The De-repression of Thiamine Biosynthesis by Adenosine

A TOOL FOR INVESTIGATING THIS BIOSYNTHETIC PATHWAY

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1. Growth of *Salmonella typhimurium* LT2 in the presence of adenosine was shown to cause enormous synthesis of thiamine in washed-cell suspensions. 2. Evidence that this was due to de-repression and not an accumulation of precursors was obtained by using a mutant blocked in the biosynthesis of the thiazole moiety, which showed a similarly large synthesis of the pyrimidine of thiamine. 3. The specific requirements for a source of energy, nitrogen and sulphur were investigated, and indicated new synthesis in this system.

Thiamine is formed in micro-organisms by the synthesis and condensation of a thiazole derivative (5- β -hydroxyethyl-4-methylthiazole) and a pyrimidine derivative (6-amino-5-hydroxymethyl-2-methylpyrimidine) (Schopfer & Jung, 1937; Robbins & Kavanagh, 1937; Nose, Ueda & Kawasaki, 1959; Suzuoki & Kobata, 1960; Camiener & Brown, 1960*a,b*; Nose, Ueda, Kawasaki, Iwashima & Fujita, 1961).

Although the reactions involved in the condensation of the two portions of the molecule have been fairly well characterized in yeast (Goodwin, 1963) and bacteria (Nose, Tokuda, Hirabayashi & Iwashima, 1964) little is known about the biosynthetic pathways of the pyrimidine and thiazole moieties. Important observations, however, are those by Pine & Guthrie (1959) that [^{14}C]formate is incorporated into the pyrimidine, and by Johnson, Howells & Goodwin (1966) that the methyl group and sulphur atom of methionine are incorporated as a unit into the thiazole. One of the main difficulties in studying these pathways is that unlike some other vitamins, e.g. riboflavin (Wilson & Pardee, 1962), thiamine is not formed in excess. Moreover, synthesis is so tightly controlled that in non-growing washed-cell suspensions negligible formation of thiamine can normally be found.

Adenosine in high concentrations (300 $\mu\text{g./ml.}$) in the culture medium inhibits the biosynthesis of the pyrimidine moiety in *Aerobacter aerogenes* (Moyed, 1964). In experiments with *Salmonella typhimurium* we have found that high concentrations of adenosine not only inhibit the formation of the pyrimidine but as a result of this cause de-repression of the biosynthesis of this compound. This phenomenon has provided a valuable tool for studying the biosynthesis of thiamine.

We performed the experiments described below in

order to understand this de-repression phenomenon before using it to elucidate the control mechanism of thiamine biosynthesis (Newell & Tucker, 1966).

MATERIALS

Organisms. *S. typhimurium* LT2 and the following derivatives of it were used: mutant T (a tryptophan auxotroph); mutant thi-1 (auxotrophic for the pyrimidine of thiamine); mutant thi-10 (auxotrophic for the thiazole of thiamine). Mutants thi-1 and thi-10 were kindly provided through Dr J. Childs from the collection of Dr M. Demerec.

The mutant thi-10/T (doubly auxotrophic for the thiazole of thiamine and tryptophan) was isolated by treatment of mutant thi-10 with the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 $\mu\text{g./ml.}$) by the method of Adelberg, Mandel & Chen (1965) and selection of an organism that required tryptophan in addition to the thiazole for growth.

Lactobacillus fermenti 36 (N.C.I.B. no. 6991; A.C.T.C. no. 9338) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

Chemicals. The pyrimidine moiety of thiamine and the 6-hydroxypyrimidine derivative were generous gifts from Dr Yasuo Abe and Dr S. Yurugi respectively, both of Takeda Chemical Industries, Osaka, Japan.

A sample of the thiazole moiety was kindly supplied by Professor B. D. Davis. The thiazole was also prepared in this Laboratory by bisulphite treatment of thiamine by the method of Williams, Waterman, Keresztesy & Buchman (1935).

Adenosine (chromatographically homogeneous) was obtained from British Drug Houses Ltd., Poole, Dorset.

DL-Tryptophan was the product of Mann Research Laboratories Inc., New York, N.Y., U.S.A.

Takadiastase and chloramphenicol B.P. were products of Parke, Davis and Co. Ltd., Hounslow, Middlesex.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Media. All strains of *S. typhimurium* were grown in

minimal medium containing glucose (0.2%) (Davis & Mingioli, 1950), enriched as indicated, in Erlenmeyer flasks on a rotary shaker in a 37° constant-temperature room.

