METABOLISM OF 2,6-DIAMINOPURINE: CONVERSION TO 5'-PHOSPHORIBOSYL-2-METHYLAMINO-6-AMINOPURINE 
BY ENZYMES OF ESCHERICHIA COLI

BY CHARLES N. REMY AND MARILYN S. SMITH

(From the General Medical Research Division, Veterans Administration Hospital, 
and the Department of Biochemistry, State University of New York, 
Medical College at Syracuse, Syracuse, New York)

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Since Hitchings et al. (1, 2) first demonstrated that 2,6-diaminopurine inhibited Lactobacillus casei, DAP has been shown to inhibit growth or multiplication in a wide variety of biological systems including mammals, tumors, birds, plants, viruses, and numerous bacteria. Although DAP is not incorporated per se into the nucleic acids, incorporation does occur after conversion to adenine and guanine (3-6). The metabolic fate of DAP generally parallels that of guanine. Thus, DAP has a dual metabolic fate, as it may serve either as an inhibitor or, when its inhibitory action is blocked, as a precursor for nucleic acid purines (5).

DAP may exert its inhibitory properties by being converted into inhibitory analogues of adenine-containing cofactors such as ATP, DPN, and FAD (5). The competition between adenine and DAP may occur either in the formation of the nucleotides or in their function. Wheeler and Skipper have shown that DAP is incorporated into the ATP fraction of mouse tissue (7). Kornberg and Pricer demonstrated that DAP riboside may be enzymatically phosphorylated to a DAP analogue of ATP (8).

To observe the phenomenon of DAP resistance in Escherichia coli, strain B, a study was conducted to determine whether growth inhibition by DAP could be correlated with the formation of DAP nucleotides. Although DAP nucleotides were isolated from the acid-soluble fraction, the isolation and identification of MeDAP and the corresponding 5'-phosphoribosyl derivative revealed that methylation of DAP at the 2-amino position was a new and quantitatively significant pathway of DAP metabolism in E. coli.

1 Abbreviations, DAP, 2,6-diaminopurine; MeDAP, 2-methylamino-6-aminopurine; meguanine, 2-methylamino-6-hydroxypurine; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; FAD, flavin adenine dinucleotide; TCA, trichloroacetic acid; E. coli/DAP, mutant of E. coli B resistant to DAP; DAPMP-5',5'-phosphoribosyl-2,6-diaminopurine; MeDAPMP-5',5'-phosphoribosyl-2-methylamino-6-aminopurine.

325
EXPERIMENTAL

Methods—E. coli B was cultured in the synthetic glucose-salts medium of Spizizen et al. (9). The inoculum was prepared by inoculating 10 ml. of the medium with the organism, carried on Difco nutrient agar slants, and incubating for 7 hours at 38° as a stationary culture. 1 ml. of inoculum was added to each 1 liter flask containing 600 ml. of salts solution and 10 ml. of 24 per cent glucose. The incubation was carried out as stationary cultures for 16 to 18 hours at 38°. The cells were harvested by centrifugation at 2°, washed with H₂O or dilute phosphate buffer, and resuspended in a small volume of 0.2 M phosphate (pH 7.0) in preparation for use as resting cell suspensions. An average of 218 mg. dry weight of cells was obtained per liter of culture medium.

The isolation of a mutant, E. coli/DAP, was accomplished by daily transfers of E. coli B through the synthetic glucose-salts medium containing 400 γ of DAP² per ml. After four such transfers, the mutant grew at a rate equal to that of the parent strain.

