Acetate		
		[1-^{14}C]	[2-^{14}C]	[U-^{14}C]
1.	Saccharomyces cerevisiae	---	---	---
2.	S. cerevisiae (39916 H.J. Bunker)	128	128	---
3.	S. cerevisiae (NCYC 1062)	---	134	---
4.	S. cerevisiae (ATCC 24903)	206	---	---
5.	Neurospora crassa	---	---	---
6.	Bacillus subtilis (ATCC 6051)	---	---	---
7.	B. subtilis (ATCC 6633)	129	129,190	129
8.	Escherichia coli B	192	180,192	---
9.	E. coli (ATCC 9637)	---	---	---
10.	E. coli K12 (mutant PTR1)	---	---	---
11.	E. coli W (mutant 83-1)	---	---	---
12.	E. coli (mutant 26 43)	---	---	---
13.	Salmonella typhimurium (LT2)	---	---	---
14.	S. typhimurium (ATCC 23592 LT2)	---	---	---

Table 1 (Continued)

	Labelled Substrate						
	Acetate					Alanine	
	[2-^{3}H]	[2-$^{2}H_3$]	[1-^{14}C,2-^{3}H]	[2-^{14}C,2-^{3}H]	[1,2-^{14}C,2-^{3}H]	[1-^{14}C]	[U-^{14}C]
1.	---	---	---	---	---	---	---
2.	---	---	---	---	---	---	128
3.	---	---	---	---	---	134	134
4.	---	---	---	---	---	---	---
5.	---	---	---	---	---	---	---
6.	---	---	---	---	---	---	---
7.	129	---	129,190	129,190	129	---	129
8.	---	207	---	---	---	---	---
9.	---	---	---	---	---	---	---
10.	---	---	---	---	---	---	---
11.	---	---	---	---	---	---	---
12.	---	---	---	---	---	---	---
13.	---	---	---	---	---	---	---
14.	---	---	---	---	---	---	143

Table 1 (Continued)

	Alanine		5-Aminoimidazole Ribonucleoside	Aspartic Acid			Carbonate
	[^{3}H]	[^{3}H,^{14}C]	[5-^{14}C]	[2-^{14}C]	[4-^{14}C]	[U-^{14}C]	[^{14}C]
1.	---	---	---	---	---	---	---
2.	---	---	---	---	---	---	---
3.	---	---	---	---	---	134	---
4.	---	---	---	---	---	---	---
5.	---	---	---	---	---	---	---
6.	---	---	---	---	---	---	---
7.	129	129	---	---	---	129	129
8.	---	---	---	---	180	180	---
9.	---	---	---	---	---	133	---
10.	---	---	---	---	---	---	---
11.	---	---	---	---	---	---	---
12.	---	---	---	181	---	---	---
13.	---	---	195,197	---	---	---	---
14.	---	---	---	---	---	---	---

Table 1 (Continued)

	Cysteine		Cystine	Formate	Fructose	Glucose		
	[3-^{14}C]	[^{35}S]	[^{35}S]	[^{14}C]	[1-^{14}C]	[1-^{14}C]	[2-^{14}C]	[6-^{14}C]
1.	---	---	---	179,184	---	---	---	---
2.	---	---	128,130	128,130	---	---	---	---
3.	---	---	---	124,125,134	---	---	---	---
4.	206	---	---	206	208	208	208	206,208
5.	---	---	---	203	---	---	---	---
6.	---	---	---	126,176	---	---	---	---
7.	---	---	---	129	---	---	---	---
8.	---	---	---	180,192	---	---	---	---
9.	---	---	131	---	---	---	---	---
10.	---	---	---	---	---	---	---	---
11.	---	---	---	---	---	---	---	---
12.	---	---	---	181	---	---	---	---
13.	---	---	---	198	---	---	---	---
14.	132	132	---	132	---	---	---	---

Table 1 (Continued)

	Labelled Substrate						
	Glucose						Glutamate
	[U-^{14}C]	[U-^{13}C]	[6-^{3}H,6-^{14}C]	[1-^{2}H]	[6,6'-$^{2}H_3$]	[5,6,6'-$^{2}H_2$]	[U-^{14}C]
1.	---	---	---	---	---	---	---
2.	---	---	---	---	---	---	---
3.	---	---	---	---	---	---	134
4.	206	---	206	---	---	---	---
5.	---	---	---	---	---	---	---
6.	---	---	---	---	---	---	---
7.	---	---	---	---	---	---	129
8.	---	207	---	207	207	207	---
9.	---	---	---	---	---	---	---
10.	---	---	---	---	---	---	---
11.	---	---	---	---	---	---	---
12.	---	---	---	---	---	---	---
13.	---	---	---	---	---	---	---
14.	---	---	---	---	---	---	132

Table 1 (Continued)

	Labelled Substrate							
	Glycerate	Glycerol				Glycine			
	[1-^{14}C]	[1,3-^{14}C]	L-[1-^{2}H]	[2-^{2}H]	L-[3-^{2}H]	[1-^{14}C]	[1-^{13}C]	[2-^{14}C]	[2-^{13}C]
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	209	---
	125	---	---	---	---	125,134	---	124,125,134	---
	---	206	---	---	---	---	---	209	---
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---
	---	---	207	207	207	---	200	180	200
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---
	---	---	---	---	---	198	---	199	---
	---	---	---	---	---	195,196	---	143,195,196	---

Table 1 (Continued)

	Labelled Substrates								
	Glycine				α-Ketoglutarate	Lactate	Methionine		
	[U-^{14}C]	[2-^{3}H,2-^{14}C]	[2-^{2}H$_2$]	[^{15}N]	[5-^{14}C]	[U-^{14}C]	[Me-^{14}C]	[2-^{14}C]	[3,4-^{14}C]
1.	---	---	---	---	---	---	---	---	---
2.	---	---	---	---	---	---	128,130,209	---	---
3.	134	---	---	134	---	---	124,134	---	134
4.	---	206	---	---	206	206	209	---	---
5.	---	---	---	---	---	---	---	---	---
6.	---	---	---	---	---	---	---	---	---
7.	---	---	---	---	---	---	129,141	---	---
8.	---	---	200	200	---	---	---	---	---
9.	---	---	---	---	---	---	---	---	---
10.	145	---	---	---	---	---	---	---	---
11.	---	---	---	---	---	---	---	---	---
12.	---	---	---	---	---	---	---	---	---
13.	---	---	---	---	---	---	---	---	---
14.	---	---	---	---	---	---	143,196	143	---

Table 1 (Continued)

Labelled Substrates

	Methionine					Mevalolactone	Mevalonate	Orotate
	[G-^{14}C]	[^{3}H]	[^{3}H,Me-^{14}C]	[^{35}S]	[Me-^{14}C,^{35}S]	[2-^{14}C]	[2-^{14}C]	[2-^{14}C]
1.	---	---	---	---	---	---	---	---
2.	---	---	---	128,130	128,130	---	---	---
3.	---	---	---	---	---	134	134	---
4.	---	---	---	---	---	---	---	---
5.	---	---	---	---	---	---	---	---
6.	---	---	---	---	---	---	---	---
7.	---	129	129	---	---	---	---	---
8.	---	---	---	---	---	---	---	180
9.	133	---	---	131	---	---	---	---
10.	---	---	---	---	---	---	---	---
11.	---	---	---	---	---	---	---	---
12.	---	---	---	---	---	---	---	---
13.	---	---	---	---	---	---	---	---
14.	196	---	---	---	---	---	---	---

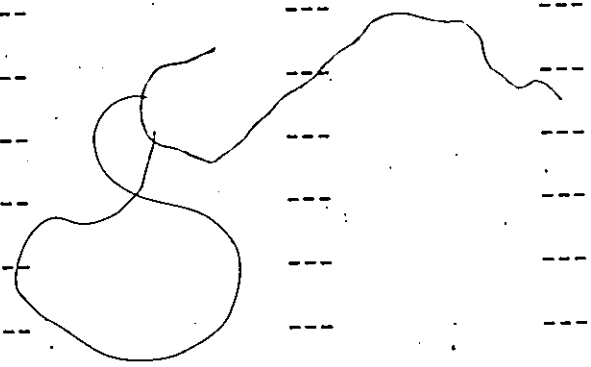


Table 1 (Continued)

	Orotate	Pyruvate		Ribose	Serine		Succinate	Sulfate
	[6-^{14}C]	[3-^{14}C]	[Me-$^{2}H_3$]	[1-^{14}C]	[3-^{14}C]	[U-^{14}C]	[1-^{14}C]	[^{35}S]
1.	---	---	---	---	---	---	---	---
2.	128	---	---	---	---	---	---	---
3.	---	---	---	125	125,134	134	---	123
4.	---	206	---	206	206	---	206	---
5.	---	---	---	---	---	---	---	---
6.	---	---	---	---	---	---	---	---
7.	---	---	---	---	---	---	---	---
8.	180	---	207	---	180	---	---	---
9.	---	---	---	---	---	133	---	133
10.	---	---	---	---	---	---	---	---
11.	---	---	---	---	---	---	---	---
12.	---	---	---	---	---	---	---	---
13.	---	---	---	---	---	---	---	---
14.	---	---	---	---	---	---	---	---

Table 1

Organism	Labelled Substrates: Tyrosine [2-^{14}C]	Tyrosine [U-^{14}C]	Tyrosine [^{15}N]	Uracil [U-^{14}C]
1. Saccharomyces cerevisiae	---	---	---	---
2. S. cerevisiae (39916 H.J. Bunker)	---	---	---	---
3. S. cerevisiae (NCYC 1062)	---	---	---	---
4. S. cerevisiae (ATCC 24903)	209	---	---	---
5. Neurospora crassa	---	---	---	---
6. Bacillus subtilis (ATCC 6051)	---	---	---	---
7. B. subtilis (ATCC 6633)	---	---	---	---
8. Escherichia coli B	---	---	148	180
9. E. coli (ATCC 9637)	---	---	---	---
10. E. coli K12 (mutant PTR1)	---	---	---	---
11. E. coli W (mutant 83-1)	147	147	---	---
12. E. coli (mutant 26 43)	---	---	---	---
13. Salmonella typhimurium (LT2)	---	---	---	---
14. S. typhimurium (ATCC 23592 LT2)	143	---	---	---

CHAPTER II

THE BIOSYNTHESIS OF THE THIAZOLE MOIETY

PART A A Re-Examination of Published Concepts and the Origin of C-2

(i) Introduction

As described in Chapter I (Section iii), several groups of workers have pursued the origin of the thiazole moiety with radioactive-tracer experiments. The results of these experiments can be divided into those concerned with the origin of C-2 and those concerned with the other five carbon atoms of the thiazole unit.

Published results support three of the hypotheses shown in Figure 5; these are reproduced in Figure 20. In support of Route D, it was reported that L-[Me-^{14}C]methionine was incorporated into the thiazole moiety of thiamin and that L-[Me-^{14}C,^{35}S]methionine was incorporated without a change in the ^{35}S/^{14}C ratio.[128,130] A strain of the yeast Saccharomyces cerevisiae (39916 H.J. Bunker) was used for these experiments. From experiments carried out with Bacillus subtilis, it was also reported that L-[Me-^{14}C]methionine was incorporated into the thiazole unit[129] and on degradation almost all of the activity was located at C-2.[141] These results were taken to show that methionine served as the precursor of the fragment C-2, S, C-5,-6,-7 of the thiazole nucleus.

Evidence contrary to this hypothesis of thiazole formation emerged from radioactive-tracer experiments carried out with a second strain of S. cerevisiae (NCYC 1062). It was found by unequivocal degradation of the

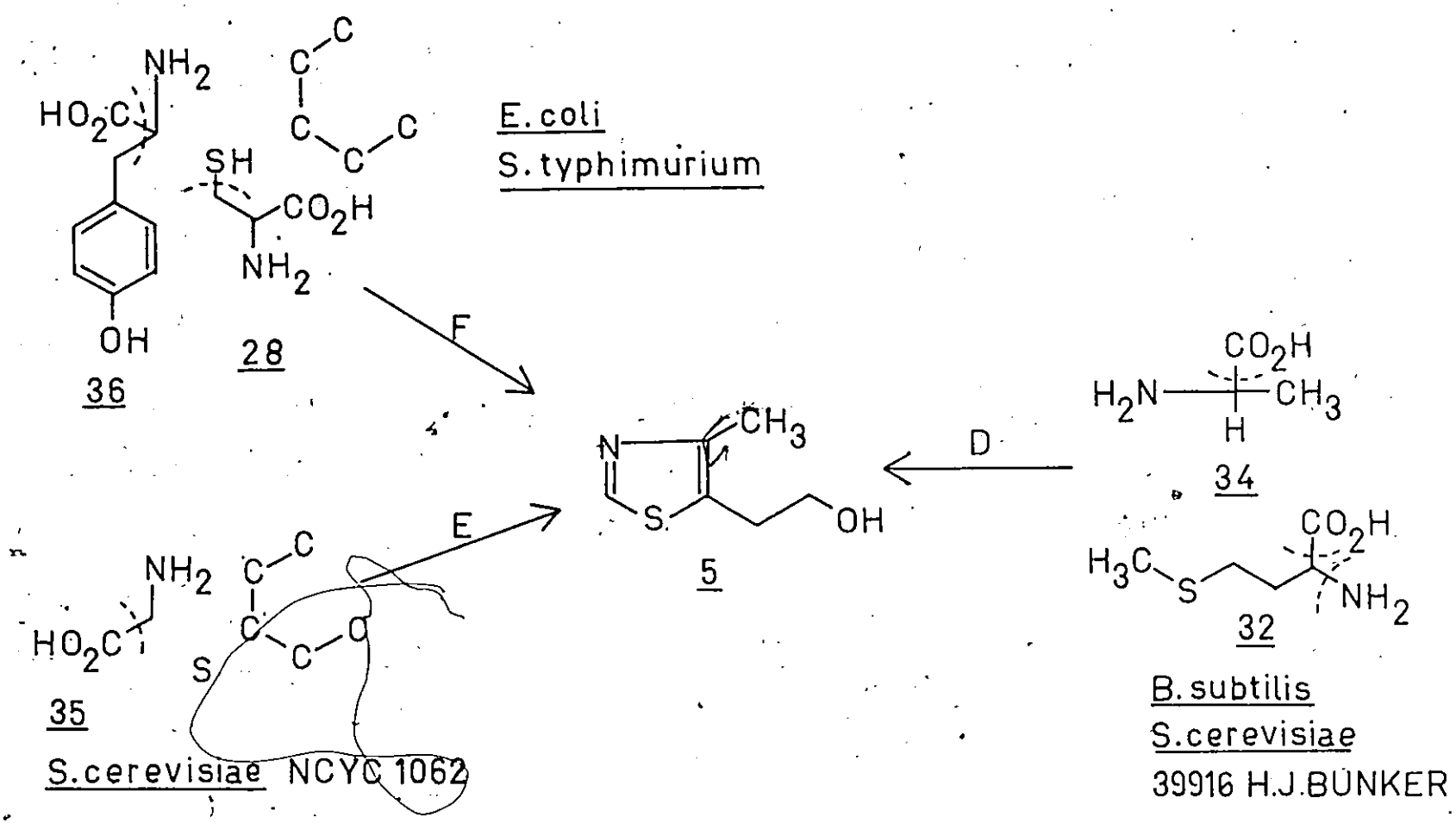


Figure 20: Contradictory hypotheses for the biogenetic origin of the thiazole moiety.

radioactively labelled thiamin, obtained from a feeding experiment with [2-^{14}C]glycine, that most of the activity of the molecule was located at C-2 of the thiazole.[124] Administration of [2-^{14}C]glycine in the presence of unlabelled methionine gave an identical result.[125] A different biosynthetic route to the thiazole unit (Route E, Figure 20) was proposed to explain the involvement of glycine.[125]

A similar hypothesis was proposed[132] to account for the incorporation of [2-^{14}C]tyrosine into C-2 of the thiazole moiety by the enteric bacteria Escherichia coli[147] and Salmonella typhimurium.[143] This scheme (Route F, Figure 20), is analogous to the glycine hypothesis and offers no explanation for the origin of the proposed C_5 unit.

Taken at face value, the results that showed methionine, glycine and tyrosine as building units of the thiazole portion of thiamin in micro-organisms are clearly incompatible.

There are three possibilities to account for the contradictory results. Either there is more than one pathway to thiamin, or C-2 of glycine, C-2 of tyrosine and the S-methyl group of methionine are metabolically interconvertible, or one or more of the reported results is in error.

Before a distinction could be made between the alternatives, a microbial system in which radioactive-tracers are incorporated into thiamin was required and to set up such a system became the initial objective of this investigation. Once the system was functional, the origin of C-2 could be established and this might provide some indication of the origin of the C_5 unit.

If methionine did serve as a precursor of C-2, then the inference[128,129] that the C_3 chain, C-2 to C-4, of methionine gives rise to C-5,-6,-7 of the thiazole moiety had to be tested by appropriate feeding experiments and chemical degradation to confirm the expected distribution of label in the thiazole moiety, The notion that C-4,-4' of the thiazole unit originates from alanine or acetate was based solely on incorporation measurements[128,129] and thus would also have to be confirmed by degradation. If, however, either glycine or tyrosine were found as the precursor of C-2, then the origin of the C_5 unit would have to be approached from first principles.

This section describes the re-investigation of some reports of incorporation of labelled compounds into thiamin and also clarifies the origin of C-2 of the thiazole moiety in S. cerevisiae.

(ii) Choice of organism and growth conditions

Although thiamin is not produced by animals, its de novo synthesis is performed by both plants and micro-organisms. Micro-organisms are more versatile than plants as an experimental organism because they are more easily cultured and feeding experiments are not limited to restricted time periods during the year. Also, if the apparant contradictions are to be resolved, similar or identical organisms to those used in the past would have to be employed.

Yeast is generally considered to be a good source of thiamin,[210-212] and this high thiamin content is due to two factors. Yeast can synthesize its metabolic requirement de novo, but if grown on a medium containing thiamin (or its two components) it will accumulate the vitamin from this source.[212-214] Thiamin levels of 0.1-2.0 mg/g dry cell weight are readily

obtained for S. cerevisiae[215] and levels as high as 17 mg/g dry cell weight (approximately 50 mg/litre of culture) have been reported for S. saké.[216] The accumulation of thiamin by yeast is very important for nutrition, but it should be avoided in a biosynthetic experiment. Yeast was therefore grown on a thiamin-free medium to encourage the de novo synthesis of thiamin.

The thiamin content of a number of yeasts has been reported as 5-100 µg/g dry cell weight.[212,217] Baker's yeast has been found to contain 9-40 µg/g dry cell weight while enriched baker's yeast contains much higher levels of thiamin (650-750 µg/g dry cell weight). Other investigators have found thiamin to be present in amounts of 7-27 µg/litre of culture[134] and 16.5 µg/g dry cell weight.[184] Consideration of such values must take into account the composition of the medium on which the organism was grown and the specificity of the method of assay used.

The thiamin content of a number of bacteria, as determined by microbiological assay, has been reported as 50-150 µg/litre of culture.[218] The medium alone, used in these experiments, elicited a response in the thiamin assay of as much as 30% of that of the culture.

Due to the uncertainties in the above values, the thiamin content of four micro-organisms was determined by thiochrome assay.[134,219] After growth on thiamin-free medium, the cells of S. cerevisiae ATCC 24903, 39916 H.J. Bunker, and ATCC 413[illegible] contained 26 ± 3, 20 ± 3, and 26 ± 3 µg/litre of culture, respectively. The value obtained for S. cerevisiae ATCC 24903 is equivalent to 10 µg/g dry weight of cells. No thiamin was found in the supernatant after the cells had been removed by centrifugation.

Although it has a greater ability to accumulate thiamin, S. cerevisiae

(ATCC 4134), also known as S. saké, produces the same amount of thiamin as S. cerevisiae (ATCC 24903 and 39916 H.J. Bunker) when grown on thiamin-free medium.

When S. cerevisiae (39916 H.J. Bunker) was grown on yeast extract medium, a thiamin content of 285 µg/litre of culture was found in the cells and the supernatant contained thiamin at a concentration of 7 µg/litre. The yeast extract used for this medium contained thiamin (150 µg/g). A one-litre culture would therefore contain 300 µg of thiamin. Most of the thiamin contained in the yeast extract, within experimental error, was recovered from the yeast cells which confirmed the ability of S. cerevisiae to accumulate thiamin from the medium.

In contrast to the published values for bacteria,[218] the cells from a one-litre culture of Bacillus subtilis 19E contained only 1.5 ± 0.2 µg/litre of culture of thiamin. No thiamin was found in the supernatant medium.

Mutants of S. typhimurium,[196] E. coli,[220] and B. subtilis[221] are known which manufacture excess amounts of the pyrimidine moiety of thiamin and excrete this compound into the medium. Such a system is very desirable since the target compound does not have to be extracted from the cells and it is produced in greater than normal quantities. No mutant which possesses similar properties for the thiazole moiety has been reported.

In this investigation, yeast was found to contain more thiamin per litre of culture than B. subtilis and it seemed likely that thiamin would be manufactured in greater quantities by the yeast. However, the amount of thiamin present still required the carrier dilution technique for its isolation from the cells in order to obtain sufficient quantities for

purification and degradation.

A second reason for the choice of yeast over bacteria was the glycine-methionine contradiction. In a baking yeast, S. cerevisiae (39916 H.J. Bunker), methionine was reported as a precursor of C-2 of the thiazole unit and in a brewing yeast, S. cerevisiae (NCYC 1062), glycine was reported as a precursor of C-2 of the thiazole moiety. A priori, it did not seem very likely that two different pathways for the biosynthesis of thiamin existed in the two strains of yeast.

(iii) Radioactive-tracer results

Radioactive-tracer experiments with [2-^{14}C]glycine and L-[Me-^{14}C]-methionine were performed with two strains of S. cerevisiae (ATCC 24903 and 39916 H.J. Bunker). All other experiments were carried out with strain ATCC 24903. Thiamin-free medium was used in each experiment and the tracer was added either at the beginning of the incubation (Expts. 1-12) or at the onset of logarithmic growth (Expts. 13-15). The cells were collected after maximum growth had been attained. A summary of these experiments and the percentage of the activity recovered in the non-cellular portion of the culture are presented in Table 2. Most of the activity, fed in the form of sodium [3-^{14}C]pyruvate (Expt. 5), D-[1-^{14}C]ribose (Expt. 6), DL-[3-^{14}C]cysteine (Expt. 9), [1-^{14}C]succinic acid (Expt. 10) and sodium 3-keto[5-^{14}C]glutarate (Expt. 11), was found in the non-cellular part of the culture.

Thiamin was extracted from the cells and, after dilution with carrier, was isolated by ion-exchange chromatography. The thiamin-containing ion-exchange fractions from experiments with D-[1-^{14}C]ribose (Expt. 6); DL-[3-^{14}C]cysteine (Expt. 9) and sodium 2-keto[5-^{14}C]glutarate

(Expt. 11) were inactive and the experiments were not continued.

The specific activity of the thiamin isolated from feedings with sodium [3-^{14}C]pyruvate (Expt. 5) and [1-^{14}C]succinic acid (Expt. 10) was too low to make degradation feasible.

If the sample of thiamin chloride hydrochloride (2) maintained a significant level of radioactivity after several recrystallizations, it was degraded by bisulfite cleavage to the pyrimidinesulfonic acid (4) and to the thiazole (5) (Figure 21). The thiazole (5), an oil at room temperature, was converted to either its phenylurethane (92) or its phthalimido (94) derivative for purification and assay of radioactivity. The results of this degradation are summarized in Tables 3 and 4.

Similar results were obtained from the experiments with L-[Me-^{14}C]-methionine with both strains of S. cerevisiae (Expts. 3 and 15) (Table 4) and from the experiments with DL-[2-^{14}C]tyrosine (Expt. 4) and sodium [1-^{14}C]acetate (Expt. 8) (Table 3). In each case, the phenylurethane (92) was completely inactive. The samples of the pyrimidinesulfonic acid (4) were either completely inactive or contained only a very small percentage of the activity present in the original thiamin.

The pyrimidinesulfonic acid (4) obtained from the sodium [^{14}C]-formate feeding (Expt. 2) retained a percentage ($\sim$ 30%) of the activity present in the original thiamin (Table 3). No activity was recovered in the thiazole portion.

Most of the activity ($\sim$ 88%) contained in the thiamin derived from DL-[3-^{14}C]serine (Expt. 12) was recovered in the thiazole phenylurethane (92) (Table 3), whereas the pyrimidinesulfonic acid (4) contained approximately 25% of the activity. Lack of material precluded further

degradation. In contrast, the pyrimidinesulfonic acid (4) derived from L-[3-^{14}C]serine (Expt. 13) contained almost all (~ 90%) of the activity of the original thiamin and the phthalimidothiazole (94) was completely inactive.

The pyrimidinesulfonic acid (4) and the thiazole phenylurethane (92), between them, appeared to contain over 300% of the activity due to ^{14}C found in the thiamin derived from [2-^{3}H,2-^{14}C]glycine (Expt. 1) (Table 3). The major part (~ 275%) was associated with the thiazole derivative (92). Practically no ^{3}H was incorporated into thiamin.

The samples of thiamin, isolated from the [2-^{14}C]glycine feeding experiments (Expts. 7 and 14), were degraded to the pyrimidinesulfonic acid (4) and the thiazole phenylurethane (92). In each case, the thiazole derivative retained more than 98% of the activity of the original thiamin. The remaining 1-2% of activity was located in the pyrimidinesulfonic acid (4). Each of the two samples of the thiazole phenylurethane (92) was further degraded according to Figure 22. Kuhn-Roth oxidation gave acetic acid (C-4,-4') which was assayed as its α-naphthylamide derivative (97), and, in each case, was completely inactive. Carbon-2 was isolated by a modification of the degradation used by Linnett and Walker.[124] Methylation of the thiazole phenylurethane gave the methiodide (95) which, in each case, retained all (99%) of the activity of the original thiamin. Reduction with sodium borohydride and hydrolysis with mercuric chloride gave C-2 as formaldehyde which, in each case, retained the activity (100%) of the original thiamin. Details of these results are summarized in Table 4.

Table 2 Uptake of radioactive tracers[a]

Expt. No.	Substrate	Nominal Total Radioactivity (μCi)	Nominal Specific Radioactivity (mCi/mmol)	Culture Size (ml)	Radioactivity Remaining in Medium (% of Total Radioactivity)
1.	[2-^{3}H,2-^{14}C]Glycine[b]	^{14}C 50 ^{3}H 450	6 9390	250	12 6
2.	Sodium [^{14}C]Formate[b]	50	7	250	11
3.	L-[Me-^{14}C]Methionine[b]	50	14(1.3)[d]	500	28
4.	DL-[2-^{14}C]Tyrosine[c]	50	46	500	56
5.	Sodium [3-^{14}C]Pyruvate[b]	50	19	500	87
6.	D-[1-^{14}C]Ribose[b]	50	50	450	100
7.	[2-^{14}C]Glycine[b]	250	41	1400	9
8.	Sodium [1-^{14}C]Acetate[b]	223	58	1250	13
9.	DL-[3-^{14}C]Cysteine[c]	100	52	700	93
10.	[1-^{14}C]Succinic Acid[b]	100	26	700	71
11.	Sodium 2-keto[5-^{14}C]Glutarate[c]	32	10	450	66
12.	DL-[3-^{14}C]Serine[b]	100	45	700	48
13.	L-[3-^{14}C]Serine[c]	125	56	700	13

Continued.....

Table 2 (Continued)

Expt. No.	Substrate	Nominal Total Radioactivity (μCi)	Nominal Specific Radioactivity (mCi/mmol)	Culture Size (ml)	Radioactivity Remaining in Medium (% of Total Radioactivity)
14.	[2-^{14}C]Glycine[b]	250	41	1400	17
15.	L-[Me-^{14}C]Methionine[b]	250	50(2.5)[d]	1400	23

a Experiments 1-13 were carried out with S. cerevisiae (ATCC 24903). Experiments 14 and 15 were performed with S. cerevisiae (39916 H.J. Bunker).

b New England Nuclear.

c Amersham/Searle.

d Calculated to allow for dilution by DL-methionine (20 mg/litre) present in the culture medium (it is assumed in the calculation that only the L-enantiomer is utilized).

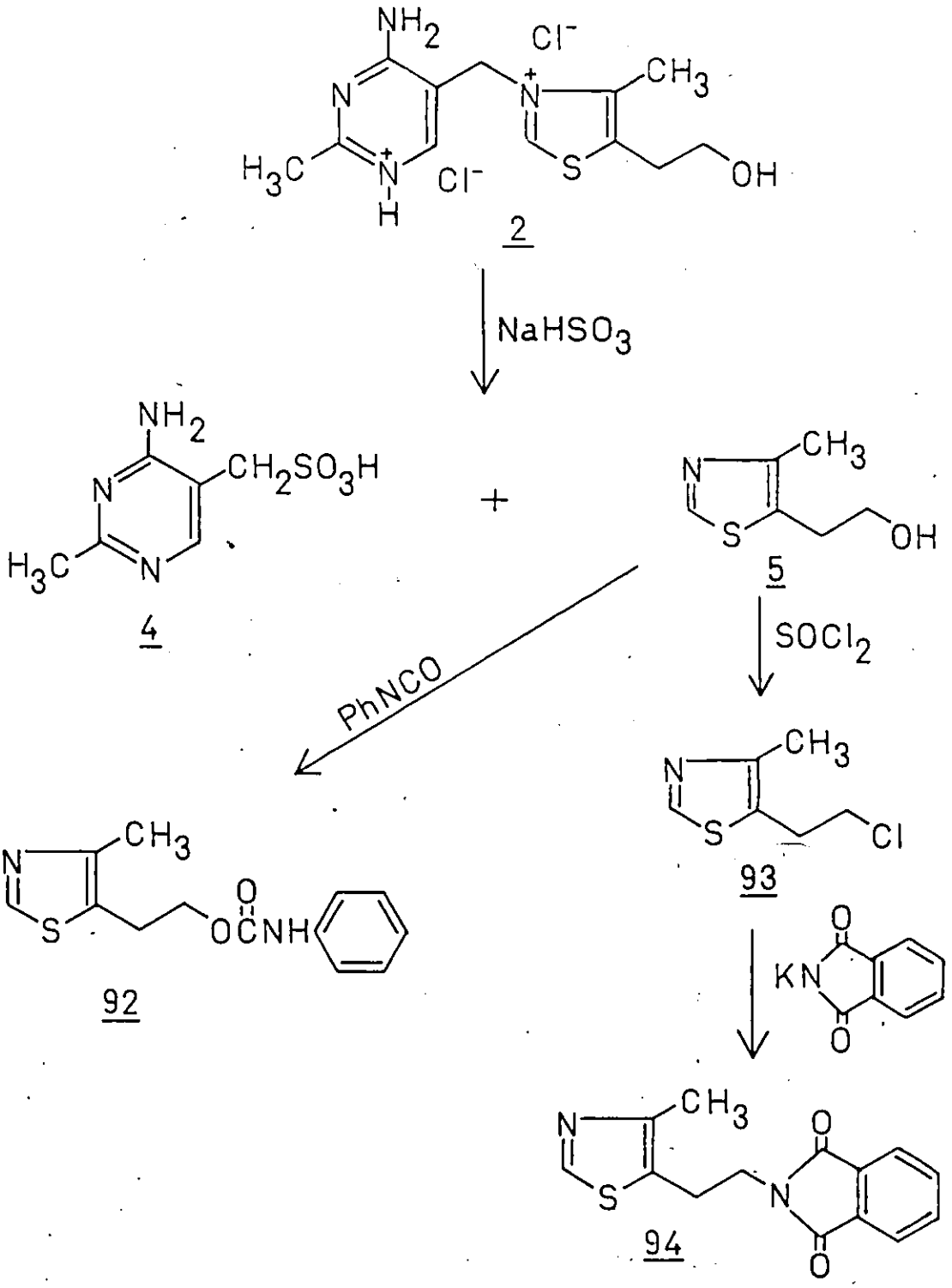


Figure 21: Bisulfite cleavage of thiamin chloride hydrochloride and the formation of solid derivatives of the thiazole moiety.

Table 3 Distribution of ^{14}C from labelled substrates between the pyrimidine and thiazole moieties of thiamin

Expt. No.	Substrate	Product: Thiamin chloride hydrochloride (2) SA[a]	RSA[b]	Pyrimidine-sulfonic acid (4) SA[a]	RSA[b]	Thiazole phenyl-urethane (92) SA[a]	RSA[b]
1.	[2-^{3}H,2-^{14}C]Glycine[c]	2.18 ± 0.03	100 ± 1	0.52 ± 0.01	24 ± 1	6.00 ± 0.03	275 ± 4
2.	Sodium [^{14}C]Formate	2.60 ± 0.02	100 ± 1	0.74 ± 0.01	28 ± 1	0.01 ± 0.01	0 ± 1
4.	DL-[2-^{14}C]Tyrosine	0.34 ± 0.02	100 ± 6	0.02 ± 0.02	12 ± 6	0.01 ± 0.02	3 ± 6
5.	Sodium [3-^{14}C]Pyruvate	0.08 ± 0.01	---	---	---	---	---
8.	Sodium [1-^{14}C]Acetate	1.31 ± 0.02	100 ± 2	0.00 ± 0.01	0 ± 1	0.00 ± 0.01	0 ± 1
10.	[1-^{14}C]Succinic Acid	0.00 ± 0.02	---	---	---	---	---
12.	DL-[3-^{14}C]Serine	0.72 ± 0.02	100 ± 3	0.18 ± 0.01	25 ± 2	0.63 ± 0.02	88 ± 4
13.	L-[3-^{14}C]Serine	0.28 ± 0.01	100 ± 5	0.24 ± 0.01	86 ± 5	0.00 ± 0.01[d]	0 ± 4

a Specific activity (dpm $mmol^{-1}$) x 10^{-4}

b Relative specific activity (%) (thiamin chloride hydrochloride = 100)

c ^{14}C results only

d Assayed as 4-methyl-5-(β-phthalimidoethyl)thiazole (94)

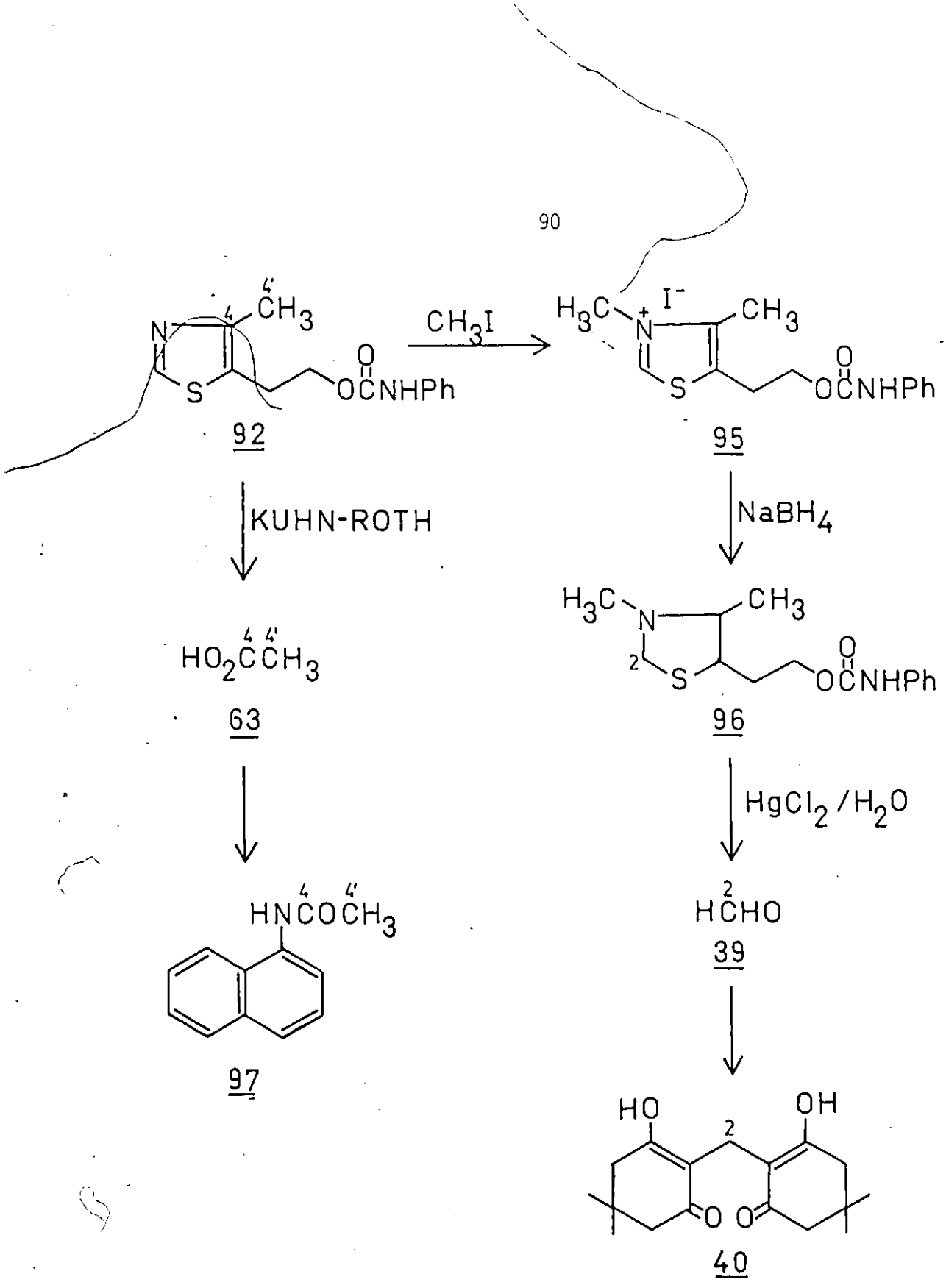


Figure 22: Degradation of the thiazole phenylurethane (92) to obtain C-4,-4' as acetic acid and C-2 as formaldehyde.

