Observations on the Biosynthesis of Thiamine in Yeast

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1. Methods are described for the isolation of radioactively pure thiamine from yeast and its degradation on a small scale to its cyclic components. 2. A degradation of the pyrimidine ring and a thin-layer method for the separation of thiamine, its derivatives and pyrimidine and thiazole residues are described. 3. [^{14}C]Formate is more effectively incorporated into the pyrimidine residue than into the thiazole residue, whereas the reverse is true with L-[Me- ^{14}C]methionine. 4. Experiments with [Me- ^{14}C , ^{35}S]methionine demonstrate that methionine provides an intact unit for the biosynthesis of the thiazole ring. 5. [6- ^{14}C]Orotic acid is insignificantly incorporated into the pyrimidine residue of thiamine. 6. Experiments with [1- ^{14}C]- and [2- ^{14}C]-acetate indicate that it is incorporated as a unit into the thiazole residue, but that only C-2 is incorporated into the pyrimidine residue. 7. L-[U- ^{14}C]Alanine is also effectively incorporated into the thiazole residue. 8. These results are discussed in relation to possible pathways of biosynthesis of the two ring components of the thiamine molecule.

Early nutrition experiments of Schopfer & Jung (1937) and Robbins & Kavanagh (1937) showed that the mould *Phycomyces blakesleeanus*, which required thiamine (I) for growth, would also grow on a mixture of the pyrimidine (4-amino-5-hydroxy-methyl-2-methylpyrimidine) (II) and the thiazole [5-(2-hydroxyethyl)-4-methylthiazole] (III) residues of thiamine. Later it was found that thiamine-requiring fungi fell into four distinct categories according to whether they required: (1) preformed thiamine; (2) both compound (II) and compound (III) preformed; (3) only compound (II) preformed; (4) only compound (III) preformed (for full details see Schopfer, 1943; Goodwin, 1963). These investi-

gations clearly indicated that the final stages of thiamine biosynthesis involved condensation of the two preformed ring systems, and recently this has been demonstrated enzymically; the reactions involve the pyrophosphorylation of compound (II), the phosphorylation of compound (III) and the condensation of these two compounds with the liberation of pyrophosphate to form thiamine monophosphate, which is converted into thiamine by a specific phosphatase (Leder, 1959; Camiener & Brown, 1959, 1960; Nose, Ueda & Kawasaki, 1959; Suzuoki & Kobata, 1960; Lewin & Brown, 1961). Much less was known of the origin of the ring systems themselves and nutrition experiments were inconclusive (see Goodwin, 1962). However, Pine & Guthrie (1959) found that *Bacillus subtilis* A.T.C.C. 6051 incorporates [^{14}C]formate specifically into the pyrimidine fragment of thiamine; somewhat similar results were reported for baker's yeast by David & Estramareix (1960, 1961), who also stated that the label was not incorporated into position 5 of the pyrimidine residue, and for *Escherichia coli* (Goldstein & Brown, 1963). Pine & Guthrie (1959) and David & Estramareix (1960, 1961) also found [^{14}C]formate to be insignificantly incorporated into the thiazole residue of thiamine in *B. subtilis* and baker's yeast.

The present investigations were undertaken in an attempt to throw more light on the origin of the two ring components in thiamine. A preliminary

short report of part of this work has already been published (Johnson, Howells & Goodwin, 1964).

EXPERIMENTAL

Organism. *Saccharomyces cerevisiae* Bunker strain no. 39916 was used throughout the investigation. It was maintained on Saboraud agar slopes that, after incubation at $28 \pm 1^\circ$ for 2 days, were stored at 4° until required. The liquid culture medium consisted of (per 100 ml.): glucose, 2.0 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.0943 g.; KH_2PO_4 , 0.05 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.03 g.; yeast extract, 0.2 g. It was dispensed (150 ml.) into Roux bottles that, after inoculation with a mother culture, were incubated for 24 hr. at $28 \pm 1^\circ$. Radioactive material was added at this point and the incubation was continued for a further required period of time before the cells were harvested in a Sharples Super centrifuge with a roll of cellophan paper inside the collecting cylinder. The resulting thick suspension was transferred to a weighed centrifuge tube and centrifuged in a bench centrifuge (Martin Christ) at 3500 rev./min. The pellet was washed thoroughly with water, frozen, transferred to a vacuum desiccator and freeze-dried over conc. H_2SO_4 . Yields were 2.6–3.0 g. of dry cells/l. of medium and the thiamine content was 160–200 $\mu\text{g./g.}$ of dry cells.

