Precursors of the Pyrimidine Moiety of Thiamine

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1. A method was devised for obtaining the pyrimidine moiety of thiamine in a pure form after its excretion into the medium by de-repressed washed-cell suspensions of mutants of *Salmonella typhimurium* LT 2. 2. By using amino acid-requiring mutants, this excretion of pyrimidine moiety was shown to be dependent on the presence of both methionine and glycine. 3. In the presence of either [*Me*-¹⁴C]-methionine or [*G*-¹⁴C]methionine, methionine-requiring mutants did not incorporate radioactivity into the pyrimidine moiety. 4. In contrast, both [*1*-¹⁴C]-glycine and [*2*-¹⁴C]glycine were incorporated into the pyrimidine moiety excreted by glycine-requiring mutants, and this occurred with little or no dilution of specific radioactivity. 5. The possible requirement for methionine as a cofactor and the significance of the incorporation of both carbon atoms of glycine are discussed.

The main difficulties encountered in studying the biosynthesis of vitamins arise because these substances are normally formed in extremely small amounts and, with the exception of riboflavine (Wilson & Pardee, 1962), are not excreted in excess into the growth medium. This necessitates the use of large amounts of radioactive material when possible radioactive precursors are being investigated, and the often difficult separation of the small quantity of the vitamin from the large excess of other compounds present in the cells. The study of the biosynthesis of the pyrimidine moiety of thiamine is normally further hampered because the usual method of cleavage of the thiamine after its isolation (Williams, Waterman, Keresztesy & Buchman, 1935) produces the sulphonated derivative of the pyrimidine, which is inactive in microbiological assays.

During investigations into the control mechanism of thiamine biosynthesis in *Salmonella typhimurium* LT 2 (Newell & Tucker, 1966*b*) it was found that the biosynthesis of the pyrimidine moiety (I) of thiamine could be de-repressed by growth of the bacteria under conditions that lowered the cellular thiamine concentration below 40 µg./mg. dry wt. Non-growing washed-cell suspensions prepared from such de-repressed cells synthesized the microbiologically active pyrimidine moiety in excess (50–100 µg./mg. dry wt.) and excreted it into the medium. This phenomenon therefore provided a valuable tool for studying the biosynthesis of the pyrimidine moiety.

Previous investigations on the biosynthesis of the pyrimidine moiety have shown that the best radioactive precursor is [¹⁴C]formate. This was demonstrated with *Bacillus subtilis* (Pine & Guthrie, 1959), with yeast (David & Estramareix, 1960, 1961; Johnson, Howells & Goodwin, 1966) and with *Escherichia coli* (Goldstein & Brown, 1963). David, Estramareix & Hirshfeld (1966) showed (by degradation of the labelled pyrimidine formed) that the formate was incorporated only into C-4 of the pyrimidine (see formula I for numbering of the ring atoms). This is by far the most important of the observations made.

Goldstein & Brown (1963) also found that [²-¹⁴C]-glycine and [²-¹⁴C]acetate were incorporated into the pyrimidine moiety in *E. coli*. The specific radioactivity of the glycine, however, was diluted 25-fold, which suggests either that radioactivity was incorporated directly or that it was incorporated via the folate-linked C₁ unit pool, since, like formate, [²-¹⁴C]glycine is a precursor of such C₁ units (Nakada, Friedman & Weinhouse, 1955). The specific radioactivity of the acetate was diluted tenfold, which made acetate a more likely direct precursor; this observation was extended by

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Tomlinson (1966), who reported that [2-¹⁴C]acetate was incorporated mainly into the methyl group on C-2 of the pyrimidine moiety. The actual incorporation of radioactivity, however, was very low in these experiments and further evidence is required to establish unequivocally that [2-¹⁴C]acetate is a direct precursor of the C-2 methyl group of the pyrimidine moiety.

