Biosynthesis of Nucleic Acid Purines in *Mycobacterium tuberculosis* H₃₇R_v

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1. The biosynthesis of nucleic acid purine in *Mycobacterium tuberculosis* H₃₇R_v has been studied by using ¹⁴C-labelled precursors. 2. The results indicate that C-2 and C-8 of the purine ring are derived most efficiently from serine and glycine and not from formate. 3. [¹⁴C]Methionine is not incorporated into the ureide carbon atoms of the purine ring.

The investigations of Sonne, Buchanan & Delluva (1946a,b, 1948a,b) on the biosynthesis of purines in the avian system have revealed that C-2 and C-8 of the purine ring originate from formate. Further studies have shown that in mammalian and avian systems these carbon atoms can be derived not only from formate and formaldehyde, but also from the α -carbon of glycine and β -carbon of serine (Reichard, 1955; Schulman, 1961; Buchanan, 1960). But, in contrast with the mammalian and avian systems in which C₁ units from any precursor are incorporated equally to positions 2 and 8 of the purine ring, micro-organisms have been shown to exhibit certain differences in the precursors of these carbon atoms. Thus, growing cells of *Pseudomonas* and *Escherichia coli* can incorporate [¹⁴C]formate into position 8 of the purine ring, but not appreciably into position 2 (Revel & Magasanik, 1958; Koch & Levy, 1955). Nester & Spizizen (1961) have also shown that in growing cells of *E. coli* formate is incorporated only into position 8 whereas isotope from [2-¹⁴C]glycine and [3-¹⁴C]serine is incorporated equally into positions 2 and 8 of the purine ring. Revel & Magasanik (1958) have shown that *Pseudomonas fluorescens* can utilize C-2 of histidine for the synthesis of C-8 of the purine ring. In micro-organisms possessing an active formate hydrogen-lyase, formate is rapidly oxidized to carbon dioxide, and in such systems other C₁ donors like serine and methionine may represent more efficient C₁ precursors than formate itself (Moat & Friedman, 1960). But few, if any, direct references to the methyl carbon of methionine as C₁ donor are available, even though several indirect implications may be found (Moat & Friedman, 1960). *Mycobacterium tuberculosis* is reported to oxidize formate (Franke & Schillinger, 1944) and hence the precursors of C-2 and C-8 of the nucleic acid purines in this organism have been the subject of the present investigation.

MATERIALS AND METHODS

Radioactive chemicals. [2-¹⁴C]Glycine (8.1 mc/m-mole), L-[3-¹⁴C]serine (4.7 mc/m-mole) and L-[Me-¹⁴C]methionine (6.6 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [¹⁴C]formate (0.97 mc/m-mole) was the product of the Atomic Energy Establishment, Trombay, India.

All other chemicals used were of the reagent grade.

Enzyme preparations. Xanthine oxidase was prepared from cream according to the method of Klenow & Emberland (1955). The second ammonium sulphate fraction (brown precipitate) was used as such.

Analytical methods. Purines were separated from extraneous materials by using ion-exchange column chromatography (Tyner, Heidelberger & LePage, 1952) and from each other by paper chromatography (Giri & Rao, 1952), identified and assayed spectrophotometrically (Wyatt, 1955). Urea was estimated by the colorimetric method of Barker (1944). All radioactive samples were plated on aluminium planchets and counted in a thin window Geiger-Müller counter. Since the amounts spotted were less than 0.03 mg. no self-absorption correction was necessary.

Culture. The human virulent strain H₃₇R_v of *M. tuberculosis* was the organism used in the present studies. The bacteria were grown at 37° on Youman's medium (Youman & Karlson, 1947) with glycerol as carbon source and asparagine as the nitrogen source. The conditions of growth were the same as described by Malathi, Sirsi, Ramakrishnan & Maller (1964).