Paper chromatography was carried out at room temperature on Whatman No. 1 paper. The following solvents have been employed (all ratios are in volume proportions): (a) n-butanol-formic acid-H₂O (77:10:13) (10), (b) isopropanol-HCl-H₂O (170:41:39) (11), (c) isoamyl alcohol layered over 5 per cent Na₂HPO₄ (12), (d) isopropanol-H₂O-NH₃ (85:15:1.3) (13), (e) 70 per cent isopropanol with 0.35 ml. of concentrated NH₃ per liter of air space placed in the bottom of the chamber (14), (f) n-propanol-NH₃-H₂O (60:30:10) (15), (g) 62 per cent n-propanol, (h) n-butanol saturated with NH₃-H₂O (1:4) (16), (j) 86 per cent n-butanol with n-butanol-H₂O-NH₃ (86:9:5) in the chamber (10), (k) 86 per cent n-butanol (10), (l) tert-butanol-constant boiling HCl-H₂O (700:132:168) (17), (m) n-butanol saturated with 10 per cent urea (12); (n) 70 per cent isopropanol. With the exception of solvents (c) and (m), descending chromatography was employed. Purines were detected on paper chromatograms by means of a Mineralight lamp and were eluted for rechromatography by descending chromatography with H₂O or 0.01 N HCl. To obtain the spectrum of a component isolated by chromatography, the desired area and a control area, for use as a blank, were eluted with 5 to 7 ml. of H₂O or 0.01 N HCl for 18 to 24 hours with occasional shaking. Absorption measurements were made with a Beckman model DU spectrophotometer.

All radioactive determinations were carried out by counting infinitely

² DAP, L-methionine, and various purines, nucleosides, and nucleotides were obtained from the California Foundation for Biochemical Research and the Nutritional Biochemicals Corporation. The Pabst Brewing Company supplied the ATP and DPN. The Schwarz Laboratories supplied the barium salts of ribose 5-phosphate and 3-phosphoglyceric acid. The barium was removed with Na₂SO₄.
thin samples in SC-16 windowless flow counter from Tracerlab, Inc. Activity has been expressed as counts per minute.

**Results**

Isolation of MeDAP—The largest amounts of MeDAP were isolated from the medium of resting cell suspensions of *E. coli* (final volume of 40 ml.) containing the following per ml.: the equivalent of 14 to 16 mg. dry weight of cells, 100 μmoles of phosphate buffer (pH 7.0), 11 μmoles of glucose, and 0.36 μmole of DAP. The amount of MeDAP isolated was approximately linear with time for reaction periods of 15 to 180 minutes. After incubation for 180 minutes at 38°, the cells were removed by centrifugation and the supernatant fluid was concentrated in vacuo to 15 ml. Phosphate was removed by addition of a slight excess of 20 per cent barium acetate while keeping the pH at 8.0 by the addition of NaOH. The phosphate-free supernatant fluid was concentrated to 2.5 ml. and hydrolyzed in N HCl for 1 hour at 100°. The purines were precipitated as the silver salts by the addition of an excess of AgNO₃ and concentrated NH₃ (18). The silver salts were washed with H₂O and then extracted with a total of 5 to 7 ml. of 0.5 N HCl. After the HCl extract was evaporated to dryness, MeDAP was separated from the bulk of contaminating purines and pyrimidines by a 24 to 36 hour chromatogram by using solvent (a). The MeDAP band, fluorescing a distinct blue, was the fastest moving major component. Xanthine, the only other identified metabolic product of DAP (19), migrated slightly faster than DAP but was well separated from the MeDAP band. Ultraviolet-absorbing contaminants overlapping the leading and trailing edge of the MeDAP band were removed by re-chromatography in solvent (b) for 48 hours. At this stage MeDAP was free of ultraviolet contaminations, as judged by a consistent absorption spectrum and absence of evidence of additional compounds by chromatography in eight different solvents.

Smaller amounts of MeDAP were isolated from the TCA-soluble nucleotide fraction of cells incubated in a medium containing per ml.: the equivalent of 7 to 8 mg. dry weight of cells, 50 μmoles of phosphate buffer (pH 7.0), 8.9 μmoles of glucose, and 1.9 μmoles of DAP. The amount isolated reached a maximum after 45 to 90 minutes of incubation and then decreased slowly. The water-washed cells were extracted with 5 per cent TCA for 30 minutes at 2°, and then washed with 5 per cent TCA, and the combined extracts were extracted with ether to remove the TCA. The aqueous fraction was adjusted to pH 8.0 and the combined nucleotide fractions were precipitated by the addition of 0.1 volume of 20 per cent barium acetate plus 4 volumes of absolute alcohol. After 16 to 20 hours at 2°, the barium nucleotides were collected, washed with absolute alcohol
and ether, and dissolved in 0.1 N HCl. Barium ions were removed with Na₂SO₄. After the nucleotides were hydrolyzed in N HCl for 1 hour at 100°, the hydrolysate was evaporated to dryness prior to the isolation of MeDAP by paper chromatography as previously described.