It appears unlikely that the pyrimidine moiety of thiamine is formed by the conventional nucleic acid pyrimidine biosynthetic pathway, since neither [¹⁴C]aspartate nor [¹⁴C]orotic acid, which are precursors of nucleic acid pyrimidines, are incorporated into the pyrimidine moiety of thiamine (Goldstein & Brown, 1963; Johnson *et al.* 1966). The fact that [¹⁴C]formate is incorporated into C-4 of the pyrimidine moiety (David *et al.* 1966) is not explicable on the conventional nucleic acid pyrimidine pathway, where C-4 arises from aspartate. It therefore seems that an entirely different pathway exists for the biosynthesis of the pyrimidine moiety of thiamine, and evidence for this is presented below.

MATERIALS

Chemicals. The pyrimidine moiety of thiamine was a generous gift from Dr Yasuo Abe of Takeda Chemical Industries, Osaka, Japan.

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

All radioactive compounds except L-[G-¹⁴C]methionine were the products of The Radiochemical Centre, Amersham, Bucks. L-[G-¹⁴C]Methionine was a product of Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

The DL-forms of valine, tryptophan, threonine, leucine

and isoleucine were from Mann Research Laboratories Inc., New York, N.Y., U.S.A. The DL-forms of phenylalanine, histidine, aspartic acid and asparagine were from Roche Products Ltd., Welwyn Garden City, Herts. DL-Ornithine was from Koch-Light Laboratories Ltd., and DL-lysine was a product of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

All other chemicals were from British Drug Houses Ltd., Poole, Dorset, and were analytical-reagent grade where possible.

Organisms. All organisms were derived from *Salmonella typhimurium* LT2. Mutants thi-1, thi-10 and thi-28 were kindly provided through Dr J. Childs from the collection of Dr M. Demerec. All other mutants were produced by mutation of these or of the wild-type of *S. typhimurium* LT2 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg, Mandel & Chen (1965). A list of the mutants used is shown in Table 1.

Minimal medium. The minimal medium of Davis & Mingioli (1950) containing glucose (0.2%) was used unless otherwise stated.

METHODS

Microbiological assay methods

The microbiological assay for the pyrimidine moiety of thiamine with mutant thi-1 and the assay for thiamine with *Lactobacillus fermenti* 36 were as described previously (Newell & Tucker, 1966a).

De-repression and preparation of washed-cell suspensions of amino acid auxotrophs of mutant thi-28/T

The original method of de-repressing thiamine biosynthesis used the principle of depleting cultures of thiamine, by allowing them to grow in the presence of high

Table 1. *S. typhimurium* LT2 mutants and their growth requirements

Mutant	Growth requirements
T	Tryptophan
T.M	Tryptophan + methionine
thi-1	Pyrimidine moiety of thiamine
thi-10	Thiazole moiety of thiamine
thi-28	Thiamine
thi-28/T	Thiamine + tryptophan
thi-28/T.M	Thiamine + tryptophan + methionine
thi-28/T.Gly	Thiamine + tryptophan + glycine or serine
thi-28/T.Thr	Thiamine + tryptophan + threonine
thi-28/T.Val + Ile	Thiamine + tryptophan + valine + isoleucine
thi-28/T.Pro	Thiamine + tryptophan + proline
thi-28/T.Glu	Thiamine + tryptophan + glutamate
thi-28/T.GluNH ₂	Thiamine + tryptophan + glutamine
thi-28/T.Asp	Thiamine + tryptophan + aspartate
thi-28/T.Lys	Thiamine + tryptophan + lysine
thi-28/T.Leu	Thiamine + tryptophan + leucine
thi-28/T.Tyr	Thiamine + tryptophan + tyrosine
thi-28/T.Phe + Tyr	Thiamine + tryptophan + phenylalanine + tyrosine
thi-28/T.His	Thiamine + tryptophan + histidine
thi-28/T.Orn	Thiamine + tryptophan + ornithine
thi-28/T.Arg	Thiamine + tryptophan + arginine
thi-28/T.U	Thiamine + tryptophan + uracil