Isolation of nucleic acid purines. The cells were collected by filtration on Whatman no. 1 filter paper and processed by the method of Marshak & Vogel (1951). The nucleic acids were extracted from the ice-cold 7% (w/v) trichloroacetic acid residue by the method of Schneider (1945). A portion (10 mg.) of the residue was heated for 15 min. at 90° with 1.5 ml. of 5% trichloroacetic acid, cooled and centrifuged. The residue was resuspended in 0.5 ml. of 5% trichloroacetic acid and centrifuged. The supernatants from both the centrifugations were pooled together and heated in a boiling-water bath for 2 hr. to decompose the trichloroacetic acid (Hitchings, 1941). The solution was evaporated to dryness under reduced pressure and the

nucleic acids were hydrolysed with 1.0 ml. of N -HCl at 100° for 1 hr. The HCl was removed under reduced pressure and the purines were separated first by ion-exchange chromatography on a Dowex 50 (X8; H^+ form) column (1 cm. $\times$ 2.5 cm.) and further purified by circular paper chromatography on Whatman no. 3 filter paper with Kirby's (1955) solvent system methanol-ethanol-11.6 N -HCl-water (50:25:6:19, by vol.). The purines were located by means of an ultraviolet lamp (Mineralight, SL2537). These areas, along with the corresponding bands in a blank area, were cut out and eluted with 4 ml. of 0.1 N -HCl and the paper was washed three times with 1.5 ml. portions of the same acid. The pooled extracts were made up to 10 ml. with acid and the concentration and radioactivity were determined as described above in Analytical methods; the specific activities of adenine and guanine were calculated.

Degradation of purines. The guanine obtained was evaporated to dryness under reduced pressure. It was taken up in 0.25 ml. of 2.5 N -H₂SO₄ and deaminated to xanthine by keeping it in a boiling-water bath and by dropwise addition of 0.2 ml. of sodium nitrite (15 mg./ml.). About 60–65% of the guanine was converted into xanthine. To this xanthine was added 10 mg. of carrier xanthine dissolved in 0.5 ml. of N -NaOH. The xanthine was precipitated twice by addition of N -H₂SO₄ with constant stirring. After washing thoroughly with water and ethanol it was dried over P₂O₅. About 2 mg. was taken to determine the radioactivity. The rest was converted into alloxan and urea,

which contains the C-8 moiety of the purine ring, by treatment with KClO₃ (Elwyn & Sprinson, 1954). Several drops of M -sodium thiosulphate were added to reduce any excess of chlorate. This urea-alloxan mixture was separated on a Dowex 50 (H^+ form) column (0.6 cm. $\times$ 10 cm.) and eluted with water (Nester & Spizizen, 1961). The first five fractions (4 ml. each) contained alloxan; fractions 15–17 contained urea. The urea fractions were pooled and concentrated. Portions were taken for the determination of concentration and radioactivity. The alloxan was further degraded to urea containing C-2 of the purine ring and the urea separated on the Dowex 50 (H^+ form) column (Nester & Spizizen, 1961). The specific activities of C-8 and C-2 moieties of the guanine were calculated, based on the radioactive and colorimetric assays of the urea resulting from the degradation.

Adenine was deaminated, exactly as described for guanine, to hypoxanthine, which was converted into uric acid by the action of xanthine oxidase at pH 7.4 and room temperature (Kalckar, 1947). To the reaction mixture, which had been freed from protein by heating in a boiling-water bath for 5 min. and centrifugation, was added 10 mg. of carrier uric acid in 0.5 ml. of N -NaOH. The uric acid was twice precipitated from alkaline solution by the addition of N -H₂SO₄. The crystalline material was washed with water and ethanol and dried over P₂O₅. A small amount of uric acid was dissolved in N -NaOH. A portion of this was diluted with water and the concentration of uric acid

Table 1. *Incorporation of sodium [¹⁴C]formate into the nucleic acid purines of Mycobacterium tuberculosis at different periods of growth*

The bacteria were grown in 75 ml. of medium in the presence of different amounts (20.6 μ moles and 41.2 μ moles) of sodium [¹⁴C]formate (310 500 counts/min./ μ mole). They were harvested at 8, 12 and 16 days. Other details are given in the text.

