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Biosynthesis of the Pyrimidine Moiety of Thiamine

A NEW ROUTE OF PYRIMIDINE BIOSYNTHESIS INVOLVING PURINE INTERMEDIATES

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1. The pattern of distribution on the purine pathway of mutants of *Salmonella typhimurium* LT2 that had the double growth requirement for a purine plus the pyrimidine moiety of thiamine (ath mutants) indicated that purines and the pyrimidine moiety of thiamine share the early part of their biosynthetic pathways, and that 4-aminoimidazole ribonucleotide (AIR) is the last common intermediate. Two mutants that at first appeared anomalous were further investigated and found not to affect this deduction. 2. The ribonucleoside form of AIR (AIR_S) satisfied the requirements both for a purine and for the pyrimidine moiety of thiamine of an ath mutant. 3. Methionine was required for the conversion of AIR into the pyrimidine moiety. 4. Radioactive AIR_S was converted into radioactive pyrimidine moiety by an ath mutant without significant dilution of specific radioactivity. 5. Possible mechanisms for pyrimidine-moiety biosynthesis from AIR are discussed.

In the preceding paper (Newell & Tucker, 1968) it was shown that both carbon atoms of glycine were incorporated into the pyrimidine moiety of thiamine, and in this respect biosynthesis of the pyrimidine moiety resembled purine biosynthesis. There are also two other phenomena that are known to relate these biosynthetic pathways: the existence of single-site mutations of enteric bacteria that resulted in a double growth requirement for a purine and for the pyrimidine of thiamine (ath mutants) (Yura, 1956; J. S. Gots, unpublished work quoted by Demerec, 1956–57), and also the ability of high concentrations of the purine adenine (or adenosine) to inhibit the biosynthesis of the pyrimidine moiety of thiamine in enteric bacteria (Brook & Magasanik, 1954; Moyed, 1964). The reason for this relationship, however, remained a mystery (Magasanik, 1962; Moyed, 1964).

A possible explanation of these phenomena was that the early part of the purine biosynthetic pathway could have been common also to the biosynthesis of the pyrimidine moiety of thiamine. Although Yura (1956) described only the genetic aspects of his work with ath mutants, his results seemed to refute such an explanation. Yura (1956) found the position on the purine pathway of several groups of ath mutants and also simple purine requirers called Ad mutants (Scheme 1). If the pyrimidine moiety of thiamine was formed by a pathway common to the early part of the purine biosynthetic pathway, then all mutants up to the point at which the two pathways branched should have been ath mutants requiring a purine plus the pyrimidine moiety of thiamine, and all mutants on the purine pathway after the branch point should have been Ad mutants requiring only a purine. Yura (1956) found that all early mutants on the purine pathway were indeed ath mutants [an apparent exception, AdD–10, was shown to be an ath mutant by H. Ozeki (see Demerec, 1955–56)], but he also found that later on the pathway an Ad group (AdC) lay between two ath groups. This

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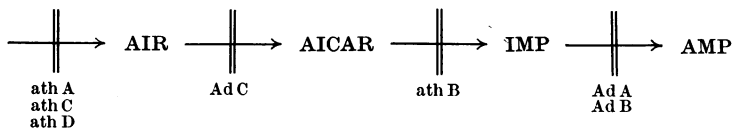


Scheme 1. Distribution on the purine pathway of genetically distinguishable groups of ath and Ad mutants shown by Yura (1956). The ath mutants required a purine plus the pyrimidine moiety of thiamine for growth, whereas Ad mutants required only a purine.

would seem to be incompatible with any hypothesis involving a simple branch in the purine pathway. Moreover, the early groups of ath mutants had their purine but not their pyrimidine moiety requirement satisfied by AICA,* implying that the two pathways could be common only as far as AICAR, yet this was contradicted by Yura's (1956) finding of an ath group (athB) that was blocked after AICAR formation.

To ascertain whether these difficulties could be resolved, further investigation into the distribution of ath and Ad mutants of *Salmonella typhimurium* LT2 on the purine pathway was undertaken, and this eventually led to proof of the hypothesis that purines and the pyrimidine moiety of thiamine share a part of their biosynthetic pathways.

MATERIALS

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

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

AICA was obtained from Calbiochem Ltd., London, W. 1.

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

Organisms. All organisms were derived from *S. 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 except thi–10/T.ath$_{38\beta}$ were produced by mutation of these or of the wild-type *S. typhimurium* LT2 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg, Mandel & Chen (1965). Mutant thi–10/T.ath$_{38\beta}$ was a spontaneous mutation from mutant thi–10/T.ath$_{38}$ to the ability to use AIR$_s$ in place of the pyrimidine moiety of thiamine for growth. A list of the mutants used is shown in Table 1.

* Abbreviations: AIR, 4-aminoimidazole ribonucleotide; AIR$_s$, 4-aminoimidazole ribonucleoside; AICA, 4-aminoimidazole-5-carboxamide; AICAR, 4-aminoimidazole-5-carboxamide ribonucleotide; C-AIR, 4-aminoimidazole-5-carboxylic acid ribonucleotide.

