Biosynthesis of Thiamine

INCORPORATION EXPERIMENTS WITH ^{14}C -LABELLED SUBSTRATES AND WITH ^{15}N GLYCINE IN *SACCHAROMYCES CEREVISIAE*

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1. Yeast was grown in a minimal synthetic medium together with a range of ^{14}C -labelled substrates under standardized conditions. After isolation, the purified thiamine was cleaved by sulphite and the pyrimidine and thiazole moieties were purified and assayed for radioactivity. 2. In order of decreasing incorporation, [^{14}C]formate, [$3\text{-}^{14}\text{C}$]serine, [$2\text{-}^{14}\text{C}$]glycine and [$2\text{-}^{14}\text{C}$]acetate supplied label for the pyrimidine, and [$2\text{-}^{14}\text{C}$]glycine, [$3\text{-}^{14}\text{C}$]serine, [$1\text{-}^{14}\text{C}$]glycine, [^{14}C]formate and [$2\text{-}^{14}\text{C}$]acetate for the thiazole. Incorporation of label into the fragments from several other ^{14}C -labelled substrates, including [$\text{Me}\text{-}^{14}\text{C}$]- and [$3,4\text{-}^{14}\text{C}_2$]-methionine, was insignificant. 3. [$3\text{-}^{14}\text{C}$]Serine was shown not to contribute label to C-2 of the thiazole ring. 4. Significant incorporation of nitrogen from [^{15}N]glycine into the thiazole moiety, but not into the pyrimidine moiety, was established. 5. It appears that C-2 and N-3 of the thiazole ring are formed from C-2 and the nitrogen atom of glycine, but the entire methionine molecule does not appear to be implicated.

The modes of biosynthesis of the pyrimidine (I) and thiazole (II) moieties of thiamine are still not well understood, though the steps involved in their linkage have been demonstrated for cell-free extracts (Camiener & Brown, 1959, 1960; Leder, 1959; Nose, Ueda & Kawasaki, 1959; Suzuoki-Zirô & Kobata, 1960).

The efficient incorporation of [^{14}C]formate into the pyrimidine (I) in various micro-organisms is well documented (Pine & Guthrie, 1959; David & Estramareix, 1960; Goldstein & Brown, 1963; Nakamura, 1965; Johnson, Howells & Goodwin, 1966; Kumaoka & Brown, 1967; Tomlinson, Kuhlman, Torrence & Tieckelmann, 1967). Degradation experiments have shown that label from [^{14}C]formate may be incorporated either into C-4 of the pyrimidine ring in yeast (David, Estramareix & Hirshfeld, 1966, 1967) or into C-2 of the pyrimidine ring in *Escherichia coli* (Kumaoka & Brown, 1967). The methyl carbon atom of acetate has been suggested as the source of the methyl group attached

to C-2 of the pyrimidine ring on the basis of double-labelling experiments with ^{14}C and ^3H (Tomlinson, 1966; Tomlinson *et al.* 1967), but Kumaoka & Brown (1967) consider this unlikely. Newell & Tucker (1967, 1968a,b) have shown that 5-aminoimidazole ribonucleotide is an intermediate in the biosynthetic pathway to the pyrimidine (I) in mutants of *Salmonella typhimurium*, though the subsequent steps are not yet established (Newell & Tucker, 1968b).

Double-labelling experiments with [$\text{Me}\text{-}^{14}\text{C}, ^{35}\text{S}$]-methionine (Johnson, Howells & Goodwin, 1964, 1966) and with [$\text{Me}\text{-}^{14}\text{C}, \text{G}\text{-}^3\text{H}$]methionine (Tomlinson *et al.* 1967) suggested the utilization of the whole molecule of methionine apart from the nitrogen atom and the carboxyl group in the biosynthesis of the thiazole (II). This pathway has been rendered unlikely by the demonstration that [$2\text{-}^{14}\text{C}$]glycine, but not [^{14}C]formate, contributes label to C-2 of the thiazole ring (Linnett & Walker, 1967). Tracer incorporation studies have suggested also the utilization of acetate and alanine in thiazole biosynthesis (Johnson *et al.* 1966; Tomlinson *et al.* 1967).

In this paper we present a study of the incorporation of label from various potential ^{14}C -labelled precursors into the thiazole and pyrimidine moieties of thiamine in yeast by using standardized culture and isolation techniques. The incorporation of label from [^{15}N]glycine into the thiazole is recorded.

(I)

(II)

MATERIALS AND METHODS

Yeast. The yeast used was *Saccharomyces cerevisiae* N.C.Y.C. 1062, obtained from the National Collection of Yeast Cultures, The Brewing Industry Research Foundation, Nutfield. The cells were grown on Sabouraud slopes at 27° for 2 days and the resulting cultures were maintained at 4° for not longer than 6 months before subculturing.

