Riboflavin biosynthesis in Staphylococcus epidermidis: studies on the enzymatic conversion of 4-ribitylamino-5-amino-2, 6-dihydroxyprimidine to 6, 7-dimethyl-8-ribityllumazine

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Riboflavin biosynthesis in *Staphylococcus epidermidis.*

Studies on the enzymatic conversion of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to 6,7-dimethyl-8-ribityllumazine

by

Hal Russell Turner

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INTRODUCTION

Studies designed to elucidate the biosynthetic pathway of riboflavin were initiated with the development of a synthetic medium capable of supporting the growth of *Eremothecium ashbyii* (56). MacLaren observed that riboflavin biosynthesis by *E. ashbyii* is enhanced when certain purines are added to the synthetic growth medium and he hypothesized that a purine is the precursor of riboflavin in microorganisms (33). Plaut (48) strengthened that hypothesis when he found growing cells of *Ashbya gossypii* incorporate radioactive carbon dioxide, glycine and formate into analogous positions of purines and riboflavin. McNutt (34, 35, 36) obtained definitive evidence for the purine-precursor concept when he discovered that with the exception of carbon-8, all the carbon and nitrogen atoms of exogenously supplied purines enter the riboflavin molecule as an intact unit. Using a purineless mutant of *Escherichia coli*, Howells and Plaut (22) demonstrated the obligatory requirement of a purine when they found that radioactivity from labeled glycine is not incorporated into riboflavin, while label from $^{14}$C-adenine enters the vitamin without dilution. In the wild-type strain of *E. coli* (capable of *de novo* purine synthesis), radioactivity from glycine is transferred into riboflavin. More recently, Baugh and Krumdieck (7) and Bacher and Mailander (4) independently obtained evidence that a guanine-compound is the purine-precursor of riboflavin in *Corynebacterium* sp. and *Aerobacter* (Enterobacter) *aerogenes*, respectively. Because both of these studies employed whole cells in their experimental design, it is impossible to ascertain whether free
guanine, a guanine-riboside or a guanine-ribotide is the actual precursor of riboflavin.

In recent years, studies on riboflavin biosynthesis have been aided greatly by the isolation from microorganisms of naturally-occurring compounds that chemically resemble the vitamin. Masuda (39, 40) isolated a green fluorescent compound from the mycelium of *E. ashbyii* and assigned to it the chemical structure 6,7-dimethyl-8-ribityllumazine (DMRL). The potential involvement of DMRL as a late intermediate in the riboflavin pathway is suggested by the similarity of its chemical structure to that of riboflavin (see Figure 1). Direct evidence for a precursor-product relationship between DMRL and riboflavin came from the demonstration that radioactively labeled DMRL is converted to riboflavin by cell-free extracts from *A. gossypii* (50, 51) and *Candida flarereri* (15). Plaut and his colleagues have thoroughly characterized the mechanism by which DMRL is converted to riboflavin (for a review see reference 52). They demonstrated that the enzymatic formation of riboflavin from DMRL occurs via the transfer of carbons 6, 7 and the methyl groups from one molecule of DMRL to a second molecule of the lumazine (18). Thus two moles of DMRL are consumed with the formation of each mole of riboflavin. The second product of the reaction is 4-ribitylamino-5-amino-2,6-dihydroxy-pyrimidine (64). The enzyme responsible for catalyzing this reaction has been named riboflavin synthetase (51), and its presence has been demonstrated in a number of plants and microorganisms (5, 15, 25, 38, 43, 44, 63).
Because carbon-8 of guanine is seemingly lost before the remaining purine-skeleton is incorporated into riboflavin, investigators suspected the 4,5-diaminopyrimidine-type compounds as potential biosynthetic intermediates in the pathway. To demonstrate the occurrence of such pyrimidines, the usual approach has been to isolate riboflavinless mutants, then identify excretion products that accumulate when the mutants are shifted from a medium containing riboflavin into one devoid of the vitamin. Thus Bacher, Oltmanns, Lingens and their associates have isolated and identified 2,4,5-triamino-6-hydroxypyrimidine (3, 6), 4-ribitylamino-2,5-diamino-6-hydroxypyrimidine (2), 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (31) and 6,7-dimethyl-8-ribityllumazine (31) from the supernatant fluid of several riboflavin-dependent mutants of *Saccharomyces cerevisiae*. Based on these findings, Bacher and Lingens (3) proposed a scheme for the biosynthesis of riboflavin (Figure 1).

In a recent study (63), several riboflavinless mutants of *Staphylococcus epidermidis* were isolated and biochemically characterized. Of ten auxotrophs isolated, eight excrete DMRL and are classified as RibA mutants. RibA mutants possess a defective riboflavin synthetase enzyme as evidenced by their inability to accomplish the *in vitro* conversion of DMRL to riboflavin. One staphylococcal mutant (Rib-7) fails to accumulate an identifiable excretion product, while the other mutant (Rib-4) excretes 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine. Thus the results obtained with the staphylococcal mutants are in agreement with those obtained by others (29, 31, 39, 40, 54, 55).
It can be seen from Figure 1 that in order for 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to be converted to DMRL, four carbon atoms must react with the pyrimidine to complete the dimethyl-substituted pyrazine ring of DMRL. When this investigation was initiated, the origin of these four carbon atoms had not been precisely determined. Evidence presented in this study indicates that diacetyl is the four-carbon unit that reacts enzymatically with 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to form DMRL.
Figure 1. Hypothetical scheme for the biosynthesis of riboflavin as proposed by Bacher and Lingens (3). I, guanine or a derivative of guanine; II, 2,4,5-triamino-6-hydroxypyrimidine or a derivative; III, 4-ribitylamino-2,5-diamino-6-hydroxypyrimidine; IV, 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine; V, 6,7-dimethyl-8-ribityllumazine; VI, riboflavin; $R_1$ and $R_2$ may be hydrogen or carbohydrate in nature.
LITERATURE REVIEW

Riboflavin biosynthesis was reviewed in 1971, 1972 and briefly in 1974 (13, 52, 53). The preparation of cell extracts, chemical synthesis of biosynthetic intermediates, assay procedures and properties of enzymes and substrates associated with riboflavin metabolism were described in a series of articles in Methods in Enzymology (32). The work described in this dissertation provides evidence that DMRL is formed enzymatically from diacetyl and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine. Consequently, this survey will deal only with the literature that examines the origin of diacetyl and the o-xylene ring of riboflavin (carbons 6, 7 and the methyl groups of DMRL).

Relationship between diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanal) and butylene glycol (2,3-butanediol)

Diacetyl, acetoin and butylene glycol are structurally-related compounds representing three different oxidation levels of the same four-carbon skeleton. The biosynthesis of acetoin was shown to proceed from pyruvate via the intermediate formation of a-acetolactic acid (acetolactate) in E. aerogenes (23), Bacillus subtilis (21), Streptococcus diacetilactis (57), Lactobacillus casei (9), Leuconostoc citrovorum (58), Pseudomonas fluorescens (11), Lactobacillus brevis, (11), Streptococcus lactis (11), and Serratia marcescens (11). In this reaction, two moles of pyruvate condense to form one mole of acetolactate and one mole of carbon dioxide. Presumably, "active acetaldehyde" (α-hydroxyethyl thiamine pyrophosphate) is involved because the reaction is thiamine pyrophosphate-dependent and requires magnesium or manganous ions for
maximal activity. In a subsequent reaction, the acetolactate is
decarboxylated to give one mole of acetoin and a second mole of carbon
dioxide. The overall reaction sequence may be summarized as follows:

\[
\begin{align*}
\text{pyruvate} & \quad \text{"active acetaldehyde"} \\
& \quad + \quad \text{acetolactate} \\
& \quad \downarrow \quad \text{pyruvate} \\
& \quad \text{acetoin} \\
& \quad \downarrow \quad \text{CO}_2
\end{align*}
\]

Acetoin synthesis in yeasts and mammalian tissues occurs by a different
mechanism \((24, 61)\). Juni \(24\) demonstrated that enzyme preparations
from \textit{S. cerevisiae} and pig heart were unable to decarboxylate acetolactate
to acetoin. Instead, pyruvate and free acetaldehyde reacted to form
acetoin without the intermediate formation of acetolactate. By
chemically degrading the acetoin formed in this manner, Juni further
demonstrated that the carbonyl carbon and adjacent methyl group arose
from pyruvate, whereas the carbinol carbon and adjacent methyl group
were derived from acetaldehyde. Similar results were obtained by
Suomalainen and Linnahalme \((61)\) and Chuang and Collins \((11)\). When
extracts from yeast were incubated with pyruvate, thiamine pyrophosphate
and magnesium ions, acetoin was produced; however, when bisulfite was
added to the reaction mixture, the synthesis of acetoin was completely
inhibited. This is not surprising in view of the fact that bisulfite
is known to bind free acetaldehyde, thus making it unavailable for
enzymatic reactions \((46)\). Bisulfite does not inhibit acetoin formation.
in microorganisms which produce acetoin via the intermediate formation of acetolactate because there is no free aldehyde group, per se, available for nucleophilic attack by the bisulfite ion. A similar mechanism for acetoin formation was demonstrated by DeLey (12) in certain species of Acetobacter, but studies with other bacteria indicated that free acetaldehyde was not involved in the biosynthesis of acetoin (21, 27, 30, 45, 58). The mechanism of acetoin formation in yeasts and mammalian tissues may be summarized as follows:

\[
\begin{align*}
\text{CO}_2 & \rightarrow \text{pyruvate} \\
& \rightarrow \text{"active acetaldehyde"} \\
& \rightarrow \text{acetoin} \\
& \rightarrow \text{free acetaldehyde}
\end{align*}
\]

Historically, it has been assumed that acetoin is the immediate precursor of both diacetyl and butylene glycol, the latter two compounds arising after the enzymatic oxidation and reduction of acetoin, respectively. However, recent evidence indicates that diacetyl biosynthesis does not occur via the intermediate formation of acetoin.

