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THE ORIGIN OF THE CARBON CHAIN IN THE THIAZOLE MOIETY OF THIAMINE IN *Escherichia coli* : INCORPORATION OF DEUTERATED 1-DEOXY-D-*threo*-2-PENTULOSE.

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Non growing washed cells of *Escherichia coli*, derepressed for the biosynthesis of thiamine, have been incubated in the presence of glucose and either 1-deoxy-D-*threo*-2-pentulose 1 or 1-déoxy-D-*erythro*-2-pentulose 2 trideuterated on the methyl group. The incorporation of deuterium into the thiazole moiety of thiamine was measured by mass spectrometry. The label of the *threo*-compound was found in more than 40% of the thiazole biosynthesized in its presence; the label of the *erythro*-compound in less than 5%. Hence it is likely that the carbon chain of 1-deoxy-D-*threo*-2-pentulose is the precursor of the five carbons chain of the thiazole moiety of the thiamine molecule in *E. coli*.

Recent published experiments by R.H. White (1,2) established that the five carbons chain of the thiazole moiety (HET) of thiamine is derived in *E. coli* from two units : the fragment C-4, C-8 originating from pyruvate and the fragment C-5, C-6, C-7 from glyceraldehyde phosphate. Consequently, it was suggested (1) that a 1-deoxypentulose-5-phosphate built up by an acyloin condensation between an activated acetaldehyde unit and glyceraldehyde phosphate could be the direct precursor of the carbon chain of HET. We wish to relate in this paper the results obtained from experiments with pentuloses labelled with deuterium at C-1, namely 1-deoxy-D-*threo*-2-pentulose 1 and 1-deoxy-D-*erythro*-2-pentulose 2, that we have prepared to test this hypothesis.

Material and methods.

Organism.- A shikimate auxotroph mutant of *E. coli* (strain 83-1) was used.

Derepression of thiamine biosynthesis.- This strain was cultivated according to a described procedure in the presence of adenosine (3,4). The cells were washed and resuspended in a minimal medium (3) with glucose (11 mM) and tyrosine (0.1 mM).

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Incubation.- To 100 ml of suspension containing 1.5 mg/ml (dry weight) of cells was added the labelled pentulose at a concentration of 3 mM. The suspension was then stirred for 1 hour at 37° and centrifuged. A blank experiment was run with unlabelled pentuloses.

Extraction and purification of thiamine.- Thiamine was extracted from cells and the extracts purified on a column of Amberlite CG 50 according to the usual procedure (4). An aliquot was taken for thiamine assay. Thiamine was purified further by linear gradient elution from Dowex 50 (H^+) (1.1 to 5.4 M HCl).

Isolation of 4-methyl-5-(β-hydroxyethyl)thiazole (HET).- Purified thiamine was then cleaved by bisulfite to HET and pyrimidine moiety (5). From the bisulfite solution (2 ml) adjusted to pH 7-8, HET was extracted with dichloromethane (5 x 2 ml). The solvent was evaporated at 30° under a stream of nitrogen. The dry residue containing HET was dissolved into 50 µl of acetone.

Gas chromatography. Mass spectrometry of HET.- A Ribermag R 10-10 apparatus was used. The column (1.5 m) was packed with 10% Carbowax 20 M on Gas-Chrom W. Aliquots of 1-3 µl were injected into the column at 150°C (injector temperature 200°C). The retention time of HET was 9.5 min. Mass spectra were recorded at 70 eV with anion source temperature of 250°. Elution of HET was detected by measuring the intensity of peaks at m/e 112, 143, 146. The greatest intensity was found during the same scan for the three peaks, indicating that no isotope fractionation occurred during the gas chromatography. The spectrum was recorded during this scan. The number of deuterium atoms in each fragment of interest was calculated from the intensity of successive peaks after subtracting the natural isotopic contribution.

Assay of thiamine.- Thiamine was assayed fluorimetrically after oxidation to thiochrome by mercuric chloride according to Morita and co-workers (6).

Chemical syntheses of 1-deoxy-D-*threo*-|1-$^2H_3$|2-pentulose 1 and 1-deoxy-D-*erythro*-|1-$^2H_3$|2-pentulose 2.

1-Deoxy-4,5-O-isopropylidene-D-*erythro*-|1-$^2H_3$|2-pentulose propylene dithioacetal 4.- This was prepared from 1,3-dithiane, |$^2H_3$|iodomethane (99.3 %, C.E.A., France) and 2,3-O-isopropylidene-D-glyceraldehyde (7) following the general procedure of Corey and Seebach (8). Recrystallization removed traces of the 3-epimer, 1-deoxy-4,5-O-isopropylidene-D-*threo*-|1-$^2H_3$|2-pentulose propylene dithioacetal 3 and gave a pure sample of dithioacetal 4 (3.5 g; 35 %), m.p. 109°C (hexane-ethyl acetate).

