Biosynthesis of Thiamine. Part II.[1] Origin of the Carbon Atom in the 2-Position of the Thiazole Component

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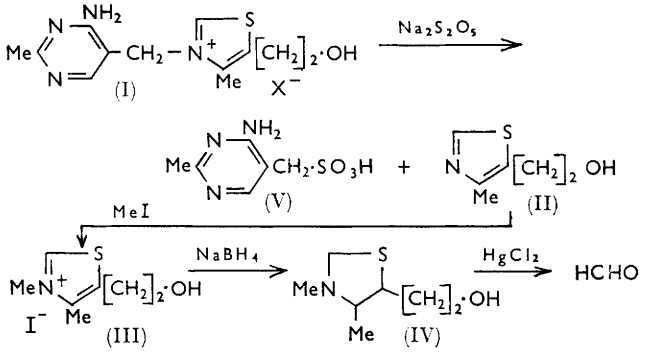
The carbon atom in the 2-position of the thiazole ring in the thiazole component of thiamine can be isolated in good yield as formaldehyde in the form of its dimedone derivative by reduction of the thiazole methiodide with sodium borohydride followed by treatment of the resulting thiazolidine with mercuric chloride and then dimedone. The procedure is suitable for tracer work, and it has been found that the 2-carbon atom of glycine is utilised by yeast as a source of the 2-carbon atom of the thiazole ring.

STUDIES on the biosynthesis of thiamine (I) suffer from the disadvantage that thiamine is not formed in excess and liberated into the culture medium, as, for example, are other microbial metabolic products such as antibiotics and riboflavin. The quantities available in biosynthetic experiments are therefore strictly limited, as synthesis is so precisely adjusted to the physiological requirements of the synthesising organism.

Although it has long been known that pre-formed pyrimidine and thiazole moieties can be combined to give thiamine in a variety of biological systems, the paths by which the two halves are themselves biosynthesised are as yet virtually unknown. Significant incorporation of [^{14}C]formate into the pyrimidine has been demonstrated in a variety of micro-organisms,[2-6] and the site of labelling has been shown to be C-4 of the pyrimidine ring.[7] The methyl carbon atom of acetate is incorporated into the pyrimidine,[6,8] providing the methyl group attached to the 2-position of the ring.[8] As far as the thiazole component is concerned, methionine has been shown to be an efficient source of the sulphur

[1] Part I, C. H. S. Hitchcock and J. Walker, *Biochem. J.*, 1961, **80**, 137.
[2] M. J. Pine and R. Guthrie, *J. Bact.*, 1959, **78**, 545.
[3] S. David and B. Estramareix, *Biochim. Biophys. Acta*, 1960, **42**, 562.
[4] G. A. Goldstein and G. M. Brown, *Arch. Biochem. Biophys.*, 1963, **103**, 449.
[5] M. Nakamura, *Vitamins, Kyoto*, 1965, **32**, 383.
[6] D. B. Johnson, D. J. Howells, and T. W. Goodwin, *Biochem. J.*, 1966, **98**, 30.
[7] S. David, B. Estramareix, and H. Hirshfeld, *Biochim. Biophys. Acta*, 1966, **127**, 264.
[8] R. V. Tomlinson, *Biochim. Biophys. Acta*, 1966, **115**, 526.

atom, both by direct labelling [6,9,10] and by isotope-competition experiments,[1] whilst Goodwin and his collaborators [6,9] have shown [*Me*-^{14}C,^{35}S]methionine to be incorporated into the thiazole moiety with unchanged ratio of ^{14}C to ^{35}S radioactivity, suggesting the possible incorporation of the methylthio residue as a unit.