Media. (a) The MYGP medium contained: malt extract (paste), 4g.; yeast extract (powder), 3g.; glucose, 10g.; peptone, 5g.; made up to 1l. with distilled water.

(b) The synthetic medium for the small-scale cultures contained: glucose, 2g.; NH_4Cl , 162mg.; KH_2PO_4 , 156mg.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 41mg.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 33mg.; Na_2SO_4 , 14mg.; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 25 μg .; KI, 10 μg .; H_3BO_3 , 10 μg .; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 5 μg .; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4 μg .; ZnCl_2 , 4 μg .; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 2 μg .; mesoinositol, 1mg.; calcium D-pantothenate, 0.5mg.; DL-biotin, 0.2 μg .; made up to a final volume of 100ml. (including subsequent additions) with deionized water.

(c) The synthetic medium for the large-scale cultures contained: glucose monohydrate, 6.6kg.; NH_4Cl , 486g.; KH_2PO_4 , 468g.; Na_2SO_4 , 150g.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 123g.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 99g.; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 75mg.; KI, 30mg.; H_3BO_3 , 30mg.; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 15mg.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 12mg.; ZnCl_2 , 12mg.; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 6mg.; mesoinositol, 3g.; calcium D-pantothenate, 1.5g.; DL-biotin, 0.6mg.; made up to a final volume of 300l. (including subsequent additions) with distilled water.

Saline. This was aq. 0.85% NaCl soln.

Cyanogen bromide. Ice-cold aq. 20% (w/v) KCN soln. was added dropwise to ice-cold saturated bromine water until the colour was just discharged (Fujiwara & Matsui, 1953). The resulting CNBr soln. was kept at 0° and used within 3hr.

Labelled compounds. All ^{14}C -labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., except for DL-[3,4- $^{14}\text{C}_2$]methionine, which was obtained from Niche Inc., Bethesda, Md., U.S.A. All were used as received.

The [^{15}N]glycine was prepared by the method of Schoenheimer & Ratner (1939) and was kindly made available by Dr A. S. McFarlane. Descending chromatography on Whatman no. 1 paper with ethanol-water (63:37, v/v) and butan-1-ol-acetic acid-water (4:1:5, by vol.) gave single ninhydrin-positive spots moving with authentic glycine. The ^{15}N content was found to be 32.06 atom % excess ^{15}N , and this value was checked by dilution with carrier glycine.

Potassium DL-[2- ^{14}C]mevalonate. This was prepared from DL-[2- ^{14}C]mevalolactone by the procedure of Levy & Popják (1960).

Determination of cell growth. Cell suspensions were examined nephelometrically at 500m μ and the concentrations of cells in terms of dry weight were estimated from a standard curve.

Determination of thiamine. The thiochrome method of Fujiwara & Matsui (1953) was adapted thus. To 0.5ml. of the 'standard extract' of thiamine in 0.1N-HCl (see below) was added 1.0ml. of CNBr soln., 0.5ml. of aq. 50% (w/v) NaOH and 3.5ml. of redistilled 2-methylpropan-1-ol. After vigorous shaking for 90sec., the two layers were separated by hand centrifugation and the upper 2-methylpropan-1-ol layer was pipetted into a 1cm. silica cuvette. The fluorescence of this solution was measured in an Aminco-Bowman

spectrophotofluorimeter with activating wavelength 370m μ and fluorescence wavelength 440m μ . The spectrophotofluorimeter was calibrated each time against a standard quinine sulphate soln. with activating wavelength 350m μ and fluorescence wavelength 450m μ . The intensity of fluorescence of the thiochrome soln. was compared with a standard curve to obtain the original thiamine concentration in the 'standard extract'. A blank carried out as above but without thiamine showed only negligible fluorescence.

Radioactivity measurements. (a) Cell suspensions and supernatants were evaporated on to tissue circles on 1cm.² polythene planchets and counted at 'infinite thinness' with an end-window counter, correction being made for background and dead time.

(b) Samples of the thiazole picrate, pyrimidinesulphonic acid and formaldehyde dimedone of over 20mg. were counted at 'infinite thickness' on 1cm.² polythene planchets with a Nuclear-Chicago Corp. (Des Plaines, Ill., U.S.A.) low-background gas-flow thin-window counter, type C115, correction being made for background (1–2 counts/min.). An 'infinitely thick' [^{14}C]polymethacrylate disk (1.0 $\mu\text{C/g.}$) on a 1cm.² polythene planchet was used as a standard, giving 1900–2200 counts/min.