concentrations of adenosine (which inhibited thiamine biosynthesis) followed by removal of the adenosine by washing (Newell & Tucker, 1966a). However, de-repression of thiamine mutants that were blocked either in the formation of the thiazole moiety (e.g. mutant thi-10) or in the reactions joining the pyrimidine moiety to the thiazole (e.g. mutant thi-28) could also be achieved by simply allowing them to grow and deplete themselves of thiamine, and this did not require the addition of any adenosine. Because of their mutational block such de-repressed cells could not form thiamine, but instead formed large quantities of the pyrimidine moiety and this was excreted into the medium. This principle was used for the de-repression of amino acid auxotrophic derivatives of mutant thi-28/T. The mutants were also blocked in tryptophan biosynthesis so that their growth could be stopped at will by deprivation of tryptophan.

The following medium in a sterile 16mm. × 150mm. plugged tube was inoculated with the particular amino acid mutant and incubated overnight at 37° on a reciprocating shaker: minimal medium containing glucose (0.02%), 5ml.; DL-tryptophan, 200μg.; thiamine, 50mμg.; amino acid required, 1mg. The culture stopped growing at a low cell density owing to the small quantity of glucose supplied. In the morning 1.5ml. of 2% (w/v) glucose was added to the tube and the culture incubated for a further 2–3hr. until it contained about 0.6–0.7mg. dry wt. of bacteria/ml. The cells were then separated and washed twice in water at 2° by centrifugation, and resuspended in minimal medium containing glucose (0.2%) either with or without the specific amino acid requirement of the mutant, but without tryptophan. The culture was incubated with shaking at 37° for 1½hr., centrifuged to separate the cells and the supernatant was assayed for pyrimidine with the pyrimidine-requiring mutant thi-1.

Biosynthesis and purification of the pyrimidine moiety of thiamine for testing possible radioactive precursors

The method described above for de-repressing mutant thi-28/T and derivatives was used, so that the bacteria excreted pyrimidine under non-growing conditions.

De-repression. The pyrimidine-excreting organisms were inoculated into 11. flasks each with 180ml. of minimal medium containing glucose (0.015%) plus the following supplements (per flask): 40mg. of DL-tryptophan, 1.5μg. of thiamine and either 5mg. of L-methionine or 10mg. of glycine (depending on the particular mutant used). Two such flasks were used per experiment with two additional control flasks to which 20μg. of thiamine was added/flask to repress the formation of the pyrimidine of thiamine.

After inoculation the flasks were incubated on a rotary shaker at 37° overnight. Owing to the low concentration of glucose in the medium the cultures ceased growing at about 0.2mg. dry wt./ml. In the morning 60ml. of 2% (w/v) glucose solution was added to each flask and the organisms were allowed to grow for a further 2–2½hr. so that they reached 0.6–0.7mg. dry wt./ml. At this stage of growth the organisms from the flasks with 20μg. of thiamine should have been repressed and those with 1.5μg. de-repressed. The cultures were then centrifuged and the bacteria washed twice with water at 2°. The repressed and de-repressed bacteria were each resuspended in 200ml. of minimal

medium containing glucose (0.6%) so that the cultures both contained 0.8mg. dry wt. of bacteria/ml. The radioactive compound was then added and the cultures were shaken in 11. flasks at 37°. Maximum synthesis of pyrimidine moiety occurred with a total of 1mg. of methionine or with 10mg. of glycine/flask. After 1½hr. incubation the cultures were centrifuged and the separated supernatants retained.

Concentration and partial purification of the pyrimidine moiety present in the supernatant. The supernatant from each flask was concentrated *in vacuo* to 20ml. in a rotary evaporator at a temperature below 70°. About 3ml. of N-KOH was then added to bring the pH to 8–9 and the alkaline solution was extracted eight times with 20ml. portions of 3-methylbutan-1-ol. The combined extracts were concentrated *in vacuo* to 1–1.5ml. at a temperature below 80°. The precipitated salt was removed by centrifugation at –10° and the solution extracted three times with 5ml. of 1M-NHCl. The combined extracts were evaporated to a final volume of 0.1ml. and any precipitate formed was removed by centrifugation.