Media. All *S. typhimurium* strains were grown in the minimal medium of Davis & Mingioli (1950) containing glucose (0·2%) unless otherwise stated.

METHODS

Microbiological assay method. The microbiological assay for the pyrimidine moiety of thiamine with mutant thi–1 was as described previously (Newell & Tucker, 1966*a*).

Chromatography. Glass plates (200mm. × 200mm.) were used with a 250μ-thick coating of MN cellulose powder 300 (Macherey, Nagel and Co., Düren, Germany). All chromatography solvents were single-phase solvents simply requiring mixing of the components, and all were run in the ascending direction: (*a*) butanol-1-ol–acetic acid–water (63:10:27, by vol.) (Mandelstam, 1958); (*b*) methanol–pentan-1-ol–benzene–aq. ammonia (sp.gr. 0·88)–water (70:35:70:8:17, by vol.); (*c*) propan-1-ol–3-methylbutan-1-ol–water–isobutyric acid–aq. 28% (w/v) ammonia (72:25:75:120:2, by vol.) (Akagi & Kumaoka, 1963); (*d*) propan-1-ol–0·2N-ammonia (3:1, v/v) (Love & Gots, 1955); (*e*) propan-1-ol–water (3:1, v/v) (Rabinowitz, 1956).

Bioautography by thin-layer chromatography. The procedure of placing a chromatogram on the surface of solid inoculated agar as was used for bioautography with paper chromatograms (Newell & Tucker, 1966*b*) could not be used with glass-backed thin-layer chromatography plates, since it was difficult to obtain a perfectly flat upper surface to the agar when it was poured at just a few degrees above its solidifying temperature. The alternative of pouring melted inoculated agar on to the thin-layer chromatogram was also unsatisfactory, since it disturbed the position of the water-soluble active spots. The procedure devised for thin-layer chromatograms was to pour the agar layer on to a clean glass plate the same size as the chromatography plate, and then to transfer the solid agar to the chromatogram as a sheet. This sheet was flexible and its bottom side was perfectly flat owing to contact with the glass plate. With this procedure 200ml. of agar-containing medium was required/thin-layer plate (200mm. × 200mm.). The medium was minimal medium with agar (British Drug Houses Ltd.) (2%, w/v), glucose (0·4%) and triphenyltetrazolium chloride (0·015%); the glucose and triphenyltetrazolium chloride were autoclaved separately and added as sterile solutions to the melted agar. The hot sterile agar was cooled to just 50° in a water bath and then the bacteria (about 5mg. dry wt. in 2ml. of water) were warmed to 50° and

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

Mutant	Growth requirements
thi–1	Pyrimidine moiety of thiamine
thi–10	Thiazole moiety of thiamine
thi–10/T	Thiazole moiety + tryptophan
thi–10/T.Ad (26 mutants)	Thiazole moiety + tryptophan + adenine
thi–10/T.ath (34 mutants)	Thiazole moiety + tryptophan + adenine + pyrimidine moiety
thi–10/T.ath$_{38\beta}$	Thiazole moiety + tryptophan + adenine + pyrimidine moiety (replaceable by AIR$_s$)
thi–10/T.ath$_{38\beta}$.M	Thiazole moiety + tryptophan + adenine + pyrimidine moiety (or AIR$_s$) + methionine
thi–10/T.ath$_{38\beta}$.Gly	Thiazole moiety + tryptophan + adenine + pyrimidine moiety (or AIR$_s$) + glycine
thi–28	Thiamine
thi–28/T	Thiamine + tryptophan
thi–28/T.Gly	Thiamine + tryptophan + glycine
thi–28/T.Gly.Ad	Thiamine + tryptophan + glycine + adenine

inoculated into the agar. This was then rapidly poured on to a clean thin-layer glass plate. To prevent the agar from flowing off the edge of the plate a strip of Lassotape (T. J. Smith and Nephew Ltd., Welwyn Garden City, Herts.) was stuck round the edge. When the agar had set, removal of the tape allowed the agar sheet to be slid off the plate and transferred to the dried chromatography plate. Incubation at 37° for 16hr. then produced bioautographs with small red spots over the active spots on the chromatogram. This method thus allowed all the advantages of thin-layer chromatography to be incorporated into the technique of bioautography.