Table 4 Distribution of ^{14}C from [2-^{14}C]glycine and L-[Me-^{14}C]methionine in thiamin

		S. cerevisiae strain			
		ATCC 24903		39916 H.J. Bunker	
		SA[a]	RSA[b]	SA[a]	RSA[b]
[2-^{14}C]Glycine	Expt. No.	7		14	
Thiamin chloride hydrochloride (2)		3.99 ± 0.04	100 ± 1	2.05 ± 0.08	100 ± 4
Pyrimidinesulfonic acid (4)		0.08 ± 0.01	2 ± 0.02	0.02 ± 0.01	1 ± 1
Thiazole phenylurethane (92)		3.91 ± 0.05	98 ± 2	2.08 ± 0.03	101 ± 4
Thiazole phenylurethane methiodide (95)		---	---	2.02 ± 0.04	99 ± 4
Kuhn-Roth acetic acid (as α-naphthylamide, 97)		0.00 ± 0.02	0 ± 1	0.00 ± 0.02	0.1 ± 0.1
Formaldehyde (as dimethone, 40)		3.94 ± 0.03	99 ± 1	2.06 ± 0.03	101 ± 4
L-[Me-^{14}C]Methionine	Expt. No.	3		15	
Thiamin chloride hydrochloride (2)		22.1 ± 0.2	100 ± 1	0.30 ± 0.03	100 ± 10
Pyrimidinesulfonic acid (4)		0.31 ± 0.01	1 ± 0.1	0.01 ± 0.01	4 ± 4
Thiazole phenylurethane (92)		0.03 ± 0.02	0.1 ± 0.1	0.01 ± 0.03	2 ± 8

[a] Specific activity (dpm $mmol^{-1}$) x 10^{-4}

[b] Relative specific activity (%) (thiamin chloride hydrochloride = 100)

(iv) Discussion

(a) Difficulties in the investigation of biosynthesis by radioactive tracers

A definitive biosynthetic investigation, which demands isolation and purification to constant radioactivity, followed by the preparation of derivatives and by chemical degradation to locate the site or sites of radioactivity, requires at least 10 mg of compound (or more, depending on the complexity of the degradation sequence). Since the scale of a radioactive-tracer experiment (volume of culture, or number of plants, per experiment) is limited by experimental facilities, it will be necessary to dilute the target compound 10^2-10^3 fold with inactive carrier, to obtain sufficient quantities of radioactively labelled material. Thus, the success of a biosynthetic tracer experiment is predicated on the selection of conditions, such that the unweighable amount (1/10 μmole or less) of target compound produced during the experimental period has a specific molar radioactivity high enough so that after dilution by a factor of 10^2-10^3 the isolated product still has a significant countrate (dpm per mg above background).

Some of the conditions required to enhance the likelihood of obtaining such a result are not under the control of the investigator: the substrate to be tested as precursor may not be available at high specific activity, and much of it may be dissipated in metabolic processes which, from the point of view of the investigation, are irrelevant and undesirable. Alternatively, the substrate may not be metabolized because membrane impermeability may prevent the transport of the substrate into the cell. The amount of target material biosynthesized during the experimental period may be less than the amount present endogenously at the start of the ex-

periment. Thus, the activity of the product, even after minimal dilution with carrier, may be at the borderline of detectability.

Another problem inherent in the handling of small quantities of labelled compounds is the presence of unweighable amounts of radioactive impurities in the product. These may not be detectable unless derivatives are prepared, but lack of material may preclude the chemical manipulation required for derivatization and degradation.

In view of these difficulties, it is understandable that many of the tracer investigations whose objective it was to identify the primary precursors of the thiazole moiety of thiamin were not pursued to a stage when definitive conclusions could be drawn. In some cases, the target compound (in this case 5-(β-hydroxyethyl)-4-methylthiazole) was not isolated, but it was assumed that a fraction eluted from a chromatogram which contained the desired product did not contain any other radioactive compound.[128,145] Alternatively, the desired compound was isolated, but no derivatives were prepared and no degradation attempted, and it was assumed that the isolated product was free of radiochemical contaminants.[129]

Results of such incomplete experiments, taken at face value, are often likely to lead to erroneous inferences.

Since it is difficult to gauge the reliability of the experimental data obtained in such incomplete investigations which lack internal checks, it is well nigh impossible to assess what credence should be given to the interpretation of their data offered by the investigators who report them. The problem is compounded by the fact that no two schools used the same strain of micro-organism for their experiments.

One series of investigations which appears to be well-designed

and thoroughly executed is that of Linnett and Walker.[124,125,134] They employed a strain of S. cerevisiae (NCYC 1062) for tracer experiments with [2-^{14}C]glycine and L-[Me-^{14}C]methionine. It was demonstrated by derivatization and degradation of the isolated thiamin that [Me-^{14}C]-methionine did not serve as a precursor, but that [2-^{14}C]glycine did, and that the entire activity of the thiamin, derived from this substrate, was present in the thiazole nucleus (isolated as the picrate of 5-(β-hydroxyethyl)-4-methylthiazole) and localized at C-2 (isolated as formaldehyde dimethone, after controlled degradation). Furthermore, heavy nitrogen from [^{15}N]glycine was located exclusively in the thiazole moiety. It can be concluded from these results that the methylene carbon atom of glycine serves as the source of C-2 of the thiazole moiety of thiamin, and it is more than likely that the C-N fragment, derived from glycine by decarboxylation, enters the thiazole moiety as a unit.

These results and their interpretation are in direct conflict with the work of Johnson et al.[128,130] These authors carried out feeding experiments with [Me-^{14}C]methionine and [Me-^{14}C,^{35}S]methionine in another strain of S. cerevisiae (39916 H.J. Bunker). They isolated thiamin, degraded it into the pyrimidine and thiazole moieties and recovered a chromatographic fraction containing the thiazole, but did not isolate, derivatize or degrade the compound. The thiazole fraction obtained in the two experiments contained, respectively, ^{14}C, and ^{14}C and ^{35}S, with a ^{35}S/^{14}C ratio identical with that of the precursor methionine. The authors concluded that the C-S unit, derived from the S-methyl group of methionine, was incorporated intact into the thiazole moiety, yielding the unit S-C-2. This result is widely quoted[59,60,65,222] and serves as

the basis for the accepted hypothesis of thiamin biosynthesis. It is considered to provide experimental support for the biogenetic ideas of Sir Charles Harington.[135]

Recent reviews of thiamin biosynthesis[59,60] summarize the experiments with yeast, as well as other investigations with bacteria,[143,147] but do not attempt a critical evaluation of the contradictory conclusions of Linnett and Walker[124] and of Johnson et al.[128] with regard to the biosynthesis of the thiazole nucleus of thiamin in yeast.

(b) Re-investigation of reported precursors

As a first step in the re-investigation of the problem, a number of the compounds suggested previously as precursors of thiamin were incubated with S. cerevisiae, and thiamin chloride hydrochloride which contained appreciable amounts of radioactivity after five or more recrystallizations was isolated. In addition, the samples appeared to be at constant specific activity. Such results imply that the radioactivity from the tracer was incorporated into thiamin and that the compounds fed were thus precursors of thiamin.

Ideally, the radiochemical purity of the isolated compound should be checked. Usually this can be carried out by the preparation of derivatives, but the bisulfite cleavage of thiamin offers an alternative and better method. Because the carbon atoms of thiamin chloride hydrochloride are all recovered in the pyrimidine (4) and thiazole (5) derivatives from the cleavage reaction, the sum of the specific activities of the two components should be identical with that of the original thiamin. Thus, the bisulfite cleavage serves not only as a check on the radiochemical purity

of thiamin, but also indicates the distribution of the label between the two moieties which comprise the vitamin.

The thiazole derivative (5) obtained from the bisulfite cleavage of thiamin chloride hydrochloride (2) (Figure 21) is an oil at room temperature. Small amounts of a liquid are difficult to purify and to separate from solvent and thus cannot be accurately weighed for assay of radioactivity. In the past, this obstacle has been overcome either by the assay of a solution of the thiazole (5),[128,129,143] or by the formation of a quaternary nitrogen salt.[124] Both the hydrochloride and methiodide salts of the thiazole (5) are hygroscopic and therefore cannot be accurately weighed. The picric acid salt of 5 is more suitable in this regard, but it is highly coloured and thus unsuitable for liquid scintillation counting.

Since a solid derivative of the thiazole (5) was required for rigorous purification by crystallization and sublimation, and the nitrogen derivatives were unsuitable, derivatives of the alcoholic function were preferred. The formation of the trityl ether and the α-naphthylurethane derivatives was attempted. However, a non-crystalline product was obtained for the former and the latter derivative was very insoluble, did not sublime and was difficult to crystallize. By treatment of the thiazole (5) with phenylisocyanate, the phenylurethane (92) (Figure 21) was obtained. This compound was colourless and easily purified by crystallization and sublimation. The phthalimidothiazole (94) was also found to be a suitable derivative. It was formed by treatment of the thiazole (5) with thionyl chloride, followed by reaction of the resulting β-chloroethylthiazole (93) with potassium phthalimide (Figure 21).

The activity associated with the thiamin chloride hydrochloride

could be due either to the incorporation of the labelled substance or to the carry-over of a radioactive impurity. For a proper interpretation, a distinction has to be made between these possibilities. It is unlikely that an impurity would be carried over *in toto* from thiamin into its two fragments and into the derivative of one of these fragments. The sum of the specific activities of the pyrimidinesulfonic acid and one or the other of the thiazole derivatives is thus a more reliable index of the specific activity of the isolated thiamin than the specific activity of the thiamin chloride hydrochloride itself.

One of the compounds whose involvement in thiamin biosynthesis is well documented is sodium [^{14}C]formate. It is a precursor of the pyrimidine moiety of thiamin,[126-129,192,198] and one report suggested that it was incorporated into the thiazole moiety.[124] The incorporation of formate is generally reported to be greater than that of most other substrates. For this reason, and to settle the question of whether or not formate is incorporated into the thiazole moiety, a trial incubation with sodium [^{14}C]formate was carried out (Expt. 2) (Table 2). Radioactive thiamin chloride hydrochloride was isolated from this experiment (Table 3) and degraded as shown in Figure 21. No activity was found in the thiazole phenylurethane (92) and only about 30% of the activity associated with the original thiamin was recovered in the pyrimidinesulfonic acid (4). Most of the radioactivity associated with the thiamin was therefore caused by small amounts of highly radioactive impurities. Further degradations to locate the exact site of labelling were not performed on the pyrimidine derivative due to lack of material. This experiment would have to be repeated to confirm that the activity found in the pyrimidinesulfonic acid (4) was due to the in-

corporation of formate and not to an impurity.

As a further preliminary step in the re-investigation of the origin of the thiazole moiety of thiamin, the apparently contradictory reports of incorporation of the methyl group of methionine and of the α-carbon atom of glycine, respectively, into C-2 of the thiazole nucleus were re-examined.

An incubation was carried out with [2-^{3}H,2-^{14}C]glycine (Expt. 1) (Table 2) and radioactivity (due to ^{14}C) was found to be associated with the isolated thiamin chloride hydrochloride (Table 3). However, no tritium was incorporated into the thiamin molecule. Either the tritium was lost en route to thiamin or it was incorporated into an exchangeable position of thiamin and lost during work up. It follows from the conclusions of Linnett and Walker,[124] that tritium on C-2 of glycine should be incorporated into thiamin and would be located at C-2 of the thiazole ring. However, Breslow[31] found that the proton at this position readily exchanges in water and the evidence presented in Chapter I (section i) indicates that this is the active site of the coenzyme, thiamin pyrophosphate. Thus, tritium would not be expected to be maintained at this position. A small amount (∿ 25%) of the ^{14}C activity of thiamin was recovered in the pyrimidinesulfonic acid (4) after bisulfite cleavage (Figure 21). The thiazole phenylurethane (92) had a specific activity almost three times that of the thiamin chloride hydrochloride (2). Under the assumption that the thiazole derivative was pure, the thiamin chloride hydrochloride must have been contaminated with a large quantity of inactive material.

There was insufficient material for further degradation, but since

the above results suggested that [2-^{14}C]glycine might be a precursor of the thiazole moiety, as reported by Linnett and Walker,[124] the experiment with glycine was repeated.

A larger culture size and [2-^{14}C]glycine with a higher specific activity were employed to increase the radioactivity associated with the isolated thiamin sufficiently so as to permit complete degradation. Samples of thiamin chloride hydrochloride, isolated by carrier dilution from the cells of two strains of S. cerevisiae which had been incubated in the presence of [2-^{14}C]glycine (Expts. 7 and 14) (Table 2), were crystallized to constant radioactivity and degraded (Figures 21 and 22) to locate the site of activity (Table 4). Bisulfite cleavage yielded the pyrimidine-sulfonic acid (4) which was non-radioactive and 5-(β-hydroxyethyl)-4-methylthiazole (5) which was converted to the crystalline phenylurethane (92) and the corresponding methiodide (95), and contained all the activity of the intact vitamin. Kuhn-Roth oxidation of the phenylurethane (92) gave acetic acid (C-4,-4' of the thiazole moiety) (isolated as the α-naphthylamide). This was not radioactive. The methiodide (95) was reduced to yield the tetrahydro derivative (96) which was not isolated but directly hydrolyzed to yield formaldehyde (C-2 of the thiazole moiety) (isolated as the dimethone) which contained all activity of the thiazole nucleus and of the intact vitamin. The methylene carbon of glycine thus serves as the progenitor of C-2 of the thiazole nucleus of thiamin and of no other C atom in S. cerevisiae (ATCC 24903 and 39916 H.J. Bunker). The same results were obtained in a third strain of S. cerevisiae (NCYC 1062) by Linnett and Walker.[124]

The reported incorporation of the methyl group of methionine was also re-investigated. Incubation of each of the two strains of S. cerevisiae (ATCC 24903 and 39916 H.J.Bunker) with L-[Me-^{14}C]methionine (Expts. 3 and 15) (Table 2) also yielded radioactive samples of thiamin chloride hydrochloride. Five recrystallizations were required in the case of these samples to reach constant activity. However, when these samples of thiamin chloride hydrochloride were subjected to bisulfite cleavage (Table 4), the two degradation fragments, the pyrimidinesulfonic acid (4) and the thiazole (5) (isolated as phenylurethane (92)), did not contain radioactivity in either case.

Since these two fragments account for all carbon atoms of the original thiamin chloride hydrochloride, it is clear that the radioactivity associated with the samples of the vitamin, isolated from experiments 3 and 15, must have been due to contamination by traces of a highly radioactive impurity which persisted despite the many purification steps. This conclusion leads us to surmise that the activity found by Johnson et al.[128] to be associated with the chromatographic fraction containing the thiazole moiety, derived from cultures of S. cerevisiae (39916 H.J. Bunker), incubated with [Me-^{14}C]- and [Me-^{14}C,^{35}S]methionine, was also due to the presence of traces of a highly radioactive impurity. If this impurity were methionine itself, or a breakdown product which still retains the -S-CH_3 function, maintenance of the $^{35}S/^{14}C$ ratio of the precursor in the contaminated but non-radioactive product would be explained.

The culture conditions in our own tracer experiments with S. cerevisiae (39916 H.J. Bunker) were somewhat different from those of Johnson et al.[128] Their experiments were carried out on a medium con-

taining yeast extract, rich in thiamin, and tracer was administered 24 h after inoculation. In our own experiments, a thiamin-free medium was used, and tracer was added 15.5 h after inoculation.

As is shown in Figure 23 (curve C), the log phase is complete after approximately 20 h incubation of S. cerevisiae (39916 H.J. Bunker) on a yeast extract medium. It is stated[59] that "de novo formation of thiamin cannot be demonstrated in non-growing cell suspensions under normal circumstances". Furthermore, it has been noted in bacteria[223] that "the enzymes for synthesizing thiamin appear to be labile and activity is completely lost after 3 h if growth is stopped". Johnson et al.[128] supplied tracer to their cultures well after logarithmic growth was complete and the culture had reached the stationary phase. It would appear that in the experiments reported by Johnson et al.[128] biosynthesis of thiamin did not take place during the time the cells were in contact with the tracer. In these experiments, a culture medium was employed which contained yeast extract (2 g per ℓ). Yeast extract contains thiamin (ca. 30-300 μg/g).

As mentioned earlier in this chapter, it is known[212-216] that S. cerevisiae accumulates thiamin from the medium on which it is grown. Furthermore,[59,101] the level of thiamin phosphate pyrophosphorylase, the enzyme responsible for the synthesis of thiamin monophosphate from the phosphorylated thiazole and pyrimidine moieties, present in commercial yeast with added thiamin, is less than 15% of that found in the same yeast produced without exogenous thiamin. The formation of the enzymes necessary to join the pyrimidine and the thiazole moieties in bacteria is also

inhibited when thiamin is present in the growth medium.[223] It seems unlikely, therefore, that significant de novo biosynthesis of thiamin takes place in yeast cells which are grown in the presence of exogenous thiamin.

The present experiments with S. cerevisiae (39916 H.J. Bunker) were carried out using a thiamin-free medium. Under these conditions (curve B, Figure 23) growth is slower and a longer lag-phase is observed than in the presence of yeast extract (curve C, Figure 23), but approximately the same cell density is reached at completion of the log phase. The addition of pyridoxal or nicotinic acid to thiamin-free glucose medium had no effect on the growth of S. cerevisiae (39916 H.J. Bunker) (curve B, Figure 24). Addition of a small amount of thiamin, however, resulted in a marked decrease in the length of the lag phase (curve A, Figure 24), which suggested that S. cerevisiae (39916 H.J. Bunker) when placed in thiamin-free medium requires some time before it can start to synthesize the vitamin. Tracer was added after 15.5 h of incubation, at the onset of active growth. Conditions for thiamin biosynthesis were thus favourable.

In summary, the results of Linnett and Walker[124,125,134] and the present work establish that, in three strains of yeast (S. cerevisiae NCYC 1062, ATCC 24903, and H.J. Bunker 39916), the methylene carbon atom of glycine serves as the biosynthetic source of C-2 of the thiazole nucleus of thiamin. Furthermore, it is likely, on the basis of the results of Linnett and Walker,[134] that an intact C-N unit, derived from glycine enters the thiazole nucleus.

(c) The origin of the C-2-N unit in bacteria

Carbon-2 of the thiazole moiety does not appear to originate from methionine in bacteria, although L-[Me-^{14}C]methionine has been reported to be incorporated into C-2 of the thiazole moiety in B. subtilis.[141] A degradation was carried out and most of the activity was reported to be at C-2, but no experimental details were given for this degradation. The enteric bacteria S. typhimurium[143] and E. coli[133] were also incubated in the presence of L-[Me-^{14}C]methionine, but no incorporation into thiamin was obtained. The evidence for methionine as a precursor of thiamin in bacteria is not strong and the role of methionine in these systems awaits clarification.

Glycine was found to replace the 5-(β-hydroxyethyl)-4-methylthiazole growth requirement of a mutant of E. coli,[144] and [U-^{14}C]glycine was reported to be incorporated into the thiazole moiety of thiamin in this mutant.[145] The activity associated with the thiazole, however, was not measured directly. The mutant produced an excess of the pyrimidine moiety and excreted it into the medium. The specific activities of both thiamin and the pyrimidine moiety were determined. The specific activity of thiamin was higher than that of the pyrimidine moiety, and it was assumed that the missing activity was located in the thiazole moiety. [2-^{14}C]Glycine was not incorporated into thiamin by S. typhimurium[143] and it has not been tested in B. subtilis. All other incubations performed with E. coli were done on mutants which only produced the pyrimidine moiety[196,198] or the incorporation into thiazole was not mentioned.[180]

Thus, a source other than glycine would appear to supply the C-2-N unit of the thiazole nucleus. In E. coli[147] and S. typhimurium,[143]

the α-carbon of tyrosine appears to supply C-2 of the thiazole unit. Each of these results is based on chemical degradation of the thiazole unit (Figure 6) and on isolation of C-2 as formaldehyde. In addition, ^{15}N from [^{15}N]tyrosine was incorporated into the thiazole moiety.[148] Although active thiamin chloride hydrochloride (2) (Table 3) was isolated from S. cerevisiae (ATCC 24903) (Expt. 4) incubated with DL-[2-^{14}C]-tyrosine, only a small percentage of the radioactivity was recovered in the pyrimidine (4) and thiazole (92) derivatives. Tyrosine does not serve as a precursor in this organism.

The incorporation of both tyrosine and glycine would be explained either if tyrosine were degraded to glycine or if glycine were used to synthesize tyrosine. However, the lack of incorporation into thiazole of tyrosine in yeast,[209] and the lack of incorporation of glycine in S. typhimurium[143] rule out the above possibilities. Since both the tyrosine[143,147,148] and glycine[124,209] results are reproducible in enteric bacteria and yeast, respectively, it is unlikely that these results are in error. The above results lead to the conclusion that the C-2-N unit of the thiazole moiety is supplied by glycine in yeast and by tyrosine in enteric bacteria. Before B. subtilis can be classified in this manner, the origin of C-2 of the thiazole unit would have to be re-investigated in this organism.

(d) The origin of the C_5 unit: a re-investigation of published concepts

No direct experimental data are available concerning the origin of the C_5 unit, either in bacteria or in yeast. Suggestions that have been made are based solely on incorporation measurements, or are purely

speculative. A number of incubations were carried out to check the validity of these ideas in S. cerevisiae (ATCC 24903).

Acetate has been reported as a precursor of both the pyrimidine[128,129,190,192] and the thiazole moieties.[128,129] A polyketide-type hypothesis can also be suggested for the origin of the C_5 unit. The thiamin chloride hydrochloride isolated from S. cerevisiae (ATCC 24903) incubated with sodium [1-^{14}C]acetate (Expt. 8) was radioactive (Table 3). However, none of the radioactivity was recovered either in the pyrimidine (4) or in the thiazole (92) degradation products. Thus, C-1 of acetate does not serve as a precursor of any of the carbon atoms of thiamin.

Parada and Ortega[149] had suggested that the C_5 unit of the thiazole moiety was formed from a pentose-like compound. In agreement with a previous report,[125] D-[1-^{14}C]ribose (Expt. 6) was not incorporated into thiamin and all of the activity fed was recovered in the non-cellular portion of the culture (Table 2). This indicates either that the labelled substance was not taken into the cell, or that the substance was taken up but not metabolized, or that it was taken up, and metabolized and the product was excreted. Measurement of the radioactivity located in the non-cellular portion of the culture cannot distinguish among these possibilities. D-Xylose is known to enter the S. cerevisiae cell but the yeast cannot use this sugar as a substrate for growth.[224] This is due to the lack of a specific kinase, dehydrogenase or enzymes that make possible its access to the common pentose phosphate pathway.[225] Thus, ribose may have been taken into the cell but it seems very likely that it was not incorporated into the main metabolic pathways. Under these

conditions, no incorporation would be expected.

Parada and Ortega[149] also suggested that this pentose-like compound might be formed from acetaldehyde and a C_3 unit by means of an aldolase- or trans-aldolase-type reaction. The intermediacy of acetaldehyde or "active acetaldehyde" was tested in the incubation of S. cerevisiae (ATCC 24903) with their precursor sodium [3-^{14}C]pyruvate (Expt. 5). Again, most of the radioactivity was recovered in the non-cellular part of the culture (Table 2), and thiamin chloride hydrochloride with only a low specific activity was isolated (Table 3).

Glutamic acid, a five-carbon amino acid, has been suggested (Route C, Figure 5) as the precursor of the C_5 unit. Incubations carried out with sodium 2-keto[5-^{14}C]glutarate (Expt. 11) and the Krebs' cycle intermediate, [1-^{14}C]succinic acid (Expt. 10) failed to yield active thiamin. Approximately 70% of the activity in each experiment was recovered in the non-cellular portion of the culture (Table 2).

That permeability of the plasma membrane is a significant factor influencing the metabolism of organic acids by yeast cells was first indicated in 1926.[226] Organic acids are known to penetrate the yeast cell by the diffusion of their undissociated forms.[214,227] The uptake of these acids would depend on the pK_a of the acid and on the pH of the medium. Thus, permeation of the membrane by pyruvate is slow in comparison to that of acetic and propionic acid, and di- and tricarboxylic acids penetrate the intact yeast cell with difficulty if at all. In fact, the poor penetrability of di- and tricarboxylic acids makes them usable in the preparation of buffer solutions. The uptake of pyruvate by Rhodotorula glutinis is also inhibited by the presence of glucose.[228]

It is very likely that these compounds were not taken up by the cell in sufficient quantities to give incorporation into thiamin and the uptake which was observed may not have been at the time of thiamin biosynthesis. As the incubation progresses, the pH of the medium and the level of glucose drop and conditions for the uptake of these acids become more favourable. However, thiamin biosynthesis may be complete at this stage of the incubation.

The carbon skeleton of cysteine has been proposed as a building block of the thiazole moiety (Routes B and C, Figure 5). DL-[3-^{14}C]-Cysteine (Expt. 9) was not taken up by S. cerevisiae (ATCC 24903) (Table 2). In an attempt to get around this problem, S. cerevisiae (ATCC 24903) was incubated with DL-[3-^{14}C]serine (Expt. 12), and radioactive thiamin chloride hydrochloride was isolated (Table 3). Degradation to the pyrimidinesulfonic acid (4) and the thiazole phenylurethane (92) led to a recovery of 113% of the activity present in the original thiamin. The major portion (88%) of this label was contained in the thiazole portion but insufficient material precluded further degradation. This incorporation of activity into the thiazole portion was encouraging, and a second experiment with L-[3-^{14}C]serine (Expt. 13) was carried out in the hope of isolating sufficient activity to allow the position of label to be determined. Radioactive thiamin chloride hydrochloride was isolated (Table 3), but a large proportion of this activity was lost on crystallization with additional carrier. Degradation gave the pyrimidinesulfonic acid (4) which retained almost all ($\sim$ 90%) of the activity present in the original thiamin. The phthalimidothiazole derivative (94) was completely inactive. Thus, serine was not a precursor of the thiazole

unit and the activity found with DL-serine (Expt. 12) could have been due to a radioactive impurity or to metabolism of D-serine. Serine dehydratases which convert D-serine to pyruvate are known,[229-232] but the presence of this enzyme in yeast has not been described. Furthermore, the recovery of radioactivity in the non-cellular portion of the culture (DL-serine, 48%, and L-serine, 13%) suggests that only the L-enantiomer is taken into the cell. Also, it has been reported[125] that DL-[3-^{14}C]serine is not incorporated into the thiazole moiety of thiamin by S. cerevisiae (NCYC 1062). The radioactivity associated with the thiazole phenylurethane in Expt. 12 was probably due to the presence of a radioactive impurity.

Experiments based on published concepts thus did not yield results which promised to offer a lead to the origin of the C_5 unit. Some of the above experiments may not have served as true indicators of the validity of these ideas, since in some instances the tracers were not absorbed during the course of the incubation.

Nevertheless, new ideas, and an approach different from that suggested by earlier investigators were evidently necessary if progress in the investigation was to be made.

(v) Experimental

(a) Micro-organisms

Two strains of Saccharomyces cerevisiae were used in radioactive tracer experiments.

S. cerevisiae (ATCC 24903) was obtained from Dr. J.J. Miller, Department of Biology, McMaster University. Stock cultures were maintained on malt extract-yeast extract-peptone-glucose (MYPG) slants which,

after incubation at 27 ± 1°C for 24 hours, were stored at 4°C and subcultured at periodic intervals.

S. cerevisiae (39916 H.J. Bunker) was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. After incubation at 27 ± 1°C for 48 hours on Sabouraud agar slopes, the stock culture was stored at 4°C and subcultured every two weeks.

Two other strains of micro-organisms were used to obtain a comparison of thiamin content.

S. cerevisiae (ATCC 4134) was obtained from The American Type Culture Collection, Rockville, Maryland, U.S.A. Stock cultures were maintained on MYPG slants using the same conditions as for S. cerevisiae (ATCC 24903).

A 1 litre culture of Bacillus subtilis 19E was supplied by Dr. I. Takahashi; Department of Biology, McMaster University. It had been grown for 1.5 to 2 days on a minimal medium (ingredients per litre: $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 14 g; KH_2PO_4, 6 g; sodium citrate, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; 50 ml of 10% glucose added after sterilization).

(b) Media

Maintenance Media:

(i) The composition of malt extract/yeast extract/peptone/glucose medium (MYPG) was as follows:[233] Difco malt extract (3 g), Difco yeast extract (3 g), Difco peptone (5 g), D-glucose (20 g), KH_2PO_4 (1 g), Difco Bacto-agar (20 g), water (1 litre).

(ii) The composition of Sabouraud agar medium[234] was: Difco neopeptone (10 g), D-glucose (40 g), Difco Bacto-agar (15 g), and water (1 litre).

Liquid culture media:

(i) Thiamin-free glucose medium was prepared from Difco Bacto vitamin-free yeast base and supplemented with myo-inositol (2000 µg/litre), calcium DL-pantothenate (400 µg/litre) and D-biotin (2 µg/litre). The composition of Difco Bacto vitamin-free yeast base[234] is: $(NH_4)_2SO_4$ (5 g), Bacto-dextrose (10 g), L-histidine monohydrochloride (10 mg), DL-methionine (20 mg), DL-tryptophan (20 mg), boric acid (500 µg), $CuSO_4$ (40 µg), KI (100 µg), $FeCl_3$ (200 µg), $MnSO_4$ (400 µg), Na_2MoO_4 (200 µg), $ZnSO_4$ (400 µg), KH_2PO_4 (1 g), $MgSO_4$ (0.5 g), NaCl (0.1 g), $CaCl_2$ (0.1 g) and water (1 litre).

(ii) Yeast extract medium[128] had the following composition: Difco yeast extract (2 g), D-glucose (20 g), $(NH_4)_2SO_4$ (0.943 g), KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.3 g), and water (1 litre).

Preparation of the cell inoculum

A fresh slant of yeast cells was prepared by inoculation from a stock culture and incubated for either 24 h (S.cerevisiae, ATCC 24903) or 48 h (S. cerevisiae, 39916 H.J. Bunker) at 27 ± 1°C. The cells were washed with sterilized water (3 x 25 ml). A portion of this cell suspension (0.5 ml, i.e., approx. 10^7 cells) was used to inoculate a liquid medium prepared by one of the following methods.

(c) Growth on thiamin-free glucose medium

Procedure a: Difco Bacto vitamin-free yeast base (16.7 g) was dissolved in water (1 litre). To give the required vitamin concentration, a stock vitamin solution (1.0 ml) (containing myo-inositol, 2000 µg/ml; calcium DL-pantothenate, 400 µg/ml; D-biotin, 2 µg/ml) was added and the medium was sterilized in 50 ml portions by filtration through Nalgene 0.20 µ disposable filter units. Each 50 ml portion was transferred asepti-

cally to a sterilized 250 ml Erlenmeyer flask.

Procedure b: Difco Bacto vitamin-free yeast base (16.7 g) was dissolved in water (100 ml) and stock vitamin solution (1.0 ml) was added. The entire medium was sterilized by filtration through an autoclaved membrane-filter holder (Sartorius GmbH Sm 16510) fitted with a membrane filter (Gelman GN-6, 0.45 μm pore diameter; Metricel, 47 mm diameter). Portions of the sterilized medium (5 ml) were then transferred aseptically by pipette to 250 ml Erlenmeyer flasks containing sterilized water (45 ml).

After inoculation from the cell suspension, the cultures were incubated with shaking (100 strokes/min, i.e., maximum aeration) at 27 ± 1°C (Warner-Chilcott Laboratories model 2156 water-bath shaker).

After a short lag phase (2-3 h), S. cerevisiae (ATCC 24903) maintained logarithmic growth for 19 h of incubation, when a cell density of approx. 8×10^7 cells/ml was attained (curve A, Figure 23). A longer lag phase (7-8 h) was observed in the case of S. cerevisiae (39916 H.J. Bunker). Logarithmic growth of this strain was slow, continuing for 48 h of incubation. A cell density of approx. 4×10^7 cells/ml was eventually attained (curve B, Figure 23).

(d) Growth on yeast-extract medium[128]

The yeast extract medium was dispensed into Roux flasks (75 ml/flask), which were then plugged with non-absorbent cotton wool and autoclaved at 15 lb/in^2 (103.5 kPa) for 25 min. One flask was chosen and inoculated with a loopful of S. cerevisiae (39916 H.J. Bunker) from a Sabouraud agar slant (grown at 27 ± 1°C for 48 h) and incubated at 27 ± 1°C for 24 h. A sample of this culture (0.5 ml) was used to

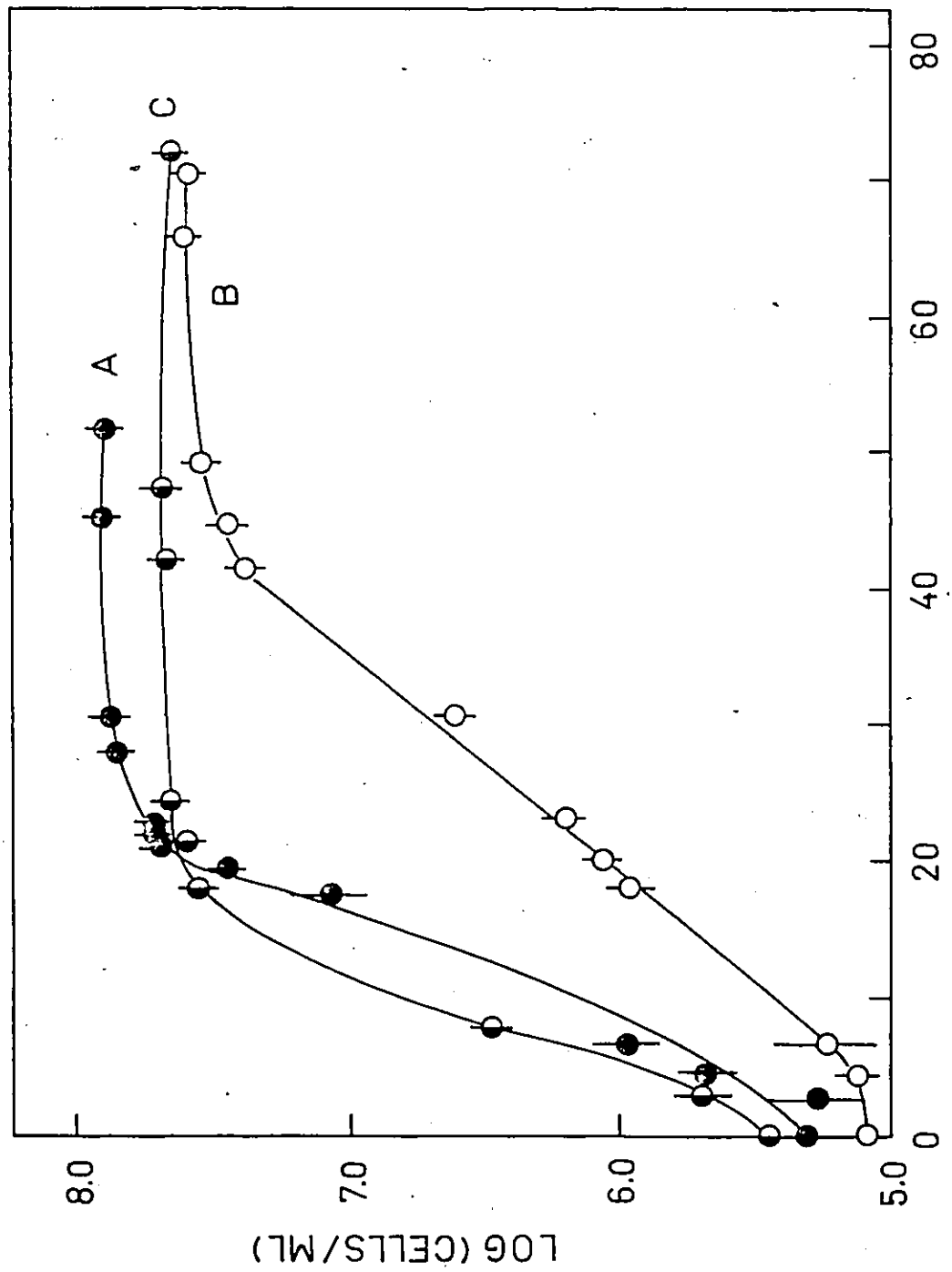


Figure 23: Growth of S. cerevisiae (ATCC 24903) in thiamin-free medium (●, curve A) and of S. cerevisiae (39916 H.J. Bunker) in thiamin-free medium (○, curve B) and yeast-extract medium (◐, curve C).

inoculate the remaining Roux flasks, which were incubated for 48 h at 27 ± 1°C. Growth of S. cerevisiae (39916 H.J. Bunker) on yeast-extract medium was not delayed and was rapid with the logarithmic phase terminating at 20 h after inoculation (curve C, Figure 23).