Indirect evidence that acetoin is not oxidized to diacetyl was obtained as early as 1936 by Michaelian and Hammer (42) when they found that the addition of acetoin to growing cultures of diacetyl-producing bacteria failed to increase the yield of diacetyl. Using a partially-purified enzyme preparation from Staphylococcus aureus, Strecker and Harary (60) found reduced nicotinamide adenine dinucleotide (NADH) was rapidly oxidized in the presence of diacetyl and acetoin. In the
same study, a partially-purified preparation from *E. aerogenes* catalyzed the reduction of NAD in the presence of butylene glycol; however, neither the staphylococcal enzyme, nor the preparation from *E. aerogenes* was able to reduce NAD in the presence of acetoin. Gabriel *et al.* (14) and Hetland, Stormer and their colleagues (10, 19, 20) obtained identical results with highly purified extracts of mouse liver and *E. aerogenes*, respectively, and Seitz *et al.* (57) have obtained similar results with crude extracts from *Streptococcus diacetilactis*. Further evidence indicating that diacetyl does not arise from acetoin was obtained in a study by Suomalainen and Linnahalme (61). Although bisulfite completely inhibited the enzymatic conversion of pyruvate and acetaldehyde to acetoin by extracts of brewer's yeast, it had no effect on the formation of diacetyl.

Recently, Speckman and Collins (59) developed a method whereby diacetyl, acetoin and butylene glycol may be separated from each other by salting-out chromatography. Using this method to separate the products of enzymatic reaction mixtures, Collins and his associates have studied the mechanism of diacetyl biosynthesis in a number of microorganisms (11, 58). When dialyzed extracts from *S. diacetilactis* and *Leuconostoc citrovorum* (58) were incubated in the presence of pyruvate-3-14C, thiamine pyrophosphate (TPP) and magnesium ions, radioactive label appeared in both diacetyl and acetoin. However, when acetolactate was the substrate, only acetoin was formed. Thus these workers were able to demonstrate that acetolactate is not involved in the biosynthesis of diacetyl. Furthermore, the addition of unlabeled
acetoin to a reaction mixture containing pyruvate-3$^{14}$C failed to dilute out the amount of radioactivity entering diacetyl. Definitive evidence concerning the mechanism of diacetyl biosynthesis came from the demonstration that extracts treated with an anion exchange resin to remove coenzyme A formed only acetoin in the presence of pyruvate, Mg$^{++}$ ions and TPP. Diacetyl-synthesizing capabilities were restored to the extract only when acetyl-CoA was added to the reaction mixture; acetyl-phosphate would not replace acetyl-CoA in the reaction. The absolute role of acetyl-CoA in diacetyl biosynthesis was confirmed in an experiment where acetyl-CoA-1$^{14}$C was included in a test system containing unlabeled pyruvate, TPP, Mg$^{++}$ ions and resin-treated extract protein. The diacetyl recovered from the reaction mixture was highly labeled, but no radioactivity was found in acetoin (58). Identical results have been obtained by Chuang and Collins (11) with extracts from Lactobacillus brevis, Pseudomonas fluorescens, E. aerogenes and S. cerevisiae.

**Origin of the o-xylene ring of riboflavin**

Riboflavin synthetase catalyzes the terminal step in the biosynthesis of riboflavin. Plaut has shown that the reaction involves the removal and transfer of four carbon atoms from one molecule of DMRL to a second molecule of the lumazine (50). Thus carbons 6, 7 and the methyl groups of DMRL become carbons 5, 6, 7, 8, 8a, 10a and the methyl groups of riboflavin (see Figure 2). It becomes apparent, then, that any study undertaken to determine the origin of the aromatic ring of riboflavin must ultimately be concerned with resolving the origin of carbons 6, 7
and the methyl groups of DMRL.

4-Ribitylamino-5-amino-2,6-dihydroxypyrimidine has been implicated as a biosynthetic intermediate in the riboflavin pathway by the demonstration that the pyrimidine accumulates in the supernatant fluid of riboflavinless mutants (31, 54, 63); moreover, the same pyrimidine is the second product of the riboflavin synthetase reaction (Figure 2). In order for 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to be converted to DMRL, four carbon atoms must be added to the pyrimidine to complete the dimethylpyrazine ring of DMRL. Maley and Plaut (37) and Birch and Moye (8) succeeded in the chemical synthesis of DMRL by condensing 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine with diacetyl. Goodwin and Treble (16) and Masuda et al. (41) found extracts from E. ashbyii were able to catalyze the formation of DMRL and riboflavin from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and acetoin. Katagiri et al. (26) confirmed these results with partially-purified extracts from E. aerogenes and E. ashbyii, but they found a very high concentration of acetoin (50 umoles) was necessary before DMRL and riboflavin could be detected in their reaction mixtures. In addition, these workers found extracts from E. aerogenes were able to convert 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to DMRL and riboflavin in the presence of pyruvate and TPP. They concluded that acetoin was probably oxidized to diacetyl by their extracts, and the diacetyl reacted non-enzymatically with the pyrimidine to form DMRL; however, this conclusion seems questionable in view of the fact that numerous workers have demonstrated E. aerogenes is unable to oxidize acetoin to
Figure 2. The riboflavin synthetase reaction; conversion of 6,7-dimethyl-8-ribityllumazine to riboflavin and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine.
2 6,7-Dimethyl-8-ribityllumazine

\[ \text{Riboflavin Synthetase} \]

\[ \text{Riboflavin} \]

4-Ribitylamino-5-amino-2,6-dihydroxypyrimidine
diacetyl (10, 11, 60).

Goodwin and Treble (16) and Goodwin and Horton (15) reported growing cells of *E. ashbyii* incorporated radioactively labeled acetoin into the o-xylene ring of riboflavin. Ali and al-Khalidi (1), on the other hand, were unable to demonstrate efficient incorporation of acetoin into riboflavin with intact cells of *E. ashbyii*. Furthermore, they demonstrated that pyruvate-2-$^{14}$C was a much more effective precursor of acetoin than was acetate-1-$^{14}$C, but the pyruvate was not incorporated into riboflavin with any greater efficiency than the radioactive acetate. These results suggest acetoin is not the source of the four carbon atoms that react with 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to form DMRL.

Plaut (19) found that growing cells of *A. gossypii* incorporated radioactivity from acetate-1-$^{14}$C primarily into carbons 6, 7, 8a and 10a of riboflavin (carbons 6 and 7 of DMRL), while radioactivity from acetate-2-$^{14}$C labeled all of the carbons of the o-xylene ring of riboflavin; however, two-thirds of the label from acetate-2-$^{14}$C appeared in carbons 5, 8 and the methyl groups of riboflavin (corresponding to the methyl groups of DMRL), and one-third of the label was found in carbons 6, 7, 8a and 10a of the vitamin. In a second experiment, glucose-1-$^{14}$C and glucose-6-$^{14}$C were incorporated with 90% efficiency into carbons 5, 8 and the methyl groups of riboflavin. For the following reasons, Plaut concluded that glucose is a more immediate precursor of the "four-carbon unit" than is acetate: If acetate was a more direct precursor than glucose, then glucose would presumably have been converted to acetate before it entered DMRL and riboflavin. Glucose-1-$^{14}$C and
glucose-6-$^{14}\text{C}$ would presumably lead to acetate-2-$^{14}\text{C}$ via glycolysis; thus the two radioactive species of glucose should have been incorporated into riboflavin with the same labeling pattern as was obtained with the authentic acetate-2-$^{14}\text{C}$. Ali and al-Khalidi (1) obtained evidence in support of this view when they demonstrated with growing and resting cells of *E. ashbyii* that unlabeled glucose extensively diluted out the amount of acetate-2-$^{14}\text{C}$ incorporated into the o-xylene ring of riboflavin. However, an alternative interpretation of the results obtained by these workers is possible and will be discussed (see DISCUSSION).
EXPERIMENTAL PROCEDURE

Stock cultures of the wild-type and riboflavin-dependent strains of *S. epidermidis* (63) were maintained on slants of brain-heart infusion agar (BHI, Difco). A riboflavinless mutant of *B. subtilis* 168 (63) was maintained on slants of trypticase-soy agar (TSA, BBL) supplemented with riboflavin (1 μg/ml). All cultures were stored at 4°C and were transferred at one-month intervals.