Radioautography. Radioactive pyrimidine in the concentrated extracts as prepared above was detected as follows: extract (about 0.01 ml.) was spotted on to a thin-layer chromatography plate coated with a 250μ-thick layer of MN cellulose powder 300 (Macherey, Nagel and Co., Düren, Germany) and the plate developed in butan-1-ol-acetic acid-water (63:10:27, by vol.; Mandelstam, 1958) for 3hr., dried and developed in the second dimension in methanol-pentan-1-ol-benzene-aq. ammonia (sp.gr. 0.88)-water (70:35:70:8:17, by vol.) for 2.5hr. The plates were dried and sheets of Kodak Royal Blue X-ray film placed firmly in contact with the surface; after about 3 weeks in the dark the X-ray films were developed with Kodak DX-80 developer.

Determination of the dilution of specific activity of [1-¹⁴C]- and [2-¹⁴C]-glycine during incorporation into the pyrimidine moiety

The specific radioactivity of the glycine supplied in the growth medium at the beginning of the experiment was determined from the radioactivity present and the amount of unlabelled and labelled glycine added. The radioactivity was measured from the counts recorded by an end-window counter from 20μl. of the uninoculated medium on a thin-layer chromatogram. The specific radioactivity recorded with different amounts of medium varied only slightly, but errors caused by absorption of radioactivity by the plate were decreased to a minimum by allowing the sample (20μl.) to spread to approximately the same area on the plate as the area of the radioactive pyrimidine counted.

To ascertain the specific radioactivity of the pyrimidine, the pyrimidine-containing spot on the two-dimensional chromatogram was first eluted with 1M-NHCl and re-chromatographed with 86% (v/v) butan-1-ol-ammonia (Lederer & Lederer, 1957) as solvent. The appropriate spot was counted with the end-window counter, then scraped off into a known volume of water for pyrimidine assay with mutant thi-1 and the plate re-counted. From the difference in counts on the plate before and after removal of the pyrimidine, the specific radioactivity of the pyrimidine moiety was calculated.

Table 2. *Pyrimidine moiety synthesized by de-repressed non-growing cultures of amino acid-requiring mutants of mutant thi-28/T in the absence of their individual amino acid requirements*

The amount of pyrimidine moiety formed was assayed by using the pyrimidine-requiring mutant thi-1.

Mutant	Amino acid supplement required by mutant	Pyrimidine moiety formed ($\mu\text{g./mg. dry wt.}$)	
		Without supplement	With supplement (50 $\mu\text{g./ml.}$)
thi-28/T.M	Methionine	0	50
thi-28/T.Gly	Glycine	1	26
thi-28/T.Val + Ile	Valine + isoleucine	38	40
thi-28/T.Pro	Proline	50	48
thi-28/T.Orn	Ornithine	63	63
thi-28/T.Arg	Arginine	54	60
thi-28/T.Asp	Aspartate	48	48
thi-28/T.Lys	Lysine	34	34
thi-28/T.Leu	Leucine	21	21
thi-28/T.Tyr	Tyrosine	68	72
thi-28/T.Phe + Tyr	Phenylalanine + tyrosine	54	68
thi-28/T.His	Histidine	18	15
thi-28/T.Thr	Threonine	19	19
thi-28/T.Glu	Glutamate	4	4
thi-28/T.GluNH ₂	Glutamine	3	4

RESULTS

Preliminary investigation of pyrimidine precursors with auxotrophic mutants

Preliminary studies with auxotrophic mutants of mutant thi-28/T were performed to demonstrate probable precursors that could then be studied by radioactive techniques.