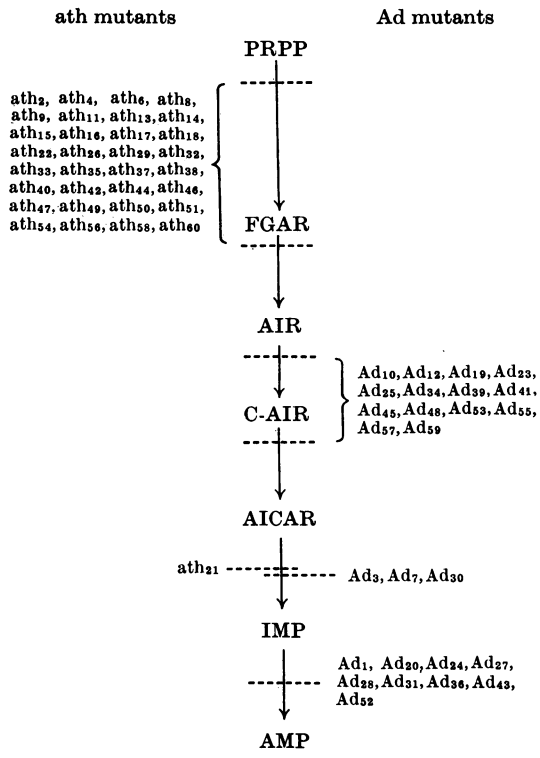
RESULTS

Distribution of ath and Ad mutants on the purine biosynthetic pathway

By using the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a mutant of *S. typhimurium* LT2 requiring the thiazole moiety of thiamine and tryptophan for growth (mutant thi–10/T) was further mutated and a total of 60 ath and Ad mutants were selected. The Ad mutants selected were those that in addition to the parental requirements needed adenine for growth, and ath mutants were those requiring adenine plus the pyrimidine moiety of thiamine for growth. To avoid selecting the same mutant more than once in an experiment, only one ath or Ad mutant was finally selected for any one mutational experiment. Mutant thi–10/T was chosen as parent because it possessed the useful ability to excrete the pyrimidine moiety of thiamine (Newell & Tucker, 1968). Mutant thi–28/T, which also had this ability, could not be used, since it required the whole thiamine molecule for growth and it would therefore have been impossible to distinguish easily between Ad and ath mutants.

The mutants obtained were first divided into categories based on the following criteria of growth abilities. (1) Their ability or inability to grow without added pyrimidine: this differentiated ath from Ad mutants. (2) Their ability to use hypoxanthine in place of adenine for growth: this differentiated mutants blocked before IMP from those blocked afterwards. (3) Their ability to use AICA in place of adenine: this distinguished mutants blocked before AICAR from those blocked afterwards.

The positions of their blocks on the purine pathway were then further studied by observing the excretions of purine intermediates when the mutants were grown on limiting adenine concentrations. The excretions were examined by the following tests. (1) The Bratton–Marshall test (Bratton & Marshall, 1939): this produced a bright purple colour with AICA having $\lambda_{max.}$ 540mμ; the earlier intermediate AIR_s or its free base aminoimidazole produced a vivid orange colour with



Scheme 2. Distribution on the purine biosynthetic pathway of ath and Ad mutants of mutant thi–10/T. PRPP, 5-phosphoribosyl 1-pyrophosphate; FGAR, formylglycinamidine ribonucleotide.

$\lambda_{max.}$ 500mμ. (2) The Pauly test (Ames & Mitchell, 1952): this was a spray test, performed after chromatography of the supernatant, in which AICA produced a blue spot and AIR_s a yellow spot; this test was valuable as the compounds were detected not only by their colours but also by their R_F values on the chromatogram.

By means of these tests the mutants were divided into classes blocked: (*a*) before AIR; (*b*) just after AIR or C-AIR; (*c*) between AICA and IMP; (*d*) between IMP and AMP. Since C-AIR is unstable and decarboxylates spontaneously to AIR (Stouthamer, de Haan & Nijkamp, 1965), mutants blocked after C-AIR are indistinguishable from those blocked after AIR. Mutants blocked just after 4-aminoimidazole-5-*N*-succinylocarboxamide ribonucleotide are also blocked after AMP at adenylosuccinase, since these two reactions share a common enzyme (Gots & Gollub, 1957). Such mutants would be included in the results as blocked between IMP and AMP.

The results are shown in Scheme 2. Apart from one mutant (ath_{21}) all the ath mutants were

blocked before AIR_s and all the Ad mutants were blocked after AIR_s. For completeness a few thi–10/T guanine mutants were also isolated, but none was ever found to require thiamine. The idea of a common pathway is supported by these results except for the presence of an ath mutant, ath_{21}, blocked between AICAR and IMP. This mutant is blocked at the same position as the B group of ath mutants described by Yura (1956). To elucidate this anomaly an investigation into the characteristics of this class of mutant was undertaken.

Characteristics of mutants ath_{21} and ath B–6

The sole member of the B class of ath mutants described by Yura (1956) was mutant ath B–6. This was obtained from Dr M. Demerec and tested along with mutant ath_{21} for its growth requirements. Both mutants required the pyrimidine moiety of thiamine for growth in concentrations similar to other ath mutants (i.e. about 2mμg. of pyrimidine/ml. of medium). They also both excreted large amounts of AICA into their medium. The amount was much greater than excreted by mutants Ad_3, Ad_7 and Ad_{30}, which like mutants ath_{21} and B–6 were thought to be blocked between AICAR and IMP. The most bewildering finding about mutants ath_{21} and ath B–6 was that, if they were taken through the procedure that de-represses pyrimidine-moiety formation in mutants of mutant thi–10/T or thi–28/T (Newell & Tucker, 1968), then they excreted large amounts of the pyrimidine moiety despite their requirement, as ath mutants, of the pyrimidine for growth. None of the other ath mutants excreted pyrimidine under these conditions. One possible explanation was that the pyrimidine-assay organism was responding to a pyrimidine precursor produced by the mutants. It was found, however, that the material produced by de-repressed washed cells of mutants ath_{21} and ath B–6 was able to substitute for authentic pyrimidine in grown tests of these same mutants. This indicated that it was unlikely that it was just a pyrimidine precursor that the mutants excreted. In addition, the R_F of the pyrimidine produced was checked by bioautography with the pyrimidine-assay mutant thi–1. In two different solvents (butan-1-ol–acetic acid–water and methanol–pentan-1-ol–benzene–ammonia–water; see the Methods section) the R_F of the pyrimidine produced was the same as that of an authentic sample of pyrimidine.

