METHYLATION OF NICOTINAMIDE WITH A SOLUBLE ENZYME SYSTEM FROM RAT LIVER*

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Handler and Dann (1) suggested that nicotinamide is methylated in vivo by methionine and excreted in the urine as N¹-methylnicotinamide (NMeN). Perlzweig, Bernheim, and Bernheim (2) demonstrated that NMeN is formed in vitro by rat liver slices. The amounts formed were quite small and the maintenance of aerobic conditions and of cellular integrity was essential; furthermore, the addition of methionine in vitro did not lead uniformly to an increased formation of NMeN. These findings have since been confirmed and extended by Ellinger (3), who, like the earlier investigators, failed to obtain methylation of nicotinamide in a cell-free extract of rat liver. The earliest observations pointing to the transfer of the methyl group of methionine to a suitable methyl acceptor in a cell-free system are those of Borsook and Dubnoff (4). These authors established that guinea pig liver homogenates supplemented with adenylic acid, an oxidizable substrate such as α-ketoglutaric acid, and oxygen formed creatine from guanidoacetic acid and L-methionine.

In recent years it has become increasingly clear that the requirement for oxygen in synthetic reactions with tissue slices or homogenates is often related to the aerobic generation of energy-rich phosphate bonds.¹ In some cases it has actually been possible to eliminate the requirement for aerobicosis by demonstrating that adenosinetriphosphate (ATP) is utilized anaerobically as a source of energy for endergonic reactions (5-9). Since methionine is the methyl donor for the methylation of both nicotinamide and guanidoacetic acid, it was considered of interest to investigate the

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¹ The symbol ~ ph will be used to designate the energy-rich phosphate bond in accordance with the usage introduced by Lipmann.
conditions required for the methylation of nicotinamide in cell-free preparations.

It has been found that a soluble enzyme system obtained from rat, pig, guinea pig, and dog liver catalyzes the formation of NMeN from methionine and nicotinamide in the presence of Mg\(^{++}\) and ATP. The pH optimum of the reaction is in the neighborhood of 7.4. The enzyme system reacts only with the L isomer of methionine. Betaine and dimethylthetin will not serve as methyl donors in the system, except when homocysteine is present. In the latter case methionine is probably the actual methyl donor. The enzyme system is specific for nicotinamide; i.e., nicotinic acid is not methylated. The requirement for ATP can be satisfied by supplying it directly or by generating this compound from phosphoglyceric acid or phosphocreatine anaerobically in the presence of catalytic amounts of ATP. As is often the case with transphosphorylating enzymes, Mg\(^{++}\) was found to be an essential requirement of the system. In the presence of an adequate phosphate donor, the reaction proceeds equally well in the presence or the absence of oxygen.\(^2\) The formation of NMeN as one of the products of the enzymatic reaction has been demonstrated in three independent ways, based on (a) its conversion to a fluorescent product by treatment with methyl ethyl ketone and alkali, (b) its ultraviolet absorption spectrum, and (c) its enzymatic oxidation to 1-methyl-3-carboxylamide-6-pyridone. The nature of the other products of the reaction is not yet known. The enzyme system for which the name “nicotinamide methylkinase” is proposed has been partially purified.

**EXPERIMENTAL**

**Enzyme Preparations**—Preparations with nicotinamide methylkinase activity were obtained from the livers of adult male albino rats, pigs, guinea pigs, and dogs, the activity of the extracts from the different species decreasing in the same order. Horse, sheep, ox, rabbit, pigeon, and duck liver did not yield active preparations. Rats, guinea pigs, pigeons, and ducks were decapitated, bled, and the livers quickly removed. Dogs and rabbits were anesthetized with nembutal and the livers removed when the animals were still alive. In all cases the livers were quickly rinsed free of excess blood with cold buffer, weighed, and homogenized in a Waring blender for 30 to 60 seconds with 3 volumes of cold buffer (sodium acetate 0.1 M, pH 5.60). Without delay the homogenate