Growth on thiamin-free glucose medium supplemented with either nicotinic acid, pyridoxol, or thiamin

Thiamin-free glucose medium was prepared as described in procedure b. Separate solutions of nicotinic acid (20 µg/ml), pyridoxol hydrochloride (20 µg/ml) and thiamin chloride hydrochloride (20 µg/ml) were sterilized by filtration through an autoclaved membrane-filter holder fitted with a membrane filter (0.45 µm pore diameter). Portions of these solutions (1.0 ml, 20 µg, or 0.1 ml, 2 µg) were transferred aseptically to separate culture flasks. After inoculation from a cell suspension of S. cerevisiae (39916 H.J. Bunker), the cultures were incubated at 27 ± 1°C on a water-bath shaker. The growth of S. cerevisiae (39916 H.J. Bunker) was not stimulated by added nicotinic acid or pyridoxol at either concentration (curve B, Figure 24). However, thiamin at either concentration caused a marked shortening of the lag phase (curve A, Figure 24).

(e) Growth curve measurements

A portion of the yeast culture was removed aseptically at varying intervals of time and was, depending on the cell density, either diluted with water or counted directly by using an American Optical Co. Brightline haemocytometer counting chamber.

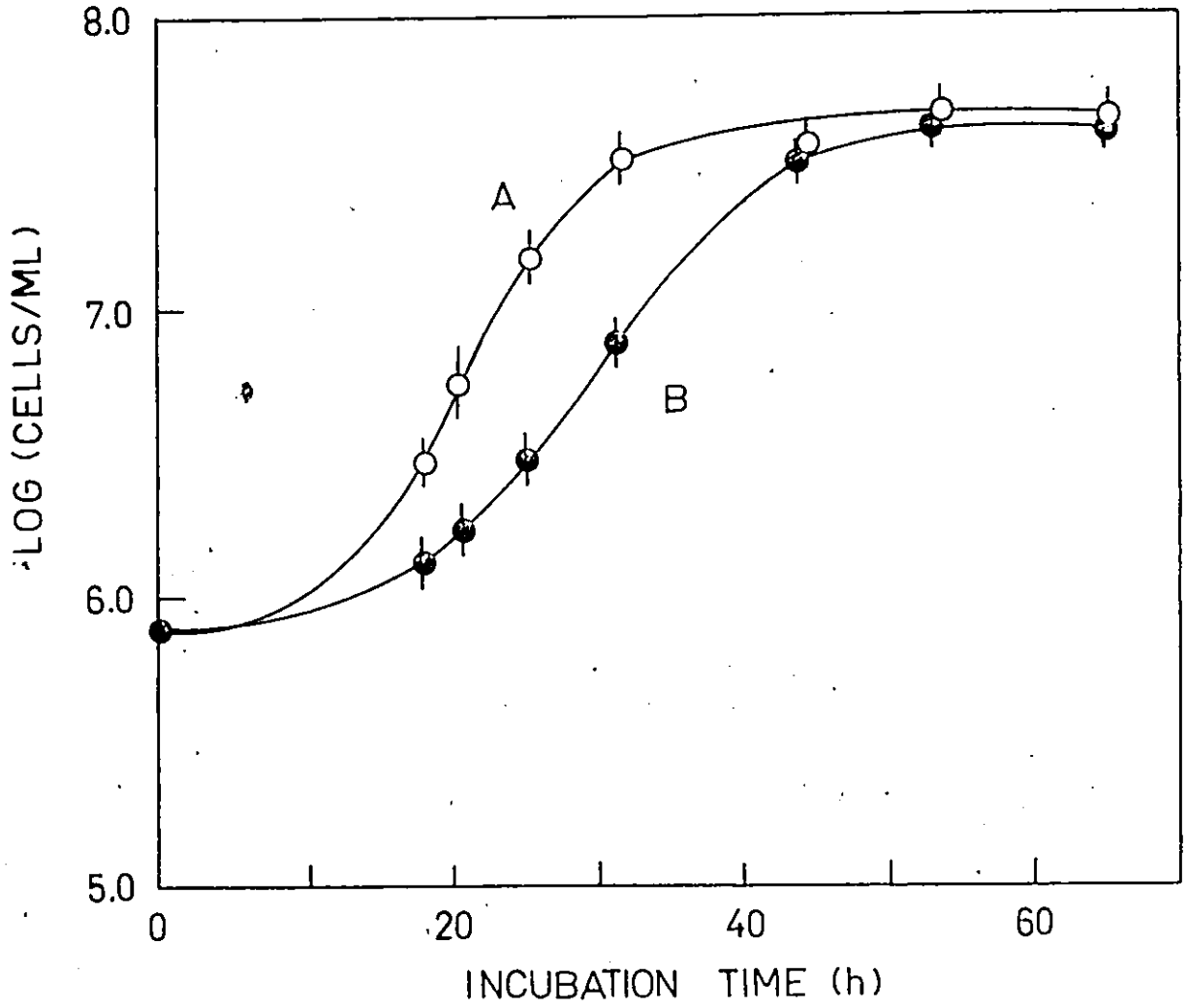


Figure 24: Growth of S. cerevisiae (39916 H.J. Bunker) on thiamin-free medium (●, curve B) and on thiamin-free medium supplemented with thiamin (○, curve A).

(f) Radioactive tracer experiments

Fifteen experiments with radioactive tracers were carried out and the details are summarized in Table 2. The labelled compound was supplied either as a solid or dissolved in aqueous ethanol. In either case, the feeding solution was prepared directly from the commercial sample. Feeding solutions of intermolecularly doubly labelled compounds were prepared by mixing together solutions of the ^{14}C tracer and the ^{3}H tracer.

(g) Addition of radioactive-tracer to the culture

Procedure a (Expts. 1-12): The radioactive compound, dissolved in water, was added to the thiamin-free glucose medium before filter sterilization. For example, in an experiment using 28 250 ml Erlenmeyer flasks, the tracer was dissolved in distilled water (50 ml). A portion of this solution (1.8 ml) was then added to each 50 ml portion of the thiamin-free glucose medium before sterilization.

Procedure b (Expts. 13-15): The radioactive compound was dissolved in sterilized distilled water (e.g., 50 ml) and the solution divided equally among the 250 ml Erlenmeyer flasks (e.g., 1.8 ml in each of 28 flasks) containing the growing yeast cultures (50 ml/flask) at the onset of logarithmic growth, i.e., when the cell density had reached 10^6 cells/ml.

In the experiment with S. cerevisiae (ATCC 24903) growing on thiamin-free glucose medium (Expt. 13) tracer solution was added after 5.5 h of incubation and the cells were collected 43 h later.

With S. cerevisiae (39916 H.J. Bunker) growing on thiamin-free glucose medium (Expts. 14 and 15) tracer solution was added after 15.5 h of incubation and the cells were collected 48 h later.

The cells were harvested by centrifugation [20 min at 10°C and 900 g (r_{av} 13 cm)] when maximum growth had been attained and were either extracted immediately or stored at -8°C until required. A portion (20 µl) of the supernatant was counted to determine the amount of radioactivity not consumed by the cells (Table 2).

(h) Extraction and isolation of thiamin[123,128,143,184,163]

The cells (e.g., from a 1400 ml culture) obtained by centrifugation as described above were heated on a steambath for 30 min. with 20 ml of 0.1 M-HCl. The mixture was centrifuged, the supernatant removed and the extraction repeated. The extracts were combined and 5 ml of 1 M-sodium acetate buffer, pH 4.7, was added. The pH was raised by dropwise addition of NaOH (50%, w/v) to about pH 4.7. Thiamin pyrophosphate was hydrolyzed by incubating this solution at 37 ± 1°C with 100 mg of α-amylase (crude-type IV-A from Aspergillus oryzae; Sigma Chemical Co., St. Louis, Mo., U.S.A.) overnight. An acid phosphatase contaminant is actually responsible for the hydrolysis.[235] The pH of the mixture was then increased to approx. pH 6 by dropwise addition of NaOH (50%, w/v). The solution was then applied to a column (1 cm x 7 cm) containing 0.8 g of cation-exchange resin (Amberlite CG-50, type 1, H^+ form, 100-200 mesh). The column was washed with water (100 ml) and eluted with about 30 ml of 1 M-HCl and the effluent collected. The desired product eluted within 10-15 ml.

(i) Thiochrome assay[134,219]

The thiamin concentration of the column eluate was determined as follows: a portion of the eluate (1.0 ml), was mixed with cyanogen

bromide solution (2.0 ml) (prepared by titrating a saturated aqueous bromine solution at 0°C with aqueous potassium cyanide (20% w/v), until colourless) and with aqueous sodium hydroxide (50% w/v, 1.0 ml). The fluorescent product was extracted into 2-methyl-1-propanol (5.0 ml) by vigorously shaking for 2 minutes. The 2-methyl-1-propanol layer was clarified by centrifugation for 30 seconds. The fluorescence of the 2-methyl-1-propanol layer was measured with an Aminco Bowman Spectrophotofluorometer using 1 cm Supracil cells. The exciting wavelength used was 370 nm and fluorescence was measured at 430 nm. A standard curve (e.g., Figure 25) was prepared for each run from solutions ranging in thiamin concentration from 0.2 to 5.0 μg/ml. With this curve, the thiamin concentration of the unknown solution was determined from its fluorescence. Before the fluorescence of each sample or standard was measured, the stability of the instrument was checked using a standard quinine sulfate solution (0.7 μg/ml in 0.05 M-H_2SO_4) and, if necessary, the value so obtained was used to correct the sample fluorescence.

(j) Purification of thiamin from the tracer experiments and dilution with inactive carrier

Variation (i) (Expts. 8-23)

Carrier thiamin (approx. 1 mg) and sodium acetate buffer (1 M, pH 4.7, 5 ml) were added to the acid eluate from the Amberlite ion-exchange column (see above). The pH of the solution was raised to ca. pH 6 by dropwise addition of sodium hydroxide solution (50% w/v). The solution was applied to an ion-exchange column (Amberlite CG-50) similar to the one described above. The column was washed with water (100 ml) and eluted with dilute hydrochloric acid (0.005 M, 500 ml), and the eluate

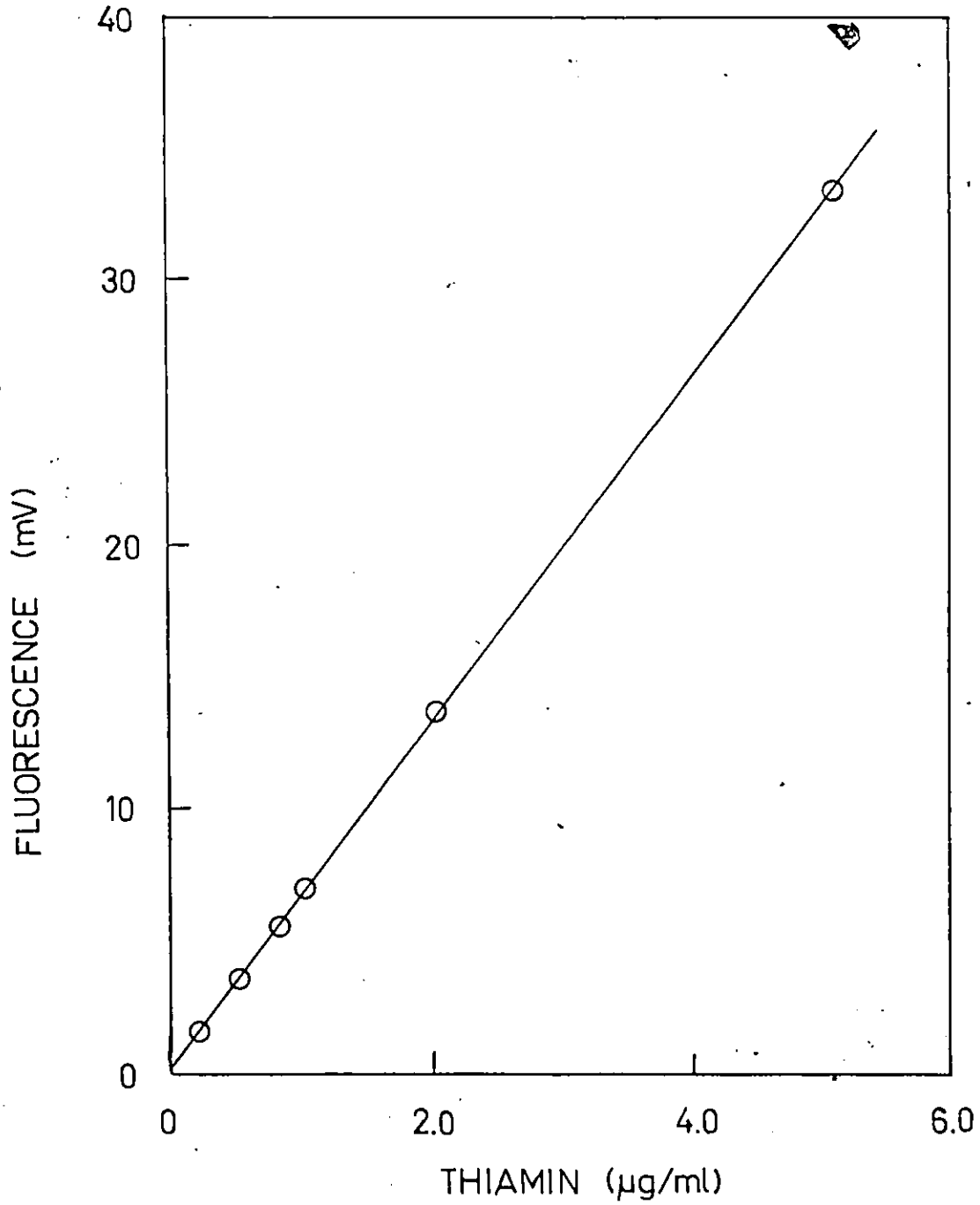


Figure 25: Standard curve obtained by thiochrome assay.

collected in 15 ml fractions. The thiamin-containing fractions (usually fractions 12-30) were combined, additional carrier thiamin (ca. 4 mg) was added and the solution was concentrated in vacuo. The residue was further purified by preparative layer chromatography on cellulose (Analtech Uniplate, 20 cm x 20 cm x 0.5 mm, MN300F cellulose Normal) using 95% ethanol:distilled water (1:1 v/v) as the solvent system. The thiamin (R_f usually 0.8 to 0.9) was located by its quenching of background fluorescence when the plate was examined under ultraviolet light. Thiamin was eluted from the cellulose with 95% ethanol. The ethanol extract was evaporated in vacuo, and additional carrier thiamin was added to the residue to give approximately 50 mg in total. This product was crystallized to constant activity from methanol/2-propanol (1:2, v/v).

The isolation procedure described above was found to be the most satisfactory of the five variations which were tried.

Variation (ii) (Expts. 2-4)

Only one ion-exchange column was used, i.e., the one described under "extraction and isolation of thiamin", and the column was eluted in succession with 0.1 M-HCl (10 ml) and 0.2 M-HCl (10 ml). The first 2 ml portion of eluate was discarded and the next 18 ml were collected; carrier thiamin (5 mg) was added and the solution was evaporated in vacuo. The residue was purified by preparative layer chromatography as described above. After carrier dilution to 50 mg with carrier thiamin, the residue was recrystallized from 95% ethanol/2-propanol to constant activity.

Variation (iii) (Expt. 1)

It differed from variation (ii) in that a second ion-exchange column (identical conditions to variation (ii)) was used before preparative layer chromatography.

Variation (iv) (Expts. 5 and 6)

The ion-exchange column described under "extraction and isolation of thiamin", was eluted with dilute HCl (0.005 M, 500 ml) and the eluate collected in 15 ml fractions. The desired fractions (12-21) were combined, diluted with carrier thiamin and run on a preparative plate using the procedure described in variation (i).

Variation (v) (Expt. 7)

Dilute HCl (0.005 M) was used to elute the column described under "extraction and isolation of thiamin". The acid eluate was further purified by the method given in variation (i).

(k) Degradation of thiamin

Additional inactive carrier was added to give a sufficient amount of thiamin (100-350 mg) for degradation while maintaining a reasonable level (> 50 dpm/mg) of radioactivity.

Separation of the pyrimidine and thiazole moieties by bisulfite cleavage[12]

Thiamin (348 mg) was dissolved in water (6 ml) and sodium bisulfite (1.70 g) was added. If necessary, the pH was adjusted to approx. pH 4.7 with sodium hydroxide (0.5 M). After heating for a few minutes on a steambath, the pH was checked and readjusted, if necessary. The reaction mixture was kept overnight at room temperature. The white

pyrimidinesulfonic acid (4) was filtered off, and recrystallized by dissolving in hot aqueous ammonia (1%) and then acidifying with hydrochloric acid (3 M). The yield was 157 mg (82%).

The filtrate was basified to pH 12 with sodium hydroxide (50% w/v) and extracted with chloroform (5 x 15 ml). The chloroform extracts were dried over anhydrous sodium sulfate and evaporated in vacuo to give 5-(β-hydroxyethyl)-4-methylthiazole (5) (125 mg, 93%) as a colourless oil. ^{1}H nmr ($CDCl_3$) δ: 2.39 (3H, s), 3.00 (2H, t), 3.56 (1H, s), 3.82 (2H, t), 8.52 (1H, s).

(1) Solid derivatives of the thiazole moiety

5-(β-Hydroxyethyl)-4-methylthiazole phenylurethane (92)

5-(β-Hydroxyethyl)-4-methylthiazole (125 mg) was dissolved in benzene (5 ml) and phenylisocyanate (0.1 ml) added. The solution was kept at room temperature overnight, the solvent was evaporated in vacuo and the thiazole phenylurethane was recrystallized from ethyl acetate. The yield was 195 mg (85%). A portion (30 mg) was separated from the diphenylurea contaminant by preparative layer chromatography (silica gel, 0.5 mm x 20 cm x 20 cm) with ether as the solvent system. A better separation was obtained by developing the chromatogram twice. The band corresponding to the thiazole phenylurethane (R_f 0.5) was eluted with ethyl acetate and sublimation of the residue at 110°C and 2 x 10^{-2} mm Hg (2.7 Pa) yielded pure product (92): m.p. 130-131°C, ^{1}H nmr (CD_3COCD_3) δ: 2.03 (s, 3H), 3.16 (t, 2H), 4.31 (t, 2H), 6.90-7.67 (m, 5H), 8.67 (s, 1H), 8.72 (s, 1H); ms m/e: 262 (M^+). Anal. calcd. for $C_{13}H_{14}N_2O_2S$: C 59.52, H 5.38, N 10.68; found: C 59.71, H 5.47, N 10.54.

5-(β-Chloroethyl)-4-methylthiazole (93)[236,237]

5-(β-Hydroxyethyl)-4-methylthiazole (5) (60 mg) was dissolved in chloroform (2 ml) at 0°C. Thionyl chloride (0.5 ml) was added dropwise over a period of a few minutes with stirring, and stirring was continued for 1 h. The solution was warmed to room temperature and evaporated to dryness *in vacuo*. The residue was freed from excess thionyl chloride by adding two portions of ether and evaporation to dryness *in vacuo*. The solid residue was dissolved in water (5 ml), neutralized with solid sodium hydrogen carbonate and extracted with ether (4 x 5 ml). The extracts were combined, dried over anhydrous magnesium sulfate and evaporated *in vacuo* to give 60 mg (88%) of colourless oil. Analysis by thin-layer chromatography (silica gel/ether) indicated that the product (R_f 0.45) was free from starting material (R_f 0.25). ^{1}H nmr ($CDCl_3$) δ: 2.42 (s, 3H), 3.23 (t, 2H), 3.70 (t, 2H), 8.64 (s, 1H).

4-Methyl-5-(β-phthalimidoethyl)thiazole (94)[238]

The above oil was dissolved in dry dimethylformamide (1 ml) and potassium phthalimide (105 mg) was added. The mixture was stirred at 100-105°C for 2 h. At this point, thin-layer chromatography (silica gel/ether) indicated that the starting material (R_f 0.45) had completely reacted and that the major spot (R_f 0.30) was due to the desired product. After the mixture had cooled to room temperature, water (5 ml) was added and the product was collected by filtration. Recrystallization from methanol-water (1:1) and sublimation at 120°C and 2 x 10^{-2} mm Hg (2.7 Pa) yielded the phthalimidothiazole (94) (68 mg, 67%): m.p. 146-147°C (Lit. 143-146°C),[238] ^{1}H nmr ($CDCl_3$) δ: 2.40 (s, 3H), 3.20 (t, 2H), 3.93 (t, 2H), 7.64-7.97 (m, 4H), 8.64 (s, 1H); ms m/e: 272 (M^+).

(m) Degradation of the thiazole moiety

C-2 as formaldehyde dimethone[124]

5-(β-Hydroxyethyl)-4-methylthiazole phenylurethane methiodide (95)

The thiazole phenylurethane (94) (69 mg) was dissolved in benzene (4 ml), excess methyl iodide was added and the mixture was refluxed for 24 h. The product was filtered off. Recrystallization from either propan-2-ol or 95% ethanol/ethyl acetate (1:3 v/v) yielded the thiazole phenylurethane methiodide (95) (76 mg, 71%): m.p. 164-165°C, ^{1}H nmr (CD_3OD) δ: 2.52 (s, 3H), 3.34 (t, 2H), 4.13 (s, 3H), 4.40 (t, 2H), 7.00-7.53 (m, 5H). Anal. calcd. for $C_{14}H_{17}IN_2O_2S$: C 41.50, H 4.24, N 6.93; found: C 41.79, H 4.35, N 6.75.

Reduction of the thiazole phenylurethane methiodide (95)

The thiazole phenylurethane methiodide (70 mg) was suspended in propan-2-ol (3 ml) at 0°C. Sodium borohydride (8 mg) in propan-2-ol (1 ml) was added with stirring, and stirring was continued for 2 h. During this period, the reaction mixture was permitted to warm up gradually to room temperature. Water (10 ml) was added, the solution was extracted with ether (5 x 5 ml), the extract was dried (anhydrous magnesium sulfate) and evaporated *in vacuo*, to yield a colourless oil (42 mg, 87%), presumably compound (96).

Hydrolysis with mercuric chloride and formation of formaldehyde dimethone

The above oil (96) was stirred overnight in water (4 ml) containing mercuric chloride (52 mg). The precipitate was filtered off and washed with water (2 x 5 ml). 5,5-Dimethylcyclohexane-1,3-dione (dimedone) (52 mg) in 95% ethanol (1 ml) was added to the filtrate and,

after heating on a steambath for 1 h, the product was filtered off. Sublimation at 95°C and 2 x 10^{-2} mm Hg (2.7 Pa) gave pure formaldehyde dimethone (40) (33 mg, 65%): m.p. 189-190°C (Lit. 191°C),[239] ms m/e: 292 (M^+).

C-4,-4' as acetic acid

Kuhn-Roth oxidation of the thiazole phenylurethane (92)[240]

The thiazole phenylurethane (92) (46 mg) was dissolved in dilute sulfuric acid (15 ml, 20% v/v) and chromium trioxide (2.1 g) in water (2 ml) was added dropwise. A slow stream of nitrogen was passed through the solution which was slowly distilled, while its volume was maintained by repeated addition of 5 ml portions of water. Over a period of 5 h, 72 ml of distillate was collected. The distillate, containing acetic acid, was titrated to pH 8 with sodium hydroxide (0.1 M). Sodium acetate (22 mg, 92%) was obtained by evaporation at 90°C in a drying oven.

Acetyl-α-naphthylamine (97)[241]

Sodium acetate (22 mg) was dissolved in water (1 ml) and an aqueous solution (2 ml) of α-naphthylamine hydrochloride (40 mg) was added, followed by 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (100 mg). On standing at room temperature, acetyl-α-naphthylamine crystallized from solution and was filtered off. Recrystallization from a benzene/cyclohexane mixture and sublimation at 95°C and 2 x 10^{-2} mm Hg (2.7 Pa) yielded pure acetyl-α-naphthylamine (97) (15 mg, 50%): m.p. 158-159°C (Lit. 159-160°C),[241] ms m/e: 185 (M^+).

(n) Determination of radioactivity

Triplicate samples (0.5-2 mg) of each compound were counted by liquid-scintillation counting (Mark I liquid scintillation computer,

Model 6860; Nuclear-Chicago Corp., Des Plaines, Il., U.S.A.). Samples were dissolved in either water, aqueous ammonia (1%), ethyl acetate, methanol, chloroform, N,N-dimethylformamide or dimethylsufoxide (approx. two drops), and the solutions dispersed in a solution of Aquasol (New England Nuclear Corp., Boston, Ma., U.S.A.). The efficiency of counting (approx. 80% for ^{14}C in samples containing only ^{14}C, and approx. 50% for ^{14}C and approx. 20% for ^{3}H in samples containing both ^{3}H and ^{14}C) was determined by external standardization with ^{133}Ba. Corrections for quenching and for background radioactivity were applied. Confidence limits shown in Tables 3 and 4 are standard deviations from the mean.

PART B The Origin of the C_5 Unit

(i) Introduction

Carbon-2 of the thiazole moiety of thiamin was found to be derived from C-2 of glycine in yeast[124,125,209] and C-2 of tyrosine in bacteria.[143,147] The nitrogen atom of each amino acid was also incorporated into the thiazole unit by the respective micro-organisms[134,148] and it seemed likely that the α-carbon and the nitrogen atom of these amino acids were incorporated as an intact unit. However, no experiments in which activity was incorporated into the C_5 portion of the thiazole moiety were reported in the above investigations and no hypothesis was put forward which explained the incorporations of glycine and tyrosine and the origin of the C_5 unit. Thus, the basic precursors of the C_5 unit were unknown and a chemically rational hypothesis was required for the origin of the thiazole portion of thiamin.

The results of the present re-investigation of suggested precursors of this C_5 unit fall into two categories. Some compounds (e.g., acetate, serine) were absorbed readily by the yeast while others (e.g., cysteine, pyruvate, ribose, α-ketoglutarate, succinate) did not seem to have been taken into the cell to any great extent. In all of these experiments, however, no significant amount of radioactivity was incorporated into the thiazole moiety.

Such results cannot be taken as proof that these compounds are not precursors of thiamin without the consideration of alternative explanations.

Although the metabolism of some of these compounds by the yeast was indicated, it is possible that they were not incorporated because

they did not reach the site of thiamin biosynthesis at the correct time. Alternatively, since there was a large concentration of glucose in the growth medium, the specific activity of the substrate supplied could have been diluted by metabolites of glucose. If this dilution were large enough, only marginal radioactivity would be detected in the isolated thiamin.

The recovery of a large fraction of the radioactivity fed in the non-cellular part of the culture probably indicated that the substance was not taken into the cell. In fact, the presence of glucose may have inhibited the uptake of these substrates.

In order to assess the role of these compounds as possible precursors in a valid fashion, conditions would have to be found under which the yeast takes up such compounds from the medium and metabolizes them. One way around this problem is to feed substrates which are converted to the desired test compound inside the cell (e.g., serine instead of cysteine). A second possible approach to this problem is to grow the yeast on a labelled carbon source and examine the distribution of activity in thiamin. An indication of the biosynthetic origins of the C_5 unit could be obtained from such an experiment.

All previous incubations were carried out with glucose as the carbon source and most of the substances tested were amino acids and compounds, such as acetate, which are directly related to the Krebs' cycle. None of the intermediates of glycolysis had been tested.

Since there is only a small amount of thiamin (1/10 μmole/1400 ml culture) produced in these experiments, the theoretical incorporation of a labelled carbon source (i.e., glucose) into thiamin ought to be

calculated. For a successful experiment, sufficient radioactivity has to be present in this small amount of thiamin to allow for carrier dilution. If 250 μCi of labelled glucose were added to a 1400 ml culture containing 14 g of glucose, then the specific activity of the glucose would be 3.2×10^{-3} μCi/μmole. If only one site of thiamin is labelled from the ^{14}C glucose then, at best, the specific activity of thiamin would be the same as that of glucose. Assuming no losses in the isolation procedure, the total amount of activity which would be isolated in thiamin is 3.2×10^{-4} μCi or 700 dpm. If 50 mg were used as carrier, then the isolated thiamin would have only 14 dpm/mg. Since this is the optimum amount of radioactivity which could be expected, then the radioactivity of the thiamin isolated from an actual experiment would be marginal.

If the specific activity of the glucose in the incubation medium were increased, the radioactivity of the isolated thiamin would also be higher. The specific activity of the glucose could be raised either by increasing the amount of radioactivity fed, or by decreasing the amount of glucose in the medium, or by a combination of the two. In order to make degradation feasible, the radioactivity of the thiamin, as calculated above, would have to be increased by a factor of at least 10. It would be impractical under Atomic Energy of Canada regulations, as well as unrealistic from a financial standpoint, to increase the quantity of radioactivity by such a large amount. The amount of glucose could be decreased by a factor of 10, but the amount of growth and the production of thiamin would be reduced under these circumstances. The relationship between these variables and the amount of activity

isolated in thiamin would not be as simple as suggested above.

A second disadvantage of labelling the carbon source is that all carbon compounds in the cell have to be manufactured from this substance. Since the carbon source is present during the entire growth phase, there is a good chance that there will be a significant amount of random incorporation.

The problems of the inhibition of tracer uptake by glucose, the dilution of the specific activity of the tracer by glucose metabolites, and the impracticality of using labelled glucose as a carbon source, can all be overcome by growing the organism on a carbon source that is quite different from glucose. The relative importances of the metabolic processes in the cell would be changed and glucose would have to be synthesized from the carbon source. Thus, it would not be present in large quantities and it could neither inhibit the uptake of tracer nor significantly dilute the radioactivity of labelled glucoses.

(ii) Search for an alternative carbon source

The energy requirements of the yeast cell growing on glucose are provided mainly by the reactions of glycolysis, even under aerobic conditions.[225] When the level of glucose drops, energy is produced from the series of reactions known as the Krebs' cycle. If a low level of glucose is desired, it seems reasonable that the cell ought to be forced to derive the carbon skeletons needed to synthesize its constituents and its energy requirements from intermediates of the Krebs' cycle.

For two reasons, a series of incubations were carried out with

amino acids, carbohydrates or related compounds as the sole source of carbon. First, a carbon source other than glucose was required, and second, if the compounds were able to support growth as the sole carbon source, it was likely that they would be metabolized when added to the culture as a radioactive tracer. Since most of the compounds tested were either on or close to the energy producing pathways of the cell, this was a reasonable assumption.

Of all the amino acids tried as an alternative carbon source, only L-glutamine was able to support growth (Table 11). Even when a small amount of glucose (1 mM) was added to stimulate growth, the yeast did not adapt to the other amino acids which were tried. The growth of S. cerevisiae (ATCC 24903) on L-glutamine (Figure 38) was peculiar in that it had two logarithmic phases separated by a long (~ 24 h) stationary period. A fairly high cell density was obtained at the end of the second logarithmic phase and at this time the cells were pink in colour. This growth was reproducible as long as the source of L-glutamine was not changed. When a new bottle of L-glutamine was used, S. cerevisiae (ATCC 24903) failed to grow. Unfortunately, the first source of L-glutamine had been exhausted so that the identity of this material could not be checked. The second logarithmic phase of growth after a stationary phase could have been due to the adaptation of the yeast from one carbon source to another, or due to the growth of a second organism in the culture. The pink coloration which developed at this stage could have been attributed to a second organism or to the large amounts of nitrogen in the culture.

The failure of S. cerevisiae (ATCC 24903) to use amino acids

as a general carbon source was in agreement with a previous study[242] in which S. cerevisiae was found to be the yeast least able to adapt to this situation.

The irreproducible results with L-glutamine meant that some other compound was required as a carbon source. Growth was obtained on acetate, lactate and ethanol (Table 12). Both sodium and potassium acetate supported a reasonable amount of growth at a 10 mM concentration (curve A, Figure 39), but higher concentrations inhibited growth. Lactic acid supported growth at concentrations of 10 and 20 mM. Growth was slow and at 20 mM lasted for a period of about 100 h (curve B, Figure 39). However, a rapid logarithmic rate of growth and a large amount of total growth was obtained when ethanol was the sole source of carbon (Figure 40). The total amount of growth increased with the concentration of ethanol up to about 0.5% (100 mM). Concentrations higher than 0.5% did not significantly increase the amount of growth. The length of the lag phase was also dependent on the concentration of ethanol: higher concentrations gave longer lag phases. As a compromise between the two variables, a 0.5% concentration was chosen for the radioactive tracer experiments as this was the smallest concentration of ethanol which gave the maximum amount of growth, and the length of the lag phase was not unreasonable.

None of the other substances tested supported growth under these conditions (Table 12). However, when the medium was supplemented with glutamic acid (1 g/litre, 5.5 mM), S. cerevisiae (ATCC 24903) was able to utilize glycerol as a carbon source[204] (curve A, Figure 41). Initial growth was slow and, as in the case of lactic acid, growth con-

tinued for more than 100 h.

The optimum conditions for growing S. cerevisiae (ATCC 24903) on a non-glucose medium appeared to be when 0.5% ethanol was used as the carbon source. Radioactive tracers could be fed to the yeast that was growing under these conditions in order to elucidate the origin of the C_5 unit.

(iii) Radioactive-tracer results

All radioactive-tracer experiments were performed with S. cerevisiae (ATCC 24903) growing on thiamin-free medium with either L-glutamine (Expts. 16 and 17) or ethanol (Expts. 18-23) as the carbon source. Radioactive tracer was added at the onset of logarithmic growth and the cells were collected after maximum growth had been attained. The experimental details and the percentage of the radioactivity recovered in the non-cellular portion of the culture are summarized in Table 5. In contrast with the experiments in Part A, only a small fraction ($\sim$ 10%) of the radioactivity was recovered in the supernatant from each feeding.

The radioactive thiamin chloride hydrochloride, isolated by carrier dilution, was degraded by treatment with bisulfite to the pyrimidinesulfonic acid (4) and the thiazole (5) (Figure 21). The thiazole (5) was converted to its crystalline phthalimido derivative (94) for assay of radioactivity. The results of these degradations are presented in Table 6.