The ability to induce cells to synthesize large amounts of pyrimidine under non-growing conditions allowed study of the effects of various auxotrophic mutations on pyrimidine synthesis without the complications of the effects of these mutations on growth. Amino acid auxotrophs were studied because amino acids seemed the most likely precursors of a molecule containing three nitrogen atoms. The mutants used and their effects on pyrimidine biosynthesis are shown in Table 2.

The results showed that starving cells of either methionine or glycine almost completely inhibited pyrimidine biosynthesis. All glycine mutants were in fact glycine-serine mutants, since they could use serine in place of glycine for growth. Serine only poorly overcame the mutants' requirements for pyrimidine biosynthesis as compared with glycine, and is therefore unlikely to be a normal intermediate. Moreover, one mutant was isolated that grew well on serine but very poorly on glycine. [This mutant presumably belonged to the group shown in *Escherichia coli* to have a decreased permeability to glycine (Simmonds & Miller, 1957).] With this

mutant, as with the others, glycine was considerably better than serine in sustaining pyrimidine biosynthesis and it is therefore concluded that glycine and not serine is the true requirement for pyrimidine biosynthesis. The other amino acid mutants (except those requiring glutamate and glutamine) showed no impairment of their ability to synthesize the pyrimidine in the absence of their amino acid requirements and it is concluded that these amino acids are not precursors of pyrimidine. The main weakness of this interpretation is the possibility that a 'leaky' mutant (i.e. a mutant whose altered enzyme is not completely inactive) could supply sufficient precursor for pyrimidine synthesis but not sufficient for growth. Because of this, only mutants that were not detectably 'leaky' (i.e. would not grow at all over prolonged incubation without their requirement) were used for this experiment.

The glutamate and glutamine auxotrophs showed only a small amount of synthesis of pyrimidine, but because this was not influenced by adding the amino acids the result was very difficult to interpret. Several cysteine-requiring mutants of mutant thi-28/T were isolated, but all proved difficult to use in this experiment as they grew slowly and often ceased growing before they were de-repressed.

A uracil mutant of mutant thi-28/T was also isolated and studied. It showed no impairment in its ability to synthesize the pyrimidine moiety of thiamine and this agrees with the results of

Goldstein & Brown (1963), who failed to show incorporation of [^{14}C]orotic acid into thiamine in *E. coli*.

It was concluded that only glycine and methionine merited further investigation as possible precursors of the pyrimidine moiety of thiamine.

Role of methionine in pyrimidine-moiety biosynthesis

The finding of a requirement for methionine in the biosynthesis of the pyrimidine moiety was not unexpected because previous work (Newell & Tucker, 1966a) had shown that a sulphur-containing compound was required for pyrimidine-moiety biosynthesis. The requirement for methionine was confirmed by means of the methionine antagonist ethionine (Fig. 1). Non-growing washed cells of *S. typhimurium* mutant T (a tryptophan auxotroph) previously de-repressed by the method of pre-incubation with adenosine (see Newell & Tucker, 1966a) were used. Ethionine (2.5 mg./ml.) produced about 50% inhibition, which was completely removed by the addition of methionine (1 mg./ml.). Excess of thiazole (100 $\mu\text{g.}/\text{ml.}$) was included in the incubation mixture to overcome the requirement for sulphur by thiazole-moiety biosynthesis. The observed effect of ethionine must therefore have been on biosynthesis of the pyrimidine moiety, confirming that this biosynthesis requires methionine. The thiamine-assay organism, *L. fermenti* 36, was shown to be unaffected by the ethionine present in the extracts being assayed.

