A Comparative Study of the Synthesis of Nicotinamide Nucleotides by Erythrocytes of some Vertebrates

BY P. G. TULPULE

Nutrition Research Laboratories, Indian Council of Medical Research, Tarnaka, Hyderabad 7, India

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Kornberg (1950) and Rowen & Kornberg (1951) were the first to demonstrate that a partially purified enzyme preparation of rat liver catalysed the synthesis of NAD from nicotinamide, and they postulated ribosylnicotinamide and NMN as intermediates in the scheme of NAD synthesis. An enzyme capable of catalysing the synthesis of NMN from nicotinamide was also demonstrated in human erythrocytes by Leder & Handler (1951). However, the equilibria of the reactions brought about by nicotinamide–adenine dinucleotide pyrophosphorylase and nicotinamide mononucleotide pyrophosphorylase in vitro were not favourable for NAD synthesis under physiological conditions (Kornberg 1950; Rowen & Kornberg, 1951). Also, NMN synthesis from nicotinamide and glucose by human erythrocytes in vitro proceeded only at an extremely high and unphysiological concentration of nicotinamide (Leder & Handler, 1951).

The observation that human erythrocytes could synthesize NAD at low concentrations of nicotinic acid and that glutamine enhanced this synthesis led Preiss & Handler (1957) to postulate an alternative scheme for NAD synthesis from nicotinic acid which did not involve the formation of free nicotinamide. The investigators also studied, with partially purified enzymes from acetone-dried and powdered human erythrocytes, rat liver and auto-lysed yeast, the intermediate steps in NAD synthesis, wherein the synthesis started from nicotinic acid and proceeded through the formation of nicotinic acid mononucleotide and nicotinic acid–adenine dinucleotide, followed by amidation at the last stage (Preiss & Handler, 1958a, b).

In studies on the metabolism of nicotinamide nucleotides, Tulpule (1958) observed that washed rat erythrocytes were unable to synthesize NAD from nicotinamide under similar conditions to those employed by Leder & Handler (1951) in studies on human erythrocytes. An attempt was therefore made to study in vitro the synthesis of nicotinamide nucleotides by different mammalian species. The erythrocytes of most other vertebrates differ morphologically from the mammalian erythrocytes in that they are elliptical, nucleated and biconvex. They are also large in size but fewer in number. In the present investigation one avian species and one amphibian species were included to observe the
effect of nucleated erythrocytes on the synthesis of nicotinamide nucleotides. Two pathways of NAD synthesis from nicotinic acid and nicotinamide, as mentioned above, were examined.

MATERIALS AND METHODS

Erythrocyte preparations. Erythrocytes from seven mammalian species, namely mouse, rat, guinea pig, rabbit, sheep, monkey (Macaca radiata) and man, and one avian and one amphibian species represented by pigeon (Columba livia) and frog (Rana tigrina) respectively, were examined. Normal, healthy adult specimens in each species were selected for the investigations. Enzyme determinations were carried out on individual blood samples for all species except mouse and pigeon, for which blood from three and two individuals respectively was pooled for each observation.

Defibrinated whole blood as well as washed erythrocytes were used in the enzyme studies. Blood was defibrinated by shaking it as soon as it was drawn in a wide test tube with two glass beads, and the fibrin clot was removed before use. The erythrocytes were separated by centrifuging at 10,000g at 3°C for 10 min. and washed four times at 3°C with Ringer glucose solution, pH 7.2, and diluted with 4 vol. of 0.01 M-phosphate buffer, pH 7.4.

Synthesis of nicotinamide-adenine dinucleotide. The reaction mixture contained 50 μmoles of phosphate buffer, pH 7.4, 125 μmoles of glucose, 20 μmoles of glutamine, 1 μmole of ATP, 1-0 ml of either defibrinated whole blood or a suspension of washed erythrocytes, and nicotinic acid or nicotinamide in quantities ranging from 0-5 μmole to 500 μmole, in the total volume of 1-5 ml. The reaction was carried out at 37°C for 20 hr. and was terminated by adding an equal volume of 10% (w/v) trichloroacetic acid. Two types of controls were run simultaneously, one containing no substrate treated in a similar way to the test solution, and the other with added substrate, where the reaction was stopped before incubation. Since the incubation period was long, aseptic conditions were ensured by adding a mixture of 20 units of penicillin and 40 units of streptomycin to each reaction mixture. Leder & Handler (1951) have shown that the antibiotics at these concentrations have no influence on nucleotide synthesis.