Growth period (days)	Wet wt. of cells (g.)	[¹⁴ C]Formate (counts/min./ μ mole)		Wet wt. of cells (g.)	[¹⁴ C]Formate (counts/min./ μ mole)	
		Adenine	Guanine		Adenine	Guanine
8	0.150	540	490	0.210	865	840
12	0.730	215	191	0.620	404	323
16	1.070	132	137	0.780	309	223

Table 2. *Incorporation of various C₁ precursors into the nucleic acid purines of Mycobacterium tuberculosis*

The bacteria were grown in 37.5 ml. of medium in the presence of various ¹⁴C-labelled compounds in the following amounts: sodium [¹⁴C]formate, 20.6 μ moles (310 500 counts/min./ μ mole); [*Me*-¹⁴C]methionine, 3.03 μ moles (2 112 000 counts/min./ μ mole); [*2*-¹⁴C]glycine, 2.45 μ moles (2 592 000 counts/min./ μ mole); [*3*-¹⁴C]serine, 4.25 μ moles (1 504 000 counts/min./ μ mole). Samples were taken at 8 and 16 days of growth. Details of the procedure are given in the text.

¹⁴ C-labelled supplement	Wet wt. of cells (g.)	8-day-old cells (counts/min./ μ mole)		Wet wt. of cells (g.)	16-day-old cells (counts/min./ μ mole)	
		Adenine	Guanine		Adenine	Guanine
Sodium [¹⁴ C]formate	0.175	1 805	1 417	0.600	357	539
[<i>Me</i> - ¹⁴ C]Methionine	0.150	377	458	0.550	0	0
[<i>2</i> - ¹⁴ C]Glycine	0.165	84 700	79 320	0.600	25 630	25 890
[<i>3</i> - ¹⁴ C]Serine	0.200	73 450	63 400	0.600	23 720	19 710

Table 3. Utilization of glycine and serine for the synthesis of C-2 and C-8 of the nucleic acid purines of *Mycobacterium tuberculosis*

Adenine and guanine, isolated from the 8-day-old cells (Table 2) grown in the presence of [2-¹⁴C]glycine and [3-¹⁴C]serine, were used for degradation. Specific activities are given in Table 2. Other details are given in the text.

¹⁴ C-labelled supplement	Wet wt. of cells (g.)	Adenine (counts/min./μmole)		Guanine (counts/min./μmole)	
		C-8	C-2	C-8	C-2
[2- ¹⁴ C]Glycine	0.165	18	18	50	58
[3- ¹⁴ C]Serine	0.200	79	68	156	171

determined from the extinction of the solution at 290 mμ. Another portion of the same solution was taken for determination of radioactivity and the specific activity of uric acid calculated. Finally the uric acid was degraded in the same manner as xanthine.

RESULTS

The initial experiments designed to determine the efficiency of the incorporation of formate revealed that formate is incorporated only to a very small extent by 1-week-old cells and the specific activities of the purines decreased with the age of the culture (Table 1). Hence the experiment was repeated with other C₁ precursors. Table 2 summarizes the data on the incorporation of four C₁ precursors: [¹⁴C]formate, [Me-¹⁴C]methionine, [2-¹⁴C]glycine and [3-¹⁴C]serine. The results indicate that the specific activities were higher with 1-week-old cells than with the 2-week-old cultures.

It is also clear that *M. tuberculosis* is able to incorporate [2-¹⁴C]glycine and [3-¹⁴C]serine to a very significant extent into the nucleic acid purines. On the other hand, formate seems to be a very poor precursor and methionine proved to be still less efficient as a purine precursor. Degradation studies were conducted with the adenine and guanine samples obtained from 1-week-old cells grown in the presence of glycine and serine to get a precise knowledge of the extent of incorporation into C-2 and C-8 of the purine molecule. The results obtained are presented in Table 3. It is clear that the isotope from both the precursors is incorporated almost equally into both C-2 and C-8 of the purine ring. Since the incorporation with serine is about five times as great as that with glycine it is evident that serine is the more efficient precursor of the ureide carbon atoms of the purine ring in this organism. The possibility of the participation of methionine as C₁ donor in purine biosynthesis can be ruled out since its incorporation is insignificant.