Quantitative studies with mutant T.M and mutant thi-28/T.M. Mutant T was mutated and a methionine auxotroph isolated (mutant T.M). This was presumably blocked between homocysteine and methionine since it grew well on methionine but not at all on homocysteine. Non-growing washed cells of mutant T.M that had been de-repressed by pre-incubation with adenosine synthesized thiamine only in the presence of added methionine, and the amount of thiamine synthesized was proportional to the amount of methionine added. Again excess of thiazole (100 $\mu\text{g.}/\text{ml.}$) was added to the incubation mixture to overcome the need for methionine by thiazole-moiety biosynthesis. A more direct demonstration of the dependence on methionine was shown by pyrimidine biosynthesis in mutant thi-28/T.M. Since this mutant formed and excreted the pyrimidine moiety there was no need to add excess of thiazole. The linear relationship between the methionine supplied and the pyrimidine excreted is shown in Fig. 2. The amount of methionine required, however, was about 70 times as great as the amount of pyrimidine synthesized. Precursors of methionine, including succinate, failed to replace the methionine requirement. Homocysteine, the immediate precursor of methionine, was inactive in

Fig. 1. Inhibition by ethionine of thiamine formation in de-repressed washed-cell suspensions of mutant T in the presence of excess of thiazole, and the antagonism of this inhibition by added methionine. ●, Without additions (control); Δ, plus ethionine (2.5 mg./ml.); ○, plus ethionine (2.5 mg./ml.) and methionine (1 mg./ml.).

Fig. 2. Relationship between the amount of pyrimidine moiety formed by the methionine-requiring mutant thi-28/T.M and the amount of methionine added to the culture medium. The cultures were non-growing washed-cell suspensions that had been previously de-repressed as described in the Methods section.

restoring thiamine synthesis in mutant T.M, which cannot convert homocysteine into methionine.

Synthesis of pyrimidine moiety in the presence of [Me- ^{14}C]methionine. Although Goldstein & Brown (1963) failed to observe incorporation of [Me- ^{14}C]methionine into the pyrimidine moiety in *E. coli*,

Johnson *et al.* (1966) showed that some incorporation did occur in yeast. Therefore further investigation into this matter seemed necessary.

The methyl group of methionine was tested as a possible pyrimidine precursor by allowing both repressed and de-repressed cultures of the methionine-requiring mutant thi-28/T.M to synthesize and excrete the pyrimidine moiety in the presence of [*Me*-¹⁴C]methionine (see the Methods section). The pattern of spots on the radioautographs of the supernatants from the repressed and de-repressed cultures should have differed solely in the presence of pyrimidine (if this was labelled) on the radioautograph from the de-repressed culture.

The results obtained showed no difference at all between the patterns of the spots on the two plates, and there was no radioactive spot corresponding to the pyrimidine on either plate. It was calculated, from the amount of [*Me*-¹⁴C]methionine added (15 μ C/flask) and the amount of pyrimidine formed, that there should have been 1400 counts/100 sec. above background on the pyrimidine spot of the chromatogram of the de-repressed culture if 1 mole of methionine was incorporated/mole of pyrimidine; about 13 counts/100 sec. above background were obtained showing that if incorporation had occurred it was less than 1%. Clearly the methyl group of methionine cannot be incorporated into the pyrimidine moiety of thiamine.

Synthesis of pyrimidine moiety in the presence of [G-¹⁴C]methionine. As the methyl group of methionine was not itself incorporated, the remainder of the molecule (excluding the sulphur atom) was tested for incorporation by using [G-¹⁴C]methionine. With the procedure described for the experiment with [*Me*-¹⁴C]methionine, no incorporation of [G-¹⁴C]methionine was observed. Since incorporation of only one atom of the molecule would have produced a dense black spot on the radioautograph, it is apparent that none of the carbon atoms of the methionine molecule are incorporated into the pyrimidine moiety of thiamine.