Definite care was exercised when examining a sample of MeDAP on a paper chromatogram with a MineraLight lamp. Visible decomposition occurred after several minutes under full intensity of the short wave ultraviolet light. The bright yellow decomposition product had an absorption maximum of 385 mμ at pH 2 which shifted to 430 mμ at pH 5. MeDAP was distinctly more stable in acid than in neutral or alkaline media.

Identification of Isolated Base As MeDAP—A comparison of the absorption spectrum of the isolated base (Fig. 1) with that of DAP revealed a marked similarity of pattern despite the increase in the relative height of the second peak as compared to the third peak and the slight shift of the third peak toward the longer wave length. Similarly, a comparison of paper chromatographic mobilities demonstrated that the isolated base consistently had higher $R_f$ values than DAP (Table I). Both findings

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**Fig. 1.** Absorption spectrum of the isolated MeDAP in neutral, alkaline, and acid solution: pH 2, 0.01 N HCl; pH 7.4, 0.05 M phosphate buffer or 0.015 M glycylglycine buffer; pH > 11, 0.01 N NaOH or N NH₃. The absorption spectrum at pH 6 (0.025 M ammonium acetate buffer) and at pH 9 (0.03 M glycylglycine buffer) is essentially identical to the spectrum at pH 7.4.
suggested an alkylamino derivative of DAP (20–22). The location and nature of the alkylamino group in the isolated base were determined by the identification of its deamination product as meguanine. The structure of the isolated base was therefore 2-methylamino-6-aminopurine. Confirmation of these findings was accomplished by direct comparison of the isolated MeDAP and its deamination product with authentic samples.3

Although DAP and guanine were easily deaminated with nitrous acid, more vigorous conditions were required for adenine and the isolated

<table>
<thead>
<tr>
<th>Purine component</th>
<th>Rf*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
</tr>
<tr>
<td>MeDAP nucleotide</td>
<td>0.01</td>
</tr>
<tr>
<td>&quot; nucleoside</td>
<td>0.21</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.41</td>
</tr>
<tr>
<td>Meguanine</td>
<td>0.27</td>
</tr>
<tr>
<td>DAP nucleotide</td>
<td>0.01</td>
</tr>
<tr>
<td>&quot; nucleoside</td>
<td>0.14</td>
</tr>
<tr>
<td>&quot; §</td>
<td>0.21</td>
</tr>
<tr>
<td>Adenyl acid§</td>
<td>0.01</td>
</tr>
<tr>
<td>Adenine§</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Owing to experimental conditions these values cannot be considered as absolute Rf values.
† For a description of the solvents see under "Methods."
‡ Ascending instead of usual descending technique.
§ Reference compounds.

MeDAP. 2 to 5 μmoles of base in a volume of 0.5 ml. were acidified with 0.8 ml. of glacial acetic acid and incubated at 56° for 5 hours. At 0, 2, and 4 hours, 0.1 ml. of barium nitrite (17 mg.) was added. At the end of the incubation period, the sample was evaporated to dryness and dissolved in H2O and barium ions were removed with 0.1 N H2SO4 (final pH 1 to 1.5). The barium-free supernatant fluid was evaporated to dryness in vacuo and chromatographed with solvent (a) (Table I). The major deamination product (Rf = 0.27) and a very minor component

3 Authentic samples of MeDAP and meguanine were kindly furnished by Dr. George H. Hitchings and Dr. Gertrude B. Elion. Data concerning their chemical properties were also furnished. Both compounds were purified by paper chromatography before their absorption spectra were obtained.
were the only two bands observed. The absence of visible amounts of MeDAP ($R_f = 0.41$) indicated an essentially complete conversion. The reduced electrophoretic mobility of the deamination product (Table II) coupled with its water insolubility indicated that the original purine had lost a basic group in the formation of a guanine derivative. After rechromatography in solvent (b), the deamination product was chromatographically pure. The absorption spectrum (Fig. 2) matched that of meguanine (21). Deamination at position 6 was unexpected, since DAP is deaminated exclusively at the 2 position to form isoguanine.