Measurement of ^{15}N . The weight of sample used was 4–12mg. for glycine and the thiazole (II) and 0.6–2.0mg. for the pyrimidinesulphonic acid (III). Samples were converted into NH_4Cl by the Kjeldahl method as modified by Steyermark (1951) with a silver-tube condenser for the steam-distillation stage to prevent cross-contamination of samples, as recommended by Newman (1966). The NH_4Cl soln. was oxidized with alkaline hypobromite (Rittenberg, 1946) and the resulting N_2 gas passed through a liquid- N_2 trap into the glass inlet system of the mass spectrometer (60° Nier-type). The intensities of the peaks at mass numbers 28, 29 and (where applicable) 30 were measured and corrected for a small 'background'. Each run was followed by a determination on N_2 of normal isotopic abundance (about 0.374 atom % ^{15}N). Values for atom % ^{15}N in the samples were calculated by the method of Rittenberg (1946).

Incorporation experiments with ^{14}C -labelled substrates. A loopful of cells was grown in 100ml. of MYGP medium with reciprocal shaking at 27° for 1.5 days. The cells were spun down, washed twice with sterile saline and then resuspended in sterile saline to give a suspension of 5mg. dry wt. of cells/ml. as the inoculum. The synthetic medium (100ml.) was autoclaved complete in a 1l. conical flask fitted with a plugged drying-tube. The ^{14}C -labelled substrate, dissolved in a suitable volume of water together with carrier material if required, was sterilized by Seitz filtration and added aseptically to the flask. After aseptic addition of 1ml. of the inoculum to the flask, the drying-tube was filled with Carbosorb granules (British Drug Houses Ltd., Poole, Dorset) to absorb any $^{14}\text{CO}_2$ produced during subsequent growth. The culture was grown for 2.5 days at 27° with reciprocal shaking. Cell growth was 60–120mg. (dry wt.).

Samples (0.1 ml.) of the cell suspension and of the supernatant after centrifugation were counted as above. These count rates gave a measure of the percentage incorporation of radioactivity into the cells. Thiamine was extracted and purified from the cells to give 5.0 ml. of a 'standard extract' of thiamine in 0.1 N-HCl by the method of Hitchcock & Walker (1961). A sample (0.5 ml.) of this 'standard extract' was assayed for thiamine content as described above. An adaptation of the sulphite cleavage reaction of Williams, Waterman, Keresztesy & Buchman (1935) was then used. To the 4.5 ml. of 'standard extract' left after the thiochrome determination (containing 0.7–2.7 μ g. of thiamine) were added 50.0 mg. of dry thiamine chloride hydrochloride as carrier, about 0.2 ml. of M-sodium acetate buffer, pH 4.7, and 100 mg. of sodium metabisulphite. The resulting solution was incubated overnight at 40°. The cooled solution was made alkaline with about 0.1 ml. of aq. 50% (w/v) NaOH and extracted with methylene dichloride (10 $\times$ 3 ml.).

The methylene dichloride extract was added directly to 4 ml. of saturated ethanolic picric acid and the resulting solution evaporated to small bulk at 40°. The crude picrate was filtered off, washed with ether and recrystallized from ethanol to constant specific activity, yielding yellow needles of 5-(2-hydroxyethyl)-4-methylthiazolium picrate, m.p. 162–164° (lit. m.p. 163–164°; Andersag & Westphal, 1937).

The aqueous layer from the sulphite cleavage was adjusted to pH 7.5 with 3 N-HCl and applied to a 13.0 cm. $\times$ 1.0 cm. column of Dowex 1(X8) resin (formate form, 100–200 mesh) according to the method of Goldstein & Brown (1963). The column was washed with 150 ml. of water and then eluted with 0.4 M-ammonium formate; 5 ml. fractions were collected and the extinction was monitored at 236 and 271 m μ . The pyrimidinesulphonic acid fractions (typically nos. 15–65) were pooled and evaporated, first on the steam bath and finally in an oven at 105° to decompose the ammonium formate. The crude pyrimidinesulphonic acid (III) was recrystallized twice from water and twice from aq. ethanol. Dilution with pure carrier (III) at this stage was followed by several more recrystallizations from aq. ethanol to constant specific activity.

Degradation of thiamine biosynthesized in the presence of [3-¹⁴C]serine to obtain C-2 of the thiazole ring. Yeast was grown in 100 ml. of synthetic medium as described above in the presence of 200 μ C (5.1 μ moles) of DL-[3-¹⁴C]serine. The pure thiazole picrate was obtained as described above and then diluted with carrier thiazole picrate, recrystallized from ethanol and counted (specific activity 6.01 μ C/mole). From 73.0 mg. of this picrate was obtained 16.6 mg. of crude formaldehyde dimedone derived from C-2 of the thiazole ring by the degradation procedure of Linnett & Walker (1967). The formaldehyde dimedone was diluted with carrier formaldehyde dimedone and recrystallized to constant specific activity from aq. ethanol (specific activity, corrected for dilution, 0.06 μ C/mole).

Incorporation experiment with [¹⁵N]glycine. A loopful of cells was grown in 100 ml. of MYGP medium for 2 days at 27°. The washed cells were suspended in sterile saline and 1 mg. dry wt. of cells was inoculated into each of two 51. flasks containing 1 l. of MYGP medium supplemented with 0.33 M-mole of thiamine. After 40 hr. growth the contents of the flasks were spun down (6.7 g. dry wt. of cells) and the cells washed three times with ice-cold sterile saline. These

cells suspended in 400 ml. of sterile saline constituted the inoculum.