\(^2\) It is of interest to note that cyanide activates the nicotinamide methylkinase system. This effect is probably due to reduction of an \(\text{—SH}\) group. The rôle of reducing agents and thiol compounds will be discussed in detail in the second paper of this series.
was centrifuged for 15 to 20 minutes at 18,000 r.p.m. in the cold. The small amount of fat which collected at the top was removed and the pH adjusted to 6.0 with 1.0 N acetic acid. The opalescent red extract was heated to 47° and kept at this temperature for 7 minutes. On the average, 5 to 8 minutes were required to bring the ice-cold extract to 47°. The extract was then chilled and the precipitate removed by centrifugation at 3000 r.p.m. for 15 minutes at 0°. The clear supernatant, which will be referred to as crude extract, was used directly, after neutralization, or was treated further, as described below. The enzyme system from rat liver was found to be stable at neutral pH for a few hours at room temperature. In the ice box it lost all activity in 48 hours. When kept frozen at −25°, the loss of activity was much slower; enzyme preparations have been kept under these conditions for a few weeks with little loss of activity. All of the forty or more preparations that were from rat liver were active; different preparations formed between 40 and 150 γ of NMeN per gm. of liver, wet weight, per hour. These figures compare favorably with the average figure of 27.3 γ of NMeN per gm. per hour reported by Ellinger (3) for rat liver slices.

The enzyme system from rat liver has been partially purified. In the experiments described below, two types of partially purified preparations were used. Preparation A was obtained by fractional precipitation with ammonium sulfate as follows: Ice-cold saturated ammonium sulfate was added to the crude extract to 38 per cent saturation. The mixture was kept at 0° for 10 minutes and the precipitate removed by centrifugation in the cold. The supernatant which contained most of the enzyme activity was brought to pH 7.5 with 1 N NaOH. Enough alkaline ammonium sulfate (containing 0.5 cc. of saturated NaOH in 100 cc. of saturated ammonium sulfate) was added to raise the concentration to 50 per cent saturation. The precipitate was separated by centrifugation as described above and dissolved in a cold buffer solution. This fraction contained between 20 and 30 per cent of the total activity present in the starting material and between 10 and 20 per cent of the total protein (Table I). As an alternative procedure (Preparation B), the crude extract was adsorbed fractionally at pH 6.0 on alumina Cγ and eluted with 0.1 M phosphate buffer at pH 6.8. In a typical experiment, 48 cc. of alumina Cγ (prepared according to Willstätter (10) and containing 10.9 mg. of Al₂O₃ per cc.) were added at 0° with vigorous mechanical stirring to 120 cc. of crude extract. Stirring was continued for 15 minutes at 0°. The suspension was centrifuged in the cold room. The supernatant was treated for a second time with 48 cc. of alumina gel as above. After centrifugation, the supernatant was discarded and the two residues were treated separately; first they were washed with 50 cc. of cold water; next
they were eluted with 25 cc. of 0.1 M phosphate buffer, pH 6.8 (Eluates 1a and 1b). The analytical results pertaining to this procedure are presented in Table I.

Other Enzyme Preparations—The rabbit muscle extract, used as a source of the glycolytic enzymes required for the transfer of the energy of the phosphate bond in phosphoglyceric acid, was the fraction of a dialyzed water extract of muscle which was precipitated by ammonium sulfate between 52 and 75 per cent saturation (11). The ammonium sulfate paste was dissolved in 20 volumes of cold water and used as such, or after dialysis against water. Approximately 0.05 cc. per cc. of reaction mixture supplied an excess of the required enzymes. I am indebted to Dr. E. Racker for a gift of this enzyme preparation. The quinine-oxidizing enzyme of rabbit liver was kindly supplied by Dr. W. E. Knox, to whom my thanks are due also for many helpful suggestions in connection with experiments on the enzymatic oxidation of NMeN. Trypsin and chymotrypsin were crystalline preparations available commercially.

Chemical Preparations—The barium salt of dl-3-phosphoglyceric acid was prepared synthetically according to Warburg and Christian (12). I

