DIFFERENTIAL DETERMINATION OF PYRIDINE NUCLEOTIDES AND $N^1$-METHYLNIAECINAMIDE IN BLOOD*

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The study of niacin and tryptophan metabolism in the animal body has been of interest to biochemists for many years. The variety of the metabolic end-products has necessitated the use of many analytical procedures for their determination. In many experiments in this laboratory as well as elsewhere, a need for a method for the simultaneous quantitative determination of small amounts of pyridine nucleotides (PN) and $N^1$-methyl- niacinamide (NMeN) in protein-containing biological materials has been indicated. The standard enzymatic assay for PN content of natural materials (1), of course, allows no measure of NMeN. The widely used fluorometric method of Huff and Perlzweig (2) was first adapted to the analysis of NMeN in urine, but it has also been used for PN analysis in blood and other tissues (3, 4). However, both constituents, with no differentiation, are measured by this method. Both of the components together are determined with the method developed by Feigelson, Williams, and Elvehjem (5) for assaying pyridine nucleotides in rat liver, but again without distinguishing between them. Carpenter and Kodicek (6) have published a procedure whereby the two components can be determined in urine. Their method consists of employing an ion exchange procedure to remove positively charged NMeN.

In studies on the formation of pyridine nucleotides in animals injected or fed tryptophan and niacin, the question of whether the substances determined were pyridine nucleotides, NMeN, or a combination of these metabolites has always existed. For that reason we have investigated the possibility of determining these substances differentially in small amounts of blood. Blood was chosen because of its ease of collection and because of its importance in clinical experiments. A method enabling this type of analysis has been developed and is reported in this paper. With slight modifications the method can probably be employed for liver and other organs. As an application of the procedure, values for the content of PN

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and NMeN in the blood of rats which have received various dietary treatments are also presented.

EXPERIMENTAL

Since it was desired to combine the use of ion exchange techniques as proposed by Carpenter and Kodieck (6) with the sensitive fluorometric analysis for the separated pyridino compounds, the main concern was to find a suitable precipitant of blood proteins which would also give complete extraction of the metabolites in question. Of the various reagents tested, including tungstic acid, trichloroacetic acid, glacial acetic-metaphosphoric acid mixture, perchloric acid, and ethanol, only 75 per cent ethanol appeared satisfactory. All other precipitants employed interfered with either the ion exchange procedure or the fluorometric analysis.

Reagents—

Absolute ethanol.

Hydrogen peroxide, 30 per cent. The peroxide served to oxidize the PN and NMeN, since the fluorometric method is not satisfactory for the reduced forms.

Decalso, 60 to 80 mesh from The Permutit Company, New York. To 100 gm. of Decalso were added 300 ml. of saturated NaCl solution. The resulting mixture was heated to 90-95° while being agitated with a mechanical stirrer to prevent bumping. After a period of 30 minutes at this temperature, the mixture was allowed to settle and the supernatant liquid decanted. The entire procedure was repeated. The Decalso was then washed with 300 ml. portions of hot, distilled water (90°) until the supernatant fluid was free from chloride ion as shown by a silver nitrate test. The Decalso was dried overnight in a vacuum oven at 60° and was stored in a desiccator. The Decalso columns were prepared just before use as follows: A small glass wool plug was placed in the bottom of each of three Hennessy tubes (Wilkens-Anderson Company, Chicago). To each tube were added 200 mg. of Decalso. Another glass wool plug was placed on top of the column to prevent floating particles.

Fluorometric assay reagents, as described by Huff and Perlzweig (2).

Standards—PN (Schwarz Laboratories) and NMeN (Delta Chemical Works) were used as standards. Neutralized standard solutions were prepared at the time of each experiment. The final concentrations used in this procedure were 20 to 25 γ of PN per ml. of standard solution and 4 to 6 γ of NMeN per ml. of standard solution.