The large-scale synthetic medium is described above. The glucose and vitamin solutions were sterilized separately and then added aseptically to the inorganic salts, which had been autoclaved *in situ* in 300 l. of solution in a stainless-steel fermenter. A solution of 3 g. of [¹⁵N]glycine (see above) in 100 ml. of water sterilized by Seitz filtration was added aseptically to the fermenter together with the inoculum. The culture was stirred at 25° with forced aeration. The fermenter was fitted with an automatic anti-foaming device with a silicone anti-foam agent (MS Silicone Antifoam Emulsion RD; Hopkin and Williams Ltd., Chadwell Heath, Essex) and the pH was automatically kept above 4.0 by the addition of N-NaOH. Growth was stopped after 86 hr. at the end of the exponential phase and the cells were spun down (wet wt. 2814 g., dry wt. approx. 845 g.). The cells were washed with 25 l. of saline and spun down again. The washed cells were boiled for 10 min. in 10 l. of 0.03 N-HCl to extract the thiamine pyrophosphate and to inactivate phosphatases. The cells were spun down and washed with 2 l. of water. The supernatant and washings were passed through a 44 cm. $\times$ 6.3 cm. column of Amberlite IRC-50 resin (Na⁺ form) to retain any free thiamine. The column was washed through with a further 1 l. of water and the eluate (approx. 13 l.) was adjusted to pH 4.7 (glass electrode) with acetic acid. Incubation with 16 g. of Taka-diastase (Parke, Davis and Co., Hounslow, Middx.) and 200 ml. of toluene at 40° for 15 hr. hydrolysed the phosphate esters of thiamine. The mixture was concentrated to about 2.3 l. below 40° in a climbing-film still with the addition of a little silicone anti-foam agent and extracted with 4 $\times$ 500 ml. of ether to remove toluene. The aqueous layer was clarified by passage through kieselguhr and then applied to a 46 cm. $\times$ 3.2 cm. column of Amberlite IRC-50 resin (H⁺ form). The column was washed with 1 l. of water and the washings were discarded. Elution with 0.08 N-HCl, 10 ml. fractions being collected, yielded thiamine in fractions 246–327 as judged by the extinction at 247 m μ , and by spotting fractions on to paper, spraying with aq. alkaline K₃Fe(CN)₆ and observing the thiochrome fluorescence under filtered u.v. light at 365 m μ . Evaporation of these fractions yielded 3.1 g. of solid residue, which contained 14.5 mg. of thiamine by a thiochrome determination on a sample as described above. The residue was diluted with 33.5 mg. of dry carrier thiamine chloride hydrochloride in 8 ml. of water. Addition of aq. 50% (w/v) NaOH to approx. pH 4.5 was followed by the addition of 1 ml. of M-sodium acetate buffer, pH 4.7, and 500 mg. of sodium metabisulphite. After incubation overnight at 40°, the solution was cooled, taken to pH 8 with aq. 50% (w/v) NaOH and extracted with 8 $\times$ 3 ml. of methylene dichloride.

The methylene dichloride extract was dried over MgSO₄ and evaporated under reduced pressure at room temperature. The residue was chromatographed on a 23 cm. $\times$ 2.4 cm. column of acid-washed alumina, which was eluted with light petroleum (b.p. 60–80°)–chloroform (1:1, v/v); 4 ml. fractions were collected and the extinction was monitored at 265 m μ . Fractions 80–113 contained the thiazole (II) and were evaporated to dryness, giving a crude yield of the thiazole (II) of 15.2 mg. The yield of pure thiazole (II) was 12.5 mg., as established from the extinction at 250 m μ of an ethanolic solution (ϵ 4378 at $\lambda_{\max}$. 250 m μ). Thin-layer chromatography on 10 cm. $\times$ 20 cm. plates of Kieselgel

GF₂₅₄ (E. Merck A.-G., Darmstadt, Germany) with ethyl acetate as developing solvent showed only a single fluorescence-quenching spot under u.v. light at 254m μ moving with authentic thiazole (II). This sample was divided into three portions, which were separately analysed for ¹⁵N content.

The aqueous layer from the sulphite cleavage at pH 8 was applied to a 32cm. $\times$ 2.4cm. column of Dowex 1(X8) resin (Cl⁻ form; 100–200 mesh). The column was washed with 2.5l. of water and then eluted with 0.007N-HCl; 5ml. fractions were collected and the extinction was monitored at 248m μ . Fractions 51–71 contained the pyrimidine-sulphonic acid (III) and were evaporated to dryness. Crystallization of the residue from aq. ethanol yielded a yellowish solid, which was treated with charcoal and further recrystallized from water to yield the pure sulphonic acid (III) as a white crystalline solid, yield 12.8mg. Thin-layer chromatography as described above with pyridine–2-methylpropan-1-ol–water (4:1:1, by vol.) as solvent (Johnson & Goodwin, 1963) showed a single fluorescence-quenching spot at R_F 0.57 moving with authentic pyrimidine-sulphonic acid (III). Three portions of this sample were separately analysed for ¹⁵N content.