1-Deoxy-4,5-O-isopropylidene-D-*threo*-|1-$^2H_3$|2-pentulose 5 and 1-deoxy-4,5-O-isopropylidene-D-*erythro*-|1-$^2H_3$|2-pentulose 6.- Oxidation of dithioacetal 4 (3.5 g) by the dimethylsulfoxide-trifluoroacetic anhydride reagent in dichloromethane at -70° gave the ketone (66 %). Sodium borohydride reduction in methanol solution afforded then a nearly equimolecular crystalline mixture of 3 and 4. This was hydrolyzed in the presence of mercuric oxide, boron trifluoride etherate and deuterated water in oxolane (9) to give an oily mixture (0.7 g) of protected pentuloses 5 and 6. Silica gel column chromatography (50 g; toluene-ethyl acetate, 65:35) separated first the *erythro* isomer (260 mg) and afterward the *threo* isomer (235 mg) as pure colorless liquids. The ^1H NMR spectra of 5 and 6 were identical with those of unlabelled samples but for the absence of the signals of 1-H protons.

Sodium borohydride reduction of unlabelled pentulose 6 gave a mixture of two protected, epimeric 1-deoxypentitols which were separated by silica gel column chromatography (chloroform-methanol, 90:10) and hydrolyzed to

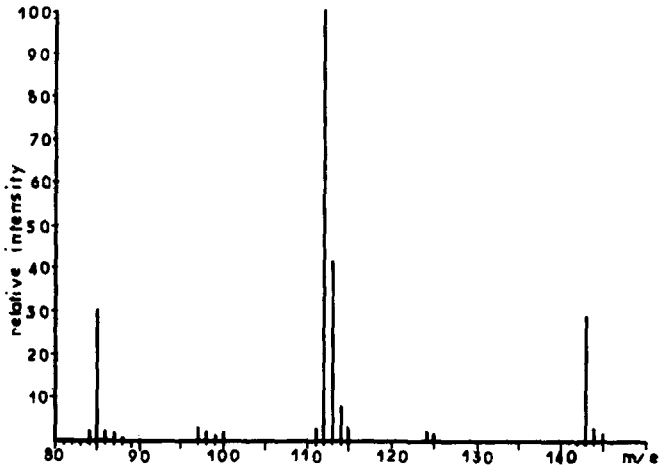


Figure 1. Mass spectrum of HET extracted from cells incubated in the presence of a mixture of unlabelled 1-deoxy-D-*threo* and 1-deoxy-D-*erythro*-2 pentuloses.

the free polyols. One of them, $|\alpha|_D^{21}$ 0° (c=1, H_2O), m.p. 128-130°C, was found identical with the known 1-deoxy-D-arabinitol, litt. (10) m.p. 130°C, $|\alpha|_D^{21}$ 1.4° and the other one, α_D^{21} + 9° (c=1, H_2O), identical with the known 1-deoxy-D-ribitol, litt. (10) $|\alpha|_D$ + 15°, (11) α_D + 9°.

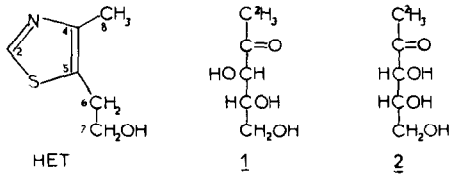
These experiments settle the configuration as D-*erythro* for derivatives 4 and 5.

1-Deoxy-D-*threo*-|1-$^2H_3$|2-pentulose 1 and 1-deoxy-D-*erythro*-|1-$^2H_3$|2-pentulose 2.

These were prepared just before use. Heating at 80° for 45 min. a solution of derivative 5 or 6 in 8:2 acetic acid-water solution, followed by evaporation *in vacuo* gave the corresponding free sugar 1, $|\alpha|_D^{20}$ +34° (c=1, water) or 2, $|\alpha|_D^{21}$ -30° (c=1, water), litt. (10) $|\alpha|_D^{21}$ -37 for the unlabelled compound. These colorless syrups were immediately dissolved in water and added to the medium.

Results

The mass spectrum of unlabelled HET (figure 1) exhibits major peaks which are practically unaffected by the background of the chromatography column, which are linked to the following ions : M, m/e 143 (molecular ion), A m/e 112 ($M-CH_2OH$), A' m/e 113 ($M-CH_2O$), B m/e 85 (A-HCN). This fragmentation scheme has already been reported by Hess and co-workers (12). The spectrum of HET



