Experiments on the Biosynthesis of Thiamine

ISOTOPE-COMPETITION EXPERIMENTS WITH [³⁵S]SULPHATE IN CULTURES OF *SACCHAROMYCES CEREVISIAE*

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Although the structure of thiamine (I) was established and its chemical synthesis accomplished twenty-five years ago (Williams & Cline, 1936; Grewe, 1936; Andersag & Westphal, 1937; Todd & Bergel, 1937), little precise knowledge of its biosynthesis has become available. Many systems have been studied which have the ability to link together the pyrimidine (II; X = OH, OEt, NH₂, Br) and thiazole (III) fragments, but the routes by which these moieties are themselves biosynthesized are quite unknown.

The presence of the thiazole-ring system has recently been demonstrated in antibiotics, such as micrococcin P (Brookes, Fuller & Walker, 1957; Brookes, Clark, Fuller, Mijović & Walker, 1960), bottromycin (Waisvisz, van der Hoeven & te Nijenhuis, 1957) and thiostrepton (Kenner, Sheppard & Stehr, 1960; Bodanszky, Sheehan, Fried, Williams & Birkhimer, 1960), and the chemically generic relationship of the structures there encountered with those found in bacitracin F (Hausmann, Weisiger & Craig, 1955; Weisiger, Hausmann & Craig, 1955) and in bacitracin A (Newton & Abraham, 1953; Craig, Hausmann & Weisiger, 1954) indicates clearly that these structures are derived from cysteine peptides by cyclization to a thiazoline for bacitracin A and by cyclization followed by dehydrogenation to thiazole rings for the others. These advances and the extent of our knowledge of the biosynthesis of the thiazolidine ring of penicillin (cf. Arnstein, 1957) have emphasized the lack of information available on the biosynthesis of the thiazole moiety (III) of thiamine, and it is with that portion of the thiamine molecule that we have concerned ourselves in particular.

The present communication describes isotopic-competition experiments in which a range of

speculative ³⁵S-containing compounds have been studied in competition with ³⁵S-labelled inorganic sulphate in order to determine the relative efficiencies of such compounds in making available the sulphur atom required for incorporation into the thiazole ring of thiamine during its biosynthesis in yeast. Although such experiments throw no direct light on the source of any of the carbon atoms required for the biosynthesis of thiamine, on account of the possibility of sulphur exchange at intermediate stages, they were expected to provide some guidance in the selection of compounds requiring study in the form of ¹⁴C-labelled substrates. No systematic application has hitherto been made of radioactive-tracer methods to the study of the biosynthesis of thiamine, though it has been shown that [¹⁴C]formate is incorporated by *Bacillus subtilis* into the pyrimidine moiety, but the absence of ¹⁴C radioactivity in the thiazole moiety was taken to indicate the absence of a single-carbon-transfer system in its synthesis (Pine & Guthrie, 1959); similar observations were made with *Saccharomyces cerevisiae* (David & Estramareix, 1960). Availability of the vitamin thiazole appears to be a limiting factor in the biosynthesis of thiamine by yeast, indicating that yeast is less capable of synthesizing the thiazole moiety than it is of synthesizing the pyrimidine half of thiamine (Van Lanen, Broquist, Johnson, Baldwin & Peterson, 1942).

MATERIALS

Yeast. Cultures were obtained from The National Collection of Yeast Cultures, The Brewing Industry Research Foundation, Nutfield, Surrey. They were maintained on agar slants at 0° and subcultured at 6-month intervals. The strain selected for use was *Saccharomyces cerevisiae* no. 1062 of the British National Collection of Yeast Cultures.

Medium. The basal medium contained per 100 ml. final volume (including any of the additions studied): glucose, 2.00 g.; NH_4Cl , 162 mg.; KH_2PO_4 , 156 mg.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 41 mg.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 33 mg.; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 25 μg .; KI, 10 μg .; H_3BO_3 , 10 μg .; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 5 μg .; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4 μg .; $\text{ZnCl}_2 \cdot \text{H}_2\text{O}$, 4 μg .; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 2 μg .; L-arginine, 53 mg.; L-leucine, 85 mg.; DL-tryptophan, 16 mg.; DL-aspartic acid, 40 mg.; calcium D-pantothenate, 500 μg .; mesoinositol, 1 mg.; DL-biotin, 0.2 μg .

Saline. This was a solution of 850 mg. of NaCl in 100 ml. of water.

Taka-diastase. A powder preparation, stated to be 'standardized by dilution with lactose', was obtained from Parke, Davis and Co., Hounslow, Middlesex; it was used without purification as a source of phosphatase.

Cyanogen bromide. A concentrated solution of potassium cyanide was added slowly dropwise to saturated bromine water at 0° until decolorization was complete. The cyanogen bromide solution was used within 3 hr.

[^{35}S]Sulphate. The solution (> 100 mc/mg. of sulphur) supplied by The Radiochemical Centre, Amersham, Bucks., was used after appropriate dilution with water.

5-(2-Hydroxyethyl)-4-methylthiazole (III). Thiamine was cleaved by means of sulphite at pH 5, and the thiazole moiety was extracted with chloroform as described by Williams, Waterman, Keresztesy & Buchman (1935). The chloroform solution was then dried (Na_2SO_4) and fractionated, and the residue was distilled *in vacuo*.

S-Adenosylmethionine. Brewer's yeast (500 g.), kindly supplied by Whitbread and Co. Ltd., was enriched with DL-methionine (15 g.) and extracted according to the method of Schlenk & DePalma (1957). The final solution (140 ml.) contained 1 m-mole of S-adenosylmethionine; paper chromatography [Whatman no. 1 paper; ethanol-water-acetic acid (65:34:1, by vol.); ascending] gave a single ultraviolet-absorbing spot of R_F 0.32. No other spot was observed under filtered ultraviolet light, or after treatment with ninhydrin or iodine vapour.

5'-Deoxy-5'-methylthioadenosine. S-Adenosylmethionine was hydrolysed under milder conditions than those used by Baddiley, Cantoni & Jamieson (1953). A solution (60 ml., pH 4) containing 0.5 m-mole of S-adenosylmethionine was heated in a steam bath for 8 hr. The mixture was then evaporated to dryness *in vacuo* and the residue was twice crystallized from water, affording 0.08 m-mole of 5'-deoxy-5'-methylthioadenosine, m.p. 204–205° (literature, m.p. 205°); paper chromatography [Whatman no. 1 paper; ethanol-water-acetic acid (65:34:1, by vol.); ascending] gave a single ultraviolet-absorbing spot of R_F 0.75.

Thiazolidine-4-carboxylic acid (IV). The method of preparation from cysteine and formaldehyde described by Ratner & Clarke (1937) was followed.

DL- α -Amino- β -(4-methylthiazol-5-yl)propionic acid (V). A specimen (Harington & Moggridge, 1939) was kindly made available by Sir Charles Harington.