Extraction, purification and assay of thiamine. A known weight (about 3 g.) of dry cells was extracted with 75 ml. of 0.1 N-HCl on a boiling-water bath for 30 min. The extract was cooled and clarified by centrifugation. The extraction vessel was washed with dilute HCl, the extract and washings were combined and the pH was adjusted to pH 4.5. The thiamine monophosphate present in the extract was hydrolysed with Taka-diestase (Parke, Davis and Co. Ltd., Hounslow, Middlesex). Taka-diestase preparations were made by suspending 6 g. of dry powder in 100 ml. of 2.5 M-sodium acetate; 5 ml. of the fresh suspension was added to the HCl extract (75 ml.) and the pH adjusted, if necessary, to 4.5. The mixture was incubated in an enzyme bath for 2.5 hr. at $45\text{--}50^\circ$ with intermittent shaking and then clarified by centrifugation (Martin Christ bench centrifuge). The supernatant was removed and the residue washed with dilute aqueous HCl (pH 4.5). The combined supernatant and washings were then purified on columns of suitably activated Decalco F resin. The activated resin was prepared by first soaking 100 g. in hot 3% (v/v) acetic acid (250 ml.) for 15 min.; the acid was drawn off under mild suction and the washing process repeated. The resin then was washed with hot 25% (w/v) KCl (250 ml.), filtered off and the acid wash again repeated. Finally, the resin was washed with hot distilled water (600 ml. portions) until free from Cl^- ions, dried at room temperature and stored in a glass jar.

Columns were made of glass tubes (inner bore 7 mm. and 30 cm. long) drawn out at one end to form constricted delivery jets. The narrow ends were plugged with non-absorbent cotton wool, and activated Decalco F (6 g.) was poured into the tubes. The delivery jets were closed with rubber tubing and screw clips, and the tubes were filled with deionized water. The open end of each tube was closed with a clean cork, and the tubes were then shaken and inverted to free the resin bed from air bubbles. The bubble-free resin was allowed to settle and plugs of non-absorbent cotton wool were placed on the top of each resin

bed, which was charged with thiamine solution and connected to dropping funnels by means of polythene tubing.

The efficiency of each batch of resin was checked by passing standard solutions (1.0–2 $\mu\text{g./ml.}$) of thiamine at pH 4.5 through two columns of the resin; the columns were washed with hot deionized water (30 ml.) and the thiamine was eluted from each column with 25% (w/v) KCl in 0.1 N-HCl (50 ml.). The columns were considered satisfactory if a recovery off the second column of 94–98% was obtained.

The extracts from the Taka-diestase incubations were purified by passing 25 ml. of extract (25–50 $\mu\text{g.}$ of thiamine) through two columns as just described. Two columns are necessary to be certain of removing all impurities that might interfere with the quantitative estimation of thiamine in an EEL fluorimeter (Association of Vitamin Chemists, 1947).

The extracts prepared as described above required further purification for radioisotope work. The eluates were desalted by a method involving extraction from the aqueous phase into phenol and re-extraction into pure water (Iacono & Johnson, 1957; Carlson & Brown, 1961). The desalted solution was evaporated to dryness in a rotary evaporator (Wrights Scientific Ltd., London), and the residue was dissolved in ethanolic HCl (pH 4.0) and made up to volume (10 or 25 ml.). It was at this stage that carrier thiamine was added if required.

Disulphite cleavage of thiamine. This reaction was described by R. J. Williams in his classical work on the structure of thiamine (see Williams, Eakin, Beerstecher & Shive, 1950). In the present investigation it was carried out by dissolving thiamine (100 mg.) in 2.6 M- Na_2SO_3 (1.5 ml.) [prepared by dissolving 32.8 g. of $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ (analytical grade) in 100 ml. of deionized water and adjusting the pH to 4.8–5.0 by bubbling SO_2 through the solution] and allowing the solution to stand in a sealed centrifuge tube for 4–5 days. The sulphonate of the pyrimidine residue (PySO_3H) crystallized out during that time and was collected by centrifugation. The precipitate was transferred to a second centrifuge tube, washed with small volumes of cold water and recrystallized in hot water.