### Table I

Preparation of Nicotinamide Methylkinase System from Rat Liver

<table>
<thead>
<tr>
<th>Preparation A. Fractionation with ammonium sulfate†</th>
<th>Volume</th>
<th>Units (total)*</th>
<th>Protein, total mg.</th>
<th>Specific activity (units per mg. protein)</th>
<th>Yield per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate buffer extract</td>
<td>340</td>
<td>11,070</td>
<td>7690</td>
<td>1.44</td>
<td>100</td>
</tr>
<tr>
<td>After heat treatment</td>
<td>290</td>
<td>11,630</td>
<td>5520</td>
<td>2.11</td>
<td>105.5</td>
</tr>
<tr>
<td>Ammonium sulfate ppt., 0-40% saturated, pH 6.0</td>
<td>58</td>
<td>1,272</td>
<td>1080</td>
<td>1.17</td>
<td>11.45</td>
</tr>
<tr>
<td>Ammonium sulfate ppt., 0-50% saturated, pH 7.5</td>
<td>33</td>
<td>2,420</td>
<td>895</td>
<td>2.7</td>
<td>22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preparation B. Adsorption and elution from alumina Cy</th>
<th>Volume</th>
<th>Units (total)*</th>
<th>Protein, total mg.</th>
<th>Specific activity (units per mg. protein)</th>
<th>Yield per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate buffer extract§</td>
<td>163</td>
<td>7,900</td>
<td>5675</td>
<td>1.39</td>
<td>100</td>
</tr>
<tr>
<td>After heat treatment‡</td>
<td>151</td>
<td>7,150</td>
<td>4130</td>
<td>1.73</td>
<td>90.5</td>
</tr>
<tr>
<td>Eluate 1a, pH 6.8</td>
<td>36.5</td>
<td>1,125</td>
<td>233</td>
<td>4.83</td>
<td>14.2</td>
</tr>
<tr>
<td>&quot; 1b, &quot; 6.8</td>
<td>52.0</td>
<td>2,160</td>
<td>277</td>
<td>7.8</td>
<td>27.3</td>
</tr>
</tbody>
</table>

* 1 unit = 1 γ of NMeN formed in 60 minutes at 38°.
† From livers of sixteen rats.
‡ 7 minutes at 47°.
§ From livers of twelve rats.
am greatly indebted to Dr. S. Ratner for a generous supply of a crystalline preparation of the acid barium salt of d-3-phosphoglyceric acid, prepared by the method of Neuberg and Lustig (13). The commercial preparations of ATP used (Armour, Schwarz, or Rohm and Haas) were found satisfactory. Occasionally, when used in large amounts (over 25 μM of easily hydrolyzable P per cc.), some blank fluorescence was observed. DL-Methionine and nicotinamide were obtained commercially. I am indebted to Dr. O. A. Bessey for a gift of the NMeN used in the experiments, to Dr. J. Stekol for a sample of DL-homocysteine, and to Dr. H. Waelsch for samples of methionine sulfoxide and ethionine. I should also like to thank Dr. A. D. Welch for a sample of dimethylthetin.

Fig. 1. Nicotinamide methylkinase activity as a function of time. Conditions, L-methionine 0.01 M; nicotinamide 0.01 M; MgCl₂, 0.02 M; 3-phosphoglyceric acid (K salt) 0.02 M; ATP with 1.5 μM easily hydrolyzable P per cc.; THAM buffer, pH 7.4, 0.025 M. Enzyme preparation, crude extract 0.25 cc. containing 7.2 mg. of protein; rabbit muscle fraction, 0.05 cc. containing 2.1 mg. of protein.

Measurement of Enzyme Activity—The reaction was carried out in small test-tubes. The chilled enzyme preparation was added to the reaction mixture at room temperature and the tubes were mixed and immersed in a water bath at 38°. Ordinarily the reaction was allowed to run for 60 minutes; Fig. 1 illustrates its time course. The reaction was stopped by the addition of 2 volumes of either 7 per cent perchloric acid or 10 per cent trichloroacetic acid. NMeN was determined on 0.1 or 0.2 cc. aliquot of the protein-free centrifugate according to the procedure of Huff² (14). The results are expressed as micrograms of NMeN formed per cc. of enzyme preparation per hour. The composition of the reaction mixture used for the assay of enzyme activity, based on experiments described in the following paragraphs, was as indicated in the legend to Fig. 1 or in Table II. The requirement for the various components is shown in Table II. Nicotinamide methylkinase shows maximal activity when the pH of

²Methyl ethyl ketone was used in place of acetone, as suggested by Lowry and Bessey (personal communication).
the reaction mixture is 7.4. The rate falls off rather sharply on the acid side and more slowly on the alkaline side of this pH. Phosphate buffers and maleate buffers were found to be inhibitory; most of the experiments were carried out in glycylglycine or tris(hydroxymethyl)aminomethane (THAM) buffer at pH 7.4. The final concentration of buffer in the reaction mixture varied in different experiments between 0.015 and 0.03 M. Ordinarily the volume of the reaction mixture was 1.0 cc., containing 0.1 to 0.3 cc. of the enzyme preparation.