A large-scale fermentation was carried out as above with glycine of normal isotopic abundance.

RESULTS

¹⁴C-incorporation experiments. It was found in preliminary experiments that yeast would grow to a reasonable extent on a synthetic medium containing glucose as the only major organic component, provided that the inoculum was prepared on MYGP medium (see the Materials and Methods section). This procedure permitted the addition of a wide range of ¹⁴C-labelled compounds to the culture medium without initial dilution of radioactivity save by the small intracellular pools introduced with the inoculum. The results for the incorporation of ¹⁴C from a range of potential precursors into the thiamine moieties are collected in Table 1.

All the ¹⁴C-labelled substrates supplied were well incorporated into the cells apart from [2-¹⁴C]mevalolactone and [2-¹⁴C] mevalonate, though the open-chain form was better incorporated than the lactone, as has been found in other systems (Fumagalli, Grossi, Poggi, Paoletti & Garattini, 1962; Durr & Shwayri, 1964). Also, DL-[3,4-¹⁴C₂] methionine was less well incorporated than L-[Me-¹⁴C]methionine into the cells: this effect may be partly due to lack of transport of the D-enantiomer through the cell wall.

General comments. The range of incorporation values found for the same labelled substrate in different experiments (see Table 1), even when similar amounts of substrate were supplied in the standardized culture procedure, shows that not too much quantitative importance should be placed on the results of single experiments. Given this variation, however, it is evident that there was a significant level of incorporation of label from

[2-¹⁴C]glycine, [3-¹⁴C]serine, [1-¹⁴C]glycine, [¹⁴C]-formate and [2-¹⁴C]acetate into the thiazole (II), in apparent order of decreasing incorporation. Similarly, label was incorporated into the pyrimidine (I) in apparent order of decreasing efficiency from [¹⁴C]formate, [3-¹⁴C]serine, [2-¹⁴C]glycine and [2-¹⁴C]acetate.

Lack of incorporation of label from [3-¹⁴C]serine into C-2 of the thiazole ring. This experiment was intended to supplement our previous work in which it was shown that there was incorporation of label from [2-¹⁴C]glycine, but not from [¹⁴C]formate, into C-2 of the thiazole ring of thiamine by yeast (Linnett & Walker, 1967). DL-[3-¹⁴C]Serine was supplied by using the same procedure employed in the other ¹⁴C-incorporation experiments, and the resulting purified thiazole picrate was diluted with carrier thiazole picrate and then degraded to formaldehyde derived from C-2 of the thiazole ring by the specific procedure of Linnett & Walker (1967). Incorporation of label into C-2 of the thiazole ring was only 1% of the incorporation into the whole of the thiazole moiety, showing that C-3 of serine, like formate, does not supply C-2 of the thiazole ring.

Incorporation of label from [¹⁵N]glycine. The use of C-2 of glycine to provide C-2 of the thiazole ring (Linnett & Walker, 1967) and the demonstration of the involvement of glycine by way of 5-amino-imidazole ribonucleotide in the biosynthesis of the pyrimidine moiety (Newell & Tucker, 1967, 1968a,b) made it desirable to test the incorporation of the nitrogen of glycine into the thiamine fragments.

The inoculum for the large-scale cultures was grown on MYGP medium (see the Materials and Methods section) supplemented with thiamine, since it had been shown that higher thiamine contents of the cells could be obtained in subsequent cultures and that label from [2-¹⁴C]glycine was incorporated into the thiazole (II) and pyrimidine (I) by thiamine-pretreated cells though at somewhat decreased levels of incorporation (Linnett, 1968). Such a lowering of incorporation into the thiamine moieties indicates that thiamine controls its own biosynthesis in yeast by repression of the enzymes involved in a manner similar to that already shown for *Salm. typhimurium* (Newell & Tucker, 1966).