**Table II**

**Paper Electrophoretic Comparison of Isolated and Authentic Purines**

<table>
<thead>
<tr>
<th>Purine</th>
<th>Mobility (cm. per hr.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.6</td>
</tr>
<tr>
<td>Adenine†</td>
<td>0.77</td>
</tr>
<tr>
<td>DAP†</td>
<td>1.56</td>
</tr>
<tr>
<td>Isolated MeDAP</td>
<td>1.83</td>
</tr>
<tr>
<td>MeDAP†</td>
<td>1.88</td>
</tr>
<tr>
<td>Isolated meguanine</td>
<td>0.23</td>
</tr>
<tr>
<td>Meguanine†</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* The electrophoretic technique involved a horizontal suspension of Whatman No. 3 MM paper (14 X 38 cm.) clamped between two heavy glass plates. Samples were applied to the center line of the paper previously saturated with 0.1 M ammonium acetate buffer and blotted free of excess buffer. A 15 minute equilibration period was allowed before applying a potential of 200 volts across the paper. All samples were allowed to migrate for 3 hours at room temperature. The trailing boundary of the DAP derivatives was difficult to outline, owing to the peculiarities of their blue color under ultraviolet light. The very low solubility and slow migration of meguanine allowed only an approximation of the mobility. All samples became more diffuse as the pH decreased.

† Authentic samples.

in essentially quantitative yields. Likewise, the expected nitrosation of the alkylamino group (20, 21) did not occur to any appreciable extent, although the minor component of the deamination of the isolated MeDAP ($R_f = 0.13$ in solvent (a)) might be the nitroso derivative. Authentic MeDAP is primarily deaminated at position 6 to form meguanine. However, some nitrosation does occur.²

The isolated MeDAP and meguanine were compared directly with authentic samples. The two pairs of compounds were found to be identical with respect to mobility on paper chromatograms in eleven different solvents (Table III) and to absorption spectrum (Figs. 1 and 2). In all solvent systems, MeDAP and meguanine characteristically had higher
Fig. 2. Absorption spectrum of the isolated meguanine: pH 2, 0.01 N HCl; pH 6, 0.03 to 0.1 M ammonium acetate buffer; pH > 11, N NH₃; 1 M NaOH.

TABLE III
Chromatographic Comparison of Isolated and Authentic Samples

<table>
<thead>
<tr>
<th>Purine</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(d)</th>
<th>(e)</th>
<th>(f)</th>
<th>(g)</th>
<th>(h)</th>
<th>(i)</th>
<th>(j)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine‡</td>
<td>0.31</td>
<td>0.37</td>
<td>0.47</td>
<td>0.53</td>
<td>0.51</td>
<td>0.62</td>
<td>0.24</td>
<td>0.26</td>
<td>0.34</td>
<td>0.32</td>
</tr>
<tr>
<td>DAP‡</td>
<td>0.18</td>
<td>0.22</td>
<td>0.26</td>
<td>0.39</td>
<td>0.37</td>
<td>0.40</td>
<td>0.11</td>
<td>0.16</td>
<td>0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>Isolated MeDAP</td>
<td>0.37</td>
<td>0.25</td>
<td>0.45</td>
<td>0.52</td>
<td>0.58</td>
<td>0.57</td>
<td>0.26</td>
<td>0.36</td>
<td>0.32</td>
<td>0.35</td>
</tr>
<tr>
<td>MeDAP‡</td>
<td>0.37</td>
<td>0.25</td>
<td>0.45</td>
<td>0.53</td>
<td>0.58</td>
<td>0.59</td>
<td>0.28</td>
<td>0.36</td>
<td>0.30</td>
<td>0.35</td>
</tr>
<tr>
<td>Guanine‡</td>
<td>0.15</td>
<td>&lt;0.05</td>
<td>0.37</td>
<td>0.30</td>
<td>0.48</td>
<td>0.07</td>
<td>0.10</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated meguanine</td>
<td>0.33</td>
<td>0.43</td>
<td>&lt;0.05</td>
<td>0.48</td>
<td>0.47</td>
<td>0.63</td>
<td>0.17</td>
<td>0.25</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Meguanine‡</td>
<td>0.33</td>
<td>0.43</td>
<td>&lt;0.05</td>
<td>0.49</td>
<td>0.45</td>
<td>0.63</td>
<td>0.18</td>
<td>0.26</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>