**Specificity**—Of the methyl compounds investigated, only L-methionine was active as a methyl donor to nicotinamide. The Michaelis constant for L-methionine, as determined with enzyme Preparation A, was

| TABLE II |
|-------------------|-----------------|
| **Methylation of Nicotinamide by Rat Liver Enzyme Components of System** |
| Complete system.  | 0.15 cc. of enzyme Preparation A containing 4.06 mg. of protein and 0.05 cc. of muscle extract in a final volume of 1.0 cc. The complete system contained nicotinamide 0.01 M, L-methionine 0.01 M, Mg++ 0.02 M, d-3-phosphoglycerate (K salt) 0.017 M, ATP with 1.45 \( \mu \)M of easily hydrolyzable P per cc., and glycylglycine buffer, pH 7.25, 0.02 M. (The concentrations are expressed as final in all the tables and figures.) Incubation time 60 minutes at 38°. The results are expressed as micrograms of NMeN formed per cc. of enzyme preparation per hour. |
| NMeN formed.       | per cent of complete system. |
| Complete system.    | 56.0 | 100 |
| No methionine.      | 11.9 | 21.3 |
| " nicotinamide.     | 2.3  | 4.1  |
| " Mg++             | 2.3  | 4.1  |
| " ATP, no phosphoglycerate. | 2.3  | 4.1  |
| " methionine, no nicotinamide. | 2.8  | 4.1  |

\( 5.0 \times 10^{-5} \) M. Fig. 2, A shows the effect of increasing concentrations of L- and DL-methionine on NMeN formation. It can be seen that the amounts formed at any one concentration of L-methionine were equal, within the limits of experimental error, to the amounts formed at twice that concentration of DL-methionine. d-Methionine, therefore, is inactive in this system. This finding is in agreement with the earlier observation of Borsook and Dubnoff (4) on the inability of d-methionine to act as a methyl donor in the synthesis of creatine.

Betaine and dimethylthetin, when used alone, were not active as methyl donors. However, when these compounds were used in conjunction with DL-homocysteine, methylation of nicotinamide occurred readily (Table III). It may be noted that the amounts of the methylated derivative
formed under these conditions were somewhat smaller than those formed when methionine was used as a methyl donor. It appears, therefore, that methionine was formed in the system through methylation of homocysteine as proposed by Borsook and Dubnoff (15, 16), and that the newly formed methionine was utilized for the synthesis of nicotinamide. Choline could not serve as a methyl donor, in either the presence or the absence of homocysteine. This is in agreement with the results of recent experiments of Dubnoff (17) and of Muntz (18). Methionine sulfoxide was investigated as a methyl donor because it has been claimed that this compound will function as a methyl donor to guanidoacetic acid even in the absence of oxygen or ATP (19). Under the conditions of the present experiments, however, it was found that methionine sulfoxide was not a methyl donor. This compound did not inhibit the formation of NMeN from methionine. Ethionine (α-amino-γ-ethiolbutyric acid) was investi-

### Table III

*Methylation of Nicotinamide by Various Compounds*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>NMeN formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>No additions</td>
<td>γ</td>
</tr>
<tr>
<td></td>
<td>L-Methionine, 0.021 M</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>DL-Homocysteine, 0.005 M</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Betaine, 0.01 M</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>Choline, 0.01 &quot;</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>DL-Homocysteine, 0.005 M and betaine, 0.01 M</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>&quot; 0.005 &quot; &quot; choline, 0.01 &quot;</td>
<td>4.7</td>
</tr>
<tr>
<td>B</td>
<td>No additions</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>DL-Methionine, 0.01 M</td>
<td>14.75</td>
</tr>
<tr>
<td></td>
<td>DL-Homocysteine, 0.0125 M</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Dimethylthetin, 0.01 M</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>DL-Homocysteine, 0.0125 M and dimethylthetin, 0.01 M</td>
<td>13.0</td>
</tr>
<tr>
<td>C</td>
<td>No additions</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>DL-Methionine, 0.0125 M</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>Methionine sulfoxide, 0.0125 M</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>&quot; 0.0125 &quot; and DL-methionine, 0.0125 M</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>DL-Ethionine, 0.0125 M</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>&quot; 0.0125 &quot; and DL-methionine, 0.0125 M</td>
<td>22.7</td>
</tr>
</tbody>
</table>
METHYLATION OF NICOTINAMIDE

gated because, in view of the recent results of Stekol et al. (20), it was of interest to see whether the enzyme system could utilize this compound as an ethyl group donor. \(N^1\)-Ethynicotinamide formation can be followed with the usual technique since \(N^1\)-ethynicotinamide yields a fluorescent condensation product when treated with methyl ethyl ketone in the presence of alkali.\(^4\) The experiment was negative; it is clear, therefore, that nicotinamide methylkinase will react only with methionine. It was also of interest to see whether ethionine as a structural analogue would inhibit the utilization of methionine in this reaction. This did not appear to be the case (Table III).