The washings were added to the supernatant and the pH was adjusted to 10 with 15% (w/v) NaOH. The solution was then extracted seven times with CHCl_3 (analytical grade) (50 ml.) and the combined extracts were re-extracted with 1.5 N-HCl. This solution was evaporated to dryness *in vacuo* and the residue extracted with ethanol; the extract was evaporated to dryness *in vacuo*, when the hydrochloride of the thiazole residue of thiamine (ThHCl) crystallized out. It was recrystallized by dissolving it in a minimum volume of ethanol, adding excess of dioxan (analytical grade) and allowing to stand.

Chromatography of thiamine and its derivatives. A thin-layer system was devised that will effectively separate thiamine, PySO_3H and ThHCl , as well as α -hydroxythiamine and thiochrome. The adsorbent was Kieselgel G (E. Merck A.-G., Darmstadt, Germany) and the solvent pyridine–2-methylpropan-1-ol (4:1:1, by vol.). Separation was complete within 2 hr. The R_F values normally obtained were: thiamine, 0.03; α -hydroxythiamine, 0.22; PySO_3H , 0.70; ThHCl , 0.30; thiochrome, 0.98. For the separation of ThHCl and PySO_3H only the solvent system pyridine–water (4:1, v/v) was very effective (R_F 0.38 and 0.65 respectively) and was used in the experiments on the disulphite cleavage of thiamine. Separation of thiamine and its two

derivatives could also be achieved by paper chromatography on Whatman no. 1 paper with water-saturated butan-1-ol containing 18% (w/v) of formic acid. The R_F values were: thiamine, 0.019; PySO_3H , 0.066; ThHCl , 0.221. If thiamine and PySO_3H were not clearly separated in this system the combined spots were eluted and run in pyridine-2-methylpropan-1-ol-water (2:3:5, by vol.), when the R_F values were: thiamine, 0.51; PySO_3H , 0.62. The advantages of the thin-layer systems over the paper systems are: (a) speed, the running time being 2 hr. compared with 24 hr.; (b) the greater sensitivity in radioautography, activities that will show up after overnight exposure on thin layers requiring some 2-3 weeks' exposure when adsorbed on paper (see Goodwin, 1964).

The compounds could be seen on the chromatograms under u.v. light (Hanovia lamp, model 16, with Chance 0×1 filter). PySO_3H , ThHCl and thiochrome spots exhibited light-blue, dark-mauve and bright-blue fluorescence respectively. Thiamine and α -hydroxythiamine show up as dark absorbing spots; they are more easily detectable if they are first converted into their thiochrome derivatives by spraying the chromatogram with a 5:5:1 (by vol.) mixture of aq. 55% (v/v) ethanol, 10% (w/v) NaOH and 2.5% (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$ (Rossi-Fanelli, Siliprandi & Fasella, 1952).

The eluted PySO_3H and ThHCl were eluted with 0.1 N-NaOH and ethanol respectively, and assayed spectrophotometrically in 0.1 N-NaOH and 0.5 N-HCl respectively: PySO_3H , λ_{max} , 235.5 ($E_{1\text{cm}}^{1\%}$, 220) and 277.5 m μ ($E_{1\text{cm}}^{1\%}$, 425); ThHCl , λ_{max} , 260 m μ ($E_{1\text{cm}}^{1\%}$, 190).

In experiments with radioactive thiamine the purity of the two ring components was always checked by making a radioautograph of a thin-layer chromatogram.

Degradation of PySO_3H . The method of Lagerkvist (1953) for the degradation of uracil was adapted for use with PySO_3H . The PySO_3H (0.1-0.15 m-mole) was dissolved in 3 ml. of 0.01 N-NaOH, and 5% (w/v) KMnO_4 was added dropwise until a faint permanganate colour persisted. The solution was acidified with 0.6 ml. of 0.1 N- H_2SO_4 and allowed to stand for 30 min. Excess of KMnO_4 was destroyed with H_2O_2 , and the precipitated MnO_2 was removed by centrifugation. The precipitate was washed with small volumes of deionized water, and the combined supernatant and washings were hydrolysed by adding 1 ml. of 5 N-NaOH and refluxing on a water bath for 3 hr. The solution was cooled, acidified with 5 N- H_2SO_4 and steam-distilled. The steam-volatile fraction was examined by gas-liquid chromatography (see the next section).