Utilization of carbon from [2-¹⁴C]glycine as a pyrimidine precursor

An experiment similar to those described with methionine was carried out to ascertain whether [2-¹⁴C]glycine was incorporated into the pyrimidine moiety, but instead of mutant thi-28/T.M the glycine mutant thi-28/T.Gly was used. Again a control flask with repressed cells was used. In contrast with the results with methionine, a spot was observed on the radioautograph from the de-repressed culture that was absent from the radioautograph from the repressed culture. This spot had the *R_F* of pyrimidine in both solvents used. The spot was eluted and further purified by

chromatography with 86% butan-1-ol-ammonia as solvent. Again the radioactivity ran with the same *R_F* as an authentic sample of the pyrimidine, showing that [2-¹⁴C]glycine had been incorporated into the pyrimidine moiety. The specific radioactivities of the isolated pyrimidine and of the added glycine counted under the same conditions were 5.0×10^7 counts/100 sec./m-mole and 4.4×10^7 counts/100 sec./m-mole respectively. Within experimental error the ratio of these specific radioactivities was unity, indicating that one of the atoms of the pyrimidine moiety was derived from the C-2 atom of glycine.

Utilization of carbon from [1-¹⁴C]glycine as a pyrimidine precursor

The incorporation of [1-¹⁴C]glycine into the pyrimidine moiety was then investigated by using the same technique as for the experiment with [2-¹⁴C]glycine. A black spot was observed on the radioautograph from the de-repressed culture that was not on that from the repressed culture. The specific radioactivities of the added [1-¹⁴C]glycine and the isolated pyrimidine were 4.9×10^7 counts/100 sec./m-mole and 3.9×10^7 counts/100 sec./m-mole respectively. Within experimental error the ratio of these specific radioactivities was unity, indicating that one of the atoms of the pyrimidine was derived from the C-1 atom of glycine.

DISCUSSION

Of the amino acids tested, the only ones found to be required for pyrimidine-moiety biosynthesis were methionine and glycine.

Whereas both the carbon atoms of glycine were incorporated into the pyrimidine moiety, none of the carbon atoms of methionine were incorporated, although methionine seemed to be an absolute requirement for pyrimidine-moiety biosynthesis. One explanation could be that it is incorporated into a pyrimidine precursor, but is later lost and so does not appear in the pyrimidine itself. Alternatively it could function as a cofactor, possibly in the form of *S*-adenosylmethionine. *S*-Adenosylmethionine is normally involved in transmethylation reactions, e.g. in the methylation of nucleic acids (Gold, Hurwitz & Anders, 1963) and in the methylation of homocysteine to form methionine (Woods, Foster & Guest, 1965). In such reactions the methyl group of the methionine moiety in *S*-adenosylmethionine is transferred to the compound being synthesized, but such could not be the case if *S*-adenosylmethionine were used in pyrimidine-moiety biosynthesis since radioactivity from [*Me*-¹⁴C]methionine was not incorporated into the pyrimidine. It has been shown by Knappe, Bohnert

& Brümmer (1965), however, that in *E. coli* the phosphoroclastic cleavage of pyruvate to acetyl phosphate and formate requires *S*-adenosylmethionine as cofactor. This is unlikely to involve transmethylation and probably represents another role of *S*-adenosylmethionine. Therefore *S*-adenosylmethionine may act as a cofactor in pyrimidine biosynthesis by a mechanism not involving the introduction of methyl groups into the molecule.

The most significant result from these studies was the finding that C-1 of glycine is incorporated into the pyrimidine moiety; C-2 of glycine is incorporated into many compounds, but incorporation of C-1 of glycine is unusual. It is presumably incorporated into serine in the glycine-serine mutants used in these experiments, but, apart from being incorporated into cysteine and tryptophan, serine has little use in biosynthetic pathways and it was thought unlikely to be an intermediate itself in pyrimidine biosynthesis. Even in porphyrin biosynthesis, which involves glycine, the C-1 from the glycine is lost before the formation of δ -amino-laevulinic acid (Radin, Rittenberg & Shemin, 1950). In the purine pathway, however, C-1 of glycine is important since it is incorporated with C-2 of glycine. The finding that both [1- 14 C]- and [2- 14 C]-glycine were also incorporated into the pyrimidine moiety suggested that the pathways of biosynthesis of the pyrimidine moiety of thiamine and biosynthesis of purines might share a common origin, and this possibility is supported by other data that closely relate these two pathways (see Newell & Tucker, 1968).

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