The effect of increasing concentrations of nicotinamide, i.e. the methyl acceptor, on NMeN formation is illustrated in Fig. 2, B. The half saturation concentration of nicotinamide was about \(6 \times 10^{-5}\) M. No formation of NMeN was detected when nicotinamide was replaced by nicotinic acid, either in the presence or in the absence of ammonia. This is in accord with the observations of Handler and Dann (1) and Unna (21). Guanidooacetate was not methylated by the nicotinamide methylkinase system.

Requirement for Energy-Rich Phosphate—As shown in Table II, \(\sim\) phosphate is one of the requirements of the nicotinamide methylkinase system. In spite of the high ATPase activity of the preparations, it was found that ATP could be used directly. Fig. 2, C shows the relationship between ATP concentration and NMeN formation. The shape of the curve suggests that, at concentrations higher than 5 \(\mu\)M per cc., the availability of ATP might be limited by the interference of inhibitory substances either present in the ATP or formed from it. In order to overcome this difficulty, it was found advantageous to use phosphoglycerate in the presence of catalytic amounts of ATP as a source of \(\sim\) ph. When phosphoglycerate was used, the liver enzyme had to be supplemented with a protein fraction obtained from an aqueous extract of rabbit muscle (11). This fraction was rich in phosphoglyceromutase, enolase, and phosphopyruvic transphosphorylase, which were either limiting or absent in the liver system. Under these conditions the rates of NMeN formation were higher than with concentrations of ATP which yielded the same amount of \(\sim\) ph (Fig. 2, C). The direct participation of ATP in the synthesis of NMeN from methionine and nicotinamide is indicated by the fact that, in the absence of catalytic amounts of ATP, no formation of NMeN was observed even in the presence of 15 \(\mu\)M of phosphoglycerate. Another high energy phosphate compound that was used for the generation of the ATP required in the biosynthetic reaction is phosphocreatine (Fig. 2, C). In these experiments also the liver en-

Personal communication from Dr. V. A. Najjar.
zyme system was supplemented with the fraction from rabbit muscle, since it contains creatine kinase.

The nicotinamide methylkinase system requires Mg++; no NMeN was formed in the absence of added magnesium. Numerous earlier findings indicate that Mg++ ions are generally needed in transphosphorylating systems. The requirement for Mg++ ions is rather high and it was neces-

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![Diagram](http://www.jbc.org/)

**Fig. 2.** Nicotinamide methylkinase activity as a function of the concentration (A) of L-methionine; (B) of nicotinamide; (C) of phosphate donor; (D) of Mg++. (A) Conditions: nicotinamide 0.005 M, MgCl₂ 0.025 M, THAM buffer, pH 7.4, 0.025 M, ATP with 26 μM of easily hydrolyzable P per cc.; methionine as indicated; enzyme Preparation A 0.23 cc. containing 4.6 mg. of protein. (B) Conditions as in (A), except that 0.01 M of dL-methionine was present; nicotinamide as indicated. (C) Conditions: L-methionine 0.01 M, nicotinamide 0.01 M, MgCl₂ 0.02 M, THAM buffer, pH 7.4, 0.02 M, enzyme Preparation A 0.143 cc. containing 7.7 mg. of protein. (D) Conditions as in (A), except that 0.01 M of dL-methionine was present; Mn++ as indicated.

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Evidence for Formation of N-Methylnicotinamide—When NMeN is treated with dialkyl ketones in an alkaline medium at room temperature and is then heated in acid, a highly fluorescent condensation product is

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necessary to raise the concentration of Mg++ ions to 0.02 M to approach maximum activity (Fig. 2, D). The effect of Mg++ is rather specific; Mn++ has only a small effect and Co++ and Zn++ are inactive.