Gas-liquid chromatography. The volatile acids from the degradation of PySO_3H were separated in an Argon Gas Chromatograph (Pye Instruments Ltd., Cambridge) with a 4 ft. column of 12% polyethylene glycol adipate in 80-100 mesh Gas-Chrom P (silicone-treated). The detector voltage was 1250 v, sensitivity $\times 10$, temp. 110° and gas flow rate 50 ml./min. Known volumes of material in ether were transferred to pads of flamed gauze from a micrometer syringe (Agla; Burroughs, Wellcome and Co., London), and the pads were dropped into place on the column top.

Radiochemical analysis. Radioactive materials were assayed in either (a) a Nuclear-Chicago Corp. automatic sample changer (model C110B) used in conjunction with a decade scaler (model 186; Nuclear-Chicago Corp.), the counting chamber being fitted with a Micromil window and flushed with proportional counting gas (argon-methane,

9:1), and the efficiency of the system being 17.5%, or (b) a Packard Tri-Carb liquid-scintillation spectrometer (model 314Ex).

Radioautography of thin layers was carried out conventionally with standard X-ray film.

The effluent from the gas chromatogram was scanned for radioactivity in a gas proportional counter (Pye Instruments Ltd.) coupled to a recording rate-meter (type N 701A; Honeywell-Brown Elektronik).

RESULTS

Incorporation of formate. The results of an experiment in which labelled formate was added to growing yeast cells are given in Table 1. This experiment was designed to give sufficient thiamine of high radioactivity that could be isolated without the addition of unlabelled carrier. This was to allow the degradation of the isolated pyrimidine-sulphonic acid and the assay of the volatile products for radioactivity. Table 1 clearly indicates that formate is significantly incorporated only into the pyrimidine residue of thiamine; the specific activity of the pyrimidine residue is 100 times that of the thiazole residue. Oxidative degradation of the PySO_3H yielded acetic acid and, probably, propionic acid as the two volatile products when examined in a gas chromatogram coupled with a radioactive scanner; essentially all the radioactivity was associated with the acetic acid peak. When the machine was overloaded with radioactivity so that the peak of activity due to acetic acid was well off the scale, a trace of activity was noted in the second zone. From this experiment it can be concluded that the methyl group attached to C-2 of the pyrimidine residue arises directly from

Table 1. *Incorporation of [^{14}C]formate into thiamine and its two ring components by baker's yeast*

A 4.0 mc sample of [^{14}C]formate (specific activity 13.4 mc/m-mole) was added to 4.5 l. (in 30 Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 96 hr. at 28°.

Yield of dry cells	18.2 g.
Yield of thiamine	3.82 mg.
Activity of thiamine	8.8×10^7 disintegrations/min.
Specific activity of thiamine	7.93×10^6 disintegrations/min./ μmole
Yield of PySO_3H	1.54 mg.
Activity of PySO_3H	5.02×10^7 disintegrations/min.
Specific activity of PySO_3H	7.85×10^6 disintegrations/min./ μmole
Yield of ThHCl	0.95 mg.
Specific activity of ThHCl	0.08×10^6 disintegrations/min./ μmole

Table 2. *Incorporation of L-[Me-¹⁴C]methionine into thiamine by baker's yeast*

A 0.1 mc sample of L-[Me-¹⁴C]methionine (specific activity 10.9 mc/m-mole) was added to 1.5 l. (in ten Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 12 hr. at 28°.

Yield of dry cells	2.59 g.
Yield of thiamine	0.619 mg.
Specific activity of thiamine	3.78×10^4 disintegrations/ min./ μ mole
Specific activity of PySO ₃ H	0.96×10^3 disintegrations/ min./ μ mole
Specific activity of ThHCl	1.93×10^3 disintegrations/ min./ μ mole
Specific activity ratio PySO ₃ H/ThHCl	0.53:1

formate, that none of the carbon atoms of the thiazole ring arises directly from formate and that the C₁ fragment attached to C-5 may or may not be derived from formate. This last conclusion remains to be settled, because it is still not clear from where in the molecule the unlabelled propionic (?) acid arises.