The mutants' abilities to form pyrimidine moiety seemed therefore to be different under different incubation conditions. Thus they required pyrimidine moiety in growth tests yet excreted pyrimidine moiety in washed-cell incubations. This suggested that their inability to make pyrimidine moiety was caused, not by a lack of one of the enzymes for its biosynthesis as in other ath mutants, but rather by an inhibition of pyrimidine-moiety biosynthesis due to an inhibitor that is removed from the cells by the washing procedure in the preparation of washed-cell suspensions.

The most likely inhibitor is AICA (which is accumulated by these mutants) or the ribonucleotide form (AICAR) from which it is derived. Mazlen & Eaton (1967) have shown that, in yeast, AICAR formed in excess by mutants blocked just after AICAR formation seems to inhibit an early step on the purine pathway. This early block does not stop the biosynthesis of AICAR, however, since the latter is also formed as a by-product of histidine biosynthesis. This explanation could also be used to explain the characteristics of mutants ath B–6 and ath_{21}. The AICA excreted by these mutants could also be derived from histidine biosynthesis and so AICA (or AICAR) when present in large amounts in the cells might then inhibit the purine pathway at an early step, thus giving a phenotypic ath mutant in normal growth studies. The washing procedure, however, would lower the AICA concentration, so that the washed cells would then produce large amounts of the pyrimidine until the concentration of AICA (or AICAR) again increased to the inhibitory level. It is probably significant that both mutants ath B–6 and ath_{21} excrete far larger amounts of AICA than the other AICA excretors that are Ad mutants.

The above explanation was tested by using the ability of histidine to inhibit its own biosynthesis and hence the formation of the by-product AICAR (Magasanik & Karibian, 1960). Addition of histidine (40μg./ml.) to the growth medium of mutants ath B–6 and ath_{21} was found to overcome the requirement for the pyrimidine moiety of thiamine, thus strongly supporting the explanation involving AICAR as the proposed inhibitor of pyrimidine-moiety formation. Histidine had no effect on other ath mutants.

It is concluded that mutants ath B–6 and ath_{21} resemble ath mutants only phenotypically, and that the existence of these mutants does not therefore disprove the hypothesis that the early part of the purine biosynthetic pathway is common to pyrimidine-moiety biosynthesis.

Branch-point compound

As all mutants blocked before AIR were ath mutants, and all AIR_s secretors were Ad mutants, it seemed likely that AIR was the last common intermediate of the purine and pyrimidine pathways. However, as mentioned above, C-AIR decarboxylates spontaneously to AIR, so that mutants blocked just after C-AIR also excrete AIR_s

and C-AIR could in fact be the last common intermediate. If this were correct, one would have expected that some at least of the 14 AIR_S-excreting mutants found would be blocked between AIR and C-AIR, and so on this scheme be ath mutants. In fact all AIR_S excretors were Ad mutants, so that AIR rather than C-AIR is thought to be the last common intermediate.

To obtain evidence for such a scheme it was clear that AIR would have to be tested as an intermediate in pyrimidine-moiety biosynthesis. This at first seemed very difficult for two reasons. (1) Purine intermediates excreted (with the exception of AICA) cannot be used by bacteria to overcome a block at an earlier step in the purine pathway (Magasanik, 1962). This is because the intermediates are excreted as the ribonucleoside or the free base, whereas the ribonucleotide is the actual intermediate, and enzymes are not available for forming the ribonucleotide from the ribonucleoside. The ribonucleotides themselves cannot penetrate bacterial cells. (2) Cell extracts that can synthesize the pyrimidine have never been produced. If this had been possible it would have overcome the difficulty of the impermeability of the ribonucleotide intermediates.

Isolation of a mutant capable of using AIR_S in place of pyrimidine. The AIR_S was nevertheless purified and added to the medium of an ath mutant (thi–10/T.ath_{38}) in the hope that it might be converted into the ribonucleotide just sufficiently to satisfy the pyrimidine-pathway requirement (which is only 0·01% of the purine requirement). The AIR_S was isolated from the supernatant of washed cells of mutant thi–10/T.Ad_{10} and purified by chromatography on Dowex 50W (NH_4^+ form) followed by elution from the column by 10mN-ammonia solution as described by Love & Levenberg (1959).