DISCUSSION

The present studies have revealed that *M. tuberculosis* is capable of new biosynthesis of nucleic

acid purines from simple precursors like glycine and serine. Formate, the classical precursor of the ureide carbon atoms of the purine ring, is not an efficient C₁ donor in this organism. Instead, both the ureide carbon atoms are derived most effectively from the β-carbon of serine and α-carbon of glycine. However, both the isotopes are incorporated almost equally into the C-2 and C-8 of the purine molecule.

Differential incorporation of the various precursors in micro-organisms has been observed by several other workers. Koch (1955) has shown that in growing cells of *E. coli* [2-¹⁴C]glycine at a concentration of 0.1 μmole/ml. labelled position 8 exclusively whereas at higher concentrations (10 μmoles/ml.) the isotope contributed equally to positions 2 and 8. In contrast with this, Nester & Spizizen (1961) have observed that in *E. coli* both glycine (1 μmole/ml.) and serine (0.25 μmole/ml.) are incorporated equally into C-2 and C-8 of the purine ring. Again, Revel & Magasanik (1958) have shown that in *P. fluorescens* and *Aerobacter aerogenes* glycine even at low concentrations (0.5 μmole/ml.) contributes equally to C-2 and C-8. In the present investigation it is observed that in *M. tuberculosis* glycine (0.066 μmole/ml.) and serine (0.12 μmole/ml.), at concentrations even lower than those used by other workers, are incorporated almost equally into both C-2 and C-8.

Formate has been shown to be incorporated only into position 8 of the purine ring in several instances. Revel & Magasanik (1958) have shown that in *P. fluorescens* formate at low concentrations (0.5 μmole/ml.) is oxidized to carbon dioxide and therefore serves more efficiently as a donor of C-6 than of C-8, whereas at higher concentration of 20 μmoles/ml. it competes with histidine as a precursor of C-8 of the purine ring. In *A. aerogenes* formate has been shown to be incorporated only into C-8 of the purine ring (Revel & Magasanik, 1958). *E. coli* and other micro-organisms possess the enzyme formate hydrogen-lyase, and thus formate is rapidly converted into carbon dioxide, a considerable portion of which can be recovered from C-6 of the purines (Moat & Friedman, 1960). Nester & Spizizen (1961)

have shown that in *E. coli* formate is incorporated mostly into C-8 of the purine ring. In the present investigations, it has been found that formate even at the same concentration as that used in *E. coli* is incorporated into nucleic acid purines in *M. tuberculosis* only to the extent of 0.9% of that reported in *E. coli*. *M. tuberculosis* has been reported to oxidize formate (Franke & Schillinger, 1944), and thus formate may not be available as the C₁ donor. However, the extremely small amount of radioactivity may be present in C-6 or C-8 of the purine ring. No attempt has been made to study this since it was considered that the radioactivity in the individual carbon atoms on degradation would be too low to work with.

In systems that do not degrade formate the conversion of free formate into formyltetrahydrofolic acid represents the most efficient system for the C₁ transfer. But in *M. tuberculosis* serine and glycine represent the most effective C₁ donors which may contribute to the formation of formyltetrahydrofolic acid without going through a free formate stage. The participation of methionine as a C₁ donor has been discussed by Moat & Friedman (1960). Even though no direct evidence is available, several indirect implications may be found. Gots & Chu (1952) have found that a *p*-aminobenzoic acid-requiring mutant of *E. coli* accumulated 5-aminoimidazole-4-carboxamide under the conditions of *p*-aminobenzoic acid deficiency, or in the presence of competitive inhibitors of *p*-aminobenzoic acid, and this accumulation is depressed by methionine, suggesting that methionine may serve as donor of C-2. However, more detailed studies by Gots & Love (1954) have shown that this depression may be more likely due to a direct inhibitory effect on the new synthesis of 5-aminoimidazole-4-carboxamide and the contribution to C-2 could be unlikely because the organism was genetically deficient in transformylase. The present studies have revealed the inertness of methionine as the precursor of the ureide carbon atoms of the purine molecule in *M. tuberculosis*.

The results presented in this paper thus show that the biosynthesis of nucleic acid purines by *M. tuberculosis* differs in some essential respects from that by mammalian tissues.

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