4-Methylthiazole. The method of Clarke & Gurin (1935) was followed.

Methanethiol. This substance was prepared by treatment of S-methylthiuronium sulphate with sodium hydroxide (cf. Windus & Shildneck, 1943), and ethanethiol was prepared similarly. The use of cupric chloride-charcoal traps (Hill & Wolfrom, 1947) was found expedient. The sodium derivatives were prepared by passing excess of mercaptan into an ethanolic solution of sodium ethoxide and precipitating the product with ether.

Mercapto alcohols. Addition of thioacetic acid to glycidol (2:3-epoxypropan-1-ol), followed by hydrolysis, gave 1-mercaptopropan-2:3-diol, b.p. 110°/7 mm., n_D^{25} 1.5247, in 45% yield (Found: C, 33.2; H, 7.6. Calc. for $\text{C}_3\text{H}_8\text{O}_2\text{S}$: C, 33.0; H, 7.4%); Sjöberg (1942) gave b.p. 100–101°/1 mm., b.p. 112°/3 mm., n_D^{20} 1.5268–1.5271.

Reaction between sodium thioacetate and 1-acetyl-1-chloropropyl 3-acetate and hydrolysis of the product gave 1-acetyl-1-mercaptopropan-3-ol (Matsukawa, Iwatsu & Yurugi, 1948).

Mercaptoacetone. Chloroacetone and NaSH afforded mercaptoacetone, m.p. 102–108°, in 75% yield under the conditions described by Jacini, Bacchetti & Rosnati (1952) and Ohta (1950); these authors gave m.p. 109° and 105–110° respectively.

4-Amino-2-methyl-5-thioformamidomethylpyrimidine (II; X = NH·CHS). The 5-aminomethylpyrimidine was thioformylated under the conditions described by Würsch (1958).

METHODS

Preparation of sulphur-deficient inoculum. The selected strain of yeast was subcultured several times in the basal medium without any addition of sulphate or other sulphur-containing compound, and then stored, if necessary, at 0°.

Isotopic-competition experiments. Each experiment employed eight 1 l. flasks, each charged with a total of 100 ml. of basal medium supplemented with equal amounts (200–400 μC , based on nominal radioactivity as supplied) of [^{35}S]sulphate. Seven flasks received supplements of the ^{32}S -containing compounds under test (Table 2, column 1) in known amounts between 1 and 100 μmoles (Table 2, column 3), and the eighth flask was kept as control; occasionally two controls were used. As a further control 5-(2-hydroxyethyl)-4-methyl[^{32}S]thiazole was tested at least once per experiment. The medium was adjusted to pH 5 if necessary, and autoclaved at 10 lb./in.² for 20 min., the glucose and [^{35}S]sulphate solutions being sterilized separately and then added; if the substance under test was heat-sensitive or volatile it was sterilized separately by Seitz filtration of its aqueous solution. About 5 mg. of washed sulphur-deficient yeast cells suspended in sterile saline was added to each flask, and the inoculated media (total, 100 ml. each) were then incubated at 25° with reciprocal shaking (60 excursions/min.) until little further growth occurred (about 60 hr.). The dry weight of cells (Table 2, column 4) obtained was estimated nephelometrically at 500 m μ with standard curves.

Radioactivity of yeast cells. Yeast suspension (0.1 ml.) was evaporated on a 1 cm.² polythene disk containing lens tissue, and the radioactivity was measured at 'infinite thinness' with an end-window Geiger counter. Under these conditions 1 μmC gave about 200 counts/min. Corrections were applied for background and dead time, and the counter was standardized with a 1 cm.² 'infinitely thick' disk containing [^{14}C]polymethacrylate of specific activity 1 $\mu\text{C/g.}$, which gave 10³ counts/min. The cells were spun off and the radioactivity of 0.1 ml. of the supernatant was similarly measured. The difference between the radioactivity of the suspension and that of the corresponding supernatant gave a measure of the radioactivity incorporated into the cells (Table 2, column 5); about 90% of the available radioactivity was normally so incorporated.

Extraction of thiamine. The cells were washed with 50 ml. of saline and then added to a boiling mixture of 2 ml. of

0.1 N-HCl and 5 ml. of water. This ensured rapid inactivation of yeast phosphatases, as recommended by Westbrink, van Dorp & Gruber (1941). After gentle boiling for 2 min. the suspension was cooled and centrifuged. The cell debris contained no thiamine. The supernatant was percolated down an 8 cm. $\times$ 0.5 cm. diameter column of washed Amberlite CG-50 (100–200 mesh) in the sodium form; this retained any free thiamine present at this stage, but normally there was none. The effluent and washings were pooled (approx. 10 ml.) and treated with about 0.1 ml. of M-sodium acetate buffer, pH 4.7, about 5 mg. of Taka-diastase and a few drops of toluene. On incubation overnight at 40° almost quantitative hydrolysis of the co-carboxylase to thiamine took place, and the resulting solution (pH 4.7) was percolated down a 8 cm. $\times$ 0.5 cm. diameter column of washed Amberlite CG-50 (100–200 mesh) in the H⁺ form. The effluent and washings (approx. 20 ml.) contained almost no thiamine, and were rejected; the column was then eluted with 5 ml. of 0.1 N-HCl, to give a 'standard' extract, containing over 95% of the thiamine originally present in the yeast.

Estimation of thiamine. A method developed from that of Fujiwara & Matsui (1953) was used. The 'standard' yeast extract (1 ml.), containing 0.01–2 μ g. of thiamine, was treated with 1 ml. of cyanogen bromide solution and then 0.5 ml. of aq. 50% NaOH solution. *iso*Butanol (5 ml.; or 1 ml. for samples of low thiamine content) was added and the mixture was shaken vigorously for 90 sec. After centrifuging by hand the clear upper layer was pipetted into cuvettes, and fluorescence was measured with a Farrand fluorimeter and a quinine sulphate solution (434.3 μ g./l. in 0.01 M-H₂SO₄) as standard. Drying of the *iso*butanol solution was unnecessary; the weight of thiochrome was estimated by reference to standard curves, and the total weight of thiamine extracted from the cells was calculated (Table 2, column 6). No blank reading was necessary. The thiamine content of unit weight of cells was also calculated for comparison purposes (Table 2, column 7).

Radioactivity of thiamine. Estimates of the radioactivity of the biosynthesized thiamine were obtained by observing the radioactivity of the derived thiochrome. *iso*Butanol solution (0.2 ml.) containing a known weight of thiochrome was transferred to a 2 cm.² polythene disk containing lens

tissue, and one drop of acetic acid was added. After desiccation the radioactivity of the solute was measured as described above. The specific activity of the thiamine (Table 2, column 8) was then calculated; it is compared with that for a corresponding control (Table 2, column 9) to give the 'relative specific activity' as a percentage (Table 2, column 10).