About 20μg. of the purified AIR_S was added to 5ml. of growth medium and incubated with mutant thi–10/T.ath_{38}. All the growth factors were supplied except pyrimidine. After 40hr. of growth the culture showed turbidity, which increased slowly. These cells were then used as an inoculum for an identical experiment with another 20μg. of AIR_S. This time the culture grew turbid after only 30hr., and after a further repetition of this experiment the organism was able to grow as fast on AIR_S as on the pyrimidine. It had not reverted, however, as without AIR_S it showed a normal requirement for the pyrimidine moiety. The organism, designated thi–10/T.$ath_{38\beta}$, had evidently spontaneously mutated, for its special ability was not lost during growth for many generations without AIR_S.

Evidence for the identity of AIR_S isolated from the culture supernatants of mutant thi–10/T.Ad_{10}. The identification of the excreted compound thought to be AIR_S was based on the characteristics shown for AIR_S by Love & Levenberg (1959), with the following results. (1) In the Bratton–Marshall test the compound gave an orange colour characteristic of AIR_S with $\lambda_{max.}$ 500mμ. (2) It was retained by Dowex 50W (NH_4^+ form) and was eluted with 10mN-ammonia as described for AIR_S. (3) It gave a yellow colour with the Pauly test (specific for imidazoles), as did AIR_S. (4) The compound gave a positive pentose test with the orcinol method of Brown (1946). The ratio of ribose to aminoimidazole (the latter calculated from the E_{500} value) was unity within experimental error. (5) It gave a negative phosphate reaction in the ammonium molybdate test for sugar phosphates (Benson, 1957). (6) When chromatographed with propan-1-ol–0·2N-ammonia it had R_F 0·6, as quoted for AIR_S (Love & Gots, 1955).

Relationship between AIR_S and compound Y

The AIR_S-containing supernatant was only partially purified by the Dowex chromatography, so that the compound present in the supernatant that overcame the pyrimidine requirement of mutant thi–10/T.$ath_{38\beta}$ (compound Y) was not necessarily AIR_S. The following evidence was obtained that compound Y was AIR_S.

Correlation between AIR_S and compound Y excretors. With mutant thi–10/T.$ath_{38\beta}$ as an assay organism for compound Y, other AIR_S-excreting mutants were investigated and all without exception were found to excrete compound Y. Mutants that were blocked in other parts of the purine pathway and did not excrete AIR_S did not excrete compound Y.

Concentration of adenine causing inhibition of AIR_S and compound Y excretion. Adenine is known to inhibit its own biosynthesis at an early step on the purine pathway and thus stop excretion of intermediates by purine mutants (Gots, 1957). If AIR_S were the same compound as compound Y then the concentration of adenine that inhibited the excretion of AIR_S should also inhibit the excretion of compound Y. AIR_S was assayed by the E_{500} value of the diazo dye formed in the Bratton–Marshall test (ϵ_{500} for AIR_S is 24600; Love & Gots, 1955). Mutant thi–10/T.$ath_{38\beta}$ was used to assay compound Y, the concentration being expressed in arbitrary units. Mutant thi–10/T.Ad_{10} was grown in test tubes from low inocula with limiting concentrations of tryptophan. Several such tubes were used and in each the adenine concentration was different, so that, when the cultures stopped growing due to lack of tryptophan, some tubes were inhibited by the adenine and excreted nothing whereas at the other end of the scale some excreted both AIR_S and compound Y. None of the cells

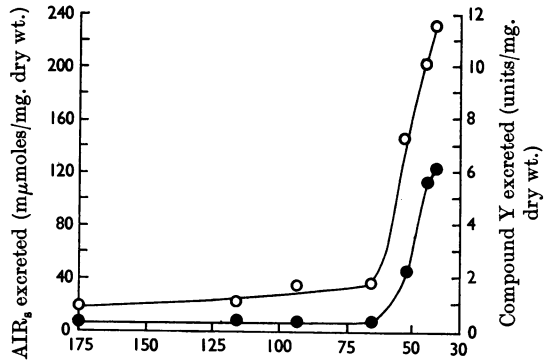


Fig. 1. Similarity in the adenine concentrations that inhibit compound Y and AIR$_s$ excretion in mutant thi–10/T.Ad$_{10}$. ●, Compound Y excreted (expressed in arbitrary units); ○, AIR$_s$ excreted.

were limited in their growth by lack of adenine. The results (Fig. 1) show that the pattern of inhibition shown by different concentrations of adenine is very similar for both AIR$_s$ and compound Y excretion. This evidence does not prove the identity of the two compounds, but it suggests that compound Y is probably closely connected with a purine intermediate.

Ratio of AIR$_s$ to compound Y during purification. If AIR$_s$ and compound Y were identical the ratio of the concentration of one to the other should have been constant during purification. This was checked in the different fractions eluted from the Dowex 50W column and within experimental error the ratio was found to be constant.