**Semi-synthetic medium**

Cell-free extracts were obtained from cells grown in a semi-synthetic medium. A stock solution of salts contained: \( K_2HPO_4 \), 70 g; \( KH_2PO_4 \), 20 g; \( Na_2\text{citrate}\cdot5H_2O \), 5 g; \( MgSO_4\cdot7H_2O \), 0.5 g; \( (NH_4)_2SO_4 \), 10 g; and enough deionized water to give a final volume of 1 liter. To avoid the formation of a precipitate, magnesium sulfate was dissolved first, then the remaining salts were added. A stock solution of vitamins contained: nicotinic acid, 100 mg; thiamine•HCl, 100 mg; D-biotin, 0.5 mg; calcium pantothenate, 25 mg; and deionized water, 100 ml. Separate stock solutions of L-tryptophan (1 mg/ml) and L-cystine (2 mg/ml) were prepared. The cystine was dissolved by adding 0.8 ml of concentrated hydrochloric acid to 200 mg of cystine suspended in 99.2 ml of water. All stock solutions were stored at 4°C.

The final growth medium contained: vitamin-free casamino acids, 6 g; glucose, 5 g; vitamins solution, 1 ml; cystine solution, 10 ml; tryptophan solution, 10 ml; and deionized water, 880 ml. The medium was autoclaved for 15 minutes at 121°C. A 100-ml aliquot of the salts
solution was autoclaved separately and aseptically added to the medium just prior to inoculation. The final pH was 7.0. Whenever a solid medium was desired, Bacto-agar was added at a final concentration of 1.5% (w/v).

Riboflavin-dependent mutants of S. epidermidis were grown in the same medium, except riboflavin was added at a final concentration of 1 μg/ml.

**Synthesis of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine**

The steps leading to the chemical synthesis of DMRL have already been described and are depicted schematically in Figure 3. 4-Ribityl-

amino-5-aminoo-2,6-dihydroxypyrimid ure (VII, Figure 3) is an intermediate in the chemical synthesis of DMRL, so this procedure was also employed to synthesize the 5-amino pyrimidine. However, because 4-ribityl-

amino-5-aminoo-2,6-dihydroxypyrimidine is highly unstable (62), the synthesis was carried only to the formation of the more stable 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine (VI, Figure 3). The pyrimidine was stored as the 5-nitroso derivative and reduced to 4-ribitylamino-5-aminoo-2,6-dihydroxypyrimidine just before it was used. The following description for the synthesis of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine is a modification of the procedure employed earlier (63).

Crystalline 4-ribitylamino-2,6-dihydroxypyrimidine (V, Figure 3; 3.5 umoles) was dissolved in 10 ml of deionized water, the pH was
Figure 3. The chemical synthesis of 6,7-dimethyl-8-ribityllumazine; I, hydroxylamine; II, ribose oxime; III, ribitylamine; IV, 4-chloro-2,6-dihydroxypyrimidine; V, 4-ribitylamino-2,6-dihydroxypyrimidine; VI, 4-ribitylamino-5-nitroso-2,6-dihydroxopyrimidine; VII, 4-ribitylamino-5-amino-2,6-dihydroxopyrimidine; VIII, diacetyl; IX, 6,7-dimethyl-8-ribityllumazine.
adjusted to 5.7 with 1 N potassium hydroxide and 1 g of sodium nitrite was added. With constant stirring, the pH of the solution was slowly adjusted to 4.3 by the dropwise-addition of 3.5 N acetic acid (45 minutes were required). The solution was allowed to stand at room temperature for approximately two hours and the entire contents of the reaction mixture were lyophilized. The reddish-orange residue remaining after lyophilization was taken up in 70 ml of deionized water and the pH was adjusted to 10.6 with concentrated ammonium hydroxide. The red solution was percolated through a column (0.8 x 25 cm) of Amberlite IRA-400 (20 to 50 mesh, formate form) and the effluent (which contained a yellow fluorescent compound) was discarded. The column was washed with 100 ml of deionized water and then with 300 ml of 0.01 M formic acid. 4-Ribitylamino-5-nitroso-2,6-dihydroxypyrimidine was eluted from the anion exchanger with 0.1 M formic acid. Ten-ml fractions were collected. Aliquots (0.1 ml) from each fraction were spotted onto sheets (15 x 45 cm) of Whatman 3MM chromatography paper and developed to a height of 25 cm with n-butanol:95% ethanol:water (50:16:35). Those fractions exhibiting a single, homogeneous UV-absorbing spot were pooled and carefully concentrated in a vacuum evaporator (Buchler Instruments, flash evaporator) until the 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine began to crystallize. The solution was then cooled in an ice bath whereupon the red crystals formed rapidly. The crystals were collected by filtration and dried in a vacuum oven at 40 C.
Preparation of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine

4-Ribitylamino-5-amino-2,6-dihydroxypyrimidine was prepared just before it was to be used by dissolving 6.5 mg (22.5 umoles) of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine in 5 ml of hot (80 to 85 °C) deionized water. Sodium hydrosulfite (13 mg) was added and the reduction of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine to the 5-amino derivative was accompanied by an immediate color change from pink to pale yellow.

Preparation of cell-free extracts

Each of six Fernbach flasks containing 1 liter of semi-synthetic medium was inoculated with 100 ml of an actively-growing culture of S. epidermidis. Cells were grown with shaking for 16 h at 37 °C. They were then harvested by centrifugation, washed three times with ice cold 0.1 M phosphate buffer (pH 7.4) and the washed cell-pellet was resuspended in 30 to 40 ml of the same buffer containing a thick slurry of Ballantini beads. The suspension was sonicated (Ratheon Sonic Oscillator, Model DF 101) for approximately 12 minutes at 2-minute intervals. The crude extract was decanted and the Ballantini beads were washed with additional 10-ml aliquots of phosphate buffer until 100 ml of crude extract had been collected. Cellular debris was removed by centrifugation at 27,000 x g for 15 minutes.

The crude cell-free extract (100 ml) was cooled in an ice bath and 57 g of solid ammonium sulfate were added. The suspension was stirred gently for one hour and the precipitate that formed was collected by centrifugation in the cold (4 °C) at 17,300 x g for 30
minutes. The precipitate was redissolved in 50 ml of 0.85% NaCl and used directly for enzyme assays.

The amount of protein in the extract was determined colorimetrically by the Biuret reaction (17).

**Enzyme assays**

Unless stated otherwise, the extracts from *S. epidermidis* were assayed in 3 ml of medium containing the appropriate substrate(s). In all cases, reactants were equilibrated to 37 C and the reactions were initiated by the addition of enzyme. Reactions were allowed to proceed for a specified period of time and were generally stopped by the addition of 0.5 ml of 25% trichloroacetic acid (TCA). For most controls, the TCA was added before the enzyme. Coagulated protein was removed from the reaction mixture by centrifugation and the amount of product that was formed during the incubation period was determined.

**Riboflavin synthetase** Reaction mixtures contained: DMRL, 0.3 umoles; NaHSO₃, 30 umoles; potassium phosphate buffer (pH 7.4), 0.3 mmoles; and extract protein (concentration varied). Reaction mixtures were incubated for one hour and the amount of riboflavin formed from DMRL was determined in a biological tube assay employing *Lactobacillus casei* ATCC 7469 (63).

**Enzymatic formation of diacetyl and acetoin** Reaction mixtures contained: Sodium pyruvate, 20 umoles; thiamine pyrophosphate, 0.25 umoles; MgSO₄·7 H₂O, 4.5 umoles; potassium phosphate buffer (pH 5.8) 0.3 mmoles; and extract protein (concentration varied). Reaction mixtures were incubated at 37 C for 30 minutes and the amount of diacetyl
and acetoin formed from pyruvate was determined using a modification of the procedure described by Westerfield (65).

**Enzymatic formation of DMRL** The complete reaction mixture contained: 4-Ribitylamino-5-amino-2,6-dihydroxypyrimidine (prepared just before use), 0.45 umoles; diacetyl, 0.45 umoles; potassium phosphate buffer (pH 7.4), 0.3 mmoles; and extract protein (concentration varied). Reactions were allowed to proceed for one hour and the amount of DMRL formed from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl was determined spectrophotometrically or in a biological tube assay employing a riboflavinless mutant of *B. subtilis* 168 (63).

**Spectrophotometric determination of DMRL**

The spectrophotometric assay described here makes use of the fact that the product of the reaction (DMRL) exhibits appreciable light absorbance at 405 nm (the molar extinction coefficient is 10,300 in neutral or acid solutions) whereas neither of the substrates (4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl) absorbs light at this wavelength.

The spectrophotometer was zeroed with a control tube to which the TCA had been added before the enzyme. The following equation was then used to determine the amount of DMRL present in the deproteinized reaction mixture:

\[
\frac{\text{optical density} @ 405 \text{ nm}}{10.3} = \text{umoles DMRL/ml of reaction mixture}
\]

To obtain the total concentration of DMRL in the reaction mixture,
the equation was multiplied by a factor of 3.5 (3-ml reaction mixture plus 0.5 ml of TCA added to stop the reaction).

Because riboflavin also exhibits considerable light absorption at 405 nm, workers should be cautioned that this equation may be used to calculate DMRL concentration only when the conversion of DMRL to riboflavin has been inhibited. Plaut (51) has described a spectrophotometric assay in which the amount of riboflavin formed in a reaction mixture may be calculated in the presence of DMRL.