Incorporation of methionine into thiamine. Table 2 shows the results of an experiment in which it is demonstrated that [Me-¹⁴C]methionine is more effectively incorporated into the thiazole residue of thiamine than into the pyrimidine residue. This strongly suggested that methionine was not functioning as a source of a C₁ unit in the thiazole residue but providing a much larger building unit. This view was supported when it was found (Table 3) that [³⁵S]methionine was better incorporated than was [³⁵S]cysteine, in spite of the fact the cysteine was more efficiently taken into the yeast cells. To make conditions as comparable as possible cells from the same batch were used under identical conditions for this experiment. Further, this precluded the need to allow for the decay of ³⁵S.

These results prompted an examination of the incorporation of L-[Me-¹⁴C,³⁵S]methionine into the thiazole residue of thiamine. As the ³⁵S/¹⁴C ratio in the added methionine was known, then, if the total counts in the isolated thiazole are measured over a considerable time (preferably longer than the half-life of ³⁵S, namely 87 days) and the logarithm of the counts plotted against time, and the activities at zero time and at 87 days determined from the graph, the ³⁵S/¹⁴C ratio in the thiazole at zero time can be determined and compared with that of the original methionine. If they are the same then methionine must be providing an intact unit for thiazole biosynthesis and not just a C₁ residue.

An experiment was carried out under our standard conditions (Table 4) in which the ¹⁴C/³⁵S ratio

Table 3. *Comparison of incorporation of DL-[³⁵S]-methionine and DL-[³⁵S]cystine into thiamine by baker's yeast*

A 0.13 mc sample of DL-[³⁵S]methionine (specific activity 15 mc/m-mole) or a 0.15 mc sample of DL-[³⁵S]cystine (specific activity 18 mc/m-mole) was added to 1.5 l. (in ten Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 12 hr. at 28°.

	With DL-[³⁵ S]methionine	With DL-[³⁵ S]cystine
Yield of dry cells	2.99 g.	3.02 g.
Yield of thiamine	0.207 mg.	0.218 mg.
Specific activity of thiamine	5.6×10^4 disintegrations/ min./ μ mole	1.32×10^3 disintegrations/ min./ μ mole
Percentage of added activity left in medium	61	36.5

Table 4. *Incorporation of L-[Me-¹⁴C,³⁵S]methionine into thiamine by baker's yeast*

A 1 mc sample of L-[Me-¹⁴C]methionine (specific activity 6.3 mc/m-mole) and a 1 mc sample of L-[³⁵S]methionine (specific activity 9.3 mc/m-mole) were added to 4.5 l. (in 30 Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 48 hr. at 28°.

Yield of dry cells	6.5 g.
Yield of thiamine	1.230 mg.
Activity of thiamine	1.53×10^8 disintegrations/ min.
Specific activity of thiamine	4.25×10^7 disintegrations/ min./ μ mole
Yield of ThHCl	0.365 mg.
Activity of ThHCl	3.54×10^7 disintegrations/ min./ μ mole
Yield of PySO ₃ H	0.520 mg.
Specific activity of PySO ₃ H	0.71×10^7 disintegrations/ min./ μ mole

in the methionine added to the culture was 1:1. Samples of the thiazole residue obtained from the thiamine synthesized in this experiment were plated out on three sets of duplicate planchets. The same amount of material was added to each planchet in the same set, but the amounts added to each set were different. Thus the same basic sample was counted at three different levels of activity. On every occasion each planchet was counted ten times on a preset count system arranged so that the counting time was approx. 10 min.; the period covered was 110 days. The results for the three series are given in Table 5 and the plot for series I is recorded in Fig. 1. From this graph $\log D_0 = 4.0025$ and $\log D_{87} = 3.8812$, so that $D_0 = 10060$ and $D_{87} = 7610$. The difference, which represents one-half the original activity of ³⁵S, is 2450; the activity of

Table 5. Decay of radioactivity of thiazole samples isolated from thiamine synthesized by baker's yeast in the presence of L-[Me- ^{14}C , ^{35}S]methionine

For details of the experiment see Table 4. Three series of two planchets were assayed at the times indicated.