L. fermenti 36 was grown in the medium of MaciasR (1957), which was prepared and autoclaved doubly concentrated with the glucose and xylose autoclaved separately. This medium gave greatly superior results to the medium of Sarett & Cheldelin (1944), which is generally used for this organism. The organism was kept as a stab culture in medium B1 (Nimmo-Smith, Lascelles & Woods, 1948), with Oxoid liver extract instead of fresh extracts. In addition 10 µg. of thiamine/ml. was added to prevent the organism from developing the ability to grow in the absence of added vitamin (Cheldelin, Bennett & Kornberg, 1946).

METHODS

Adenosine preincubation of mutant T and mutant thi-10/T. An overnight culture whose growth had been restricted by limiting concentration of glucose was diluted to 0.035 mg. dry wt./ml. in 200 ml. of minimal medium containing glucose (0.2%) and DL-tryptophan (100 µg./ml.). Then 300 µg. of adenosine/ml. was added and the culture aerated on a rotary shaker in a 1 l. flask for precisely 2.5 hr. at 37°. Cells were then chilled rapidly by swirling the flasks in icy water and washed suspensions prepared immediately. (Cells appear to lose their ability to synthesize the pyrimidine moiety within 2–3 hr.)

Washed-cell suspensions. These were prepared by centrifuging and resuspending the adenosine-preincubated cells twice in water at 2°. After centrifuging once more the cells were added to flasks containing minimal medium without added tryptophan. The flasks were then incubated with shaking at 37°. During this time samples were removed to measure the thiamine or the pyrimidine produced, and to measure E_{420} , the latter to determine the weight of organisms in suspension by using a predetermined relationship.

Thiamine assay. This was a microbiological assay with *L. fermenti* 36. This organism was used since it gave no response in 20 hr. incubation to the pyrimidine and thiazole moieties even at 100 times the normal assay concentration.

The inoculum for the assay was an 18 hr. culture in medium B2 (Nimmo-Smith *et al.* 1948) with Oxoid liver extract instead of fresh extracts. The organisms were washed three times with sterile water and 0.1 ml. of 1:20 dilution was used to inoculate each assay tube. The assay was performed in stagnant cultures of 6.5 ml. total volume in upright sterile plugged tubes (16 mm. × 150 mm.).

A standard curve was performed with each experiment with thiamine concentrations between 2 and 30 µg./assay tube. Growth of the organism was measured after 20 hr. incubation by using an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral-density filter.

The cells of *S. typhimurium* LT2 were found to contain virtually all the thiamine in the culture and unless otherwise stated the culture supernatant was not assayed.

Thiamine was extracted from the cells by boiling for 5 min. in 0.05 M-sodium acetate buffer adjusted to pH 5.0 with acetic acid. This was followed by incubation with Takadiastase (1 mg./ml.) at 45° for 4 hr. to convert any thiamine pyrophosphate into thiamine (Kinnersley & Peters, 1938). The latter treatment is necessary since the pyrophosphate has about 30% more activity than an

equimolar concentration of thiamine (Sarett & Cheldelin, 1944).

Pyrimidine assay. This was performed with the *S. typhimurium* LT2 mutant thi-1 that required the pyrimidine of thiamine or thiamine itself for growth. The specificity for pyrimidines was checked by using uracil, cytosine and 6-hydroxy-5-hydroxymethyl-2-methylpyrimidine (the 6-hydroxy derivative of thiamine pyrimidine), all of which had no activity even at 1000 times the normal assay concentration.

The cell extraction procedure was exactly as for thiamine except that the culture supernatant was retained for assay, as the pyrimidine is not all contained inside the cell.

The organisms were grown in 6.5 ml. of minimal medium including assay material in plugged sterile tubes (16 mm. × 150 mm.), tilted and shaken on a reciprocating shaker at 37° for 16 hr. The density of growth was measured in an EEL photoelectric colorimeter with a neutral-density filter.

Since mutant thi-1 grows with either the pyrimidine or thiamine a correction must be made for the thiamine content of the material that is being assayed for the pyrimidine. Standard curves for the pyrimidine and for the thiamine were run with mutant thi-1 so that the response due to thiamine (measured independently with the *L. fermenti* assay) could be subtracted from that given by a mixture of the two substances.