RESULTS

Reliability of assay method. The thiamine in the 'standard' extract accounted for only about 10% of the total radioactivity of the extract, but the *iso*butanol solution of the derived thiochrome gave a single radioactive spot on paper chromatograms. It was therefore considered desirable to assess the reliability of the method by studying the radioactivity of the thiochrome obtained after the application of different methods of purification to the thiamine, and, by comparison with the results of an independent method for the assay of the specific radioactivity of the biosynthesized thiamine, to permit the selection of conditions suitable for routine use.

Yeast was grown under the standard conditions described above in the presence of (i) [³⁵S]sulphate, and (ii) [³⁵S]sulphate plus 5-(2-hydroxyethyl)-4-methyl[³²S]thiazole (1 μ mole/100 ml. of medium); the inoculum in both cases consisted of about 4 mg. of washed yeast cells, and the dry weight of cells at the end of 5 days was 29 mg. in both cases. 'Standard' extracts (each 5 ml.) were prepared from the washed cells and processed side by side in the following ways: (a) 1 ml. portions of the 'standard' extracts were treated with cyanogen bromide, and the specific activities of the thiamine estimated as thiochrome. The remaining 4 ml. portions of the 'standard' extract were treated with 50 μ g. of [³²S]thiamine and freeze-dried. The residues were taken up in 80% ethanol and each

Table 1. *Specific activities of biosynthesized [³⁵S]thiamine after various treatments of the 'standard' extract of yeast grown in the presence of (i) [³⁵S]sulphate and (ii) [³⁵S]sulphate plus 5-[2-hydroxyethyl]-4-methyl[³²S]thiazole*

Paper chromatography: Whatman no. 1; ethanol-water-acetic acid (65:34:1, by vol.), ascending flow.
Paper electrophoresis: Whatman no. 54; 10% formic acid under CCl₄; 27–33 v/cm.; 45 min.

Treatment of 'standard' extract	Form assayed	Specific activity of biosynthesized thiamine (counts/min./ μ mole)	
		(i)	(ii)
(a) None; thiamine oxidized directly	Thiochrome	37.3	8.7
(b) Dilution with thiamine; sulphite cleavage of thiamine; 5-[2-hydroxyethyl]-4-methylthiazole picrate crystallized to constant radioactivity	5-[2-Hydroxyethyl]-4-methylthiazole picrate	37.3	7.4
(c) Dilution with thiamine; freeze-dried; thiamine oxidized directly	Thiochrome	37.3	10.9
(d) Dilution with thiamine; paper chromatography as thiamine	Thiochrome	37.3	3.4
(e) Dilution with thiamine; paper electrophoresis as thiamine	Thiochrome	37.6	4.0
(f) Thiochrome from (b) chromatographed and recovered	Thiochrome	32.0	7.4
(g) Thiochrome from (f) subjected to electrophoresis and recovered	Thiochrome	31.6	9.7

solution was divided into four portions, which were treated in pairs in the following ways: (b) dilution with 50 mg. of [^{32}S]thiamine, cleavage with sulphite and assay as 5-(2-hydroxyethyl)-4-methylthiazole picrate, recrystallized to constant radioactivity; (c) direct conversion into thiochrome and assay as before; (d) paper chromatography, automatic scanning of chromatogram for radioactivity, elution, conversion into thiochrome, and assay; (e) paper electrophoresis, automatic scanning for radioactivity, elution, conversion into thiochrome, and assay. In addition, the thiochrome obtained in (c) was (f) chromatographed on paper, eluted and again assayed, and the recovered thiochrome was then (g) subjected to electrophoresis on paper, eluted and again assayed. The results are recorded in Table 1.

The excellent agreement between the values for the specific activities of biosynthesized thiamine given in (i) (a)–(e) showed that direct oxidation of thiamine in the 'standard' extract and assay as thiochrome was adequate for routine use. The fluctuations of specific activity observed in experiment (ii) are attributable to the lower counts obtained in that experiment. The lower specific activities observed in (i) (f) and (i) (g) may have been due to some further purification of the thiochrome, or, more probably, to some instrumental error in view of the good agreement shown between (i) (a) and (i) (b)–(e).

Isotopic competition experiments

Yeast uses [^{35}S]sulphate efficiently as a source of sulphur for the biosynthesis of thiamine, as shown by Table 1, Expt. (i), and if the medium also contains the [^{32}S]thiazole moiety of thiamine the specific activity of the biosynthesized thiamine is substantially decreased, as shown by Table 1, Expt. (ii). The presence of a non-radioactive sulphur source in the medium will result in a lower specific activity of biosynthesized thiamine only if: (a) the non-radioactive sulphur compound is absorbed by the yeast and transported, either as such or in a modified form, to the appropriate site(s) inside the cell where required; (b) the sulphur atom, with or without attached carbon atoms, is then available for incorporation into the thiamine molecule. Although the method can only be expected to detect substances capable of providing the sulphur atom of thiamine, if a significantly low specific activity is observed the compound under test must satisfy conditions (a) and (b) and may in addition be a possible precursor of the thiazole moiety of thiamine; in this way the selection of possible ^{14}C -labelled compounds suitable for further studies could be facilitated.

For convenience we define the relative specific activity due to a compound as the ratio (expressed

as a percentage) of the specific activity of thiamine from yeast grown in a medium containing [^{35}S]sulphate and a given concentration of the particular ^{32}S -labelled compound to that of the thiamine from yeast grown in an identical 'control' medium containing no non-radioactive sulphur competitor; the figures so obtained give a measure of the efficiency with which conditions (a) and (b) are met. Table 2 gives the results of experiments designed to determine the relative specific activities due to various non-radioactive sulphur-containing compounds at different concentrations. In three cases (Expts. 1–3) where controls were carried out in duplicate, the reproducibility of the experiment was satisfactory, variations of 6–8% being observed.