The distribution of activity in the C_5 unit of the thiazole moiety was determined via the degradation outlined in Figure 26. A portion of the thiazole (5) was oxidized by pyridinium dichromate to 5-formyl-

Table 5 Uptake of labelled substrates by S. cerevisiae (ATCC 24903)

Expt. No.	Substrate	Nominal Total Radioactivity (μCi)	Nominal Specific Radioactivity (mCi/mmol)	Culture Size (ml)	Radioactivity Remaining in Medium (% of Total Radioactivity)
16.	Sodium L-[U-^{14}C]Lactate[a]	100	139	1400	7
17.	D-[6-^{14}C]Glucose[a]	250	53	1350	14
18.	D-[U-^{14}C]Glucose[a]	250	313	1400	11
19.	D-[1-^{14}C]Glucose[a]	500	56	2800	6
20.	D-[1-^{14}C]Fructose[b]	500	57	2800	9
21.	D-[6-^{3}H,6-^{14}C]Glucose[a]	^{14}C 500 ^{3}H 5000	53 33990	2800	15 39
22.	D-[2-^{14}C]Glucose[a]	500	53	2800	9
23.	[1,3-^{14}C]Glycerol[a]	500	56	2800	11

[a] New England Nuclear

[b] Amersham/Searle

Table 6 Distribution of ^{14}C from labelled substrates between the pyrimidine and thiazole moieties of thiamin

Expt. No.	Substrate	Product: Thiamin chloride hydrochloride (2) SA[a]	RSA[b]	Pyrimidinesulfonic acid (4) SA[a]	RSA[b]	Phthalimidothiazole (94) SA[a]	RSA[b]
16.	Sodium L-[U-^{14}C]Lactate	0.29 ± 0.02	---	---	---	---	---
17.	D-[6-^{14}C]Glucose	1.42 ± 0.04	100 ± 3	0.69 ± 0.02	49 ± 2	0.72 ± 0.02	51 ± 2
18.	D-[U-^{14}C]Glucose	1.96 ± 0.04	100 ± 2	1.10 ± 0.01	56 ± 1	1.06 ± 0.03	54 ± 2
19.	D-[1-^{14}C]Glucose	2.22 ± 0.03	100 ± 1	1.34 ± 0.02	60 ± 1	0.97 ± 0.02	44 ± 1
20.	D-[1-^{14}C]Fructose	2.38 ± 0.03	100 ± 1	1.30 ± 0.01	55 ± 1	1.06 ± 0.02	45 ± 1
21.	D-[6-^{3}H,6-^{14}C]Glucose[c]	2.91 ± 0.07	100 ± 2	1.43 ± 0.04	49 ± 2	1.52 ± 0.03	52 ± 2
22.	D-[2-^{14}C]Glucose	5.21 ± 0.08	100 ± 2	3.10 ± 0.03	60 ± 1	2.09 ± 0.02	40 ± 1
23.	[1,3-^{14}C]Glycerol	3.35 ± 0.04	100 ± 1	1.92 ± 0.03	57 ± 1	1.49 ± 0.03	45 ± 1

[a] Specific activity (dpm $mmol^{-1}$) x 10^{-4}

[b] Relative specific activity (%) (thiamin chloride hydrochloride = 100)

[c] ^{14}C results only

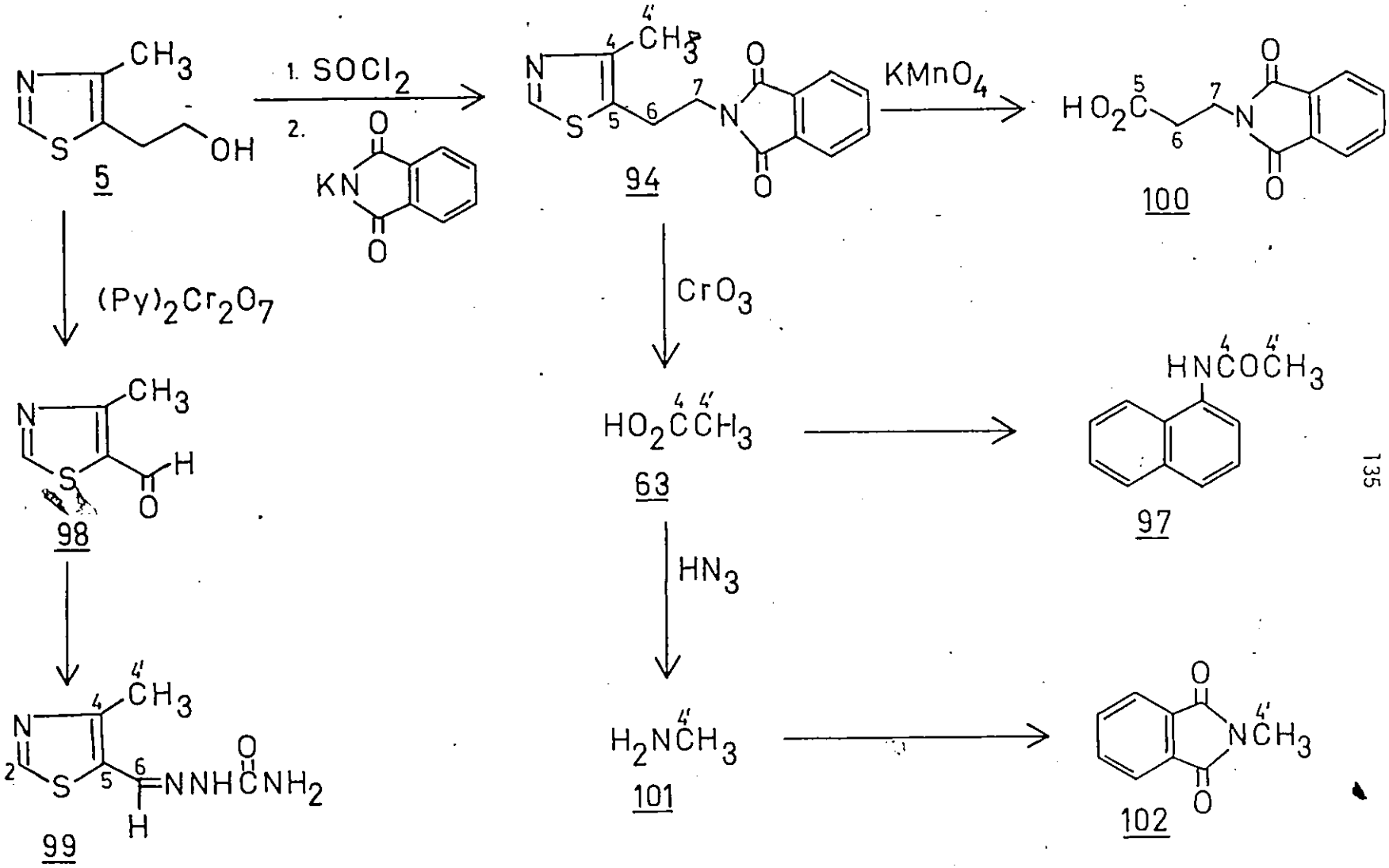


Figure 26: Degradation of 5-(β-hydroxyethyl)-4-methylthiazole to separate the individual carbons of the C_5 unit.

4-methylthiazole (98) (loss of C-7) which was purified by conversion to its semicarbazone derivative (99). The remainder of the thiazole (5) was converted via its β-chloroethyl derivative (93) to the crystalline phthalimidothiazole (94). N-Phthaloyl-β-alanine (100) (C-5,-6,-7) was obtained by permanganate oxidation of the phthalimido derivative (94). Kuhn-Roth oxidation of the same derivative gave acetic acid from the C-methyl group (C-4,-4'). A portion of the acetic acid was isolated as its α-naphthylamide (97) and the remainder was subjected to a Schmidt degradation. The methylamine (101) (C-4') was converted to N-methylphthalimide (102) by treatment with N-carbethoxyphthalimide (Nefkens' Reagent).

The above degradation permits the determination of radioactivity at four individual carbon atoms: C-4' directly (N-methylphthalimide (102)), C-4 by difference (acetyl-α-naphthylamine (97) minus N-methylphthalimide (102)), C-7 by difference (phthalimidothiazole (94) minus thiazole semicarbazone (99)), and C-2 by difference (phthalimidothiazole (94) minus acetyl-α-naphthylamine (97) minus N-phthaloyl-β-alanine (100)). The activity located at C-5,-6 cannot be determined for each individual atom, but two difference methods (either thiazole semicarbazone (99) minus acetyl-α-naphthylamine (97) minus C-2, or N-phthaloyl-β-alanine (100) minus C-7) can be used to determine the sum of the activities contained in these two positions.

The results of this degradation are summarized in Table 7 and are presented pictorially in Figures 27-31.

The thiamin chloride hydrochloride that was isolated from a feeding with sodium L-[U-^{14}C]lactate (Expt. 16) had a low specific

Table 7 Incorporation of hexoses and glycerol into the thiazole unit of thiamin.

Products	C-Atoms of The Thiazole Moiety	PRECURSORS													
		Expt. 18 D-[U-^{14}C]-Glucose		Expt. 17 D-[6-^{14}C]-Glucose		Expt. 21 D-[6-^{3}H,6-^{14}C] Glucose[c]		Expt. 22 D-[2-^{14}C]-Glucose		Expt. 19 D-[1-^{14}C]-Glucose		Expt. 20 D-[1-^{14}C]-Fructose		Expt. 23 [1,3]-Glycerol	
		SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]
4-Methyl-5-(β-phthalimidoethyl)thiazole (84)	All	1.06 ± 0.03	100 ± 2	0.72 ± 0.02	100 ± 3	1.52 ± 0.03	100 ± 2	2.09 ± 0.02	100 ± 1	0.97 ± 0.02	100 ± 2	1.06 ± 0.02	100 ± 2	1.49 ± 0.03	100 ± 2
5-Formyl-4-methylthiazole semicarbazone (99)	All but C-7	---	---	---	---	0.25 ± 0.01	17 ± 1	---	---	---	---	---	---	1.04 ± 0.01	70 ± 2
Acetyl-α-naphthylamine (97)	C-4,-4'	---	---	---	---	---	---	2.00 ± 0.02	96 ± 1	0.89 ± 0.02	92 ± 3	0.89 ± 0.02	84 ± 2	0.59 ± 0.03	40 ± 2
N-Methylphthalimide (102)	C-4'	---	---	---	---	0.24 ± 0.02	16 ± 2	0.96 ± 0.02	46 ± 1	0.90 ± 0.01	93 ± 2	0.96 ± 0.02	91 ± 3	0.41 ± 0.01	28 ± 1
N-Phthaloyl-β-alanine (100)	C-5,-6,-7	0.36 ± 0.01	34 ± 2	0.64 ± 0.03	93 ± 5	1.28 ± 0.02	84 ± 2	---	---	0.05 ± 0.01	6 ± 1	0.05 ± 0.01	5 ± 1	0.89 ± 0.02	60 ± 2

[a] Specific activity (dpm $mmol^{-1}$) x 10^{-4}

[b] Relative specific activity (%) (4-methyl-5-(β-phthalimidoethyl)thiazole = 100)

[c] ^{14}C results only

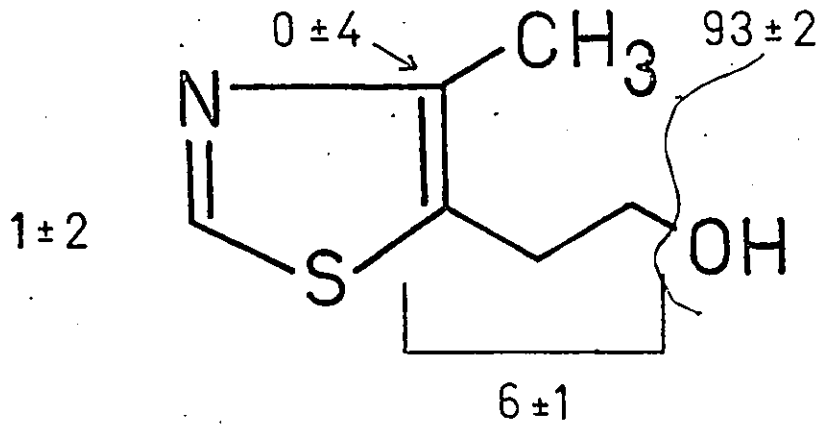


Figure 27: Distribution of activity (%) in the thiazole moiety derived from D-[1-^{14}C]glucose (Table 7).

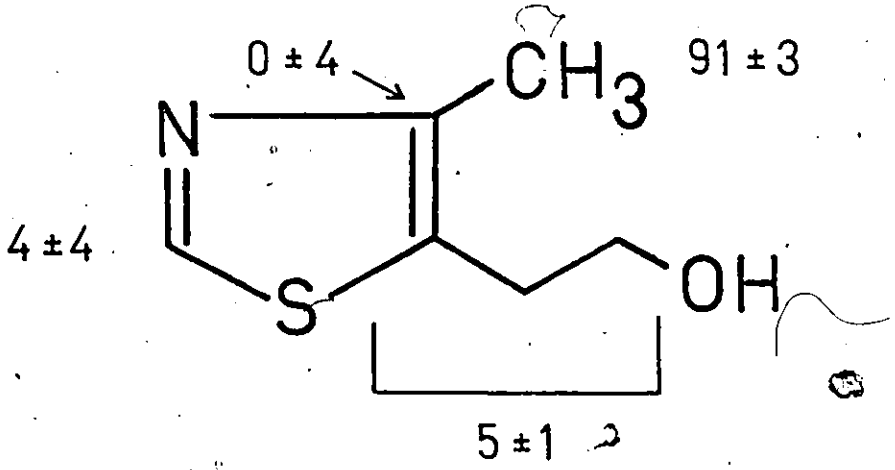


Figure 28: Distribution of activity (%) in the thiazole moiety derived from D-[1-^{14}C]fructose (Table 7).

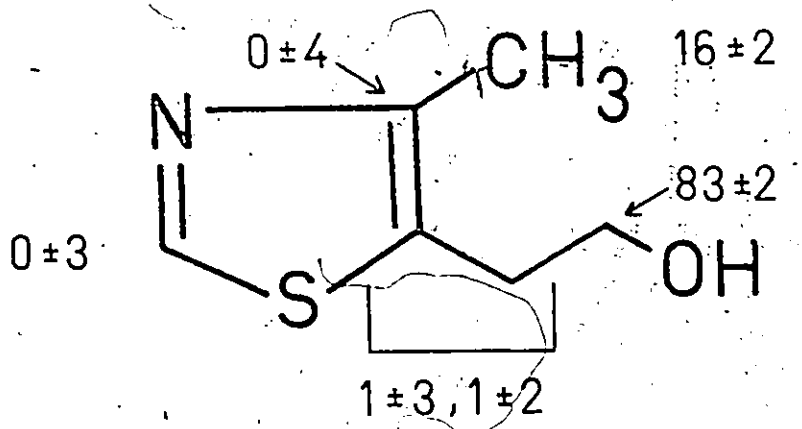


Figure 29: Distribution of ^{14}C activity (%) in the thiazole moiety derived from D-[6-^{14}C,6-^{3}H]glucose (Table 7).

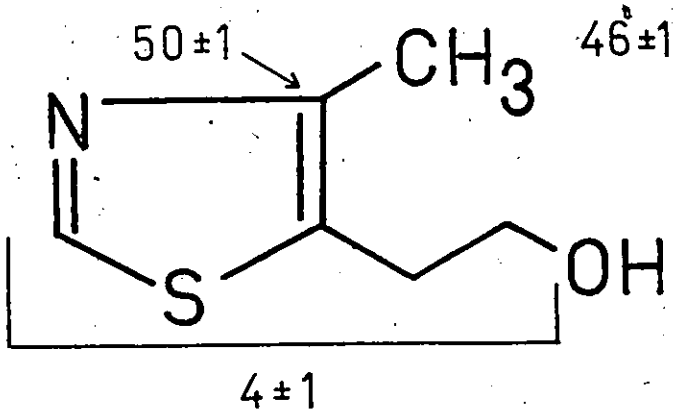


Figure 30: Distribution of activity (%) in the thiazole moiety derived from D-[2-^{14}C]glucose (Table 7).

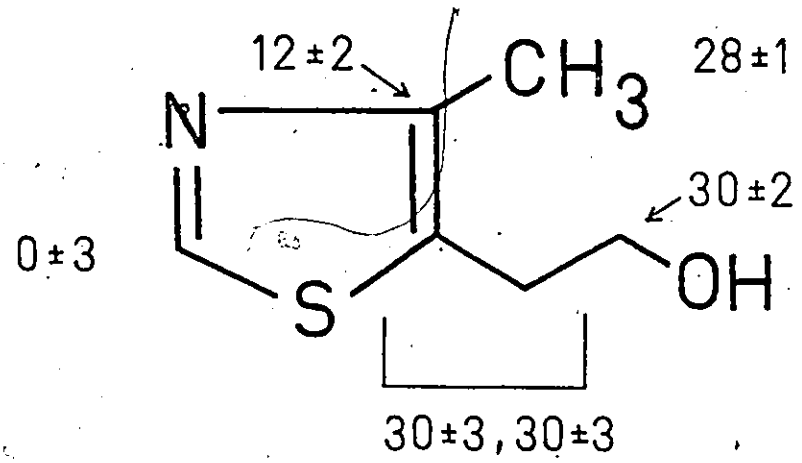


Figure 31: Distribution of activity (%) in the thiazole moiety derived from [1,3-^{14}C]glycerol (Table 7).

activity (Table 6) and was not further degraded.

The activity in thiamin chloride hydrochloride that was derived from D-[U-^{14}C]glucose (Expt. 18) was evenly divided over the thiazole (94) and pyrimidine (4) moieties (Table 6). Degradation of the phthalimidothiazole (94) yielded N-phthaloyl-β-alanine (100) (C-5,-6,-7) which contained about one-third of the activity present in the thiazole nucleus (Table 7).

Incubations in the presence of D-[6-^{14}C]glucose (Expt. 17) and D-[6-^{3}H,6-^{14}C]glucose (Expt. 21) yielded thiamin in which the radioactivity due to ^{14}C was evenly distributed between the pyrimidine (4) and thiazole (94) derivatives (Table 6). Most of the activity (> 80%) of the thiazole unit was contained in C-5,-6,-7 (N-phthaloyl-β-alanine (100)) (Table 7). Lack of material precluded further degradation of the D-[6-^{14}C]glucose-derived thiazole derivative. The remaining activity (< 20%) in the thiazole derived from D-[6-^{3}H,6-^{14}C]glucose was recovered in C-4' (N-methylphthalimide (102)). The same amount of activity was located in the thiazole semicarbazone (99) and thus the activity recovered in N-phthaloyl-β-alanine (100) was located exclusively at C-7. These results are summarized in Figure 29.

A small portion (< 1%) of the D-[6-^{3}H,6-^{14}C]glucose feeding solution was diluted with carrier glucose. One portion of this sample was converted to β-D-glucose pentaacetate and a second part was subjected to periodate oxidation to liberate C-6 of glucose as formaldehyde. The formaldehyde was trapped as its dimethone derivative and the specific activity due to ^{14}C of this substance was identical with that of the pentaacetate derivative (Table 8). Within the limits of error, the

Table 8 Distribution of ^{14}C in D-[6-^{3}H,6-^{14}C]Glucose (Expt. 21)

	C-atoms of D-glucose	SA[a]	RSA[b]
β-D-Glucose pentaacetate	All	2.55 ± 0.04	100 ± 1
Formaldehyde dimethone	C-6	2.55 ± 0.01	100 ± 1

[a] Specific activity (dpm $mmol^{-1}$) x 10^{6}

[b] Relative specific activity (%) (β-D-Glucose pentaacetate = 100)

$^{3}H/^{14}C$ ratios of the feeding solution and the two compounds formed from glucose were the same (Table 9). This ratio was maintained on incorporation into C-5,-6,-7 (N-phthaloyl-β-alanine (100)) of the thiazole moiety, but ^{14}C and practically no tritium was found at C-4' (N-methylphthalimide (102) or thiazole semicarbazone (99)).

The pyrimidinesulfonic acid (4) that was obtained from the cleavage of thiamin chloride hydrochloride which had been derived from D-[1-^{14}C]glucose (Expt. 19) and D-[1-^{14}C]fructose (Expt. 20), contained slightly more than half of the activity present in the original thiamin. The remaining activity was located in the phthalimidothiazole (94) (Table 6). More than 90% of the activity of the thiazole derivative was recovered in acetyl-α-naphthylamine (97) (C-4,-4') and in N-methylphthalimide (102) (C-4') (Table 7, Figures 27 and 28). The missing activity was found in N-phthaloyl-β-alanine (100) (C-5,-6,-7). Lack of material prevented a more complete degradation.

More activity was located in the pyrimidinesulfonic acid (4) than in the phthalimidothiazole (94) of the thiamin chloride hydrochloride (2) which had been derived from D-[2-^{14}C]glucose (Expt. 22) (Table 6). The Kuhn-Roth acetate (C-4,-4') obtained from the thiazole derivative (94) contained most of the activity (96%) present in the thiazole unit (Table 7, Figure 30). Approximately half of the activity was located at C-4' (N-methylphthalimide (102)).

An incubation with [1,3-^{14}C]glycerol (Expt. 23) yielded thiamin with more activity located in the pyrimidine (4) nucleus than in the thiazole (94) nucleus (Table 6). Approximately 40% of the activity of the thiazole derivative (94) was recovered in the Kuhn-Roth acetate

Table 9 $^{3}H/^{14}C$ ratios of tracer and degradation products from D-[6-^{3}H,6-^{14}C]glucose feeding (Expt. 21).

Compound	$^{3}H/^{14}C$ ratio
D-Glucose Feeding Solution	9.09 ± 0.24
β-D-Glucose Pentaacetate	8.96 ± 0.24
Formaldehyde Dimethone	9.09 ± 0.04
Thiamin chloride hydrochloride (2)	6.34 ± 0.26
Pyrimidinesulfonic acid (4)	5.52 ± 0.16
4-Methyl-5-(β-phthalimidoethyl)thiazole (94)	7.47 ± 0.34
5-Formyl-4-methylthiazole semicarbazone (99)	0.18 ± 0.09
N-Methylphthalimide (102)	0.30 ± 0.15
N-Phthaloyl-β-alanine (100)	9.13 ± 0.19

(C-4,-4') (Table 7). Most of this activity (28%) was located in C-4' (N-methylphthalimide (102)). Pyridinium dichromate oxidation yielded the 5-formylthiazole (98) which contained 70% of the activity present in the thiazole (94). N-Phthaloyl-β-alanine, which contained 60% of the activity, was obtained from the phthalimidothiazole (94). These results are summarized in Figure 31.

(iv) Discussion

(a) Pyruvic acid as a precursor of the thiazole moiety

The impermeability of the yeast cell membrane to pyruvate prevented a definite conclusion to be drawn on the role of pyruvic acid in thiamin biosynthesis. One solution to this problem is to feed substances which are taken into the cell and transformed into pyruvate. Alanine, serine and lactic acid are known in some organisms to be converted to pyruvate and, if these transformations take place, the organism should be able to utilize these compounds as a sole source of carbon. Of these substances, only lactic acid was found to support the growth of S. cerevisiae (ATCC 24903) (Tables 11 and 12). If lactic acid was supplied in tracer amounts, it would seem reasonable that it would be taken into the cell and metabolized. This was confirmed in a feeding experiment with L-[U-^{14}C]lactate (Expt. 16).

However, the thiamin chloride hydrochloride isolated from this experiment had only a low specific activity and was therefore not degraded. This result can be taken as evidence that pyruvate is not a precursor of thiamin, and that other sources for the C_5 unit of the thiazole nucleus have to be considered.

(b) Biosynthesis of the thiazole moiety from glycine and a 2-ketopentose

No chemically rational hypothesis which implicates glycine in the biosynthesis of the thiazole moiety has been previously proposed. Such a scheme is presented in Figure 32. It is very possible that the thiazole unit of thiamin is derived from one of the stereoisomers of the Schiff base (104), which is generated by condensation of glycine (35) with a phosphoketopentose (103). The Schiff base (104) is converted to the thiazole phosphate (24) in a multistep sequence comprising dehydration (or elimination of phosphate) (A), dehydration and tautomerization (B and C) and addition of sulfur (D), followed by ring closure (E) and concomitant decarboxylation and dehydration. There are a number of variants for the sequence of these steps and these are indicated in Figure 32. The interesting features of this scheme are that no oxidations or reductions are involved and that the immediate precursor of thiamin, thiazole phosphate (24), is generated.

(c) Possible origins of the 2-ketopentose

The 2-ketopentose (103), postulated as a precursor of the C_5 unit, could be any one of sixteen possible compounds. Four stereoisomers are possible because of the two chiral centres and twelve other compounds can be generated by the substitution of the four stereoisomers, with phosphate at either the 1 or the 5 position, or at both. All four of these stereoisomers are known natural substances and their structures are shown in Figure 33. However, of the twelve phosphorylated compounds, only the four 5-phospho derivatives and the 1,5-diphospho derivatives of the D-isomers are known natural compounds. A priori, it seems likely that one of these ten naturally occurring compounds is involved in thiamin biosynthesis.

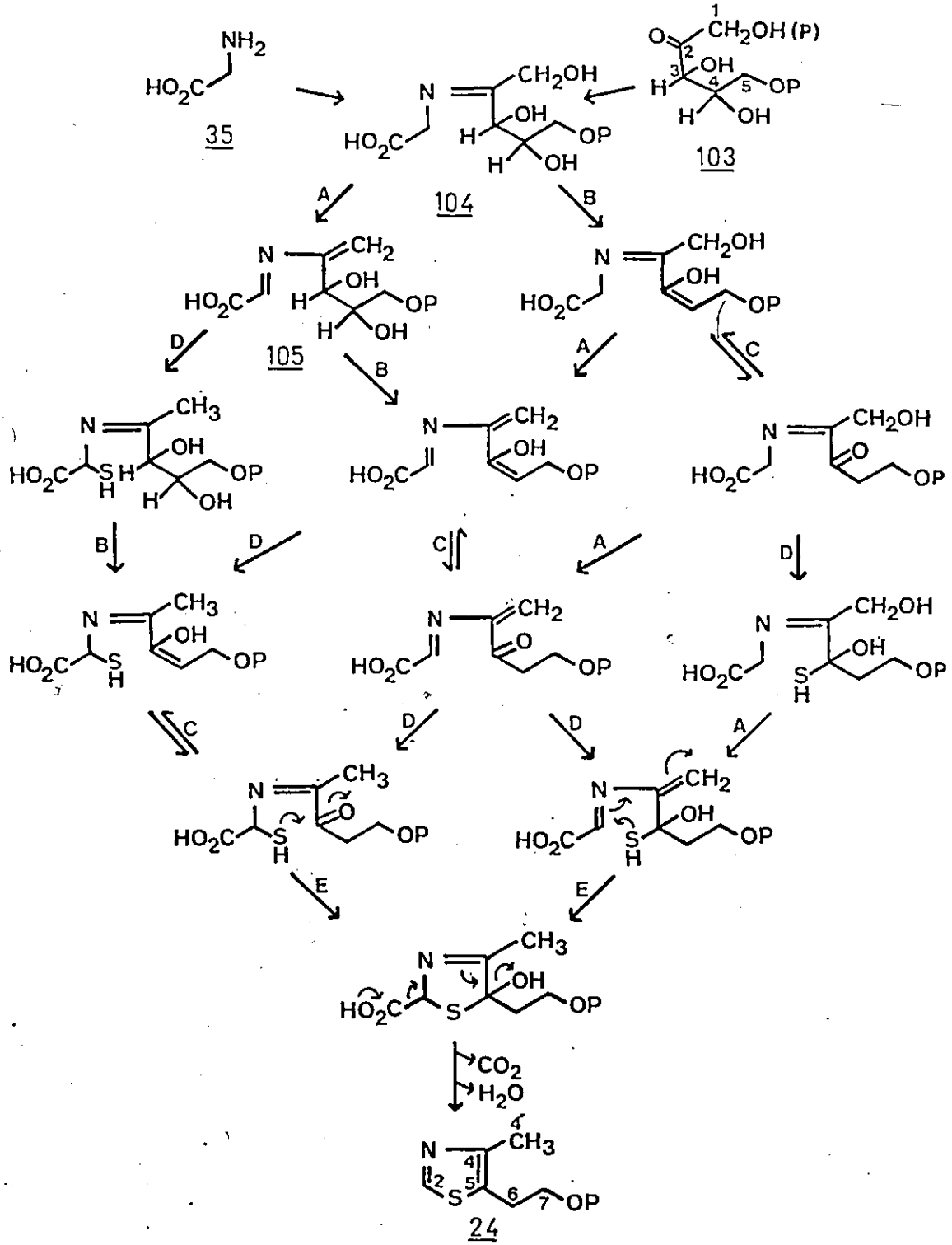
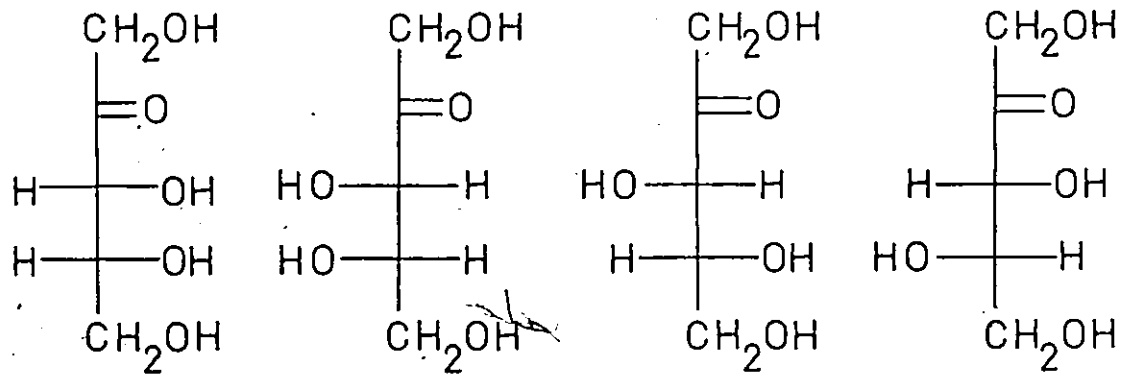


Figure 32: Biogenesis of the thiazole unit of thiamin, in yeast, from glycine (35) and a phosphoketopentose (103).



106 107 108 109

D-ribulose L-ribulose D-xylulose L-xylulose

Figure 33: The four possible 2-ketopentoses.

The involvement of these ketopentoses could be tested by incubating the yeast in the presence of one of these labelled compounds, but this approach is not feasible for two main reasons. Labelled 2-ketopentoses are not commercially available and, more importantly, they would probably not be incorporated into the metabolism of the cell by S. cerevisiae. S. cerevisiae (ATCC 24903) did not utilize D-ribulose or any of the other pentoses tried as the sole source of carbon (Table 12). This non-fermentation of pentoses by S. cerevisiae is well-known[243] and serves as a classification test.[244,245] Thus, it appears that one or more of the enzymes required for the metabolism of these compounds is not present in S. cerevisiae.

On the other hand, the 5-phospho esters of these ketopentoses are well-known intermediates of metabolism, but it is unlikely that a feeding experiment with labelled phosphoesters would be successful as cell membranes are not usually permeable to such compounds. In fact, the cell prevents the diffusion of substances into the medium by phosphorylating them.

However, the yeast does contain the necessary enzymes for the metabolism of glucose and fructose, and the conversion of these hexoses into pentoses is well-established. D-Ribulose 5-P and D-xylulose 5-P are known intermediates of the pentose pathway[246,247] and this pathway is known to account for up to 27% of the catabolism of glucose when S. cerevisiae is grown on glucose.[248] In fact, the enzymes hexokinase,[249] phosphofructokinase,[250] fructose diphosphate aldolase,[251] transketolase,[252] glucose 6-P dehydrogenase,[253] 6-phosphogluconate dehydrogenase,[254] and D-ribulose 5-P 3-epimerase[255] have been isolated and purified from yeast.

The transformation of D-glucose 6-P (110) to D-ribulose 5-P (113) and D-xylulose 5-P (116) is shown in Figure 34. The predicted incorporation of the carbon atoms of glucose into the thiazole moiety via the scheme in Figure 32 is also indicated.

D-Ribulose 5-P (113) is derived from D-glucose 6-P (110) via an oxidative decarboxylation route involving D-gluconic acid 6-P (111) and 3-keto D-gluconic acid 6-P (112). A pentose generated in this fashion would be derived from carbons 2 to 6 of glucose and C-4',-4,-5,-6,-7 of the thiazole moiety would be derived from carbons 2 to 6 of glucose, respectively.

A non-oxidative route from D-glucose 6-P (110) also exists, which, except for one enzyme, comprises the reactions of glycolysis up to the formation of dihydroxyacetone P (115), and D-glyceraldehyde 3-P (18). The enzyme transketolase, which requires thiamin pyrophosphate as a coenzyme, transfers a C_2 unit (C-1,-2) of D-fructose 6-P (14) onto D-glyceraldehyde 3-P (18) to form D-xylulose 5-P (116). The pentose generated in this fashion would be derived from C-1,-2,-4,-5,-6 of glucose and would then be incorporated into C-4',-4,-5,-6,-7, respectively, of the thiazole nucleus.

D-Ribulose 5-P (113) and D-xylulose 5-P (116) are also interconvertible. Thus, it would not be possible to distinguish which one of these compounds is the immediate precursor of the thiazole moiety on the basis of feeding experiments with labelled glucoses.

L-Xylulose (109) can be formed directly from D-glucose (117) via D-glucuronic acid (118) and L-gulonic acid (119) as shown in Figure 35. In this route, carbon-6 of D-glucose is oxidized and lost as carbon

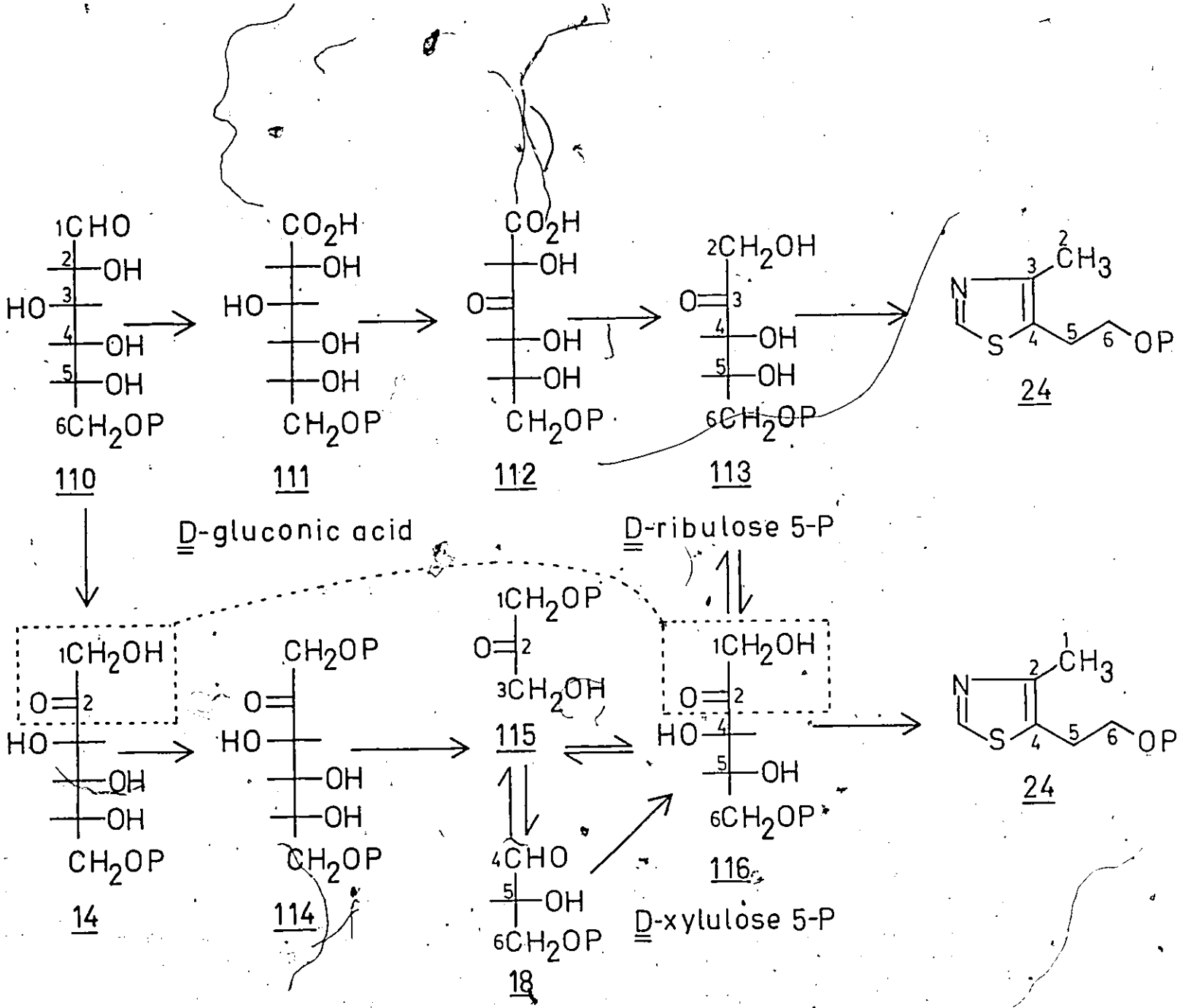


Figure 34: Predicted incorporation of the carbon atoms of D-glucose into the thiazole moiety via D-ribulose 5-P and D-xylulose 5-P.

