THE METHYLATION OF NICOTINAMIDE BY RAT LIVER IN VITRO*

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During recent years several attempts made by workers in these laboratories to demonstrate the methylation of nicotinic acid or of its amide in vitro by tissues of animals which were known to excrete "trigonelline" after doses of these compounds were unsuccessful. This failure was ascribed largely to the technical difficulty of determining very small amounts of trigonelline in the presence of a relatively large excess of unused nicotinic acid, for the only method for the determination of trigonelline was based on its transformation into nicotinic acid by means of alkaline hydrolysis in the presence of urea (1) and the measurement of the increment in the total nicotinic acid present. The method of Kodicek and Wang (2) for the direct determination of "trigonelline" appeared inadequate and inaccurate in the preliminary form in which it was published. In recent months, Fox, McNeil, and Field (3) and also Sarett (4) elaborated more workable modifications of this method. At about the same time, it evolved that the urinary fluorescent material, F2, of Najjar and collaborators (5) is identical with N1-methylnicotinamide (6, 7). Our studies (7) indicate, furthermore, that at least a large proportion of the metabolite appearing in the urine after the ingestion of nicotinic acid or of its amide which was hitherto designated as "trigonelline" consists in fact of the amide of trigonelline, or of N1-methylnicotinamide. It was, therefore, decided to reinvestigate this problem by means of the newer methods for determining the methylated derivatives of nicotinic acid.

EXPERIMENTAL

Mature rats of both sexes, of the Vanderbilt strain, were killed by decapitation and exsanguinated. The livers were removed immediately, and slices, 0.2 to 0.5 mm. thick, were made by hand, washed in two changes of Ringer’s solution, blotted, and 150 to 450 mg. weighed rapidly on a torsion

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balance, and the slices placed in 50 ml. Erlenmeyer flasks with 4.0 ml. of Ringer-bicarbonate solution (as used by Krebs and Henseleit (8)) and substrate substances; the solution was equilibrated with a mixture of 95 per cent O₂ and 5 per cent CO₂; the flasks were stoppered and agitated in a Warburg bath at 37° for varying periods of time. In the anaerobic experiment, the gas space was equilibrated with a mixture of 95 per cent N₂ and 5 per cent CO₂. The controls and experimental combinations were run in duplicate or triplicate. Immediately upon removal from the bath, the supernatant fluids were transferred, with small washings of water, to 15 ml. graduated tubes to a volume of 4.5 to 5.0 ml. and 0.5 ml. of 20 per cent trichloroacetic acid was added. The whole was mixed, heated in a water bath at 75-80° for 30 minutes, cooled, and made up to 5.5 ml. with water. The precipitated proteins were removed by centrifugation, and the supernatant fluid was used for fluorescence or colorimetric analyses directly. Adsorption on permutit and elution for fluorescence analysis were omitted after it was ascertained that the tissue extracts, unlike urine, contained relatively small concentrations of interfering pigments. In each experiment the control value for tissue slices alone without substrate was determined, and the increment due to the substrates was obtained by subtracting the control value. Added N¹-methylnicotinamide and trigonelline were recovered quantitatively under the above conditions.

The fluorescence analysis for F₂ (N¹-methylnicotinamide) was carried out essentially by the modified (7) procedure of Najjar et al. (5) except that the transfer of F₂ from the aqueous to the butanol phase was further facilitated by dissolving 1.8 gm. of anhydrous Na₂SO₄ in the 5 ml. aliquots of the tissue extracts. This salting-out procedure was found to result in an increase of the transfer of F₂ to the butanol phase, so that the galvanometer reading obtained was 5 to 6 divisions per microgram of F₂ present in the aqueous phase, as compared with a reading of 3 divisions per microgram obtained in the transfer from 25 per cent KCl solution, as used in the case of eluates from urine. Unfortunately, concentrated Na₂SO₄ solutions cannot be successfully used at room temperatures below 25° without troublesome crystallization and resulting diminution of the transfer of F₂ to the butanol.