Evidence for Formation of N-Methylnicotinamide—When NMeN is treated with dialkyl ketones in an alkaline medium at room temperature and is then heated in acid, a highly fluorescent condensation product is
formed (22). This reaction forms the basis of the method of Huff (14), which was found valuable for the quantitative estimation of NMeN formed enzymatically. However, the formation of a fluorescent condensation product is characteristic of N-substituted nicotinamide derivatives in general, and is in no way specific for methyl substitution. Therefore, additional evidence that NMeN is the product formed by the enzymatic reaction under investigation had to be obtained. The ultraviolet absorption spectrum of nicotinamide differs sufficiently from that of NMeN (Fig. 3) for identification purposes. A reaction mixture was prepared containing L-methionine 0.0025 M, nicotinamide 0.00025 M, d-3-phosphoglycerate 0.01 M, MgCl₂ 0.02 M, ATP with 1.5 μM of easily hydrolyzable P, THAM buffer, pH 7.3, 0.015 M, enzyme Preparation A 0.6 cc. (containing 26 mg. of protein), and muscle fraction 0.05 cc.; final volume, 3 cc.

A control without nicotinamide was also prepared. 0.5 cc. of each reaction mixture was removed at zero time and after incubation at 37° in an atmosphere of nitrogen for 4 hours, and deproteinized by addition of 0.2 cc. of 25 per cent BaCl₂, 1.3 cc. of 5 per cent ZnSO₄, and 0.65 cc. of saturated Ba(OH)₂. The mixture was shaken after each addition and allowed to stand at room temperature for a few minutes. The ZnSO₄-Ba(OH)₂ mixture was selected as a deproteinizing agent because it largely removes adenine nucleotides, thus reducing or eliminating ultraviolet absorption of these compounds, which would otherwise interfere with the estimation of NMeN and nicotinamide. For the same reason, phospho-

Fig. 3. Ultraviolet absorption spectra of nicotinamide (●) and N¹-methyl-nicotinamide chloride (○). The concentration of both compounds was 2 × 10⁻⁴ M, in water, at pH 7.0.

5 The incubation was carried out in the apparatus designed by Dubnoff for non-nanometric studies.

6 Suggested by Dr. N. O. Kaplan.
glycerate in the presence of a suboptimal catalytic amount of ATP was used as a source of ~ phosphate. The absorption of the four samples was determined after filtration through No. 1 Whatman paper. The curves illustrated in Fig. 4, A were obtained by subtracting the values of the control samples from those of the corresponding experimental samples. It can be seen that there was a definite shift in maximum absorption from 260 to 265 m\(\mu\), as well as an increase in the optical density at 265. The amount of NMeN formed during incubation was determined fluorometrically on a second aliquot deproteinized with trichloroacetic acid as usual. A calculation based on the figure thus obtained indicated that, at the end of the experiment, the reaction mixture contained 59.7 \(\gamma\) of nicotinamide and 44.7 \(\gamma\) of NMeN. A mixture containing the pure compounds in the same proportions and after comparable dilution would have an optical density of 0.148 at 265 m\(\mu\). This figure agrees quite closely with the observed value of 0.154.

The above results suggest that NMeN is formed in the course of the reaction. However, since the changes observed were not large, it appeared desirable to demonstrate methylation of nicotinamide in still another way. It was possible to take advantage of the observation of Knox and Grossman (23) that NMeN is oxidized to the corresponding 6-pyridone derivative by the quinine-oxidizing enzyme of rabbit liver. The 6-pyridone has a characteristic ultraviolet absorption spectrum. An
experiment was performed essentially as the one described above. The reaction mixture had the same composition and the same enzyme preparation was used. After incubation in an atmosphere of nitrogen for 240 minutes at 37°, the reaction mixtures were supplemented with 0.15 cc. of quinine-oxidizing enzyme (containing 43 units per cc.) (24) and the reaction was allowed to continue for 60 minutes in an atmosphere of oxygen. Appropriate samples were taken at 0, 240, and 300 minutes for the fluorometric determination of NMeN, and at 0 and 300 minutes for measurements of ultraviolet absorption spectrum. The absorption spectra obtained by subtracting the values of the controls (without nicotinamide) from the experimental samples are shown in Fig. 4, B. It can be seen that there is a very marked change in the absorption spectrum upon incubation; a comparison of Curve 2, Fig. 4, B, with Curve 2, Fig. 4, A, will indicate what fraction of this change can be ascribed to the addition of the quinine-oxidizing enzyme. It might be pointed out that Curve 2, Fig. 4, B, has the plateau between 280 and 300 mμ characteristic of the 6-pyridone derivative of NMeN. In regard to the quantitative aspects, the results of the fluorometric determinations indicated that, in the anaerobic phase of the experiment, 69.9 γ of NMeN were formed. At the end of incubation with the quinine-oxidizing enzyme, the amount of NMeN had decreased to 9.8 γ. It should be noted that the oxidation product of NMeN is not fluorescent under the conditions used to determine NMeN. The fluorometric data therefore indicated that as much as 54.6 γ of the 6-pyridone derivative could have been formed by the action of the quinine oxidizing enzyme. From the molecular extinction coefficient of the 6-pyridone derivative at 260 mμ (13,000) (23), it was calculated that 78 γ of it had been formed. This amount is somewhat larger than that estimated by the fluorometric data. However, in view of the shape of the time curve (Fig. 1), the discrepancy should be attributed, in part at least, to a continuation of the biosynthesis of NMeN, followed by its oxidation, during the 60 minutes incubation with the quinine-oxidizing enzyme. The results of the experiment prove conclusively that NMeN is formed in the course of the experiment and is subsequently oxidized to 1-methyl-3-carboxylamide-6-pyridone. 