The ¹⁵N contents of the thiamine fragments obtained in the normal glycine and [¹⁵N]glycine experiments are summarized in Table 2. A slight enrichment of ¹⁵N content is apparent in the values obtained for the thiazole and pyrimidine moieties grown in the presence of glycine of normal isotopic abundance; it is not certain whether this result was due to isotope effects in the various biosynthetic steps leading to the moieties or was within the experimental error of the method. In the experiment with ¹⁵N-enriched glycine the ¹⁵N content of

Table 1. *Incorporation of label from ^{14}C -labelled substrates into the thiamine moieties by yeast*

Yeast was grown in 100 ml. of synthetic medium supplemented with the ^{14}C -labelled substrate under test for 2.5 days at 27°. The cells were harvested, and the thiamine was extracted, purified and diluted with 50 mg. of carrier thiamine. Sulphite cleavage yielded the thiazole (II) picrate and the pyrimidinesulphonic acid (III), which were each purified to constant specific activity. Incorporation of label into the cells is the ratio of the radioactivity of the cells after growth to that of the cells plus supernatant. Incorporation of label into the moiety is the ratio of its specific activity as biosynthesized (obtained from the measured specific activity and corrected for previous dilution) to the specific activity of the substrate as supplied (both specific activities being on a molar basis). Experimental details are given in the Materials and Methods section.

Labelled substrate	Sp. activity of substrate supplied ($\mu\text{C}/\mu\text{mole}$)	Amount of substrate supplied (μmoles)	Incorporation of label into cells (%)	Incorporation into the thiazole (%)	Incorporation into the pyrimidine (%)
DL-[2- ^{14}C]Mevalolactone	5.0	9.0	0.3	Zero	0.03*
Potassium DL-[2- ^{14}C]mevalonate	5.0	8.5	1.3	0.06*	0.02*
L-[U- ^{14}C]Glutamic acid	3.9	6.3	73	0.10*	0.05*
L-[U- ^{14}C]Aspartic acid	2.3	42.3	67	Zero	0.9*
L-[U- ^{14}C]Alanine	7.2	7.0	65	0.03*	0.11
DL-[1- ^{14}C]Alanine	10.0	10.0	22	0.06*	0.04*
Sodium [2- ^{14}C]acetate	8.3	12.0	84	0.03	0.43
Sodium [2- ^{14}C]acetate	10.4	33.6	89	0.12	0.68
Sodium [^{14}C]formate	10.8	11.6	95	3.8	32.3
Sodium [^{14}C]formate	30.5	9.8	83	0.96	27.5
L-[Me- ^{14}C]Methionine	3.6	7.0	64	0.03*	0.06*
L-[Me- ^{14}C]Methionine	3.6	7.0	59	0.25*	0.11*
L-[Me- ^{14}C]Methionine	26.1	5.3	64	0.31	0.09
DL-[3,4- $^{14}\text{C}_2$]Methionine	0.89	11.2	14	0.14*	0.35*
DL-[3,4- $^{14}\text{C}_2$]Methionine	0.89	28.0	3	0.18*	Not measured
L-[U- ^{14}C]Serine	4.8	2.1	72	1.9	1.8
L-[U- ^{14}C]Serine	2.4	18.9	62	3.9	<9.0
L-[U- ^{14}C]Serine	1.8	11.9	57	1.4	5.4
L-[3- ^{14}C]Serine	4.7	7.9	77	5.7	9.2
DL-[3- ^{14}C]Serine	38.9	5.1	45	2.6	1.5
[U- ^{14}C]Glycine	3.9	12.4	52	6.4	6.7
[2- ^{14}C]Glycine	6.3	7.9	23†	33.5†	7.8†
[2- ^{14}C]Glycine	3.5	7.1	67	11.6	2.4
[2- ^{14}C]Glycine	31.7	0.5	85	0.6	0.06
[2- ^{14}C]Glycine	31.7	6.3	90	5.2	1.4
[1- ^{14}C]Glycine	7.9	6.3	60	4.4	0.02
[1- ^{14}C]Glycine	3.5	7.2	55	0.6	0.20*

* Represents limit of resolution under the experimental conditions used, i.e. when the count rate of the sample tended towards the background count rate.

† Cell growth less than usual (22 mg. dry wt. of cells).

the pyrimidine moiety was similar to that in the normal glycine experiment. The extent of incorporation of nitrogen from [^{15}N]glycine into the thiazole (II), however, was definitely significant, being well above the 'background' level for the ^{15}N content of the thiazole obtained in the normal glycine experiment and well above the calculated value for the total ^{15}N content of the medium (about 0.14 atom % excess ^{15}N).

DISCUSSION

Biosynthesis of the pyrimidine moiety. The lack of appreciable incorporation of label from [^{14}C]-

uracil or [^{14}C]orotate (Goldstein & Brown, 1963; Johnson *et al.* 1966) into the pyrimidine (I) suggests that the well-known biosynthetic pathway to the nucleic acid pyrimidines by way of *N*-carbamoyl-aspartate and UMP (see, e.g., Crosbie, 1960) is not involved. It has been shown in this work and in that of Goldstein & Brown (1963) that [^{14}C]aspartate is poorly incorporated into the pyrimidine residue. The more significant incorporations from [2- ^{14}C]aspartate (Nakamura, 1965) and from [U- ^{14}C]aspartate (Tomlinson *et al.* 1967) into the pyrimidine by other systems, however, suggest that aspartate may supply some of the carbon atoms of the pyrimidine moiety either directly, with loss of one

Table 2. ^{15}N contents of the thiazole and pyrimidine moieties of thiamine biosynthesized by yeast in the presence of ^{15}N glycine and normal glycine

Yeast was grown in 300l. of synthetic medium plus 3g. of either ^{15}N glycine (32.1 atom % excess ^{15}N) or normal glycine (0.374 atom % ^{15}N). The procedures for culturing, extraction of thiamine and subsequent purification of the thiazole and pyrimidine moieties are described in the Materials and Methods section. Each sample was split into three parts and converted separately into N_2 gas for analysis in the mass spectrometer. The values in parentheses indicate the spread of measured values of ^{15}N content around the mean value for each sample.