RESULTS AND DISCUSSION

Effect of preincubation with adenosine on the biosynthesis of thiamine in washed cells. Growing cultures of *S. typhimurium* LT2 and the tryptophan-requiring mutant T normally contain 40 µg. of thiamine/mg. dry wt. of cells. When washed suspensions of mutant T were incubated in minimal medium, in the absence of tryptophan to prevent further growth, there was no increase in the thiamine content of the cells and no excretion of the vitamin into the medium. When, however, the washed suspensions were prepared from bacteria grown in the presence of 300 µg. of adenosine/ml. for 2.5 hr. the organisms produced 4–5 times their normal content of thiamine in 1.5 hr. (Fig. 1). This was all found in the cells and none was detected in the medium. The conditions for adenosine preincubation are critical since too short an incubation decreases the synthesis of thiamine and with too long an incubation the adenosine is converted into hypoxanthine, via inosine, both of which are inactive.

When growth was restricted during preincubation by depriving the mutant T of tryptophan or by adding chloramphenicol (100 µg./ml.) no effect of adenosine was observed (Fig. 1). This clearly indicates the need for growth or protein synthesis with adenosine for the latter to have any effect.

Effect of incubation with adenosine on the cell content of thiamine. The cell content of thiamine was followed during the adenosine preincubation (Fig. 2). Cultures prepared for preincubation as

Fig. 1. Synthesis of thiamine by non-growing washed-cell suspensions of mutant T that had been preincubated for 2.5 hr. in minimal growth medium with tryptophan and the following supplements: ▲, no supplements (control); ○, adenosine (300 $\mu\text{g./ml.}$); ●, adenosine (300 $\mu\text{g./ml.}$) + chloramphenicol (100 $\mu\text{g./ml.}$); △, adenosine (300 $\mu\text{g./ml.}$), but with tryptophan omitted.

Fig. 2. Thiamine concentration in mutant T during preincubation: ○, with adenosine (300 $\mu\text{g./ml.}$); ●, without adenosine.

described in the Methods section consisted of organisms deprived of glucose, and generally contained less thiamine than growing cells. Moreover, the initial fall in thiamine content when growth was restarted was very characteristic of such cells. In the control flask, incubated without adenosine, thiamine synthesis started abruptly after this fall and rose to the normal level as shown. The flask with adenosine showed no rise until about 2.5 hr. (harvesting time), when adenosine is nearly all converted into inactive hypoxanthine.

Mechanism of adenosine action. Since adenosine inhibits the biosynthesis of the pyrimidine moiety of thiamine (Moyed, 1964) it would be expected that its action as shown above occurred through some effect on the pyrimidine pathway. It could either cause the accumulation of some precursor of the pyrimidine that was subsequently converted into thiamine, or it could de-repress the controlling enzymes on this pathway. The following evidence strongly favours de-repression.

Preincubation with adenosine depletes organisms of thiamine. If the action of adenosine in causing large amounts of thiamine to be synthesized in washed-cell suspensions were solely caused by growth in the presence of lowered concentrations of thiamine, then a similar phenomenon should also occur if a mutant blocked in the formation of the thiazole portion (and which cannot therefore form thiamine) was depleted of thiamine by growth in the absence of adenosine. Such a mutant, thi-10/T (a thiazole-tryptophan double auxotroph), was grown for 2.5 hr. without added thiamine or adenosine and then washed-cell suspensions were made as before. During preincubation mutant thi-10/T produced the pyrimidine moiety and this was excreted into the medium. Since no thiazole was formed no thiamine was made and the cellular concentration of thiamine fell, just as it did with adenosine preincubation of mutant T. Washed-cell suspensions now produced large amounts of the pyrimidine in similar molar proportion to the thiamine produced by mutant T. Since mutant thi-10/T had no block in formation of the pyrimidine as mutant T cells had in the presence of adenosine and yet formed large amounts of thiamine pyrimidine in washed-cell suspensions, any explanation of the effect of adenosine by assuming an accumulation of pyrimidine precursors is rendered very unlikely. Moreover, in view of the enormous synthesis found both after adenosine preincubation of mutant T and after thiamine depletion of mutant thi-10/T washed cells, which is far greater than the total thiamine synthesized in the control flask (Figs. 1 and 2), and in view of the inability of thiamine to inhibit its own synthesis in washed-cell suspensions, it is concluded that lowering the thiamine concentration below normal causes de-repression of the thiamine biosynthetic pathway.

Requirements for thiamine biosynthesis by washed-cell suspensions. To demonstrate further that new synthesis of thiamine must be occurring, experiments designed to show the nutritional requirements for thiamine synthesis were undertaken. De-repressed cultures of mutant T were prepared by preincubation with adenosine and washed-cell suspensions were incubated in minimal medium lacking various components.