Hitherto, no method has been available for the separation of the various atoms of the thiazole component (II) suitable for tracer studies, and we now illustrate a method for isolating the carbon atom in the 2-position of the thiazole ring. In view of the known lability of the thiazolidine ring towards mercuric chloride,[11] and the fact that thiamine [12] and other thiazolium salts [13] can be reduced to thiazolidines with sodium borohydride, it appeared promising to apply these reactions to the methiodide (III) of the thiazole (II) obtained by bisulphite fission [14] of thiamine. 5-(2′-Hydroxyethyl)-4-methylthiazole methiodide (III) was smoothly reduced by sodium borohydride to give the oily thiazolidine (IV), which was characterised as its methiodide. The thiazolidine (IV), when treated with aqueous mercuric chloride, first deposited a copious white precipitate, presumably of an addition compound, which redissolved slowly, and the resulting solution, on treatment with an ethanolic solution of dimedone, readily gave the formaldehyde derivative. In subsequent tracer experiments in which the thiazole (II) was isolated as the picrate, the formaldehyde dimedone derivative was isolated in 47—76% yield based on the thiazole picrate. As a check on the efficiency of the procedure [*thiazole*-2-^{14}C]thiamine hydrochloride, prepared unequivocally [15] from [^{14}C]carbon disulphide, was suitably diluted with carrier thiamine and cleaved with bisulphite. The thiazole (II) picrate was isolated and decomposed on an alumina column prior to degradation of the thiazole in the manner described above; the resulting formaldehyde dimedone derivative had 91% of the specific activity found for the thiazole picrate.

In experiments [16] in which [2-^{14}C]glycine or sodium [^{14}C]formate were fed to growing cultures of *Saccharomyces cerevisiae* in a minimal medium, the biosynthesised thiamine was isolated, diluted with carrier thiamine, and cleaved with bisulphite to give 4-amino-2-methylpyrimidin-5-ylmethanesulphonic acid (V) and the thiazole (II) which was isolated as its picrate. The thiazole picrates were degraded in the manner described above, and the results are summarised in the Table. The incorporation of radioactivity from [2-^{14}C]glycine into the thiazole depended upon the level of feeding, and reached about 10%,[16] and incorporation from [*Me*-^{14}C]-methionine into the thiazole in the same system was about 0·2%; [16] the low level of activity and the scale of the experiment in the latter case precluded an estimation of the radioactivity of C-2 of the thiazole ring.

Utilisation of labels from [2-^{14}C]glycine and sodium [^{14}C]formate in providing the thiazole 2-carbon atom in thiamine

Precursor	Degradation product	Specific activity (μc/mole)	Relative activity at the thiazole 2-position compared with total activity of thiazole (II)
[2-^{14}C]Glycine	Thiazole (II) picrate	14·0	74%
	Formaldehyde dimedone deriv.	10·4	
Sodium [^{14}C]formate...	Thiazole (II) picrate	5·98	0·3%
	Formaldehyde dimedone deriv.	0·02	

These results suggest that it is C-2 of glycine which provides the carbon atom in the 2-position of the thiazole ring and not the *S*-methyl carbon atom of methionine as required by the theory of the thiazole biosynthesis proposed by Harington and Moggridge [17] and by Buchman and Richardson.[18] Goodwin and his collaborators [6,9] in their double-labelling experiment suggested that it was reasonable to suppose utilisation of the whole methionine molecule. The results presented here, however, suggest a different interpretation, namely, that the *S*-methyl carbon atom of methionine is incorporated into some other position in the thiazole moiety than C-2 of the ring. The lack of incorporation of formate into the 2-position of the thiazole ring, but its incorporation into the thiazole moiety as a whole (about 2% [16]) * suggests that a one-carbon precursor

* The results of Johnson *et al.*[6] also appear to show a greater incorporation of radioactivity from [^{14}C]formate than from [*Me*-^{14}C]methionine into the thiazole.

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[10] H. Kumaoka, *J. Vitamins*, 1963, **9**, 188.
[11] Cf., *e.g.*, J. M. Sprague and A. H. Land, "Heterocyclic Compounds," ed. R. C. Elderfield, Wiley, New York, 1957, vol. 5, p. 701.
[12] G. E. Bonvicino and D. J. Hennessy, *J. Amer. Chem. Soc.*, 1957, **79**, 6325.
[13] G. M. Clarke and P. Sykes, *Chem. Comm.*, 1965, 370.
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[15] M. Tomita, S. Uyeo, H. Inouye, H. Sakurai, and S. Moriguchi, *J. Pharm. Soc. Japan*, 1948, **68**, 151.
[16] P. E. Linnett and J. Walker, to be published.
[17] C. R. Harington and R. C. G. Moggridge, *J. Chem. Soc.*, 1939, 443; *Biochem. J.*, 1940, **34**, 685.
[18] E. R. Buchman and E. M. Richardson, *J. Amer. Chem. Soc.*, 1939, **61**, 891.

provides some other carbon atom in the thiazole moiety than C-2 of the ring. It is possible that the *S*-methyl carbon atom of methionine acts as a one-carbon donor to this other position as well as formate, since both act as one-carbon donors to the folic acid pool[19] although at different oxidation levels. The 2-carbon atom of glycine can also contribute to the one-carbon pool as in its incorporation into the 2- and 8-positions of purines,[20] but the lack of incorporation of formate into the 2-position of the thiazole ring of thiamine suggests the need for the intact glycine molecule or some close relative followed by subsequent loss of the carboxyl carbon atom.