![Table 1. Synthesis of nicotinamide nucleotides from nicotinamide and glucose by erythrocytes of some mammalian species.](image)

Table 1. Synthesis of nicotinamide nucleotides from nicotinamide and glucose by erythrocytes of some mammalian species

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of observations</th>
<th>Conc. of nicotinamide nucleotides (μg./ml.)</th>
<th>Synthesis of nicotinamide nucleotides (μg./ml. of erythrocyte suspension)</th>
<th>Synthesis of ribose (μg./ml. of erythrocyte suspension/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>8</td>
<td>30.0±1.394</td>
<td>62.5±4.263</td>
<td>314±18-623</td>
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<td>Monkey</td>
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<td>63.1±8.101</td>
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<td>0-0</td>
</tr>
<tr>
<td>Rat</td>
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<td>94.2±4.621</td>
<td>205.0±6.840</td>
<td>0-0</td>
</tr>
<tr>
<td>Mouse</td>
<td>2</td>
<td>194.1</td>
<td>407.5</td>
<td>186.0±5.56</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>6</td>
<td>37.8±1.536</td>
<td>77.1±1.910</td>
<td>147.0±9.625</td>
</tr>
<tr>
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<td>59.5</td>
<td>131.8</td>
<td>0-0</td>
</tr>
<tr>
<td>Sheep</td>
<td>3</td>
<td>—</td>
<td>192.1±9.297</td>
<td>0-0</td>
</tr>
</tbody>
</table>

Experimental details are given in the text. The results are given as means±s.e.m. (where appropriate). —, Not done.

RESULTS

Formation of nicotinamide nucleotides from nicotinamide and glucose by erythrocytes of seven mammalian species. In these experiments the procedure described by Leder & Handler (1951) for the synthesis of nicotinamide nucleotides by human erythrocytes was followed with 200 μmoles of nicotinamide in the reaction mixture. Glutamine was omitted from the reaction mixture.

The results (Table 1) demonstrate that, of the seven species studied, only man and guinea-pig erythrocytes were capable of synthesizing nicotinamide nucleotides under the conditions used. However, all the species that were unable to carry out this synthesis had a higher blood concentration of nicotinamide nucleotides than guinea pig and man. The results also demonstrate the formation of ribose phosphate from glucose 6-phosphate in rat and guinea-pig erythrocytes, and also that the ribose concentrations of the whole blood of these two species were almost equal. The ribose phosphate did not therefore seem to be a limiting factor in the synthesis of nicotinamide nucleotides in rat erythrocytes.

Formation of nicotinamide nucleotides from nicotinic acid and nicotinamide by erythrocytes at different concentrations of the substrates. Preiss & Handler (1957) showed that with low concentrations of nicotinic acid, in the presence of glucose and...
glutamine, there was appreciable synthesis of nicotinamide nucleotides by human blood, whereas much higher concentrations of nicotinamide were required to attain their synthesis in comparable quantities. In our experiments the capacity of the defibrinated whole blood of man, monkey, guinea pig, rat, pigeon and frog to synthesize nicotinamide nucleotides from nicotinic acid and nicotinamide, at various concentrations ranging from 0.5 \( \mu \) mole to 500 \( \mu \) moles, was tested.

Defibrinated human blood could synthesize these nucleotides from nicotinamide, as well as from nicotinic acid (Fig. 1). The formation of nicotinamide nucleotides from nicotinamide increased with the amount of substrate present in the reaction mixture. At concentrations of 0.5 to 1.0 \( \mu \) mole of nicotinic acid/1.5 ml. of reaction mixture there was appreciable synthesis of nicotinamide nucleotides, but as the amount of the substrate was increased to 10 \( \mu \) moles the amount of nicotinamide nucleotides synthesized was decreased. At higher concentrations of nicotinamide there is a much greater synthesis of nicotinamide nucleotides than in the presence of optimum amounts of nicotinic acid. The results were essentially the same with defibrinated whole blood and isolated erythrocytes. It was also observed (Fig. 2) that the maximum synthesis of nicotinamide nucleotides could be obtained when 1 \( \mu \) mole of nicotinic acid was added to the reaction mixture, and at a similar concentration nicotinamide could synthesize only one-tenth of that quantity. These results agree with the observations of Preiss & Handler (1957) and support the view of these authors that NAD synthesis from nicotinic acid through nicotinic acid mononucleotide and nicotinic acid–adenine dinucleotide represents a physiologically operative pathway in human erythrocytes.