Colorimetric determination of diacetyl and acetoin

The amount of diacetyl and acetoin present in the reaction mixtures was determined by a modification of the procedure described by Westerfield (65). At the end of the incubation period, 0.2 ml of the deproteinized reaction mixture was withdrawn and transferred to 0.8 ml of deionized water. To this were added in the following order: 1 ml of 0.5% creatine (aqueous; w/v) and 1 ml of 5% α-naphthol (5 g colorless α-naphthol/100 ml of 2.5 N NaOH; prepared just before use). The color was developed for 15 minutes at 37°C and the absorbance was read at 530 nm with a colorimeter (Bausch and Lomb, Spectronic 20). For each assay, a standard curve was constructed in which optical density was plotted against concentration of diacetyl (0 to 15 μg/tube).
RESULTS

Characteristics of synthetic 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine

A UV- and visible-light absorption spectrum of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine·HCl, as prepared by the method of Wacker et al. (64), exhibited a severe depression in the absorbance of light at 268 nm ($a_m = 8,900$ vs $24,500$). This indicated that the preparation contained a contaminating substance, and paper chromatography revealed the presence of a yellow fluorescent compound in addition to the UV-absorbing 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine. Regardless of variations in the procedure of synthesis and precautions taken to preclude the possibility of photochemical decomposition, preparations of the hydrochloride salt of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine always contained the contaminating yellow fluorescent compound. However, the fluorescent contaminant was never present on paper chromatograms when 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine was prepared freshly by hydrosulfite reduction of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine; moreover, a preliminary experiment demonstrated that sodium hydrosulfite did not interfere with riboflavin synthetase activity. Consequently, the pyrimidine was stored as 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine was prepared just before it was used.

Table 1 shows the $R_f$ values of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine in several different solvent systems. In addition, the $R_f$ values of
the products formed when freshly prepared 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine was condensed with diacetyl and glyoxal (63) are compared to the $R_f$ values obtained with authentic samples of DMRL and 8-ribityllumazine. In each of the solvent systems tested, the two different pyrimidines appeared individually as homogeneous spots when chromatograms were examined under ultraviolet light. Chemical condensation of the 5-amino derivative with diacetyl and glyoxal gave products whose chromatographic properties were identical to those of DMRL and 8-ribityllumazine, respectively.

The spectral properties of the two different pyrimidines are shown in Figures 4 and 5. In 0.1 N HCl, 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine (Figure 4) exhibits absorption maxima at 315 nm ($\epsilon_m = 9,000$) and 226 nm ($\epsilon_m = 15,100$) with a shoulder at 252 nm. In neutral and basic solutions, the pyrimidine has a single peak at 310 nm ($\epsilon_m = 17,100$). Katagiri et al. (26) reported that their synthetic 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine had a maximum peak at 312 nm in water; however, these workers did not report a molar extinction coefficient. The spectrum of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (Figure 5) exhibits characteristics that are identical to those reported by Wacker et al. (64). The single light-absorbing peak at 268 nm in 0.1 N HCl has a molar extinction coefficient of 24,500.
Table 1. Paper Chromatography. Mobility characteristics of 4-ribitylamino-5-nitroso-2,6-
dihydroxypyrimidine (4-RNP), 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (4-RAP),
and a comparison of the Rf values obtained after 4-ribitylamino-5-amino-2,6-dihy-
droxyprpyrimidine was chemically condensed with diacetyl and glyoxal to those obtained
with authentic DMRL and 8-ribityllumazine.

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>4-RNP</th>
<th>4-RAP</th>
<th>4-RAP + Diacetyl</th>
<th>DMRL</th>
<th>4-RAP + Glyoxal</th>
<th>8-Ribityllumazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Potassium Phosphate Buffer (pH 7.4)</td>
<td>0.65</td>
<td>0.30</td>
<td>0.70</td>
<td>0.69</td>
<td>0.68</td>
<td>0.69</td>
</tr>
<tr>
<td>3% NH₄Cl</td>
<td>0.68</td>
<td>-</td>
<td>0.74</td>
<td>0.74</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td>t-BuOH:NH₄OH:H₂O</td>
<td>0.52</td>
<td>0.38</td>
<td>0.40</td>
<td>0.39</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>60 : 5 : 35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-BuOH:Pyridine:H₂O</td>
<td>0.17</td>
<td>0.09</td>
<td>0.22</td>
<td>0.23</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>60 : 15 : 25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-BuOH:H₂O</td>
<td>0.36</td>
<td>0.29</td>
<td>0.38</td>
<td>0.38</td>
<td>0.32</td>
<td>0.31</td>
</tr>
<tr>
<td>6 : 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-BuOH:Pyridine:H₂O</td>
<td>0.59</td>
<td>0.48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 : 4 : 7</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Samples (50 µg) were spotted onto sheets (15 x 45 cm) of Whatman 3MM paper and developed to a height of 25 cm in the dark. Chromatograms were examined in the dark with ultraviolet (UV) light; the pyrimidines (U-RNP and 4-RAF) were identified as UV-absorbing spots and all other compounds as fluorescent spots.

The following abbreviations are used in this table: t-BuOH, tertiary-butanol; NH₄OH, concentrated ammonium hydroxide; n-BuOH: primary-butanol; EtOH, 95% ethanol; PrOH, primary-propanol; HAc, glacial acetic acid.

<table>
<thead>
<tr>
<th>n-BuOH:HAc:H₂O</th>
<th>0.47</th>
<th>-</th>
<th>0.57</th>
<th>0.57</th>
<th>0.49</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-BuOH:EtOH:H₂O</td>
<td>0.25</td>
<td>0.18</td>
<td>0.32</td>
<td>0.31</td>
<td>0.24</td>
<td>0.23</td>
</tr>
<tr>
<td>PrOH:NH₄OH:H₂O</td>
<td>0.51</td>
<td>0.31</td>
<td>0.57</td>
<td>0.57</td>
<td>0.49</td>
<td>0.49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>-</th>
<th>0.57</th>
<th>0.57</th>
<th>0.49</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-BuOH:EtOH:H₂O</td>
<td>0.25</td>
<td>0.18</td>
<td>0.32</td>
<td>0.31</td>
<td>0.24</td>
<td>0.23</td>
</tr>
<tr>
<td>PrOH:NH₄OH:H₂O</td>
<td>0.51</td>
<td>0.31</td>
<td>0.57</td>
<td>0.57</td>
<td>0.49</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Samples (50 µg) were spotted onto sheets (15 x 45 cm) of Whatman 3MM paper and developed to a height of 25 cm in the dark. Chromatograms were examined in the dark with ultraviolet (UV) light; the pyrimidines (U-RNP and 4-RAF) were identified as UV-absorbing spots and all other compounds as fluorescent spots.

The following abbreviations are used in this table: t-BuOH, tertiary-butanol; NH₄OH, concentrated ammonium hydroxide; n-BuOH: primary-butanol; EtOH, 95% ethanol; PrOH, primary-propanol; HAc, glacial acetic acid.

<table>
<thead>
<tr>
<th>n-BuOH:HAc:H₂O</th>
<th>0.47</th>
<th>-</th>
<th>0.57</th>
<th>0.57</th>
<th>0.49</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-BuOH:EtOH:H₂O</td>
<td>0.25</td>
<td>0.18</td>
<td>0.32</td>
<td>0.31</td>
<td>0.24</td>
<td>0.23</td>
</tr>
<tr>
<td>PrOH:NH₄OH:H₂O</td>
<td>0.51</td>
<td>0.31</td>
<td>0.57</td>
<td>0.57</td>
<td>0.49</td>
<td>0.49</td>
</tr>
</tbody>
</table>

The following abbreviations are used in this table: t-BuOH, tertiary-butanol; NH₄OH, concentrated ammonium hydroxide; n-BuOH: primary-butanol; EtOH, 95% ethanol; PrOH, primary-propanol; HAc, glacial acetic acid.

Not determined.
Figure 4. Absorption spectrum of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine (0.0345 umoles per ml); ————, 0.1 N HCl; ————, 0.1 M potassium phosphate buffer (pH 7.0); ······, 0.1 N NaOH.
Figure 5. Absorption spectrum of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (0.0172 umoles per ml); --------, 0.1 N HCl; --------, 0.1 M potassium phosphate buffer (pH 7.0); ···········, 0.1 N NaOH.
Enzymatic formation of riboflavin from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine.

It is now well-documented that DMRL is the immediate precursor of riboflavin (52). 4-Ribitylamino-5-amino-2,6-dihydroxypyrimidine has been implicated as a biosynthetic intermediate in the riboflavin pathway for two reasons: (1) the pyrimidine is excreted by riboflavin-dependent mutants (31, 54, 63); and (2) the pyrimidine is structurally quite similar to DMRL. Only four carbon atoms are required to convert 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to DMRL.

Table 2 shows the results of an experiment designed to gain some information concerning the nature of those four carbon atoms. Extracts from the wild-type strain of S. epidermidis were incubated with 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and potential four-carbon compounds or precursors of four-carbon compounds. Reaction mixtures were incubated for 60 minutes at 37 C and the amount of riboflavin formed from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and potential four-carbon donors was determined in a biological assay with L. casei ATCC 7469 (63). Controls contained heat-inactivated enzyme.

The DMRL-control is simply an assay for riboflavin synthetase activity and indicates that any DMRL formed from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine is capable of being further converted to riboflavin. The eighteen amino acids tested in this assay were the L-isomers of: phenylalanine, tryptophan, tyrosine, isoleucine, leucine, valine, aspartic acid, glutamic acid, arginine, histidine, lysine, alanine, serine, methionine, cystine, proline and threonine. The effect of glycine was also examined. It is apparent from Table 2 that 4-ribityl-
amino-5-amino-2,6-dihydroxypyrimidine is converted to riboflavin only when diacetyl is present in the reaction mixture.