Sulphate and 5-(2-hydroxyethyl)-4-methylthiazole. When $\text{Na}_2^{32}\text{SO}_4$ is used as non-radioactive competitor the effect is to lower the specific activity of the [^{35}S]sulphate in the medium, and conditions (a) and (b) must both be fulfilled equally well by both species. Non-radioactive sulphate at $1\text{ }\mu\text{mole}/100\text{ ml.}$ resulted in a relative specific activity of 62%, and $10\text{ }\mu\text{moles}/100\text{ ml.}$ in a relative specific activity of 7%. These figures correspond to a control medium containing about $1\text{ }\mu\text{mole}$ of total available sulphur/100 ml.; this would include sulphur transferred to the medium with the inoculum (which would depend on the history of the inoculum) and impurities in the medium, as well as the radioactive sulphur (less than $0.2\text{ }\mu\text{mole}/100\text{ ml.}$) added. The amount of [^{35}S]sulphate varied from experiment to experiment between 200 and $400\text{ }\mu\text{C}$, but was constant throughout any one experiment, and each experiment included a control. The concentration in the medium of available sulphur from all sources was not accurately known, but the concentration of adventitious sulphur-containing material was kept as low as possible and as constant as practicable; nevertheless the specific activity of the thiamine isolated from the controls varied over a considerable range ($34\text{--}106\text{ counts/min.}/\mu\text{mole}$), reflecting changes in the available sulphur content of the media, and, in particular, in the ratio of ^{35}S to ^{32}S . These fluctuations had, however, no effect on the relative specific activities as defined above; thus the relative specific activities of thiamine obtained from cultures containing $1\text{ }\mu\text{mole}$ of the non-radioactive thiazole/100 ml. of medium were relatively constant at 20–29%, confirming that the relative specific activity values obtained for the same substance in the same concentration but in different experiments are comparable. By inference, therefore, the relative specific activities of thiamine biosynthesized in the presence of different compounds in different experiments may be compared in assessing the relative efficiencies of various com-

pounds as sources of the sulphur atom for the biosynthesis of thiamine.

If the effects of 1 μ mole of the thiazole and of [32 S]sulphate/100 ml. are compared, it is apparent that the thiazole is about twice as efficient as sulphate in the provision of the sulphur atom for thiamine biosynthesis. At concentrations of 10 μ moles/100 ml., however, the relative specific activity due to the thiazole does not change, whereas that due to [32 S]sulphate falls below the expected value of 14 %, based on the value of the relative specific activity observed for 1 μ mole/100 ml. Whereas the sulphate must continue to fulfil conditions (a) and (b), the additional thiazole evidently cannot meet condition (a), since if all the thiazole were available for thiamine biosynthesis a fall in relative specific activity to about 3 % would result. Biosynthesis directly from sulphate of about one-quarter of the total thiamine therefore occurs when the medium contains 500 or even 5000 times the amount of the thiazole required for full incorporation. On the other hand, if only 0.05 μ mole of the thiazole/100 ml. (equivalent to 25 times the weight of thiamine biosynthesized) was provided, the thiamine obtained had a relative specific activity of 40 %, implying a greater efficiency of incorporation at higher dilution. These results emphasize the importance of condition (a) and the inefficiency (relative to sulphate) of transport of the thiazole to the thiamine-producing sites in the cell; they also confirm that the thiazole is incorporated by the yeast into the thiamine molecule.

Although it is generally believed that sulphate must first be reduced before the sulphur can become available for biosynthetic processes in yeast, sodium sulphide appeared to be less effective than sodium sulphate at equivalent concentrations.

Methionine, homocysteine, homocystine, cysteine. It is evident from the values for relative specific activity recorded in Table 2 that methionine is more efficient than cysteine, homocysteine or homocystine in providing the sulphur atom of thiamine. In spite of the known metabolic interrelationships, the differences in comparable relative specific activities are significant (DL-methionine: 14, 13; DL-homocysteine: 50; DL-homocystine: 97 %. L-Methionine: 15, 5, 10; L-cysteine, 78, 83, 85, 69 %). In comparing the values for relative specific activity due to DL- and to L-methionine, it seems probable that the D-enantiomorph has little effect. These and other comparisons suggest that the sulphur atom is incorporated into thiamine most directly from L-methionine. Homocysteine is less efficient as a source of sulphur, and methylation to methionine may be an obligatory stage. The inefficiency of homocystine may be due to inefficient transport, since, once inside the cell,

rapid reduction to homocysteine would be expected. It is well known that disulphides are reduced to thiols by metabolizing yeast (Neuberg & Schwenk, 1915), and it has been reported by Sandegren, Ekström & Nielsen (1950) that in the yeast *Rhodotorula gracilis* growing on a limited sulphur supply all the polarographically active sulphur present is in the thiol form. These results run roughly parallel with the observations on protein synthesis in yeast (*Torulopsis utilis*) by Roberts, Cowie, Abelson, Bolton & Britten (1955), who found cysteine to be a more effective competitor than cystine and also noted the relative inefficiency of homocysteine and the greater inefficiency of homocystine, which they attributed to inability of the yeast cell to reduce the disulphide forms.

Cysteine is also much less efficient than homocysteine or methionine, suggesting that it can provide sulphur for incorporation into thiamine only via the cystathionine-homocysteine-methionine pathway. Djenkolic acid (relative specific activity, 108 %) was without effect, and the relative specific activity due to ethionine could not be determined, since the presence of this amino acid in the medium inhibited yeast growth.

Glutathione. Comparison of the relative specific activity values due to sulphate, glutathione and cysteine at 1 μ mole/100 ml. (62, 57 and 85 % respectively) or at 10 μ moles/100 ml. (7, 5 and 69–85 % respectively) shows that the two former compounds can supply the sulphur atom required for thiamine biosynthesis much more efficiently than can cysteine, and the provision of glutamic acid and glycine as well as cysteine in the medium did not affect the relative specific activity appreciably. Since glutathione is biosynthesized from L-cysteine the marked difference in their competitive powers is surprising.

S-Adenosylmethionine and 5'-deoxy-5'-methylthioadenosine. The success with which methionine competed with sulphate as the source of the sulphur for incorporation into the thiamine molecule prompted the examination of *S*-adenosylmethionine and of 5'-deoxy-5'-methylthioadenosine; both entered into competition with sulphate, the former slightly more efficiently and the latter less efficiently than methionine.

Thiazolidine-4-carboxylic acid (IV). Neither cysteine nor 4-methylthiazole gave relative specific activities for biosynthesized thiamine, suggesting that they were of significance in yeast, but the values for relative specific activity obtained with thiazolidine-4-carboxylic acid showed that it acted as a sulphur donor as efficiently as methionine; by comparison with the inefficiency of cysteine this result was unexpected. The more direct observations of Korte, Weitkamp & Vogel (1959), using

Table 2. *Relative specific activities of thiamine biosynthesized by yeast in isotopic-competition experiments between 200–400 μ C of [35 S]sulphate/100 ml. and various compounds containing non-radioactive sulphur*