The procedure is briefly as follows: 5.00 ml. of the protein-free tissue extract are measured into 125 ml. conical separatory funnels, 1.8 gm. of anhydrous Na₂SO₄ are added and dissolved by agitation or with the aid of the pipette carrying the air current which is used for stirring as described below, and 12.0 ml. of n-butanol are added. While the two liquids are being stirred with a brisk air current passing through them, 1 ml. of 10 N NaOH is blown into the emulsion with the aid of a rubber bulb at the end of the pipette. The air current is continued for 1 to 1.5 minutes after the addition of the alkali and stopped. The aqueous phase is drained off after a brief period of separation; the butanol extract is shaken with about 2 gm.
of anhydrous Na₂SO₄ and decanted into the test-tubes or cuvettes and read at once in the fluorophotometer and again after standing 15 to 20 minutes in the dark. In this study, we used the Coleman electric fluorophotometer, model 12, equipped with the two filters designed for the determination of thiochrome.

A summary of the data is presented in Table I. These recorded observations demonstrate conclusively that rat liver slices methylate nicotinamide readily to N¹-methylnicotinamide, since the fluorescence method used does not include trigonelline and presumably is specific for N¹-methyl-

<table>
<thead>
<tr>
<th>Table I</th>
<th>Synthesis of N¹-Methylnicotinamide by Rat Tissues in Vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>No. of experiments</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Liver</td>
<td>20</td>
</tr>
<tr>
<td>&quot;</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>6</td>
</tr>
<tr>
<td>Kidney</td>
<td>2</td>
</tr>
<tr>
<td>&quot;</td>
<td>2</td>
</tr>
<tr>
<td>Muscle, diaphragm</td>
<td>1</td>
</tr>
</tbody>
</table>

* The figures in parentheses are averages.
	nicotinamide. In several experiments parallel determinations were kindly made by Dr. Sarett on the liver extracts after incubation with nicotinamide by his modification of Kodicek and Wang's method (4) for total methylated nicotinic acid derivatives. The values so obtained corresponded, within ±10 per cent, to those obtained by fluorescence analysis, indicating that no appreciable amounts of trigonelline were formed by liver slices from nicotinamide. In experiments with liver slices from twenty-two rats not a single negative result was obtained.

The process appears to be strictly aerobic, for in the incubation of liver slices with nicotinamide in an atmosphere of nitrogen no trace of the methyl-
ated product could be found. That the intact cells are required was shown likewise by totally negative results when broken cells (liver brei) were employed in the presence of oxygen.

Kidney and diaphragm muscle slices produced with nicotinamide insignificant amounts, or none at all, of the methylated compound. The same was true of rat brain brei.

Nicotinic acid on incubation with liver or kidney slices did not yield significant amounts either of N¹-methylnicotinamide or of trigonelline and other methylated derivatives, as shown by Dr. Sarett's analyses in which his method (4) referred to above is employed.

Methionine when added to nicotinamide incubated with liver slices increased the methylation product by 25 to 50 per cent above that with nicotinamide alone in five out of ten experiments, whereas in five experiments this effect was completely absent. The same inconstancy of result was observed by Handler and Bernheim (9) in their study of creatine formation by liver slices in the presence of methionine.

Choline, when substituted for methionine, enhanced the methylation of nicotinamide in one experiment but failed to do so in another. It is planned to obtain more definite data on this aspect of the problem.

The time factor in the methylation of nicotinamide by liver slices in vitro at 37° is illustrated by the following values obtained with 370 to 380 mg. of liver incubated with 0.1 mg. of nicotinamide: 1 hour 3 γ, 2 hours 7.5 γ, 3 hours 12 γ, 4 hours 15 γ, per 1 gm. of liver. Methylation apparently proceeds at a fairly constant rate during the first 3 hours and slows up thereafter.