* As a means of obtaining more consistent \( R_F \) values and to minimize the effects of salts, the sample was applied to the paper and the reference purine (usually DAP or adenine) was applied to the same spot as an “internal” reference.
† For a description of the solvents see under “Methods.”
‡ Authentic samples.

\( R_F \) values than DAP and guanine, respectively. The paper electrophoretic mobilities of the isolated and authentic samples compared favorably (Table II).

Direct evidence that the isolated MeDAP was a metabolic product of the exogenous DAP was obtained by the isolation of MeDAP-C\(^{14}\) from the...
medium of a resting cell suspension containing 11.8 μmoles of DAP-8-C\textsuperscript{14} (53,300 c.p.m. per μmole)\textsuperscript{4} incubated for 3 hours. As there is no evidence for the endogenous synthesis of DAP or for the formation of MeDAP in the absence of DAP, the specific activity of the isolated MeDAP was assumed to be that of the exogenous DAP-C\textsuperscript{14}. Chromatography of the HCl extract of the silver purines in solvent (a) and subsequent elution of the MeDAP band revealed the presence of 2.2 μmoles of MeDAP as judged by radioactivity. Since the isolation procedure was not quantitative, there was a minimum of 18.7 per cent conversion of DAP to MeDAP during the 3 hour incubation. By determining the optical density of a solution of purified MeDAP of known specific activity, the molar extinction coefficient was approximated to be 8610 at 288 μm and pH 2.

Since glucose served as the sole carbon source for E. coli, the formation of the methyl group in vivo was demonstrated by the incorporation of the carbon of D-glucose-1-6-C\textsuperscript{14} into MeDAP (Table IV). The specific activity of the isolated MeDAP remained constant (within experimental error) after chromatography in four solvents. The carbon of glucose but not of formate was incorporated into MeDAP. Glucose definitely was not the limiting factor in MeDAP synthesis, since washed E. coli cells were able to carry out the synthesis in the absence of exogenous glucose.

Isolation and Identification of MeDAPMP-5'—The nucleotides of the TCA-soluble fraction of E. coli cells incubated with DAP were separated into barium salts which were insoluble in water and those which were precipitated from solution by alcohol. The latter was dissolved by adjusting the pH to 3, freed of barium ions with Na\textsubscript{2}SO\textsubscript{4}, and chromatographed in solvent (c). No evidence for the presence of the free base, MeDAP, was found. The MeDAPMP-5' was located on the paper as a blue band just overlapping the trailing edge of adenylic acid (Table I). To remove the phosphate of the solvent, the nucleotide band was eluted with H\textsubscript{2}O, adsorbed on a minimum of Norit A, and eluted with ethanol-NH\textsubscript{3}-H\textsubscript{2}O (5:3:2). Further purification was accomplished by rechromatography in solvents (b), (e), and (g). The absorption spectrum of MeDAPMP-5' is given in Fig. 3. The nucleotide has also been purified by elution from a Dowex 1 (chloride form, 10 per cent cross-linkage, 200 to 400 mesh) column with 0.002 N HCl.