Procedure—The differential determination of the PN and NMeN content of blood was performed in the following manner. To three 15 ml. centrifuge tubes, respectively, were added 0.75 ml. of water, 0.75 ml. of PN standard, and 0.75 ml. of NMeN standard. Into each tube were pipetted
2.25 ml. of absolute ethanol to give a final concentration of 75 per cent ethanol. To each of the three tubes was added 0.1 ml. of blood obtained either by heart puncture or directly from the tail of the rat. The pipette used to measure the blood was washed by drawing the alcohol mixture into the pipette and expelling it several times. After the addition of 0.05 ml. of 30 per cent hydrogen peroxide to each tube, the contents were stirred thoroughly and allowed to stand for 25 minutes to obtain complete extraction of PN and NMeN.

At this time, the precipitated protein of each sample was removed by centrifugation. Centrifuging was continued until clear supernatant solutions were obtained. The supernatant liquids were collected in graduated centrifuge tubes, and each of the protein mats was washed successively with 1 and 2 ml. portions of water by resuspending the precipitates and recentrifuging. This liquid was added to the corresponding original supernatant solutions, the total volume was read, and, after thorough mixing, 3 ml. of each of the resulting fluids were placed on the Decalso columns. Portions of the original sample and the effluent were then used for analysis of the PN and NMeN content.

Fluorometric Analysis—In each of two tubes was placed 1 ml. of each solution not treated with Decalso. 1 ml. portions of the corresponding treated solutions were added to two other tubes. One tube from each set of two tubes served as the blank. In the other tube the fluorescence of the PN and NMeN present was developed as described by Huff and Perlzweig (2). The only change which was made in their final assay procedure was to employ a blank containing acetone but no alkali, as discussed by Carpenter and Kodicek (6). The fluorescence which was developed was read in a Coleman fluorometer with the filters ordinarily employed for determination of thiamine.

When the blood samples were taken, duplicate samples were drawn for hemoglobin analysis by the oxyhemoglobin method (7). We have expressed our values on the basis of hemoglobin content as well as per ml. of whole blood, since the red blood cells have been reported to contain all but a negligible amount of blood PN (8).

Calculations—Calculations involve mainly a determination of the number of divisions on the fluorometer scale equivalent to a known amount of standard. One can then calculate the amount of PN or NMeN in a sample in which the concentration of these materials is unknown. After correction for blanks all fluorometer readings were multiplied by the total volume of supernatant solution plus the washings. The number of fluorometer scale divisions equivalent to 1 γ of standard was determined by subtracting the reading for blood alone from that for blood plus each added standard and dividing the resulting figure by the concentration of the
respective standards. This procedure was carried out for each sample and for its corresponding equivalent treated with Decalso.

The fluorometer reading for blood alone after Decalso treatment was divided by the number of fluorometer divisions per microgram of PN to give the content of PN in 0.1 ml. of blood. The figure obtained by subtracting the reading for blood after Decalso treatment from the reading before the Decalso treatment was divided by the number of fluorometer divisions per microgram of NMeN to give the NMeN of 0.1 ml. of blood. The values for PN and NMeN per ml. of blood divided by the hemoglobin content per ml. of blood gave the concentration of these substances in micrograms per gm. of hemoglobin.

A comparison of the fluorometer divisions per microgram of PN standard before and after Decalso treatment was employed to indicate the percentage recovery of the added PN. Similarly, a comparison of the fluorometer readings before and after adsorption of the samples containing added NMeN standard indicated whether the NMeN was completely removed from the sample.

Application of Method—The method reported here for differentiation of NMeN and PN in blood seems both reliable and reproducible. The recovery of added PN standard after Decalso treatment has averaged 98.4 ± 1.3 per cent in 51 experiments by the procedure.

The procedure has been applied to a determination of the PN and NMeN content of the blood of normal rats on a complete stock ration. The same rats were then fed a non-protein diet to deplete them of tissue pyridine nucleotides (9), and PN and NMeN were again determined. The deficient animals were then fed a supplement of niacin or tryptophan, and the effect on the levels of PN and NMeN was observed.

Adult, male, albino rats of the Sprague-Dawley strain weighing 200 to 250 gm. were used for these experiments. After determination of the PN and NMeN content of the blood when the animals were receiving a good stock ration, they were fed a non-protein diet. The composition of the diet was 5 per cent corn oil, 4 per cent Salts 4 (10), 2 per cent vitamin mix containing all of the known vitamins except niacin (11), 1.5 per cent sulfasuxidine to depress intestinal synthesis of niacin, and 87.5 per cent sucrose. The animals were housed in screen-bottomed cages and received water and food ad libitum.