Days after addition of labelled methionine	Radioactivity (disintegrations/min.)		
	Series I	Series II	Series III
29	9175	16410	15400
30	9130	16220	15370
31	9120	16130	15310
35	8960	15940	15120
48	8590	15230	14510
49	8561	15140	14400
52	8511	15050	14290
55	8417	14880	14180
57	8366	14790	14050
59	8270	14710	13960
63	8220	14450	13850
65	8175	14380	13680
106	7160	12520	11950
107	7120	12470	11930
108	7110	12390	11890
109	7095	12370	11840

^{35}S at time zero is thus 4900, and that of ^{14}C is $10060 - 4900 = 5160$; so the $^{14}\text{C}/^{35}\text{S}$ ratio is 1.05:1. The ratios calculated in the same way for series II and III were 0.93:1 and 1.00:1 respectively. From this it can be concluded that methionine is providing a building unit for the biosynthesis of the thiazole ring.

Table 4 shows that some activity from [^{14}C]methionine is incorporated into the pyrimidine residue, and this confirms the results given in Table 2 and demonstrates that to carry out the doubly labelled experiment just described it is necessary to work on the isolated thiazole residue and not on the intact thiamine molecule.

Incorporation of [6- ^{14}C]orotic acid into thiamine. If the pyrimidine residue of thiamine arises by the conventional pathway of pyrimidine synthesis then labelled orotic acid would be expected to be actively incorporated into the pyrimidine residue. Table 6 shows that only an insignificant incorporation, 1270 disintegrations/min. from 0.2mc, was observed. This is at least 1000-fold less than the incorporation of [^{14}C]formate (see Table 1) and agrees with the observations of Goldstein & Brown (1963) with an orotic acid-dependent mutant of *E. coli*. The little activity that was observed in thiamine appeared to be confined to the pyrimidine residue.

Incorporation of acetate and alanine into thiamine. The observation that methionine was contributing a unit to the thiazole residue of thiamine suggested that N-3, C-4 and the methyl group attached to

Fig. 1. Rate of decay of thiazole sample I (Table 5) isolated from thiamine synthesized by baker's yeast in the presence of L-[Me- ^{14}C , ^{35}S]methionine ($^{14}\text{C}/^{35}\text{S}$ ratio 1:1 at time zero). For details of the experiment see Table 4.

Table 6. Incorporation of [6- ^{14}C]orotate into thiamine by baker's yeast

A 0.2mc sample of [6- ^{14}C]orotate (specific activity 32.6mc/m-mole) was added to 4.5l. (in 30 Roux bottles) of yeast cells 24hr. after inoculation; the cells were incubated for a further 48hr. at 28°.

Yield of thiamine	1.00 mg.
Activity of thiamine	1270 disintegrations/min.
Specific activity of thiamine	
(a)*	428 disintegrations/min./ μmole
(b)†	285 disintegrations/min./ μmole
Yield of PySO_3H	0.64 mg.
Activity of PySO_3H	760 disintegrations/min.
Specific activity of PySO_3H	285 disintegrations/min./ μmole

* Before dilution with carrier thiamine.

† After dilution with carrier thiamine for degradation.

C-4 (see compound I) arose from a source other than methionine. Further, the failure to incorporate formate to any significant extent into the thiazole residue (Table 1) indicated that the methyl group did not arise from a C_1 unit. The possibility was therefore tested whether C-4 and its attached methyl group could arise from a C_2 unit (acetate) and whether alanine could supply these two carbon atoms and the nitrogen atom at position 3. An experiment with [1- ^{14}C]acetate and [2- ^{14}C]acetate demonstrated that both were incorporated into thiamine, that, under the same cultural conditions, [1- ^{14}C]- and [2- ^{14}C]-acetate were incorporated into the thiazole residue to an equal extent, that [1- ^{14}C]-acetate was insignificantly incorporated into the pyrimidine residue, and that [2- ^{14}C]acetate was

Table 7. Incorporation of [1-¹⁴C]- and [2-¹⁴C]-acetate into thiamine in baker's yeast

A 0.1 mc sample of [1-¹⁴C]acetate (specific activity 12 mc/m-mole) or a 0.1 mc sample of [2-¹⁴C]acetate (specific activity 10 mc/m-mole) was added to 1.5 l. (in ten Roux bottles) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 48 hr. at 28°. To make conditions as comparable as possible the two batches were inoculated from the same suspension of yeast cells and incubated side by side.

