Thiamin Biosynthesis in *Saccharomyces cerevisiae*

ORIGIN OF CARBON-2 OF THE THIAZOLE MOIETY

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(Received 2 October 1978)

Radioactivity from [2-¹⁴C]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-¹⁴C]methionine or from DL-[2-¹⁴C]tyrosine does not enter thiamin.

Although the structure of thiamin (vitamin B₁) 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).

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-¹⁴C]methionine was incorporated into the thiazole moiety of thiamin and that the ³⁵S/¹⁴C ratio of L-[Me-¹⁴C,³⁵S]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 [1-¹⁴C]- and [2-¹⁴C]-acetate and from L-[U-¹⁴C]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 1. The final stages of the biosynthesis of thiamin pyrophosphate

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 $[2-^{14}\text{C}]$ -glycine that most of the activity of the molecule was localized at C-2 of the thiazole nucleus (Linnett & Walker, 1967). Administration of $[2-^{14}\text{C}]$ -glycine in the presence of unlabelled methionine gave an identical result (Linnett & Walker, 1969), and, in an experiment with $[^{15}\text{N}]$ -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 $27 \pm 1^\circ\text{C}$ for 24 h, 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 $27 \pm 1^\circ\text{C}$ for 48 h 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 (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 (Difco Manual, 1953) was Difco neopeptone (10 g), D-glucose (40 g), Difco Bacto-agar (15 g), 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 (2000 $\mu\text{g/litre}$), calcium DL-pantothenate (400 $\mu\text{g/litre}$) and D-biotin (2 $\mu\text{g/litre}$). The composition of Difco Bacto vitamin-free yeast base (Difco Manual, 1953) is: $(\text{NH}_4)_2\text{SO}_4$ (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).

Yeast-extract medium (Johnson *et al.*, 1966). The composition of this medium was Difco yeast extract (2 g), D-glucose (20 g), $(\text{NH}_4)_2\text{SO}_4$ (0.943 g), KH_2PO_4 (0.5 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.3 g), and water (1 litre). Difco yeast extract contains thiamin (150 $\mu\text{g/g}$).

Methods

Growth on thiamin-free medium. A fresh slant of yeast cells was prepared by inoculation from a stock culture and incubated for either 24 h (*S. cerevisiae*, A.T.C.C. 24903) or 48 h (*S. cerevisiae*, 39916, H. J. Bunker) at $27 \pm 1^\circ\text{C}$. The cells were washed with sterilized water ($3 \times 25\text{ ml}$). A portion of this cell suspension (0.5 ml, i.e., approx. 10^7 cells) was used to inoculate each 50 ml portion of the liquid medium, prepared as follows. Difco Bacto vitamin-free yeast base (16.7 g) was dissolved in water (100 ml) and stock vitamin solution (1.0 ml) (containing 2000 μg of *myo*-inositol/ml, 400 μg of calcium DL-pantothenate/ml, and 2 μg 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, 0.45 μm pore diameter; Metrical, 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).

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 $27 \pm 1^\circ\text{C}$) (Warner-Chilcott Laboratories model 2156 water-bath shaker).

After a short lag phase (2–3 h), *S. cerevisiae* (A.T.C.C. 24903) maintained logarithmic growth for 19 h of incubation, when a cell density of approx. 8×10^7 cells/ml was attained (Fig. 1, curve A). 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 (Fig. 1, curve B).

Growth on yeast-extract medium (cf. Johnson *et al.*, 1966). The yeast-extract medium was dispensed into Roux flasks (75 ml/flask), which were then plugged with non-absorbent cottonwool and autoclaved at 15 lb/in² (103.5 kPa) for 20 min. One flask was chosen and inoculated with a loopful of *S. cerevisiae* (39916, H. J. Bunker) from a Sabouraud agar slant (grown at $27 \pm 1^\circ\text{C}$ for 48 h) and incubated at $27 \pm 1^\circ\text{C}$ for 24 h. A sample of this culture (0.5 ml) was used to inoculate the remaining Roux flasks, which were incubated for 48 h at $27 \pm 1^\circ\text{C}$. 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 (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. 10^6 cells/ml. Radioactive tracer solution was added after 5.5 h of incubation and the cells collected 43 h 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 15.5 h of incubation and the cells were collected 48 h later. The cells were collected by centrifugation [20 min at 10°C and 900g (r_{av} , 13 cm)]. 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 30 min with 20 ml of 0.1 M-HCl. The mixture was centrifuged and the residue re-extracted in the same way. 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 hydrolysed by incubating this solution at $37 \pm 1^\circ\text{C}$ 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 (Myrbäck & Neumüller, 1950). 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 $\times$ 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.