MeDAPMP-5' was dephosphorylated by incubation with 12 γ of snake venom protein,\textsuperscript{5} containing a specific 5'-nucleotidase (23), 0.8 μmole of DAP-8-C\textsuperscript{14} and L-methionine-CH\textsubscript{3}-C\textsuperscript{14} were obtained from the Isotopes Specialties Company, Inc. D-Glucose-1-6-C\textsuperscript{14} was furnished by the New England Nuclear Corporation. Formate-C\textsuperscript{14} was obtained from Dr. Martin P. Schulman.\textsuperscript{4} Lyophilized Crotilus adamanteus venom as supplied by Ross Allen's Reptile Institute.
MgC\textsubscript{2}, and 8 \( \mu \)moles of glycine buffer (pH 8.5) in a total volume of 0.4 ml. After an incubation of 35 minutes at 38\(^\circ\), the reaction was stopped by heating in boiling H\textsubscript{2}O for 2 minutes. Control experiments with yeast adenylic and guanylic acids demonstrated that the 2\(^{\prime}\) - and 3\(^{\prime}\)-phosphonucleosides were not appreciably dephosphorylated under these conditions. The nucleoside of MeDAP was initially isolated by chromatography in solvent (a) (Table I). No detectable amounts of free base were liberated. Rechromatography with solvents (b), (c), (d), and (m) confirmed the presence of a component having the expected \( R_f \) of a nucleoside. Presence of ribose in the nucleoside was confirmed by the orcinol method of Mejbaum as modified by Umbreit \textit{et al.} (24). The spectrum of the nucleoside coincided with that of the nucleotide (Fig. 3). Hydrolysis of either the nucleoside or nucleotide in \( N \) HCl for 60 minutes at 100\(^\circ\) yielded a product whose \( R_f \) and spectrum were identical to those of MeDAP.

When the water-insoluble barium salt fraction was dissolved in 0.1 \( N \)
HCl, freed of barium ions, and chromatographed in solvent (c), another distinct MeDAP-containing nucleotide was isolated. This component had a higher $R_f$ (0.69 versus 0.60) than the monophosphonucleoside of MeDAP. Although complete identification has not been carried out, it would appear to be either the di- or triphosphonucleoside of MeDAP as judged by paper and ion exchange chromatography. Visual examination of paper chromatograms strongly suggested that more of the polyphosphate than the monophosphate component was present.

Isolation and Identification of DAPMP-5'—The monophospho- and presumably the polyphosphoriboside of DAP have also been isolated from the TCA-soluble fraction of E. coli by the methods described for the MeDAP derivatives. Location of the monophosphonucleoside on chromatograms was facilitated by virtue of its distinctly blue appearance and by the fact that, as in the case of the free bases, the nucleotide of DAP had a lower $R_f$ than the MeDAP nucleotide (Table I). Identity of the nucleotide as DAPMP-5' was based on a comparison of $R_f$ and spectral characteristics of the isolated nucleotide, nucleoside, and free base. The latter two were obtained from the nucleotide by enzymatic and acid hydrolysis, respectively, by methods outlined for MeDAPMP-5'. The spectral differences between MeDAP and DAP persist in the corresponding nucleotides. The mobility of the nucleoside in a two-dimensional chromatogram with solvents (c) and (m) was similar to that reported by Wheeler and Skipper (7). The evidence for a polyphosphonucleotide was based on

![Absorption spectrum of MeDAP nucleotide in acid and alkaline solution](https://example.com/absorption_spectrum.png)

**Fig. 3.** Absorption spectrum of MeDAP nucleotide in acid and alkaline solution: pH 2, 0.01 N HCl; pH 11 to 12, 0.01 N NH₃ and 0.01 N NaOH. The spectrum at pH 5 and pH 7 is the same as that at pH 2 and pH 11 to 12, respectively. The absorption spectra of MeDAP and DAPMP-5' are identical.
the isolation of a different DAP-containing component after paper and ion exchange chromatography of the barium-insoluble nucleotide fraction. Although all experiments were carried out under presumably similar conditions, it was not possible to predict whether the nucleotides of DAP or MeDAP would be isolated as the major component.

**Inhibition of DAP and MeDAP Incorporation**—In regard to the mechanism whereby adenine is able to prevent the growth inhibition of E. coli by DAP, it was of interest to find that E. coli cells did not incorporate either DAP or MeDAP into the nucleotide fraction when the cells were incubated in a medium containing both DAP and adenine (50 μmoles of phosphate buffer, 8.9 μmoles of glucose, 1.92 μmoles of DAP, and 0.38 μmole of adenine per ml.). However, incorporation did occur under identical conditions when adenine was omitted from the medium. Since these results were obtained from visual observation of chromatograms, slight incorporation would be undetectable.