	With [1- ¹⁴ C]acetate	With [2- ¹⁴ C]acetate
Yield of thiamine	0.336 mg.	0.477 mg.
Specific activity of thiamine after dilution	2.64×10^4 disintegrations/min./μmole	4.03×10^4 disintegrations/min./μmole
Yield of ThHCl	60 μg.	60 μg.
Specific activity of ThHCl	(a)* 2.64×10^4 disintegrations/min./μmole	3.33×10^4 disintegrations/min./μmole
	(b)† 1.69×10^5 disintegrations/min./μmole	1.80×10^5 disintegrations/min./μmole
Yield of PySO ₃ H	140 μg.	120 μg.
Specific activity of PySO ₃ H*	0	7.0×10^3 disintegrations/min./μmole

* Derived from undiluted thiamine.

† Calculated for undiluted material.

Table 8. Incorporation of L-[U-¹⁴C]alanine into thiamine by baker's yeast

A 0.1 mc sample of L-[U-¹⁴C]alanine (specific activity 15.2 mc/m-mole) was added to 500 ml. (in ten Erlenmeyer flasks) of yeast cells 24 hr. after inoculation; the cells were incubated for a further 24 hr. with shaking at 28°.

Yield of thiamine	150 μg.
Specific activity of thiamine	
(a)*	1.80×10^5 disintegrations/min./μmole
(b)†	2.35×10^4 disintegrations/min./μmole
Yield of ThHCl	36 μg.
Specific activity of ThHCl	
(a)‡	2.28×10^4 disintegrations/min./μmole
(b)§	1.75×10^5 disintegrations/min./μmole
Yield of PySO ₃ H	30 μg.
Specific activity of PySO ₃ H§	0.07×10^4 disintegrations/min./μmole

* Before dilution with carrier thiamine.

† After dilution with carrier thiamine for degradation.

‡ Derived from diluted thiamine.

§ Calculated for undiluted material.

incorporated into the pyrimidine residue but to a smaller extent than into the thiazole residue (thiazole/pyrimidine specific activity ratio 4.7:1) (Table 7). An experiment with L-[U-¹⁴C]alanine

(Table 8) showed clearly that the label is very effectively incorporated into the thiazole residue of thiamine, whereas only little activity was found in the pyrimidine residue, the specific activity of the thiazole residue being only about one-thirtieth that of the pyrimidine residue.

DISCUSSION

Origin of the thiazole residue. Harington & Moggridge (1940) suggested that the thiazole ring in thiamine arose from α-amino-β-(4-methylthiazol-5-yl)propionic acid (IV), which in turn could be considered formally to arise from methionine, acetaldehyde and an amino group (reaction 1); support for this view came from the observation that compound (IV) stimulated thiamine production in yeast (Harington & Moggridge, 1939) and pea roots (Bonner & Buchman, 1938); isotope competition experiments, however, indicated that compound (IV) was not an effective precursor, but nutritional studies with isolated pea roots, which require preformed thiazole, suggested that the C₂ side chain must be intact (see Bonner & Bonner, 1948). Our present experiments (Table 4) demonstrate that the CH₃-S grouping of methionine is incorporated intact into the thiazole residue, and thus it is reasonable to assume that C-5 and the hydroxyethyl side chain also come from methionine, in agreement with the proposals of Harington & Moggridge (1940). The failure of yeast to incorporate [¹⁴C]formate into the thiazole residue (Table 1) indicates that the methyl group at C-4 is not derived from a C₁ unit, and the equal incorporation of [1-¹⁴C]- and [2-¹⁴C]-acetate into this residue (Table 7) suggests that acetate is being incorporated into the thiazole residue intact and thus is a logical precursor of C-4 and its attached methyl group; on modern biochemical views this is more likely than acetaldehyde, which was the first C₂ unit proposed. However, this does not mean that acetate is the normal precursor *in vivo*, and the effective incorporation of L-[U-¹⁴C]alanine into the thiazole residue (Table 8) makes this amino acid a good candidate for the source of all the atoms not arising from methionine, although the activity may be due to its conversion into pyruvate, which, via