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 (1.0 ml) was mixed with CNBr solution (2.0 ml) [prepared by titrating a saturated aq. Br_2 solution at 0°C with aq. KCN (20% w/v), until colourless] and 1.0 ml of aq. NaOH (50%, w/v). The fluorescent product was extracted into 2-methylpropan-1-ol (5.0 ml) by vigorously shaking for 2 min. The 2-methylpropan-1-ol layer was clarified by centrifugation for 30 s. The fluorescence of the 2-methylpropan-1-ol layer was measured with an Aminco-Bowman spectrophotofluorimeter in 1 cm Supracil cells. The exciting wavelength was 370 nm and

fluorescence was measured at 430 nm. A standard curve was prepared for each run from solutions ranging in thiamin concentration from 0.2 to $5.0 \mu\text{g/ml}$. 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 ($0.7 \mu\text{g/ml}$ in 0.05 M- 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. 1 mg) and 5 ml of 1 M-sodium acetate buffer, pH 4.7, were added to the acid eluate from the Amberlite ion-exchange column (see above). The pH of the solution was increased to about pH 6 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 (100 ml) and eluted with 500 ml of 0.005 M-HCl, and the eluate collected in 15 ml fractions. The thiamin-containing fractions (usually fractions 12–30) were combined, additional carrier thiamin (approx. 4 mg) 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, 20 cm $\times$ 20 cm $\times$ 0.5 mm) 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. 50 mg. This product was crystallized to constant radioactivity from methanol/propan-2-ol (1:2, v/v).

Determination of radioactivity. Triplicate samples (1–2 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 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 (100–300 mg) was added to the thiamin before degradation.

(i) Separation of the pyrimidine and thiazole

moieties by bisulphite cleavage (cf. Williams *et al.*, 1935). Thiamin (348 mg) was dissolved in water (6 ml) and NaHSO₃ (1.70 g) 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 157 mg (82 %).

The filtrate was titrated to pH 12 with NaOH (50 %, w/v) and extracted with chloroform (5 × 15 ml). The chloroform extracts were dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to give 5-(β-hydroxyethyl)-4-methylthiazole (2) (125 mg, 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 (5 ml) and phenyl isocyanate (0.1 ml) added. The solution was kept at room temperature (25°C) overnight, the solvent was evaporated *in vacuo* and the thiazole phenylurethane recrystallized from ethyl acetate. The yield was 195 mg (85 %). A portion (30 mg) was separated from the diphenylurea contaminant by preparative thin-layer chromatography (silica-gel plate, 0.5 mm × 20 cm × 20 cm) 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 (2.7 Pa). 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; C₁₃H₁₄N₂O₂S 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) (69 mg) was dissolved in benzene (4 ml), excess methyl iodide was added and the mixture was refluxed for 24 h. 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 76 mg; 71 %). The methiodide (8) had m.p. 164–165°C; δ (p.p.m.) ([²H]methanol) 2.52 (3 H, 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; C₁₄H₁₇IN₂O₂S requires C, 41.50; H, 4.24; N, 6.93 %).

Table 1. Feeding experiments with radioactive tracers

Substrate	<i>S. cerevisiae</i> strain ... A.T.C.C. 24903					39916, H. J. Bunker				
	Expt. no.	Total radio-activity (μCi)	Sp. radio-activity (mCi/mmol)	Culture size (ml)	Radioactivity remaining in medium (% of total activity)	Expt. no.	Total radio-activity (μCi)	Sp. radio-activity (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	500	29	4	250	49.7	1400	23
			(1.3)†					(2.5)‡		
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 (20 mg/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*

Substrate	A.T.C.C. 24903		39916, H. J. Bunker	
	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) (70 mg) was suspended in propan-2-ol (3 ml) at 0°C. NaBH₄ (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 allowed to warm up gradually to room temperature (25°C). Water (10 ml) was added, the solution was extracted with diethyl ether (5 × 5 ml), the extract dried with