**Distribution of MeDAP Synthesis**—To determine whether MeDAP synthesis occurred in other microorganisms, Streptococcus faecalis, L. casei, and Saccharomyces carlsbergensis were grown as stationary cultures (the first two in trypticase soy broth, the third in mycophil broth, Baltimore Biological Laboratory) for 16, 22, and 96 hours, respectively. Resting cell suspensions (final volume of 30 ml.) contained per ml.: 14 to 35 mg. dry weight of cells, 125 μmoles of phosphate (pH 7.0), 11 μmoles of glucose, 2 μmoles of L-methionine, and 0.36 or 1.96 μmoles of DAP. Control vessels contained, in addition, 0.36 or 0.81 μmole per ml. of adenine. After a 3 hour incubation at 38°, the cells were collected by centrifugation. The purine fraction of the medium was isolated as previously described. The TCA-soluble fraction of the cells was passed through a column of Dowex 1 (chloride form) having the dimensions 0.64 sq. cm. × 3.5 cm. The bases and nucleosides were eluted with 0.01 N NH₄Cl and the combined nucleotide fraction was eluted with N HCl. The HCl eluate was hydrolyzed and the purines were purified through the silver salts. The presence of DAP and MeDAP was determined by two-dimensional chromatograms with solvent (a) or (m) followed by solvent (c). Although none of the three organisms synthesized MeDAP in amounts detectable on paper chromatograms, they did incorporate DAP into the nucleotide fraction of the cell. The degree of incorporation was proportional to the DAP concentration in the medium. In each case, adenine prevented or greatly decreased the incorporation of DAP.

**Formation of MeDAP and DAP Nucleotides by E. coli/DAP**—No evidence was obtained that the mutant, E. coli/DAP, could incorporate either DAP or MeDAP into the TCA-soluble nucleotide fraction when resting cell suspensions were incubated in a medium containing DAP.
However, isolation of MeDAP from the medium demonstrated that the resistant mutant converted DAP to MeDAP at 60 to 90 per cent of the rate of normal cells. The mutant differed from the parent strain in its inability to convert either the exogenous DAP or the endogenously synthesized MeDAP into the nucleotide fraction of the cells.

**Synthesis of MeDAP by Cell-Free System**—Investigations as to the requirements for MeDAP synthesis were carried out with extracts of 16 to 18 hour stationary cultures of *E. coli* B grown in Difco brain heart infusion medium or trypticase soy broth. Washed cells were ground with aluminum oxide, and then diluted with buffer, and the cellular debris was removed by centrifugation at 18,000 × g for 15 minutes. The supernatant fluid was recentrifuged for 25 minutes at 25,000 × g. This crude enzyme extract contained approximately 15 to 18 mg. of protein per ml. The degree of MeDAP synthesis was determined by the conversion of DAP-8-C^{14} to MeDAP-8-C^{14}. After an incubation of 3 hours, the reaction was stopped by addition of TCA. The purine fraction was hydrolyzed in N HCl, purified through the silver salts, and isolated by paper chromatography in solvent (a). The MeDAP area was eluted and the concentration of MeDAP-C^{14} was determined. In a reaction mixture containing (per ml.) 1.5 μmoles of DAP, 6.8 μmoles of ATP, 20 μmoles of MgCl₂, 10 μmoles of L-methionine, 100 μmoles of phosphate (pH 7.4), and 6 to 8 mg. of enzyme, it was possible to demonstrate an essentially absolute requirement for each of the reactants except phosphate. The methyl group of L-methionine-CH₃-C^{14} was incorporated into MeDAP by such a system. The participation of adenosylmethionine (25) as the primary methyl donor is being investigated.