Attempts to study the effect of the concentration of the enzyme involved in this process by varying the weight of liver slices taken from 120 to 450 mg. gave somewhat erratic results which cannot be interpreted. Varying the concentration of the substrate (nicotinamide) in the presence of 200 mg. of liver slices gave the maximal effect with 0.25 mg. which was not increased by raising the amount of nicotinamide to 2.0 mg. in 4 ml. of Ringer's solution.

The stability of the methylation product in the system was indicated by the quantitative, 96 to 100 per cent, recovery of 100 γ of N¹-methylnicotinamide added to 200 to 400 mg. of liver slices in 4 ml. of Ringer's solution, agitated at 37° for 4 hours. Nor was trigonelline destroyed under these conditions. An illustrative experiment gave the following values.

<table>
<thead>
<tr>
<th>Galvanometer units</th>
<th>Increments</th>
</tr>
</thead>
<tbody>
<tr>
<td>240 mg. liver control</td>
<td>22</td>
</tr>
<tr>
<td>240 &quot; + 10 γ N¹-methylnicotinamide</td>
<td>75 53</td>
</tr>
<tr>
<td>Reagent blank</td>
<td>11</td>
</tr>
<tr>
<td>10 γ N¹-methylnicotinamide</td>
<td>66 55</td>
</tr>
</tbody>
</table>
It has been repeatedly observed in the studies on the metabolism of nicotinic acid in rats that individual rats vary widely in their daily excretion of methylated nicotinic acid compounds when maintained on ordinary nicotinic acid-free diets. Two such rats showing a very wide difference in excretion after a 3 mg. dose of nicotinamide were sacrificed and their livers tested with nicotinamide with and without added methionine. The results are shown in Table II. Apparently the rate of excretion of the methylated product parallels the rate of its formation in the liver.

**DISCUSSION**

Another methylation system has been demonstrated for rat liver *in vitro*: nicotinamide to N1-methylnicotinamide, in addition to the established process of creatine formation (10). In the absence of this mechanism in rat kidney and muscle, in its aerobic character, and in the rôle played by methionine it resembles the creatine system, thus making it likely that the methylation process in the animal tissues follows a well defined pattern.

**TABLE II**

<table>
<thead>
<tr>
<th>Rat</th>
<th>$F_2$ in 24 hr. urine</th>
<th>$F_2$ produced per gm. liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver + nicotinamide</td>
</tr>
<tr>
<td>C</td>
<td>1030</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>355</td>
<td>17</td>
</tr>
</tbody>
</table>

It has been shown by Handler and Dann (11) that the feeding of large amounts of nicotinamide to rats is capable of inhibiting their growth by exhausting the supply of labile methyl groups, since the inhibition was overcome by methionine. It may now be presumed that the "trigonelline" determined in the urine of their rats was really N1-methylnicotinamide.

The methylation of nicotinamide, but not of nicotinic acid, by liver slices raises the obvious question of the site of transformation *in vivo* of nicotinic acid into its amide prior to methylation. Recent observations in these laboratories show that individual rats excrete almost identical amounts of the methylated amide in 24 hours after ingestion or parenteral injection of small amounts (3 mg.) of either nicotinic acid or nicotinamide. The problem as to whether the rat does or does not methylate nicotinic acid to trigonelline must also await solution in further experimentation. The data at hand indicate that most, if not all, of the methylated products in the urine of rats on nicotinic acid-free diets are in the form of methylated nicotinamide.

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nicotinamide. After a large dose of nicotinic acid, however, there does appear to be, in certain cases, a considerable fraction of the total methylated product which is not the amide. This problem is being studied further.

**SUMMARY**

Rat liver slices when incubated with nicotinamide at 37° were shown to synthesize N¹-methylnicotinamide (F₂). This process is strictly aerobic, requires unbroken cells, and is usually, but not always, enhanced by the addition of methionine. Liver slices do not methylate nicotinic acid. Rat kidney and muscle do not exhibit, in vitro, the capacity to methylate nicotinamide. Suggestive evidence is presented to indicate that the individual variations in the rate of excretion of N¹-methylnicotinamide in the urine of rats are possibly related to the rate of methylation of nicotinamide in their livers.

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