Table 2. The ability of several compounds to react enzymatically with 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to form riboflavin.

<table>
<thead>
<tr>
<th>Compounds Tested</th>
<th>Concentration per Reaction Mixture (umoles)</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acids (18)</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Amino Acids + pyridoxal-PO₄</td>
<td>0.45 0.25</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Malic Acid</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fumaric Acid</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Oxalacetic Acid</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Succinic Acid</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>0.45</td>
<td>1.72</td>
</tr>
<tr>
<td>Acetoin</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Acetoin + NAD or NADP</td>
<td>0.45 0.15</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DMRL-Control</td>
<td>0.45</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Assays were performed at 37 °C in 3 ml of medium containing: 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine, 0.45 umoles; potassium phosphate buffer (pH 7.4), 0.3 mmoles; extract protein, 6.5 mg; and potential four-carbon donors at the concentrations indicated. Specific activity is expressed as millimoles riboflavin formed/mg protein/hour.
Evidence for the enzymatic formation of DMRL from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl

The structural gene coding for riboflavin synthetase is defective in the RibA class of staphylococcal mutants and they excrete DMRL (63). Because the RibA mutants are unable to accomplish the in vitro conversion of DMRL to riboflavin, they represent a source of enzyme that is ideal for studying the enzymatic conversion of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine to DMRL. Table 3 shows the results of an experiment in which extracts from *S. epidermidis* Rib-4 and the RibA mutants were incubated with 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl. Controls for each reaction mixture contained heat-inactivated (boiling water bath, 20 minutes) enzyme. The amount of DMRL formed in each reaction mixture was determined biologically with a riboflavinless mutant of *B. subtilis* 168, while the amount of riboflavin formed from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine was determined in a biological tube assay employing *L. casei* ATCC 7469 (63). The *B. subtilis* mutant is known to exhibit a dose response to both DMRL and riboflavin; however, *L. casei* will respond only to riboflavin. The results obtained with the RibA mutants indicate that the growth exhibited by the *B. subtilis* mutant was in response to DMRL only. Less than 0.05 nmoles of riboflavin was produced in the reaction mixtures containing extracts of RibA mutants.

Although controls in this experiment contained heat-inactivated enzyme, it might be argued that the DMRL to which the *B. subtilis* mutant responded was not formed enzymatically, but arose spontaneously as a result of the autoclaving process in preparation for the biological
Table 3. The formation of DMRL from L-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl by RibA mutants of S. epidermidis and by mutant Rib-4

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Riboflavin Formed</th>
<th>DMRL Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>RibA1</td>
<td>&lt;0.05</td>
<td>2.34</td>
</tr>
<tr>
<td>RibA2</td>
<td>&lt;0.05</td>
<td>1.78</td>
</tr>
<tr>
<td>RibA5</td>
<td>&lt;0.05</td>
<td>3.25</td>
</tr>
<tr>
<td>RibA6</td>
<td>&lt;0.05</td>
<td>3.36</td>
</tr>
<tr>
<td>RibA8</td>
<td>&lt;0.05</td>
<td>2.25</td>
</tr>
<tr>
<td>RibA16</td>
<td>&lt;0.05</td>
<td>6.45</td>
</tr>
<tr>
<td>RibA17</td>
<td>&lt;0.05</td>
<td>4.50</td>
</tr>
<tr>
<td>Rib-4</td>
<td>0.97</td>
<td>2.35</td>
</tr>
</tbody>
</table>

All reactions were performed at 37 °C in 3.0 ml of medium containing: L-ribitylamino-5-amino-2,6-dihydroxypyrimidine, 0.45 umoles; diacetyl, 0.45 umoles; potassium phosphate buffer, 0.3 mmoles; and extract protein (concentration varied between 3 and 6 mg extract protein per reaction mixture). Reactions were allowed to proceed for 1 hour and figures refer to the nmoles of riboflavin or DMRL formed/mg protein/hour.

In order to circumvent this criticism, L-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl were separated from DMRL by percolating reaction mixtures through a column of activated magnesium silicate (Florisil, 60 to 100 mesh; J. T. Baker Chemical Company).

Figure 6 shows the elution profile of authentic samples of L-ribitylamino-5-amino-2,6-dihydroxypyrimidine, diacetyl and DMRL from a column (1 x 3 cm) of Florisil. Samples (0.45 umoles in 3.5 ml of
Figure 6. Elution profile of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (RAP), diacetyl and 6,7-dimethyl-8-ribityllumazine (DMRL) from a column (1 x 3 cm) of Florisil.
3.6% TCA) of each compound were individually added to the column and it was washed first with 10 ml of deionized water, then with 10 ml of 50% aqueous acetone (v/v). One-ml fractions were collected; each acetone-containing fraction was placed onto a watch glass, dried in a stream of warm (45 C) air and the residue was redissolved in 1 ml of deionized water. The elution of diacetyl from the Florisil column was monitored by analyzing each fraction according to the modified Westerfield procedure. DMRL and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine were identified by monitoring the absorbance of light at 405 nm and 268 nm, respectively. Neither diacetyl nor 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine demonstrated an affinity for the Florisil and both compounds were eluted in fractions 2 through 8. DMRL, on the other hand, was retained on the Florisil and could only be eluted by the addition of 50% aqueous acetone.

The Florisil column was used to separate components of reactions mixtures that contained extracts from S. epidermidis RibAl6. At the end of the incubation period, TCA was added to each of the reaction mixtures, coagulated protein was removed by centrifugation and the entire contents of the deproteinized reaction mixture were immediately percolated through a column of Florisil. Fractions 14 through 22 were pooled, dried, and the residue that remained after drying was redissolved in 1 ml of deionized water. The amount of DMRL formed from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl in each of the reaction mixtures was determined spectrophotometrically. The results shown in Table 4 indicate that DMRL was formed only in the
Table 4. Enzymatic formation of 6,7-dimethyl-8-ribityllumazine from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (4-RAP) and diacetyl with partially-purified extracts from S. epidermidis RibA16.

<table>
<thead>
<tr>
<th>Reaction Mixture Components</th>
<th>Concentration (umoles)</th>
<th>Enzymatic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-RAP</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Diacetyl</td>
<td>0.45</td>
<td>9.0</td>
</tr>
<tr>
<td>Phosphate buffer (pH 7.4)</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Extract protein (2.8 mg)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Controls

- RAP omitted
- Diacetyl omitted
- Extract protein heat-inactivated
- Extract protein TCA-inactivated
- Bovine serum albumin replaced extract protein

Expressed as nmoles of DMRL formed/mg protein/h.

To insure that the compound eluted from the Florisil in fractions 14 through 22 was DMRL, and to further demonstrate that DMRL was formed only in the complete reaction mixture, aliquots of each reaction mixture were subjected to paper chromatography. Samples (0.1 ml) were spotted onto sheets (15 x 45 cm) of Whatman 3MM chromatography paper and developed to a height of 25 cm in 0.1 M potassium phosphate buffer (pH 7.0) and n-butanol:glacial acetic acid:water (200:50:75). When the chromatograms were viewed under ultraviolet light, a fluorescent spot was detected only in the sample that corresponded to the complete reaction mixture. Furthermore, the compound co-chromato-
graphed with authentic DMRL (n-butanol:glacial acetic acid:water, Rf 0.31; 0.1 M potassium phosphate buffer, Rf 0.69). Further evidence that DMRL is not formed spontaneously under the conditions described in Table 4 comes from the observation that no DMRL could be detected in a reaction mixture lacking cell-free extracts even after the substrates had been incubated for four hours at 37 C.

Figure 7 illustrates the results of an experiment in which the concentration of extract protein in several reaction mixtures was varied, while all other variables remained constant. The graph shows that there is a linear relationship between protein concentration and the amount of DMRL formed per hour.

The optimum pH for the enzymatic formation of DMRL from 4-ribityl-amino-5-amino-2,6-dihydroxypyrimidine and diacetyl was determined by varying the pH over a range from 4.5 to 8.5 with potassium phosphate buffer. As shown in Figure 8, the enzyme exhibited a broad range of activity with maximum DMRL formation occurring at pH 7.2.

**Combined biosynthesis of diacetyl and acetoin**

In most bacteria, acetoin is produced from pyruvate via the intermediate formation of acetolactate (21, 24, 58). For many years, it was presumed that diacetyl arose from acetoin by enzymatic oxidation; however, recent studies indicate that diacetyl is biosynthesized by a direct reaction between acetyl-CoA and an "active acetaldehyde" moiety derived from pyruvate (11, 58). The results of experiments in this study indicate that diacetyl is directly involved in riboflavin
Figure 7. Effect of protein concentration on the conversion of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl to 6,7-dimethyl-8-ribityllumazine (DMRL). Reactions were performed at 37 C in 3.0 ml of medium containing: Diacetyl, 0.45 umoles; 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine, 0.45 umoles; and potassium phosphate buffer (pH 7.4), 0.3 mmoles. The amount of DMRL formed in each reaction mixture was determined spectrophotometrically. Extracts were obtained from S. epidermidis RibAl6.
Figure 8. Effect of pH on the conversion of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and diacetyl to 6,7-dimethyl-8-ribityllumazine by extracts from S. epidermidis RibA16. The reaction conditions were the same as described in Figure 7, except each reaction mixture contained 5.5 mg of extract protein. The amount of DMRL formed in each reaction mixture was determined spectrophotometrically.
nmoles DMRL formed / mg protein / hour

pH
biosynthesis; consequently, it became necessary to determine whether or not diacetyl is synthesized by *S. epidermidis*. Unfortunately, both diacetyl and acetoin arise from the same precursor (pyruvate), and the modified Westerfield procedure employed to determine the amount of acetoin and diacetyl present in reaction mixtures does not differentiate between the two compounds. Thus readers must realize that the results presented in the following figures and tables actually represent the sum of diacetyl and acetoin produced in the reaction mixtures.