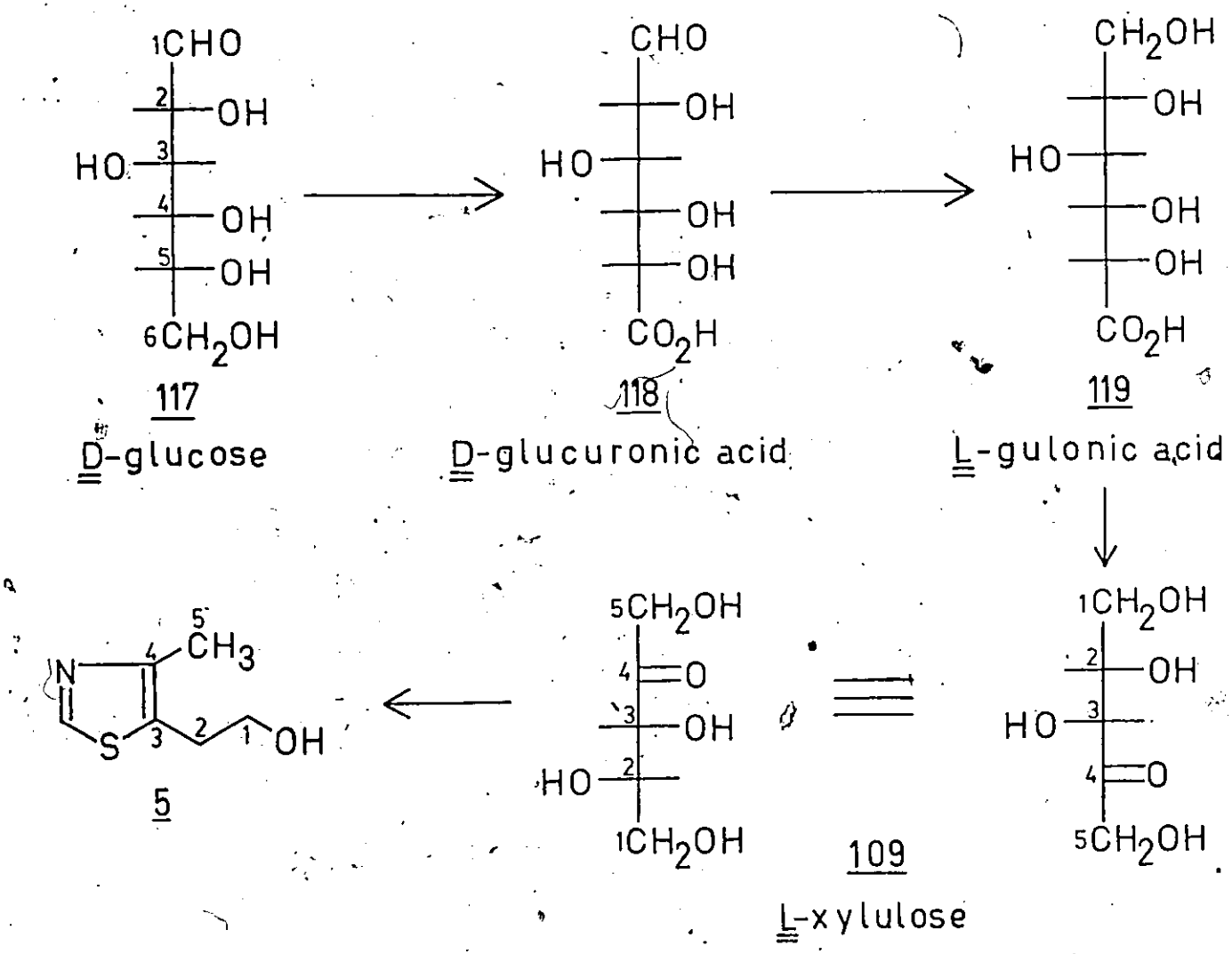


Figure 35: Predicted incorporation of the carbon atoms of D-glucose into the thiazole moiety via L-xylulose.

dioxide and the resulting pentose is thus derived from carbons 1 to 5 of D-glucose. Incorporation of L-xylulose (109) into the thiazole moiety via the scheme in Figure 32 would place C-5,-4,-3,-2,-1 of glucose at C-4',-4,-5,-6,-7 of the thiazole unit.

Neither L-ribulose (107) nor its 5-phosphoester is formed directly from D-glucose or D-glucose 6-P, but rather via the above pentoses.

(d) Hexose feeding experiments

The incorporation of radioactivity from specifically labelled hexoses into the thiazole nucleus of thiamin is thus predicted on the basis of the above known metabolic routes to 2-ketopentoses, and the mode of incorporation should allow a distinction to be made between the involvement of L-xylulose (109) and D-ribulose 5-P (113) or D-xylulose 5-P (116).

As a preliminary experiment, D-[U-^{14}C]glucose was administered to S. cerevisiae (ATCC 24903) and non-random incorporation of activity into the thiazole nucleus was found. Only one-third of the activity was located in C-5,-6,-7 (N-phthaloyl-β-alanine), i.e., in one-half of the carbon atoms. If it is assumed that an equal amount of activity is located at each of the six carbon atoms of D-[U-^{14}C]glucose and that glucose enters only the C_5 unit according to the routes described above, then the three carbon atoms recovered by degradation would have been expected to contain approximately 60% of the activity contained in the thiazole nucleus. In order to explain the low recovery of activity in the C_3 unit, C-5,-6,-7, the growth of the yeast on ethanol must first be examined.

In order to synthesize hexoses and related cell constituents, the cell requires a method of transforming the C_2 units of the carbon source, ethanol, into the intermediates of glycolysis. Ethanol is oxidized by an inducible alcohol dehydrogenase[256] and is further transformed to acetyl CoA, which is incorporated into the constituents of the cell via the Krebs' and Glyoxylate cycles.[257] The Glyoxylate cycle is required to replenish the Krebs' cycle intermediates, which are removed for synthetic purposes. The two enzymes, malate synthase and isocitrate lyase, which are responsible for this cycle are induced under these growth conditions.[256] The transformation of oxaloacetate to phosphoenolpyruvate connects these cycles to glycolysis. Reversal of the glycolytic reactions from phosphoenolpyruvate generates D-glyceraldehyde 3-P, which after conversion to, and combination with dihydroxyacetone P, yields hexoses.

The D-[U-^{14}C]glucose results indicate that the C_5 unit is derived from glucose by a route in which some substances are diluted to a greater extent than others. This inference could be validated by Kuhn-Roth degradation of the thiazole phenylurethane (94), C-4,-4'. The remaining two-thirds of the thiazole activity would be expected in this fragment. The incorporation of label via the transketolase route to D-xylulose 5-P (116) would be consistent with these results since the C_3 unit (C-5,-6,-7) isolated by degradation would be derived from D-glyceraldehyde 3-P (18) and C-4,-4' would be supplied from D-fructose 6-P (14). Since label from glucose requires two extra steps to enter D-glyceraldehyde 3-P (18), and sinc D-glyceraldehyde 3-P (18) is closer than D-fructose 6-P (14) to the carbon source on the glycolytic pathway, it is reasonable to expect that the activity in this C_3 unit should be diluted to a greater extent than the activity in the C_2 unit supplied directly from D-fructose 6-P (14).

The indication of the involvement of the transketolase route in the generation of a precursor of the thiazole moiety was confirmed by experiments with specifically labelled hexoses.

Activity from D-[1-^{14}C]glucose and D-[1-^{14}C]fructose is located almost entirely (> 90%) in the C-methyl group (C-4') of the thiazole unit (Figures 27 and 28). The remainder of the activity (< 10%) was located at C-5, C-6 and/or C-7. Lack of material precluded further degradation to determine the exact location of label. This distribution of activity is as predicted by the transketolase pathway (Figure 34), and it immediately rules out the glucuronic-gulonic acid pathway to L-xylulose (Figure 35).

The incorporation of label into more than one position of the thiazole nucleus can be explained on the basis of the triosephosphate isomerase catalyzed interconversion of the triose phosphates, D-glyceraldehyde 3-P (18) and dihydroxyacetone P (115). This interconversion would tend to place label from C-1 and C-6 of glucose into the same positions and thus the distribution of activity in D-xylulose derived from D-glucose via the transketolase route would not be exactly as shown in Figure 34, but would depend on the extent of the interconversion of the trioses. It is very likely that the second site of labelling from [1-^{14}C]hexoses was C-7.

A complementary result was obtained when either D-[6-^{14}C]- or D-[6-^{3}H,6-^{14}C]glucose was added to the growing yeast culture (Figure 30). The label was located mainly at C-7 (> 80%), while the C-methyl group contained the remainder of the activity due to ^{14}C (< 20%) in the D-[6-^{3}H,6-^{14}C]glucose experiment. This distribution can be explained

on the basis of the interconversion of the triose phosphates, followed by resynthesis to hexoses. Fructose 6-P aldolase can catalyze both the synthesis and the cleavage of D-fructose 1,6-diP (114), whereas the interconversion of D-fructose 6-P (14) and D-fructose 1,6-diP (114) is mediated by two enzymes. Fructose 1,6-diP phosphatase is present only in small amounts when yeast is grown on glucose, but when gluconeogenesis is required, this enzyme is present in appreciable quantities.[258] The latter conditions are used in these feeding experiments. Label from a [6-^{14}C]hexose would thus be moved to the 1 position where the transketolase reaction would transfer it to C-1 of D-xylulose 5-P or to C-4' of the thiazole moiety.

The larger amount of randomization, compared to the [1-^{14}C]-hexose feedings, can be rationalized on the basis of the triose phosphate equilibrium. [6-^{14}C]Hexose primarily labels D-glyceraldehyde 3-P (18) while [1-^{14}C]hexose primarily yields labelled dihydroxyacetone P (115). The equilibrium constant favours the formation of dihydroxyacetone P and the flow of carbon from the ethanol carbon source is also moving in this direction. Thus, more label would be expected to move from D-glyceraldehyde 3-P to dihydroxyacetone P than vice versa, and this is what is observed.

The ^{3}H/^{14}C ratio of D-[6-^{3}H,6-^{14}C]glucose was maintained on incorporation into C-7 of the thiazole moiety (Table 9). However, only ^{14}C was randomized into the C-methyl carbon. This result was obtained in both the pyridinium dichromate oxidation and the Kuhn-Roth-Schmidt sequence, it is therefore unlikely that loss of tritium occurred by exchange during degradation. Also, it has been reported that at least

90% of the tritium is retained in the Schmidt reaction.[191] The $^{3}H/^{14}C$ ratio of the phthalimidothiazole (94) is also consistent with ^{14}C being located at two positions and ^{3}H only at one. Since the tritium is lost, it must be exchanged during the conversion of the hexose to the thiazole. In many of the intermediates, the C-1 protons are α to a ketone functionality and the scheme in Figure 32 places these protons in the exchangeable positions of an enamine.

The transketolase pathway is consistent with all the results discussed above. The glucuronic-gulonic acid route (Figure 35), which is known to operate in animals,[247] can be eliminated on the basis of these results. Label from D-[6-^{14}C]- and D-[6-^{3}H,6-^{14}C]glucose can be incorporated by both the transketolase and the gluconic acid route, but label from [1-^{14}C]hexoses cannot be incorporated by the gluconic acid pathway. However, the results of these experiments would not be inconsistent with the use of both the transketolase and the gluconic acid route to generate pentoses. This situation would be best illustrated by a D-[2-^{14}C]glucose feeding experiment and the distribution of activity in the thiazole moiety can be calculated from the [1-^{14}C]-glucose results as shown in Table 10. The distribution of the activity at positions 4 and 4' would serve as an indication of the amount of label delivered to pentoses from hexoses over the two routes. When the experiment with D-[2-^{14}C]glucose was carried out, and the thiazole was degraded, the Kuhn-Roth acetate contained 96% and the C-methyl (C-4') was found to have 46% of the activity of the original thiazole. By difference, 50% of the activity was located at C-4. Presumably, the remainder of the activity (4%) was at C-6, but a degradation was not

Table 10 Predicted distribution of activity in the thiazole moiety from D-[2-^{14}C]Glucose as a function of pathway

Pathway		Distribution of activity in the thiazole moiety					
Transketolase	Gluconic Acid	C-2	C-4	C-4'	C-5	C-6	C-7
100	0	0	92	0	0	8	0
75	25	0	69	25	0	6	0
54	46	0	50	46	0	4	0
50	50	0	46	50	0	4	0
25	75	0	23	75	0	2	0
0	100	0	0	100	0	0	0

performed to confirm this. If 54% of the label from D-glucose had been delivered by the transketolase route, then these results are exactly as predicted in Table 10.

The distribution of label from D-[1-^{14}C]-, D-[2-^{14}C]- and D-[6-^{14}C]glucose in ribose,[259] D-arabitol[260] and within the ribitol moiety of riboflavin[261] has also been interpreted on the basis of the transketolase and the gluconic acid pathways operating concurrently. The contribution from each pathway was found to depend on the manner in which the organism was grown, the stage of growth of the culture and the nature of the carbon source.[262]

If the triose phosphate which is utilized in the transketolase reaction could be labelled equally in its terminal carbon atoms, then the three carbon atoms of the C_5 unit, which are derived from this substance, could be identified. Glycerol is metabolized to dihydroxyacetone P (115) via L-α-glycerol P in yeast.[263] Therefore, an experiment was performed with [1,3-^{14}C]glycerol. On the basis of the hexose feedings, equal amounts of activity would be expected at C-5 and C-7 of the thiazole moiety, whereas C-6 would be inactive. Activity would also be expected in C-4' as well as at C-4, since some synthesis of hexoses from the labelled triose phosphates would occur. The transketolase reaction would then place activity at C-4' of the thiazole unit and the gluconic acid route would account for the activity at C-4. The thiazole moiety derived from [1,3-^{14}C]glycerol had the predicted distribution of activity. Equal amounts of activity were found at C-7 and C-5,6. Unfortunately, C-5 and C-6 could not be distinguished by the degradation which was employed and it must be assumed that the label was located

solely at C-5. The amount of activity found at C-4' was not greatly different from that at C-5 or C-7, but the presence of activity at C-4 again indicates that it is C-5,-6,-7 which is directly derived from a triose phosphate.

In summary, the results of the [^{14}C]hexose and [1,3-^{14}C]glycerol experiments are consistent with the formation of the thiazole moiety of thiamin in yeast from glycine (35) and a 2-ketopentose (103) as outlined in Figure 32. It is likely that the 2-ketopentose is D-ribulose 5-P (113), or D-xylulose 5-P (116), or possibly a diphosphate derivative of one of these compounds. Additional experiments are required to distinguish among these possibilities.

(e) Origin of the C_5 unit in bacteria

In bacteria, the C-2-N fragment of the thiazole moiety of thiamin has been shown to be derived from the α-carbon[143,147] and the nitrogen[148] of tyrosine. The scheme in Figure 32 can be adapted to explain this incorporation. Elimination of the p-hydroxybenzyl moiety from the Schiff base (120), formed from tyrosine and the 2-ketopentose (103), as shown in Figure 36, would generate the intermediate (105) that was proposed in Figure 32. The unstable quinonemethide (121), formed by the elimination of the p-hydroxybenzyl group, would rapidly react with water to form p-hydroxybenzyl alcohol (122). The latter compound has been isolated from bacteria and its synthesis has been found to parallel the production of thiamin in this organism.[264] Similar fragmentations of Schiff bases of the pentulose (103) with the amino acids tryptophan (123), threonine (124), serine (126), histidine (125),

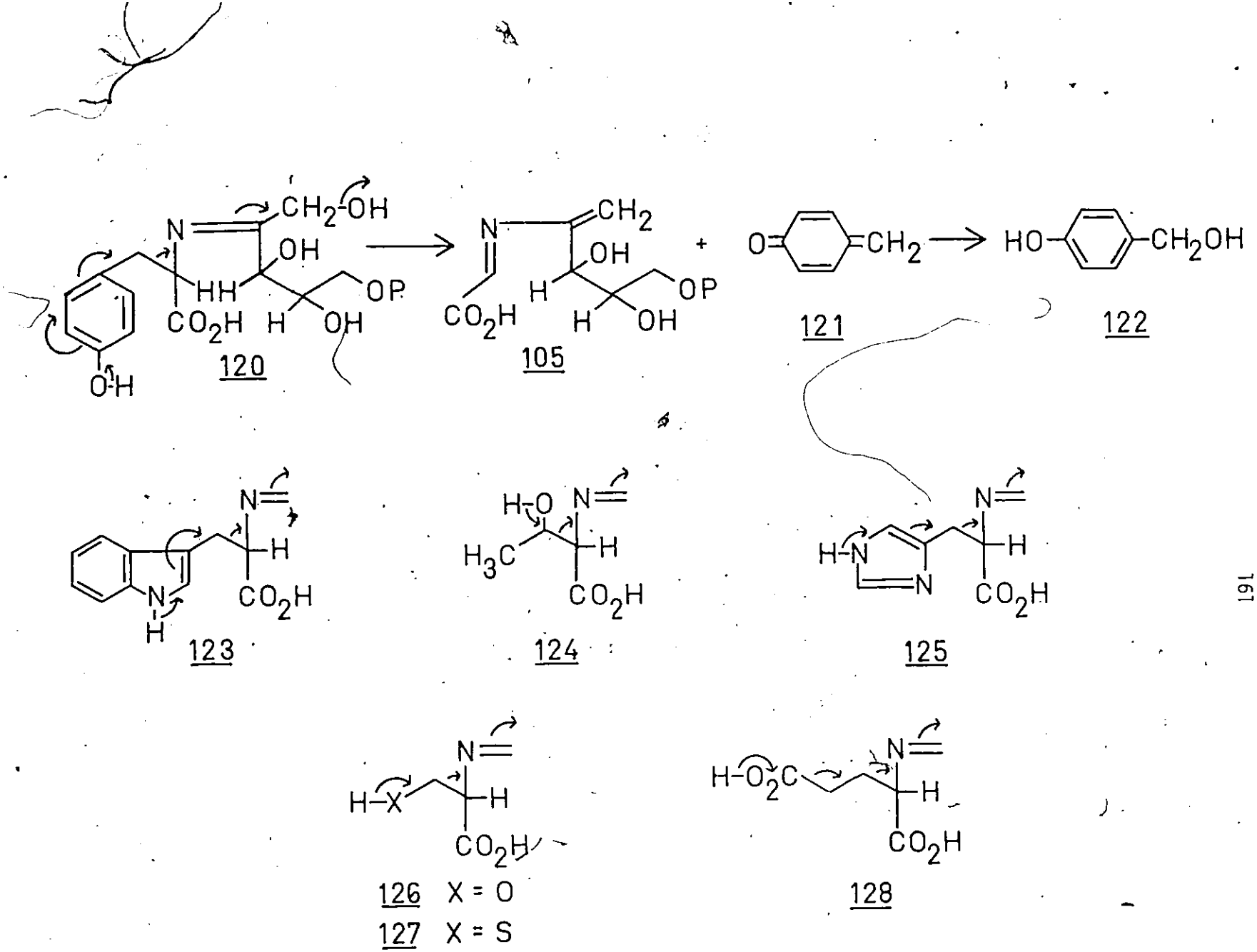


Figure 36: Amino acids as the source of the C-2-N portion of the thiazole moiety.

glutamate (128) and cysteine (127) can also be envisaged as shown in Figure 36. In each case, the C-2-N fragment of the thiazole nucleus is supplied by the amino acid. Feeding experiments with the above amino acids labelled in their α-carbon have not been carried out in either bacteria or yeast. It is possible that the C-2-N fragment of the thiazole moiety is derived from more than one amino acid in the same organism, or that separate organisms employ different amino acids as the source of this portion of thiamin. The available evidence for glycine and tyrosine suggests the latter.

Phenylalanine has been found to inhibit the biosynthesis of the thiazole unit of thiamin in E. coli[146] and this inhibition was overcome by tyrosine. If phenylalanine, instead of tyrosine were to form a Schiff base with a 2-ketopentose, the further transformations of this intermediate would be blocked as there is no facile method of eliminating the benzyl moiety from this intermediate. Tyrosine could overcome this inhibition by displacing phenylalanine from the Schiff base intermediate.

The hypothesis (Figure 32) for the origin of the thiazole moiety in yeast can be modified to account for the incorporation of tyrosine, but the available evidence for the C_5 unit in bacteria suggests a different origin.[207] E. coli was grown on substances labelled with either ^{2}H or ^{13}C, and the thiazole moiety was isolated and analyzed by gas chromatography and mass spectrometry. Experiments with D-[6,6'-$^{2}H_2$]-, D-[5,6,6'-$^{2}H_3$]-, and D-[U-^{13}C]glucose indicated that the C_5 unit was synthesized from a C_2 and a C_3 compound. These results are consistent with the transketolase route to the 2-ketopentoses. On the basis of

the published results, the operation of the decarboxylation route via gluconic acid cannot be judged. Label from L-[3-^{2}H]glycerol was incorporated into the two positions predicted by the transketolase pathway. Also consistent with this was the finding that the deuterium atoms from L-[1-^{2}H]- and [2-^{2}H]glycerol were not incorporated. [2-$^2H_3$]Acetate showed only random incorporation, but label from [3-$^2H_3$]pyruvate was incorporated into the C-methyl group with retention of up to three deuterium atoms. Resynthesis of triose phosphate by gluconeogenesis from pyruvate accounts for the incorporation of label into the C_3 derived fragment. But, this intact incorporation of a CD_3-group from pyruvate cannot be explained on the basis of the incorporation of 2-ketopentoses, as shown in Figure 32. An alternative scheme which attempts to rationalize the derivation of the C-methyl group of the thiazole unit from pyruvate was proposed by White[207] and is shown in Figure 37. The first step in the pathway involved an acyloin-type condensation of an activated C_2 unit derived from pyruvate (6) with D-glyceraldehyde 3-P (18). It was further suggested that the desoxy-2-ketopentose (129) that was formed by this condensation, underwent dehydration and tautomerization to yield the diketone (130), which then combines in an unspecified manner with tyrosine (36) and a sulfur donor, to form the thiazole moiety.

From studies with N. crassa mutants, Harris[81] suggested two pathways for the biosynthesis of thiamin. One route was postulated to involve the condensation of a thiazole precursor with the pyrimidine moiety to give an intermediate which is then converted to thiamin. The p-hydroxybenzyl derivatives (131) and (132) were suggested by White[207] as these intermediates. Two points make this proposal unreasonable. The evidence on which Harris based his proposal has been disputed by

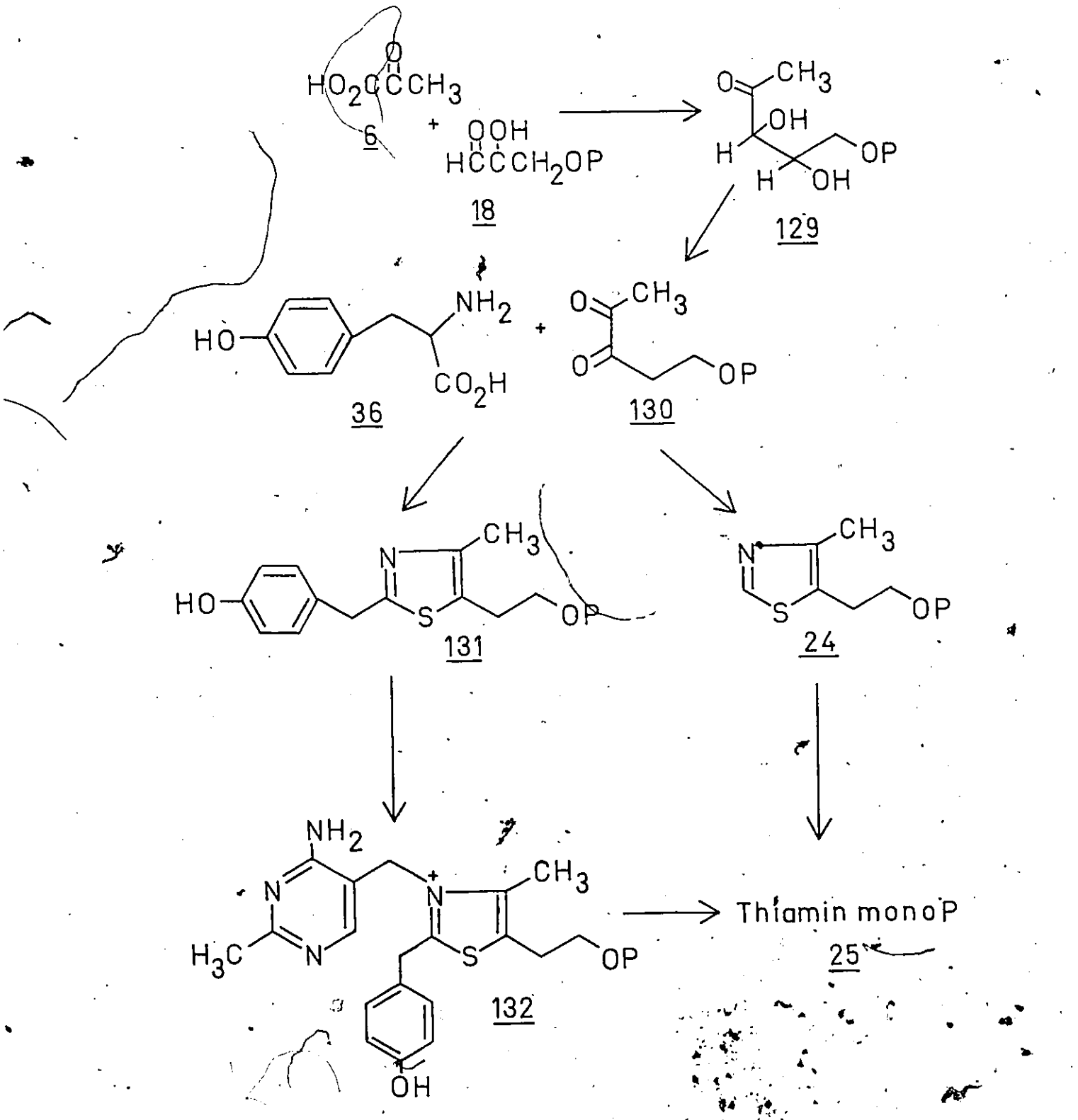


Figure 37: Proposed biosynthesis of 5-(β-hydroxyethyl)-4-methylthiazole and thiamin in E. coli.

Eberhart and Tatum[82] and in addition, the C-2-N precursor of the thiazole unit is not known in N. crassa. It is clear that if glycine supplied these atoms in N. crassa, then the postulated intermediates (131) and (132) would not be involved in thiamin biosynthesis.

Although the series of reactions leading to the thiazole moiety were not defined, a suggestion for the incorporation of tyrosine based on the biosynthesis and reactions of glucosinolates was proposed[207]. Alternatively, a scheme involving dehydrations, decarboxylation, and elimination of the p-hydroxybenzyl moiety can be constructed if an oxidation step is included.

Pyruvate cannot be a direct precursor of the thiazole nucleus in yeast. Not only did the negative results obtained in the experiment with pyruvate (Expt. 5) and lactate (Expt. 16) suggest this conclusion, but the mode of incorporation of activity from labelled hexoses is not consistent with the direct incorporation of pyruvate. The C-methyl group of the thiazole unit is derived from C-1 of glucose, and C-7 of the thiazole nucleus is derived from C-6 of glucose. If pyruvate and D-glyceraldehyde 3-P had been the precursors, then these two carbon atoms would have been equally labelled in each of the experiments with D-[1-^{14}C]- and D-[6-^{14}C]glucose, since D-glyceraldehyde 3-P is an intermediate of the conversion of D-glucose to pyruvate. One major difference between the present investigation and that carried out in E. coli[207] is the method of administration of the labelled compounds. In the present experiments, the labelled substrate was added only in trace amounts, whereas in the experiments with E. coli, the labelled

substrate was also the carbon source and was present in a concentration of 4 g/100 ml of medium. Before a definitive conclusion can be drawn on the status of pyruvate in thiamin biosynthesis in E. coli, its incorporation should be confirmed by an experiment in which labelled pyruvate is added only in trace amounts.

The available evidence suggests that two routes to the thiazole moiety of thiamin are in operation: one in yeast and one in E. coli and S. typhimurium. The details of these routes and their occurrence in other organisms are subjects for future investigation.

(v) Experimental

All experiments were carried out with S. cerevisiae (ATCC 24903) which was maintained as described in Part A.

(a) Liquid culture medium

A basal salts medium was prepared with the same composition as the thiamin-free glucose medium except that glucose was not added. The inorganic salts ($(NH_4)_2SO_4$, 5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g) and the amino acids (L-histidine monohydrochloride, 10 mg; DL-methionine, 20 mg; DL-tryptophan, 20 mg) were weighed out before each experiment and dissolved in water (98 ml). A stock vitamin solution (1.0 ml, containing myo-inositol 2000 µg/ml; calcium DL-pantothenate, 400 µg/ml; D-biotin 2 µg/ml) and a trace element solution (1.0 ml, containing boric acid, 500 µg/ml; $CuSO_4 \cdot 5H_2O$, 40 µg/ml; KI, 100 µg/ml; $FeCl_3$, 200 µg/ml; $MnSO_4 \cdot H_2O$, 400 µg/ml; $Na_2MoO_4 \cdot 2H_2O$, 200 µg/ml; $ZnSO_4 \cdot 7H_2O$, 400 µg/ml) were added.

Preparation of the cell inoculum

A suspension of S. cerevisiae (ATCC 24903) in sterilized water was prepared according to the procedure described in Part A.

(b) Growth on various carbon sources (Tables 11 and 12)

The carbon source (e.g., alanine, 178 mg) was dissolved in water (20 ml) and filter sterilized (0.45 μm pore diameter membrane). A portion (5 ml) was transferred aseptically by pipette to a 250 ml Erlenmeyer culture flask, fitted with a side-arm for optical density measurements, which contained sterilized water (40 ml). Previously, the filter sterilized (0.45 μm pore diameter membrane) basal salts medium (5.0 ml) had been transferred aseptically to the culture flask. The usual concentration of carbon source tested was 10 mM. In an effort to stimulate the yeast to adapt to the carbon source, the medium was supplemented with a small amount of D-glucose (1 mM).[242] After inoculation from a cell suspension of S. cerevisiae (ATCC 24903) the cultures were incubated at 27 ± 1°C on a water-bath shaker.

Experiments which tested the ability of amino acids to support growth are summarized in Table 11. Of those tested, only L-glutamine supported growth. After a lag phase of 4-6 h, logarithmic growth continued for approx. 16 h followed by a stationary phase of approx. 24 h and then a second logarithmic phase of approx. 20 h (Figure 38). The cells became pink in colour during the second logarithmic phase and attained a cell density of approx. 6×10^7 cells/ml.

Table 12 summarizes the experiments in which carbohydrates and related compounds were tested as carbon sources. Potassium acetate

Table 11 Growth of S. cerevisiae (ATCC 24903) on various amino acids

Amino Acid	Concentration (mmol/litre)	Time of Incubation (h)	Amount of Growth (Klett Units)	Growth Stimulated 1 mM Glucose
L-Alanine	10	43	0	--
L-Arginine	10	97	0	No
L-Aspartic Acid	10	150	0	No
L-Asparagine	10	150	0	No
L-Glutamic Acid	10	97	0	No
L-Glutamine	25	70	208	--
	50	70	256	--
Glycine	28	50	0	--
L-Leucine	10	140	0	--
L-Methionine	10	50	0	--
L-Proline	10	97	0	No
DL-Serine	20	140	0	--
DL-Valine	20	140	0	--

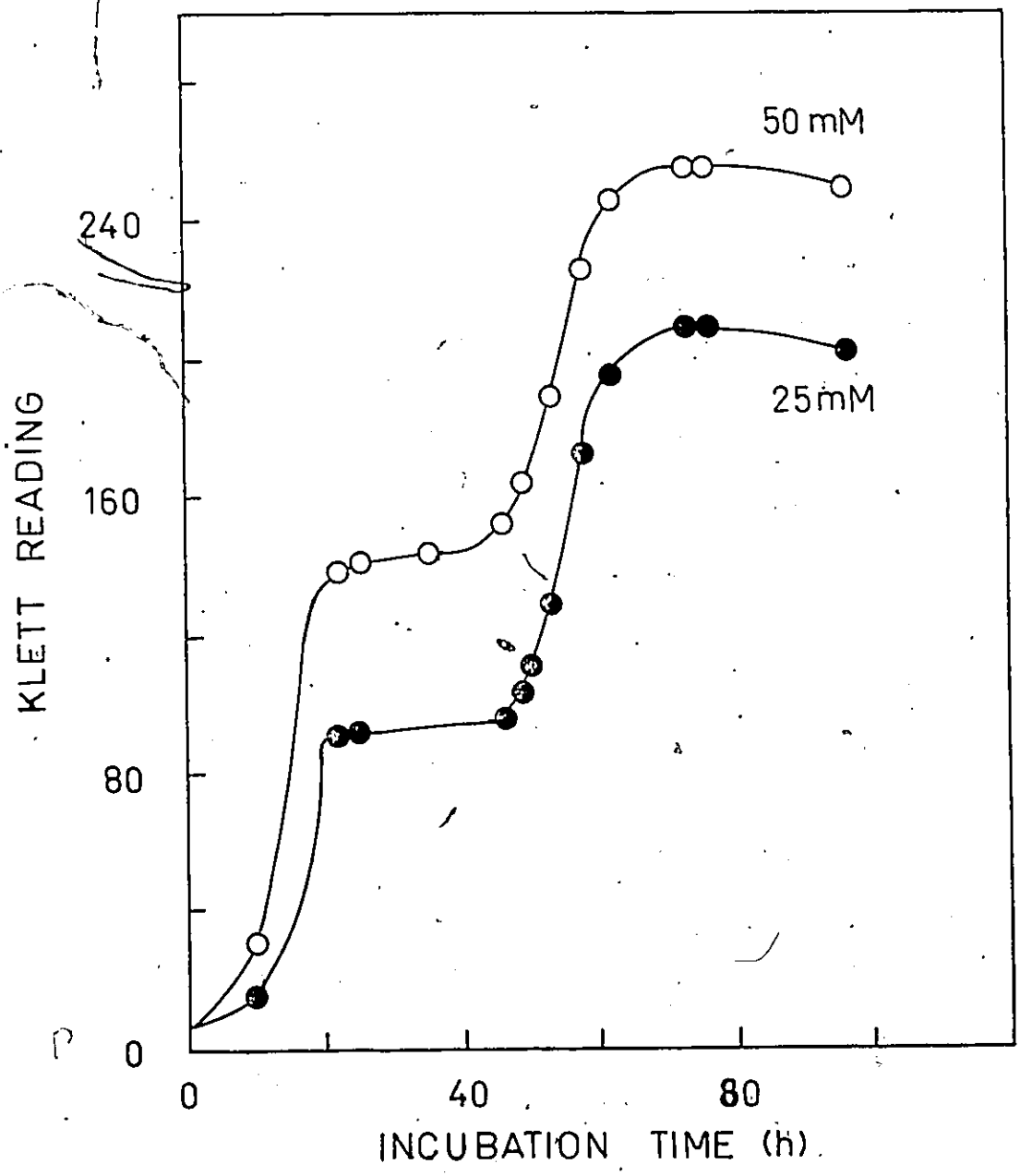


Figure 38: Growth of S. cerevisiae (ATCC 24903) with L-glutamine (50 mM, ○ ; 25 mM, ●) as the carbon source.