Monkey blood synthesized nicotinamide nucleotides in vitro from nicotinic acid with low amounts of the substrate (1 \( \mu \) mole) when the incubation mixture contained glutamine (Fig. 3), and the enzyme catalysing this synthesis was inhibited completely when the concentration of substrate was increased to 40 \( \mu \) moles/1.5 ml. of reaction mixture. Results with nicotinamide and glucose confirmed the findings reported above that the monkey blood could not use nicotinamide directly for the synthesis of nicotinamide nucleotides. However, when the reaction mixture containing nicotinamide and glucose was fortified with glutamine appreciable synthesis of nicotinamide nucleotides was observed, which reached a maximum when 100 \( \mu \) moles of the substrate were used and

![Graph](image)

**Fig. 1.** Effect of substrate concentration on the synthesis of nicotinamide nucleotides by washed human erythrocytes diluted to the original volume of blood (O) and by defibrinated whole blood (\( \nabla \)). (a) Reaction with nicotinamide as substrate; (b) reaction with nicotinic acid as substrate in the presence of glutamine. Each point represents the mean of six observations. The range of values at each point is indicated by the horizontal lines below and above each average value.

![Graph](image)

**Fig. 2.** Synthesis of nicotinamide nucleotides by human erythrocytes at physiological concentrations of the substrates. O, \( \nabla \) and □. Values for three different blood samples. Continuous line, nicotinic acid as substrate; broken line, nicotinamide as substrate.
then decreased when the substrate concentration was further increased. It seemed possible, therefore, that only one pathway of NAD synthesis, whereby amidation occurred after the nicotinic acid had been converted into nicotinic acid mononucleotide, was operative in monkey blood, and that nicotinamide could participate in the synthesis in vitro only after its primary conversion into nicotinic acid.

The results of studies on guinea-pig blood are represented in Fig. 4. The guinea-pig blood differed from the human and monkey blood in that it was unable to synthesize nicotinamide nucleotides from nicotinic acid in the presence of glucose and glutamine. However, in the presence of nicotinamide and glucose, NAD was synthesized. A progressive increase in the synthesis of nicotinamide nucleotides was observed, the rate of the reaction being proportional to the nicotinamide concentration up to 100 μmoles/1.5 ml. of reaction mixture.

Studies with rat blood showed that the synthesis of nicotinamide nucleotides did not occur in the presence of either nicotinamide or nicotinic acid. They suggested the total absence of the enzyme in this tissue of rat.

Of the two vertebrate species, namely pigeon and frog, selected to provide nucleated erythrocytes, both were capable of synthesizing nicotinamide nucleotides from nicotinamide and nicotinic acid (Table 2). The pattern of synthesis of nicotinamide nucleotides at different substrate concentrations was almost the same as that observed with human erythrocytes. When nicotinamide was used as substrate the amount of nicotinamide nucleotides synthesized by pigeon and frog erythrocytes at any particular concentration of the substrate was about one-fifth of that synthesized by human or guinea-pig erythrocytes. However, the synthesis of nicotinamide nucleotides from nicotinic acid in the presence of glutamine was only slightly lower in the pigeon erythrocytes. These results clearly indicated that the nicotinic acid pathway of the synthesis of nicotinamide nucleotides is probably an important mechanism in nucleated erythrocytes. Rajagopalan, Sundaram & Sarma (1958) have demonstrated a nicotinamide deamidase in the tissues of vertebrates like pigeon and chick. It may be that in these species the nicotinic acid needed for the synthesis of nicotinamide nucleotides is formed in vivo.

**SUMMARY**

1. Seven mammalian, one avian and one amphibian species were studied for the capacity of their erythrocytes to synthesize nicotinamide nucleotides in vitro.

2. Under the conditions used, only human erythrocytes and nucleated erythrocytes of pigeon and
frog were capable of synthesizing nicotinamide nucleotides from both nicotinamide and nicotinic acid. On the other hand, rat blood was unable to form nicotinamide nucleotides from either of these substrates.

3. Human, guinea-pig, pigeon and frog erythrocytes synthesized nicotinamide nucleotides from nicotinamide and glucose. Monkey erythrocytes synthesized nicotinamide nucleotides from nicotinamide only in the presence of glutamine, suggesting that in this species the conversion of nicotinamide into nicotinic acid was an essential step in the formation of nicotinamide nucleotides.

4. Human, monkey, pigeon and frog erythrocytes could synthesize appreciable amounts of nicotinamide nucleotides from nicotinic acid, glucose and glutamine, whereas this did not occur in guinea-pig erythrocytes. Nicotinic acid was an effective precursor of nicotinamide nucleotides only at low concentrations.

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REFERENCES


Studies on Metabolism of Vitamin A

1. THE BIOLOGICAL ACTIVITY OF VITAMIN A ACID IN RATS

BY P. MALATHI, K. SUBBA RAO, P. SESHA DRI SASTRY* AND J. GANGULY

Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

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Since Dowling & Wald (1960) demonstrated that rats grow normally on continued dosage of vitamin A acid, but that they become blind, it has become increasingly apparent that vitamin A may exist in multiple forms with different biological functions. This view has further been strengthened by claims that the acid fails to meet the reproductive requirements of either male or female rats (Thompson, Bioch. 1963, 87