Chromatographic evidence for the identity of AIR$_s$ and compound Y. Although Love & Levenberg (1959) regarded the AIR$_s$ obtained by their method as pure, other compounds with similar weakly basic properties to AIR$_s$ might have been present. This was particularly important since the pyrimidine itself was one such compound. Thin-layer cellulose chromatography coupled with bioautography with mutant thi–10/T.ath$_{38\beta}$ to show up spots of compound Y activity were chosen as the best means of showing the identity between AIR$_s$ and compound Y. The three solvents chosen were: (i) butan-1-ol–acetic acid–water; (ii) propan-1-ol–3-methylbutan-1-ol–water–isobutyric acid–ammonia; (iii) propan-1-ol–water (see the Methods section). The R_F of AIR$_s$ in each solvent was found by running another chromatogram in the same tank as the chromatogram used for bioautography, and developing it with the Pauly reagent.

The R_F of AIR$_s$ was identical with that of the main active spot on the bioautograph in each of the three solvents, and was also clearly distinguishable from the R_F values of thiamine and the pyrimidine moiety of thiamine.

It was concluded that compound Y was identical with AIR$_s$. For later experiments in which AIR$_s$ was employed it was always further purified by thin-layer chromatography with butan-1-ol–acetic acid–water after the Dowex purification.

Evidence that AIR is a common intermediate

Overcoming the double requirement. The mutant thi–10/T.ath$_{38\beta}$ that could use AIR$_s$ in place of the pyrimidine moiety having been obtained, it seemed possible that AIR$_s$ would satisfy both the mutant's purine and pyrimidine-moiety requirements. This was found to be correct. In the presence of AIR$_s$ (20 μg./ml.) normal growth was obtained without added pyrimidine moiety or purine and the yield of cells was similar to that expected from the same amount of adenine. The parent mutant (thi–10/T.ath$_{38}$) failed to respond, as did the controls without AIR$_s$. This experiment showed that AIR was common to both purine and pyrimidine-moiety biosynthetic pathways. (Although AIR$_s$ was the actual compound excreted and absorbed it must have been derived from AIR, the ribonucleotide form, and the latter is therefore suggested as the branch-point intermediate.)

Conversion of AIR$_s$ into pyrimidine moiety in washed cells. When mutant thi–10/T$_{38\beta}$ was taken through the steps that normally de-repress thi–10 or thi–28 mutants (Newell & Tucker, 1968) and washed cells were prepared, they synthesized no pyrimidine moiety since the mutant was an ath mutant, blocked in the biosynthesis of the pyrimidine moiety. If AIR$_s$ (4 μg./ml.) was supplied, however, then large amounts of pyrimidine moiety were synthesized and excreted into the medium and about 1% of the AIR$_s$ was converted into the pyrimidine. The process did not occur with repressed cells and the concentration of thiamine that was needed for repression was roughly the same as for repression in mutant thi–10/T. This was the first demonstration of the synthesis of the pyrimidine moiety from a purine intermediate.

Positions of the methionine and glycine requirements. Biosynthesis of the pyrimidine moiety requires methionine and glycine (Newell & Tucker, 1968). The conversion of AIR$_s$ into pyrimidine moiety effectively divided the pyrimidine biosynthesis into two regions, namely from 5-phosphoribosyl 1-pyrophosphate to AIR$_s$ and from AIR$_s$ to pyrimidine moiety. It was therefore decided to see which part had the methionine and glycine requirements. The results provided strong evidence for AIR$_s$ being an intermediate in pyrimidine-moiety biosynthesis.

For this purpose thi–10/T.ath$_{38\beta}$ was further

Table 2. *Pyrimidine moiety synthesized from AIR_s by the methionine auxotroph thi-10/T.ath$_{38\beta}$.M in the presence and absence of methionine and the pyrimidine moiety synthesized from AIR_s by the glycine auxotroph thi-10/T.ath$_{38\beta}$.Gly in the presence and absence of glycine*

The mutants were de-repressed in tubes by growth with suboptimum concentrations of thiamine and washed cells were prepared. To some of the tubes was added AIR_s either with or without methionine or glycine, and the amount of pyrimidine moiety excreted into the medium by the mutants was assayed with mutant thi-1.

Mutant	Supplements (μg./ml.)	Pyrimidine moiety formed (mμg./mg. dry wt. of cells)
thi-10/T.ath$_{38\beta}$.M	Methionine (5) (control)	1·4
thi-10/T.ath$_{38\beta}$.M	AIR_s (4)	1·4
thi-10/T.ath$_{38\beta}$.M	AIR_s (4)+methionine (5)	75
thi-10/T.ath$_{38\beta}$.Gly	Glycine (50) (control)	3
thi-10/T.ath$_{38\beta}$.Gly	AIR_s (4)	60
thi-10/T.ath$_{38\beta}$.Gly	AIR_s (4)+glycine (50)	60

mutated and a glycine auxotroph and a methionine auxotroph were selected. These mutants (thi-10/T.ath$_{38\beta}$.Gly and thi-10/T.ath$_{38\beta}$.M) were de-repressed in tubes by growth with suboptimum concentrations of thiamine (Newell & Tucker, 1968) and washed cells were prepared. To some of the tubes was added AIR_s either with or without methionine or glycine, and the amount of pyrimidine excreted into the medium by the mutants was assayed with mutant thi-1. The results are shown in Table 2. The methionine-requiring mutant synthesized very little pyrimidine moiety from AIR_s in the absence of methionine, yet synthesized it in good yield in its presence. In contrast, the glycine mutant synthesized pyrimidine moiety from AIR_s with or without added glycine. It was concluded therefore that the methionine-requiring step is on the part of the pathway from AIR to pyrimidine moiety. Further, this part of the pathway has no glycine requirement. As the overall biosynthesis of pyrimidine moiety has a glycine requirement the formation of AIR apparently requires glycine, as shown by Goldthwait, Peabody & Greenberg (1955). This experiment also provides strong evidence that the AIR_s was acting as a precursor of pyrimidine moiety rather than just a cofactor, since it replaced the glycine requirement for this biosynthesis (Scheme 3).