Table 12 Growth of *S. cerevisiae* (ATCC 24903) on carbohydrates and related compounds

Compound	Concentration (mmol/litre)	Time of Incubation (h)	Amount of Growth (Klett Units)	Stimulated by 1 mM Glucose
Potassium Acetate	10	40	55	--
Sodium Acetate	6	38	50	--
Ethanol	100	90	350	--
Glyoxylic acid	10	97	0	No
Lactic acid	10	100	50	--
	20	190	105	--
Glycerol	220	150	0	--
*	220	150	390	--
D-Arabinose	10	76	0	--
L-Arabinose	10	46	0	--
D-Arabitol	10	76	0	--
L-Arabitol	10	46	0	--
D-Ribose	25	150	0	--
*	25	150	0	--
D-Ribulose	7	170	0	No
Xylitol	10	46	0	--
D-Xylose	10	46	0	--
L-Xylose	10	76	0	--
D-Gluconic acid	10	48	0	No
D-Glucose	10	25	196	--
D-Glucuronic acid	10	46	0	--

* Supplemented with L-glutamic acid hydrochloride (1 g/litre).

(curve A, Figure 39), sodium acetate and lactic acid (curve B, Figure 39) supported various amounts of growth. When ethanol was used as the source of carbon, the lag phase and the amount of growth obtained were quite dependent upon the concentration of ethanol in the culture medium (Figure 40) but the rate of logarithmic growth and the total growth were second only to glucose. Glycerol did not support growth unless the medium was supplemented with L-glutamic acid (5.5 mM) (Figure 41).

(c) Growth on thiamin-free L-glutamine medium

The basal salts medium was supplemented with L-glutamine (3.65 g/100 ml) and filter sterilized (0.45 µm pore diameter membrane). Portions of the sterilized medium (5 ml) were transferred aseptically by pipette to 250 ml Erlenmeyer flasks that contained sterilized water (45 ml). After inoculation from a cell suspension of S. cerevisiae (ATCC 24903) (0.5 ml), the cultures were incubated at 27 ± 1°C on a water-bath shaker.

(d) Growth on thiamin-free ethanol medium

The ingredients of the basal salts medium were dissolved in water (95 ml) and ethanol (95%, 5 ml) was added to bring the volume of the medium to the standard size (100 ml) before filter sterilization (0.45 µm pore diameter membrane). Portions of the sterilized medium (5 ml) were transferred aseptically by pipette to 250 ml Erlenmeyer flasks that contained sterilized water (45 ml). After inoculation from a cell suspension of S. cerevisiae (ATCC 24903) (0.5 ml), the cultures were incubated at 27 ± 1°C on a water-bath shaker.

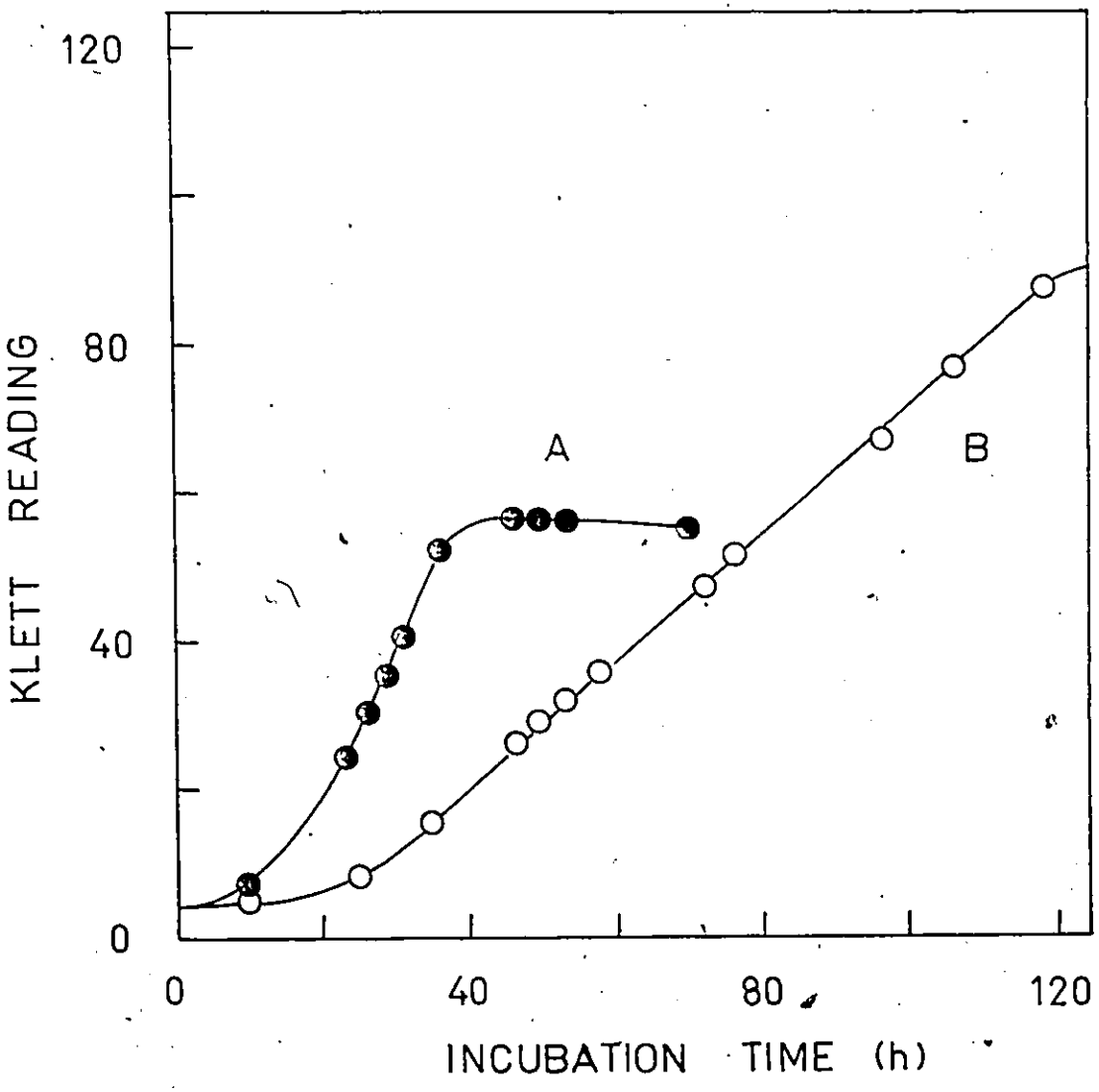


Figure 39: Growth of S. cerevisiae (ATCC 24903) with potassium acetate (10 mM; curve A, ●) or lactic acid (20 mM; curve B, O) as the carbon source.

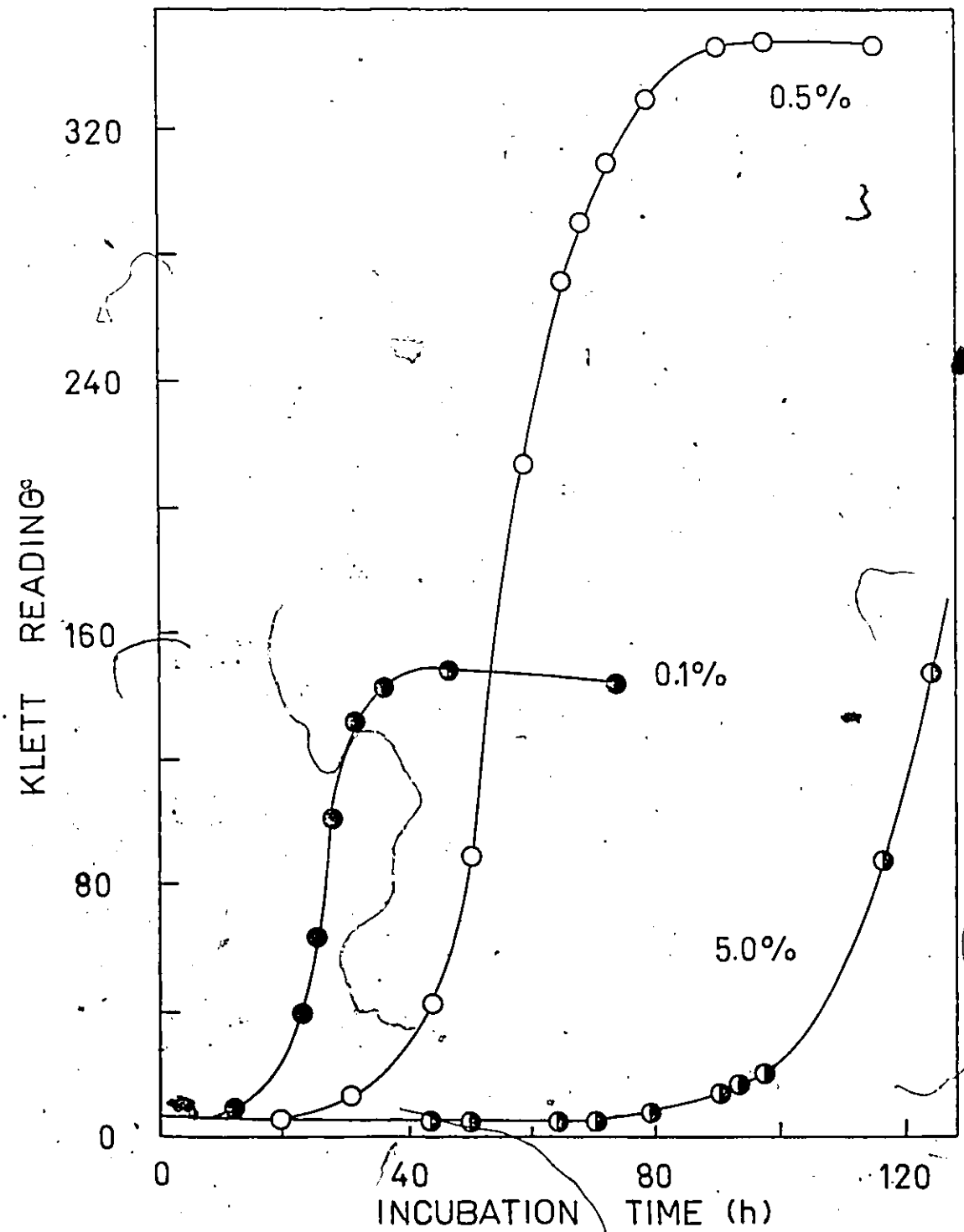


Figure 40: Growth of S. cerevisiae (ATCC 24903) with ethanol (0.1%, ● ; 0.5%, ○ ; 5.0%, ◑) as the carbon source.

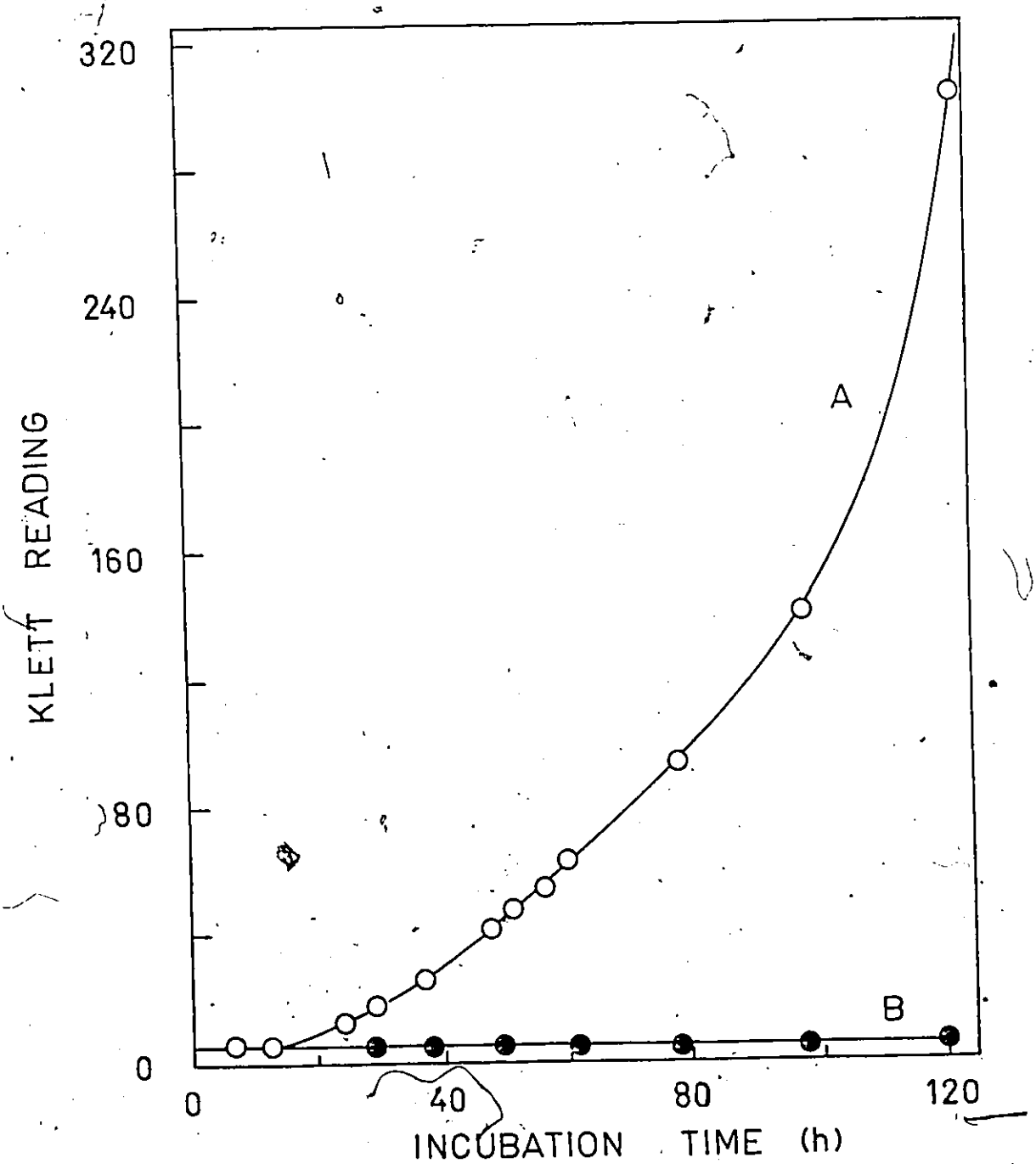


Figure 41: Growth of S. cerevisiae (ATCC 24903) with glycerol as the carbon source (curve B, ●) with glycerol (220 mM) supplemented with L-glutamic acid (5.5 mM) (curve A, O).

(e) Growth on thiamin-free glutamic acid-ethanol medium

The basal salts medium which contained ethanol (95%, 5 ml/100 ml) and L-glutamic acid hydrochloride (1 g/100 ml) was prepared and filter sterilized (0.45 μm pore diameter membrane). Portions of the sterilized medium (5 ml) were transferred aseptically by pipette to 250 ml Erlenmeyer flasks containing sterilized water (45 ml). After inoculation from a cell suspension of S. cerevisiae (ATCC 24903) (0.5 ml), the cultures were incubated at 27 ± 1°C on a water-bath shaker.

(f) Growth curve measurements

The yeast cultures for which growth was monitored by optical density measurements were grown in 250 ml Erlenmeyer flasks which had been modified to include a side-arm which fitted the Klett-Summerson Photoelectric Colorimeter, Model 900-3. The colorimeter was fitted with a blue filter (Number 42) and the optical density was recorded at 400-465 nm in Klett Units. A Klett Unit is related to absorbance by the following equation:[265]

$$\text{Absorbance} = \frac{\text{Klett Unit} \times 2}{1000}$$

This procedure eliminated the need to remove a sample of the culture and thus lowered the chance of contamination. At the conclusion of these experiments, the cell density was determined with an American Optical Co. Brightline haemocytometer and the culture was examined visually and microscopically for contamination.

(g) Radioactive-tracer experiments

Eight experiments with radioactive tracers were carried out

and the details are summarized in Table 5. Solutions of the ^{14}C tracer and the ^{3}H tracer were mixed to prepare a feeding solution of an intermolecularly doubly labelled compound.

(h) Addition of radioactive tracer to the culture

The commercially supplied radioactive compound was dissolved in sterilized distilled water (e.g., 50 ml). The solution was divided equally among the 250 ml Erlenmeyer flasks (e.g., 1.8 ml in each of 28 flasks) that contained the growing yeast cultures (50 ml/flask) at the onset of logarithmic growth, i.e., when the cell density had reached approximately 10^6 cells/ml or 10 Klett units. The culture was examined microscopically to check for contamination before the tracer was added.

Tracer solution was added to S. cerevisiae (ATCC 24903) growing on thiamin-free L-glutamine medium (Expts. 16 and 17) after 6 h of incubation and the cells were collected 66 h later.

After approx. 35 h of incubation, tracer solution was added to S. cerevisiae (ATCC 24903) growing on thiamin-free ethanol medium (Expts. 18-22) and the cells were collected 40 h later.

When L-glutamic acid was added to the thiamin-free ethanol medium (Expt. 23), the logarithmic growth of S. cerevisiae (ATCC 24903) not only started earlier in the incubation, but proceeded at a faster rate. Thus, the tracer solution was added after approx. 26 h of incubation and the cells were harvested 34 h later.

The cells were harvested by centrifugation [20 min at 10°C and 900 g (r_{av} 13 cm)] when maximum growth had been attained and were either extracted immediately or stored at -8°C until required. A portion (20 µl) of the supernatant was counted to determine the amount of radio-

activity that had not been consumed by the cells (Table 5).

(i) Extraction and isolation of thiamin

Thiamin was extracted from the cells, isolated, purified and diluted with carrier thiamin as described in Part A.

(j) Degradation of thiamin

Thiamin was degraded by bisulfite cleavage and the thiazole unit (5) was converted to its phthalimido derivative (94) as described in Part A.

(k) Degradation of 5-(β-hydroxyethyl)-4-methylthiazole (5) C-7 by difference

Preparation of pyridinium dichromate[266]

Pyridine (41 ml, 0.5 mol) was added gradually to a cooled solution of chromium trioxide (50 g, 0.5 mol) in water (50 ml) while the temperature was kept at less than 30°. Acetone (200 ml) was added and the mixture was cooled to 0°C. The orange crystals were filtered off and dried to a constant weight in vacuo giving 68 g (73%): m.p. 138-140°C (Lit. 144-146°C).[266]

Oxidation of 5-(β-hydroxyethyl)-4-methylthiazole (5) with pyridinium dichromate[266]

Pyridinium dichromate (380 mg, 1 mmol) was suspended in methylene chloride (1.5 ml). One drop of water (approx. 50 mg) was added and the mixture was stirred until the reagent and the water formed a homogeneous phase. 5-(β-Hydroxyethyl)-4-methylthiazole (24 mg, 0.16 mmol) in methylene chloride (1.5 ml) was added and stirring was continued at

room temperature for 24 h. Ether (10 ml) was added and the reaction mixture was decanted. The black, gummy residue was extracted with ether (3 x 10 ml). All extracts were combined and filtered through silica gel (60-200 mesh). The colourless filtrate was evaporated to dryness in vacuo. Sublimation at room temperature and 2 x 10^{-2} mmHg (2.7 Pa) yielded 5-formyl-4-methylthiazole (98) (11 mg, 52%): m.p. 66-70°C (Lit. 72.5°C[135] and 75°C[137]), ^{1}H nmr ($CDCl_3$) δ: 2.79 (s, 3H), 9.02 (s, 1H), 10.18 (s, 1H); ms m/e: 127 (M^+).

5-Formyl-4-methylthiazole semicarbazone (99)

5-Formyl-4-methylthiazole (7 mg) was dissolved, along with sodium acetate (10 mg) and semicarbazide hydrochloride (8 mg), in water (0.5 ml). A white precipitate formed immediately. It was dissolved in hydrochloric acid (0.1 M), and neutralization with ammonia (10% v/v) gave 5-formyl-4-methylthiazole semicarbazone (7 mg, 69%): m.p. 240-241°C (Lit. 241°C)[135] ^{1}H nmr (CD_3SOCD_3) δ: 2.41 (s, 3H), 6.28 (s, 2H), 8.13 (s, 1H), 8.96 (s, 1H), 10.26 (s, 1H); ms m/e: 184 (M^+).

(1) Degradation of 4-methyl-5-(β-phthalimidoethyl)thiazole (94) C-4,-4' as acetic acid

Kuhn-Roth oxidation of 4-methyl-5-(β-phthalimidoethyl)thiazole (94)[240]

The Kuhn-Roth procedure described in Part A was followed. Typically, 4-methyl-5-(β-phthalimidoethyl)thiazole (57 mg) was oxidized to sodium acetate (26 mg) in yields (92%) comparable to those of the thiazole phenylurethane (92).

C4' as methylamine

Schmidt reaction on acetic acid[267] and conversion of resulting methylamine into N-methylphthalimide[268]

Sodium acetate (18 mg) was dissolved in conc. sulfuric acid (1 ml). Sodium azide (60 mg) was added and the mixture was heated on a steambath in a flask attached to a system of three gas traps. The carbon dioxide which evolved was passed by means of a stream of nitrogen into potassium hydroxide solution (15%, w/v). When gas evolution had ceased (approx. 4 h), the potassium hydroxide solutions in the traps were replaced by hydrochloric acid (1 M, 1 ml per trap). The acidic reaction mixture was cooled to near its freezing point in a dry ice-acetone bath, basified (to pH > 12) by addition of potassium hydroxide solution (15%, w/v), and was then heated 1.5 h on the steambath while the system was flushed with nitrogen.

N-Carbethoxyphthalimide (32 mg) was added to the hydrochloric acid solutions contained in the first two traps. The mixture was stirred and solid sodium carbonate was added to bring the mixture to about pH 9 and it was stirred at room temperature for 1 h. The precipitate was collected by filtration and then sublimed at 75°C and 2 x 10^{-2} mmHg (2.7 Pa). Recrystallization from methanol yielded pure N-methylphthalimide (9 mg, 42%). Thin layer chromatography (silica gel/ethyl acetate: cyclohexane, 1:4, v/v) was used to monitor the presence of N-carbethoxyphthalimide (R_f 0.15) in N-methylphthalimide (R_f 0.27). M.p. 134-135°C (Lit. 134°C),[269] ^{1}H nmr ($CDCl_3$) δ: 3.17 (s, 3H), 7.61-7.94 (m, 4H); ms m/e: 161 (M^+). Potassium phthalimide and methyl iodide were stirred

in dimethylformamide for 3 h at room temperature. N-Methylphthalimide so formed was identical to the above sample.

C-5,-6,-7 as N-phthaloyl-β-alanine (100)

Oxidation of 4-methyl-5-(β-phthalimidoethyl)thiazole (94) with potassium permanganate[270]

4-Methyl-5-(β-phthalimidoethyl)thiazole (94) (21 mg) was suspended in dilute sulfuric acid (0.5 M, 1.5 ml) at room temperature. Potassium permanganate (80 mg) was added in portions over a 1 h period and the mixture was stirred for an additional 2 h. It was decolourized by adding sodium hydrogen sulfite, and extracted with ether (4 x 5 ml). The ether extracts were combined, washed with water (1 x 5 ml), dried over anhydrous magnesium sulfate and evaporated in vacuo to dryness. The residue was sublimed at 105°C and 2 x 10^{-2} mm Hg (2.7 Pa) and recrystallized from water to yield pure N-phthaloyl-β-alanine (9 mg, 53%): m.p. 150-151°C (Lit. 150-151°C),[271] ^{1}H nmr (CD_3COCD_3) δ: 2.74 (t, 2H), 3.94 (t, 2H), 7.83 (s, 4H); ms m/e: 219 (M^+). This sample was identical with one obtained by fusion[272] of β-alanine with phthalic anhydride at 170°C for 15 min.

(m) Degradation of D-[6-^{3}H,6-^{14}C]glucose (Expt. 21)

Carrier D-glucose (500 mg) was added to a small portion (approx. 3 μCi) of the D-[6-^{3}H,6-^{14}C]glucose feeding solution and this mixture was diluted to 100 ml with water.

β-D-Glucose pentaacetate[273]

A portion (approx. 10 ml) of the above D-[6-^{3}H,6-^{14}C]glucose

solution was evaporated to dryness in vacuo. Anhydrous sodium acetate (47 mg) and acetic anhydride (1 ml) were added to this noncrystalline residue (56 mg). The mixture was heated on a steambath for 2.5 h and ice water (5 ml) was added. An oil separated from solution at this point, but when it stood at 4°C for 2 days, it became crystalline. The crystals were filtered off and sublimed at 120°C and 2 x 10^{-2} mm Hg (2.7 Pa) to yield pure β-D-glucose pentaacetate (42 mg, 34%): m.p. 131-132°C (Lit. 131-132°C).[273]

C-6 of D-[6-^{3}H,6-^{14}C]glucose as formaldehyde dimethone[274]

A small amount (approx. 6 ml) of the above D-[6-^{3}H,6-^{14}C]-glucose solution was evaporated to dryness in vacuo. The noncrystalline residue (33 mg) was redissolved in water (2 ml), sodium hydrogen carbonate solution (1 M, 2 ml) and sodium metaperiodate (200 mg) in water (2 ml) were added, and the mixture was kept at room temperature for 1 h. 5,5-Dimethylcyclohexane-1,3-dione (dimedone) (100 mg) in 95% ethanol (1 ml) was added and, after 10 min at room temperature, the white product was collected by filtration. Sublimation at 110°C and 2 x 10^{-2} mm Hg (2.7 Pa) yielded pure formaldehyde dimethone (53 mg, 98%): m.p. 190-192°C (Lit. 191°C).[239]

PART C Future Investigations

Identification of the basic precursors is a necessary first step towards the complete elucidation of a biosynthetic pathway. The present work has provided a good deal of evidence about the nature of the precursors of the thiazole unit of thiamin. However, the intact incorporation of a C-N unit from glycine and the exact identity of the pentose precursor are two aspects of thiazole biosynthesis which require more evidence before they can be considered proved.

The incorporation of both the methylene carbon[124,209] and the nitrogen atom[134] of glycine into the thiazole moiety suggests that these atoms are incorporated as an intact unit. However, the cleavage of the C-N bond of glycine and incorporation of the atoms by separate routes cannot by ruled out by the above experiments. A feeding experiment carried out with [^{15}N,2-^{13}C]glycine would conclusively prove, or disprove, the intact incorporation of the C-2-N fragment of glycine. If the unit enters intact, analysis of thiamin by ^{13}C nuclear magnetic resonance spectroscopy would show the resonance due to C-2 of the thiazole ring to be a doublet due to the neighbouring ^{15}N atom. A complementary result would be seen in the ^{15}N nuclear magnetic resonance spectrum. The success of this experiment depends not only on a good incorporation of [^{15}N,2-^{13}C]glycine into thiamin, but also on the magnitude of the coupling. The coupling constant between the ^{13}C and ^{15}N nuclei in these positions would have to be large enough for the doublet signals to be resolved.

The scheme in Figure 32 requires the loss of only one of the methylene protons of glycine and it is possible that this loss is stereo-

specific. It would be interesting to determine whether the proton retained in the thiazole from glycine is the pro-R proton, i.e., the proton which corresponds in stereochemistry to the α-proton of L-tyrosine. In order to carry out this experiment, a system would be required which produces only the thiazole moiety, not thiamin, because of the ready exchange of the proton at this position in thiamin.

Additional experiments with ^{14}C labelled glucoses would provide no additional information, but would only confirm the origin of a 2-ketopentose by the two pathways. In any case, D-[3-^{14}C]-, D-[4-^{14}C]- and D-[5-^{14}C]glucose are not commercially available and the cost (> $4000.00 per experiment) of D-[3,4-^{14}C]glucose makes the use of this precursor unattractive. The incorporation of the hydrogen atoms of glucose into the thiazole nucleus can be tested with tritiated glucoses and these compounds are readily available. Activity from both D-[1-^{3}H]- and D-[5-^{3}H]-glucose, if exchange does not occur, should be incorporated, but label from D-[3-^{3}H]- and D-[4-^{3}H]glucose would be lost as these positions of glucose become quaternary sites in the thiazole moiety. Label from D-[2-^{3}H]glucose, barring exchange, should be incorporated into the thiazole unit via the gluconic acid pathway but not the transketolase route of 2-ketopentose formation.

Additional evidence could be obtained from a [2-^{14}C]glycerol experiment. The results of this experiment would complement those of the [1,3-^{14}C]glycerol feeding.

None of these feedings would prove conclusively that a 2-ketopentose is a direct precursor of thiamin. This could be shown by feeding labelled pentoses, but these compounds are not fermented by S. cerevisiae

due to the absence of the required kinases. Thus, if a phosphorylated 2-ketopentose is the immediate precursor of the thiazole unit, then no incorporation would be expected in these experiments. There are two ways around this problem: either an organism could be chosen which ferments pentoses (e.g., Candida utilis); or, cell-free extracts of S. cerevisiae could be used. The use of C. utilis or a similar organism would require preliminary experiments to determine whether the basic precursors of the thiazole moiety are the same as in S. cerevisiae. Chemical synthesis of labelled pentoses would also be necessary, since D-[1-^{14}C]ribose is the only specifically labelled pentose that is commercially available. Investigations which employ a cell-free extract would require the establishment of such a system before feeding experiments could be carried out. In addition, the preparation of specifically labelled phosphorylated pentoses may be required as the unphosphorylated pentoses might not be metabolized.

After some preliminary development, either of these two new approaches may yield results which extend the knowledge gained by the present work.

CHAPTER III

PRELIMINARY CONCLUSIONS ON THE BIOSYNTHESIS OF THE PYRIMIDINE MOIETY

(i) Glycine as a precursor

The incorporation of glycine into the pyrimidine moiety of thiamin has been shown in S. typhimurium[195,196,198,199] and E. coli.[145,200] The results of these experiments have been consistent with Route F in Figure 16. Incubation of S. cerevisiae (ATCC 24903 and 39916 H.J. Bunker) with [2-^{14}C]glycine yielded radioactive thiamin, but the activity was located completely (~ 99%) in C-2 of the thiazole moiety (Table 4). This result indicates that glycine is not a precursor of the pyrimidine nucleus in yeast.

(ii) Aspartic acid as a precursor

Aspartic acid (56) has also been suggested as a precursor of the pyrimidine nucleus (Figure 11), but the hypothesis based on the nucleic acid pathway (Route A, Figure 11) was rejected since uracil (58)[180] and orotic acid (57)[128,180] were found not to be incorporated into thiamin. These results do not exclude the incorporation of aspartate (56) in a manner different from its entry into the nucleic acid pyrimidines. Such a proposal (Route C, Figure 11) was put forth by Tomlinson et al.[129] who reported that [U-^{14}C]aspartate was incorporated into the pyrimidine moiety. On degradation, they recovered most of the activity in the region of the predicted incorporation. The incorporation of aspartate was also reported by Nakamura et al.[181] However,

other reports have indicated that aspartate is not incorporated into the pyrimidine unit.[133,134,180]

A third hypothesis (Figure 42) can be proposed to account for the incorporation of aspartic acid (56). In contrast to the nucleic acid hypothesis (Route A, Figure 11), C-7, rather than C-4, is predicted as the position derived from C-4 of aspartate. The hypothesis of Tomlinson et al. (Route C, Figure 11) suggested that C-6 of the pyrimidine ring would be labelled by [4-^{14}C]aspartate. Both the hypothesis in Figure 41 and the Tomlinson proposal require a C_2 unit, for C-2,-2' of the pyrimidine nucleus, and a C_1 unit, such as formate, as the precursor of C-4 of the pyrimidine ring. The latter carbon atom has been shown to be derived from formate in yeast.[184]

The incorporation of aspartic acid (56) as indicated in Figure 42 is not without precedent. A portion of the nicotinic acid molecule is derived from aspartic acid in such a manner.[275] The relationship of the proposed intermediates (133, 134, and 135) is based on similar transformations found in the biosynthesis of the nucleic acid pyrimidines and in nicotinic acid biosynthesis.

A feeding experiment carried out with [4-^{14}C]aspartic acid and S. cerevisiae (ATCC 24903) would confirm the incorporation or the non-incorporation of aspartate. If incorporation were obtained, degradation of the labelled thiamin would distinguish between the two alternative hypotheses.

A significant incorporation of activity into the pyrimidine moiety was obtained when S. cerevisiae (ATCC 24903) was grown in thiamin-free ethanol medium in the presence of [^{14}C]hexoses or

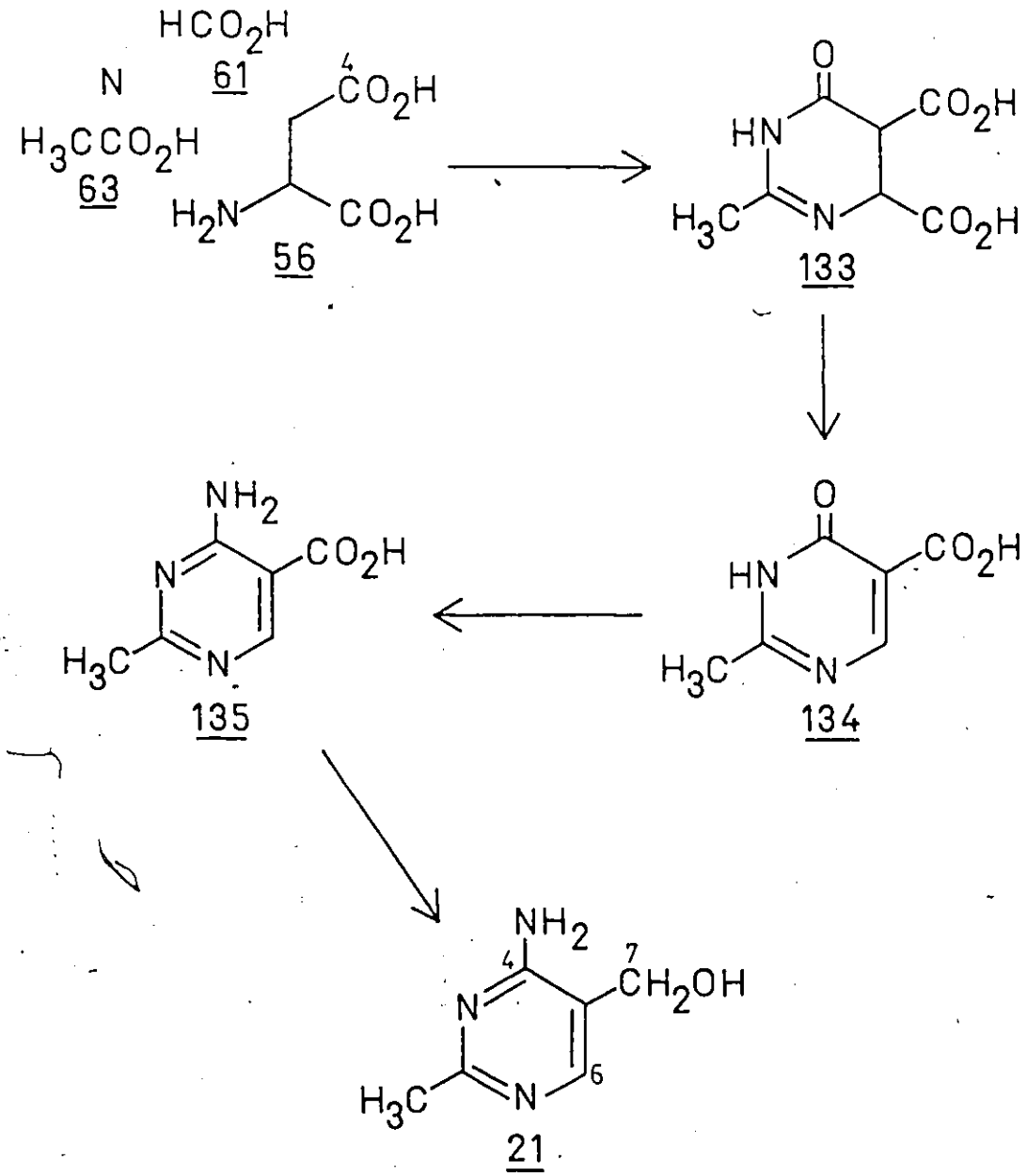


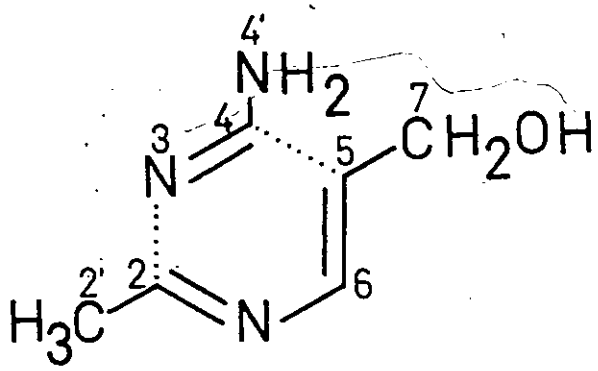
Figure 42: Biogenetic hypothesis for the origin of the pyrimidine moiety of thiamin from aspartic acid.