The optimum pH for the formation of diacetyl and acetoin was determined with partially-purified extracts from the wild-type strain of *S. epidermidis*. The pH was increased by units of 0.2 to 0.3 over a range from 4.2 to 7.0 using 0.1 M potassium phosphate buffer. As shown in Figure 9, the enzymes involved in the combined biosynthesis of diacetyl and acetoin exhibited highest activity between pH 5.5 and 6.1 with maximum combined activity occurring at pH 5.8.

Partially-purified extracts from several riboflavin-dependent mutants of *S. epidermidis* were also assayed for their ability to produce acetoin and diacetyl. The results shown in Table 5 indicate that all the strains can produce diacetyl, acetoin, or diacetyl and acetoin.

Table 6 shows the effect that each of the components of a reaction mixture has on the combined formation of diacetyl and acetoin. When components of a complete reaction mixture were individually omitted, there was a substantial decrease in the formation of acetoin and diacetyl. A specific activity of 0.05 represents the lower limit
Figure 9. The effect of pH on the combined formation of diacetyl and acetoin. Reactions were carried out at 37°C in 3.0 ml of medium containing: sodium pyruvate, 20 umoles; thiamine pyrophosphate, 0.25 umoles; MgSO4·7 H2O, 4.5 umoles; extract protein, 5.75 mg; and potassium phosphate buffer at the indicated pH, 0.3 mmoles. The combined amount of diacetyl and acetoin produced in each reaction mixture was determined by the modified Westerfield procedure.
of detection of acetoin and diacetyl when the modified Westerfield procedure is employed to quantify the two compounds.

Table 5. The ability of partially-purified extracts from several riboflavin-dependent mutants and the wild-type S. epidermidis to produce acetoin and diacetyl from pyruvate.\(^a\)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Specific Activity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RibA1</td>
<td>0.98</td>
</tr>
<tr>
<td>RibA2</td>
<td>1.12</td>
</tr>
<tr>
<td>RibA5</td>
<td>0.93</td>
</tr>
<tr>
<td>RibA6</td>
<td>0.95</td>
</tr>
<tr>
<td>RibA8</td>
<td>1.03</td>
</tr>
<tr>
<td>RibA16</td>
<td>0.97</td>
</tr>
<tr>
<td>RibA17</td>
<td>0.97</td>
</tr>
<tr>
<td>Rlb-4</td>
<td>0.84</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.09</td>
</tr>
</tbody>
</table>

\(^a\) Conditions of the reaction are described in Figure 9.

\(^b\) Expressed as the combined umoles diacetyl and acetoin formed/mg protein/hour.
Table 6. Conditions necessary for maximum combined formation of diacetyl and acetoin by extracts from the wild-type *S. epidermidis*.

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>Component Concentration (umoles)</th>
<th>Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent of Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complete Mixture</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine pyrophosphate</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>4.5</td>
<td>3.13</td>
<td>100</td>
</tr>
<tr>
<td>Phosphate buffer (pH 6.1)</td>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract protein (5.75 mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Omissions from Complete</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.10</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Thiamine pyrophosphate</td>
<td>0.05</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>1.69</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Extract protein (TCA-inactivated)</td>
<td>0.05</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as the combined umoles of diacetyl and acetoin formed/mg protein/hour.
DISCUSSION

Any worker attempting to elucidate an unknown biosynthetic pathway has at his disposal only a few experimental approaches that may be employed effectively. In studying the biosynthesis of riboflavin, one of the most effective approaches has involved the isolation and identification of naturally-occurring compounds that are excreted by riboflavin auxotrophs when the mutants are shifted from a medium containing riboflavin to one devoid of the vitamin. Thus 2,4,5-triamino-6-hydroxypyrimidine (3, 6), 4-ribitylamino-2,5-diamino-6-hydroxypyrimidine (2), 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (4-RAP; 31, 54, 63) and DMRL (29, 31, 40, 55, 63) have been implicated as biosynthetic intermediates in the riboflavin pathway (Figure 1). Despite this seeming wealth of information, it is generally recognized that these compounds cannot be claimed as actual biosynthetic intermediates because the methods of isolation do not preclude the possibility that the compounds are modified before or during the isolation procedure. Ultimately, before any compound may be claimed as an in vivo biosynthetic intermediate, it must be shown that the compound is enzymatically converted to another compound whose role in the pathway is already defined. As a result, the sequence of biochemical reactions leading to the formation of a given end product is often determined in reverse. In the riboflavin pathway, for example, only the terminal enzymatic reaction has been described. Riboflavin synthetase catalyzes the removal and transfer of carbons 6, 7 and the methyl groups from one
molecule of DMRL to a second molecule of the lumazine (52). The reaction yields one molecule of riboflavin and one molecule of 4-RAP (Figure 2).

4-RAP has been implicated as a biosynthetic intermediate in the riboflavin pathway for the following reasons: (i) the pyrimidine is excreted by riboflavin-dependent mutants (31, 54, 63); (ii) 4-RAP satisfies the growth requirement for a riboflavin auxotroph of Aspergillus nidulans (54); and (iii) 4-RAP is one of the products of the riboflavin synthetase reaction (65). In order for 4-RAP to be converted to DMRL (a well-established in vivo intermediate), only four carbon atoms must be inserted into the pyrimidine to complete the dimethylpyrazine ring of the lumazine. On structural grounds, then, 4-RAP is an attractive candidate for the immediate precursor of DMRL. This investigation has revealed that 4-RAP and diacetyl react enzymatically to form DMRL in S. epidermidis.

Chemical synthesis of biological intermediates

Wacker et al. (64) and Kishi et al. (28) succeeded in the chemical synthesis of the hydrochloride salt of 4-RAP by exposing aqueous solutions of the free pyrimidine to gaseous HCl and subsequent crystallization of the pyrimidine from 80% ethanol. I was unable to crystallize 4-RAP·HCl from ethanol and my preparations were always contaminated with a yellow fluorescent compound. Wacker et al. (64) and Kishi et al. (28) did not report the presence of a contaminating substance in their preparations, so it is likely that the
contaminating compound remained in the mother liquor when 4-RAP·HCl was crystallized from ethanol. It was necessary to eliminate the contaminant from my preparations of the pyrimidine because the fluorescent compound represented a potential inhibitor of enzymatic activity and interfered with the spectrophotometric assay for DMRL.

When 4-RAP was prepared freshly by hydrosulfite reduction of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine, no contaminating substance was detected by paper chromatography (Table 1), and the spectral properties of the freshly prepared pyrimidine (Figure 5) were identical to those reported by Wacker et al. (64). In addition, a chemical condensation of freshly prepared 4-RAP with glyoxal and diacetyl gave the expected products—namely 8-ribityllumazine and DMRL, respectively (Table 1). Molar extinction coefficients have not been reported for 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine, but Katagiri et al. (26) reported their synthetic 5-nitrosopyrimidine to have an absorption maximum of 312 nm in water. In potassium phosphate buffer (pH 7), my synthetic 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine exhibited an absorption maximum of 310 nm (Figure 4), and the pyrimidine appeared as a homogeneous spot when chromatographed in several different solvent systems (Table 1). Taken collectively, these results attest to the high purity of both of the pyrimidines.

Freshly-prepared solutions of 4-RAP appeared to remain stable for at least five hours, and the presence of sodium hydrosulfite in
solutions of the pyrimidine did not appear to have any deleterious effects on enzymatic activity. Thus the pyrimidine was stored as 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine and 4-RAP was prepared just prior to its use as a substrate. It is noteworthy that during the 10 to 12 months during which this investigation has progressed, the spectral and chromatographic properties of the synthetic 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine have not changed.

Enzymatic formation of DMRL

The role played by acetoin as the four-carbon moiety inserted into 4-RAP to form DMRL and subsequently riboflavin has been viewed with a great deal of caution. Several groups of workers claimed to have demonstrated the enzymatic synthesis of riboflavin from 4-RAP and acetoin, but others failed to confirm these results. Thus Kishi et al. (28) and Katagiri et al. (26) reported that extracts of E. ashbyii catalyzed the formation of riboflavin from 4-RAP and acetoin. The latter group of workers also reported that extracts from E. aerogenes accomplished the same reaction. Goodwin and Treble (16) reported that growing cells from E. ashbyii incorporated acetoin-2-\(^{14}\)C into carbons 5, 8 and the methyl groups of riboflavin. Their results seem curious in view of the fact that one would expect acetoin-2-\(^{14}\)C to label carbons 6, 7, 8a and 10a of riboflavin. In a later paper, Goodwin and Horton (15) cited Goodwin and Treble (16) and reported that the radioactive species of acetoin was labeled in
carbon-1. Ali and al-Khalidi (1) were unable to demonstrate the incorporation of radioactive acetoin into growing or resting cells of E. ashbyii. Their results are in direct conflict with those of Goodwin and Treble (16). The reason for the difference is not clear as Goodwin and Treble have never reported the complete details of their experiments.