Non-radioactive-sulphur- containing competitor (1)	Expt. no. (2)	Concn. of competitor in medium (μ moles/ 100 ml.) (3)	Dry wt. of harvested cells (mg./ 100 ml.) (4)	Total radioactivity of cells (mega-counts/ min.) (5)	Wt. of thiamine isolated* (μ m-moles) (6)	Thiamine content of cells* (μ g./g.) (7)	Sp. activity of thiamine* (counts/min./ μ mole) (8)	Sp. activity of corresponding control (counts/min./ μ mole) (9)	Relative sp. activity of bio- synthesized thiamine (%) (10)
None†	1	0	41	55.1	1.37†	—	43.8	—	97
	1	0	41	62.0	2.17	18	45.9 (av.)	—	103
	2	0	37	54.0	1.97	18	45.9	—	104
	2	0	37	59.6	1.78	17	42.7 (av.)	—	96
	3	0	20	52.1	1.10	19	106.1	—	103
	3	0	22	51.7	1.10	17	99.8 (av.)	—	97
	4	0	30	68.4	1.81	21	51.8	—	—
	5	0	29	56.9	2.02	24	48.8	—	—
	6	0	24	40.4	1.04	15	89.2	—	—
	7	0	32	41.7	1.31	14	57.0	—	—
	8	0	24	40.9	1.14	16	60.1	—	—
	9	0	32	58.8	2.30	25	38.0	—	—
	10	0	22	44.3	1.53	24	45.8	—	—
Sodium sulphate	11	0	15	27.4	0.95	20	51.5	—	—
	12	0	12	28.0	0.87	20	55.7	—	—
	13	0	22	17.2	1.39	22	34.6	—	—
	8	1	40	41.8	2.15	19	37.4	60.1	62
	3	10	122	71.5	13.15	37	7.3	103.0	7
	1	0.05	41	57.4	2.37	20	17.8	44.6	40
	3	1	22	56.6	2.37	20	8.9	44.8	20
5-(2-Hydroxyethyl)-4-methylthiazole	3	1	22	57.9	1.10	17	29.7	103.0	29
	6	1	22	39.9	1.18	16	24.5	103.0	24
	7	1	30	39.3	1.04	18	22.2	89.2	25
	9	1	33	56.9	1.58	24	13.2	57.0	23
	10	1	21	45.0	2.31	19	8.8	38.0	23
	11	1	14	27.1	1.39	22	11.1	45.8	24
	12	1	12	29.0	0.78	19	12.1	51.5	24
	2	10	41	58.2	0.88	19	11.8	55.7	21
	8	10	26	40.7	2.11	18	10.4	44.3	23
	13	10	26	17.7	1.14	15	15.1	60.1	25
	1	2	53	60.5	1.44	19	7.3	34.6	21
	9	2	51	61.5	3.51	23	27.2	44.6	61
DL-Methionine	9	2	115	3.57	10.55	24	25.4	38.0	67
	10	20	89	40.1	9.40	36	5.8	38.0	14
	5	1	43	57.5	3.15	25	34.7	48.8	71
	2	10	106	72.4	6.85	22	6.8	48.8	15
L-Methionine	7	10	85	40.4	4.94	22	2.8	44.3	5
	8	10	144	47.9	11.27	27	6.3	57.0	10
	13	20	39	19.9	2.60	23	17.3	34.6	50
	11	100	106	32.4	8.83	29	4.2	51.5	8
DL-Homocysteine	1	2	41	58.1	2.56	22	41.9	44.6	94
	13	10	25	17.9	1.39	19	32.9	34.6	95
	5	20	36	54.2	2.37	23	47.3	48.8	97
	8	100	47	41.9	3.01	22	31.1	60.1	52
L-Cysteine	1	1	41	60.2	2.67	23	37.8	44.6	85
	2	10	37	57.4	2.07	19	34.7	44.3	78
	8	10	29	39.8	1.37	16	50.0	60.1	83
	11	10	18	25.0	1.16	20	43.6	51.5	86
	13	10	41	19.4	2.49	21	23.9	34.6	69
	12	100	40	28.7	2.86	25	19.4	55.7	35

Table 2 (cont.)

	Expt. no. (2)	Concn. of competitor in medium (μmoles/100 ml.) (3)	Dry wt. of harvested cells (mg./100 ml.) (4)	Total radioactivity of cells (mega-counts/min.) (5)	Wt. of thiamine isolated* (μm-moles) (6)	Thiamine content (μg./g.) (7)	Sp. activity of thiamine* (counts/min./μmole) (8)	Sp. activity of corresponding control (counts/min./μmole) (9)	Relative sp. activity of bio-synthesized thiamine (%) (10)
Non-radioactive-sulphur-containing competitor (1)									
Djenkolic acid	4	10	30	64.7	2.07	24	56.0	51.8	108
Glutathione	1	1	49	62.1	3.11	22	25.5	44.6	57
	3	10	117	71.2	12.57	37	5.4	103.0	5
	10	10	73	49.4	8.10	36	3.9	45.8	8
Glutamic acid+cysteine+glycine	11	10 each	17	26.7	1.07	19	45.3	51.5	88
S-Adenosylmethionine	9	2	65	60.6	5.26	28	15.1	38.0	40
	9	20	147	67.9	14.31	34	3.0	38.0	8
5'-Deoxy-5'-methylthioadenosine	9	20	104	64.3	8.67	29	11.5	38.0	30
Thiazolidine-4-carboxylic acid	13	1	28	19.1	1.88	23	22.8	34.6	66
	13	10	58	17.7	6.13	37	3.8	34.6	11
	12	100	128	29.3	10.7	29	0.6	55.7	1
4-Methylthiazole	12	100	17	29.4	1.53	26	37.3	55.7	67
	13	10	34	18.8	2.14	22	22.2	34.6	63
DL-α-Amino-β-(4-methylthiazol-5-γ)propionic acid	6	2	22	43.9	0.88	14	68.5	89.2	77
	6	20	24	35.9	1.06	15	36.9	89.2	41
	7	20	33	41.2	1.14	12	20.5	57.0	36
(4-Methylthiazol-5-γ)acetic acid	1	1	41	60.7	2.31	19	43.4	44.6	97
Benzylpenicillin	10	100	25	45.1	1.47	20	43.5	45.8	95
DL-Biotin	8	20	26	40.7	1.23	16	59.8	60.1	100
Methanethiol	5	1	43	64.3	3.24	26	38.5	48.8	79
	5	10	51	54.2	3.76	26	33.0	48.8	68
	2	100§	106	58.2	14.3	47	1.4	44.3	3
Sodium methyl sulphide	6	100§	68	44.1	6.33	32	23.5	89.2	26
	7	100§	79	41.1	4.16	18	15.7	57.0	28
Ethanethiol	4	10	36	69.6	4.42	42	18.6	51.8	36
Sodium ethyl sulphide	6	100	78	45.5	4.86	22	14.0	89.2	16
S-Methylthiuronium chloride	4	10	30	65.0	2.04	24	54.6	51.8	105
Thiourea	1	1	39	57.4	2.07	18	46.0	44.6	103
Thioacetic acid	4	10	11.5	44.9	1.39	42	17.0	51.8	33
	10	10	37	35.9	3.70	34	14.7	45.8	32
Thioglycolic acid	4	10	30	66.0	2.60	30	46.5	51.8	90
Taurine	2	1	41	64.4	2.01	17	40.7	44.3	92
1,2-Dimercaptopropan-3-ol (BAL)	5	10	31	49.8	2.25	25	40.2	48.8	82
1-Mercaptopropan-2,3-diol	7	100	27	37.3	1.70	22	49.5	57.0	87
	8	100	28	38.6	1.23	15	36.4	60.1	64
1-Acetyl-1-mercaptopropan-3-ol	6	100	22	46.1	1.42	22	45.2	89.2	51
4-Amino-2-methyl-5-thioformamidomethyl-pyrimidine	11	10	25	27.5	2.31	34	33.6	51.5	65
	12	100§	50	33.5	6.70	45	13.8	55.7	25
Sodium sulphide	4	10	53	66.0	5.36	35	21.6	51.8	42
Thiamine	2	0.24	46	59.6	64.2	483	0.3	44.3	1
	4	0.003	22	57.8	1.82	30	38.0	51.8	73

* Estimated as thiochrome.