After 6 weeks on this diet, the blood of these rats was analyzed again for the amount of PN and NMeN present. At this time, they were divided into two groups of four animals each and were fed non-protein rations containing either niacin (163 mg. per cent) or tryptophan (270 mg. per cent) at equimolar levels. After the rats had received the supplements for 1

1 Standard error of the mean.
week, the analyses were performed for a third time. The results of these experiments are presented in Table I.

For comparison of the results for Groups I and II it may be seen that after the animals received a non-protein diet for 6 weeks the PN content of the blood was decreased to almost 50 per cent of normal. However, a change in the NMeN concentration was not apparent. These values for the content of PN in the blood of normal animals are somewhat higher than are those previously reported (8). The higher values obtained in these experiments are probably nearer the true values for blood PN, since both oxidized and reduced PN were measured in these experiments. This was accomplished by the addition of the hydrogen peroxide which immediately oxidizes reduced PN. A similar increase in liver PN has been observed by the addition of peroxide (5). After supplementation with niacin (Group III) the blood PN content was increased to the level present when the animals received the normal diet, as shown by a value of 805 γ per gm. of hemoglobin. Feeding tryptophan (Group IV) did not produce as great an increase in PN levels as did the niacin supplementation.

**Table I**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Ration</th>
<th>No. of rats</th>
<th>PN per ml. blood</th>
<th>PN per gm. blood hemoglobin</th>
<th>NMeN* per ml. blood</th>
<th>NMeN per gm. blood hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Stock</td>
<td>14</td>
<td>109</td>
<td>755</td>
<td>2.64 (8)†</td>
<td>19.2</td>
</tr>
<tr>
<td>II</td>
<td>Non-protein</td>
<td>8</td>
<td>58</td>
<td>457</td>
<td>2.12</td>
<td>16.4</td>
</tr>
<tr>
<td>III</td>
<td>&quot; + niacin</td>
<td>4</td>
<td>88</td>
<td>805</td>
<td>2.91</td>
<td>26.4</td>
</tr>
<tr>
<td>IV</td>
<td>&quot; + tryptophan</td>
<td>4</td>
<td>67</td>
<td>583</td>
<td>3.15</td>
<td>27.0</td>
</tr>
</tbody>
</table>

* NMeN is expressed as N1-methylniacinamide hydrochloride.
† The figure in parentheses indicates the number of rats used for this determination.

This differential assay as applied to blood may give the investigator a better insight into the actual course of niacin and tryptophan metabolism in the rat than has heretofore been possible. In many instances, changes in these metabolites have been attributed solely to alterations in the pyridine nucleotide content. It is true that under the experimental conditions reported here the changes in NMeN content appeared negligible and did not seem to change to any great degree during the feeding procedure described. However, it is evident that this may not always be the case.
PN AND N\textsuperscript{1}-METHYLNIAINAMIDE

For example, in certain experiments in which niacin and tryptophan are injected in single large doses, it is conceivable that an apparent rise in blood pyridine nucleotides as measured by a non-differential assay could be the result of a far from negligible rise in NMeN.

The amount of NMeN which may be removed by the 200 mg. Decalso columns is much greater than the amount presented for adsorption from the blood samples being analyzed. In experiments designed to give an approximation of the capacity, 80 \( \gamma \) of NMeN were removed completely, and, even after the addition of 300 \( \gamma \) of NMeN to the column, only a very small amount appeared in the effluent. From these data it can be seen that there would be very little danger of incomplete removal of NMeN from any blood sample under consideration.

**SUMMARY**

1. A method for the quantitative differentiation of PN and NMeN in single samples of blood has been described. The main features of the method consist of precipitation of blood proteins and concurrent extraction of PN and NMeN by 75 per cent ethanol and removal of NMeN from the resulting extract by use of a 200 mg. Decalso column.

2. Recovery of PN from the effluent after the Decalso treatment has averaged 98.4 ± 1.3 per cent in 51 experiments.

3. Application of the method has been made to a study of the effects of dietary tryptophan and niacin on the content of PN and NMeN in the blood of the rat.

**BIBLIOGRAPHY**

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