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

(iii) Hydrolysis with HgCl_2 . The oil (9) was stirred overnight in water (4 ml) containing HgCl_2 (52 mg). The precipitate was filtered off and washed with water (2×5 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 steam bath for 1 h, the formaldehyde dimethone was filtered off and sublimed at 95°C and 2×10^{-2} mm Hg (2.7 Pa). The yield obtained was 33 mg (65%), with m.p. $189\text{--}190^\circ\text{C}$ [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) (46 mg) was dissolved in 15 ml of 20% (v/v) H_2SO_4 and CrO_3 (2.1 g) in water (2 ml) 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 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 0.1 M-NaOH. Sodium acetate (22 mg, 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 (1 ml) and an aqueous solution (2 ml) of α -naphthylamine hydrochloride (40 mg) was added, followed by 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide (100 mg). 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 (2.7 Pa). The yield obtained was 15 mg [46% based on the thiazole phenylurethane (7)] with m.p. $158\text{--}159^\circ\text{C}$ [reported m.p. $159\text{--}160^\circ\text{C}$ (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 $\pm$ s.d.) $\mu\text{g/litre}$ 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 300 $\mu\text{g/litre}$ of culture.

Radioactive-tracer experiments with $[2\text{-}^{14}\text{C}]\text{glycine}$

and $\text{L-}[Me\text{-}^{14}\text{C}]\text{methionine}$ were performed with each of the two yeast strains. A further experiment with $\text{DL-}[2\text{-}^{14}\text{C}]\text{tyrosine}$ 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 $\text{L-}[Me\text{-}^{14}\text{C}]\text{methionine}$ and $\text{DL-}[2\text{-}^{14}\text{C}]\text{tyrosine}$

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 $\text{L-}[Me\text{-}^{14}\text{C}]\text{methionine}$ with both strains of *S. cerevisiae* (Expts. 3 and 4) and in the experiment with $\text{DL-}[2\text{-}^{14}\text{C}]\text{tyrosine}$ 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 $[2\text{-}^{14}\text{C}]\text{glycine}$

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'.

<i>S. cerevisiae</i> strain ...		A.T.C.C. 24903		H. J. Bunker, 39916	
		$10^{-4} \times \text{Sp. radioactivity (d.p.m./mmol)}$	Relative sp. radioactivity (%) [*]	$10^{-4} \times \text{Sp. radioactivity (d.p.m./mmol)}$	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 $\mu\text{g/g}$ dry cell wt., i.e. 20–200 $\mu\text{g/litre}$ of culture (Kutsky, 1973), and higher plants 0.1–10 $\mu\text{g/g}$ fresh wt. (Kutsky, 1973; Robinson, 1966). Since for a definitive biosynthetic investi-

gation, which demands isolation and purification to constant radioactivity, followed by derivatization and chemical degradation to locate the site or sites of radioactivity, at least 10mg 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 B₁ 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 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 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 [2-¹⁴C]glycine and L-[Me-¹⁴C]-methionine. It was demonstrated by preparation of derivatives and degradation of the isolated thiamin that [Me-¹⁴C]methionine did not serve as a precursor, but that [2-¹⁴C]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, ¹⁵N from [¹⁵N]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-¹⁴C]methionine and [Me-¹⁴C,³⁵S]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 derivatives of or degrade the compound. The thiazole fraction obtained in the two experiments contained respectively ¹⁴C, and ¹⁴C and ³⁵S, with a ³⁵S/¹⁴C 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 B₁ 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 [2-¹⁴C]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-¹⁴C]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-¹⁴C]- and [Me-¹⁴C,³⁵S]-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-CH₃ function, maintenance of the ³⁵S/¹⁴C 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 24 h after inoculation. In the present experiments, a thiamin-free medium was used, and radioactive tracer was added 15.5 h after inoculation.

As is shown in Fig. 1 (curve C), logarithmic phase is complete after approx. 20 h 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 (2 g/litre). Yeast extract contains thiamin (approx. 30–300 µg/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 15.5 h 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-¹⁴C]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. [2-¹⁴C]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-¹⁴C]-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

C₅ unit (C-4', C-4, C-5, C-6, and C-7) of the thiazole moiety in yeast and in bacteria awaits clarification.

We thank Professor John J. Miller, Department of Biology, McMaster University, for his unfailing advice and help with yeast culturing, and for the hospitality of his laboratory. We thank Professor T. W. Goodwin, C.B.E., F.R.S., Department of Biochemistry, University of Liverpool, Liverpool, U.K., for assistance in locating a culture of *S. cerevisiae* strain H. J. Bunker, 39916. This work was supported by the National Research Council of Canada.

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