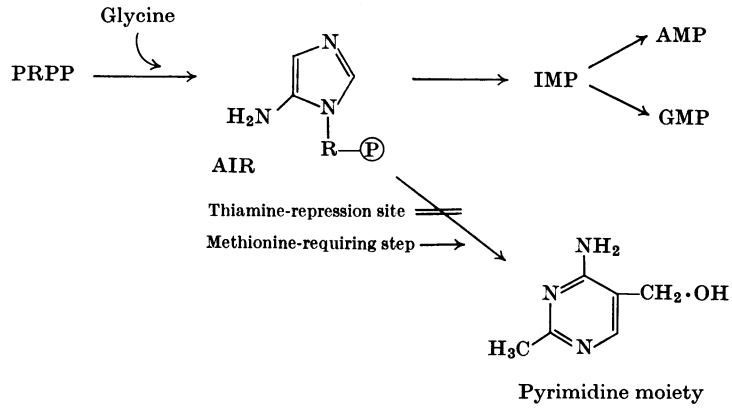
Incorporation of radioactivity into pyrimidine moiety from ^{14}C-labelled AIR_s. AIR was confirmed as an intermediate on the pyrimidine pathway by preparing purified ^{14}C-labelled AIR_s from [^{14}C]-glycine and converting this into labelled pyrimidine.

An AIR_s-excreting mutant was required that also had a requirement for glycine. Such an auxotroph was obtained by the easiest route, namely by mutating mutant thi-28/T.Gly and isolating an Ad mutant excreting AIR_s. This organism (thi-28/T.Gly.Ad) was grown with limiting concentrations of adenine (14 μg./ml.) until its growth rate started to slow down and washed cells were prepared. To a flask containing 100 ml. of bacterial suspension was added 5 mg. of unlabelled glycine with 50 μc of [1-^{14}C]glycine and the culture aerated at 37°. The ^{14}C-labelled AIR_s excreted into the supernatant was purified first by the method of Love & Levenberg (1959) and then by chromatography with butan-1-ol–acetic acid–water as solvent to remove any radioactive glycine still present. The purified AIR_s was then added to two flasks containing washed cells of mutant thi-10/T.ath$_{38\beta}$: one flask with cells repressed by excess of thiamine and one flask with cells de-repressed by lack of thiamine. After incubation for 1·5 hr. the supernatants of these flasks were concentrated, purified and chromatographed as previously described and radioautographs developed from them (Newell & Tucker, 1968). The results of the radioautographs showed that a spot corresponding to the pyrimidine moiety was present on the radioautograph from the de-repressed culture that was absent from that from the repressed culture. It is therefore concluded that AIR_s labelled by the incorporation of [1-^{14}C]glycine is converted into labelled pyrimidine. The specific radioactivity of the [1-^{14}C]glycine used was $1{\cdot}36 \times 10^8$ counts/100 sec./m-mole. The specific radioactivity of the ^{14}C-labelled AIR_s formed was $1{\cdot}25 \times 10^8$ counts/100 sec./m-mole. The specific radioactivity of the pyrimidine formed was $1{\cdot}15 \times 10^8$ counts/100 sec./m-mole.

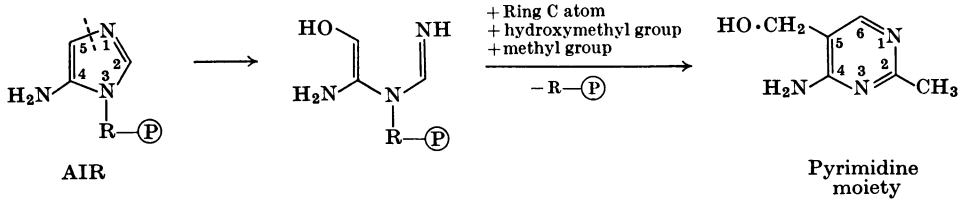
These results indicate that the ^{14}C-labelled AIR_s was a direct precursor of the pyrimidine moiety and that AIR is on the pathway of pyrimidine-moiety biosynthesis.

DISCUSSION

The evidence presented indicates that the pyrimidine moiety of thiamine is synthesized from the purine intermediate AIR (Scheme 3).



Scheme 3. Simplified scheme of the purine biosynthetic pathway showing the branch leading to the formation of the pyrimidine moiety of thiamine. The order of the thiamine-repression and methionine-requiring steps is unknown. PRPP, 5-phosphoribosyl 1-pyrophosphate.