† Controls for numbered experiments.

‡ Some yeast accidentally lost.

§ Concentration uncertain owing to volatility and/or insolubility.

4-methyl[2-¹⁴C]thiazole, showed that that substance remained unchanged in cultures of a range of micro-organisms capable of effecting partial synthesis of thiamine.

DL- α -Amino- β -(4-methylthiazol-5-yl)propionic acid (V). The observed relative specific activity values due to the DL-amino acid confirm utilization by yeast to some extent for thiamine biosynthesis, but, when compared with the corresponding values due to 5-(2-hydroxyethyl)-4-methylthiazole itself, suggest that the acid does not lie on the normal route, since 1 μ mole of the DL-amino acid/100 ml. (relative specific activity of biosynthesized thiamine, 77 %) is much less efficient than an equivalent concentration of 5-(2-hydroxyethyl)-4-methylthiazole (relative specific activity of biosynthesized thiamine, 25 %).

4-Methylthiazol-5-ylacetic acid had no effect on thiamine biosynthesis.

Benzylpenicillin. The relative specific activity of thiamine (97 %) biosynthesized in the presence of 100 μ moles of benzylpenicillin/100 ml. of medium shows that it is ineffective as a source for the supply of the sulphur atom of thiamine.

Biotin. The standard medium always contained about 1 μ m-mole of DL-biotin/100 ml., which was essential for growth. When it was supplemented with 20 μ moles there was little change in the relative specific activity of the thiamine (91 %), showing that the sulphur of biotin is not available as a source of sulphur for incorporation into thiamine.

Alkanethiols. The thiols were either added to the medium as such or as their solid sodium derivatives; in either case some thiol was lost. Tight cotton-wool plugs and cupric chloride-impregnated charcoal traps were used, and any thiol in the gas phase was continuously shaken up with the yeast suspension. The concentration of thiol available in the medium was therefore uncertain, and the figures given in Table 2 relate to the quantities of thiol initially added. Nevertheless, it is apparent from the relative specific activity values that both thiols compete with sulphate for the provision of the sulphur required for thiamine biosynthesis. Neither *S*-methylthiuronium chloride nor thiourea had any significant effect.

Miscellaneous compounds. At a concentration of 10 μ moles/100 ml. thioacetic acid ($\text{CH}_3\cdot\text{CO}\cdot\text{SH}$) gave a relative specific activity of 32–33 % for biosynthesized thiamine showing some degree of incorporation of its sulphur atom, possibly by way of a thioacetylation mechanism; thioglycollic acid ($\text{HS}\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$) at the same concentration was almost ineffective (relative specific activity of biosynthesized thiamine, 89 %), as also was taurine.

Other compounds tested were 1:2-dimercaptopropan-3-ol (dimercaprol, BAL), 1-mercaptopro-

pan-2:3-diol and 1-acetyl-1-mercaptopropan-3-ol, which might have been capable of conversion into the thiazole moiety of thiamine. Although effects were observed in the presence of large amounts, in no case did the relative specific activity of biosynthesized thiamine fall to a level indicating noteworthy incorporation of sulphur from these sources. The presence of mercaptoacetone in the medium inhibited yeast growth, and no reliable relative specific activity values could be obtained.

The thioformyl derivative of the pyrimidine moiety of thiamine [4-amino-2-methyl-5-thioformamidomethylpyrimidine (II; $\text{X} = \text{NH}\cdot\text{CHS}$)] gave values of relative specific activity for biosynthesized thiamine indicating utilization of the sulphur to some extent; this might have been due to partial decomposition during incubation and subsequent utilization of the fragments, as is also observed in pea roots (Bonner & Buchman, 1938).

DISCUSSION

As the strain of yeast selected for use was non-exacting (B. L. Brady, personal communication, 1957) it was expected to be capable of synthesizing all its sulphur compounds from inorganic sulphate, and when given [³⁵S]sulphate it was found to synthesize thiamine of high specific activity, the only dilution suffered by the carrier-free radioisotope coming from the limiting amount of endogenous sulphur present in the inoculum and in the medium, not all of which would be expected to be available for thiamine biosynthesis. The specific activity of the biosynthesized thiamine was in fact of the order of 1 mc/mg., compared with about 120 μ C/mg. for thiamine synthesized from [³⁵S]-thiourea (Würsch, 1958), so that biosynthesis affords a convenient method for the preparation of small amounts of [³⁵S]thiamine of high specific activity. The amount of thiamine synthesized by yeast, however, is extremely small and most of the sulphur is used for other purposes; on the other hand [³⁵S]sulphate is relatively cheap. When grown in the synthetic medium described, the yeast normally contained about 25 μ g. of thiamine (as cocarboxylase) per gram (dry weight) of cells (Table 2, column 7); this figure is within the range quoted for non-enriched yeasts (Van Lanen & Tanner, 1948). The thiamine content of the yeast could be increased only by providing thiamine or both its moieties in the medium; in these circumstances less thiamine was biosynthesized, and it incorporated less label, though it was present in larger quantities. This was demonstrated by the use of thiamine itself as non-radioactive competitor for the [³⁵S]sulphate, when both the specific and total activities of the extracted thiamine were lower than those of controls. Moreover, not all the

added vitamin could be accounted for, suggesting that some of it had been metabolized (cf. Bonner & Buchman, 1939; Tatum, 1944). No advantage would therefore be gained in the routine use of thiamine-enriched yeast.