[1,3-^{14}C]glycerol (Table 6). In each experiment, the incorporation was equal to or greater than that found in the thiazole nucleus. Under the conditions of the experiments, the activity from glucose, fructose, or glycerol probably would have been diluted by the carbon source, ethanol, before it could have labelled the intermediates of the Krebs' cycle. It is very likely that aspartic acid (56), which is synthesized from oxaloacetate, would not be labelled to the same extent as the thiazole precursors. Thus, aspartic acid does not appear to be a precursor of the pyrimidine unit.

(iii) A new hypothesis based on compounds derived from glucose

The incorporation of activity from hexoses and glycerol in both units of thiamin suggests that the precursors of these two units are the same or that they are closely related. An indication as to the identity of the pyrimidine precursors may be obtained by the comparison of the structure of the pyrimidine moiety of thiamin to that of pyridoxol (136) (Figure 43). A C_5N unit is common to both structures, but the unit which occupies positions 3, 4 and 4' is different. The molecules are dissected to illustrate these features. It is possible that the identical structural units have a similar biosynthetic origin.

The fragmentation shown in Figure 43 suggests that C-4 of the pyrimidine ring is derived from a C_1 unit, and sodium [^{14}C]formate has been incorporated into this position.[184] The preliminary experiment reported in Chapter IIA (Expt. 2) indicated that formate was incorporated, but the feeding should be repeated to obtain sufficient



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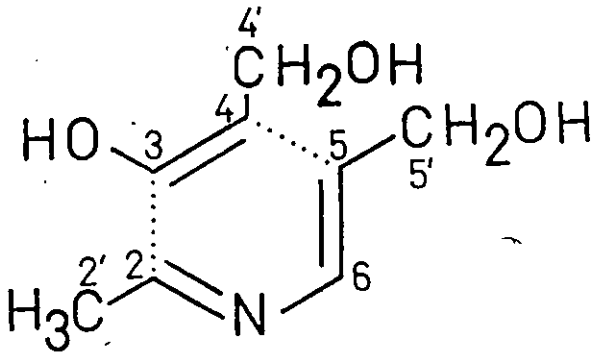

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Figure 43:. Structural similarities of the pyrimidine moiety of thiamin and pyridoxol.

activity for degradation and determination of the exact site of the label in the pyrimidine moiety. The conversion of serine to glycine donates C-3 of serine to the C_1 pool. The activity located in the pyrimidine nucleus from the L-[3-^{14}C]serine feeding (Expt. 13) could have been incorporated by this route.

A new hypothesis based on the reported incorporation of formate and the available evidence for the biosynthesis of pyridoxol (136),[267] is presented in Figure 44. Activity from both [^{14}C]glycerol and [^{14}C]-glucose is incorporated into the pyridoxol molecule and the incorporation of these substrates might be expected to follow a similar pattern in the biosynthesis of the pyrimidine moiety. Carbons 5, 5' and 6 of pyridoxol are derived from C-4,-5,-6 of glucose, whereas the remaining carbon atoms are more closely related to C-1,-2,-3 of glucose.[276] On this basis, most of the activity in the pyrimidine derived from D-[6-^{14}C]glucose would be located in C-7 while most of the activity derived from D-[1-^{14}C]glucose would be located in C-2'. These results would be consistent with D-glyceraldehyde 3-P (18) and acetyl phosphate (20) as the basic precursors (Figure 44).

As described in Chapter I, acetyl phosphate (20) is formed from either D-fructose 6-P (14) or D-xylulose 5-P (115) via the phosphoketolase reaction (Figure 3). If D-fructose 6-P (14) were the substrate for this reaction, then the activity from the D-[2-^{14}C]glucose experiment would be located mostly in C-2 of the pyrimidine nucleus. On the other hand, if D-xylulose 5-P (115) were the immediate precursor of acetyl phosphate (20), then the activity would be distributed over C-2 and C-2' in a ratio which would reflect the relative contributions

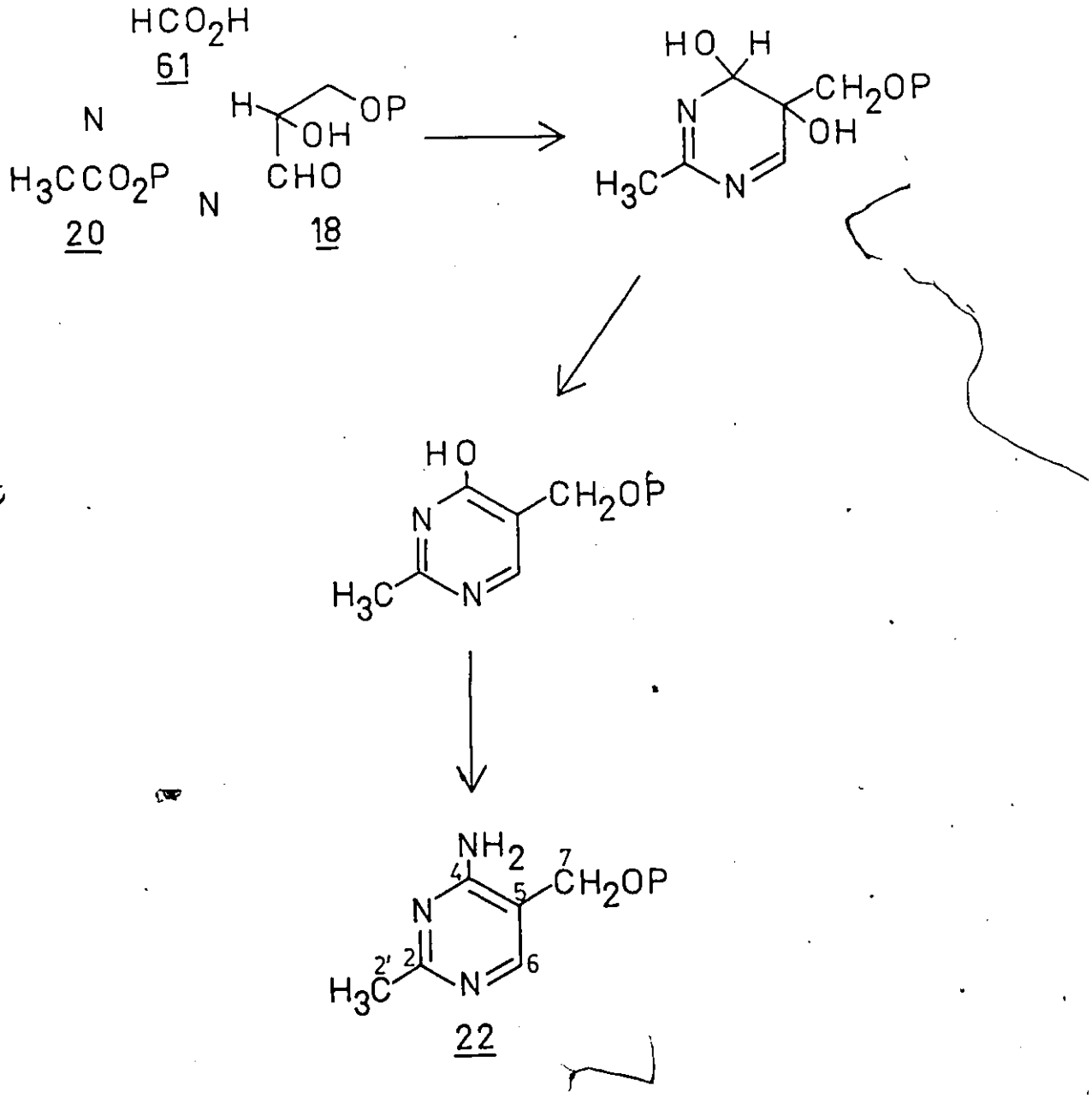

Figure 44: Proposed biogenesis of the pyrimidine moiety of thiamin from acetyl phosphate, formic acid and D-glyceraldehyde 3-phosphate.

of the transketolase and the gluconic acid pathways of pentose formation. This ratio would be expected to be similar to that found in the thiazole moiety from the same feeding.

The degradation of the labelled pyrimidinesulfonic acids, to determine the exact location of the label, is required. However, this constitutes a complete separate investigation. The results of this degradation will either confirm the hypothesis presented here or they will provide evidence for another route. In either instance, the occurrence of different pathways for the biosynthesis of the pyrimidine nucleus of thiamin in yeast and bacteria will be confirmed.

CHAPTER IV

SUMMARY

The biosynthesis of thiamin was investigated by the administration of radioactively labelled compounds to growing yeast cultures. Thiamin chloride hydrochloride was extracted from the yeast cells and, after dilution with carrier, was isolated by ion-exchange and preparative layer chromatography. The radiochemical purity of the recrystallized thiamin chloride hydrochloride was checked by the bisulfite cleavage reaction. The formation of either the crystalline phenylurethane or phthalimido derivatives of 5-(β-hydroxyethyl)-4-methylthiazole improved the purification procedure of this compound. Consequently, activity from the reported precursors, ([1-^{14}C]acetate, L-[Me-^{14}C]-methionine, and DL-[2-^{14}C]tyrosine), and from the suggested precursors, (2-keto[5-^{14}C]glutarate, sodium [3-^{14}C]pyruvate, L-[U-^{14}C]lactate, D-[1-^{14}C]ribose, [1-^{14}C]succinic acid, and DL-[3-^{14}C]cysteine) was found not to enter thiamin in S. cerevisiae (ATCC 24903).

A modified literature procedure was used to isolate C-2 of the thiazole moiety as formaldehyde. Label from [2-^{14}C]glycine, in two strains of S. cerevisiae (ATCC 24903 and 39916 H.J. Bunker), was located only at this position. In contradiction to previous results obtained with S. cerevisiae (39916 H.J. Bunker), L-[Me-^{14}C]methionine did not serve as a precursor of thiamin in this organism. However, the present results were in agreement with those found in a third strain of S. cerevisiae (NCYC 1062).

A degradation procedure was developed to determine the distribution of activity within the C_5 unit of the thiazole moiety. Pyridinium dichromate oxidation of 5-(β-hydroxyethyl)-4-methylthiazole gave 5-formyl-4-methylthiazole (C-7 by difference). Kuhn-Roth oxidation of 4-methyl-5-(β-phthalimidoethyl)thiazole yielded acetic acid (C-4,-4') which was transformed to methylamine (C-4') in a Schmidt reaction. Permanganate oxidation of this derivative yielded N-phthaloyl-β-alanine (C-5,-6,-7). Thus, the activity located in C-4, C-4', C-7 and C-5,-6 could be determined.

A new hypothesis was proposed for the biosynthesis of the thiazole nucleus of thiamin. The formation of a Schiff base by glycine and a 2-ketopentose was suggested as the first step of this scheme. The Schiff base is converted to the thiazole precursor of thiamin in a multistep sequence which comprises dehydration (or elimination of phosphate), dehydration and tautomerization, addition of sulfur, ring closure and concomitant decarboxylation and dehydration. Several variants of this route are shown in Figure 32.

The hypothesis was further tested by the incubation of S. cerevisiae (ATCC 24903) in the presence of labelled substrates. Since ethanol, rather than D-glucose, was used as the carbon source, labelled substrates of glycolysis could be added as tracers. Thus, activity from [1,3-^{14}C]glycerol, D-[1-^{14}C]fructose, D-[U-^{14}C]-, D-[1-^{14}C]-, D-[2-^{14}C]-, D-[6-^{14}C]- and D-[6-^{3}H,6-^{14}C]glucose was incorporated non-randomly into the C_5 portion of the thiazole molecule. The mode of incorporation of [1,3-^{14}C]glycerol and the labelled hexoses indicated that the 2-ketopentose was formed from glycolytic intermediates by two path-

ways: the transketolase route; and the decarboxylation route via D-gluconic acid. The 2-ketopentose is most likely D-ribulose 5-phosphate, or D-xylulose 5-phosphate, or a compound which is closely related to these.

The observed incorporation of sodium [^{14}C]formate and L-[3-^{14}C]-serine into the pyrimidine moiety agreed with previous reports, whereas the incorporation of [1,3-^{14}C]glycerol and the labelled hexoses did not fit the existing theories. A new hypothesis for the biosynthesis of the pyrimidine nucleus of thiamin was proposed to account for these incorporations.

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Thiamin Biosynthesis in *Saccharomyces cerevisiae*

ORIGIN OF CARBON-2 OF THE THIAZOLE MOIETY

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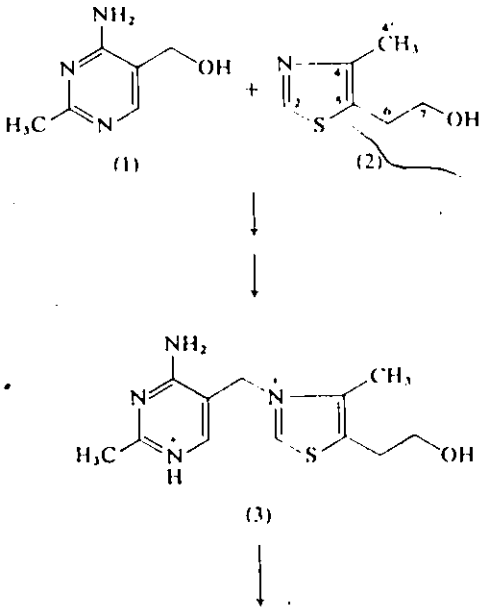
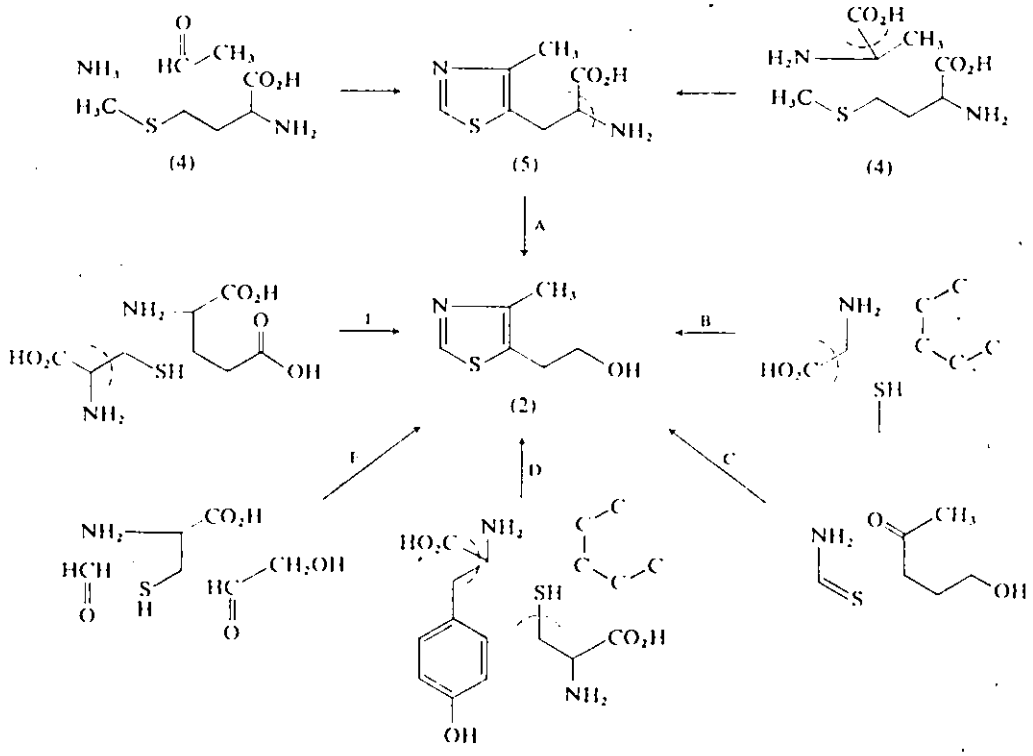
Radioactivity from [2-^{14}C]glycine enters C-2 of the thiazole moiety of thiamin and no other site, in *Saccharomyces cerevisiae* (strains A.T.C.C. 24903 and 39916, H. J. Bunker). Radioactivity from L-[*Me*-^{14}C]methionine or from DL-[2-^{14}C]tyrosine does not enter thiamin.

Although the structure of thiamin (vitamin B_1) has been known for over 40 years (Williams, 1936; Grewe, 1936) and its chemical synthesis accomplished soon thereafter (Williams & Cline, 1936; Andersag & Westphal, 1937; Todd & Bergel, 1937), the biosynthetic pathway to thiamin (3) has still not been elucidated completely. It is well established that the pyrimidine (1) and thiazole (2) moieties are biosynthesized independently and are then joined to give thiamin (Scheme 1) (Leder, 1975). However, there is no general agreement concerning the basic precursors either of the pyrimidine or of the thiazole moieties. Thus, in the case of the thiazole moiety, it has been reported that C-2 of the thiazole nucleus is derived respectively from the α-carbon atom of tyrosine in *Escherichia coli* (Estramareix & Therisod, 1972) and *Salmonella typhimurium* (Bellion *et al.*, 1976), from the *S*-methyl group of methionine in *Bacillus subtilis* (Torrence & Tieckelmann, 1968) and *Saccharomyces cerevisiae* (Johnson *et al.*, 1966), and from C-2 of glycine, also in *S. cerevisiae* (Linnett & Walker, 1967).



Scheme 1. *The final stages of the biosynthesis of thiamin pyrophosphate*

These conflicting experimental claims reflect the several hypotheses (shown in Scheme 2) that have been proposed to account for the origin of the thiazole moiety of thiamin. Thus, Harington & Moggridge (1939) suggested that the thiazole moiety could arise from ammonia, acetaldehyde and methionine (4) via the intermediate α-amino-β-(4-methylthiazol-5-yl)propionic acid (5) (Scheme 2A). They found that this compound was converted into the thiazole moiety by fermenting yeast (Harington & Moggridge, 1940). In further support of this Scheme, it was reported that radioactive label from L-[*Me*-^{14}C]methionine was incorporated into the thiazole moiety of thiamin and that the ^{35}S:^{14}C ratio of L-[*Me*-^{14}C,^{35}S]methionine was maintained in the thiazole moiety (Johnson *et al.*, 1966). From these results it was inferred that the carbon atoms of methionine (other than the carboxy group) accounted for C-5 and the β-hydroxyethyl side chain. Activity from [1-^{14}C]- and [2-^{14}C]-acetate and from L-[U-^{14}C]alanine was also reported to be incorporated and it was assumed that these compounds served as precursors of the C-methyl group and C-4 of the thiazole moiety. Degradations to locate the site of radioactive label were not carried out, however. Evidence contrary to the above view of thiamin biosynthesis emerged from radioactive-tracer experiments with glycine. It was found by unequivocal degradation of the radioactively labelled thiamin



Scheme 2. *Six hypothetical schemes accounting for the biogenetic origin of the thiazole moiety of thiamin (vitamin B_1)* Route A (Harington & Moggridge, 1939; Buchman & Richardson, 1939); Route B (Linnett & Walker, 1969); Route C (Bonner & Buchman, 1938); Route D (Bellion & Kirkley, 1977); Route E (Nakayama, 1956); Route F (Plaut, 1961).

obtained from a feeding experiment with [2-^{14}C]-glycine that most of the activity of the molecule was localized at C-2 of the thiazole nucleus (Linnett & Walker, 1967). Administration of [2-^{14}C]glycine in the presence of unlabelled methionine gave an identical result (Linnett & Walker, 1969), and, in an experiment with [^{15}N]glycine, thiamin was isolated whose thiazole moiety (but not the pyrimidine unit) was enriched with ^{15}N (Linnett & Walker, 1968). Glycine is thus implicated in the biosynthesis of the thiazole unit (Scheme 2B).

Taken at face value the results showing methionine on the one hand and glycine on the other as building units of the thiazole portion of thiamin in *S. cerevisiae* are clearly incompatible.

There are three possibilities to account for these contradictory results. Either there is more than one pathway to thiamin in *S. cerevisiae*, or C-2 of glycine can serve as a precursor of the *S*-methyl group of methionine, or one or the other of the reported results is in error.

As a preliminary step in our own investigation of the biosynthesis of the thiazole moiety of thiamin in *S. cerevisiae*, the origin of C-2 had to be reinvestigated. It is now confirmed that, in yeast, this carbon atom is derived from the methylene group of glycine (Linnett & Walker, 1967, 1968, 1969), and that, contrary to previous reports (Johnson *et al.*, 1966), the *S*-methyl group of methionine is not incorporated. Nor does the α-carbon atom of tyrosine, reportedly a source of C-2 of the thiazole nucleus in bacteria (Estramareix & Therisod, 1972; Bellion *et al.*, 1976), enter this site.

Experimental

Materials

Micro-organisms. Two strains of *Saccharomyces cerevisiae* were used in radioactive-tracer experiments.

S. cerevisiae (A.T.C.C. 24903) was obtained from Dr. J. J. Miller, Department of Biology, McMaster University. Stock cultures were maintained on malt extract/yeast extract/peptone/glucose slants that, after incubation at 27±1 C for 24h, were stored at 4 C and subcultured at periodic intervals.

S. cerevisiae (H. J. Bunker, 39916) was obtained from the Commonwealth Mycological Institute, Kew, Surrey, U.K. After incubation at 27±1 C for 48h on Sabouraud agar slopes, the stock culture was stored at 4 C and subcultured every 2 weeks.

Media. Maintenance media. (i) The composition of malt extract/yeast extract peptone glucose medium (Patel & Miller, 1972) was as follows: Difco malt extract (3g), Difco yeast extract (3g), Difco peptone (5g), D-glucose (20g), KH_2PO_4 (1g), Difco Bacto-agar (20g), water (1 litre). (ii) The composition of Sabouraud agar (Difco Manual, 1953) was Difco neopeptone (10g), D-glucose (40g), Difco Bacto-agar (15g), and water (1 litre).

Thiamin-free medium. A liquid medium, containing no thiamin, was used for all tracer experiments with radioactive substances. It was prepared from Difco Bacto vitamin-free yeast base and supplemented with *myo*-inositol (2000μg/litre), calcium DL-pantothenate (400μg/litre) and D-biotin (2μg/litre). The composition of Difco Bacto vitamin-free yeast base (Difco Manual, 1953) is: $(NH_4)_2SO_4$ (5g), Bacto-dextrose (10g), L-histidine monohydrochloride (10mg), DL-methionine (20mg), DL-tryptophan (20mg), boric acid (500μg), $CuSO_4$ (40μg), KI (100μg), $FeCl_3$ (200μg), $MnSO_4$ (400μg), Na_2MoO_4 (200μg), $ZnSO_4$ (400μg), KH_2PO_4 (1g), $MgSO_4$ (0.5g), NaCl (0.1g), $CaCl_2$ (0.1g), and water (1 litre).

Yeast-extract medium (Johnson *et al.*, 1966). The composition of this medium was Difco yeast extract (2g), D-glucose (20g), $(NH_4)_2SO_4$ (0.943g), KH_2PO_4 (0.5g), $MgSO_4,7H_2O$ (0.5g), $CaCl_2,2H_2O$ (0.3g), and water (1 litre). Difco yeast extract contains thiamin (150μg/g).

Methods

Growth on thiamin-free medium. A fresh slant of yeast cells was prepared by inoculation from a stock culture and incubated for either 24h (*S. cerevisiae*, A.T.C.C. 24903) or 48h (*S. cerevisiae*, 39916, H. J. Bunker) at 27±1 C. The cells were washed with sterilized water (3×25ml). A portion of this cell suspension (0.5ml, i.e., approx. 10^7 cells) was used to inoculate each 50ml portion of the liquid medium, prepared as follows. Difco Bacto vitamin-free yeast base (16.7g) was dissolved in water (100ml) and stock vitamin solution (1.0ml) (containing 2000μg of *myo*-inositol/ml, 400μg of calcium DL-pantothenate/ml, and 2μg of D-biotin/ml) was added. The entire medium was sterilized by filtration through an autoclaved membrane-filter holder (Sartorius G.m.b.H. Sm16510) fitted with a membrane filter (Gelman GN-6, 0.45μm pore diameter; Metricel, 47mm diameter). Portions of the sterilized medium (5ml) were then transferred aseptically by pipette to 250ml Erlenmeyer flasks containing sterilized water (45ml).

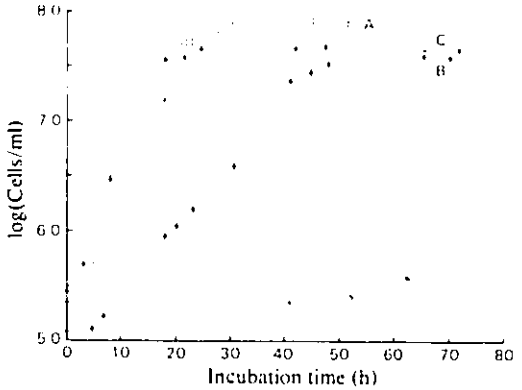


Fig. 1. *Growth of S. cerevisiae (A.T.C.C. 24903) in thiamin-free medium (curve A) and of S. cerevisiae (H. J. Bunker, 39916) in thiamin-free medium (curve B) and yeast-extract medium (curve C)*

After inoculation from the cell suspension, the cultures were incubated with shaking (100 strokes/min, i.e. maximum aeration, at 27±1 C) (Warner–Chilcott Laboratories model 2156 water-bath shaker).

After a short lag phase (2–3h), *S. cerevisiae* (A.T.C.C. 24903) maintained logarithmic growth for 19h of incubation, when a cell density of approx. 8×10^7 cells/ml was attained (Fig. 1, curve A). A longer lag phase (7–8h) was observed in the case of *S. cerevisiae* (39916, H. J. Bunker). Logarithmic growth of this strain was slow, continuing for 48h of incubation. A cell density of approx. 4×10^7 cells/ml was eventually attained (Fig. 1, curve B).

Growth on yeast-extract medium (cf. Johnson et al., 1966). The yeast-extract medium was dispensed into Roux flasks (75ml/flask), which were then plugged with non-absorbent cottonwool and autoclaved at 15lb/in^2 (103.5kPa) for 20min. One flask was chosen and inoculated with a loopful of *S. cerevisiae* (39916, H. J. Bunker) from a Sabouraud agar slant (grown at 27±1 C for 48h) and incubated at 27±1 C for 24h. A sample of this culture (0.5ml) was used to inoculate the remaining Roux flasks, which were incubated for 48h at 27±1 C. Growth of *S. cerevisiae* (39916, H. J. Bunker) on yeast-extract medium was not delayed and was rapid with the logarithmic phase terminating at 20h after inoculation (Fig. 1, curve C).

Growth-curve measurements. A portion of the yeast culture was removed aseptically at various times and was, depending on cell density, either diluted with water or counted directly by using an American Optical Co. Brightline haemocytometer counting chamber.

Radioactive-tracer experiments. All experiments with radioactive tracers were carried out with cultures growing on thiamin-free medium. Details of these experiments are summarized in Table 1. Radioactive

tracer was added at the onset of logarithmic growth and cells were harvested when maximum growth had been attained. The radioactive compound was dissolved in sterilized water and the solution divided equally among 250ml Erlenmeyer flasks containing the growing yeast cultures (50ml flask) when the cell density had reached approx. 10^8 cells/ml. Radioactive tracer solution was added after 5.5h of incubation and the cells collected 43h later in the experiments with *S. cerevisiae* (A.T.C.C. 24903). With *S. cerevisiae* (39916, H. J. Bunker), however, radioactive-tracer solution was added after 15.5h of incubation and the cells were collected 48h later. The cells were collected by centrifugation [20min at 10°C and 900*g* (r_{av} 13cm)]. The different times were necessary because the growth rates of the two organisms were different.

The cells obtained from each experiment were either extracted immediately or stored at −8°C until needed.

Extraction and isolation of thiamin (*cf. Hitchcock & Walker*, 1961; *Johnson et al.*, 1966; *David et al.*, 1967; *Kumaoka & Brown*, 1967; *Bellion et al.*, 1976). The cells obtained by centrifugation as described above were heated on a steambath for 30min with 20ml of 0.1M-HCl. The mixture was centrifuged and the residue re-extracted in the same way. The extracts were combined and 5ml of 1M-sodium acetate buffer, pH4.7, was added. The pH was raised by dropwise addition of NaOH (50%, w/v) to about pH4.7. Thiamin pyrophosphate was hydrolysed by incubating this solution at 37±1°C with 100mg of α-amylase (crude-type IV-A from *Aspergillus oryzae*; Sigma Chemical Co., St. Louis, MO, U.S.A.) overnight. An acid phosphatase contaminant is actually responsible for the hydrolysis (Myrbäck & Neumüller, 1950). The pH of the mixture was then increased to approx. pH6 by dropwise addition of NaOH (50%, w/v). The solution was then applied to a column (1cm×7cm) containing 0.8g of cation-exchange resin (Amberlite CG-50, type I, H^+ form, 100–200 mesh). The column was washed with water (100ml) and eluted with about 30ml of 1M-HCl and the effluent collected. The desired product eluted within 10–15ml.

Thiochrome assay (*cf. Fujiwara & Matsui*, 1953; *Linnett & Walker*, 1968). The thiamin concentration of the column eluate was determined as follows. A portion of the eluate (1.0ml) was mixed with CNBr solution (2.0ml) [prepared by titrating a saturated aq. Br_2 solution at 0°C with aq. KCN (20%, w/v), until colourless] and 1.0ml of aq. NaOH (5%, w/v). The fluorescent product was extracted into 2-methylpropan-1-ol (5.0ml) by vigorously shaking for 2min. The 2-methylpropan-1-ol layer was clarified by centrifugation for 30s. The fluorescence of the 2-methylpropan-1-ol layer was measured with an Aminco-Bowman spectrophotofluorimeter in 1cm Supracil cells. The exciting wavelength was 370nm and fluorescence was measured at 430nm. A standard curve was prepared for each run from solutions ranging in thiamin concentration from 0.2 to 5.0μg/ml. With this curve, the thiamin concentration of the unknown solution was determined from its fluorescence. Before the fluorescence of each sample or standard was measured, the stability of the instrument was checked by using a standard quinine sulphate solution (0.7μg/ml in 0.05M-H_2SO_4) and, if necessary, the value so obtained was used to correct the sample fluorescence.

Purification of thiamin from the radioactive-tracer experiments and dilution with inactive carrier. Carrier thiamin (approx. 1mg) and 5ml of 1M-sodium acetate buffer, pH4.7, were added to the acid eluate from the Amberlite ion-exchange column (see above). The pH of the solution was increased to about pH6 by dropwise addition of NaOH solution (50%, w/v). The solution was applied to an ion-exchange column (Amberlite CG-50) similar to the one described above. The column was washed with water (100ml) and eluted with 500ml of 0.005M-HCl, and the eluate collected in 15ml fractions. The thiamin-containing fractions (usually fractions 12–30) were combined, additional carrier thiamin (approx. 4mg) was added and the solution concentrated *in vacuo*. The residue was further purified by preparative thin-layer chromatography on cellulose (MN300F Normal cellulose on Analtech Uniplate, 20cm×20cm×0.5mm) with 95% ethanol/water (1:1, v/v) as the solvent system. The thiamin (R_f usually 0.8–0.9) was located by its quenching of background fluorescence when the plate was examined under u.v. light. Thiamin was eluted from the cellulose with 95% ethanol. The ethanol extract was evaporated *in vacuo*, and additional carrier thiamin was added to the residue to give a total weight of approx. 50mg. This product was crystallized to constant radioactivity from methanol/propan-2-ol (1:2, v/v).

Determination of radioactivity. Triplicate samples (1–2mg) of each compound were counted by liquid-scintillation counting (Mark I liquid-scintillation computer, model 6860; Nuclear-Chicago Corp., Des Plaines, IL, U.S.A.). Samples were dissolved in water, aq. NH_3 (1%), ethyl acetate, methanol, chloroform or *NN*-dimethylformamide (approx. two drops) and the solutions dispersed in a solution of Aquasol (New England Nuclear Corp., Boston, MA, U.S.A.). The efficiency of counting (approx. 80% for ^{14}C) was determined by external standardization with ^{133}Ba. The usual corrections for quenching and for background radioactivity were applied. Confidence limits shown in the Tables are standard deviations of the mean.

Degradation of thiamin (*see Scheme* 3). Additional inactive carrier (100–300mg) was added to the thiamin before degradation.

(i) Separation of the pyrimidine and thiazole

moieties by bisulphite cleavage (cf. Williams *et al.*, 1935). Thiamin (348mg) was dissolved in water (6ml) and $NaHSO_3$ (1.70g) was added. If necessary, the pH was adjusted to a value of about 4.7 with 0.5M-NaOH. After heating for a few minutes on a steam bath, the pH was checked and readjusted if necessary. The reaction mixture was kept overnight at room temperature (25°C). The white (4-amino-2-methylpyrimidin-5-yl)methanesulphonic acid (6) was filtered off, and recrystallized by dissolving in hot aq. ammonia (1%) and then acidifying with 3M-HCl. The yield was 157mg (82%).

The filtrate was titrated to pH12 with NaOH (50%, w/v) and extracted with chloroform (5×15ml). The chloroform extracts were dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give 5-(β-hydroxyethyl)-4-methylthiazole (2) (125mg, 93%) as a colourless oil. ^1H n.m.r. spectroscopy gave δ (p.p.m.) ([^2H]chloroform) 2.39 (3 H, s), 3.00 (2 H, t), 3.56 (1 H, s), 3.82 (2 H, t), 8.52 (1 H, s).

(ii) Phenylcarbamoyl derivative (7) of 5-(β-hydroxyethyl)-4-methylthiazole [5-(β-hydroxyethyl)-4-methylthiazole phenylurethane]. The thiazole (2) obtained above was dissolved in benzene (5ml) and phenyl isocyanate (0.1ml) added. The solution was kept at room temperature (25°C) overnight, the solvent was evaporated *in vacuo* and the thizaole phenylurethane recrystallized from ethyl acetate. The yield was 195mg (85%). A portion (30mg) was separated from the diphenylurea contaminant by preparative thin-layer chromatography (silica-gel plate, 0.5mm×20cm×20cm) with diethyl ether as the solvent system. A better separation was obtained by developing the chromatogram twice. The band corresponding to the thiazole phenylurethane (R_f 0.5) was eluted with ethyl acetate and the product sublimed at 110°C and 2×10^{-2}mm Hg (2.7Pa). The phenylcarbamoyl derivative (7) of 5-(β-hydroxyethyl)-4-methylthiazole has m.p. 130–131°C; *m/e* 262 (M^+); δ (p.p.m.) ([^2H]acetone) 2.03 (3 H, s), 3.16 (2 H, t), 4.31 (2 H, t), 6.90–7.67 (5 H, m), 8.67 (1 H, s), 8.72 (1 H, s) (Found: C, 59.71; H, 5.47; N, 10.54; $C_{13}H_{14}N_2O_2S$ requires C, 59.52; H, 5.38; N, 10.68%).

Degradation of the thiazole moiety. (a) C-2 as formaldehyde dimethone (cf. Linnett & Walker, 1967). (i) 5-(β-Hydroxyethyl)-4-methylthiazole phenylurethane methiodide (8). The thiazole phenylurethane (7) (69mg) was dissolved in benzene (4ml), excess methyl iodide was added and the mixture was refluxed for 24h. The thiazole phenylurethane methiodide (8) was filtered off and recrystallized either from propan-2-ol or 95% ethanol/ethyl acetate (1:3, v/v) (yield 76mg; 71%). The methiodide (8) had m.p. 164–165°C; δ (p.p.m.) ([^2H]methanol) 2.52 (3H, s), 3.34 (2 H, t), 4.13 (3 H, s), 4.40 (2 H, t), 7.00–7.53 (5 H, m) (Found: C, 41.79; H, 4.35; N, 6.75; $C_{14}H_{17}IN_2O_2S$ requires C, 41.50; H, 4.24; N, 6.93%).