The formation of DAP and MeDAP nucleotides was demonstrated in a reaction mixture containing per ml.: 1.3 μmoles of DAP, 4 μmoles of ribose 5-phosphate, 1.4 μmoles of ATP, 20 μmoles of MgCl₂, 15 μmoles of L-methionine, 2 μmoles of 3-phosphoglyceric acid, 10 μmoles of glucose, 1.2 μmoles of DPN, 80 μmoles of phosphate (pH 7.4), and 6 to 8 mg. of enzyme. After a 3 hour incubation at 38°, the reaction was stopped with TCA. The nucleotide fraction was isolated via small Dowex 1 columns (chloride form) and the corresponding free purines were separated and identified by two-dimensional chromatography by methods previously stated. Both DAP and MeDAP nucleotides were formed in the above reaction. At a concentration of 3.4 μmoles per ml., ATP was distinctly inhibitory. The omission of methionine resulted in considerable loss of MeDAP nucleotide formation without affecting the synthesis of DAP nucleotides. At a level of 1 μmole per ml., adenine completely inhibited the formation of both DAP and MeDAP nucleotides. No inhibition occurred at a level of 0.2 μmole of adenine per ml. However, adenine did not inhibit the synthesis of the free base, MeDAP.
C. N. REMY AND M. S. SMITH

DISCUSSION

In intact *E. coli* cells, DAP is deaminated to xanthine, converted to mono- and polyphosphonucleosides, and methylated to yield MeDAP which in turn is incorporated into similar nucleotides. Methylation of DAP appears to occur at the base rather than at the nucleotide level, since *E. coli* DAP converts DAP to MeDAP but fails to convert either DAP or MeDAP to the corresponding nucleotide. The observation that adenine not only overcomes the growth inhibition of DAP but also prevents the formation of DAP and MeDAP nucleotides may indicate that the three bases are converted to the nucleotide form via the same enzyme and that adenine prevents the incorporation of DAP and MeDAP by successfully competing for the enzyme. Whether MeDAP nucleotides have any specific inhibitory role is not known at present. These findings support the view that DAP inhibition is the result of the incorporation of DAP into an analogue of a specific adenine-containing cofactor via the same route by which adenine is incorporated and that it is the DAP analogue which is the active inhibitor (5, 26).

The observation that *E. coli* DAP is unable to incorporate either DAP or MeDAP into the nucleotide fraction likewise supports the data indicating that resistance to DAP in *L. casei* occurred as a result of the loss of an enzyme system responsible for the incorporation of DAP into the nucleotide form (26, 27). The mechanism bestowing resistance to DAP in *E. coli* is not related to MeDAP formation, as both the wild and resistant strains are capable of carrying out its synthesis.

Other methylamino derivatives of purines that have been isolated from biological systems include 6-methylaminopurine, a constituent of deoxy-nucleic acids of certain strains of *E. coli* (20), 6-dimethylaminopurine, a component of Puromycin (28), and N²-methylguanine, a normal constituent of human urine (21). The finding of MeDAP further emphasizes an increasingly significant role for these purine derivatives. The present study revealed that MeDAP is considerably less inhibitory than DAP for *E. coli* B. A similar finding has been reported for *L. casei* (29). The methylation of DAP may be considered as a method of detoxification of the inhibitor.

SUMMARY

1. *Escherichia coli* B not only incorporated 2,6-diaminopurine (DAP) into 5'-nucleotides of the trichloroacetic acid-soluble nucleotide fraction, but also metabolized it to 2-methylamino-6-aminopurine (MeDAP) and converted the latter to 5'-nucleotides. Adenine prevented the incorporation of DAP and MeDAP into the nucleotide form.

2. A DAP-resistant mutant of *E. coli* B was able to form MeDAP but was unable to convert either DAP or MeDAP to nucleotides.
3. A crude enzyme preparation of *E. coli* required methionine, adenosine triphosphate, magnesium ions, and DAP for MeDAP synthesis. Methionine acted as a methyl donor in this reaction. The requirements for DAP and MeDAP nucleotide formation were determined.

BIBLIOGRAPHY

METABOLISM OF 2,6-DIAMINOPURINE: CONVERSION TO 5'-PHOSPHORIBOSYL-2-METHYLAMINO-6-AMINOPURINE BY ENZYMES OF ESCHERICHIA COLI

Charles N. Remy and Marilyn S. Smith