When glucose was omitted no synthesis of thia-

Fig. 3. Synthesis of thiamine in the absence of glucose by non-growing washed-cell suspensions of mutant T previously incubated with adenosine (300 µg./ml.). The following were added to the non-growing washed-cell suspensions: Δ , no addition; $\blacksquare$, thiazole moiety (15 mµg./ml.); $\blacktriangle$, pyrimidine moiety (15 mµg./ml.); $\circ$, pyrimidine moiety + thiazole moiety (each 15 mµg./ml.); $\bullet$, control with glucose (0.2%, w/v).

mine occurred (Fig. 3). Addition of the thiazole moiety to the incubation mixture had negligible effect and the pyrimidine moiety only partially overcame the lack of glucose. Addition of both components together gave initially rapid synthesis, which later slowed down, probably owing to the exhaustion of endogenous energy supplies. It was further noted that vigorous aeration is essential for fast synthesis even with glucose present.

When nitrogen-containing compounds were omitted from the medium in which the washed cells were suspended no synthesis of thiamine occurred (Fig. 4). Neither of the moieties (which both contain nitrogen) restored synthesis on its own, but both together gave excellent synthesis, the quantity being governed by the amounts of the two moieties added. None of the amino acids tested as substitutes for the pyrimidine was more effective than ammonium salts (per mole of nitrogen) except aspartate, which was always at least as active and sometimes more active than ammonium sulphate. Glutamate gave slow synthesis, and all other amino

Fig. 4. Synthesis of thiamine in the absence of nitrogen-containing salts by non-growing washed-cell suspensions of mutant T previously incubated with adenosine (300 µg./ml.). The following were added to the non-growing washed-cell suspensions: $\blacktriangle$, no addition; $\blacktriangle$, thiazole moiety (15 mµg./ml.) (points coincident with 'no addition'); Δ , pyrimidine moiety (15 mµg./ml.); $\circ$, pyrimidine moiety + thiazole moiety (each 15 mµg./ml.); $\bullet$, control with ammonium sulphate (1 mg./ml.).

Fig. 5. Synthesis of thiamine in the absence of nitrogen-containing salts by non-growing washed-cell suspensions of mutant T previously incubated with adenosine (300 µg./ml.). The following were added to the non-growing washed-cell suspensions: $\blacktriangle$, no addition; Δ , glycine (0.1 mM); $\blacksquare$, glutamate (0.1 mM); $\circ$, aspartate (0.1 mM); $\bullet$, control with ammonium sulphate (0.05 mM).

Fig. 6. Synthesis of thiamine in the absence of sulphur-containing salts by non-growing washed-cell suspensions of mutant T previously incubated with adenosine (300 µg./ml.). The following were added to the non-growing washed-cell suspensions: ▲, no addition; ■, pyrimidine moiety (15 mµg./ml.); Δ, thiazole moiety (15 mµg./ml.); ○, pyrimidine moiety + thiazole moiety (each 15 mµg./ml.); ●, control with ammonium sulphate (1 mg./ml.).

acids were almost inactive (Fig. 5). The significance of aspartate's acting as a good nitrogen source for the pyrimidine is hard to assess from such an experiment since it might just equilibrate rapidly with the transaminase system or enter the cell more easily than other amino acids. On the other hand, it might indicate a role of aspartate in the synthesis of the pyrimidine of thiamine similar to its role in nucleic acid pyrimidine biosynthesis.

Sulphur compounds were also left out of the medium to determine the requirement for this element (Fig. 6). To deplete the organisms of sulphur compounds before washing, the last 0.5 hr. of preincubation was also in sulphur-free medium, a similar depletion procedure being carried out with the control culture. The results were unexpected. The thiazole alone did not overcome this lack of sulphur although the thiazole plus the pyrimidine fully overcame it. This implies that sulphur compounds are required for the synthesis of the pyrimidine as well as for making the sulphur-containing

thiazole, which is noteworthy in view of the methyl group at C-2 of the pyrimidine, which might be donated by methionine.

Adenosine preincubation thus provides a mechanism for freeing thiamine biosynthesis of its limiting control mechanism, thereby allowing rapid new synthesis in non-growing cells. This system has proved extremely useful in the study of the control mechanism of thiamine biosynthesis (Newell & Tucker, 1966) and it should also be invaluable when radioactive tracer studies are undertaken to determine the biosynthesis of thiamine.

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