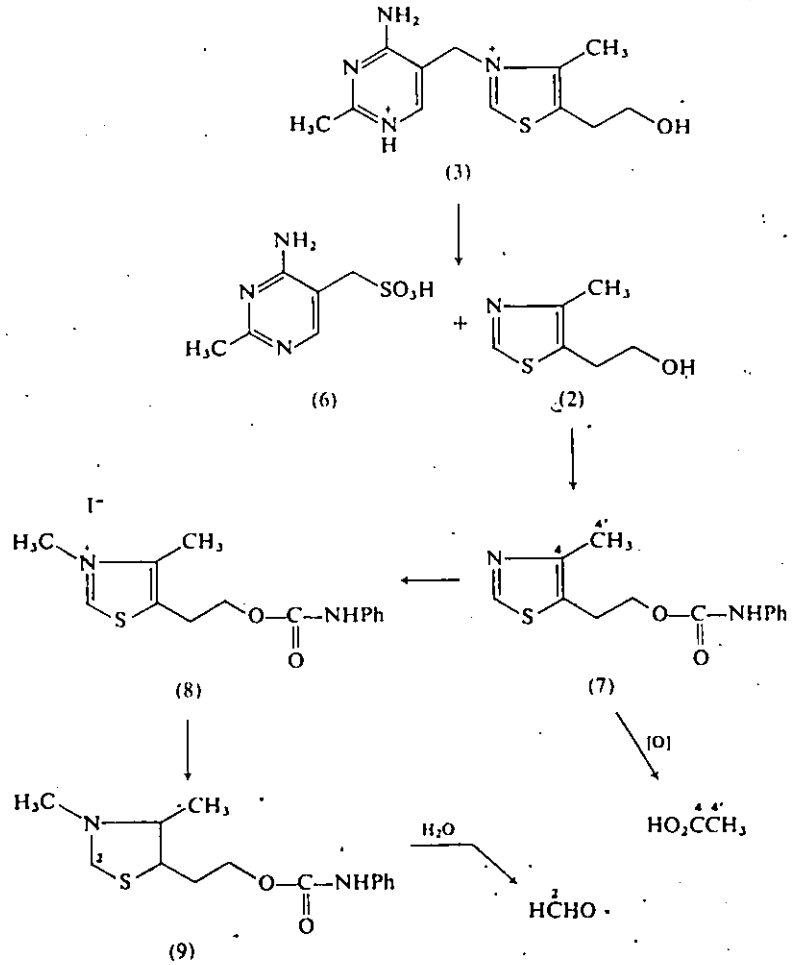
Table 1. *Feeding experiments with radioactive tracers*

Substrate	*S. cerevisiae* strain ... A.T.C.C. 24903					39916, H. J. Bunker				
	Expt. no.	Total radioactivity (μCi)	Sp. radioactivity (mCi/mmol)	Culture size (ml)	Radioactivity remaining in medium (% of total activity)	Expt. no.	Total radioactivity (μCi)	Sp. radioactivity (mCi/mmol)	Culture size (ml)	Radioactivity remaining in medium (% of total activity)
[2-^{14}C]Glycine*	1	250	40.7	1400	9	2	250	40.7	1400	17
L-[*Me*-^{14}C]Methionine*	3	50	13.6 (1.3)‡	500	29	4	250	49.7 (2.5)‡	1400	23
DL-[2-^{14}C]Tyrosine†	5	50	46	500	56					

* Obtained from New England Nuclear Corp., Boston, MA, U.S.A.
† Obtained from Amersham/Searle, Oakville, Ont., Canada.
‡ Calculated to allow for dilution by DL-methionine (20mg/litre) present in the culture medium (it is assumed in the calculation that only the L-enantiomer is utilized).

Table 2. *Specific radioactivity of thiamin chloride hydrochloride after carrier dilution and recrystallization to constant radioactivity*

S. cerevisiae strain ...	A.T.C.C. 24903		39916, H. J. Bunker	
Substrate	Expt. no.	Sp. radioactivity (d.p.m./mmol)	Expt. no.	Sp. radioactivity (d.p.m./mmol)
[2-^{14}C]Glycine	1	$3.99 (\pm 0.04) \times 10^4$	2	$2.05 (\pm 0.08) \times 10^4$
L-[*Me*-^{14}C]Methionine	3	$2.21 (\pm 0.02) \times 10^5$	4	$3.00 (\pm 0.30) \times 10^3$
DL-[2-^{14}C]Tyrosine	5	$3.43 (\pm 0.2) \times 10^3$		



Scheme 3. *Chemical degradation of the thiazole moiety of thiamin*

(ii) Reduction of the methiodide (8), The methiodide (8) (70mg) was suspended in propan-2-ol (3ml) at 0°C. $NaBH_4$ (8mg) in propan-2-ol (1ml) was added with stirring and stirring was continued for 2h. During this period, the reaction mixture was allowed to warm up gradually to room temperature (25°C). Water (10ml) was added, the solution was extracted with diethyl ether (5×5ml), the extract dried with

anhydrous $MgSO_4$ and evaporated *in vacuo* to yield a colourless oil (yield 42mg, 87%), presumably compound (9).

(iii) Hydrolysis with $HgCl_2$. The oil (9) was stirred overnight in water (4ml) containing $HgCl_2$ (52mg). The precipitate was filtered off and washed with water (2×5ml). 5,5-Dimethylcyclohexane-1,3-dione (dimedone) (52mg) in 95% ethanol (1ml) was added to the filtrate and, after heating on a steam bath for 1h, the formaldehyde dimethone was filtered off and sublimed at 95 C and 2×10^{-2}mm Hg (2.7Pa). The yield obtained was 33mg (65%), with m.p. 189–190 C [reported m.p. 191 C (Rappoport, 1967)] and *m/e* 292 (M^+).

(b) C-4′ and C-4 as acetic acid. (i) Kuhn–Roth oxidation of the thiazole phenylurethane (7) (cf. Kuhn & Wendt, 1939). The thiazole phenylurethane (7) (46mg) was dissolved in 15ml of 20% (v/v) H_2SO_4 and CrO_3 (2.1g) in water (2ml) was added dropwise. A slow stream of N_2 was passed through the solution, which was slowly distilled, while its volume was maintained by repeated addition of 5ml portions of water. Over a period of 5h, 72ml of distillate was collected. The distillate, containing acetic acid, was titrated to pH8 with 0.1M-NaOH. Sodium acetate (22mg, 92%) was obtained by evaporation at 90 C in a drying oven.

(ii) Acetyl-α-naphthylamine (cf. Leete *et al.*, 1965). The sodium acetate was dissolved in water (1ml) and an aqueous solution (2ml) of α-naphthylamine hydrochloride (40mg) was added, followed by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide (100mg). On standing at room temperature (25°C), acetyl-α-naphthylamine crystallized from solution and was filtered off. It was then recrystallized from a benzene/cyclohexane mixture and sublimed at 95°C and 2×10^{-2}mm Hg (2.7Pa). The yield obtained was 15mg [46% based on the thiazole phenylurethane (7)] with m.p. 158–159°C [reported m.p. 159–160°C (Leete *et al.*, 1965)] and *m/e* 185 (M^+).

Results

Radioactive-tracer experiments were carried out with two strains of *S. cerevisiae* (A.T.C.C. 24903 and H. J. Bunker, 39916), growing on thiamin-free medium. Radioactive tracer was added at the onset of logarithmic growth and cells were collected after maximum growth had been attained. The thiamin content of the harvested cells grown under these conditions was similar for both strains [26±3 and 20±3 (mean±S.D.) μg/litre of culture respectively, as determined by thiochrome assay]. When grown on yeast-extract medium, however, *S. cerevisiae* (H. J. Bunker, 39916) contained thiamin in amounts greater than 300μg/litre of culture.

Radioactive-tracer experiments with [2-^{14}C]glycine and L-[*Me*-^{14}C]methionine were performed with each of the two yeast strains. A further experiment with DL-[2-^{14}C]tyrosine was carried out with strain A.T.C.C. 24903. A summary of these experiments is presented in Table 1. Thiamin was isolated from each of these cultures by carrier dilution, and in each case maintained radioactivity after several recrystallizations (Table 2). The sample of thiamin obtained from each feeding experiment was degraded, by the reactions shown in Scheme 3, which permitted separate assay of radioactivity within the pyrimidine and the thiazole moieties of thiamin, and at individual carbon atoms of the thiazole nucleus.

Experiments with L-[*Me*-^{14}C]*methionine and* DL-[2-^{14}C]*tyrosine*

The samples of thiamin isolated from Expts. 3, 4 and 5 were degraded by bisulphite cleavage to give the pyrimidinesulphonic acid (6) and the thiazole derivative (2) (Scheme 3). The thiazole derivative (2), an oil at room temperature, was converted into the crystalline phenylurethane (7). Similar results were obtained in the experiments with L-[*Me*-^{14}C]-methionine with both strains of *S. cerevisiae* (Expts. 3 and 4) and in the experiment with DL-[2-^{14}C]tyrosine with *S. cerevisiae* (A.T.C.C. 24903) (Expt. 5). In each case, the phenylurethane (7) was completely inactive. The samples of the pyrimidinesulphonic acid (6) contained a small percentage of the activity present in the original thiamin.

Experiments with [2-^{14}C]*glycine*

The samples of thiamin isolated from Expts. 1 and 2 were cleaved with bisulphite to give the pyrimidinesulphonic acid (6) and the thiazole derivative (2), which was converted into the phenylurethane (7). In each case, the thiazole derivative retained more than 98% of the activity of the original thiamin. The remaining 1–2% of activity was located in the pyrimidinesulphonic acid (6). Each of the two samples of the thiazole phenylurethane (7) was further degraded according to Scheme 3. Kuhn–Roth oxidation gave acetic acid (derived from C-4 and C-4′ of the thiazole moiety), which was assayed as its α-naphthylamide derivative, and, in each case, was completely inactive. C-2 was isolated by a modification of the degradation used by Linnett & Walker (1967). Methylation of the thiazole phenylurethane gave the methiodide (8) that, in each case, retained all (99%) of the activity of the original thiamin. Reduction with $NaBH_4$ and hydrolysis with $HgCl_2$ gave C-2 as formaldehyde, which was isolated as the dimethone and in each case retained the activity (100%) of the original thiamin.

Details of these results are summarized in Table 3.

Table 3. *Distribution of ^{14}C from labelled substrates in thiamin in two strains of S. cerevisiae*
For details of confidence limits, see under 'Determination of radioactivity'.

S. cerevisiae strain ...	A.T.C.C. 24903		H. J. Bunker, 39916	
	$10^{-4}\times$ Sp. radioactivity (d.p.m./mmol)	Relative sp. radioactivity (%)*	$10^{-4}\times$ Sp. radioactivity (d.p.m./mmol)	Relative sp. radioactivity (%)*
[2-^{14}C]Glycine Expt. no. ...	1		2	
Thiamin chloride hydrochloride (3)	3.99±0.04	100±1	2.05±0.08	100±4
Pyrimidinesulphonic acid (6)	0.08±0.01	2±0.2	0.02±0.01	1±1
Thiazole phenylurethane (7)	3.91±0.05	98±2	2.08±0.03	101±4
Thiazole phenylurethane methiodide (8)	—	—	2.02±0.04	99±4
Kuhn–Roth acetic acid (as α-naphthylamide)	0.00±0.02	0±1	0.00±0.02	0.1±0.1
Formaldehyde (as the dimethone)	3.94±0.03	99±1	2.06±0.03	101±4
L-[*Me*-^{14}C]Methionine Expt. no. ...	3		4	
Thiamin chloride hydrochloride (3)	22.1±0.2	100±1	0.30±0.03	100±10
Pyrimidinesulphonic acid (6)	0.31±0.01	1±0.1	0.01±0.01	4±4
Thiazole phenylurethane (7)	0.03±0.02	0.1±0.1	0.01±0.03	2±8
DL-[2-^{14}C]Tyrosine Expt. no. ...	5			
Thiamin chloride hydrochloride (3)	0.34±0.02	100±6		
Pyrimidinesulphonic acid (6)	0.04±0.02	12±6		
Thiazole phenylurethane (7)	0.01±0.02	3±6		

* Thiamin chloride hydrochloride = 100%.

Discussion

It may appear surprising that, at a time when primary precursor–product relationships are well established for most naturally occurring compounds, and the present focus of biosynthetic investigations of highly complex molecules such as the porphyrins and corrins (C_{30}–C_{45}), the steroids (C_{18}–C_{30}) and diterpenes (C_{20}), the *Vinca* (C_{20}–C_{45}) and opium alkaloids (C_{16}–C_{20}), to name but a few, lies in establishing biosynthetic intermediates, and in elucidating the stereochemistry and probing the mechanism of individual steps of the pathways, it should be necessary to carry out an investigation to secure information regarding the identity of the primary precursors of the thiazole unit of thiamin (vitamin B_1), a C_6 compound.

There are several factors that have contributed to the delay in establishing unequivocally, by radioactive-tracer methods, the identity of the basic building blocks of thiamin, and its thiazole moiety in particular.

The major obstacle is the small amount of thiamin which is present in tissues that produce it. Thus micro-organisms contain 10–100 μg/g dry cell wt., i.e. 20–200 μg/litre of culture (Kutsky, 1973), and higher plants 0.1–10 μg/g fresh wt. (Kutsky, 1973; Robinson, 1966). Since for a definitive biosynthetic investigation, which demands isolation and purification to constant radioactivity, followed by derivatization and chemical degradation to locate the site or sites of radioactivity, at least 10 mg of compound (or more, depending on the complexity of the degradation sequence) is required, and since the scale of a radioactive-tracer experiment (volume of culture, or number of plants, per experiment) is limited by experimental facilities, it will be necessary to dilute the target compound 10^2–10^3-fold with inactive carrier to obtain sufficient quantities of radioactively labelled material. Thus the success of a biosynthetic radioactive-tracer experiment is predicated on selecting conditions, such that the unweighable amount of target compound produced during the experimental period has a specific molar radioactivity high enough so that after dilution by a factor of 10^2–10^3 the isolated product still has a significant count rate (d.p.m./mg, above background).

Some of the conditions required to enhance the likelihood of obtaining such a result are not under the control of the investigator. Thus the substrate to be tested as precursor may not be available at high specific activity, and much of it may be dissipated in metabolic processes which, from the point of view of the investigation, are irrelevant and undesirable. The amount of target material biosynthesized during the experimental period may be less than the amount

present endogenously at the start of the experiment. Thus the activity of the product, even after minimal dilution with carrier, may be at the borderline of detectability.

Another problem inherent in the handling of small quantities of labelled compounds is the presence of, unweighable amounts of radioactive impurities in the product. These may not be detectable unless derivatives are prepared, but lack of material may preclude the chemical manipulation required for the preparation of derivatives and for degradation.

In view of these difficulties, it is understandable that many of the radioactive-tracer investigations whose objective it was to identify the primary precursors of the thiazole moiety of vitamin B_1 were not pursued to a stage when definitive conclusions could be drawn. In some cases, the target compound [in this case 5-(β-hydroxyethyl)-4-methylthiazole] was not isolated, but it was assumed that a chromatographic fraction containing the desired product did not contain any other radioactive compound (Johnson *et al.*, 1966; Iwashima & Nose, 1971). Alternatively, the desired compound was isolated, but no derivatives were prepared and no degradation attempted, and it was assumed that the isolated product was free of radiochemical contaminants (Tomlinson *et al.*, 1967).

Results of such incomplete experiments, taken at face value, are often likely to lead to erroneous inferences.

Since it is difficult to gauge the reliability of the experimental data obtained in such incomplete investigations lacking internal checks, it is well nigh impossible to assess what credence should be given to the interpretation of the data offered by the investigators who report them. The problem is compounded by the fact that no two schools used the same strain of micro-organism for their experiments.

One series of investigations that appears to be well-designed and thoroughly executed is that of Linnett & Walker (1967, 1968, 1969). They used a strain of *S. cerevisiae* (N.C.Y.C. 1062) for radioactive-tracer experiments with [2-^{14}C]glycine and L-[*Me*-^{14}C]-methionine. It was demonstrated by preparation of derivatives and degradation of the isolated thiamin that [*Me*-^{14}C]methionine did not serve as a precursor, but that [2-^{14}C]glycine did, and that the entire activity of the thiamin, derived from this substrate, was present in the thiazole nucleus [isolated as the picrate of 5-(β-hydroxyethyl)-4-methylthiazole] and localized at C-2 (isolated as formaldehyde dimethone, after controlled degradation). Furthermore, ^{15}N from [^{15}N]glycine was located exclusively in the thiazole moiety. It can be concluded from these results that the methylene carbon atom of glycine serves as the source of C-2 of the thiazole moiety of thiamin, and it is more than likely that the C–N fragment, derived from glycine by decarboxylation, enters the thiazole moiety as a unit.

These results and their interpretation are in direct conflict with the work of Johnson *et al.* (1966). These authors carried out feeding experiments with [*Me*-^{14}C]methionine and [*Me*-^{14}C,^{35}S]methionine in another strain of *S. cerevisiae* (H. J. Bunker, 39916). They isolated thiamin, degraded it into the pyrimidine and thiazole moieties and recovered a chromatographic fraction containing the thiazole, but did not isolate, prepare derivates of or degrade the compound. The thiazole fraction obtained in the two experiments contained respectively ^{14}C, and ^{14}C and ^{35}S, with a ^{35}S/^{14}C ratio identical with that of the precursor methionine. The authors concluded that the C–S unit, derived from the *S*-methyl group of methionine, was incorporated intact into the thiazole moiety, yielding the unit S–C-2. This result is widely quoted (e.g. Goodwin, 1963; Luckner, 1972; Brown, 1972; Leder, 1975) and serves as the basis of the accepted hypothesis of thiamin biosynthesis. It is seen as providing experimental support for the biogenetic ideas of Sir Charles Harington (Harington & Moggridge, 1939).

Reviews of thiamin biosynthesis (Brown, 1972; Leder, 1975) summarize the experiments with yeast, as well as other investigations with bacteria (Estramareix & Therisod, 1972; Bellion *et al.*, 1976), but do not attempt a critical evaluation of the conflicting conclusions of Linnett & Walker (1967) and of Johnson *et al.* (1966) concerning the biosynthesis of the thiazole nucleus of vitamin B_1 in yeast.

The present results with two strains of *S. cerevisiae* (A.T.C.C. 24903 and H. J. Bunker, 39916) confirm those of Linnett & Walker. Samples of thiamin chloride hydrochloride, isolated by carrier dilution from the cells of two strains of *S. cerevisiae* that had been incubated in the presence of [2-^{14}C]glycine (Expts. 1 and 2) (Table 1), were crystallized to constant radioactivity (Table 2) and degraded (Scheme 3) to locate the site of activity (Table 3). Bisulphite cleavage yielded the pyrimidinesulphonic acid (6), which was non-radioactive, and 5-(β-hydroxyethyl)-4-methylthiazole (2), which was converted into the crystalline phenylurethane (7) and the corresponding methiodide (8), and contained all the activity of the intact vitamin. Kuhn–Roth oxidation of the phenylurethane (7) gave acetic acid (C-4 and C-4′ of the thiazole moiety) (isolated as the α-naphthylamide). This was not radioactive. The methiodide (8) was reduced to yield the tetrahydro derivative (9), which was not isolated but directly hydrolysed, yielding formaldehyde (C-2 of the thiazole moiety) (isolated as the dimethone), which contained all activity of the thiazole nucleus and of the intact vitamin. The methylene carbon atom of glycine thus serves as the progenitor of C-2 of the thiazole nucleus of thiamin and of no other C atom.

Incubation of each of the two strains of *S. cerevisiae* (A.T.C.C. 24903 and H. J. Bunker, 39916) with L-[*Me*-^{14}C]methionine (Expts. 3 and 4, Table 1) also yielded radioactive samples of thiamin chloride hydrochloride (Table 2). Five recrystallizations were required in the case of these samples to reach constant radioactivity. However, when these samples of thiamin chloride hydrochloride were subjected to bisulphite cleavage (Table 3), the two degradation fragments, the pyrimidinesulphonic acid (6) and the thiazole (2) isolated as the phenylurethane (7) did not contain radioactivity in either case.

Since these two fragments account for all carbon atoms of the original thiamin chloride hydrochloride, it is clear that the radioactivity associated with the samples of the vitamin, isolated from Expts. 3 and 4, must have been due to contamination by traces of a highly radioactive impurity, which persisted despite the many purification steps. This conclusion leads us to surmise that the activity found by Johnson *et al.* (1966) to be associated with the chromatographic fraction containing the thiazole moiety, derived from cultures of *S. saccharomyces* (H. J. Bunker, 39916) incubated with [*Me*-^{14}C]- and [*Me*-^{14}C,^{35}S]-methionine, was also due to the presence of traces of a highly radioactive impurity. If this impurity were methionine itself, or a breakdown product that still retains the $-S-CH_3$ function, maintenance of the $^{35}S/^{14}C$ ratio of the precursor in the contaminated, but non-radioactive, product would be explained.

The culture conditions in our own radioactive-tracer experiments with *S. cerevisiae* (H. J. Bunker, 39916) were somewhat different from those of Johnson *et al.* (1966). Their experiments were carried out on a medium containing yeast extract, rich in thiamin, and radioactive tracer was administered 24h after inoculation. In the present experiments, a thiamin-free medium was used, and radioactive tracer was added 15.5h after inoculation.

As is shown in Fig. 1 (curve C), logarithmic phase is complete after approx. 20h incubation of *S. cerevisiae* (H. J. Bunker, 39916) on a yeast-extract medium. It has been stated by Leder (1975) that '*de novo* formation of thiamin cannot be demonstrated in non-growing cell suspensions under normal circumstances'. Johnson *et al.* (1966) supplied radioactive tracer to their cultures well after logarithmic growth was complete and the culture had reached stationary phase. It would seem likely that in the experiments reported by Johnson *et al.* (1966) biosynthesis of thiamin was not taking place during the time the cells were in contact with the radioactive tracer.

In these experiments, a culture medium was employed that contained yeast extract (2g/litre). Yeast extract contains thiamin (approx. 30–300μg/g).

It has been shown (Suomalainen & Oura, 1971; Stieglitz *et al.*, 1974) that *S. cerevisiae* accumulates thiamin from the medium on which it is grown. Furthermore, the level of thiamin phosphate pyrophosphorylase, the enzyme responsible for the synthesis of thiamin monophosphate from the phosphorylated thiazole and pyrimidine moieties, present in commercial yeast cultivated with added thiamin, is less than 15% of that found in the same yeast produced without exogenous thiamin (Leder, 1970, 1975). It seems unlikely, therefore, that significant biosynthesis of thiamin takes place *de novo* in yeast cells that are grown in the presence of exogenous thiamin.

The present experiments with *S. cerevisiae* were carried out in a thiamin-free medium. Under these conditions (curve B, Fig. 1) growth is slower than in the presence of thiamin (curve C, Fig. 1), but approximately the same cell density is reached at completion of logarithmic phase. Radioactive tracer was added after 15.5h of incubation, i.e. at the onset of active growth. Conditions for thiamin biosynthesis were thus favourable.

The results of Linnett & Walker (1967, 1968, 1969) and the present work establish that, in three strains of yeast (*S. cerevisiae* N.C.Y.C. 1062, A.T.C.C. 24903, and H. J. Bunker, 39916), the methylene carbon atom of glycine serves as the biosynthetic source of C-2 of the thiazole nucleus of thiamin. Furthermore, it is likely, on the basis of the results of Linnett & Walker (1968), that an intact C–N unit derived from glycine enters the thiazole nucleus.

In bacteria, a carbon source other than glycine would appear to supply C-2 of the thiazole nucleus, but conflicting results remain to be evaluated. In *E. coli* (Estramareix & Therisod, 1972) and *Salm. typhimurium* (Bellion *et al.*, 1976), the α-carbon atom of tyrosine appears to supply C-2 of the thiazole unit. This substrate does not serve as a precursor in *S. cerevisiae* (A.T.C.C. 24903) (Expt. 5, Tables 1–3). Radioactive label from L-[*Me*-^{14}C]methionine is not incorporated into the vitamin in these two bacteria (Bellion *et al.*, 1976; Estramareix *et al.*, 1977). Yet, in *B. subtilis*, the *S*-methyl group of methionine is reported to serve as a source of C-2 (Torrence & Tieckelmann, 1968). Each of these results is based on chemical degradation of the thiazole unit and on isolation of C-2 as formaldehyde. [2-^{14}C]Glycine did not serve as a thiamin precursor in *Salm. typhimurium* (Bellion *et al.*, 1976). It was not tested as a substrate in *B. subtilis*. Glycine was found to replace 5-(β-hydroxyethyl)-4-methylthiazole in promoting the growth of a mutant of *E. coli* requiring this thiazole derivative (Iwashima & Nose, 1970), and [U-^{14}C]-glycine was reported to be incorporated into the thiazole moiety of thiamin in this mutant (Iwashima & Nose, 1971).

Thus, the source of C-2 of the thiazole nucleus of thiamin in bacteria, and the origin of the remaining

C_5 unit (C-4', C-4, C-5, C-6, and C-7) of the thiazole moiety in yeast and in bacteria awaits clarification.

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Biosynthesis of Vitamin B_1 in Yeast. Origin of the Thiazole Unit

Sir:

More than forty years after the elucidation of the structure of vitamin B_1 (thiamin),[1,2] the primary precursors of its thiazole unit (5) are still in dispute[3] and a chemically rational hypothesis of the biosynthesis of this unit has not yet been formulated. We now advance such a hypothesis and present some evidence which is consistent with it.

It is our view that, in yeast, the thiazole unit of vitamin B_1 is derived from one of the stereoisomers of the Schiff base 3, which is generated by condensation of glycine (1) with a phosphoketopentose (2). The Schiff base 3 is converted into the thiazole derivative 4[5] in a multistep sequence (Scheme I) comprising dehydration (or elimination of phosphate) (A), dehydration and tautomerization (B and C), and addition of sulfur (D), followed by ring closure (E) and concerted decarboxylation and dehydration. Several variants of this route are shown in Scheme I.

In support of this scheme, we now present experimental evidence which demonstrates the participation of a C_5 unit (2) derived from glucose in the biosynthesis of the thiazole unit of thiamin in yeast. The incorporation of C-2 and N of glycine (1) into the thiazole unit of thiamin in yeast, in accord with Scheme I, has been documented.[4,10]

Origin of the C_5 Unit 2. Yeast (*Saccharomyces cerevisiae*) does not utilize ribose or other pentoses[11] when these are supplied to the culture medium. Evidence for the participation of a pentose in thiamin biosynthesis was therefore obtained indirectly, by testing the mode of incorporation of glucose and fructose. These hexoses are known to be utilized and to yield pentoses in vivo. In separate experiments,[12] D-[1-^{14}C]-, D-[2-^{14}C]-, and D-[6-^{14}C]glucose and D-[1-^{14}C]fructose were administered to growing yeast cultures (*S. cerevisiae* A.T.C.C. 24903)[13] at the onset of logarithmic growth. The cells were collected when logarithmic growth had ceased and radioactive thiamin was isolated[4] by carrier dilution. Bisulfite cleavage[9] yielded the thiazole moiety (5-(β-hydroxyethyl)-4-methylthiazole) (5) as an oil, some of which was oxidized[14] to 5-formyl-4-methylthiazole[15] (isolated as the semicarbazone[15]) and some of which was converted, via the 5-(β-chloroethyl) derivative,[16,17] into the 5-(-β-phthalimidoethyl) derivative.[18] This

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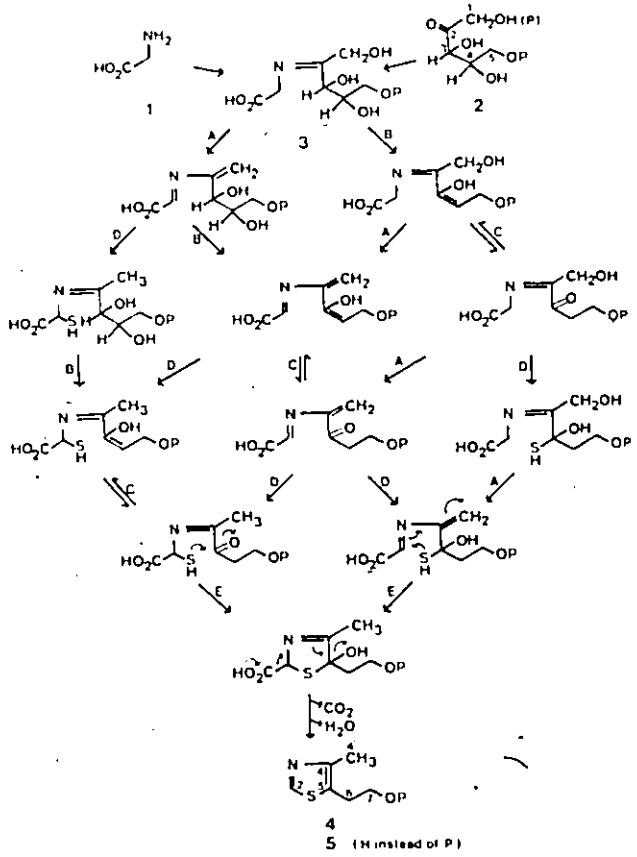
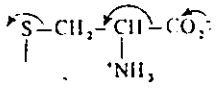
Scheme I. Biogenesis of the Thiazole Unit (4) of Vitamin B_1 in yeast, from Glycine (1) and a Phosphoketopentose (2)[a]



[a] (A) Dehydration (or elimination of phosphate) (P = $-PO(OH)_2$); (B) dehydration; (C) tautomerization; (D) addition of sulfur. The S donor which, for simplicity, is represented as H_2S, is more likely to be cysteine; if so, ring closure (E) would be accompanied by the fragmentation process



was purified to constant radioactivity and was further degraded. Kuhn-Roth oxidation[19] yielded acetic acid (C-4′,C-4 of the thiazole moiety) (isolated as the α-naphthylamide[20]) which was converted into methylamine (C-4′) (isolated as *N*-methylphthalimide[21]) by a Schmidt reaction.[22] Acid permanganate oxidation[23] of the phthalimidoethyl derivative gave N-phthaloyl-β-alanine (C-5,-6,-7).

The results of these experiments are shown in Table I. Activity from D-[1-^{14}C]glucose and from D-[1-^{14}C]fructose is located almost entirely (>90%) in the *C*-methyl group (C-4′) of the thiazole unit (*N*-methylphthalimide). The remaining activity (<10%) was located at C-5, C-6, and/or C-7 (*N*-phthaloyl-β-alanine). Lack of material precluded further degradation to determine the exact location of label. It is very likely, however, that C-7 is the site of labeling. A complementary result was obtained when D-[6-^{14}C]glucose served as substrate. The label was located mostly at C-7 (~80%), while the *C*-methyl group contained the remaining activity (~20%). The label from D-[2-^{14}C]glucose was confined to the C_2 unit, C-4,C-4′ (acetyl-α-naphthylamine, 96%), and was almost equally divided between these two carbons (C-4′, methylphthalimide, 46%; C-4, 50%, by difference).

Several phosphoketopentoses (**2**), all metabolically interconvertible without skeletal rearrangement, are derivable from glucose in the course of metabolism in yeast.[24] Thus, D-ribulose 5-phosphate (**2**, 3-*R*, 4-*R*) is generated from D-glucose, via D-glucose-6-phosphate and 6-phospho-D-gluconic acid, by oxidation and decarboxylation of the latter. Generation of **2** in this way would, in accord with observation (Table I), deliver activity from [6-^{14}C]glucose into C-7 and from [2-^{14}C]glucose into C-4′ of the thiazole nucleus, but would, contrary to observation, lead to unlabeled thiazole from [1-^{14}C]glucose. D-Xylulose 5-phosphate (**2**, 3-*S*, 4-*R*) is produced by a transketolase reaction which transfers the C_2 unit, C-1,C-2, of fructose 6-phosphate (derivable from glucose or fructose) onto glyceraldehyde 3-phosphate, which is derived, primarily, from the C_3 unit, C-4,C-5,C-6, of glucose or fructose, via fructose 1,6-diphosphate. Generation of **2** by this route would deliver activity from [6-^{14}C]glucose into C-7, from [2-^{14}C]glucose into C-4, and from [1-^{14}C]glucose and [1-^{14}C]fructose into C-4′ of the thiazole nucleus. Neither the oxidative route via 6-phospho-D-gluconic acid by itself nor the transketolase pathway alone can account for the observed distribution of activity, derived from labeled hexoses, within the C_5 unit of the thiazole. However, the two pathways operating concurrently[25] will lead to a pool of labeled pentoses which, upon metabolic interconversion,[24] would show a labeling pattern corresponding to that observed in the C_5 unit of the thiazole moiety of thiamin, isolated in each of the four experiments.

Several other possibilities for the generation of **2**, e.g., a contraction of the hexose chain similar to that occurring in the biosynthesis of streptose,[27] followed by elimination of C-3 or C-4 of glucose, are less likely but cannot yet be ruled out.

Glycine (1). It was reported in 1967 that glycine (**1**) is implicated in the biosynthesis of the thiazole moiety of thiamin in yeast.[10] This report contradicted accepted dogma that the thiazole unit is derived from methionine and alanine.[28] We have recently shown[4] that published results[28] concerning the incorporation, in yeast (*S. cerevisiae* 39916, H. J. Bunker), of the *S*-methyl group of methionine into C-2 of the thiazole unit are in error. We also found that the α-carbon atom of tyrosine, reportedly the source of C-2 of the thiazole unit in bacteria,[29-31] does not serve as a precursor in yeast (*S. cerevisiae* A.T.C.C. 24903). We demonstrated that, in these two yeast strains, the methylene carbon of glycine serves as the specific precursor of C-2 of the thiazole unit of thiamin, and of no other

Table I. Incorporation of Hexoses into the Thiazole Unit of Thiamin

products	C atoms of the thiazole moiety	relative specific activity %[a]			
		D-[6-^{14}C] glucose[b]	D-[2-^{14}C] glucose[b]	D-[1-^{14}C] glucose[b]	D-[1-^{14}C] fructose[b]
5-(β-hydroxyethyl)-4-methylthiazole[c]	all	100 ± 2	100 ± 1	100 ± 3	100 ± 2
5-formyl-4-methylthiazole	all except C-7	17 ± 1			
acetyl-α-naphthylamine	C-4, -4′		96 ± 1	92 ± 3	84 ± 2
N-methylphthalimide	C-4′	16 ± 2	46 ± 1	93 ± 2	91 ± 3
N-phthaloyl-β-alanine	C-5, -6, -7	84 ± 2		6 ± 1	5 ± 1

[a] The specific activity (disintegrations per minute/millimole) of the thiazole derivative **5**, derived from thiamin isolated by carrier dilution in each of the four experiments, is normalized to 100. The specific activity of each degradation product is expressed as percent of the molar specific activity of the thiazole derivative from which it was obtained. [b] Precursor. [c] Assayed as the phthalimidoethyl derivative.

carbon atom.[4] This evidence reinforces the findings of Linnett and Walker[10] that, in yeast (*S. cerevisiae* N.C.Y.C. 1062), the methylene group of glycine supplies C-2, and the amino nitrogen of glycine supplies the nitrogen atom of the thiazole nucleus of vitamin B_1. It is very likely therefore that an intact C-N unit, derived from glycine (**1**), enters the thiazole nucleus. Scheme I is consistent with these results.

The origin of the thiazole moiety of thiamin in bacteria differs from that in yeast in two respects. Firstly, the unit C-2,N is derived from tyrosine[29,30,32] and not from glycine. A simple modification[33] of the present biogenetic scheme can accommodate this difference. Secondly, the C_5 precursor is generated by condensation of 3-phosphoglyceraldehyde with a C_2 unit derived from C-3,C-2 of pyruvate[34] rather than from C-1,C-2 of fructose 6-phosphate. An acyloin condensation was proposed to account for the formation of the C_5 unit from these precursors. The distribution of label observed in the present work is not consistent with such a proposal.

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