In a typical experiment, 250 μC of [^{35}S]sulphate was added to 100 ml. of the basal medium; the 25 mg. of yeast obtained had incorporated 220 μC , of which about 25 μC was extracted by the boiling acid. A radioactivity of 5 μC was retained by the first chromatographic treatment and 15 μC was not retained by the second. The 5 ml. of 'standard' extract contained 5 μC of which [^{35}S]thiamine accounted for about 0.5 μC . Since 25 mg. of yeast cells contained only about 0.5 μg . of thiamine, its specific activity had to be high to enable its total activity to be measured. The specific activity of the [^{35}S]sulphate in the medium had therefore to be high; a basal medium was therefore chosen which contained no sulphur other than added [^{35}S]sulphate and sulphur intrinsically present in the inoculum. The use of [^{35}S]sulphate of high activity (> 100 mc/mg. of sulphur), and of small volumes of inoculum consisting of washed yeast cells, which were themselves sulphur-starved, minimized introduction of extraneous sulphur, and yielded thiamine whose specific activity was conveniently measurable. Preliminary experiments confirmed the observations of Kleinzeller, Kotyk & Kováč (1959) that both glucose and ammonium salts must be present in the medium for maximum uptake of sulphate by non-proliferating yeast. Under these conditions [^{35}S]thiamine is found in non-proliferating cells kept in the presence of [^{35}S]sulphate, demonstrating thiamine turnover, but the label is necessarily diluted by the non-radioactive sulphur already present in the yeast.

Cowie, Bolton & Sands (1951) have demonstrated that the presence of non-radioactive sulphur compounds, in a medium containing [^{35}S]sulphate and enough total sulphur for optimum growth, may eliminate or suppress uptake of the label by *Escherichia coli* cells. The presence of a sulphur-containing compound in a medium containing little sulphur as sulphate (about 1 $\mu\text{mole}/100\text{ ml.}$) in which sulphur-starved yeast is growing may well have other effects than on incorporation of the extra sulphur into the thiamine of the yeast; indeed the general sulphur metabolism in such yeast may be abnormal. Table 2, column 5, gives the total activity of cells grown in such a medium and shows that the effect observed with *E. coli* is not obvious with yeast. Variations in the specific activity of biosynthesized thiamine are therefore not due to selective uptake of sulphur compounds by the yeast cell. It is likely that all the non-radioactive compounds tested are also absorbed by the cell and are there available to compete more or

less efficiently with [^{35}S]sulphate, but it is not known to what extent selective transport occurs within the cell, and it is in this respect that the competition may not be a fair one. The major proportion of a competitor might remain near the cell wall while the [^{35}S]sulphate reached thiamine-producing sites relatively unhindered. In this connexion the localization of *S*-adenosylmethionine in enriched *Torulopsis utilis* cells is of interest; this yeast stores large quantities of 'active methionine' in the vacuole (Svihla & Schlenk, 1959; 1960). The addition of some ^{32}S -containing compounds caused greatly enhanced yeast growth (Table 2, column 4), thus confirming their utilization by the yeast, but hindering comparisons. The thiamine content of unit weight of yeast, however, did not vary widely (Table 2, column 7), confirming the inability of the yeast to synthesize and store extra thiamine unless provided with the vitamin itself or its moieties. It may be that the extra vitamin (much of which is stored as free thiamine) is stored without turnover and is not in equilibrium with thiamine biosynthesized *de novo*. One & Ichikawa (1954) have suggested that exogenous thiamine is not absorbed through the cell wall, and electron microscopy indicates that it is located on the cell wall (Ichikawa, 1956).

The isotopic-competition methods described here do not allow rigorous quantitative treatment of the results, and even qualitative interpretation may be misleading. Nevertheless, in the group of sulphur-containing amino acids, L-methionine appeared to be about twice as effective as the DL-form, which, in turn, was much more effective than DL-homocysteine in supplying the sulphur atom for incorporation into the thiazole moiety of thiamine. Fukui (1950) reported that *Mucor racemosus* can form thiamine from 4-amino-5-aminomethyl-2-methylpyrimidine (II, $\text{X} = \text{NH}_2$) and methionine in the Reader medium, whereas *Aspergillus oryzae* and *Saccharomyces saké* can also do so if the medium is supplemented with peptone, so that methionine appears to be a significant precursor. The fact that efficiencies as sulphur donors for thiamine biosynthesis appeared to follow the order homocysteine < methionine < *S*-adenosylmethionine > 5'-deoxy-5'-methylthioadenosine seems to indicate that *S*-adenosylmethionine is more directly concerned with the biosynthesis than are the other members of the series. Nakayama (1956) has reported that mutant strains of *Escherichia coli* and *Neurospora crassa*, which both require 5-(2-hydroxyethyl)-4-methylthiazole (III) for growth, responded moderately to 4-methylthiazole; cysteine and thiazolidine-4-carboxylic acid (IV) maintained the growth of both strains, but no nutritive activity was observed in homocysteine, methionine or methionol. As thiamine and 5-(2-hydroxyethyl)-4-methyl-

thiazole were detected in *Neurospora* cultures that had been incubated with cystine or thiazolidine-4-carboxylic acid, it was assumed (Nakayama, 1956) that biosynthesis of thiamine took place from cystine via thiazolidine-4-carboxylic acid and 4-methylthiazole. In yeast, however, only thiazolidine-4-carboxylic acid appeared to be of significance as a sulphur donor, and it was comparable with methionine in efficiency. The apparent inefficiency of cysteine and the effectiveness of glutathione are at first sight difficult to reconcile with each other, but it may be a reflexion of a wider range of uses by yeast for cysteine, and the same weighting factor no doubt applies with other substrates to unknown and varying extents.

α -Amino- β -(4-methylthiazol-5-yl)propionic acid (V) has been suggested as a precursor of 5- β -hydroxyethyl-4-methylthiazole (Harington & Moggridge, 1939; Buchman & Richardson, 1939) and it could be derived from methionine, acetaldehyde and ammonia (Harington & Moggridge, 1939). Homocysteine has been postulated as a precursor of the thiazole moiety of thiamine in a *Neurospora* mutant, possibly by conversion into S-formyl-homocysteine, which could condense with a -C.C.N- fragment to give the same amino acid (V) (Doudney & Wagner, 1953). It has been shown that the L-form of this amino acid is converted into 5-(2-hydroxyethyl)-4-methylthiazole (III) by brewer's yeast, leaving the D(-)-form unchanged (Harington & Moggridge, 1940); conversion into 5-(2-hydroxyethyl)-4-methylthiazole also occurs in pea roots (Bonner & Buchman, 1938) but not in *Phycomyces blakesleeanus* (Bonner, quoted by Buchman & Richardson, 1939), in *Staphylococcus aureus* (Knight, quoted by Harington & Moggridge, 1939), or in *Neurospora crassa* (Tatum & Bell, 1946), as the amino acid cannot replace 5-(2-hydroxyethyl)-4-methylthiazole in these systems. In yeast it would not appear that the amino acid (V) lies on the direct route of thiamine biosynthesis, although it is utilized to some extent. Bonner & Buchman (1938) also found that pea roots could apparently synthesize thiamine when given its pyrimidine moiety and (i) 5-(2-hydroxyethyl)-4-methylthiazole, or (ii) thioformamide and 1-acetyl-1-chloropropan-3-ol, or (iii) thioformamide and 1-acetylpropan-3-ol. Tatum & Bell (1946), however, showed that such mixtures [(ii) and (iii)] could not replace 5-(2-hydroxyethyl)-4-methylthiazole in an exacting