Extracts of S. epidermidis do not catalyze the conversion of 4-RAP and acetoin to riboflavin (Table 2). Plaut (52) has suggested that in experiments where acetoin was supposed to have served as the four-carbon insertion compound, the microbial extracts simply catalyzed the oxidation of acetoin to diacetyl and the diacetyl then reacted non-enzymatically with 4-RAP to form DMRL. This is not the case in S. epidermidis unless a coenzyme other than NAD or NADP is utilized as the hydrogen-electron acceptor required for the enzymatic oxidation of acetoin to diacetyl (Table 2). Further evidence rendering Plaut's suggestion unlikely comes from the fact that several workers have been unable to demonstrate the oxidation of acetoin to diacetyl with cell-free extracts of microorganisms (10, 57, 58, 60).

Of the compounds examined in this study, only diacetyl was able to effect a net increase in the formation of riboflavin. The results presented in Table 2 do not permit a final conclusion concerning the role of diacetyl in the biosynthesis of riboflavin because the reaction mixtures contained extracts from the wild-type strain of S. epidermidis. The possibility that diacetyl and 4-RAP reacted spontaneously to yield DMRL cannot be precluded; any DMRL formed in this manner could presumably be converted to riboflavin by extracts from the wild-type strain.
In a previous study (63), ten riboflavin-dependent mutants of *S. epidermidis* were isolated and biochemically characterized. A summary of their excretion characteristics is shown in Figure 10. The RibA mutants excrete DMRL and are unable to accomplish the *in vitro* conversion of DMRL to riboflavin. Consequently, extracts from these mutants were employed as a source of enzyme to study the enzymatic formation of DMRL from 4-RAP and diacetyl. The fact that the mutants lacked riboflavin synthetase made it possible to study the enzymatic formation of DMRL with relatively crude enzyme preparations. The Rib-4 strain of *S. epidermidis* excretes 4-RAP and is presumed to lack either the enzyme catalyzing the insertion of a preformed four-carbon moiety into 4-RAP or a structural gene coding for the synthesis of the four-carbon unit.

Extracts from Rib-4 and all the RibA mutants of *S. epidermidis* were able to catalyze the conversion of 4-RAP to DMRL when diacetyl was present in reaction mixtures (Table 3). In view of these results, it does not seem likely that *S. epidermidis* Rib-4 lacks the enzyme responsible for inserting a preformed four-carbon moiety into 4-RAP.

The major concern regarding the role of diacetyl as the four-carbon unit inserted into 4-RAP to form DMRL is that the dicarbonyl condenses spontaneously with ortho-diamines under relatively mild chemical conditions (see Figure 3). The data shown in Table 3 were obtained through the use of biological assays employing *L. casei* ATCC 7469 (for riboflavin determinations) and a riboflavin auxotroph of *B. subtilis* 168 (for DMRL determinations). The *B. subtilis*
Figure 10. An abbreviated scheme for the biosynthesis of riboflavin in *S. epidermidis* showing excretion products and possible defective enzymes (X) in riboflavinless mutants.
Guanine derivative

4-Ribitylamino-5-amino-2,6-dihydroxypyrimidin (RAP)

Rib-4

4 Carbons

6,7-Dimethyl-8-ribityl-lumazine

RibA mutants

Riboflavin + RAP
mutant exhibits a dose response to both DMRL and riboflavin, but \textit{L. casei} will respond only to riboflavin (63). In the preparation of biological assays, aliquots of each reaction mixture were added to a medium (riboflavin assay medium, Gibco) devoid of riboflavin and DMRL. The medium was then autoclave-sterilized, cooled, and inoculated with the individual assay organisms. Thus the biological assay tubes contained 4-RAP and diacetyl during the autoclaving process; under these conditions (12 minutes at 121°C), the 4-RAP and diacetyl may have been spontaneously converted to DMRL. For this reason, a procedure was developed whereby 4-RAP and diacetyl were separated from DMRL (Figure 6).

Extracts from \textit{S. epidermidis} RibAl6 produced DMRL only when both 4-RAP and diacetyl were present in the reaction mixture (Table 4). When either of the substrates was omitted from the reaction mixture, no DMRL was formed. Furthermore, DMRL formation from 4-RAP and diacetyl was dependent upon the presence of active enzyme. No DMRL was produced in reaction mixtures containing heat- or TCA-inactivated enzyme, and bovine serum albumin would not replace the microbial extracts as a source of enzyme.

When the amount of extract protein in a set of reaction mixtures was varied while the concentrations of 4-RAP and diacetyl were held constant, there was a direct relationship between the amount of DMRL formed and the amount of protein in each reaction mixture (Figure 7). In this experiment, if 4-RAP and diacetyl had condensed spontaneously to produce DMRL, then the amount of DMRL present in each reaction
mixture should have remained constant. These results clearly establish the enzymatic nature of the reaction. Further evidence in support of the view that the reaction is enzymatic comes from the fact that the optimum pH for the enzymatic conversion of 4-RAP and diacetyl to DMRL is 7.2 (Figure 8); deviations from this pH resulted in lower yields of DMRL. Under the same conditions, but in the absence of enzyme, no DMRL was formed from 4-RAP and diacetyl even after the two compounds had been incubated together for four hours. The chemical condensation of the pyrimidine with diacetyl occurs best at pH 4 and at a temperature between 80 and 85 °C (37).

The biosynthesis of diacetyl

The results of this study have clearly established diacetyl as the four-carbon moiety reacting enzymatically with 4-RAP to form DMRL in S. epidermidis. If diacetyl is the only compound able to react enzymatically with 4-RAP to form DMRL, then it follows that S. epidermidis must be able to accomplish the de novo biosynthesis of diacetyl.

Historically, acetoin and diacetyl have been considered together from a biochemical point of view. It was presumed for many years that acetoin was an intermediate in the biosynthesis of diacetyl, the dicarbonyl arising from acetoin via enzymatic oxidation; however, this view became questionable when many workers were unable to demonstrate the enzymatic oxidation of acetoin to diacetyl in vitro (10, 57, 58, 60). Unfortunately, the most commonly employed procedure to assay for
acetoin and diacetyl does not differentiate between the two compounds (65) and results have always been expressed as the sum of diacetyl and acetoin. However, with the development of a technique whereby diacetyl and acetoin may be separated from each other, Speckman and Collins (58) have shown that diacetyl arises via a direct reaction between a molecule of acetyl-CoA and an "active acetaldehyde" moiety derived from pyruvate. Neither acetolactate nor acetoin was found as a biosynthetic intermediate in the formation of diacetyl.

The mechanism of diacetyl biosynthesis of *S. epidermidis* remains unclear. The results shown in Table 5 indicate that the RibA, Rib-4 and wild-type strains of *S. epidermidis* are capable of synthesizing diacetyl, acetoin or both of the compounds; however, attempts to separate diacetyl from acetoin by the method of Speckman and Collins (59) were unsuccessful. In order to achieve good separation of the two different compounds, Speckman and Collins required at least 25 ug of each four-carbon compound. It is possible that such a large quantity of diacetyl was not produced with extracts of *S. epidermidis*.

Insofar as the qualitative requirements for the combined formation of acetoin and diacetyl are concerned, the results obtained in this study are in agreement with results obtained by others (9, 21, 23, 57, 60). Single omissions of pyruvate, thiamine pyrophosphate or extract protein from reaction mixtures decreased the combined synthesis of diacetyl and acetoin by as much as 98%. When magnesium ions were omitted from the reaction mixture, the combined formation of diacetyl and acetoin was decreased by approximately 50%; however, undialyzed
extracts were employed for these determinations and it is likely that the preparation contained endogenous magnesium ions (Table 6).

The results obtained with extracts from the Rib-4 strain of S. epidermidis are somewhat confusing. The mutant excretes 4-RAP and it was presumed that this mutant either possessed a defective enzyme for the insertion of diacetyl into 4-RAP or lacked a structural gene coding for the synthesis of diacetyl. The results shown in Table 3 indicate that extracts from the Rib-4 mutant are able to accomplish the insertion reaction; however, the results shown in Table 5 indicate that extracts from this mutant are also able to synthesize either acetoin, diacetyl or both of the four-carbon compounds. In view of the fact that the method of assaying for diacetyl and acetoin does not differentiate between the two compounds, it is not possible to make a final conclusion regarding the nature of the defective enzyme in this mutant. It is not likely that the Rib-4 mutant lacks the pyruvic acid decarboxylase system (which leads to the formation of both "active acetaldehyde" and acetyl-CoA) as such a mutation would not be expressed as simple riboflavin auxotrophy. It is much more likely that this mutant is unable to effect a direct reaction between an "active acetaldehyde" moiety and acetyl-CoA, if such a mechanism of diacetyl biosynthesis occurs in S. epidermidis. A definitive answer awaits a more sensitive method for differentiating between diacetyl and acetoin.