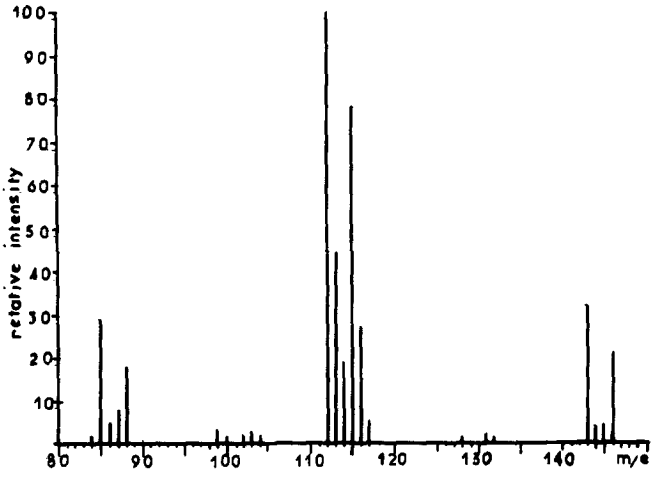


Figure 2. Mass spectrum of HET extracted from cells incubated in the presence of 1-deoxy-D-*threo*-|1-$^{2}H_3$|2-pentulose 1.

biosynthesized in the presence of the trideuterated 1-deoxy-D-*threo*-pentulose (1) showed intense peaks at m/e 146, 115, 116 and 88 (fig. 2). From numeric data the number of deuterium atoms in M, A, A' and B peaks were calculated. In these calculations, an identical isotopic composition of ions A and A' was assumed, as presumably the extra hydrogen atom in A' is the labile one of the hydroxy group. As recorded in table 1, the same heavy incorporation of deuterium was found in M and A, A' : more than 35% of these ions were trideuterated. Ion B was expected to have the same isotopic composition as A, since the hydrogen atom at C-2 is labile in thiamine. The observed, slight difference reflects the higher contribution from the background noise

Table 1. Incorporation of 1-deoxy-D-*threo*-|1-$^{2}H_3$| 2-pentulose into HET.

Number of ^{2}H	Percentage of total in ions		
	M (m/e 143)	A (m/e 112)	B (m/e 85)
0	54	53	51
1	4	4	7
2	5	5	11
3	37	38	31
4	0	0	0

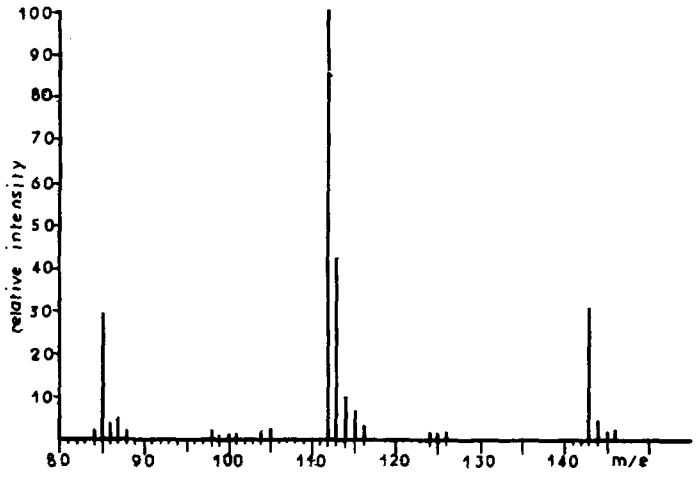


Figure 3. Mass spectrum of HET extracted from cells incubated in the presence of 1-deoxy-D-*erythro*-[1-$^2H_3$]2-pentulose 2.

to peaks, m/e 85 to 88. Only a much smaller incorporation was found from the labelled 1-deoxy-D-*erythro*-pentulose 2 (figure 3 and table 2).

Discussion

Although these mass spectrometric experiments do not indicate in a rigorous way the site of incorporation of deuterium, it can be assumed that it is located on carbons 6 or 8 of HET from the fact that ions M and A have the same isotopic composition. Moreover, less than 5% of HET was found to have incorporated only one or two deuterium atoms. Presumably, the three deuterium atoms of the trideuteromethyl group of the *threo* pentulose 1 are incorporated together in biosynthetic HET.

Table 2. Incorporation of 1-deoxy-D-*erythro*-[1-$^2H_3$] 2-pentulose into HET.

Number of ^2H	Percentage of total in ions		
	M (m/e 143)	A (m/e 112)	B (m/e 85)
0	93	91	83
1	2.5	3.5	7
2	0	1.5	8
3	4.5	4	2
4	0	0	0

Assays of thiamine indicated that 14% of the thiamine extracted in these experiments were already present in the cells before the incubation with labelled 1. Therefore, nearly 45% of HET synthesized in the presence of glucose and methyl labelled 1-deoxy-D-*threo*-pentulose originate from this last compound. It is well known that glucose is used more readily, even at weaker concentration, than any other exogene metabolite to supply the broad metabolic intermediates. Therefore it is unlikely that the label in pentulose 1 would be incorporated so heavily if the molecule had to be broken first to some glycolytic intermediate. The observed results are a proof that this compound 1 is a specific precursor of the carbon chain of HET.

The weak incorporation rate of the *erythro*-pentulose 2 can be explained in two ways : perhaps its partial epimerisation has occured chemically or biochemically; perhaps it can replace partially the *threo*-compound in a non fully stereospecific reaction.

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