Stability of Enzyme System—The nicotinamide methylkinase system is rapidly destroyed by heating to temperatures above 50°. Exposure to acid (pH values lower than 5.5) leads, even in the cold, to flocculation and irreversible inactivation of the enzyme system. The preparation is more stable on the alkaline side of neutrality and little or no loss of activity occurs following incubation at pH 9.0 at room temperature for 60 minutes. Prolonged dialysis of the crude liver extract against phosphate or THAM buffers in both the presence and the absence of methionine and nicotin-
amide leads to a great loss in activity. Enzyme Preparation B is somewhat more stable and can be dialyzed in the cold against 0.01 M phosphate buffer, pH 7.4, for 4 hours without loss of activity. The system is inactivated by incubation with trypsin or chymotrypsin. The proteolytic inactivation was not diminished in the presence of methionine.

DISCUSSION

On the basis of the important contributions of Borsook and Dubnoff (4, 15, 16) and of du Vigneaud and his school (25, 26), reactions involving methyl group transfer can be divided into two different types. The difference lies both in the nature of the methyl donor and in the requirement for energy-rich phosphate. On the one hand there is a type which results in the methylation of homocysteine and which proceeds without the addition of ~ ph. The methyl group is supplied by compounds like betaine, dimethylthetin, and dimethylpropiothetin, whose molecules are characterized by the fact that the methyl radicals are attached directly to an onium pole (quaternary N or ternary S). On the other hand, there are methylation reactions such as those involved in the biosynthesis of creatine and NMeN. It is characteristic of these reactions that L-methionine is the source of the methyl group, and that they require ~ ph supplied directly as ATP, or made available indirectly through the generation of ~ ph, by glycolysis or aerobic phosphorylation. It is indeed possible that the formation of epinephrine (27) and of choline may be found to follow a similar course. In the absence of more precise information one can only speculate on the specific function of phosphate bond energy in these reactions; ~ ph could be required to satisfy the energy needs of these synthetic reactions; regardless of what the energy relationship may prove to be, intermediate phosphorylation of one of the substrates appears to be obligatory. In addition, it seems probable that ATP is utilized to activate the methyl radical in methionine, perhaps by bringing about the conversion of the amino acid to an onium compound.

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SUMMARY

1. The methylation of nicotinamide in cell-free preparations of rat liver has been studied.

2. Biosynthesis of N\textsuperscript{1}-methylnicotinamide proceeds anaerobically from L-methionine and nicotinamide in the presence of Mg\textsuperscript{++} and a source of
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~ ph; adenosinetriphosphate and phosphoglycerate or phosphocreatine in
the presence of catalytic amounts of adenosinetriphosphate can be utilized
by the enzyme system to satisfy the requirements for energy-rich phos-
phate.

3. N¹-Methylnicotinamide has been identified as a reaction product
(a) by fluorometric determination, (b) by means of its ultraviolet ab-
sorption spectrum, and (c) by its enzymatic oxidation to 1-methyl-3-
carboxylamide-6-pyridone.

4. The enzyme system, for which the name of nicotinamide methyl-
kinase is suggested, has been partially purified.

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