A re-examination of the literature

Ali and al-Khalidi (1) were unable to demonstrate efficient incorporation of radioactive acetoin into riboflavin with intact cells.
of *E. ashbyii*. Furthermore, they demonstrated that resting cells of *E. ashbyii* incorporated pyruvate-2-\(^{14}\)C into acetoin 60 times as effectively as acetate-1-\(^{14}\)C, but the pyruvate was incorporated into riboflavin only 1.5 times as effectively as the acetate-1-\(^{14}\)C. These results were rightly taken as evidence excluding acetoin as a direct precursor of the o-xylene ring of riboflavin; however, these results in no way exclude diacetyl as the four-carbon unit inserted into 4-RAP to form DMRL (and subsequently the aromatic ring of riboflavin) if diacetyl biosynthesis in *E. ashbyii* occurs by a direct condensation reaction between a molecule of acetyl-CoA and an "active acetaldehyde" moiety derived from pyruvate (58).

In re-evaluating the experiments of Ali and al-Khalidi (1), readers must realize that the workers employed resting cells in their experimental design. Aside from endogenous precursors, the radioactive acetate or pyruvate added to the reaction vessels was the only source of carbohydrate available to the cells. Presumably, exogenously supplied acetate-1-\(^{14}\)C would be converted directly to acetyl-CoA-1-\(^{14}\)C. The passage of carboxyl-labeled acetyl-CoA through the tricarboxylic acid cycle and subsequent conversion to pyruvate would lead to the appearance of label only in the carboxyl group of pyruvate (66). The conversion of pyruvate-1-\(^{14}\)C to an "active acetaldehyde" moiety would result in the loss of the radioactive carbon as carbon dioxide. A direct reaction between the "active acetaldehyde" moiety generated in this manner with a second molecule of acetyl-CoA-1-\(^{14}\)C would lead to diacetyl labeled in only one of the carbonyl carbons. Pyruvate-2-\(^{14}\)C,
on the other hand, would lead to diacetyl labeled in both of the carbonyl carbons. The pyruvate decarboxylase system would be expected to decarboxylate pyruvate-2-$^{14}$C to acetyl-CoA-1-$^{14}$C with the intermediate formation of an "active acetaldehyde" moiety labeled in the "aldehyde" carbon. A direct reaction between the two radioactive species would generate a diacetyl molecule labeled in both of the carbonyl carbons. Accordingly, pyruvate-2-$^{14}$C would be expected to label riboflavin twice as effectively as acetate-1-$^{14}$C. Experimentally, Ali and al-Khalidi found that pyruvate-2-$^{14}$C labeled riboflavin 1.5 times as effectively as acetate-1-$^{14}$C. The experimental value of 1.5 to 1 is somewhat lower than the theoretical value of 2 to 1; however, the difference might be explained by the fact that pyruvate-2-$^{14}$C could actually generate additional pyruvate-1-$^{14}$C (via the tricarboxylic acid cycle). As pointed out earlier, pyruvate-1-$^{14}$C would not be expected to contribute any radioactivity to a diacetyl molecule. Thus it is possible that after several turns of the tricarboxylic acid cycle, the exogeneously supplied pyruvate-2-$^{14}$C could be in competition with pyruvate-1-$^{14}$C generated during the experiment.

Acetate-2-$^{14}$C was incorporated into riboflavin 2.5 times as effectively as pyruvate-2-$^{14}$C. These results are not surprising in view of the fact that the passage of acetate-2-$^{14}$C through the tricarboxylic acid cycle would generate pyruvate-2-$^{14}$C and pyruvate-3-$^{14}$C. Both radioactive species of pyruvate could then be converted to diacetyl without the loss of radioactive carbon; pyruvate-2-$^{14}$C would be expected
to label one of the carbonyl carbons of diacetyl, while pyruvate-3-^{14}C would label one of the methyl carbons of diacetyl. Similarly, the exogenously supplied acetate-2-^{14}C would be converted to methyl-labeled acetyl-CoA and radioactivity would appear in one of the methyl carbons of diacetyl. Unfortunately, Ali and al-Khalidi (1) were unable to isolate enough riboflavin to perform a Kuhn-Roth degradation (49); consequently, the position of radioactivity in the riboflavin molecule could not be determined.

A chemical degradation of the riboflavin produced when *A. gossypii* was grown in the presence of acetate-1-^{14}C demonstrated that the radioactive acetate entered carbons 6 plus 7 and 8a plus 10a with 90% efficiency (49). Furthermore, glucose-1-^{14}C and glucose-6-^{14}C were incorporated with 97% efficiency into carbons 5, 8 and the methyl groups of riboflavin. These are the results one would expect if diacetyl is the four-carbon insertion compound and is biosynthesized in *A. gossypii* by a direct reaction between "active acetaldehyde" and acetyl-CoA. Radioactive label from acetate-2-^{14}C was found in all the carbons of the o-xylene ring of riboflavin; however, 66% of the radioactivity appeared in carbons 5, 8 and the methyl groups of the vitamin and 34% of the radioactivity appeared in carbons 6, 7, 8a and 10a. As pointed out by Plaut (49), the more diffuse distribution of radioactivity obtained with the acetate-2-^{14}C may have been caused by randomization of the label in the tricarboxylic acid cycle. Ali and al-Khalidi (1) reasoned that glucose was a more immediate precursor of riboflavin than acetate because unlabeled glucose diluted out the amount of radio-
active acetate incorporated into riboflavin with resting cells of *E. asbyii*; however, it is important to recognize that the proportion of unlabeled glucose to radioactive acetate was 555 to 1 (on a molar basis). Theoretically, each mole of glucose could yield two moles of acetate. This would increase the ratio to greater than 1000 to 1. Thus it is not surprising that glucose diluted the amount of acetate incorporated into the aromatic ring of riboflavin.

I am not aware of any studies that have been undertaken to determine the mechanism of diacetyl biosynthesis of *E. asbyii* or *A. gossypii*. Plaut (49) and Ali and al-Khalidi (1) were unable to make any absolute conclusions concerning the origin of carbons 6, 7 and the methyl groups of DMRL; however, the results obtained by these workers may be readily explained if diacetyl is biosynthesized in *E. asbyii* and *A. gossypii* via a direct reaction between a molecule of "active acetaldehyde" and a molecule of acetyl-CoA. In view of the fact that diacetyl reacts enzymatically with 4-RAP to produce DMRL in *S. epidermidis*, a study to determine the mechanism of diacetyl biosynthesis in riboflavin-producing microorganisms seems in order.

Oltmanns et al. (47) have isolated two genetically distinct riboflavin-dependent mutants of *S. cerevisiae*, both of which excrete 4-RAP. It was supposed that one of the mutants lacked the enzyme necessary for the insertion of a four-carbon moiety into 4-RAP and the other was unable to synthesize the four-carbon moiety. It would be interesting to examine the enzymatic properties of these mutants in some
detail as Chuang and Collins (11) have established the mechanism by which diacetyl is synthesized in *S. cerevisiae*.

**The riboflavin pathway, future direction**

This investigation has clearly established the enzymatic nature of the reaction whereby diacetyl is inserted into 4-RAP to form DMRL. If diacetyl is the only compound capable of reacting with the pyrimidine to form DMRL, it follows that *S. epidermidis* must be able to biosynthesize the diacarbonyl. Definitive evidence in this area is lacking.

Although no data are presented, preliminary experiments indicate that acetoin is not oxidized to diacetyl by extracts of *S. epidermidis*. Extracts of *S. epidermidis* are able to produce acetoin, diacetyl or both acetoin and diacetyl from pyruvate; however, the modified Westerfield assay employed during the latter parts of this investigation does not differentiate between the two compounds and attempts to separate diacetyl and acetoin were not successful. Likewise, attempts to couple the enzyme system synthesizing diacetyl from pyruvate to the enzyme responsible for inserting diacetyl into 4-RAP gave inconsistent results.

In my opinion, then, definitive evidence must be obtained regarding the mechanism of diacetyl biosynthesis in riboflavin-producing microorganisms. Preliminary experiments dictate the need for a more sensitive method to quantify diacetyl. Indeed, the most commonly employed procedures of assaying for diacetyl require microgram quantities of the dicarbonyl while well-regulated microorganisms may produce the compound in only nanogram quantities.
SUMMARY

A method for the chemical synthesis of 4-ribitylamino-5-nitroso-2,6-dihydroxypyrimidine and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (4-RAP) is described. The chromatographic and spectral properties of both compounds are given.

Diacetyl is the four-carbon fragment that reacts enzymatically with 4-RAP to produce DMRL in S. epidermidis. The enzymatic nature of the reaction was proven by the demonstration that no DMRL is produced from 4-RAP and diacetyl in the absence of enzyme. Also, there is a direct relationship between the amount of enzyme present in reaction mixtures and the amount of DMRL produced. The optimum pH for the reaction is 7.2, and only 4-RAP, diacetyl, and enzyme are necessary for the production of DMRL.

The mechanism by which diacetyl is biosynthesized in S. epidermidis is less clear. Cell-free extracts of the wild type and several riboflavin-dependent strains of S. epidermidis catalyze the formation of diacetyl, acetoin, or both of the compounds; however, efforts to separate and identify the two compounds failed. Definitive evidence concerning the nature of the biosynthesis of diacetyl and acetoin awaits more reliable and sensitive methods for their separation and detection.
LITERATURE CITED


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