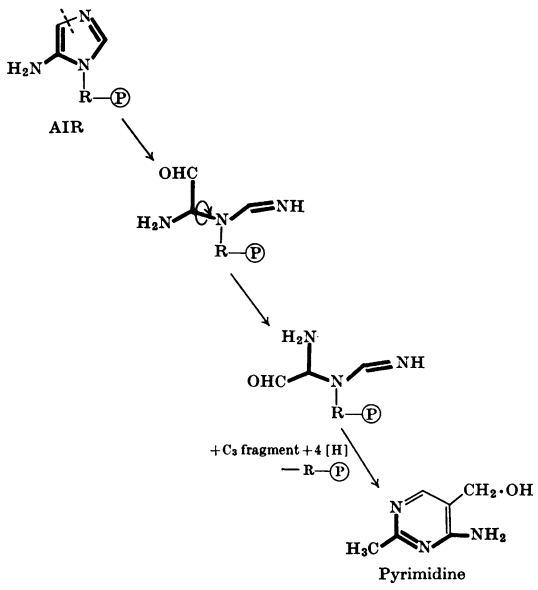


Scheme 4. First mechanism conceived for the biosynthesis of the pyrimidine moiety from AIR. Evidence against this type of mechanism is discussed in the text.

Such a branched purine pathway explains both the early ath mutants and the phenomenon of inhibition of pyrimidine-moiety biosynthesis by adenine and derivatives. A mutational block before the branch point results in a requirement for a purine plus the pyrimidine moiety of thiamine, i.e. a requirement for the products of both of the branches. The same is also true when adenine derivatives inhibit the purine pathway. Nierlich & Magasanik (1965) showed that adenine derivatives inhibit the first reaction specific to the purine pathway, namely 5 - phosphoribosyl 1 - pyrophosphate amidotransferase. Normally the control is exercised by adenine plus guanine derivatives acting synergistically, but adenine derivatives alone can inhibit the pathway if in sufficiently high concentration. It is concluded therefore that the common pathway shared by purines and pyrimidine moiety explains both the early ath mutants and the inhibition of pyrimidine-moiety biosynthesis by adenosine or adenine.

During the conversion of AIR into pyrimidine moiety the ribose phosphate is lost, an extra carbon atom is added to the ring and two other carbon atoms are added adjacent to the ring.

One obvious way in which this could happen would be by rupture of the imidazole ring at the bond between atoms 1 and 5 and insertion of an extra ring carbon atom. Addition of a methyl group and a hydroxymethyl group and removal of ribose phosphate then gives pyrimidine (Scheme 4). Two major objections may be raised against such a scheme. (1) It involves the addition of a methyl group. This one would expect to be derived from the methyl group of methionine or from the folate-linked C_1 unit pool. However, methionine does not donate a methyl group to pyrimidine (Newell & Tucker, 1968), and formate (a good source of C_1 groups for the folate pool) does not contribute to this methyl group either (David, Estramareix & Hirshfeld, 1966). (2) David *et al.* (1966) showed that formate does contribute to pyrimidine moiety but only at C-4. This is the carbon atom with the amino group. It is also known that formate is the precursor of C-2 of AIR, so that one can predict that C-2 of AIR becomes C-4



Scheme 5. Postulated mechanism for the biosynthesis of pyrimidine moiety from AIR. The portion of the pyrimidine moiety derived from the AIR molecule is indicated by the bonds drawn as thick lines.

of pyrimidine moiety. In Scheme 4, however, C-2 of AIR becomes C-2 of pyrimidine moiety, which would contradict the evidence of David *et al.* (1966).

The imposition that C-2 of AIR becomes C-4 of pyrimidine moiety decreases the number of possible mechanisms. Both of these atoms are bonded to two nitrogen atoms, and this suggests that, unless a nitrogen atom is removed and another added again, the amino nitrogen atom of the pyrimidine moiety is derived from one of the ring nitrogen atoms of AIR. Therefore the amino group produced on breaking the AIR ring is probably not included in the ring when the ring of the pyrimidine moiety is formed. Instead the amino group attached to C-4 of AIR would be free to rotate and become included in the pyrimidine ring (Scheme 5).

This type of reaction mechanism is illustrated in nicotinic acid biosynthesis (Goodwin, 1963), in which the ring of 3-hydroxyanthranilic acid opens to produce an intermediate with amino and carboxyl groups free to rotate about their bonding carbon atom. When the ring is re-closed the amino group is included in the ring in place of the carboxyl group.

The scheme outlined for pyrimidine-moiety biosynthesis (Scheme 5) introduces a C_3 fragment. This could be a molecule such as glycerol or α-glycerophosphate, but no evidence has been obtained on this point. Besides explaining the mechanism of C-2 of AIR becoming C-4 of pyrimidine moiety, Scheme 5 provides a mechanism for the formation of a methyl group not derived from methionine or the folate-linked C_1 unit pool. Instead this methyl group would be derived in this scheme from C-2 of glycine, by the reduction of the aldehyde group produced when the AIR ring opens.

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