acetate, is then incorporated. This would also require the existence of a specific amino donor, e.g. glutamate, as a source of N-3. However, the fact that the labelling pattern in the pyrimidine residue with L-[U-¹⁴C]alanine is different from that with [2-¹⁴C]acetate argues against the prior degradation of alanine. So it would appear that the cyclic precursor of the thiamine thiazole residue arises from combination of L-alanine and L-methionine. The nature of this precursor remains to be established but, as indicated above, nutritional studies with pea roots suggest that the C₂ side chain must be intact. On the other hand, Nakayama (1956*a,b*, 1957*a,b*) proposed reaction (2) for the biosynthesis of the thiazole residue as a result of investigations on thiamine-requiring mutants of *E. coli* and *Neurospora crassa*, which can utilize the thiazole residue. The mutants respond to a limited extent to 4-methylthiazole (VI), and can be maintained in the absence of the thiazole residue by either cysteine or thiazolidene-4-carboxylic acid (V) but not by homocysteine, methionine or methanol. Our results contrast sharply with these because we find that methionine is actively incorporated into the thiazole residue (Tables 2, 3 and 4), that [³⁵S]-methionine is more effectively incorporated than is [³⁵S]-cysteine (Table 4) and that formate is ineffectively incorporated. Further, Korte & Weitkamp (1959) and Korte, Weitkamp & Vogel (1959) reported that 4-methyl[2-¹⁴C]thiazole is not incorporated into thiamine in a number of microorganisms, and Hitchcock & Walker (1961) found that methionine dilutes out the incorporation of ³⁵SO₄²⁻ into thiamine more effectively than does cysteine.

Origin of the pyrimidine residue. A number of observations raise doubts as to whether the pyrimidine residue arises via the conventional biosynthetic pathway, i.e. through carbamoylaspartate, orotic acid and uridine monophosphate, followed by a C₁ transfer similar to that involved in thymine biosynthesis. Goldstein & Brown (1963) demonstrated that in an orotic acid-dependent *E. coli* only a very small incorporation of [¹⁴C]orotic acid was observed and, further, somewhat greater incorporation was obtained with [2-¹⁴C]orotic acid than with [6-¹⁴C]orotic acid, which suggests that the ring is metabolized before the ¹⁴C was incor-

porated into thiamine. Very low incorporation was also obtained by Goldstein & Brown (1963) with [U-¹⁴C]uracil, [U-¹⁴C]aspartic acid and [4-¹⁴C]-aspartic acid. Our observations with [6-¹⁴C]orotic acid (Table 6) showed that in yeast, as in the *E. coli* mutant, orotic acid was not an effective precursor of the pyrimidine residue of thiamine.

A pathway for which there is no direct evidence but which fits all the known facts is one that would involve β-methylaspartate instead of aspartate in the basic pathway, to give: carbamoyl-β-methylaspartate → 5-methylorotic acid → 5-methyluridine monophosphate. This is compatible with: (a) the failure to incorporate [¹⁴C]orotic acid into the thiamine pyrimidine residue (Table 6; and Goldstein & Brown, 1963); (b) the much greater activity observed in the pyrimidine residue with [2-¹⁴C]-acetate than with [1-¹⁴C]acetate; this could be due to the incorporation of acetate into glutamate via the tricarboxylic acid cycle, which would involve much greater loss of C-1 than C-2 of acetate; glutamate could then be converted into β-methylaspartate by the vitamin B₁₂ coenzyme-dependent isomerase (Barker, 1961); (c) the possible failure of incorporation of [¹⁴C]formate into the 5-hydroxymethyl group of the thiamine pyrimidine residue (David & Estramareix, 1961; and Table 1) [although David & Estramareix (1964) reported the presence of label from [¹⁴C]formate in a derivative of the pyrimidine containing C-4, C-5, C-6 and the C attached to 3-5]. Although this may represent the general pathway of synthesis of the thiamine pyrimidine residue many details remain to be settled, e.g. the point at which the ribose phosphate is split off the base, because it is the free pyrimidine diphosphate that is condensed with the thiazole residue; also, the point at which the methyl group is inserted still remains unknown.

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