EXPERIMENTAL

[^{14}C]Labelled compounds were obtained from the Radiochemical Centre, Amersham, and used as supplied. The culture conditions for the strain of yeast used and the isolation of biosynthesised thiamine were similar to those already described;[1] further details will be published separately.[16]

Radioactive samples were counted with a Nuclear-Chicago low background, gas-flow counter at "infinite thickness," *i.e.*, over 20 mg./cm.^{2}, on 1-cm.^{2} Polythene planchettes with background correction, and specific activities were calculated by comparison with the count rate for a 1-cm.^{2} "infinitely thick" disc of [^{14}C]polymethacrylate (1·0 μc/g.; Radiochemical Centre, Amersham) on a 1-cm.^{2} Polythene planchette. Each sample was counted to at least 2×10^5 counts.

5-(2′-*Hydroxyethyl*)-3,4-*dimethylthiazolidine* (IV).—(*a*) The thiazole (II) (15·1 g.) was heated under reflux in ethanol (30 c.c.) with methyl iodide (30 c.c.) for 45 min. Evaporation and recrystallisation of the residue from methanol–ethyl acetate afforded the methiodide as stout colourless prisms (23·9 g., 79%), m. p. 82—84° (lit.,[21] 89°).

(*b*) To a solution of the thiazole methiodide (14·3 g.) in water (50 c.c.) at 0° was added slowly with stirring cold N-sodium hydroxide (25 c.c.). With the stirred solution kept at 0°, a cold solution of sodium borohydride (1·90 g.) in water (25 c.c.) was added dropwise, and the solution stirred until it reached room temperature (*ca.* 30 min.). Extraction with chloroform (5 times), drying of the extract ($MgSO_4$), and evaporation yielded an oil, affording, on vacuum distillation, the thiazolidine as a colourless liquid (7·17 g., 89%), b. p. 106—109°/0·5 mm., n_D^{23} 1·5244, $\nu_{max.}$ (film) 3300br (OH), 2780sh (NMe), 1054 cm.$^{-1}$ (C–OH), with no absorption between 1500 and 1600 cm.$^{-1}$ (*i.e.*, neither C:C nor C:N).

The thiazolidine was characterised as the methiodide by refluxing with an excess of methyl iodide for 1 hr. Recrystallisation from methanol–ethyl acetate afforded colourless needles of 5-(2′-*hydroxyethyl*)-3,3,4-*trimethylthiazolidinium iodide*, m. p. 188—189° (Found: C, 31·8; H, 6·0; N, 4·2; S, 10·5. $C_8H_{18}INOS$ requires C, 31·7; H, 5·9; N, 4·6; S, 10·6%).

Degradation of [thiazole-2-^{14}C]*Thiamine Hydrochloride*.—(*a*) An aqueous solution (1·0 c.c.) of the labelled thiamine hydrochloride (0·1 μc, 1·26 μg.) was diluted with carrier thiamine hydrochloride (200 mg.) in aqueous solution (10 c.c.) and cleaved by an adaptation of the method of Williams *et al.*[14] Sodium pyrosulphite (500 mg.) was added followed by M-acetate buffer (*ca.* 0·2 c.c.) to pH 4·7, and the solution was either set aside overnight at 40° or heated for 2 hr. on a steam-bath; both methods gave equally satisfactory results. The cooled solution was made alkaline by dropwise addition of 50% aqueous sodium hydroxide, and extracted with methylene chloride (7×5 c.c.). The methylene chloride extracts were added directly to saturated ethanolic picric acid (10 c.c.), and the mixture was reduced to small bulk at 40°. On cooling, the crude thiazole (II) picrate was collected and washed with ether. The product was recrystallised (4 times) to constant radio-activity (specific activity 159 μc/mole), m. p. 162—164° (lit.,[22] 163—164°).