Precursor supplied	Moiety	^{15}N content of moiety		^{15}N content of moiety as biosynthesized (i.e. corrected for dilution of thiamine with carrier) (atom % excess ^{15}N)
		(atom % as measured)	(atom % excess)	
^{15}N Glycine	Thiazole (II)	0.477 (± 0.006)	0.103	0.34
^{15}N Glycine	Pyrimidine (III)	0.384 (± 0.003)	0.010	0.03
Normal glycine	Thiazole (II)	0.395 (± 0.003)	0.021	0.05
Normal glycine	Pyrimidine (III)	0.389 (± 0.002)	0.015	0.04

or more of its carbon atoms, or indirectly by prior metabolism to the actual precursor.

The pathway to the pyrimidine moiety proposed by Newell & Tucker (1968b) requires the incorporation of both carbon atoms of glycine into C-4 and C-5 respectively of 5-aminoimidazole ribonucleotide (see, e.g., Schulman, 1961), as indeed was shown by Newell & Tucker (1968a) in their experiments with *Salm. typhimurium* mutants. In growing yeast, however, one might expect C-2 of glycine to contribute to a certain extent to the folic acid pool of C_1 intermediates (see Jaenicke & Kutzbach, 1963) and thus to contribute label to some extent to C-2 as well as to C-4 of 5-aminoimidazole ribonucleotide. Thus a better incorporation from $[2\text{-}^{14}\text{C}]$ glycine than from $[1\text{-}^{14}\text{C}]$ glycine into the pyrimidine (I) could be expected, as found in this work. The nitrogen atom of glycine would become the amino group attached to the C-4 of the pyrimidine ring on the pathway postulated by Newell & Tucker (1968b). The apparent lack of incorporation of ^{15}N from ^{15}N glycine into the pyrimidine moiety found here could be due to the lack of sensitivity of the ^{15}N measurement, since there are two other nitrogen atoms in each molecule of the pyrimidine (I) not expected to be derived from glycine, and to the possible exchange of the C-4 amino group with ammonia in the cell. Alternatively the results may reflect a somewhat different pathway for pyrimidine biosynthesis in yeast.

The significant incorporation of label from $[3\text{-}^{14}\text{C}]$ serine into the pyrimidine moiety, as found also by Goldstein & Brown (1963), suggests that C-3 of serine may be metabolized to formate or its equivalent folic acid derivative (Jaenicke & Kutzbach, 1963) before incorporation into the pyrimidine.

There was a significant but small amount of incorporation from $[2\text{-}^{14}\text{C}]$ acetate into the pyrimidine moiety found here and elsewhere (Goldstein &

Brown, 1963; Johnson *et al.* 1966; Tomlinson, 1966; Kumaoka & Brown, 1967; Tomlinson *et al.* 1967). Double-labelling experiments suggested the incorporation of the methyl group of acetate into the methyl group attached to C-2 of the pyrimidine ring (Tomlinson, 1966; Tomlinson *et al.* 1967), but the results of the formate competition experiment of Kumaoka & Brown (1967) make it possible that acetate is first metabolized to a C_1 precursor before incorporation.

Biosynthesis of the thiazole moiety. Alanine has been shown to be incorporated into the thiazole moiety to a certain extent in yeast (Johnson *et al.* 1966) and in *Bacillus subtilis* (Tomlinson *et al.* 1967), but the poor incorporation found here suggests that alanine may not be an immediate precursor of the thiazole (II). The incorporation of label from $[2\text{-}^{14}\text{C}]$ acetate found here, though low, together with the incorporation found elsewhere for $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]$ -acetate (Johnson *et al.* 1966; Tomlinson *et al.* 1967), but not for $[\text{Me-}^3\text{H}]$ acetate (Tomlinson *et al.* 1967), suggests that acetate may either be incorporated directly into the thiazole with loss of its methyl hydrogen atoms or may be metabolized to another C_2 precursor before incorporation.

The lack of incorporation from $[\text{U-}^{14}\text{C}]$ glutamate into the thiazole moiety found here, together with its poor incorporation into thiamine (Tomlinson *et al.* 1967), suggests that the pathway envisaged by Plaut (1961) by way of cysteine and glutamate does not operate in the biosynthesis of the thiazole (II).