mutant strain of *Neurospora*, and Louis (1951) found isolated pea roots capable of synthesizing thiamine from the intact pyrimidine and thiazole moieties but not from the pyrimidine, thioformamide and 1-acetyl-1-chloropropan-3-ol. *Mucor racemosus*, *Saccharomyces saké* and *Serratia marcescens* are said to synthesize thiamine from 4-amino-5-formamidomethyl-2-methylpyrimidine (II; X = NH $\cdot$ CHO) and 1-acetyl-1-mercapto-propan-3-ol, and *M. racemosus*, *S. saké* and *Bacillus vulgatus* are said to produce thiamine from the formamidomethylpyrimidine and 1-acetyl-1-chloropropan-3-ol (Fukui & Kishibe, 1951). It has been pointed out, however, that, some of these mixtures may give thiamine *in vitro* under mild conditions without enzymic intervention (Matsukawa & Yurugi, 1951; Bonner & Buchman, 1938), and the components appear unlikely ones in natural biosynthesis. Neither 4-amino-2-methyl-5-thioformamidopyrimidine nor 1-acetyl-1-mercapto-propan-3-ol appeared to be capable of contributing sulphur effectively for thiamine biosynthesis in yeast, as shown by the high concentrations necessary to affect appreciably the relative specific activities of biosynthesized thiamine in the present work.

A number of cases have been described in which a substance other than the vitamin thiazole (III) has appeared to be capable of replacing the latter in growth tests on what have been regarded as exacting organisms, but it is difficult to assess the significance of such growth tests. For example, penicillin in 10⁶-fold concentration is capable of replacing 5-(2-hydroxyethyl)-4-methylthiazole as a growth factor for *Phycomyces blakesleeanus* (Shulman, Dick & Farrer, 1957), and a similar effect has been described for bacitracin, particularly for specimens liable to contain bacitracin F (Ebringer, 1960). There can be no question of conversion of these substances into the vitamin thiazole, and benzylpenicillin is certainly incapable of supplying the sulphur atom required for thiamine biosynthesis.

The fairly inefficient use of methane- and ethane-thiol as sources of the sulphur atom in thiamine is of interest. It has been reported that methane- and ethane-thiol are incorporated into S-adenosyl-methionine and S-adenosylethionine by yeast (*Torulopsis utilis*) (Schlenk, 1957; Schlenk & Tillotson, 1954); on the other hand, it has also been reported that methane-[³⁵S]thiol is converted by yeast into S-methyl[³⁵S]cysteine and that the major known sulphur compounds, such as cysteine, glutathione and methionine, do not become labelled (Black, Downey & Wolff, 1957). The comparable behaviour of methane- and ethane-thiol in the present experiments would seem to indicate that a process of sulphur exchange operates, and the relative efficiencies as competitors of the various

compounds examined may be a measure of the relative abilities of the yeast to metabolize these substances to give a common, and at present unidentified, sulphur-containing intermediate in the biosynthesis of thiamine. Apart from the vitamin thiazole itself, 5-(2-hydroxyethyl)-4-methylthiazole, none of the compounds examined can be recognized as a major precursor of the thiazolium ring of thiamine. The mechanism by which these compounds that can compete with sulphate make sulphur available for biosynthesis of thiamine is by no means clear; whether carbon is simultaneously incorporated from any of these compounds can only be determined by experiments with ^{14}C -labelled materials.

In conclusion, the prosthetic group of crystalline rabbit-muscle α -glycerophosphate dehydrogenase has properties which suggest that it has the quaternary thiazole structure (VI); it can replace the thiazole moiety of thiamine in growth tests on *Leuconostoc mesenteroides*, and thiamine deficiency in rats results in lowering of soluble glycerophosphate dehydrogenase (van Eys, 1960). What may be the relationship of this substance, which has been named thiamic acid, to the thiazole moiety of thiamine remains to be seen.

SUMMARY

1. Yeast grown on a defined medium containing [^{35}S]sulphate produces [^{35}S]thiamine of high specific activity in small amounts.

2. Yeast has been grown in a defined medium containing [^{35}S]sulphate and a non-radioactive sulphur-containing substance as sole sulphur sources. These two substances may, or may not, compete in contributing the sulphur required for incorporation into the thiazole ring of thiamine; the specific activity of the biosynthesized thiamine compared with controls gives a measure of the efficiency of the non-radioactive sulphur-containing compound under test as a donor of sulphur for the biosynthesis of thiamine.

3. The following compounds are utilized by the yeast, in the presence of sulphate, as sources of sulphur for the biosynthesis of thiamine: very efficiently: 5-(2-hydroxyethyl)-4-methylthiazole; efficiently: *S*-adenosylmethionine, sodium sulphate,

glutathione, methionine, thiazolidine-4-carboxylic acid, 5'-deoxy-5'-methylthioadenosine; fairly efficiently: α -amino- β -(4-methylthiazol-5-yl)propionic acid, sodium sulphide, thioacetic acid; inefficiently: cysteine, homocysteine, 4-amino-2-methyl-5-thioformamidomethylpyrimidine, methanethiol and ethanethiol.

4. Other compounds tested which do not appear to compete significantly with sulphate are: homocystine, thiourea, biotin, thioglycolic acid, djenkolic acid, *S*-methylthiuronium chloride, 4-methylthiazole, (4-methylthiazol-5-yl)acetic acid, benzylpenicillin, taurine, 1:2-dimercaptopropan-3-ol (BAL), 1-mercaptopropan-2:3-diol, 1-acetyl-1-mercaptopropan-3-ol.

5. Of the compounds examined, none—apart from 5-(2-hydroxyethyl)-4-methylthiazole—could be recognized as a major precursor of the thiazolium ring in thiamine.

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Metabolism of Collagen

APPEARANCE AND DISAPPEARANCE OF [¹⁴C]HYDROXYLYSINE IN RAT CONNECTIVE TISSUE

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The technique of labelling collagen with the radioisotope of a distinctive amino acid, hydroxylysine, simplifies the study of collagen metabolism. In collagen from adult animals this amino acid is derived from the hydroxylation of lysine after the 'parent' amino acid has been incorporated into a polypeptide (Sinex & Van Slyke, 1955, 1957; Sinex, Van Slyke & Christmas, 1959).

The metabolism of collagen has previously been studied by isotopically labelling a constituent amino acid. [¹⁴C]Glycine was used in investigations on the rabbit corium (Harkness, Marko, Muir & Neuberg, 1954) and by Jackson (1957) on the water- and salt-soluble and -insoluble fractions of the carageenin granuloma. However, glycine, though present in high concentrations in collagen,