(*b*) The thiazole picrate (81·7 mg., 0·22 mmole) was dissolved in ethanol (5 c.c.), diluted with ethyl acetate (45 c.c.), and applied to a column ($11·5 \times 1·0$ cm.) of alumina (Spence, type H). The column was further eluted with ethanol–ethyl acetate [1 : 9 (v/v); total, 150 c.c.], leaving the picric acid on the column. The eluate was evaporated, leaving the crude thiazole (II) as an oil, which was refluxed with methyl iodide (1·0 c.c.) and ethanol (0·5 c.c.) at about 70° (bath) for 1 hr.; evaporation left the crude thiazole methiodide (III) (54·4 mg., 87% on picrate).

(*c*) A solution of the methiodide in water (1·0 c.c.) was cooled in melting ice, with stirring, and cold N-sodium hydroxide (0·1 c.c.) was added followed dropwise by cold M-sodium borohydride (0·19 c.c.). After stirring to room temperature (*ca.* 15 min.) the solution was extracted with chloroform (6×3 c.c.), and the extracts were dried ($MgSO_4$) and evaporated, to give the crude thiazolidine as a colourless oil (29·3 mg., 83% on picrate).

(*d*) An aqueous solution (1·0 c.c.) of the thiazolidine was stirred in a stoppered flask with mercuric chloride (49·4 mg.) in water (1·0 c.c.) for about 20 hr. at room temperature. The almost clear solution was filtered, and the filtrate and washings (*ca.* 10 c.c.) were treated with dimedone (50·9 mg.) in ethanol (0·5 c.c.). The resulting solution was heated on a steam-bath for 1 hr., and cooled in ice, and the crude formaldehyde dimedone derivative was collected, well washed with water, and dried (30·4 mg., 47% on picrate). Three recrystallisations from aqueous ethanol yielded material of constant radioactivity (specific activity 145 μc/mole), m. p. 192—192·5° not depressed on admixture with an authentic specimen (m. p. 194—195°). The infrared spectrum (KCl disc) was identical with that of an authentic specimen of formaldehyde dimedone. The radioactivity was further checked by dilution ($\times 4$) with carrier formaldehyde dimedone.

Degradation of Thiamine Biosynthesised in Cultures of Saccharomyces cerevisiae *containing* [2-^{14}C]*Glycine or Sodium* [^{14}C]*Formate*.—An inoculum of washed yeast cells (5 mg., dry weight) was grown in a minimal defined medium[16] (100 c.c.) for 2½ days at 26° in the presence of [2-^{14}C]glycine (15—200 μc; 0·5—7·9 μmoles in four separate experiments) or of sodium [^{14}C]formate (125, 300 μc; 11·6, 9·8 μmoles in two separate experiments). The cells from each culture were harvested and worked up, to give a "standard" extract of thiamine in 0·1N-hydrochloric acid (4·5 c.c.).[1,16] This solution (containing 1—2 μg. of thiamine hydrochloride) was diluted with unlabelled carrier thiamine hydrochloride

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[20] V. G. Malathi and T. Ramakrishnan, *Biochem. J.*, 1966, **98**, 594, and references there cited.

[21] E. R. Buchman, *J. Amer. Chem. Soc.*, 1936, **58**, 1803.

[22] H. Andersag and K. Westphal, *Ber.*, 1937, **70**, 2035.

(50·0 mg.) and cleaved with sodium pyrosulphite (100 mg.) in the presence of M-acetate buffer, pH 4·7 (*ca.* 0·2 c.c.) as described above. After basification the solution was extracted with methylene chloride (10 × 3 c.c.), and the extract was treated with saturated ethanolic picric acid (4 c.c.). The thiazole picrate from each experiment was recrystallised (usually 4 times) to constant radioactivity.

The thiazole (II) picrates from the four experiments with [2-^{14}C]glycine were then bulked (88·5 mg.), as were those from the two experiments with sodium [^{14}C]formate (73·5 mg., including 21·2% of added carrier picrate). Each bulked thiazole picrate sample was recrystallised and counted prior to degradation to formaldehyde and isolation of the formaldehyde dimedone derivative in the manner described above. The results are summarised in the Table.

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