The lack of incorporation from $[2\text{-}^{14}\text{C}]$ mevalonate rules out its utilization by methyl group migration and carboxyl group elimination to form the entire C_5 part of the thiazole moiety comprising C-4, C-5 and their attached side chains.

The results presented here do not support the conclusion reached by Johnson *et al.* (1964, 1966) and Tomlinson *et al.* (1967) that the thiazole (II)

(IV)

is biosynthesized from methionine by the pathway proposed by Harington & Moggridge (1939, 1940) via the thiazolylalanine (IV) (see also Buchman & Richardson, 1939). Though nutritional experiments have shown that the thiazolylethanol (II) can be utilized to provide the thiazolylethanol (II) in various systems (Bonner & Buchman, 1938; Harington & Moggridge, 1940; Robbins, 1940; Bonner & Bonner, 1948; Akagi, Kumaoka, Uchida & Mukaiyama, 1963), it would appear from the [^{35}S]sulphate competition experiments of Hitchcock & Walker (1961) and from the results of Fujii, Esaki & Kawasaki (1967) with *E. coli* that the amino acid (IV) does not lie on the normal biosynthetic pathway to the thiazole (II). Methionine has been shown to be a reasonably efficient source of sulphur for thiazole biosynthesis (Hitchcock & Walker, 1961; Kumaoka, 1963; Johnson *et al.* 1964, 1966). The efficient utilization of C-2 of glycine to provide C-2 of the thiazole ring (Linnett & Walker, 1967), which is the position expected to be occupied by the methyl carbon atom of methionine on the Harington & Moggridge (1939, 1940) pathway, and the lack of significant incorporation from [$\text{Me-}^{14}\text{C}$] and from [$3,4\text{-}^{14}\text{C}_2$]methionine into the thiazole moiety found here suggest that the Harington & Moggridge (1939, 1940) pathway to the thiazole is not followed in yeast. Since neither [^{14}C]formate (Linnett & Walker, 1967) nor [$3\text{-}^{14}\text{C}$]serine provide label for C-2 of the thiazole ring, though they are significantly incorporated elsewhere into the thiazole moiety, C-2 of glycine is not first metabolized to a C_1 intermediate, as presumably are formate and C-3 of serine (Jaenicke & Kutzbach, 1963), before incorporation into the thiazole (II). These results suggest that formate, C-3 of serine and the methyl carbon atom of methionine provide a C_1 precursor to some position other than C-2 of the thiazole ring.

In the light of the above data, the experiment of Johnson *et al.* (1964, 1966), which showed that [$\text{Me-}^{14}\text{C}$, ^{35}S]methionine was incorporated into the thiazole (II) with retention of the $^{14}\text{C}/^{35}\text{S}$ specific activity ratio, could indicate either utilization of the methylthio residue as a unit to provide C-5 and the sulphur atom of the thiazole ring or the fortuitous incorporation of the methyl carbon atom of methionine as one of the side-chain carbon atoms of the thiazole (II) at the same dilution of activity as the sulphur atom. Austin & Meyers (1966) have

reported a case of such an anomalous retention of isotope ratios in the biosynthesis of skimmin. Tomlinson *et al.* (1967) found that [$\text{Me-}^{14}\text{C}$, $\text{G-}^3\text{H}$]methionine ($^{14}\text{C}/^3\text{H}$ ratio 1:1) was incorporated into the thiazole fragment with a resultant $^{14}\text{C}/^3\text{H}$ ratio 1:0.3. This result, though being consistent with the Harington & Moggridge (1939, 1940) pathway, as pointed out by Tomlinson *et al.* (1967), is also consistent with the incorporation of just the methyl carbon atom of methionine together with its hydrogen atoms into any of the three side-chain positions of the thiazole (II), each of which bears non-exchangeable hydrogen atoms.

The significant incorporation of nitrogen from glycine into the thiazole fragment found here, together with the incorporation of C-2 of glycine into C-2 of the thiazole ring (Linnett & Walker, 1967), suggests either that the aminomethyl group of glycine provides C-2 and N-3 of the thiazole ring as a unit or that these atoms of the thiazole ring are supplied from two separate glycine molecules. It is impossible to decide between these two routes from the available data, except to note that the incorporation of label found here from [$1\text{-}^{14}\text{C}$]glycine into the thiazole (II), though at a lower level than that from [$2\text{-}^{14}\text{C}$]glycine, and the location of about a quarter of the radioactivity from [$2\text{-}^{14}\text{C}$]glycine elsewhere in the thiazole moiety than at C-2 of the ring (Linnett & Walker, 1967), suggest that glycine might contribute to C-4 of the thiazole ring and either to C-5 of the ring or to the methyl group attached to C-4, as well as supplying C-2 of the thiazole ring. Further work will be needed to elucidate fully the part played by glycine and by C_1 donors in the biosynthesis of the thiazole moiety.

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